VOLUME 30 • NUMBERS 1–2 • 1995

acta

Phytopathologica et Entomologica Hungarica

AKADÉMIAI KIADÓ
BUDAPEST

Acta Phytopathologica et Entomologica Hungarica

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Acta Phytopathologica publishes original papers on phytopathology, entomology and pesticide chemistry mainly in English.

Acta Phytopathologica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

H-1117 Budapest, Prielle Kornélia u. 19–35

Manuscripts and editorial correspondence should be sent to the Editorial Office

H-1525 Budapest, P.O. Box 102

Subscription information

Orders should be addressed to

AKADÉMIAI KIADÓ

H-1519 Budapest, P.O. Box 245

Subscription price for Volume 30 (1995) in 4 issues US$ 104.00, including normal postage, airmail delivery US$ 20.00.

Acta Phytopathologica is indexed in Current Contents

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Salt Stress in *Plantago* The Role of Membranes, Channels and Pumps

H. B. A. PRINS

University of Groningen, Biological Center, Department of Plant Biology, Kerklaan 30
P.O. Box 14, NL 9750 AA Haren, The Netherlands

In the present article the cellular mechanism of Na^+ transport across the plasma membrane and tonoplast of root cells of *Plantago media* (salt sensitive) and *Plantago maritima* (salt tolerant) is discussed based on findings obtained mainly by patch clamp technique. It is conluded that the combination of Na^+ induced channel closure together with the Na^+ induced Na^+/H^+ antiporter forms an effective mechanism for sequestering Na^+ in the vacuole of *P. maritima*, while tha salt-sensitive *P. media* lacks the ability to pump Na^+ into the vacuole.

Species of the genus *Plantago* differ widely in their response to salt (NaCl) stress. While *P. maritima* and, to a lesser extent, *P. coronopus* can withstand high external NaCl levels in the external medium, uptill respectively 300 and 150 mM, growth of *P. media* can already be severely inhibited at 20 mM external NaCl. At 50 mM the latter species is generally damaged and often killed (Erdei and Kuiper, 1979; De Boer, 1985). Under natural conditions *P. media* grows in nutrient poor soils with a low electrical conductivity, while *P. maritima* grows typically in coastal or otherwise saline regions.

The two species showed a different physiological response towards an applied salt stress. When placed in a Na^+ containing solution, transport of Na^+ to the sprout started immediately in *P. maritima*, while in *P. media* a lag phase was observed (Erdei and Kuiper, 1979). Subsequently De Boer (1985) showed that this lag phase in *P. media* resulted from the decreased water potential at high levels of NaCl, resulting in a reduced rate of water transpiration flow and from retention of Na^+ in the root. In contrast to *P. media*, *P. maritima* readily took up Na^+ from the external medium, even at very low concentrations. As a result addition of Na^+ to the medium apparently had less of an osmotic effect in that species. However, also in the absence of any osmotic effect retention in the root was found.

This latter finding indicates that the plasma membrane between the xylem vessels and the surrounding xylem parenchyma cells can act as a barrier for effective Na^+ transport to the shoot and that excretion into and resorption from the xylem vessels can effectively regulate the distribution of Na^+ between root and shoot.

There was also a clear difference between the salt tolerant and salt sensitive species with respect to the effect of salinity on lipid composition. The salt sensitive *P. media* showed already a decrease of the root levels of galacto-, sulpho-, and phospholipids at low, < 20 mM, of external NaCl. The salt tolerant *P. maritima* and *P. coronopus* also showed a similar change in lipid levels in response to NaCl stress but only at much

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higher NaCl concentrations (Erdei et al., 1980). The same authors observed that the effect of salt stress on lipid composition was very much affected by the nutritional level of the growth medium. At high nutritional levels salt stress had far less effect than under low nutrient conditions.

As judged from the effect on phospholipids a mild NaCl stress, e.g. NaCl < 75 mM and a high level of nutrients, appeared to be even beneficial for *P. maritima*. The importance of the nutritional level for salt tolerance/sensitivity was also indicated by experiments of Maathuis (1991) who repeated some of the above-mentioned growth experiments (De Boer, 1985) at more favourable nutrient conditions. In these experiments an applied salt stress had far less negative effect on growth of *P. media* and *P. maritima*, although the relative differences between the two species remained. Up to 20 mM NaCl in the growth medium stimulated growth not only of *P. maritima* but also of *P. media*.

Despite this finding the author concluded that *P. maritima* should not be considered a true halophyte, but that this species, and to a lesser extent *P. coronopus*, should be called salt tolerant and *P. media* salt sensitive (Greenway and Munns, 1980; Maathuis, 1991).

In *Plantago* salt stress is caused by the presence of Na^+ and not Cl^- as NaCl and Na_2SO_4 seem to cause the same stress response (Maathuis, 1991).

Sorbitol apparently serves as a compatible solute to balance the osmotic potential of the cytoplasm against the vacuole and the external medium in case of high salinity (Ahmad et al., 1979; Jefferies et al., 1979; Lambers et al., 1981).

In the present article we discuss the cellular mechanism of Na^+ transport across the plasma membrane and tonoplast of root cells of *P. media* and *P. maritima* and its physiological significance for tolerance to salt stress.

The role of membranes in salt stress

As major plant cell membranes, plasma membrane (PM) and tonoplast play a vital role in compartmentation, translocation and extrusion. The tonoplast is crucial in compartmentation of Na^+, which serves the purpose of minimising cytoplasmic Na^+ levels and simultaneously lowering the osmotic potential of the cell.

In a number of salt tolerant and halophytic species this mechanism involves a Na^+ inducible Na^+/H^+ antiporter (Blumwald and Poole, 1985; Garbarino and Dupont, 1988). This is also the case in *P. maritima* (Staal et al., 1991). This transporter, in concert with a low ion channel conductance (Maathuis and Prins, 1990, Maathuis et al., 1992) assures effective sequestering of Na^+ ions in the vacuole.

The PM serves an even more pivotal role in minimising NaCl stress, as it constitutes the primary barrier between cytoplasm and external compartment or root environment. As outlined below, PM ion channels and other transport systems are involved in both Na^+ influx and Na^+ efflux processes.

The role of plasma membrane transport

The root cells form a symplast surrounded by a plasma membrane. At the inner side of the root core this PM forms the interface between xylem vessel content and root symplast. At the outer side this membrane separates the symplast from the outer medium. Suberization of the cell wall of the endodermis and of older cortical cells electrically isolates the xylem vessel content from the outer medium and prevents ion transport through the free space of the cell walls between xylem vessels and external solution. Physiologically the xylem vessel and the outer medium can both be considered as exterior compartments with respect to the symplast but isolated from each other.

Plasma membrane ATPase proton pumps (PM-ATPase) are located at the PM at the inner side, pumping protons into the xylem vessels, and at the outer, cortical, side pumping protons in the direction of the external medium (Hanson, 1978; De Boer et al., 1983, 1984, 1985). Active excretion of protons by the PM-ATPase is an electrogenic process, thus at the cortical side PM-ATPases contribute to the cell electrical potential difference (PD) between the symplast and the outer medium, while the xylem parenchyma or inner PM-ATPases generate an electrical PD between symplast and the xylem vessel content. Both PM-ATPases contribute to the electrical PD between xylem content and the outer medium, but in opposite direction. When the shoot is removed this latter electrical PD can be measured as the xylem exudate potential (XPD). Numerically XPD is the difference between both cell electrical PD's. Both PM-ATPases not only create an electrical PD but also establish a ΔpH and thus a proton motive force (pmf).

Na^+ influx pathways

Na^+ present in the outer medium enters the root symplast by passing the cortical, and in younger parts the epidermal, PM via a uniport system. Figure 1 gives an oversight of the relevant Na^+ transport pathways in a root cell. Na^+ experiences a large inwardly directed driving force because of the generally high external concentration and the negative membrane potential, typically –l00 to –170 mV at 1 mM KCl or NaCl (de Boer, 1985; Maathuis 1991). It is generally assumed that Na^+ enters the symplast via PM cation conducting channels. Yet, no Na^+ selective channel has been reported in *Plantago* or other plants, but several PM K^+ channels show a Na^+ conductance large enough to account for considerable Na^+ uptake (Schroeder et al., 1987; Schachtman et al., 1991; Schubert and Duchli, 1993; Murata et al., 1994). Additionally other channel·types and possibly carriers may also contribute to inward Na^+ passage. Murata et al. (1994) found evidence of K^+ and Na^+ conducting channels in tobacco suspension cells which showed a reduced permeability when the cells were adapted to high, 50 mM, NaCl.

In *Plantago* the situation is·less clear. Patch clamp experiments with root cortical cells of *P. media* revealed a number of PM channels (Vogelzang and Prins, 1992, 1994). In the cell attached configuration (CAP) 5 outward rectifying channels (ORC) and 6 inward rectifying channels (IRC) were identified. In the outside out patch configuration (OOP) of these one ORC and 4 IRC's were also observed. The dominant channel in both

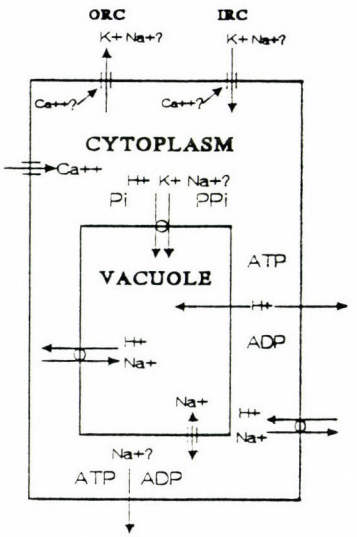

Fig. 1

configurations was a K^+ conducting ORC, called after its typical flickering behaviour ORC-f. Voltage dependent activation of this channel shifted with the Nernst potential for K^+ (E_K). Its threshold potential for activation lay between 25 and 50 mV more negative than E_K. Thus at potentials more negative than E_K, physiologically probably the "normal" state of the cell, this channel can conduct an inward current carried by K^+. In principle this would make this channel a candidate for a Na^+ entry pathway. It was estimated that $P_{K^+}/_{PNa^+}$ was 30 or higher (Vogelzang and Prins, 1992). In more recent experiments, however, addition of 100 mM Na^+ to the extracellular side of the PM without any Na^+ at the cytoplasmic side did not significantly change the reversal potential of this channel in OOP (Vogelzang and Prins, 1994). This would indicate that the ORC-f is highly selective for K^+ and probably not a pathway for Na^+. The IRC's seem far less selective and thus, also because of their inward rectifying nature, seem more likely candidates for Na^+ entry. This awaits future reseach.

Na^+ efflux pathways

At an external Na^+ concentration of 10 mM and a membrane potential of –120 mV, the cytoplasmic [Na^+] would rise to somewhat less than 1 M in case of passive ion distribution. To prevent this lethal Na^+ level should involve active, i.e. directly energised, Na^+ extrusion. This was strongly indicated by experiments with metabolic inhibitors. The addition of diethyl-stilbesterol (DES) or carbonyl-cyanide m-chlorophenyl hydrazone (CCCP) (De Boer, 1985; Maathuis, 1991) resulted in an increase of intracellular Na^+.

The nature of active extrusion into the external medium is unknown. Staal et al. (1991), using PM vesicles, failed to find any evidence for Na^+/H^+ antiport at the PM, a mechanism present in the tonoplast of *P. maritima*. The pmf generated across the inner PM, between root symplast and xylem vessel content, can be used for Na^+ extrusion into the vessels as well as for Na^+ resorption from the xylem sap into the surrounding symplast (De Boer et al., 1983, 1985).

Despite the central role of the PM-ATPase in providing the driving force for Na^+ transport across the PM both at the inner side between xylem and symplast and at the outer side between symplast and outer medium, there seems to be no relation between salt stress and the kinetic parameters of isolated PM-ATPase in *Plantago* species (Brüggemann and Janiesch, 1987, 1988, 1989). This might indicate that, if there is any regulation of PM-ATPase activity by an applied salt stress, it is reversible and depends on a cytoplasmic factor.

The role of tonoplast transport

The overall concentration of Na^+ in root and shoot of *P. maritima* can be very high without any serious inhibition of growth. At 300 mM NaCl in the external solution, root and shoot Na^+ concentrations on a fresh weight basis were 200 and 600 mM respectively (Erdei and Kuiper, 1979). These high overall concentrations indicate that Na^+ is sequestered in the vacuole, as these high concentrations in the cytoplasm would seriously hamper the normal functioning of the plant. Jeschke (1984) also concluded that *P. maritima* efficiently sequesters Na^+ ions in the cell vacuoles, and that this is part of the mechanism of salt tolerance in this species.

Data from growth experiments with *P. media* (De Boer, 1985) indicated that this salt sensitive species lacks the ability to transport Na^+ effectively into the vacuole. In this species overall Na^+ concentrations, which would stimulate growth in *P. maritima*, were toxic.

Maathuis and colleagues (Maathuis and Prins, 1989, 1990, 1991; Staal et al., 1991) studied Na^+ transport across the tonoplast of *P. media* and *P. maritima* in detail using the patch clamp technique and isolated tonoplast vesicles. Using acridine orange fluorescence as an indicator for the ΔpH across the membrane of these vesicles, it was found that in both species, *P. media* and *P. maritima*, a ΔpH could be generated by a tonoplast bound ATPase and a pyrophosphatase. Addition of Na^+ after the build up of the ΔpH resulted in dissipation of this gradient in vesicles prepared from *P. maritima* grown in the presence of Na^+, but not when grown in the absence of Na^+. In *P. media*, whether grown in the absence or presence of Na^+, the addition of Na^+ to the vesicles did not result in dissipation of the ΔpH. Apparently a Na^+/H^+ antiport system is expressed in the tonoplast of *P. maritima* root cells when grown in the presence of Na^+. The obvious function of this antiporter is to sequester Na^+ in the vacuole (Staal et al., 1991). Comparable tonoplast Na^+/H^+ antiporters have been found in other salt tolerant species as well (Blumwald and Poole, 1985; Garbarino and DuPont, 1988, 1989).

In order to sequester Na^+ effectively in the vacuole there should be no pathway for a back-flux of Na^+ from the vacuole to the cytoplasm. The vacuolar electrical potential is circa 20 mV positive with respect to the cytoplasm. Together with the high vacuolar Na* concentration this forms a very strong driving force for Na^+ in the direction of the cytoplasm. Such a pathway could be formed by a Na^+ conducting tonoplast channel. It was therefore rather unexpected that patch clamp experiments showed the existence of a Na^+ conducting channel in the tonoplast of both species (Maathuis and Prins, 1990). In both the dominant tonoplast channel was a 60 to 70 pico Siemens channel with a low selectivity. The conductance of this channel for Na^+ was the same as for K^+ ($P_{K^+}/P_{Na^+} = 1$). The cation to anion selectivity (P_{K^+}/P_{Cl^-}) was around 5. A Na^+/H^+ antiport system cannot effectively transport Na^+ into the vacuole with this channel activated. Closer examination of this channel revealed, however, that the activity of this cation channel very much depended on the growth conditions of the plant material from which the vacuoles were derived.

Channel opening occurred in bursts. In vacuole attached experiments the frequency of these bursts was drastically reduced when the plants had been grown in the presence of Na^+. Only the frequency of the bursts was reduced not the single channel characteristics. This Na^+ induced closure of the channels was also apparent in a strong reduction of the whole vacuole currents (Maathuis and Prins, 1989, 1990, 1991).

The combination of Na^+ induced channel closure together with the Na^+ induced Na^+/H^+ antiporter forms an effective mechanism for sequestering Na^+ in the vacuole of *P. maritima*.

In *P. media* the function of the Na^+ induced channel closure , however, is not yet clear as this species lacks the ability to pump Na^+ into the vacuole. It may be that the Na^+ induced channel closure is a general reaction to osmotic stress. It is conceivable that in *P. media* other ions than Na^+, e.g. K^+, are transported to the vacuole for osmotic purposes. In that case it could be functional, also for *P. media*, to sequester K^+ (or other cations) in the vacuole by channel closure induced by osmotic stress.

Future research

Future research on the role of Na^+ transport in salt stress should concentrate on the plasma membrane. Basic guestions regarding the uptake routes of Na^+ ions, their cytoplasmic status, modes of efflux and the various involved transport systems, are still unanswered. A specific hypothesis to be tested, is the possibility of plant membrane systems responsible for Na^+ extrusion and compartmentation, different from the well established Na^+/H^+ antiporter. We propose to investigate, Na^+ stress related, transport processes using Na^+ dependent fluorometry and to combine this technology with electrophysiological techniques (Fig. 2).

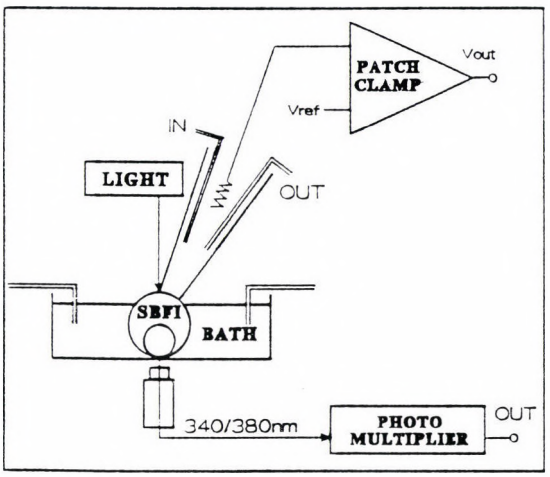

Fig. 2

Four separate research goals can be defined:

(A) To establish the precise uptake pathway(s) for Na^+.

(B) To test the hypothesis that apart from Na^+/H^+ antiport, alternative Na^+ pumping systems are present in plant membranes.

(C) To study how expression and regulation of various Na^+ pathways are affected by growth of plants at different Na^+ levels.

(D) To develop a Na^+ monitoring technique, based on Na^+ dependent fluorescence and applicable at the single cell level, which can directly monitor Na^+ fluxes in plant cells.

Acknowledgement

This research was supported ECOTRANS B.V. ECOTRANS publication #51.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 15–19 (1995)

Role of PPi and H^+-PPiase in Maintaining Vacuolar Proton Gradient in Metabolic Inhibitor-treated Cells

A. VIANELLO[1], M. ZANCANI[1], E. PETRUSSA[1], P. DELL'ANTONE[2] and F. MACRÌ[1]

[1]Cattedre di Fisiologia Vegetale e Biochimica Vegetale,
Università di Udine, via del Cotonificio 108, I-33100 Udine, Italy
[2]Dipartimento di Scienze Biomediche Sperimentali,
Università di Padova, via Trieste 75, I-35100 Padova, Italy

In *Acer pseudoplatanus* cells, the role of ATP and PPi in keeping the vacuolar ΔpH was investigated after treatments with 2-deoxy-D-glucose, inhibitor of glycolysis, and KCN, inhibitor of respiration, in order to simulate a condition of glucose startvation and anoxia. It was concluded that the tonoplast H^+-PPiase is responsible for the maintenance of vacuolar ΔpH and that this enyme is the major consumer of PPi in metabolic inhibitor-treated cells.

Differently from animal cells, the plant cells survive for long periods of hypoxia, because they carry out only a transient lactic fermentation which provides the signal to trigger that ethanolic [1]. As known, the latter does not result in a severe acidosis [2]. Among plant cells, tolerance to short periods of anaerobiosis depends on their ability to maintain a constant vacuolar pH [3, 4]. In non-tolerant plants, indeed, cell death is caused by a leakage of acids from vacuoles which determines a cytoplasmic acidosis [5].

The functionality of vacuoles is linked to the activities of H^+-ATPase (EC 3.6.1.3) and H^+-PPiase (EC 3.6.1.1) that keep an electrochemical gradient across tonoplast, utilizing ATP or pyrophosphate (PPi) as substrates [6]. Since cytoplasmic PPi level, conversely to ATP, does not change when tissues are subjected to anoxia or respiratory poisons [7, 8], it has been suggested that this energy source is utilized by H^+-PPiase to provide a system for the maintenance of vacuolar compartmentation, despite temporary metabolic perturbations [9]. In addition, this enzyme, having a high affinity for PPi, can also be active at very low concentrations of substrate [6] and appears to be inducible by either anoxia or chilling [10].

In this paper, it is examined the role of ATP or PPi in keeping the vacuolar ΔpH in *Acer pseudoplatanus* cells, treated by 2-deoxy-D-glucose (inhibitor of glycolysis) and KCN (inhibitor of respiration) to simulate a condition of glucose starvation or anoxia.

Effect of metabolic inhibitors on H^+ extrusion and vacuolar ΔpH (NH_4^+-induced release of AO) in A. pseudoplatanus cells

The activity of vacuolar proton pumps, both ATP and PPi-driven, depends on the continuous supply of these substrates. For this reason, the effect of the metabolic inhibitors on H^+ extrusion and vacuolar ΔpH was checked (Table 1).

0238–1249/95/$ 5.00

Table 1

Effect of metabolic inhibitors on proton extrusion and vacuolar ΔpH in *A. pseudoplatanus* cells after 24 h incubation

Additions	H^+ extrusion	ΔpH[a]	
	Ext. pH	$\Delta A_{495-540}$	
Control (0 h)	7.50	0.088[b]	–
Control (24 h)	7.22	0.042	0.077[c]
10 mM KCN	7.47	–	0.063
5 mM 2-deoxy-D-glucose	7.25	–	0.072
KCN+2-deoxy-D-glucose	7.52	–	0.061

[a] ΔpH was measured as NH_4^+-induced release of AO, after 15 min incubation of cells with dye.
[b] Without correction of external pH.
[c] With correction at pH 7.5 of external pH.

Pre-incubation of control cells for 24 h, before performing dye uptake, determines an acidification of the incubation medium that is associated with an apparent decrease in vacuolar ΔpH. However, when the external pH is restored to the initial value (7.5), the proton gradient is very near to that detected before pre-incubation. Potassium cyanide inhibits H^+-extrusion, while only slightly lowers the vacuolar ΔpH detected after pH correction. On the contrary, 2-deoxy-D-glucose causes negligible changes in H^+-extrusion and vacuolar ΔpH, indicating that the role of ATP, produced by glycolysis, in keeping the vacuolar proton gradient and to fuel plasmalemma H^+-ATPase is minor. In agreement KCN plus 2-deoxy-D-glucose show an effect comparable to that caused by KCN alone.

Effect of metabolic inhibitors on ATP and PPi content of A. pseudoplatanus cells

The low impairment of vacuolar ΔpH, caused by KCN, raises the question whether this could be kept by PPi and/or ATP utilized by vacuolar H^+-PPiase and H^+-ATPase. In anaerobiosis, pyrophosphate level is maintained constant [7], while ATP may be yielded by glycolytic reactions [1].

Table 2 shows that the ΔpH of isolated vacuoles is approx. 50 % decreased after 3 h of incubation. The additions of ATP and PPi, alone or together, partially (PPi) or almost completely (ATP) restore this ΔpH. The presence of ATP or PPi appears, hence, to be crucial in keeping the proton gradient across the tonoplast. In agreement, bafilomycin A_1 (BAF) or imidodiphosphate (IDP) inhibit, respectively, the ATP or PPi-dependent restoration of ΔpH, thus confirming the involvement of H^+-ATPase and H^+-PPiase is maintaining the appropriate vacuolar ΔpH.

The incubation of control cells for 24 h induces a negligible decrease of ATP amount and an increase of PPi level. Potassium cyanide alone or plus 2-deoxy-D-glucose

Table 2

Effect of ATP and PPi on ΔpH of isolated *A. pseudoplatanus* vacuoles after 3 h incubation

Additions	ΔpH[a]
	$\Delta A_{495-540}$
Control (0 h)	0.102
Control (3 h)	0.053
1 mM ATP	0.090
500 μM PPi	0.068
1 mM ATP + 500 μM PPi	0.094
1 mM ATP + 10 μM BAF	0.042
500 μM PPi + 500 μM IDP	0.038

[a] ΔpH was evaluated as NH_4^+-induced release of AO after 15 min incubation of vacuoles with dye.

causes, respectively, approx. 95% and 38% decrease in cellular concentrations of ATP and PPi (Table 3).

The large drop in ATP level, caused by metabolic inhibitors, is a consequence of respiration inhibition and permits to explain the lack of acidification of the medium in KCN-treated cells (Table 1), owing to the diminished availability of ATP that limits the activity of the plasmalemma H^+-ATPase. The decreased level of PPi might be linked to the drop in ATP content, because several cell biosynthetic reactions, yielding PPi, depend on the continuous supply of nucleotide triphosphates [11]. However, the drop in PPi level opens the question on its contribution to the maintenance of a ΔpH in cells treated with KCN plus 2-deoxy-D-glucose.

The decrease of PPi level in metabolic inhibitor-treated cells could be linked to its consumption to fuel H^+-PPiase, whose activity is necessary to maintain the pH gradient.

Table 3

Effect of metabolic inhibitors on ATP and PPi level of *A. pseudoplatanus* cells after 24 h incubation

Additions	ATP level	PPi level
	nmol g^{-1} FW	nmol/g^{-1} FW
Control (0 h)	128	13
Control (24 h)	101	21
10 mM KCN	7	11
10 mM KCN + 5 mM 2-deoxy-D-glucose	5	8

Table 4

Effect of KF and IDP on vacuolar ΔpH and PPi level in metabolic inhibitor-treated *A. pseudoplatanus* cells after 24 h incubation

Additions	ΔpH[a]	PPi level
	$\Delta A_{495-540}$	nmol/g^{-1} FW
Control (0 h)	0.090[b]	11
Control (24 h)	0.080[c]	20
10 mM KCN + 5 mM 2-deoxy-D-glucose	0.066	7
5 mM KF	0.064	52
KCN + 2-deoxy-D-glucose + KF	0.018	23
5 mM IDP	0.040	87
KCN + 2-deoxy-D-glucose + IDP	0.020	81

[a] ΔpH was measured as NH_4^+-induced release of AO, after 15 min of cell incubation with dye.
[b] Without correction of external pH.
[c] With correction at pH 7.5 of external pH.

To test this possibility, the effect of the two PPiase inhibitors (KF and IDP) on PPi level and vacuolar ΔpH in metabolic inhibitor-treated cells was assayed (Table 4). Fluoride and IDP, *per se*, as previously shown [12, 13], strongly increase PPi level in untreated cells. Conversely, these inhibitors lower the proton gradient. In KCN plus 2-deoxy-D-glucose-treated cells, the level of PPi is partially (KF) or almost completely (IDP) restored by PPiase inhibitors, whereas ΔpH is stronger lowered. Therefore, the above rationale appears to be supported.

Role of PPi and H^+-PPiase in maintaining vacuolar ΔpH in metabolic inhibitor-treated plant cells

Metabolic inhibitors (KCN and 2-deoxy-D-glucose), used alone or together, have only a little inhibitory effect on vacuolar ΔpH. On the contrary, KCN or KCN plus 2-deoxy-D-glucose strongly lower cellular ATP level, while halve PPi content. The vacuolar ΔpH is, hence, maintained even in cells depleted of ATP by metabolic inhibitors, according to what was found in cells grown in anaerobiosis [3, 4]. While the drop of ATP in metabolic inhibitor-treated cells is expected, the decrease of PPi is, at least in part, surprising, because it does not change in tissues subjected to anoxia [7]. As PPi is synthesized through several biosynthetic reactions requiring ATP [11], its decreased level can depend on the low availability of the latter. On the other hand, PPi can be consumed to sustain vacuolar H^+-PPiase activity which, as suggested [9], would maintain the proton

gradient in anoxic cells. The latter hypothesis is supported by the experiment with inhibitors of phosphatases (KF and IDP).

In agreement with the results obtained by others [12, 13], KF and IDP increase PPi content in untreated cells. In addition, these inhibitors restore PPi level in metabolic inhibitor-treated cells, while decrease the vacuolar ΔpH by inhibiting H^+-PPiase.

It is, therefore, concluded that the tonoplast H^+-PPiase is especially responsible for the maintenance of vacuolar ΔpH and that this enzyme is the major consumer of PPi in metabolic inhibitor-treated cells. This conclusion is supported by the observation that H^+-PPiase is induced by anoxia or chilling and, hence, plays a key role in maintaining vacuolar proton gradient and in limiting cytoplasmic acidosis [10].

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 21–25 (1995)

Effect of Salinity in the Early Stages of Tomato Fruit Growth

C. VESPASIANI , C. ARIAS and E. TALEISNIK

Instituto de Fitopatología y Fisiología Vegetal, INTA, Estafeta Postal Coronel, Olmedo, Córdoba, Argentina

In tomato fruits, fruit fresh weight as well as the accumulation of dry matter as sugars or starch are markedly affected by salinity and drought. These changes are already apparent three weeks after fruit set, but the effects of salinity on earlier stages of fruit development had not been characterized. The accumulation of soluble sugars and starch, and the activity of ADPG pyrophosphorylase (enzyme which controls a rate-limiting step of starch synthesis) were studied in the first twenty days after fruit set, in fruits of two tomato cultivars which differ in salt tolerance.

Plants of tomato *Lycopersicon esculentum* Mill. cvs. Ace, salt sensitive, and Edkawi, salt tolerant, were subject to high salinity levels (200 mM NaCl) in sand culture. Flowers were hand-pollinated, and fruits were harvested on day 5,10,15 and 20 after pollination (DAP).

As expected, salinity decreased fruit fresh weight, in both cultivars. Starch and soluble sugar content per fruit fresh weight increased as a function of fruit age in this period, and were significantly higher in fruits from salinized plants than in controls. ADPG pyrophosphorylase activity did not change significantly in fruits from non-salinized plants in the course of this study and it increased under salinity in cv. Ace. Starch accumulation, however, was not related to the *in vitro* activity of ADPG pyrophosphorylase.

It is concluded that carbohydrate metabolism in the very early stages of fruit development is sensitive to saline stress. However, no correlation between salt tolerance and carbohydrate accumulation could be observed in this period.

Tomato fruit growth and quality are influenced by genetic and environmental factors (Yelle et al., 1988, Rudich et al., 1977, Mizrahi et al., 1988, Ehret and Ho, 1986). Salinity affects tomato yields through a reduction of fruit size in the first four weeks of harvest and later, through reductions in both fruit size and number (Adams and Ho, 1989).

Positive correlations between relative growth rate and starch level (Walker and Thornley, 1977) and between growth and the rate of starch accumulation (Guan and Janes, 1991) have been reported in tomato fruit. Starch accumulation early in fruit development correlated with carbon import rate (Hewitt et al., 1982). However, under salinity and water deficit, reduced fresh weight, increased starch and decreased hexose accumulation were observed in experiments where tomato fruits were analyzed starting from the third week after anthesis (Mitchell et al., 1991).

ADPG[1] pyrophosphorylase is a key enzyme in starch synthesis (Preiss, 1982). The transient accumulation of starch which occurs in the first stages of fruit development has

[1] ADPG: adenosine diphosphoglucose, DAP: days after pollination, DTT: dithiothreitol, EDTA: ethylene diamine tetra-acetic acid, EGTA: ethylene glycol tetra-acetic acid, NADP: nicotinamine adenine dinucleotide phosphate, PPi: inorganic pyrophosphate.

0238–1249/95/$ 5.00

been suggested to be related to ADPG pyrophosphorylase activity rather than to starch degradation (Robinson et al., 1988). Though it is known that stress conditions can alter carbohydrate accumulation patterns in tomato fruit (Ehret and Ho, 1986; Mitchell et al., 1991) less information is available on the effects of stress on this enzyme. In wheat grains, ADPG pyrophosphorylase activity was shown to be sensitive to water stress but not to high temperatures (Caley et al., 1990).

The purpose of the present work was to compare the effect of salinity on starch and soluble carbohydrate accumulation, in the early stages of fruit development in two tomato cultivars which differ in salt tolerance.

Materials and Methods

Tomato, *Lycopersicon esculentum* Mill., cvs Edkawi and Ace were used. Edkawi which is considered salt tolerant, is grown in salt-affected soils at the north coast of Egypt (Mahmoud et al., 1986). Both are determinate plants, and fruit is of similar shape, thus providing for adequate comparisons.

Seedlings were grown on vermiculite and were transferred at 4 leaf-stage to sand culture in a naturally illuminated greenhouse. Three times a day they were irrigated automatically with Hoagland solution (Hoagland and Arnon, 1953). Half of the plants were salinized by adding NaCl to the irrigation solution in 50 mM weekly increments, until a final concentration of 200 mM NaCl was reached.

Flowers were hand-pollinated and fruits were harvested 5, 10, 15 and 20 days after pollination (DAP). Fruits were weighed and stored at –70 °C. Extracts were prepared essentially as described by Guan and Janes (1991). Approximately one gram of fresh tissue of whole fruit was ground into powder with the addition of liquid air and then homogeneized in an OmniMixer 17106 with 1 volume of extraction buffer. It contained 50 mM Hepes KOH (pH 8.3), 2 mM EDTA, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM DTT as described by Robinson et al. (1988). The homogenate was centrifuged at 17 000 rpm for 20 min at 4 °C and the resulting supernatant and pellet were stored at –70 °C until enzyme activity was assayed. This double freezing process did not affect enzyme activity.

Protein concentration was determined according to Bradford (1976) using bovine albumin as a standard. ADPG pyrophosphorylase activity was determined in desalted extracts as described by Guan and Janes (1991). A blank without PPi was prepared for each extract. Total soluble carbohydrates were determined in non-desalted supernatant with the Anthrone reagent (Fales, 1951) using sucrose as a standard. Starch was determined in the pellet; after hydrolysis with amyloglucosidase, glucose was determined by a two-step enzyme assay involving the formation of reduced NADP, which was read at 340 nm.

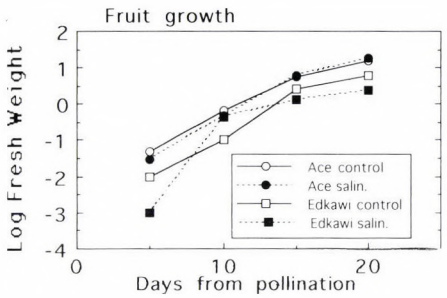

Fig. 1. Initial growth of fruit of tomato cvs. Ace and Edkawi grown under non-salinized or salinized conditions (200 mM NaCl, closed symbols)

Results and Discussion

Fruits of tomato cvs. Ace and Edkawi grew at a similar rate between 5 and 20 DAP (Fig. 1). Growth rates were only transiently affected by salinity.

Total soluble sugars increased initially as a function of fruit age (Fig. 2, TOTAL SOLUBLE SUGARS) and a significant increase in sugar accumulation produced by salinity was observed on day 20 after pollination. Starch increased in both cvs. as a function of fruit age, except in cv. Edkawi at 20 DAP (Fig. 2, STARCH). Starch content in fruits from salinized plants was higher than in non-salinized controls. These results agree with those of Mitchell et al. (1991) for immature fruits that were analyzed starting 3 weeks after anthesis and underscore the fact that carbon metabolism in immature fruits is sensitive to saline conditions from the very early stages of development.

Protein concentration was higher in Edkawi than in Ace and it tended to decrease with fruit age (Fig. 2, PROTEIN). No significant effects of salinity were observed. ADPG pyrophosphorylase specific activity was similar in both cultivars under control conditions and did not change significantly in the course of this study (Fig. 2: ADPG PYROPHOSPHORYLASE). Under salinity, both cultivars exhibited different behavior: ADPG pyrophosphorylase specific activity increased in Ace but it remained constant in Edkawi.

The significance of the increased starch accumulation in tomato fruit observed under stress conditions has been discussed by other authors (Guan and Janes, 1991, Mitchell, 1991). Increased starch accumulation may ensure the transformation of imported sucrose and thus maintain a continued sucrose gradient for further carbohydrate import. In that case, a correlation with ADPG pyrophosphorylase activity was to be expected, as the biochemical regulation of starch synthesis is centered predominantly on this enzyme, such expectation, however, was not met. The changes in ADPG pyrophosphorylase activity did not correlate with starch content, suggesting that increased starch accumulation under salinity is not associated with ADPG pyrophosphorylase specific activity. Alternatively, the *in vitro* activity of the enzyme might not reflect the *in vivo*

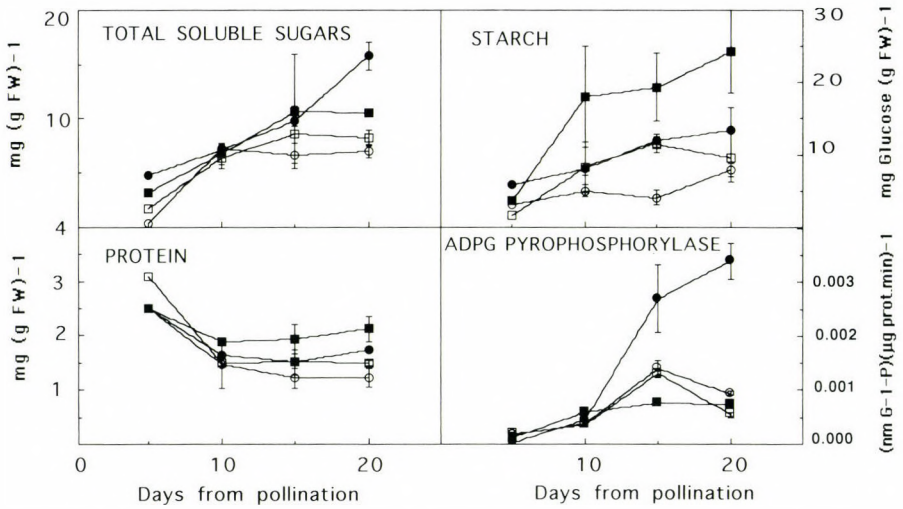

Fig. 2. Concentration of total soluble sugars, strach and protein, and ADPG pyrophosphorylase activity in fruits of tomato cvs. Ace (0) and Edkawi (0) grown under non-salinized (open symbols) or salinized conditions (200 mM NaCl, closed symbols) and harvested at various times after pollination. Results are means ±SE of 4 samples

activity. ADPG pyrophosphorylase is activated by physiological concentrations of 3-phosphoglycerate (see Beck and Ziegler, 1989, and references therein), which was not measured in this study; stress conditions might alter the availability of this regulator.

No association could be found between the salt tolerance of these two cultivars and initial fruit growth and carbohydrate accumulation. This suggests that the reported salt tolerance of Edkawi (Mahmoud et al., 1986) is apparently not related to a differential response to salinity in the early stages of fruit develoument.

Acknowledgements

Support from the International Foundation for Science and Consejo de Investigaciones de la Provincia de Córdoba is gratefully acknowledged.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 27–37 (1995)

Adaptive Responses of Plants under Stress Conditions

L. ERDEI, ANIKÓ PESTENÁCZ, KATALIN BARABÁS and ZS. SZEGLETES

Institute of Biophysics, Biological Resarch Center, Hungarian Academy of Sciences
P.O. Box 521, H-6701 Szeged, Hungary

The effects of NaCl salinity and osmotic stress (administered as polyethylene glycol treatment) were investigated on the protein kinase activity and on the accumulation of cations and polyamines in maize and sorghum grown hydroponically. The influence of excess UV-B irradiation as oxidative stress, on the glutathione antioxidant system was followed in young wheat plants. Concerning the response to osmotic stress of the protein kinase activity in sorghum and maize, results suggest that substantial differences may exist between the two species in their signal reception/transduction mechanisms leading to divergent drought stress tolerance. Potassium preference under NaCl stress as well as polyamine accumulation under both salt and osmotic stresses were regarded as adaptive responses in sorghum which traits were less expressed in maize. Changes in glutathione levels and the glutathione reductase activity correlated with the adaptation of wheat plants to excess UV-B irradiation.

The consequence of the stratospheric ozone depletion (Stolarsky et al., 1992) is an increased UV-B radiation reaching the biosphere (Webb, 1991). Together with substances of anthropogenic origin, their effects result in increasing oxidizing capacity of the atmosphere (Thompson, 1992) that could give a rise of so far unknown chemical and biochemical perturbations in plants, animals and man (Smith et al., 1992; van der Leun and Tevini, 1992). Especially terrestrial plants are subjected to additional abiotic environmental stresses like high or low salinity, drought or metal pollutants. The mechanisms of defense and stress tolerance, and the signal – response relationship under complex stress situation, however, are not well understood and require urgent and detailed studies.

In the present research, factors and mechanisms involved in salinity and drought stress (simulated with polyethylene glycol as osmoticum) were studied in maize, sorghum and wheat which species differ in stress tolerance. Salinity and osmotic stresses were combined with oxygen toxicity as induced by excess of UV-B irradiation. Our particular working hypothesis was that the immediate stress for the organism is an oxidative stress and antioxidants indeed play an important role in tolerance. Therefore, antioxidant defense mechanisms were also investigated.

0238–1249/95/$ 5.00

Materials and Methods

Plant material and treatments

Maize (*Zea mays* L. cv. Pioneer 3950) and sorghum (*Sorghum bicolor* (L.) Moench cv. ICSV 112), a drought tolerant line from Hyderabad, India (Masojidek et al., 1991) were hydroponically cultivated in complete nutrient solution with 0.3 mM KCl (Erdei et al., 1984) in phytotron (Conviron PGW 36, Canada) under the light conditions detailed below. For non-ionic osmotic and ionic stress treatments, PEG 6000 and NaCl were added, respectively, to the nutrient solution at equivalent osmotic concentrations of 50, 100, 200,300 and 400 mOsm (i.e. 0.12, 0.24, 0.49, 0.73 and 0.97 MPa) at the 8th day of growth in the phytotron and the treatments lasted for 3 days. Measurements for water potential and sampling for polyamine determination took place after 72 h treatments.

Wheat (*Triticum aestivum* L.) cv. Tiszatáj (drought tolerant) were cultivated similarly, with exception that 3-d old germs were transferred into hydroponics. Osmotic stress was applied by using 200 mOsm concentration of polyethylene glycol 6000 (PEG 6000). Osmolarity of the PEG solution was confirmed with an osmometer. The PEG level was chosen based upon previous works (Trolinder and Shang, 1991; Szabó-Nagy et al., 1992; Erdei and Taleisnik, 1993). Salinity stress was imposed using NaCl at 100 mM, i.e. at the same osmolarity as PEG 6000.

For studying the effects of UV-B irradiation, wheat seedlings were cultivated hydroponically in two phytotrons (Conviron PGW 36) with 23/18 °C and 13/11 hours day/night period for 11 days. One phytotron served as control while another was supplemented with UV-B emitting tubes. In both phytotrons, irradiance was 60 Wm^{-2} at shoot level (Sylvania type power tubes F48/T12/CW/VHO and Gro-Lux WS F48/T12/GRO/VHO/WS). In one of the phytotrons, the set of tubes was supplemented with Philips sun lamps (TL 100W/01) providing 2.5 Wm^{-2} radiation of major spectral emittance between 310 and 315 nm (λ_{max} = 311/312 nm) (Santos et al., 1993). For studying early responses to UV-B treatment, sampling was made daily between day 4 and 8 and at the 11th day of growth in both phytotrons.

Determination of cations

Na^+, K^+, Ca^{2+}, Mg^{2+} contents were measured by atomic absportion/emission spectrophotometry.

Determination of polyamines

Polyamines were analyzed by high performance liquid chromatography as described by Flores and Galston (1984) and Erdei et al. (1990). Authentic polyamine standards (Sigma Chemical Co.) were prepared similarly to plant samples. Polyamines were

eluted with a mixture of acetonitrile : water = 45 : 55 (by vol) through a Hi-Pore reversed phase column (250 mm × 4.6 mm) at 35 °C and monitored at 254 nm. The HPLC instrumentation was a BIO-RAD isocratic system with workstation.

Determination of glutathione

Samples of plant tissues (2 g FW) were placed in liquid nitrogen, pulverized to fine powder with a mortar and pestle, and quickly transferred to 8 ml of cold aqueous solution of 1 M (10%) perchloric acid (PCA) and 1 mM of bathophenanthroline disulfonic acid (BPDS). BPDS prevents thiol oxidation and thiol disulfide interchange and does not interfere with the quantification of oxidized form of glutathione (Reed et al., 1980). The extracts were centrifuged at 12 100 g for 20 min at 4 °C, and supernatants were collected (Siller-Cepeda et al., 1991).

For the determination of reduced and oxidized forms of glutathione (GSH and GSSG, respectively), PCA extracts were carboxy-methylated. After complete carboxymethylation, the samples were derivatized with 2,4-dinitro-1-fluorobenzene (Fariss and Reed, 1987).

Chromatographic conditions: dinitro-phenyl derivatives were separated and measured using a gradient HPLC (BioRad) system equipped with a BioRad Amino-SS column and UV detector (365 nm). Following a 100 µl injection of the centrifuged solution containing derivatized sample, the mobile phase was 75% solution A and 25% solution B for 10 min followed by a 30 min linear gradient to 5% A and 95% B. This mobile phase concentration was held for 10 min, then the mobile phase was returned to the initial conditions. Flow rate was 1.0 ml/min. Total run time for each sample including re-equilibration was 70 min. Solution A contained 80 vol.% methanol, solution B was composed of 0.55 M sodium acetate, 12.6 vol.% acetic acid and 64 vol.% methanol. Retention times, peak symmetry and peak areas in same concentrations of compounds were highly reproducible.

Enzyme assays

Protein kinase activity was determined using the cytoplasmic protein fraction obtained by differential centrifugation (Hodges and Leonard, 1974). The total protein content was determined by the method of Lowry et al. (1951). In vitro protein autophosphorylation was carried out at room temperature with a reaction mixture containing 10 mM $MgCl_2$, 2 mM EGTA or 0.1 mM $CaCl_2$ in 25 mM Tris-MES (pH 7.5) buffer solution and 20 g protein extract. Reaction was initiated by [γ-^{32}P] ATP (spec. act.: 110 TBq/mmol) in each sample and stopped after 3 min by adding SDS sample buffer. Samples were boiled for 1 min before using for SDS-PAGE. SDS-PAGE was performed according to the standard method (Laemmli, 1970), using 3 % stacking gel and 11 % separating gel to determine the molecular weight of the phosphorylated proteins. The labelled proteins were detected by autoradiography at –70 °C for 3 days.

Glutathione reductase activity was measured as absorbance increment at 412 nm when Ellman's reagent (DTNB, *5,5'-dithiobis(2-nitrobenzoic acid)* is reduced by GSH, which is generated from the GSSG. The standard reaction mixture contained 1.0 ml 0.2 M potassium phosphate (pH = 7.5) containing 1 mM EDTA, 0.5 ml 3 mM DTNB in 0.01 M phosphate buffer, 0.25 ml H_2O, 0.1 ml 2 mM NADPH, 0.05 ml yeast glutathione reductase (1 U/ml) for standard or 0.5 ml crude extract and 0.1 ml 20 mM GSSG. The developed color was measured at 412 nm. The rate was calculated from the initial slope of the curve and expressed as a rate/5 min.

Results and Discussion

Signal transduction

Stress tolerance can be regarded as the sum of traits of adaptive value expressed at different levels of organization. Biochemical, physiological and structural features should all be considered, therefore, as determining factors.

Reduced water potential is a common consequence of both drought and salinity. Under either of these conditions plants have to reduce their internal water potential to avoid desiccation. This is achieved by the accumulation of inorganic ions and/or the synthesis and accumulation of organic osmoprotectants. Under salt stress the former, while under drought the latter, may be more significant (Flowers and Yeo, 1986). However, depending on species and varieties, damages and responses are different to the same degree of stress.

Between the appearance of the signal (decreased water potential) and the physiological response, a cascade of events are involved. Definitely, the signal, i.e. the loss of turgor, is of physical or mechanical nature, whereas the the first response is chemical or possibly electrical. It can be stated, therefore, that the first line in the signal reception is the plasma membrane with its receptors and/or ion channels. These are the targets for plant hormones, especially for abscisic acid. As a consequence of the changes in membrane function/structure, Ca^{2+} ions, owing to their 10^{-4}–10^{-3}-fold concentration gradient betwen the external region and the inner side, enter the cytosol. The elevated Ca^{2+} concentration triggers the calcium-calmodulin dependent cascade reactions as a second line in the signal tranduction mechanism, resulting in the activation of calcium and calmodulin dependent enzymes like the Ca^{2+}-ATPase and protein kinases (Poovaiah, 1993). Phosphorylated proteins can play their role as messengers afterwards, carrying the signal towards the nucleus.

In our present experiments we raised the question whether protein kinases in sorghum and maize are responsive or not to osmotic signal. Concerning the calcium-dependent 49 kDa protein kinase, we found that its activity was stimulated after only 1 h osmotic treatment in sorghum but not in maize (Fig. 1). This stimulation of the protein kinase activity was prevented by 3-d-long pretreatment with abscisic acid showing that the plants became tolerant for osmotic stress in advance of the real occurrence of the

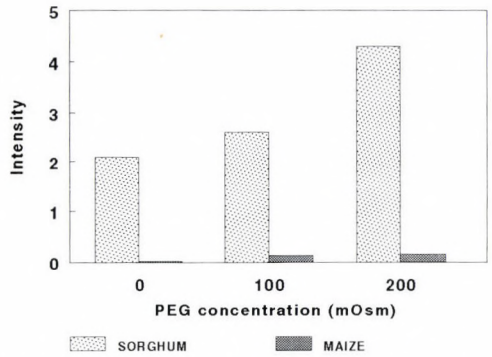

Fig. 1. Effects of polyethylene glycol 6000 treatment on the activity of the 49 kDa Ca^{2+} dependent protein kinase in the roots of maize and sorghum

stress (data not shown). This is in agreement with our earlier observations for ABA effects on water relation parameters (Erdei and Taleisnik, 1993). The present results suggest that substantial differences may exist between sorghum and maize in their signal reception/transduction mechanisms leading to divergent drought stress tolerance of these two species.

Cation accumulation

Plant analysis for internal cations showed that under salinity, Na^+ was more intensively accumulated in the roots of maize than in those of sorghum, however, shoot Na^+ levels were near the same in both species (Fig. 2). Internal K^+ concentration was always higher in sorghum, both in roots and shoots, than in those of maize (Fig. 3). Thus, under high salinity, the ratio of K^+ to Na^+ was 2–4-fold higher in the roots of sorghum as compared to that in maize. In shoots, although less spectacularly, similar trend was observed. It is interesting to mention that internal Mg^{2+} in the roots of sorghum was about twice of that found in maize (data not shown).

Accumulation of essential ions leading to lower water potential and retaining water, can be a common property in salt and osmotic tolerance. There is increasing evidence that the maintenance and accumulation of K^+ under saline conditions is one of the traits linked to stress tolerance in some plant species. This feature was observed in a salt tolerant cell line of citrus (Ben-Hayyim, 1987), in salt tolerant genotypes of wheat (Sharma, 1987; Erdei and Trivedi, 1989; Trivedi et al., 1991) and in Indian mustard where K^+ was accompanied by a high level of Mg^{2+} (Kumar, 1984). In our present experiments, sorghum possessed the capability for maintaining higher K^+ levels than maize under both osmotic and salt stresses. In maize, the role of potassium in osmotic adjustment may vary

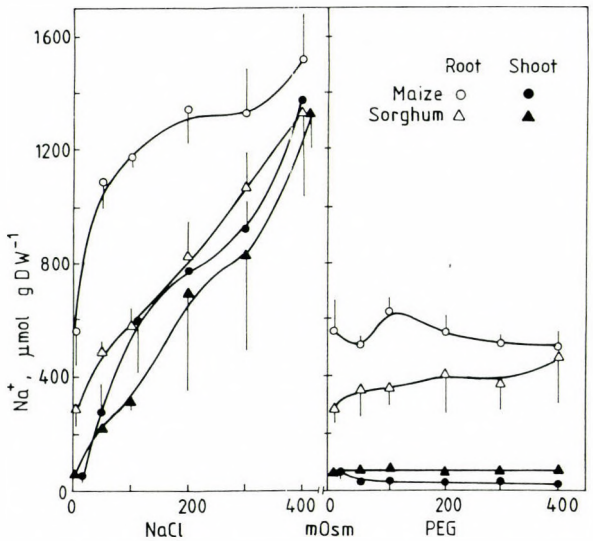

Fig. 2. Effects of NaCl salinity and polyethylene glycol 6000 treatment on the internal Na^+ concentration in roots and shoots of maize and sorghum. Mean±SE (n=5)

with plant part and/or with the cultivars, since Sharp et al. (1990) found no K^+ accumulation, except the apical 2 mm zone in the root, while significant accumulation of K^+ was shown in the leaves of five other cultivars (Premachandra et al., 1992) at low water potential.

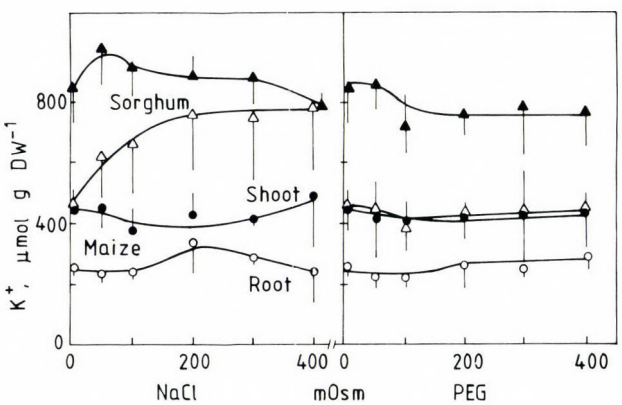

Fig. 3. Effects of NaCl salinity and polyethylene glycol treatment on the internal K^+ concentration in roots and shoots of maize and sorghum. Mean±SE (n=5)

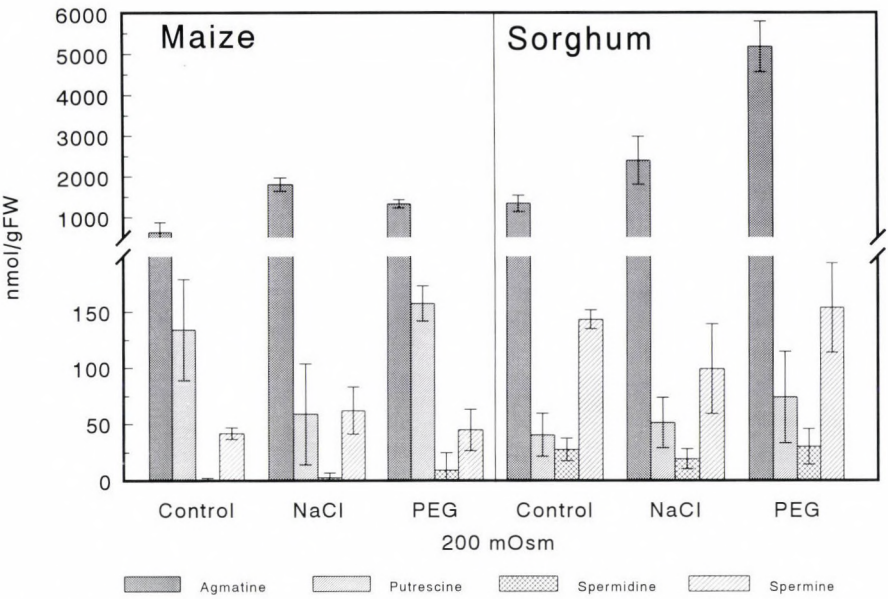

Fig. 4. Changes in polyamine titers in the leaves of maize and sorghum treated for 3 d with 200 mOsm NaCl or polyethylene glycol 6000. Mean±SE (n=5)

Polyamine accumulation

Although under salt stress the accumulation of inorganic ions is of primary importance, under non-ionic osmotic (drought) stress the synthesis and accumulation of organic substances may be more significant. Polyamines are thought to play a role in maintaining ionic homeostasis and membrane integrity, as well as in protecting macromolecular structure under most of the stress conditions (Slocum et al., 1984; Smith, 1984). In general, but most prominently in grasses, the accumulation of polyamines is characteristic under osmotic shock (Flores and Galston, 1982; Erdei et al., 1990), but to our best knowledge, so far no comparative work has been done for sorghum and maize in this respect. From our data (Fig. 4) it is seen that in maize, both NaCl and osmotic stresses were effective in inducing polyamine accumulation, while in sorghum, osmotic stress was the more effective as compared to salt treatment. This is indicated by the high titers of agmatine. In maize, the absence of spermidine may refer to its fast turnover. In sorghum, the accumulation of di- and polyamines was proportional to the strength of the osmotic stress applied and the impairment of polyamine synthesis was also observable at high degrees of stress.

Antioxidant systems

Oxidative stress arises from deleterious but inevitable reactions of oxygen (Cadenas, 1989). Such reactions lead to the formation of reduced oxygen species such as hydrogen peroxide and superoxide radicals. These species can lead to the formation of damaging free radicals which process can be catalyzed by metal ions. The reactive radicals cause lipid peroxidation and denaturing of proteins.

A variety of antioxidant systems exists in plants which work by eliminating precursors of hydroxyl radicals (i.e. hydrogen peroxide, superoxide radical). One general antioxidant category which may be involved in abiotic stress tolerance includes the enzymatic/peptidic scavenging systems. These are catalase and peroxidase converting hydrogen peroxide into water and oxygen, superoxide dismutase that catalyzes the conversion of the superoxide radical to hydrogen peroxide and oxygen (Larson, 1988; Monk et al., 1989; Vianello et al., 1990; Vianello and Macri, 1991) and, the antioxidative enzymes ascorbate peroxidase, monodehydroascorbate reductase and glutathione reductase (Pastori and Trippi, 1992). In the Halliwell-Asada activated oxygen detoxification pathway (Asada, 1992) hydrogen peroxide is scavenged by the ascorbate-dependent actions of ascorbate peroxidase, NADPH-dependent monodehydroascorbate reductase and glutathione dehydroascorbate reductase.

The activity of this hydrogen peroxide-scavenging system has been reported to increase during drought (Smirnoff and Colombé, 1988). During desiccation, a drought-tolerant moss exhibited increased activities of both superoxide dismutase and catalase with a concomitant reduction in lipid peroxidation compared to the drought-intolerant species (Dhindsa and Matowe, 1981). Similarly, it has been reported that drought tolerance in maize was correlated with high activities of both superoxide dismutase and glutathione reductase (Malan, 1990). High activity of either enzyme alone, however, did not confer drought tolerance.

Figure 5 describes the effects of UV-B irradiation on reduced (GSH) and oxidized (GSSG) glutathione levels as well as on glutathione reductase (GR) activity in the leaves of young wheat plants. It is seen that GSSG level increased and reached its maximal values at the 5th and 6th days of UV-B irradiation while after the 7th day it decreased to a low level. During the same period, the levels of GSH showed an opposite pattern, reaching the maximum values on the 7th day. The GR activity increased during ageing, and significantly, its activity on day 6 was higher in the UV-B irradiated leaves than in the control. The increase in activity and/or induction of GR both renew GSH for further scavenging of free radicals, and prevent GSSG from accumulating in toxic amounts (Navari-Izzo and Izzo, 1993). These results concerning the glutathione defence system are in good agreement with growth patterns of irradiated and control plants when the initially retarded plants reached their controls by the 11th day of the experiment (data not shown). It can be concluded that young intact wheat plants possess the capability of the restoration/activation their defence system against moderate UV-B irradiation. The defence mechanism, however, may include multiple steps whose detection may be difficult

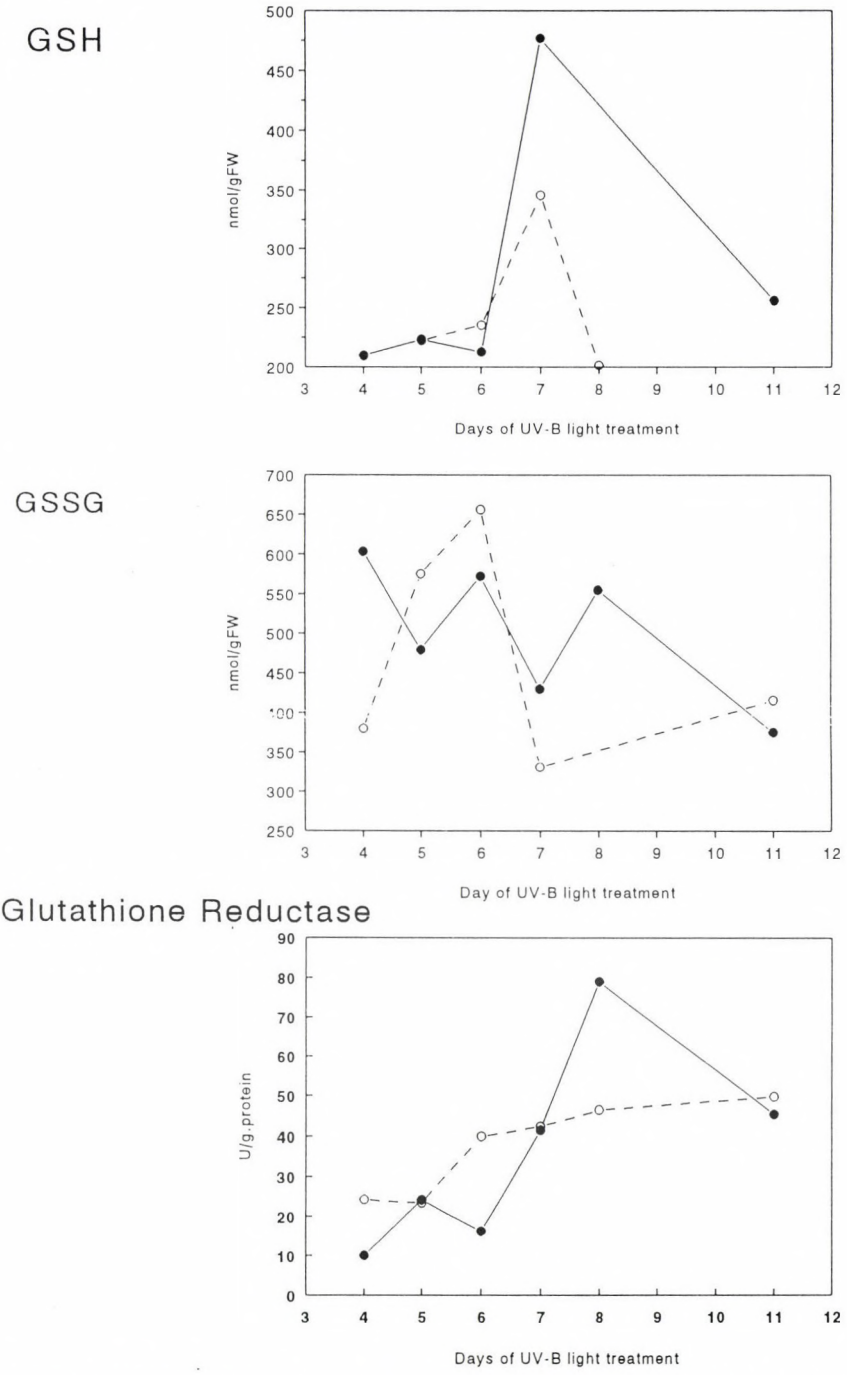

Fig. 5. Effects of UV-B irradiation on the levels of reduced (GSH) and oxidized (GSSG) glutathione and on the glutathione reductase activity in the leaves of young wheat (cv. Tiszatáj) plants
Symbols: •, control; ○, treated.

in isolated, *in vitro* systems. These results suggest that the increased/or induced antioxidant defence activity is an adaptive response in plants under excess of UV-B irradiation.

Acknowledgements

This work was supported by the National Committee for Technological Development (OMFB)-PHARE ACCORD project contract No. H 9112-0163. Lecture presented on the *Biostress '94 - Bioexpo '96 Workshop on Plant Responses to Environmental Stress, July 11–12, 1994, Szeged, Hungary.*

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 39–45 (1995)

Role of Antioxidant Systems and Juvenility in Tolerance of Plants to Diseases and Abiotic Stresses

B. BARNA, A. L. ÁDÁM, G. GULLNER and Z. KIRÁLY

Plant Protection Institute, Hungarian Academy of Sciences,
H-1525 Budapest, P.O. Box 102.

Juvenility of plant tissues enhances resistance to toxins, cell wall-degrading enzymes, autolysis of membrane lipids and thereby to necrotrophic pathogens. Moreover, inhibition of senescence by high doses of NO_3-nitrogen or decapitation increased superoxide dismutase (SOD) enzyme activity and decreased the rate of lipid peroxidation in tobacco plants. The oxidative stress caused by the herbicide acifluorfen treatment induced a higher increase in level of non-protein thiols and in activity of the antioxidant enzymes glutathione-reductase, GSH S-transferase and ascorbate-peroxidase in leaves of superoxide (paraquat) tolerant (PT) than of sensitive (PS) tobaccos. PT plants showed not only enhanced juvenility, but also increased tolerance to many biotic and abiotic stresses. On the other hand, decapitation, or treatment of tobacco plants by kinetin or benzyladenine increased the tolerance to the oxidative stress caused by paraquat.

The importance of juvenility of plant tissues in resistance to biotic and abiotic stresses was emphasized by the elevated tolerance to paraquat, fusaric acid and to *B. cinerea* of transformed tomato plants with enhanced cytokinin and auxin production.

Juvenility and senescence

Induction of juvenility (delay of senescence) by external application of cytokinins (Király and Szirmai, 1964; Lloyd, 1972; Novacky, 1972; Balázs et al., 1976), by high doses of nitrate nitrogen (Huber and Watson, 1974; Gilly et al., 1979; Sarhan et al., 1982) or by removal of the terminal bud (Király et al., 1968; Ádám et al., 1990) enhances resistance of plants to necrosis caused by viruses, bacteria or fungi. Natural tissue senescence itself increases sensitivity of leaves to necrotization by virus (Király et al., 1968) or fungal infection (Stavely and Slana, 1971; Barna and Györgyi, 1992). As regards the mechanism, juvenile plant tissues are more tolerant to toxins, cell wall-degrading enzymes produced by pathogens and to autolysis of membrane lipids during tissue damage caused by pathogens (Barna et al., 1983, 1985; Barna and Györgyi, 1992). It is generally accepted that membranes play a crucial role in the development of necrosis. A correlation has been found between resistance to necrotization caused by pathogens and the lower free sterol/phospholipid (St/Pl) ratio and microviscosity of membranes in juvenile leaves, induced by high nitrogen doses or decapitation (Barna et al., 1985; Ádám et al., 1990a).

Oxidative stress and antioxidants

Ageing also correlates with enhanced oxy free radical formation, higher rate of lipid peroxidation and lipoxygenase activity, lower enzymatic and nonenzymatic antioxidant capacity of plant tissues (Leshem, 1988). Consequently, by inhibiting senescence,

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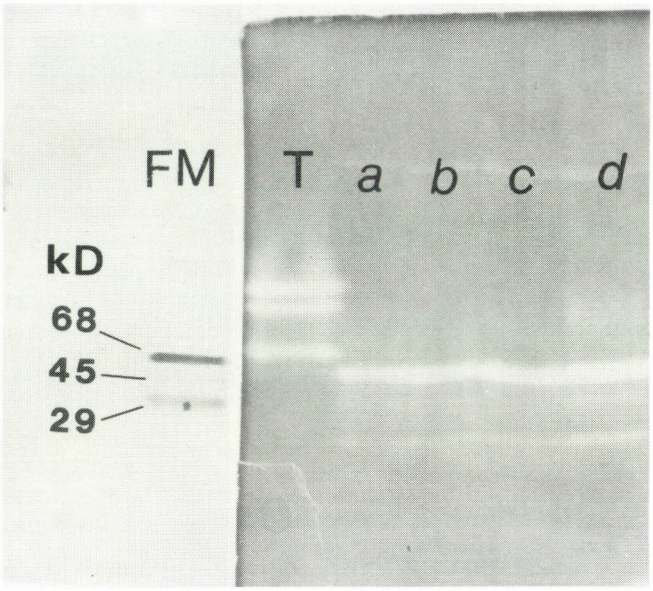

Fig. 1. SOD activities in leaf extracts from Xanthi nc tobaccos supplied with various doses of nitrogen after gradient PAGE. Treatments, electrophoresis and SOD staining were carried out as described earlier (Sarhan et al., 1982; Barna et al., 1993). FM = molecular mass standards, T = horse-radish SOD (5 µg), a = 70, b = 280, c = 630, d = 1050 ppm nitrogen

the antioxidant capacity of plants can be increased. This hypothesis was supported by the augmented superoxide dismutase (SOD) enzyme activity of tobacco plants supplied with high doses of NO_3-nitrogen (Fig. 1). Similarly, decapitated tobacco plants showed lower rate of lipid peroxidation (MDA content) and higher SOD activity as compared to the

Table 1

The effect of decapitation on the malondialdehyde (MDA) content and SOD activity* in leaves of Xanthi nc tobacco

	MDA	SOD Cyanide-sensitive	SOD Cyanide-resistant	SOD Total
	n mol g^{-1}	(EU g^{-1} protein)		
Control	4.50	13.7 ± 2.1	7.6 ± 1.6	21.3 ± 1.4
Decapitated	1.92	16.8 ± 2.2	20.5 ± 2.6	37.3 ± 1.8

* The methods are described by Ádám et al. (1990b).

control plants. Especially the cyanide-resistant activity (MnSOD + FeSOD) increased after decapitation (Table 1).

It was suggested that oxy free radicals may play a significant role in inducing plant cell and tissue death during the hypersensitive necrotic reaction caused by plant pathogens (Doke, 1983; Ádám et al., 1989; Sutherland, 1991; Abdou et al., 1993).

Therefore, plants with high antioxidant capacity should tolerate the necrotization induced by pathogenic attack. Indeed, in vitro selected and regenerated paraquat (superoxide-producing herbicide) tolerant (PT) tobacco (Furusawa et al., 1984) shows tolerance to necrotic symptoms induced by bacteria (Ádám et al., 1989) viruses or fungi (Barna et al., 1993). In addition, PT plants were more tolerant to treatments with sulfur dioxide (Tanaka et al., 1988), acifluorfen, which is a singlet oxygen and superoxide producing herbicide (Gullner et al., 1991), mercuric chloride, fusaric acid toxin, freezing or heat (Barna et al., 1993) as compared to the normal sensitive (PS) tobacco.

Abiotic stresses, like herbicides (paraquat, acifluorfen), air pollutants (ozone and sulfur dioxide), heavy metals, drought, extreme temperature and light conditions also increase the formation of the reduced and active derivatives of molecular oxygen in higher plants (Elstner, 1982). It has became more and more evident that these oxidative processes play an important role in the damaging effects of environmental stresses (Aioub et al., 1993; Schmidt and Kunert, 1986). Various antioxidative systems in plants are supposed to be activated and involved in mediating the response of plant cells to oxidative stress caused by different environmental effects (Kenyon and Duke, 1985; Shaaltiel et al., 1988).

Tolerance to oxidative stress and necrosis

Our results support this hypothesis because PT tobacco leaf extracts have higher SOD (especially MnSOD) activity (Barna et al., 1993), and activity of some antioxidant and detoxifying enzymes as well as the level of nonprotein thiols were particularly elevated in PT plants as compared to PS plants (Table 2). The above parameters were much less affected by paraquat. The only significant changes observed at low concentrations of this herbicide were the increased thiol content and glutathione S-transferase activity in tolerant tobacco leaves.

However, even if we consider that the augmented enzymatic and nonenzymatic antioxidant activities may participate in tolerance to oxidative stresses caused by herbicidal and environmental oxidants or tissue necrosis induced by pathogens, it remains to be seen what is the cause of a general tolerance of PT tobacco plants to several stresses including freezing and heat treatments? Another aspect of the problem has been investigated when it was realized that PT plants exhibit characteristic symptoms of juvenility. Tolerant plants have much higher chlorophyll, protein, galacto- and phospholipid and lower free sterol and thiobarbituric acid reactive substance (TBA-RS) contents than the sensitive (PS) plants (Barna et al., 1993).

The low free sterol/phospholipid ratio is not only a typical feature of juvenile plant membranes, but it is an important factor of cold resistance (Vígh et al., 1979). PT

Table 2

Effect of the herbicide acifluorfen (5×10^{-5} M) on ascorbic acid and thiol levels and on the activities of three antioxidant enzymes in leaves of tolerant and sensitive tobacco plants after 0, 2 and 4 days (0 d, 2 d, 4 d) of exposure. Treatments and measurements of antioxidant levels and activities were carried out as described earlier (Gullner et al., 1991)

	Antioxidant levels and activities					
	PS			PT		
	0 d	2 d	4 d	0 d	2 d	4 d
Ascorbic acid (µmol/g FW)	1.92	3.09	1.82	2.43	3.11	2.24
Non-proteins thiols (µmol/g FW)	0.31	0.72	0.44	0.30	1.08	0.66
Glutathione-reductase (µmol NADPH/g FW/min)	0.32	0.74	1.06	0.30	1.10	2.28
Glutathione S-transferase (µmol conj./g FW/min)	0.16	2.27	2.21	0.15	4.20	3.25
Ascorbate-peroxidase (µmol asc./g FW/min)	22.30	22.10	21.40	24.70	34.80	117.00

plants flower 2–3 weeks later and grow significantly slower. The augmented SOD activity in leaves of PT tobacco seems to be in correlation with higher degree of juvenility, since young leaves have higher SOD activity which gradually decreases during senescence. All these data lend support to the hypothesis that juvenile plant tissues have a general tolerance to necrobiosis as compared to the senescent ones.

On the other hand, development of necrosis is associated with (or induced by) the formation of oxy free radicals. Therefore, inhibition of necrobiosis by inducing juvenility should reduce the damaging effect of oxy free radicals. Indeed, decapitation or treatments of tobacco plants with kinetin or benzyladenine increased the tolerance of leaves to superoxide (Fig. 2).

The early data of Engelbrecht and Mothes (1964) also reported on the protective effect of cytokinins against heat shock. These results support the view that inhibition of senescence of plant tissues increases their tolerance to oxidative damage caused not only by biotic but by abiotic stresses as well.

Increasing plant juvenility and stress tolerance by genetic engineering

The above conclusion may indicate that by genetic engineering one can create juvenile plants and thereby breeding crops for agriculture with tolerance to a wide spectrum of stresses and diseases. In order to check this hypothesis tomato plants regenerated from *Agrobacterium tumefaciens* T37 (p TiT37) tumors (Necasek et al., 1988) were tested for stress tolerance. The regenerated plants contained nopaline synthase activity, suggesting the incorporation of T-DNA with *ipt* gene responsible for enhanced cytokinin (and *iaaM* and *iaaH* genes for enhaced auxin) production. The transformed plants and their offsprings exhibited elevated tolerance to paraquat or fusaric acid toxin treatments and to infection by *Botrytis cinerea* (Table 3).

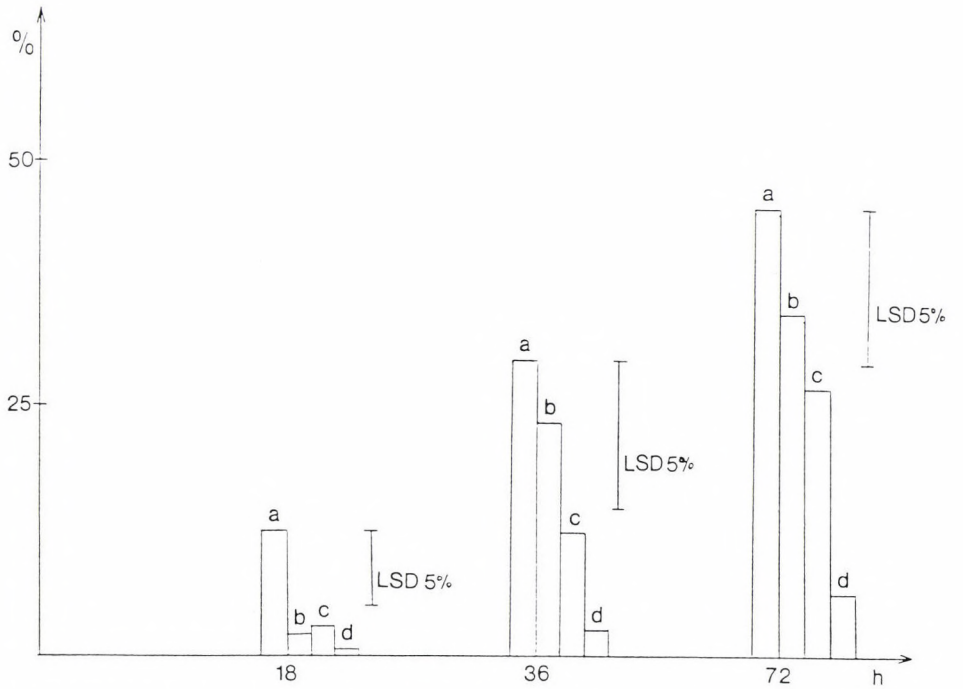

Fig. 2. Effect of paraquat (5×10^{-5} M) treatment on leaf disks of control (a), 20 ppm benzyladenine (b), 20 ppm kinetin (d) treated or decapitated (c) Xanthi nc tobacco. Data presented show the percentage of the damaged leaf area. Plants were treated every day for 2 weeks before paraquat test

We conclude that juvenility plays a significant role in tolerance of plants to necrotic diseases and to several abiotic stresses. Juvenility, as a complex term, generally means the lower content of oxy free radicals and ethylene, lower rates of lipid peroxida-

Table 3

Effect of paraquat (5×10^{-5} M) and fusaric acid (2.5×10^{-3} M) treatments or *Botrytis cinerea* infection on control (L XXIV) and transformed tomato leaves

Leaves of tomato strains	Paraquat Ion leakage (μS)	Fusaric acid Ion leakage (μS)	B. cinerea Diseased area (mm^2)
L XXIV (control)	75.2 ± 0.3	50.3 ± 1.5	30.8 ± 8.4
22 R_0	44.4 ± 0.0	39.9 ± 3.1	14.1 ± 4.4
35 R_0	57.3 ± 1.5	44.1 ± 0.4	21.2 ± 5.9
8 R_2	57.8 ± 0.8	41.2 ± 4.9	18.7 ± 2.9

tion and lipoxygenase activity, higher enzymatic and non-enzymatic antioxidant capacity, more fluid and stable membranes etc. All of these factors can reduce the damage caused by several stresses. Recently, tobacco plants transformed with *A. tumefaciens* GV 3101 (pMP90RK) containing *p35S* IPT are tested for tolerance by various stresses and necrosis inducing pathogens.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 47–49 (1995)

Ultraviolet-B Radiation Induced Damage to the Function and Structure of Photosystem II

I. VASS[1], CORNELIA SPETEA[1], ÉVA HIDEG[1], R. BARBATO[2] and V. PETROULEAS[3]

[1] Institute of Plant Biology, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary
[2] Department of Biology, University of Padova, Padova, Italy
[3] Institute of Materials Sciences, National Centre for Scientific Research "Demokritos", Aghia Paraskevi, Attikis, Greece

The effects of Ultraviolet-B radiation on the structure and function of the PSII complex has been investigated in isolated PSII membrane particles. By combined application of thermoluminescence, chlorophyll fluorescence and EPR we show that the primary action site of UV-B is the water-oxidizing complex. Experiments on the UV-B induced degradation of the D1 and D2 protein subunits of the PSII reaction centre show that contrary to earlier suggestions quinones are not needed to sensitize the protein damage.

Ultraviolet-B (280–320 nm) light has long been known to inhibit photosynthetic electron transport. Several studies have shown that UV-B primarily attacks photosystem II (PSII) in the photosynthetic apparatus (reviewed in Ref. 1). However, the precise molecular targets within PSII are not yet clarified. The impairment of the water-splitting function [2], damage to the acceptor side quinone components [3, 4] and destruction of the redox-active tyrosines [5] have equally been suggested. An important consequence of UV-B irradiation is the degradation of the D1 and D2 reaction centre proteins [4, 6–8]. Based on the action spectrum of protein degradation, plastosemiquinones have been proposed to act as UV-activated species responsible for the protein cleavage [6]. However, protein constituents such as tyrosines, whose absorption is very similar to those of semiquinones [9], cannot be excluded either as UV-sensitizers of D1 and D2 protein degradation.

Results and Discussion

Here we applied thermoluminescence (TL), chlorophyll fluorescence and EPR measurements on thylakoids and PSII membrane particles in combination with protein analysis to identify the molecular targets of UV-B radiation in PSII. TL measurements on thylakoid membranes have revealed that charge recombinations from the $S_2Q_B^-$, $S_2Q_A^-$ and Tyr-$D^+Q_A^-$ states show differential sensitivity to UV-B irradiation. The loss of the TL component from the $S_2Q_B^-$ recombination was faster than from the $S_2Q_A^-$ recombination, indicating a destabilization of Q_B^- binding in the UV-irradiated thylakoids. This effect was confirmed by direct measurements of electron transfer between Q_A and Q_B by flash-induced chlorophyll fluorescence. In contrast to the gradual loss of TL signals from the water-oxidizing complex ($S_2Q_B^-$ and S_2Q_A recombinations), the TL signal arising from the

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accessory donor Tyr-D ($Tyr\text{-}D^{+}Q_A^{-}$ recombination) was transiently enhanced at the beginning of the irradiation period. This observation is consistent with the inactivation of the water-oxidizing function in the early phase of UV-B irradiation, and a consequently stimulated recombination from $Tyr\text{-}D^{+}$.

The functioning of PSII redox components in UV-B irradiated PSII membranes was also followed by EPR spectroscopy. In agreement with the TL results, the loss of light-induced S_2-state formation, as indicated by the loss of the multiline EPR signal, was the earliest event during the course of the UV-B irradiation. This was followed by the distinctly slower loss of Signal II_{slow} from $Tyr\text{-}D^{+}$. The inhibition of Q_A functioning, monitored by the loss of the $Q_A^{-}Fe^{2+}$ EPR signal, was similar to that of $Tyr\text{-}D^{+}$. This effect is clearly related to the damage of Q_A itself, since the $Q_A^{-}Fe^{2+}$ EPR signal declined to the same extent after inducing Q_A reduction by illumination or by dithionite treatment. In addition, the redox functioning of the non-heme iron, as indicated by the ability of ferricyanide to convert it to the Fe^{3+} form, was lost to a smaller extent than the $Q_A^{-}Fe^{2+}$ EPR signal. A further consequence of UV-B irradiation was the gradual shift of the redox potential of cytochrome b-559 from the high potential to the low potential form, as indicated by the accumulation of the EPR signal characteristic for low potential oxidized cytochrome b-559. These results demonstrate that the primary action site of UV-B radiation in PSII is the water-oxidizing complex. The redox functioning of Tyr-D and of Q_B and Q_A are also modified or lost, but these events are subsequent to the inactivation of water oxidation.

UV-B radiation damages not only the redox processes but also the protein structure of PSII. In order to clarify the putative role of quinone electron acceptors as UV sensitizers in the protein degradation process [6] we followed the UV-B induced protein loss in PSII preparations of different integrity. In thylakoid membranes, which contain the whole PSII complex together with the quinone electron acceptors, we identified the primary site of UV-B induced D1 protein cleavage in the middle of the second transmembrane helix [8]. In isolated core complexes of PSII, which retain only the Q_A quinone electron acceptor on the D2 protein but not the Q_B acceptor on the D1 protein, the rate of D2 protein loss is slower than that of the D1. Furthermore, in isolated reaction centre complexes, in which neither Q_A nor Q_B is present, both D1 and D2 protein are degraded by UV-B to a similar extent. The presence of the quinone analog DBMIB has only a minor effect on the rate of the protein degradation. These results strongly suggest that quinones are not absolutely required to sensitize the UV-B induced degradation of the D1 and D2 reaction centre proteins. We also showed that the absence of oxygen or low temperatures (0 °C) does not block the protein loss which indicates that UV-B has a direct damaging effect on the D1 and D2 proteins.

Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 51–57 (1995)

EPR Spectroscopy Detection of Active Oxygen and Free Radicals in Thylakoids Exposed to Photoinhibition

ÉVA HIDEG, CORNELIA SPETEA and I. VASS

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, P. O. Box 521, Hungary

High intensity illumination of thylakoids results in the well-characterized impairment of Photosystem II electron transport (photoinhibition), followed by the degradation of the D1 reaction centre protein. The time course and features of photodamage are different in fully functional thylakoid membranes, when photoinhibition is invoked by impairment of Photosystem II acceptor side electron transport, and in thylakoids which are unable to oxidize water, when the damage is a consequence of inactivation of Photosystem II donor side.

In the present study we followed the production of singlet oxygen and free radicals during both types of photoinhibition by EPR spectroscopy. Singlet oxygen was detected by following the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl, a stable nitroxide radical yielded in the reaction of singlet oxygen with the sterically hindered amine 2,2,6,6-tetramethylpiperidine. Free radicals were detected as spin adducts of the spin trap 5,5-dimethyl-1-pyrrolin-*N*-oxide, and identified on the basis of hyperfine splitting constants of the EPR spectra.

We found that (i) singlet oxygen, a non-radical form of active oxygen was detectable only in samples undergoing acceptor side induced photodamage. (ii) The acceptor side induced process was accompanied by the oxygen dependent production of carbon centred (alkyl or hydroxyalkyl) radicals, probably from the reaction of singlet oxygen with histidine residues. (iii) Donor side induced photoinhibition was dominated by hydroxyl radicals, which were produced in anaerobic samples, too. The production rate of these radicals, as well as D1 protein degradation, was dependent on the possibility of electron donation from manganese ions to Photosystem II.

The marked distinction between the active oxygen forms produced in acceptor and donor side induced photoinhibition are in agreement with earlier reports on the different mechanism of these processes.

Photoinhibition, the damage induced by high intensity illumination results in the impairment of the electron transport in photosystem (PS*) II, followed by the degradation of the D1 reaction centre protein (for reviews see Refs.1–4) The time course and features of photodamage are different in fully functional thylakoid membranes, when photoinhibition is invoked by the impairment of PS II acceptor side electron transport, and in thylakoids with defective water oxidizing complex, when the damage is a consequence of inactivation of PS II donor side (Ref. 4 and references therein).

If photosynthetically active thylakoid membranes are illuminated with high intensity light, photoinhibition is promoted by reactions occurring at the inhibited acceptor

* Abbreviations: API, acceptor side induced photoinhibition; DMPO, 5,5-dimethyl-1-pyrrolin-*N*-oxide; DMPO-OH, Hydroxyl radical adduct of DMPO; DMPO-R, Carbon centred radical adduct of DMPO; DPI, donor side induced photoinhibition; EDTA, Ethylenediamine tetraacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; PS, Photosystem; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol

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side. In this process, the light induced over-reduction of the acceptor side results in an unusual, double reduction of the first quinone electron acceptor Q_A [5–7]. This state, which is not present during normal photosynthesis, is inactive in mediating the photosynthetic electron transfer but promotes the formation of triplet chlorophyll ($^3P_{680}$) in the reaction centre with a high yield [8–10]. Triplet chlorophyll is known to react with the triplet ground state of oxygen, producing the highly reactive singlet oxygen [11]. Singlet oxygen may directly attack important amino acids, such as histidines, of the D1 protein or may induce a conformational change of the reaction centre complex by damaging its chlorophyll or carotenoid components and thus expose the D1 protein to the proteolytic activity [12]. Also, singlet oxygen, which is a powerful active oxygen form but not a free radical, may induce the formation of oxygen free radicals [11].

An other pathway, different from the above described mechanism, is the photoinhibition invoked by the photodamage in PS II donor side. This occurs if the electron transport from the water splitting enzyme to P^+_{680} is significantly slower than the rate of electron withdrawal from the reaction centre. In this case the damage is attributed to the generation of abnormal long lived states of cation radicals P^+_{680} and Tyr^+_Z which could attack chlorophylls, carotenoids or amino acids (Refs. 2, 4 and references therein).

In the present study we followed active oxygen production during both types of photoinhibition by spin trapping EPR spectroscopy. This technique is based on the fact that the spin trap itself is diamagnetic (has no EPR signal), but upon reacting with a free radical its radical adduct is paramagnetic (well detectable with EPR). The shape of the EPR spectrum, determined by the hyperfine splitting constants, is characteristic to the trapped radical (for review see Ref. 13).

Materials and Methods

Thylakoid membranes were isolated from market spinach as described by Takahashi and Asada [14] and suspended in a Hepes buffer (40 mM, pH 7.5) containing 0.4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$.

Tris-washing, which removes Mn and the water soluble subunits of the water splitting enzyme was performed as described in Ref. 15. As a result of this treatment, the oxygen evolving ability of the samples was decreased by more than 85%.

Untreated or Tris-treated samples, diluted with the Hepes buffer to 100 µg chlorophyll/ml, were exposed to high-intensity (1000 $\mu E/m^2/s$) illumination from a Tungsten lamp while stirred in a temperature controlled glass cuvette at 22 °C.

Singlet oxygen was determined according to the method of Lion et al. [16], in samples containing 10 mM TEMP, by measuring the EPR absorption of the stable nitroxide radical, TEMPO, which is produced from the reaction between $^1O_2$ and TEMP, as described earlier [17]. Free radicals were detected as spin adducts of the spin trap DMPO, and identified on the basis of hyperfine splitting constants of the EPR spectra [13, 18].

EPR spectra were measured with a Brucker ECS-106 spectrometer. X-band spectra were recorded at room temperature with 9.45 GHz microwave frequency,16 mW microwave power and 100 kHz modulation frequency, as described earlier [19, 20].

Results and Discussion

Our data provide direct experimental evidence for the production of free radicals during both types of photoinhibition (Figs. 1 and 2).

In thylakoids, the acceptor side induced process was accompanied by the oxygen dependent production of carbon centred, probably alkyl or alkoxyl, radicals (Fig. 1). The free radical dominant after 50 minutes and DMPO yield a nitroxide radical which gives a six line EPR spectrum (marked with asterisks). The hyperfine splitting constants obtained from this spectrum (a_N=1.47 mT, a_H^β=2.26 mT) are characteristic to a carbon centred, probably alkyl or hydroxyalkyl, adduct of DMPO (DMPO-R) [21]. In thylakoids photoinhibited for more than 50 minutes, another type of nitroxide radical (marked with circles) appears besides DMPO-R. Its hyperfine splitting constants ($a_N = a_H^\beta$ = 1.47 mT) are the same as the ones reported for DMPO-OH [13, 21], demonstrating that a small amount of hydroxyl radicals are produced in the latter phase of photoinhibition. Free radical

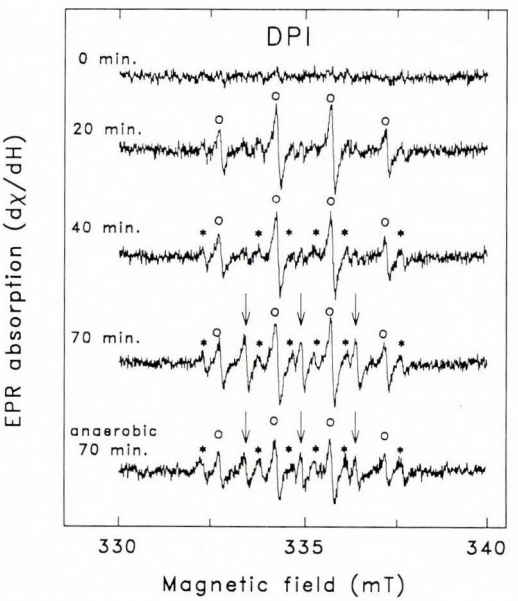

Fig. 1. EPR detection of free radicals trapped by DMPO in spinach thylakoids exposed to photoinhibition for the time indicated in the figures. Asterisks mark EPR spectra of the carbon centred radical adduct of DMPO, DMPO-R, circles indicate spectra of DMPO-OH. The lowest trace labelled as anaerobic represent samples exposed to photoinhibition while bubbled with argon

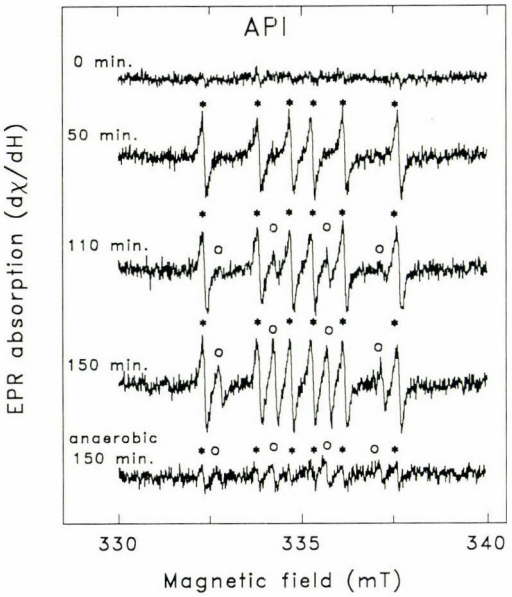

Fig. 2. EPR detection of free radicals trapped by DMPO in Tris-washed spinach thylakoids exposed to photoinhibition for the time indicated in the figures. Asterisks mark EPR spectra of the carbon centred radical adduct, DMPO-R, circles indicate spectra of DMPO-OH. The lowest trace labelled as anaerobic represent samples exposed to photoinhibition while bubbled with argon. Downward arrows mark the triplet EPR spectrum from damaged DMPO

production is markedly smaller in samples undergoing anaerobic photoinhibition (compare the last two traces in Fig. l). This indicates that the radicals trapped during the acceptor side induced process, both oxygen and carbon centred ones, are produced in a reaction requiring oxygen.

Free radicals are also produced during the donor side induced photoinhibition occurring in Tris-washed thylakoids (Fig. 2). However, contrary to the acceptor side induced reactions, the hydroxyl radicals dominate this process. More prolonged photoinhibition results in the appearance of other free radicals, too. The EPR spectra in Fig. 2 illustrate that besides DMPO-OH (circles), some DMPO-R (asterisks) is also present. The last spectrum in Fig. 2 shows that, contrary to the acceptor side induced process, these radicals are produced at a high yield even in the absence of oxygen. The three line EPR spectrum (marked with arrows in Fig. 2) is probably from DMPO molecules damaged by some product of donor side induced photoinhibition.

Singlet oxygen, a non-radical from of active oxygen was detectable only in samples undergoing acceptor side induced photodamage (Fig. 3).

The above differences between the active oxygen forms produced in the two types of photoinhibition are in agreement with earlier reports on the distinction of these processes.

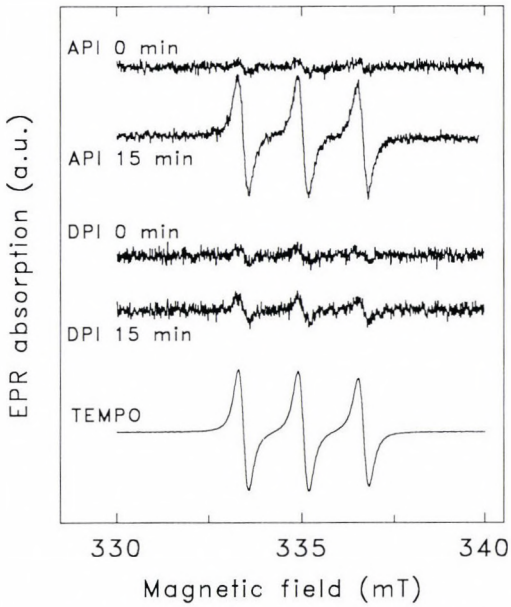

Fig. 3. EPR spectra of the nitroxide radical formed in the reaction of TEMP and singlet oxygen in thylakoids undergoing acceptor side induced photoinhibition (API) and in Tris-washed thylakoids undergoing donor side induced photoinhibition (DPI). The lowest trace represents the EPR spectrum of TEMPO, the synthesized singlet oxygen adduct of TEMP

Our experiments with the spin trap DMPO prove that both acceptor side and donor side induced photoinhibition are accompanied by free radical production (Fig. 1). This is in agreement with earlier suggestions that free radicals may be involved in the photodamage of the D1 reaction centre protein [22–24]. The detected radicals were among the ones found earlier in chloroplasts exposed to an other type of stress: methylviologen poisoning [25, 26].

Comparing the kinetics of D1 reaction centre protein damage and the yield of DMPO adducts demonstrates that, during the progress of both types of photoinhibition, the production rate of the dominant free radical increases prior to the loss of D1 (data not shown). This implies that the radicals are formed in PS II centres damaged by photoinhibition and may be involved in the sequence of events leading to D1 degradation. This is supported by the observation that radical production during acceptor side induced photoinhibition requires oxygen (Fig. 1), similarly to singlet oxygen formation and D1 degradation [3, 10], while both D1 damage [27] and free radical production (Fig. 2) proceeds in the absence of oxygen during the donor side induced process. Participation of free radicals in damaging the D1 protein would also be in line with earlier reports on the partial protection of D1 against photoinhibition by free radical scavengers [22–24].

In acceptor side photoinhibition singlet oxygen or its products were suggested to initiate the D1 protein degradation (reviewed in Ref. 4). Since high yields of radical production were observed earlier than the start of D1 protein degradation but later than the

onset of singlet oxygen production [17], it is possible that the radicals are produced in a radical cascade initiated by $^1O_2$ and participate in the attack on D1. This is supported by our observation that the attack of $^1O_2$ on histidine yields carbon centred radicals in a model experiment [20]. This way, the observation of this radical in thylakoids during acceptor side induced photoinhibition suggests the following model: Acceptor side impairment of PS II results in the formation of $^3P_{680}$ [7–10] and consequently $^1O_2$ [10, 17, 28, 29]. Singlet oxygen attacks crucial histidine residues of the reaction centre (e.g. those which bind chlorophylls). The breakdown of histidine yields carbon centred radicals and results in a (possibly conformational) modification of the reaction centre making D1 accessible to proteolytic degradation. The carbon centred radicals may participate in the further fragmentation of the D1 protein but the precise mechanism of this process has yet to be established.

In Tris-washed thylakoids photoinhibition is dominated by hydroxyl radicals. Because the most characteristic feature of donor side induced photodamage is the formation of the unusually long-lived, strong oxidizing radicals (P^+_{680} and $Tyr^{\cdot}_Z$), it has been suggested that they could initiate the damage *via* oxidizing nearby amino acid residues or electron transport components [2–4]. A plausible explanation of our data is that the observed hydroxyl radicals are produced in these oxidizing reactions. Supporting this assumption, our data demonstrate that hydroxyl radicals are produced and the time course of their production rate, as well as that of D1 degradation, was delayed by the increase and accelerated by the decrease in the life time of the oxidizing cation radicals [20].

Acknowledgement

This work was supported by research grants from the Hungarian Academy of Sciences (OTKA/III-888, OTKA/6241) and UNIDO-ICGEB(GE/GLO/89/001 No. 91/54).

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 59–70 (1995)

Wheat as an Ozone Sensitive Crop

G. SOJA[1] and ANNA-MARIA SOJA[2]

[1] Research Center Seibersdorf, Dept. of Agricultural Research, A-2444 Seibersdorf, Austria
[2] Agricultural University Vienna, Institute of Agronomy and Plant Breeding, A-1180 Wien, Austria

Several independent studies have confirmed evidence that under European conditions prevailing concentrations of tropospheric ozone may decrease grain yield of wheat significantly. Based on results from the European Open-Top Chamber Project, a critical level of 5.3 ppm.h (AOT40 = accumulated exposure over 40 ppb) for a 10% yield reduction has been recently established at a workshop in Switzerland. Although this value is currently the best approach to quantify ozone effects on vegetation, important modifiers of ozone effects (genotype, drought stress, other pollutant stresses) are not taken into consideration because of the lack of experimental data. If once these interactions are better known, revisions of the critical value might become necessary.

We present results on a closed-chamber fumigation experiment that had the aim to study sensitivity differences during plant development of winter wheat cv. Perlo. Ear growth was most affected, followed by stem growth and total leaf production. Maintaining a high proportion of green leaves proved to be critical for determining total productivity reduction. Comparing two ozone treatments (78 vs. 15 ppb for 8 h/d), early growth stages responded to ozone with a rapid loss of green leaf area. In later growth stages the decrease of green leaf area proceeded with similar speed in control and fumigated plants, indicating higher sensitivity in early growth stages. Apparently the shortage in photoassimilates affected the growth of reproductive parts most. Measurements of chlorophyll fluorescence showed decreased f_v/f_m-values under ozone, indicating impaired photochemical reactions also in green leaves without visible injury.

Although many gaseous air pollutants are known to have potentially phytotoxic effects, frequently they are only of local importance. After the successful reductions of SO_2-emissions, in Middle and South European countries there remains one pollutant, that is believed to be a more limiting factor for agricultural production than other gaseous air pollutants: tropospheric ozone (Näf und Fuhrer, 1992; Unsworth and Geissler, 1992).

In the USA research about ozone effects on plants has started already several years earlier than in Europe, mainly in the frame of the National Crop Loss Assessment Network (NCLAN). During this program a wide range of crop plants have been investigated for their ozone susceptibility, with wheat being in the top group of sensitive plants, and being the only crop that is of prominent economic importance in Central Europe (Sommerville et al., 1989). So this paper wants to present firstly an overview on current research and open questions concerning ozone impact on wheat, and secondly some new results on phenological differences in ozone sensitivity of wheat.

0238–1249/95/$ 5.00

Ozone effects on yield and yield parameters of wheat

Based on experiments with open-top chambers (OTC) performed in Europe in the years 1986–1991, several dose-response functions for the effects of ozone on wheat yield have been established:

$y = 99 + 0.117\ x - 0.007\ x^2$, using a wider range of data (Skärby et al., 1992) or

$y = 105 - 0.131\ x - 0.00605\ x^2$, using only Swedish and Swiss data (Skärby et al., 1992)

with y being the relative yield based on results attained in charcoal-filtered OTC and x being the 8-h mean value in ppb. The reference ($y = 100$ %) was set at a level of 20 ppb. Based on a 7-h mean value in ppm, Fuhrer et al. (1989b) used the function $y = \exp[-(x/0.089)^{2.46}]$.

Further analysis of data from the European open-top chamber network concentrated on the use of sum indices for characterizing ozone exposure. The best fit of data was found with the AOT40-value (accumulated exposure over threshold of 40 ppb). Relating yield reduction and AOT40, a yield loss of 10% was calculated to occur at 5.3 ppm.h (daylight hours only), derived from the function $y = 99.6 - 1.804\ x$, with x being the AOT40 in ppm.h. This exposure index is presently regarded to be the most realistic critical level for ozone injury on wheat (Fuhrer and Achermann, 1994).

Other agricultural crops have not been investigated so extensively under European conditions, perhaps except bush bean. Nevertheless, even in wheat several questions remain open for discussion and would require further research. An important issue is the possibilitiy of interactions of ozone with other stress factors that act as modifiers of ozone impact. The role of these modifiers may be different in diverse crop species and their importance to influence the critical level for ozone has yet to be established. Additionally to stress factors, genotypic differences may play an important role in determining crop sensitivity to ozone; the functions given above mainly were derived by averaging the results of experiments with different cultivars. Velissariou et al. (1992) compared wheat cultivars having been introduced between 1930 and 1980 and found more modern cultivars to be more ozone sensitive than older cultivars. When Adaros et al. (1991) compared two spring wheat cultivars, there appeared cultivar differences in ozone susceptibility but they were not consistent over the years. Analysing crop loss in wheat, corn and soybean with data from NCLAN in the Tennessee Valley, Brewer and Parkhurst (1988) stated that the differences in cultivar sensitivity to ozone could account for more variation in crop loss estimates than annual or regional differences in ozone exposure. In experiments of Mulchi et al. (1986) six winter wheat cultivars showed significant intervarietal differences in the effects to ozone, especially in grain characteristics. Studying three cultivars of winter wheat, Kress et al. (1985) found significant interactions between ozone treatment and cultivars in one year, but not in the other. Also Decoteau and Craker (1981), Heagle et al. (1979) and Shannon and Mulchi (1974) reported cultivar differences in wheat sensitivity. The relative sensitivity of cultivars as young plants was no useful indicator for prediction ozone effects on seed yield (Heagle et al., 1979). In a survey of the OTC experiments performed in the USA as well as in Europe, Miller (1992)

concludes that the apparent genetic differences in ozone sensitivity of wheat need further exploration. The uncertainty in actual yield loss before a cultivar has been individually tested may reach from 4 to 28% at moderate (SUM06 20 ppm.h) and from 30 to 62% (25th and 75th percentile, resp.) at more severe (SUM06 40 ppm.h) ozone stress (Hogsett et al., 1992).

Drought is another important factor influencing ozone effects. As drought stress is accompanied by decreased stomatal conductance, less ozone reaches the inner leaf tissues, thereby decreasing ozone injury to the leaves. Even differences in relative air humidity may affect ozone response, decreasing leaf injury at lower air humidity (Mortensen, 1990; Thompson and Olszyk, 1988). The importance of soil moisture in altering ozone sensitivity of agricultural crops is also reported by Brewer et al. (1988). King (1987, 1988) predicted the drought-induced reduction in ozone sensitivity of approximately 20% in a modeling study. However, Fangmeier et al. (1994) failed to detect a water supply by ozone interaction in their fumigation experiments.

Although some studies have concentrated on the interactions of ozone with other pollutants (Bender and Weigel, 1992), a general response pattern of crops to a mixture of air pollutants has not yet emerged. The authors summarize that additive effects occur more frequently than significant interactions, and in the case of interactions, the mode of interaction was mostly antagonistic. Clearly more research is needed before the modifying effects of low levels of SO_2 and/or NO_2 on ozone impact can be generally assessed.

Ozone effects on yield parameters have been investigated considerably less frequently than the effects on total crop productivity. In experiments with separate determinations of straw and grain yield of wheat, the productivity reductions of straw yield were less pronounced than the decreases in grain yield. The individual grain weight was similar or more affected by ozone than the number of grains per ear (Fuhrer et al., 1989a, 1992). Analysing the period of grain filling as influenced by ozone in greater detail, Slaughter et al. (1993) observed a decreased grain growth rate and assimilate utilization under increasing ozone concentrations, but no effect on grain-fill duration and on effective filling period.

Ozone effects on photosynthesis of wheat

A reduction in net photosynthesis of wheat due to exposure to increased ozone concentrations has been described by Reich and Amundson (1985) and Lehnherr et al. (1988); the concomitant increase in respiration rate is mainly caused by increases m maintenance respiration and is at least partly responsible for the decrease in apparent photosynthesis (Amthor, 1989). Gutser et al. (1990) observed in their fumigation experiments that yield was only in those treatments decreased that showed reduced photosynthetic rate. Barton and Knoppik (1990) report that only above a certain threshold exposure a linear decrease of photosynthetic rate with received dose of ozone could be observed; the injury threshold was < 30 ppb for younger leaves and > 30 ppb for flag leaves. However, when Nie et al. (1993) analysed different leaf sections of the same leaf, they found the highest impairment of several photosynthetic parameters in older tissues

at the leaf tip and no effect in tissues of the middle and basal section. They conclude that the ozone exposure had no effect on the photosynthetic apparatus during leaf expansion but that it promoted the onset of premature senescence in fully expanded tissues.

The question after the main cause for photosynthetic impairment in wheat has evoked further research. Analysing the $\sigma\ ^{13}C$ of lant material as an integrated measure of the plant response to pollutant exposure, Martin et al. (1988) and Saurer et al. (1991) found less negative values with increasing ozone concentrations. They interpreted these findings as an indication of greater stomatal limitation of photosynthesis; a result which was not always confirmed by gas exchange and biochemical studies (Bender, 1992). Grimm and Fuhrer (1992) suggest that only in young leaves ozone affects the stomata directly and in senescent leaves the limitation of photosynthesis is primarily caused by reduced carboxylation. Lehnherr et al. (1988) concluded that the decrease in stomatal conductance did not account for the decrease in photosynthesis and that ozone rather affects ribulose bisphosphate carboxylation. They also suggested a limitation of photosynthesis by an ozone effect on pentose phosphate reductive cycle activity. Based on short-term acute ozone fumigation experiments, also Farage et al. (1991) consider stomatal limitation to play a minor role in the decrease in light-saturated rate of CO_2-uptake. They suggest the apparent carboxylation efficiency to be the initial cause for a decline in photosynthesis and that the regeneration of ribulose bisphosphate is less susceptible to ozone. The authors also conclude from their chlorophyll fluorescence measurements that photochemical efficiency of photosystem II is not primarily impaired and that photochemical reactions are not responsible for the initial inhibition of CO_2 uptake. In similar analyses Grimm and Fuhrer (1992) observed a stimulation of the decline in F_v/F_0 by ozone, but this effect could not be detected during night. This is an indication of the reversibility of ozone effects on photosynthetic structures and suggests that ozone rather causes metabolic changes than damages to structural components.

Ozone effects on vegetative and generative development of wheat

Leaf development

Although many studies have investigated the ozone effects on productivity patterns of wheat at full maturity, not much information is available about ozone sensitivity at different developmental stages. This question is important because it could be that differences in ozone tolerance during ontogeny might require different levels of protection during the vegetation cycle. For studying this question, a closed-chamber fumigation experiment has been set up with two ozone levels and a cultivar of winter wheat known for its ozone sensitivity.

Winter wheat "Perlo" was germinated at + 20 °C for 24 hours and immediately afterwards vernalized for 60 days at + 1 °C. These vernalized seedlings were planted in pots with a standard soil mix (Frux ED 73) and thinned to 15 plants per pot (0.81 soil per plant). The fumigation experiment was performed in greenhouse compartments adapted as fumigation chambers. Temperature was held at 15/10 °C (day/night) during the first three weeks after emergence, afterwards at 20/12 °C. Plants were irrigated as necessary.

Table 1

Ozone fumigation as characterized by 8-hour mean value (MV8) in ppb and integrated AOT40 ppm.h for the fumigated period (9 a.m. till 5 p.m.) calculated for the various harvest dates (d.a.e. = days after emergence)

d.a.e.	MV8 (ppb) control	MV8 (ppb) ozone treatment	integrated AOT40 (ppm.h) control	integrated AOT40 (ppm.h) ozone treatment
30–52	4	75	0	6.8
53–79	10	77	0	14.8
80–107	16	77	0.1	23.0
108–134	28	81	0.6	30.2

Fumigation started one month after emergence (stage 21) and lasted for three months (stage 71). Ozone was generated from pure oxygen by electric discharge (Fischer 502, Meckenheim, Germany). Ozone concentrations were held constant at 80 ppb (± 6 ppb) from 9 a.m. to 5 p.m with a continously registrating ozone immission analyzer (Horiba APOA 350E, Horiba Europe, Langenfeld, Germany) controlling the output of the ozone generator. With the same instrument the ozone levels in control chambers and in ambient air were measured with an electronically controlled sample mainfold in hourly intervals. The effective ozone concentrations and ozone doses relevant for this experiment are given in Table 1. Plant material was harvested for dry weight determinations (70 °C) of green, senescent and dead leaf dry matter, stems and ears. Statistical analysis (ANOVA, n = 5) was performed with WinSTAT (G. Greulich Software, Staufen, Germany).

Ozone affected leaf growth in each stage of plant development, showing some parallels and some differences to natural leaf senesence (Table 2). In comparison to the considerable decrease of total dry matter production under ozone stress, leaf dry matter remained at a relatively stable level. Although ozone stress was severe for cultivar "Perlo", even after 30 ppm.h ozone total leaf dry matter amounted to not less than 70-80% of unstressed plants, whereas stem and ear productivity had been much more reduced. This shows the high priority of leaf production for the wheat plants which favor allocation of assimilates for leaf growth. Probably under a low level of assimilate supply stem growth and storage of carbohydrates in the stem is depressed, consequently acropetal translocation of assimilates for storage in the ear is also decreased. After flowering stage in ozone-stressed plants leaves contributed a higher proportion to total dry matter (48%) than control plants (33%). This could be partly explained with the decreased storage pools in stems and ears, but also the delay in generative development (in spite of enhanced senescence of vegetative parts) could be a cause for a slower or unfinished export of leaf constituents. At final harvest (134 d.a.e., AOT40 30 ppm.h) stressed plants had produced about 30% less total leaf dry matter than control plants.

Table 2

Growth parameters of winter wheat cv. Perlo at two fumigation levels (see Table 1) and at different developmental stages (d.a.e.=days after emergence, DM=dry matter). Parameters in $g.m^{-2}$ if not indicated otherwise

d.a.e.	parameter	control	ozone	t-test
52	height (cm)	39 ± 4	48 ± 2	***
	leaf area ($dm^2 \cdot m^{-2}$)	215 ± 10	107 ± 13	***
	green leaf DM	76 ± 3	44 ± 6	***
	senescent leaf DM	1.9± 0.7	8.6± 1.2	***
	dead leaf DM	1.7± 0.5	7.9± 1.4	***
	stem DM	18 ± 1	17 ± 2	ns
	total DM	98 ± 4	77 ± 8	**
	specific leaf area ($cm^2 \cdot g^{-1}$)	281 ± 10	245 ± 19	*
	leaf area ratio ($cm^2 \cdot g^{-1}$)	219 ± 6	139 ± 10	***
79	height (cm)	62 ± 4	59 ± 3	ns
	leaf area ($dm^2 \cdot m^{-2}$)	896 ± 47	359 ± 52	***
	green leaf DM	284 ± 12	158 ± 23	***
	senescent leaf DM	27 ± 6	56 ± 3	**
	dead leaf DM	33 ± 7	70 ± 11	***
	stem DM	121 ± 14	134 ± 13	ns
	total DM	465 ± 27	419 ± 47	ns
	specific leaf area ($cm^2 \cdot g^{-1}$)	315 ± 15	227 ± 16	**
	leaf area ratio ($cm^2 \cdot g^{-1}$)	193 ± 11	86 ± 7	***
107	height (cm)	64 ± 2	72 ± 4	*
	leaf area ($dm^2 \cdot m^{-2}$)	629 ±100	260 ± 65	**
	green leaf DM	236 ± 50	115 ± 29	*
	senescent leaf DM	67 ± 13	92 ± 21	ns
	dead leaf DM	219 ± 26	175 ± 13	**
	stem DM	267 ± 25	203 ± 48	ns
	total DM	789 ± 88	584 ± 99	*
	specific leaf area ($cm^2 \cdot g^{-1}$)	269 ± 13	227 ± 22	*
	leaf area ratio ($cm^2 \cdot g^{-1}$)	80 ± 7	44 ± 8	***
134	height (cm)	93 ± 7	75 ± 5	**
	leaf area ($dm^2 \cdot m^{-2}$)	410 ±259	72 ± 66	ns
	green leaf DM	176 ± 60	40 ± 36	*
	senescent leaf DM	169 ± 20	85 ± 36	**
	dead leaf DM	410 ± 48	382 ± 91	ns
	stem DM	1080 ±160	413 ±108	**
	total DM	2300 ±339	1050 ±257	**
	specific leaf area ($cm^2 \cdot g^{-1}$)	220 ± 94	175 ± 25	ns
	leaf area ratio ($cm^2 \cdot g^{-1}$)	19 ± 13	6 ± 5	ns
	number of ears (m^{-2})	689 ± 53	374 ± 68	***
	ear DM	465 ±218	127 ± 25	ns

The effects of ozone on the senescence of healthy leaves was more pronounced than on total leaf productivity. Although the percentage 2 healthy leaves decreased linearly both in ozone-stressed leaves with accumulated AOT40 ($r^2 = 0.99$) and in control leaves with age ($r^2=0.97$), the decrease started later in control plants and proceeded afterwards with a similar pace. Calculated on the basis of total leaf dry matter, the first 7 ppm.h of ozone reduced the proportion of healthy leaves by 27 %, whereas aging alone caused a loss of only 5% of total leaves. During further plant development the percentage of healthy leaf dry matter in control plants decreased from 95% of total leaf dry matter 52 d.a.e. to 23% at milky grain stage (fumigated plants: 73 and 8%, resp.). This can be explained with a higher sensitivity of young wheat plants compared to older ones, but also a decrease in the difference of ozone exposure (expressed as multi-hour mean value) of control and fumigated plants could have been contributed to decreased sensitivity. The 8-h mean value for control plants increased from 4 to 28 ppb (fumigated plants: 75 to 81 ppb), but it is generally believed that concentrations below 30 ppb ozone do not injure even sensitive crop plants. The AOT40 of control plants was also lower than 0.7 ppm.h what is considered to be a threshold for visible ozone injury on leaves.

In fumigated plants, the proportion of senescent leaves remained relatively constant at 15–25% of total leaf dry matter. However, during natural senescence the proportion of senescent leaves increased potentially ($y = a*x\text{^}b$; $r^2 = 0.99$) with plant age. Dead leaves increased exponentially ($y = a*\exp(b*x)$) and more pronounced in fumigated plants ($r^2 = 1.00$) than in control plants.

Although young wheat plants probably are less tolerant to ozone than generative plants, the dynamics of leaf growth suggest that the post-flowering stage is the most vulnerable period for ozone injury. This period is very important for carbon assimilation and translocation to the ear, but if green leaf area is reduced due to former or recent ozone injury and if additionally the reproductive stage is delayed, yield reductions because of decreased carbon gain are inevitable. When Decoteau and Craker (1981) stated that in their experiments a reduction in leaf area was not always associated with reduced yield, this apparently only applied for less sensitive wheat cultivars or for lower ozone doses. But at higher oxidative stress levels our results suggest a predominant role of maintaining green leaf area for determining yield effects of ozone.

Stem and ear development

Stem development (including the leaf sheaves) remained at first relatively unaffected by ozone stress, but only till flowering stage. During the vegetative stages, stem (and pseudo-stem) dry matter of fumigated plants had been even less decreased than total leaf dry matter. Starting with flowering stage, stem dry matter of ozone-exposed plants increased considerably slower than in control plants. The increase in stem dry matter (given as proportion of total dry matter) corresponded best to an exponential function ($y = a*\exp(b*x)$) in non-fumigated plants ($r^2 = 1.00$) and to a hyperbolic function ($y = x/(a + b*x)$) in ozone-stressed wheat ($r^2 = 0.99$).

Ozone effects on ear development were still more pronounced than on stem growth. At milky grain stage (134 d.a.e.), at an AOT40 of 30 ppm.h, ozone-stressed wheat had produced about 60% less stem dry matter and 70% less ear dry matter than control plants. Both the number of ears and the weight per ear were decreased (by 45 and 50%, resp.). Applying the critical level approach to the exposure characteristics of this experiment, a decrease in grain yield of about 55% would be expected (Fuhrer and Achermann, 1994); using the data based on the seasonal 8-hour mean values, the yield reduction might range from 40–45% (Skärby et al., 1992). This is not necessarily in disagreement with our data as only the yield data till stage 73 are shown, additionally the cultivar "Perlo" is supposed to be more sensitive than most other wheat cultivars (unpublished data).

These results of growth analysis in a wheat cultivar known for special sensitivity to ozone show a pattern of ozone effects that reflects the hierarchy for assimilate distribution. The higher sensitivity of young leaves compared to mature leaves makes wheat especially vulnerable in early growth stages. Periodic ozone effects during tillering and early shooting stage might harm leaf development so seriously, that the loss of green leaf area cannot be compensated till full maturity. Fortunately in Europe during these early growth stages serious ozone problems very infrequently occur. But these findings about early ozone sensitivity of wheat leaves give some indication that wheat could be used for active bioindication of ozone. Apart from the increase in the proportion of dead and senescent leaves, the appearance of chlorotic and necrotic leaf spots could be used for an assessment of ozone injury.

Although ozone-stressed plants show a similar leaf productivity than unstressed plants, the revenue in form of photoassimilates is dramatically decreased due to the loss of green leaf area. Additionally to decreased photosynthetical carbon gain, the possibility of assimilate loss because of increased respiration for repair mechanisms (Amthor, 1989) should also be taken into consideration. Consequently, stem dry matter production is more depressed because it is dependent on assimilate supply from the leaves. As stem length is less affected than stem weight, the lack in dry matter is apparently caused by missing storage carbohydrates. So it is not astonishing that ear growth is negatively influenced because less assimilates for translocation can be provided. The lower number of ears indicates disturbances during the early stages of reproductivity, suggesting interferences of ozone with ear development after tillering.

Fast kinetics of chlorophyll fluorescence

Chlorophyll fluorescence is a frequently used tool for studying environmental effects on photosynthetic functions. By analysing the initial fluorescence induction curve with mobile equipment in the field, screening for the fast kinetics of fluorescence may provide information on the extent of photochemical impairment. From the diverse parameters that can be measured in the course of a induction curve of chlorophyll fluorescence, especially the f_v/f_m-ratio is regarded to be a good indicator of photochemical efficiency (Bolhàr-Nordenkampf and Öquist, 1993).

Fluorescence measurements were made with a mobile non-modulated system (PEA, Hansatech Instruments Ltd., King's Lynn, England) after a pre-darkening period of 30 minutes at both sides of the leaves. Attention was given to uniformity of leaf position relative to the sun and to uniformity of measured leaf section. The curves given in Fig. 1 are representative for each treatment (eight measurements per treatment).

As exemplified with some characteristic induction curves in Fig. 1, f_v/f_m was significantly decreased under the influence of ozone, the lower side of the leaf being more distinctly affected than the upper side. The difference was rather due to a decrease in maximal fluorescence (f_m) than to an increase in initial fluorescence (f_0). Consequently also the area under the fluorescence curve was decreased, giving evidence for a decreased pool size of plastoquinone electron acceptors. As these measurements were taken at the uppermost fully developed leaf (middle section without visual ozone injury) that is the most important leaf for the carbon gain, the negative influence of ozone on photosynthetic performance of wheat becomes evident. So it can be concluded that decreased productivity due to ozone is not only caused by a decrease in green leaf area but also by a lowered efficiency of photosynthetic processes. These results do not confirm the findings of Farage et al. (1991) who did not observe decreased f_v/f_m after ozone exposition. The differences might be due to the short fumigation duration and high concentrations of ozone used in their study, whereas in our experiments the plants had been exposed to lower concentrations for a longer time (28 days at the time of measurement).

Conclusions

Although wheat is the best investigated agricultural crop in Europe, concerning its ozone sensitivity, large uncertainties remain if the economic effects of this sensitivity are to be calculated. For detailed quantification cultivar screening seems to be indispensible with the cultivars being grown till maturity. More research will also be needed on interactions of ozone with phenology and drought sensitivity. With these results, the concept of fixed critical levels probably can be modified to a concept of variable, locally and temporarily adaptable critical levels. Although our results suggest increased sensitivity of wheat at early growth stages, usually in Europe no elevated ozone concentrations occur during this time. Because sensitivity does not change dramatically in later growth stages, it is concluded that a revision of the existing critical levels of ozone to adapt them to developmental tolerance characteristics of wheat would bring no big advantages.

Yield depressions clearly are correlated with losses of green leaf area due to enhanced senescence and with decreases in photochemical efficiency of the remaining green leaves, thereby reducing total carbon gain. Up to now EPR-spectroscopy has been the only means of analysing radical action in ozone-injured leaves *in situ*. After having attained to measure directly the effects of ozone on photosynthetic parameters, it could be one of the next aims in photosynthesis research to develop techniques that are useful for distinguishing ozone-enhanced senescence from natural senescence.

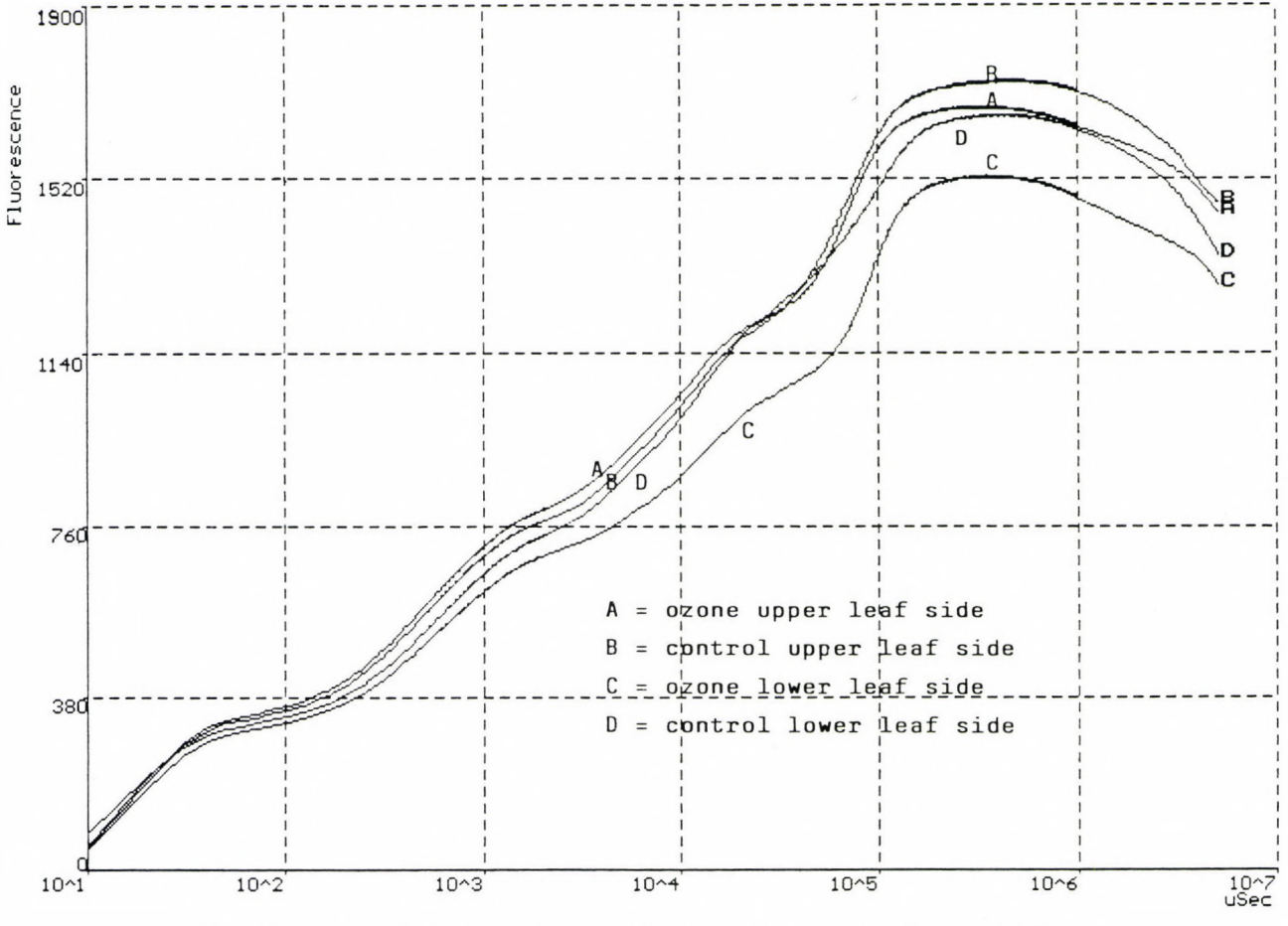

Fig. 1. Fluorescence induction curves for upper and lower leaf sides uppermost fully expanded wheat leaves 58 d.a.e. (ozone treatment for 28 days, treatments see Table 1)

Acknowledgements

The authors gratefully acknowledge financial support by the Austrian Ministry for Science and Research (TSP-Programm) and by the Jubiläumsfonds der Österreichischen Nationalbank (project nr. 4112).

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 71–80 (1995)

Photoinhibition in CO_2-depleted Green Algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii*

S. DEMETER, T. JANDA and L. KOVÁCS

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

Cultivation of the green algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii* in CO_2-depleted medium at moderate light intensity resulted in an inhibition of Photosystem II electron transport from water to diaminodurene (acceptor at the plastoquinone pool) but only slightly affected the electron flow from water to 2,6-dichlorobenzoquinone (acceptor at the primary quinone electron acceptor, Q_A). The intermediary fluorescence level, F_i was raised to the maximum level of fluorescence, F_m. The initial level of fluorescence, F_0 was considerably enhanced. The observations suggest that illumination of CO_2-depleted green alga cells results in the development of an inhibition of electron transport between the primary (Q_A) and secondary (Q_B) quinone electron acceptor. Prolonged photoinhibitory illumination of the CO_2-depleted cells led to an irreversible loss of variable fluorescence and electron transport. The photoinactivation developed slower in the CO_2-depleted than in the CO_2-containing cells. Consequently, in the bicarbonate-depleted redox state the photosystem II reaction center is less susceptible to photoinhibition than in the bicarbonate-containing state.

Photoinhibition in photosynthetic organisms occurs when the light energy funneled to the reaction center chlorophyll exceeds the capacity of the photosynthetic system to utilize this energy in metabolic reactions or dissipate it in harmless processes. Photoinhibition is manifested as an inhibition of photosystem II (PS II)-catalysed electron transport and results in a loss of the variable part of fluorescence emission from PS II, as well [1, 2].

Inhibition of the Q_A to Q_B electron transfer by DCMU-type inhibitors confers protection of the PS II reaction centers against photoinhibition [3]. Moreover, it has been observed that the Q_B nonreducing centers are highly resistant to photoinhibition [4, 5]. Photoinhibition was also alleviated when the oxidation of Q_A was retarded by low temperature during exposure of *Chl. reinhardtii* cells to high intensity light at 5 °C [6]. These observations suggest that other treatments inhibiting the oxidation of Q_A^- by Q_B may also ease photoinhibition.

Bicarbonate is a ligand to the non-heme iron in the Q_A-Fe-Q_B quinone-iron complex of PS II [7]. It can be removed from its binding site by several monovalent anions, of which formate is the most effective [7]. In the bicarbonate-depleted electron transport

Abbreviations: Dl, polypeptide of the reaction center; DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_0, initial fluorescence; F_i, intermediate fluorescence level; F_m, maximum fluorescence; F_v, variable fluorescence; P680, primary electron donor chlorophyll of PS II; PS I, Photosystem I; PS II, Photosystem II; Q_A and Q_B, primary and secondary quinone electron acceptor of PS II; Y_Z^+, redox active tyrosinei-161 of the D_1 protein.

0238–1249/95/$ 5.00

chain the electron transport is inhibited between Q_A and Q_B. Consequently, we assumed that bicarbonate/CO_2-depletion which inhibits the oxidation of Q_A^- by Q_B or Q_B^- [7] would also provide protection against photoinhibition.

In the present work we observed that in vivo CO_2-depletion of *Chl. stellata* and *Chl. reinhardtii* green alga cells by bubbling the culture medium with CO_2-free air resulted in an inhibition of electron transport from Q_A to the plastoquinone pool demonstrating the requirement of bound bicarbonate in an uninhibited electron flow. It was also found that CO_2-depleted green alga cells are less susceptible to photoinhibition than cells in the non-depleted state.

Materials and Methods

Chl. stellata and *Chl. reinhardtii* green algae were cultivated at pH 7.0 under continuous light (10 W/m^2) by bubbling the cultures with a mixture of 5% CO_2 and 95% air (v/v) [8]. CO_2-depletion was achieved by purging the alga suspensions with CO_2-free air.

The rate of steady state oxygen evolution was measured in the culture medium by using a Clark-type electrode.

Fluorescence induction transients were measured in a home-built setup.

Photoinhibitory illumination of *Chl. stellata* and *Chl. reinhardtii* alga cultures was carried out at room temperature with white light at light intensities of 300 and 1000 W/m^2, respectively. After photoinhibitory illumination the CO_2-depleted culture was bubbled for 5 min with CO_2-containing air. Afterwards, both the CO_2-depleted and CO_2-containing cells were kept under the cultivation light for 60 min preceding fluorescence induction and electron transport rate measurements.

Results

Effects of CO_2-depletion on the steady state electron transport of PS II

Figure 1 shows the electron transport rates of PS II under various experimental conditions in the course of CO_2 depletion of *Chl. stellata*. Without any addition the photosynthetic electron transport was almost completely inhibited in the first hour of CO_2-deprivation. The rate of PS II electron flow was also measured in the H_2O to oxidized diaminodurene (DAD_{ox}) reaction. DAD_{ox} probably intercepts electrons at the plastoquinone pool [9]. To exclude the influence of CO_2 fixation (Calvin cycle) 1 μM dibromothymoquinone (DBMIB) which blocks electron transport between the plastoquinone pool and the cytochrome b/f complex was added to the assay medium.

The $H_2O \rightarrow DAD_{ox}$ reaction was completely inhibited by CO_2 depletion (Fig. 1). To clarify that the inhibition is not caused by an impairment of the water-splitting system the rate of PS II electron flow was measured from H_2O to dichlorobenzoquinone (DCBQ), which can accept electrons from Q_A even in the presence of DCMU [10]. The

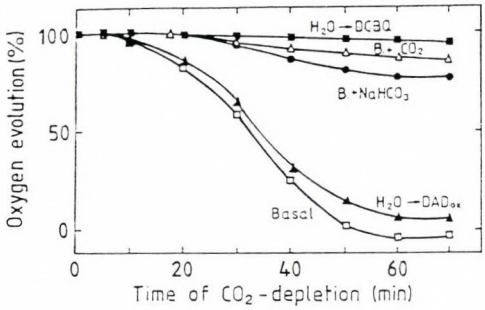

Fig. 1. Partial electron transport rate measurements of the green alga, *Chl. stellata* during CO_2-depletion in the light (10 W/m^2). □–□ basal electron transport (abbreviated as B); ▲–▲, $H_2O \rightarrow$ 200 μM oxidized diaminodurene (DADox + 1 μM DBMIB); ■–■, $H_2O \rightarrow$ 200 μM 1,6-dichloro-p-benzoquinone (DCBQ + 1 μM DBMIB); ●–●, basal electron transport + 5 mM $NaHCO_3$ incubated for 5 min); △–△, basal electron transport + CO_2 (bubbled with CO_2-containing air for 5 min). The absolute rates of O_2 evolution in the autotrophic alga before CO_2-depletion in the same order as the partial electron transport reactions were: 130 μM; 78 μM; 160 μM; 110 μM; 130 μM oxygen evolved/mg Chl h

$H_2O \rightarrow$ DCBQ Hill reaction was only very slightly influenced by the removal of CO_2 showing that the activity of the oxygen-evolving system is not changed by short-term CO_2 depletion. Thus, we can conclude that CO_2 depletion results in an inhibition between the electron accepting site of DCBQ and DAD_{ox} that is between Q_A and the plastoquinone pool.

Bubbling the CO_2-free culture with strong flow of CO_2-containing (5%) air for 5 min completely restored the original uninhibited rate both in the basic electron transport (Fig. 1) and in the $H_2O \rightarrow DAD_{ox}$ reaction (not shown). Bicarbonate addition was less efficient than CO_2. Incubation of the alga, which was cultivated for 70 min in CO_2-free air, for 5 min in the presence of 5mM $NaHCO_3$ restored about 80% of the basic electron transport rate (Fig. 1) and 60% of Hill reaction rate in the $H_2O \rightarrow DAD_{ox}$ reaction (not shown). The electron transport rate measurements demonstrat'e that in contrast to [11] in vivo depletion of CO_2 in the absence of formate results in an almost complete inhibition of electron flow. Thus, bicarbonate is a requirement for uninhibited electron transport of PS II.

Effects of CO_2-depletion on fluorescence induction
Reversible photoinhibition of the CO_2-depleted alga cells

Bicarbonate depletion also changed the fluorescence transients of the green alga cells. Figure 2 (upper left corner) shows the fluorescence induction of *Chl. stellata* cultivated in CO_2-containing medium. The fast rise of fluorescence yield from the initial level, F_0 to the intermediately level, F_i is followed by a dip, F_D and a maximum, F_p. DCMU which blocks the electron transport between Q_A and Q_B quickly raised the intermiediary level, F_i to the maximum level of fluorescence, F_m. Dibromothymoquinone

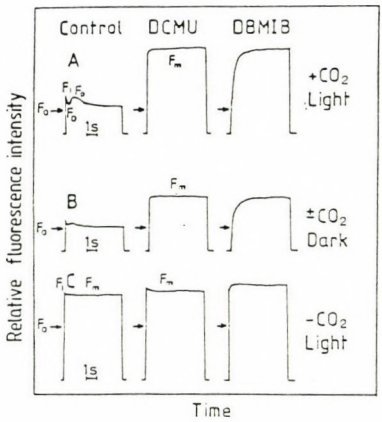

Fig. 2. Effect of cultivation conditions and inhibitors on the fluorescence induction of autotrophic *Chl. stellata*. A: alga was cultivated in white light (10 W/m^2) by bubbling the medium with a mixture of 5% CO_2 and 95% air (v/v). B: alga was cultivated in the dark for 1 h either in the presence or absence of CO_2. C: the same as A except that the cultivation medium had been purged with CO_2-free air for 1 h. Samples were taken directly from the culture and kept in the dark for 1 min before fluorescence measurements. Additions: 10 μM DCMU or 2 μM DBMIB. Designations: F_0, initial; F_i, intermediary; F_m, maximal and F_v, variable fluorescence. F_D is a dip in the fluorescence rise

(DBMIB) which inhibits the oxidation of plastohydroquinone also increased the F_i level but the rise was slower and the F_m level was somewhat lower.

Dark adaptation of alga either in CO_2-containing or CO_2-free medium caused a large decrease in the F_m level (Fig. 2, middle part). The F_0 level remained almost the same but the variable fluorescence, F_v decreased approximately to the half. The lowered F_m level can be accounted for by a change in light energy distribution between the two photosystems. Our observations are in agreement with other reports showing that green algae are in the low fluorescence State II in the dark and convert to the high fluorescence State I upon illumination [12, 13]. It has to be emphasized that no difference could be observed between the fluorescence characteristics of alga dark adapted either in CO_2-containing or CO_2-depleted medium. This is in agreement with the suggestion that in HCO_3^--depleted thylakoids the rate-limiting, step in electron transfer is the protonation of Q_B^- and Q_B^{2-} thus the inhibition of electron flow develops only gradually in the light after several turnovers of the reaction center [7]. Since dissociation of bound HCO_3^- is enhanced in the light [7] it can be also assumed that in the dark the firmly bound endogenous HCO_3^- molecules cannot be completely removed during the CO_2 depletion procedure. In order to achieve complete removal of bound HCO_3^-/CO_2 in addition to a reduction of partial CO_2 pressure outside the cell a light-induced photosynthetic utilization of HCO_3^-/CO_2 inside the cell may also be necessary. Moreover, in the dark the internal CO_2 production may inhibit the removal of HCO_3^- from the green alga cells purged with CO_2-free air.

Illumination of the dark adapted CO_2-containing culture with light of moderate intensity (10 W/m^2) restored the original light adapted pattern of fluorescence induction (Fig. 2, upper part). However, exposure of the CO_2-depleted culture to light induced large changes in the fluorescence characteristics. Similarly, as after DCMU addition, the intermediary fluorescence level, F_i was raised to the F_m level (Fig. 2, lower part) suggesting an inhibition of electron transport between Q_A and Q_B. Moreover, the initial level of fluorescence, F_0 considerably increased, simultaneously with about 50% decrease in the extent of variable fluorescence ($F_v = F_m - F_0$). The same phenomenon could be observed when CO_2 depletion of the alga was carried out in the light. The F_0 rise also developed in *Chl. reinhardtii* cells during bubbling the alga culture with CO_2-free air at moderate light intensity (not shown). The elevated F_0 level can be interpreted by a reduced state of the Q_A pool which is stable in the dark for a certain time [1, 14–17]. Supporting this interpretation the F_0 rise was not developed when the alga was cultivated in the presence of an electron acceptor, 2,6-dichlorobenzoquinone (not shown).

Addition of DCMU or DBMIB to the CO_2-depleted alga increased the F_m level a little further (Fig. 2, lower part) suggesting that neither the Q_A to Q_B nor the Q_B to plastoquinone electron transfer are completely inhibited in the CO_2-depleted alga.

The buildup of enhanced F_0 level was almost the same at pH 7.0 and pH 6.0 (not shown). However, it developed faster and its extent was much higher at pH 5.0 (Fig. 3).

The original F_0 level could be restored by readdition of CO_2. The increased F_0 yield returned to the control level more slowly at pH 5.0 than at pH 7.0. Since the extent and decay of the elevated F_0 level depends on the pH of the medium we can assume that in the CO_2-depleted alga cells the F_0 rise reflects a protonated state of the reduced Q_A

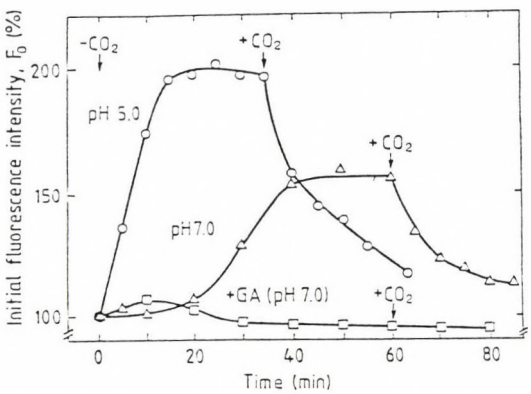

Fig. 3. Effect of pH and glutaraldehyde on the buildup of the enhanced F_0 level in *Chl. stellata*. At the times indicated by the experimental points samples were taken from the alga cultures and the fluorescence was measured after 1 min dark adaptation. First arrow indicates the beginning of CO_2-depletion. Glutaraldehyde treatment (0.002%) was etarted simultaneously with the CO_2-depletion in the cultivation vessel. From the moments shown by arrows the cultures were bubbled with 5% CO_2 and 95% air (v/v)

acceptor pool. At pH 5.0 the enhanced F_0 level was about 60–80% of the F_m level. Chemical cross-linking of proteins by the addition of 0.02% glutaraldehyde completely inhibited the development of the F_0 rise (Fig. 3).

Irreversible photoinhibition of the CO_2-depleted alga

The first part of photoinhibition which appears as a reversible F_0 rise is followed by a second irreversible part which is represented as a gradual decrease of the maximal, F_m and variable fluorescence, F_v [14]. In the cultivation light of moderate intensity the F_m level of CO_2-depleted alga decreased very slowly during several hours. The rate of irreversible damage was accelerated by increasing the light intensity from 10 W/m^2 to 300 W/m^2 during cultivation of *Chl. stellata* and to 1000 W/m^2 for the *Chl. reinhardtii*. A significant part of variable fluorescence was apparently lost due to the rise of the F_0 level and not due to irreversible photoinhibition. In order to determine the real yield of variable fluorescence CO_2 was resupplied to the cultures after photoinhibitory illumination. This restored the original F_0 level and the uninhibited electron flow. The rate of electron transport and the loss of F_v as a function of photoinhibition were measured in the restored cultures of *Chl. stellata* and *Chl. reinhardtii* and the results are depicted in Figs 4 and 5.

The inhibition of electron transport was the same in the presence and absence of the PS II acceptor, phenyl-p-benzoquinone, PpBQ showing that in our experiments

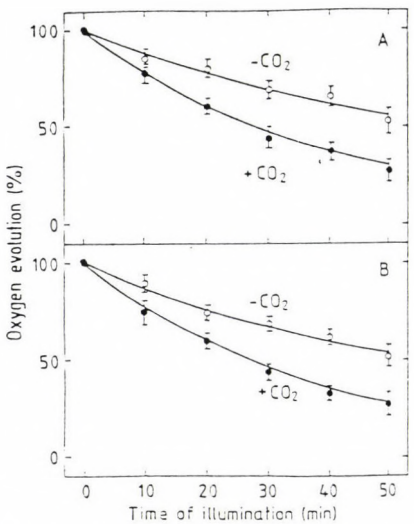

Fig. 4. Effect of photoinhibitory light treatment on the electron transport rate measured from water to 200 μM phenyl-p-benzoquinone in control and CO_2-depleted *Chl. stellata* (Part A; light intensity: 300 W/m^2) and in control and CO_2-depleted *Chl. reinhardtii* (Part B; light intensity: 1000 W/m^2) alga cells. The points and bars show the means of three measurements with the standard deviations. Other experimental details are described in the Materials and Methods

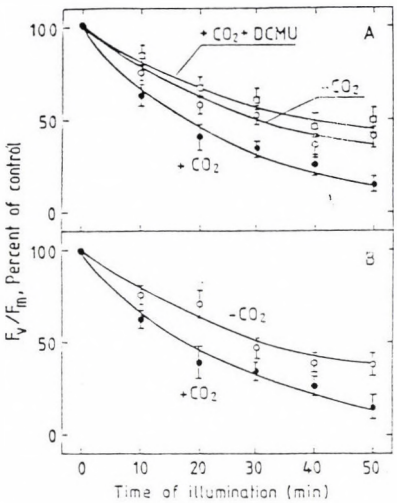

Fig: 5. Effect of photoinhibitory light treatment on the variable fluorescence (F_v/F_m) in control, DCMU-treated and CO_2-depleted *Chl. stellata* (Part A; light intensity: 300 W/m^2) and in control and CO_2-depleted *Chl. reinhardtii* (Part B; light intensity: 1000 W/m^2). The points and bars represent the means of three measurements with the standard deviations

photoinhibition is associated only with PS II and not with PS I. The results demonstrate that both F_v and the electron transportI rate decreased slower in the CO_2-depleted alga as compared to the nondepleted alga. If the CO_2-containing cells were illuminated in the presence of DCMU the photoinhibitory damage was deccelerated and comparable to that observed in the absence of CO_2 (Fig. 5). This is consistent with the protective effect of DCMU-type inhibitors against photoinhibition [3, 18]. Consequently, inhibition of electron transport between Q_A and Q_B either by CO_2 depletion or by DCMU provided protection against irreversible photoinhibitory damage.

Discussion

The present work demonstrates that *Chl. stellata* and *Chl. reinhardtii* alga cells can be depleted of CO_2 without the use of inhibitory anions simply by purging the medium with CO_2-free air at moderate light intensity. We observed that CO_2-depletion completely inhibited the electron transport from water to diaminodurene (an acceptor at the plastoquinone pool) but only slightly influenced the water to dichlorobenzoquinone (acceptor at Q_A) partial reaction. The intermediary level of fluorescence, F_i was lifted to the maximal level, F_m and it was not changed further by DCMU addition. All of these observations confirm the earlier suggestion [7,19] that CO_2/HCO_3^- is a requirement for an in vivo uninhibited electron flow between Q_A and Q_B.

We can assume that inhibition of electron transport in the CO_2-depleted alga is caused by the development of a reduced state of Q_B. Since bicarbonate is probably involved in the protonation of Q_B^{2-} [7], in the bicarbonate-depleted thylakoids the protonation process determines the rate of electron transport. The retarded protonation process of Q_B^{2-} molecules can result in an "overreduction" of the Q_B pool inhibiting the electron transfer between Q_A and Q_B.

It is natural to assume that due to the "overreduced" state of the Q_B pool the steady state forward electron flow leads to accumulation of electrons in the Q_A pool, as well, even at moderate light intensity. The reduced state of a certain portion of the Q_A pool is reflected in an elevated level of initial fluorescence, F_0. Supporting this interpretation in alga cells cultivated in the presence of the electron acceptor, dichlorobenzoquinone, the F_0 rise was not developed.

Continuous illumination of the CO_2-depleted alga cells which keeps a portion of the Q_A pool in permanently reduced state probably allows a slow protonation of the singly-reduced Q_A molecules to occur. This notion is corroborated by the observation that the extent and decay of the enhanced F_0 level depends on the pH of the medium. At pH 5.0 we observed about 100% increase of the F_0 level (about 60–80% of F_m) suggesting an almost complete reduction and protonation of the Q_A pool. The elevated F_0 level was also considered as a manifestation of a protonated stable reduced state of the Q_A acceptor pool in previous reports [6, 14, 16, 17].

In the CO_2-depleted green alga cells the development of the enhanced F_0 level was not accompanied by a lowering in the maximal fluorescence yield, F_m. Therefore, the enhanced F_0 level can be ascribed to protonated singly-reduced Q_A molecules. Double reduction and protonation of Q_A would lead to a lowered yield of F_m [17, 20].

In spinach thylakoids the F_0 rise could be observed only in anaerobic conditions independently of the presence or absence of bicarbonate [15]. As our present observations demonstrate the elevated F_0 level can also be induced by depletion of CO_2 from the alga cells even under aerobic conditions (in the presence of oxygen). Thus, in green alga cells the appearance of the stable, but reversible state of the Q_A pool and the enhanced F_0 level are not restricted to anaerobiosis as observed in spinach thylakoids [15].

It has been established that photoinhibition can be initiated both on the acceptor- and donor-side of PS II [1, 2]. In strong light irreversible photinhibition is probably induced by "overreduction" of the acceptor side of PS II ("acceptor-side photoinhibition") [1, 2]. Charge neutralization of the singly [16, 17] or doubly [20] reduced Q_A forms by protonation can promote the generation of ^{3}P680 triplet molecules. It has been suggested as a possible mechanism of irreversible photoinhibition that in the interaction of ^{3}P680 and molecular oxygen, singlet oxygen is produced [16, 21, 22] which can cause rapid abolishment of the stable reduced Q_A species and the F_0 rise [2, 16, 17]. In contrast to this expectation, in the CO_2-depleted green alga cells the enhanced F_0 and F_m levels were constant during several hours of illumination at moderate light intensity. The original F_0 and F_m levels and the uninhibited electron transport rate could be completely restored by readdition of CO_2 (or bicarbonate). It can be inferred that formation of charge neutralized singly reduced Q_A species (enhanced F_0 level), which are suggested to be also tri-

plet promoting [16, 17], is not enough to induce photodamage at moderate light intensity.

At photoinhibitory light intensity the loss of electron transport and variable fluorescence was slower in the CO_2-depleted cells (possessing enhanced F_0 level) than in the CO_2-containing ones. The F_0 rise was also accompanied with partial protection against irreversible photoinhibitory damage in *Chl. reinhardtii* cells illuminated under aerobic conditions at low temperature (5 °C) and in spinach thylakoids illuminated under anaerobic conditions [6, 15]. Taken together we suggest that CO_2-depletion and other treatments which inhibit electron transport between Q_A and Q_B and result in accumulation of stable reduced Q_A species during illumination, can provide partial protection against irreversible photoinhibition.

In the present work we found that inhibition of electron transport between Q_A and Q_B either by CO_2-depletion or DCMU addition provides partial protection against irreversible photoinhibition. It is a possible explanation that due to the block of electron transport at Q_A the electrons cannot reach the primary action site of photoinhibition, where the main damaging mechanism is triggered, thus the extent of photoinhibition is diminished. In agreement with this assumption recent flash-induced fluorescence yield [23] and EPR measurements [24] provided strong evidence that the non-heme iron is the target of the primary photoinhibitory damage.

Several observations indicate that under physiological conditions donor-side photoinhibition might be predominant [25, 26]. Light-induced formation of high ΔpH and acidification of the thylakoid lumen may induce Ca^{2+} release from the waters-plitting system [27]. Due to the impaired water-splitting system illumination can cause overoxidation of the donor side of PS II and generation of very positive reactive radicals, Y_Z^+ and $P\ 680^+$ [1, 2]. In the CO_2-depleted alga cells limited electron flow at the acceptor side of PS II, due to inhibition of electron transport between Q_A and Q_B and formation of stable reduced Q_A molecules, prevents the overoxidation of the donor side. Thus CO_2-depletion can provide protection against donor-side photoinhibition similarly to the DCMU provided protection in donor-side inactivated systems [1]. The protective effect of CO_2-depletion against photoinhibition observed in the present work might be explained by the in vivo existence of a donor-side mechanism of photoinhibition.

Acknowledgements

This work was supported by the Hungarian National Committee for Technological Development in the PHARE ACCORD programmes, No. H9112-0147 and No. H9112-0150 as well as by the Hungarian National Science Foundation OTKA I/3 2667/1991 and OTKA I/3 2668/1991.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 81–87 (1995)

Lipoxygenase Activity of Plasmalemma and its Relation to Plant Cell Senescence and Stress Response

F. MACRÌ, E. BRAIDOT, E. PETRUSSA and A. VIANELLO

Cattedre di Fisiologia Vegetale e Biochimica Vegetale,
Università degli Studi di Udine, via Cotonificio 108, I-33100 Udine, Italy

Lipoxygenase activity was investigated on the surface of sunftower (*Helianthus annuus* L.) protoplasts and in purified soybean (*Glycine max* L.) plasma membranes. The enzyme activity was inhibited by salicylhydroxamic acid, propyl gallate and butylated hydroxytoluene; while calcium, hydrogen peroxide and nucleotide triphosphates were stimulatory. It is concluded that although lipoxygenase is regarded as a soluble enzyme common in plants, its association with membranes may constitute an important step in the chain of events leading to utilization of this enzyme in stress reponses.

Lipoxygenase(s) (LOX) is an ubiquitous enzyme in eukaryotes. It is an iron non-heme containing protein that catalyzes the oxidation of fatty acids with a *cis,cis*-1,4-pentadiene configuration to hydroperoxides that contain *cis,trans* conjugated double bonds [1]. The molecular ·structure and functions of several mammalian LOX have been described [2]. These enzymes utilize arachidonic acid as the main substrate to produce precursors of chemical messengers, e.g. leukotrienes and lipoxins. In plants, LOX pathway is in many respects similar to the "arachidonic acid cascade" in animals. An array of enzymes further metabolize hydroperoxides produced by lipoxygenases: hydroperoxide dehydrase, hydroperoxide lyase, hydroperoxide isomerase, hydroperoxide-dependent lipoxygenase and lipoxygenase itself, giving rise to a variety of compounds (e.g. traumatin, jasmonic acid and hydroxy acids) [3]. For these LOX different functions have been postulated. They appear to be involved in plant growth, development, senescence, as well as in defense mechanisms, synthesis of regulatory molecules and responses to wounding [4].

Hitherto, plant LOX have been studied in soluble and particulate fractions. The available evidence suggests that the bulk of LOX is localized in the cytosol [4], although some reports indicate their presence in mitochondria [5–8], vacuoles [9], chloroplasts [7, 10, 11] and microsomal membranes [5, 8, 12–14]. Only in a recent paper a plasmalemma-bound LOX from tomato pericarp has been described [15].

The fine definition of the subcellular localization is a difficult objective, since soluble enzymes might adhere non-specifically to membranes. On the other hand, their presence on the plasmalemma could suggest a more direct involvement in plant senescence, response to wounding and resistance to disease [4]. This task was reached by two different approaches. First, LOX was measured at the surface of plant protoplasts. Sec-

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ond, the activity was also evaluated in plasma membranes isolated using aqueous polymer two-phase partitioning, which permits to obtain highly purified preparations [16].

Lipoxygenase activity at the surface of sunflower protoplasts

Sunflower (*Helianthus annuus* L.) protoplasts are a suitable system to evaluate LOX activity at their surface. After cell wall digestion, the plasmalemma remains intact and contamination with cytoplasmic LOX is easily amended. In fact, no LOX activity was detected in the resuspending medium after protoplasts have been removed. On the contrary, in the presence of protoplasts a lipoxygenase-like activity may be revealed by two independent assay methods (Fig. 1). The addition of linolenic acid to protoplasts induces an increase of conjugated diene formation (Panel A, trace a) and an oxygen consumption (Panel B, trace a). After an initial lag-phase, linolenic acid-induced conjugated diene formation increases linearly up to 4 min. Salicylhydroxamic acid SHAM (trace b) and propyl gallate (trace c), two typical inhibitors of LOX, and the antioxidant butylated hydroxytoluene, BHT (trace d) are inhibitory. External calcium stimulates this activity since, in the presence of EGTA or EDTA (trace e), linolenic-acid dependent diene formation is lower and identical to the control without $CaCl_2$ (trace f).

Lipoxygenase activity in isolated soybean plasma membranes

Figure 2 shows that the addition of linolenic acid to isolated soybean (*Glycine max* L.) membranes also induces the formation of conjugated diene (Panel A, trace a) and an oxygen consumption (Panel B, trace a), with a stoichiometric ratio that approximated to one. The double reciprocal plot of diene formation versus concentration of linolenic acid shows that the enzyme had a K_m value of ca. 200 μM and a V_{max} of ca. 1400 nmol · (mg protein · min)$^{-1}$ (inset of panel A). Again the typical inhibitors of LOX, such as SHAM (trace b), propyl gallate (trace c) and nordihydroguaiaretic acid, NDGA (trace d), loweres both linolenic acid-dependent activities. Linoleic acid also induces diene formation and oxygen uptake, with a K_m very similar to that estimated for linolenic acid, but with a lower V_{max} value: ca. 200 nmol · (mg protein · min)$^{-1}$ (inset of panel A). Thus, it seems that the hydroperoxide produced may act as a non-competitive inhibitor for this reaction [17]. Similar results were also obtained using purified plasma membranes from sunflower hypocotyls (not shown).

To determine the orientation of the enzyme in the vesicles, the effect of *p*-chloromercuryphenylsufonate PCMPS, an impermeant inhibitor of plasma membrane redox systems [18], on linolenic acid-dependent oxygen consumption was assayed. PCMPS does not inhibit this activity, but when the vesicles are permeabilized by 0.01 % Triton X-100, which *per se* lowered oxygen consumption, the activity is ca. 65% inhibited. These results, hence, suggest for the presence of a LOX in soybean isolated plasma membranes, with the catalytic site inside the vesicles.

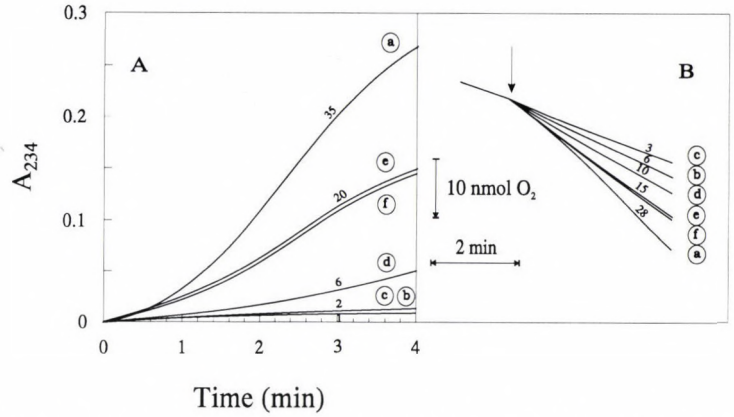

Fig. 1. Conjugated diene formation (Panel A) and oxygen consumption (Panel B) induced by linolenic acid addition in sunflower protoplasts. a) Control; b) 2 mM SHAM; c) 2 mM propyl gallate; d) 1 mM BHT; e) 1 mM EGTA or 1 mM EDTA; f) without 1 mM $CaCl_2$. Values next to each trace are expressed as nmol · · $(10^6$ protoplasts · min)$^{-1}$. These rates were calculated by considering only the linear segment of the traces. The arrow indicates the addition of linolenic acid

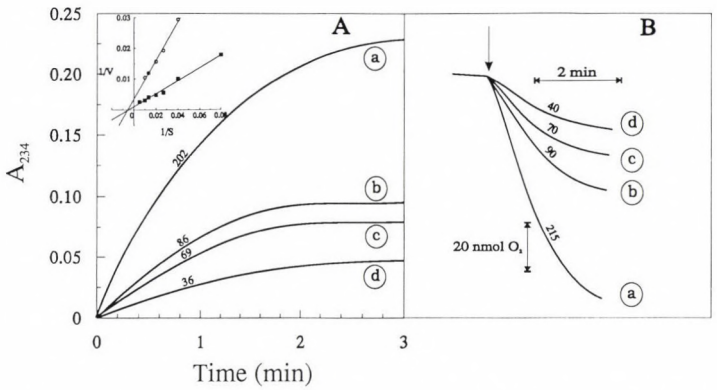

Fig. 2. Conjugated diene formation (Panel A) and oxygen consumption (Panel B) induced by linolenic acid in soybean plasma membranes. a) Control; b) 200 μM SHAM; c) 100 μM propyl gallate; d) 5 μM NDGA. Figures next to each trace, indicating the initial rate, are expressed as nmol · (mg protein · min)$^{-1}$. Inset of panel A represents the double reciprocal plot of diene formation *versus* linolenic (■) and linoleic acid (○) concentration. The arrow indicates the addition of linolenic acid

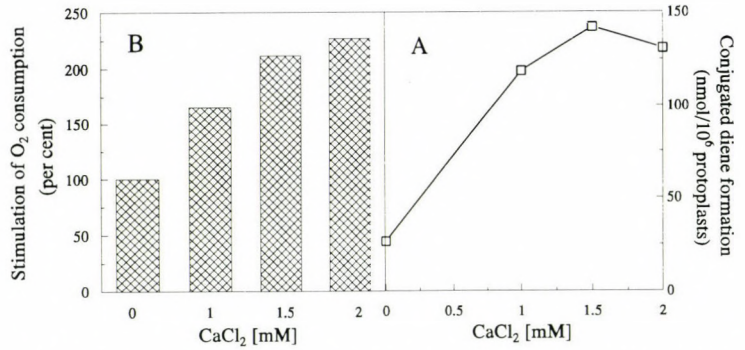

Fig. 3. Effect of $CaCl_2$ concentration on conjugated diene formation (Panel A) or oxygen consumption (Panel B) induced by linolenic acid in sunflower protoplasts. Conjugated diene were determined after 4 min; oxygen consumption (initial rate) is expressed as per cent values by considering the linolenic acid induced O_2 uptake as 100

Modulation of lipoxygenase activity of plasmalemma by calcium, hydrogen peroxide and nucleotide triphosphates

In an attempt to further characterize this plasmalemma-bound lipoxygenase, the effect of some molecules known to affect this activity was tested [2]. As previously observed, protoplast LOX is stimulated by addition of external $CaCl_2$ up to 1.5 mM (Fig. 3). In addition, LOX activity is stimulated by ATP and the effect is more marked in the presence of calcium than in its absence (results not shown). Hydrogen peroxide also stimulates this activity over the concentration range of 2 to 5 nM, while higher concentrations result in a lower extent of enzyme stimulation, to become then inhibitory (Fig. 4).

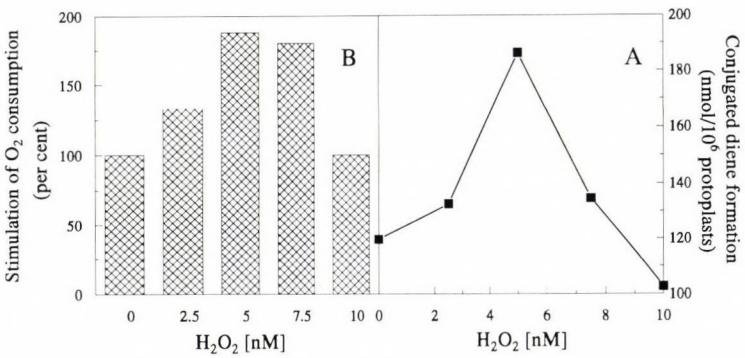

Fig. 4. Effect of H_2O_2 concentration on conjugated diene formation (Panel A) or oxygen consumption (Panel B) induced by linolenic acid in sunflower protoplasts in the presence of 1 mM EGTA. Conjugated diene were determined after 4 min; oxygen consumption (initial rate) is expressed as per cent values by considering the linolenic acid induced O_2 uptake as 100

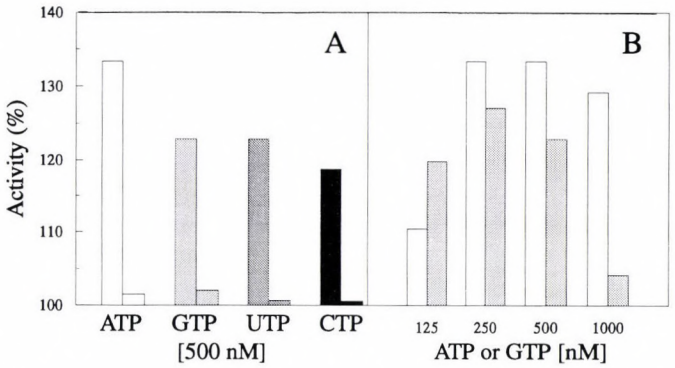

Fig. 5. Effect of nucleotide triphosphates on the initial rate of linolenic acid dependent oxygen consumption in soybean plasma membranes (bars on the left) or soluble fraction (bars on the right) (Panel A). Effect of ATP or GTP concentrations on the initial rate of linolenic acid dependent oxygen consumption in soybean plasma membranes (Panel B). ATP (open bars); GTP (light dotted bars); UTP (heavy dotted bars); CTP (solid bars). Per cent stimulation was calculated by considering as 100 control value [186 nmol $O_2 \cdot (\text{mg protein} \cdot \text{min})^{-1}$]

These results are confirmed using purified soybean plasma membranes. LOX activity is also modulated by calcium, hydrogen peroxide and some nucleotide triphosphates. The latter stimulate LOX in the following order: ATP > GTP = UTP > CTP (Fig. 5, panel A, bars on the left). Both ATP and GTP effects depend on their concentration (Panel B), being stimulatory over the concentration range of 125 to 1000 nM, with a maximum at 250 nM. LOX activity is more sensitive to GTP than ATP concentration variations. Conversely, soluble LOX is not affected by nucleotide triphosphates (Panel A, bars on the right) and, therefore, this feature permits to distinguish it from the membrane-bound one.

Involvement of plasmalemma lipoxygenase in senescence and response of plants to stress

Plant LOX have been regarded for a long time as soluble enzymes. Several isoforms have been identified and their occurrence, structure, pH optima and calcium requirements are variable, albeit their mode of action appears to be very similar [4].

The problem of subcellular localization is far to be resolved. Besides its cytosolic localization, LOX have been detected in different types of cellular membranes [5–15] and, in some cases, appear to be tightly bound to reconstituted membranes [5, 8, 11–15], or present in specific organelles (chloroplasts or mitochondria) [5–8, 10]. In particular, membrane-bound LOX, found in microsomal vesicles from tomato fruit and senescing carnation petals have been partially characterized [12, 13]. Both utilize linoleic acid as substrate, have a pH optimum near to 6.0 and are distinguishable from their soluble

counterpart. Membrane fractionation studies indicate that the tomato fruit enzyme is associated with thylakoid membranes [11]. In a recent paper, another membrane-associated LOX from tomato pericarp has been demonstrated [15]. This appears to be present in plasmalemma as well as in tonoplast, is polymorphic and, similar to the soluble enzyme, has a molecular size of 92 kDa. It seems that charge modification may allow association of soluble LOX to membranes.

The latter results are confirmed by the findings that LOX activity is also associated to soybean plasma membrane and is present at the surface of sunflower protoplasts. According to others [15], the characteristics of soluble and membrane-bound LOX are similar, but the latter appears to differ from the former because it is stimulated by nucleotide triphosphates.

The physiological role of LOX is still controversial. As outlined in previous reviews, LOX may be involved in plant growth and development, biosynthesis of regulatory molecules and plant responses to wounding and pathogen infection [4, 19]. However, if this enzyme is, in some way, involved in some of these phenomena, such as senescence or defense mechanisms, one would expect that it is attached to membranes [4]. Indeed, lipolytic enzymes liberate polyunsaturated fatty acids that trigger lipoxygenase activity and, subsequently, other enzymic and nonenzymic reactions, leading to membrane deterioration and disassembly [20, 21]. LOX found in soybean membrane and in sunflower protoplasts can better accomplish this role, since it is plasma membrane-bound.

LOX activity is modulated by calcium, hydrogen peroxide and nucleotide triphosphates. A rise in Ca^{2+} concentration is one of the initial events that characterizes plant senescence [20]. This increase stimulates membrane phospholipase but also LOX that, therefore, appear to be modulated by the same signal. The effect of H_2O_2 has been already shown for animal [3] and soybean [22] LOX, and may be related to the ability of this peroxide to convert Fe^{2+}, at the active site of the resting enzyme, to Fe^{3+} [4]. Since hydrogen peroxide appears to be generated in the initial phases of pathogen infection [23], this molecule may function as a signal to subsequently induce LOX activity, which is also activated during this type of responses [24]. Stimulation by ATP has also been shown for animal LOX [3]. Soybean plasma membrane LOX is stimulated not only by ATP, but also by GTP, UTP and CTP in a concentration dependent manner. The activity is very sensitive to GTP concentration variation and this stimulation resembles that exerted by guanine (and other) nucleotides in the regulation of auxin-stimulated NADH oxidase of soybean plasma membrane [25].

Undoubtedly LOX is a soluble enzyme widespreadly distributed in plants. Its presence in plasma membranes and perhaps in organelles raises, however, the possibility for a more specific role in plant cell processes that imply a rapid phospholipid membrane metabolism. In animal cell a Ca^{2+}-dependent translocation of 5-lipoxygenase from the cytosol to a membrane-bound site, where it is utilized for leukotriene synthesis, has been described [26]. It is possible to speculate that also in plant cells, possessing a LOX with similar characteristics [27], membrane association may constitute an important step in the chain of events leading to utilization of this enzyme in response to stress. The similarity

between membrane-bound and soluble LOX and the observation that this enzyme is not an integral membrane protein [15], support this contention. On the other hand, its presence in phloem exudates opens the possibility that LOX may traverse the membranes to assume a systemic function [28].

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 89–92 (1995)

The Role of Lipid Desaturation in Protection Mechanism Against Temperature Stresses

Z. GOMBOS[1], ÉVA HIDEG[1], O. ZSIROS[1], HAJIME WADA[2] and NORIO MURATA[2]

[1] Institute of Plant Biology, Biological Research Center, Hung. Acad. of Sciences, Szeged, Hungary
[2] National Institute for Basic Biology, Okazaki, Japan

The sequential elimination of fatty-acid desaturases of membrane glycerol lipids resulted in mutant strains of cyanobacterium, *Synechocystis PCC6803*, with well defined fatty-acid compositions. The physiological studies on these strains revealed that polyunsaturated fatty acids are essential in adaptation to low temperatures and in tolerance to low-temperature stresses. In contrast to these findings we were able to demonstrate that fatty-acid desaturation has minor if any affect on high-temperature tolerance. The complete elimination of polyunsaturated fatty-acid enhanced the susceptibility of cells to low-temperature photoinhibition. Our results suggest a determinative role of membrane structure in recovery processes following the photoinhibitory inactivation of photosynthetic machinery.

Glycerolipidis of thylakoid membranes not only serve as a major constituent of the membrane-forming bilayers, but they also provide hydrophobic ligands to membranous proteins (Doyle and Yu, 1985). It has been suggested that the four most abundant glycerolipids (MGD, DGD, SQDG and PG) of thylakoid membranes in the chloroplast of higher plants and in the cells of cyanobacteria play important roles maintaining in the photosynthetic electron transport machinery.

Cyanobacterial cells because of their similarity to chloroplasts of higher plants with respect to cellular structure and glycerolipid composition can be used as a simple in vivo model system to study the correlation of membrane structure and its physical characteristics to thermal behaviors of photosynthetic functions. The degree of unsaturation of acyl residues of glycerolipids determines the physical characteristics of membranes and, consequently, the molecular motions of the lipids in the membranes (Quinn et al., 1989). Therefore one can postulate that fatty-acid unsaturation of the glycerolipid should affect various functions of photosynthetic membranes. To examine how the fatty-acid unsaturation is related to thermal tolerance of photosynthesis we developed a unique experimental system in which the desaturation of fatty-acids can be eliminated in a stepwise manner (Wada et al., 1992). Two mutants, Fad^{6} and Fad^{12}, which were defective in desaturation of fatty-acids, were isolated from Synechocystis PCC6803, a transformable cyanobacterial strain (Wada and Murata, 1990). The selection was based on the growth rate of the strains at 22 °C. Fad^{12} mutant, defective in desaturation at the $delta^{12}$ position, grow much slower than the wild type. In order to clone a gene for the desaturation at the $delta^{12}$ position a genomic library was constructed and screened with the capability of complementing Fad^{12} in the growth at low temperature. A 1.5-kbp fragment was found to complement Fad^{12}. It contained one open-reading frame of 1053 bp corresponding to 351

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amino acid residues. This gene, termed *desA*, encodes an acyl-lipid desaturase, which can introduce the second double bond at the delta^{12} position. The genetic manipulation of fatty-acid desaturation was carried out with *desA*. The *desA* gene interrupted by the Km^r cartridge was constructed to allow manipulation of the extent of desaturation of fatty-acids in membrane lipids of *Synechocystis PCC6803*. The wild type and Fad^6 mutant, defective in desaturation at delta^6 position, were transformed with this disrupted gene. *DesA* was used to transform a low-temperature sensitive cyanobacterial strain, Anacystis nidulans R2-Spc in this strain there is only one fatty-acid desaturase which can introduce double bond at delta^9 position. The transformation of this strain with *desA* resulted in the introduction an extra double bond at delta^{12} position.

These transformant and wild-type strains were used for the study of membrane structure and its relationship to thermal properties of photosynthesis.

Effect of Fatty-Acid Unsaturation on Chilling Tolerance and Heat Tolerance

Chilling tolerance. We investigated the effects of unsaturation of glycerolipids of photosynthetic membranes on the tolerance to low temperatures of the cyanobacteria, *Synechocystis PCC6803* a chilling-resistant and *Anacystis nidulans R2-Spc* a chilling-sensitive strains.

The complete elimination of polyunsaturated fatty-acids from *Synechocystis PCC6803* cells by genetic manipulation did not alter significantly the low-temperature tolerance of transformant cells compared to that of the wild type. However, the susceptibility of the Fad^6 cells transformed with disrupted *desA* to low temperature photoinhibition enhanced remarkably (Gombos et al., 1992). Our experiments with isolated thylakoids which do not possess any recovery capability pointed out that the damage site of photoinhibitory process is not affected by the alteration of elimination of the polyunsaturated fatty-acids from the photosynthetic membranes. Nevertheless, the recovery process, which involves the synthesis of the proteins of the photosynthetic reaction center and their insertion into the photosynthetic membranes, was severely affected by the modification of fatty-acid composition of the photosynthetic membranes (Gombos et al., unpublished).

The transformation of the chilling sensitive *A. nidulans R2-Spc* with *desA* resulted in a transformant strain possessing dienoic fatty-acids while in the wild-type cells there are only monoenoic fatty-acids. The emergence of polyunsaturated fatty-acids manifested in enhanced tolerance to low-temperature (Wada et al., 1990). The temperature of phase transition, from the liquid-crystalline to the phase separated state, of the cytoplasmic membranes in the transformant cells appeared at 4 °C and that of the wild type at 6 °C. This result emphasized that the fatty-acid unsaturation plays an important role in chilling tolerance.

Heat tolerance. The effect of the unsaturation of glycerolipids of thylakoid membranes on the heat tolerance of photosynthesis was studied *in vivo* by mutants and transformants of *Synechocystis PCC6803* and *A. nidulans R2-Spc*.

Table

Modification of fatty acid unsaturation by transformation of *Anacystis nidulans R2-Spc* and *Synechocystis PCC6803* with *desA* and the disrupted *desA*, respectively

Strain	Fatty-acid									
	16:0	16:1	16:2	18:0	18:1	18:2 6, 9	18:2 9, 12	18:3 6, 9, 12	18:3 9, 12, 15	18:4
Synechocystis PCC6803										
WT	+			+	+	+	+	+	+	+
WT/*desA*$^-$	+			+	+	+	–	–	–	–
Fad^6	+			+	+	–	+	–	+	–
Fad^6/*desA*$^-$	+			+	+	–	–	–	–	–
Anacystis nidulans R2-Spc										
WT	+	+	–	+	+	–				
WT/*desA*$^+$	+	+	+	+	+	+				

+ symbol indicates the existing fatty acids in the strains.

The thermal properties of the photosynthetic activities of Fad^{12} mutant of *Synechocystis PCC6803* lacking delta^{12} desaturase, consequently it does not contain trienoic fatty-acids, were compared with those of wild type (Gombos et al., 1991). Despite great diversity in the extent of unsaturation of fatty-acids between the wild type and Fad^{12} no significant differences were found in the heat stability of photosynthetic activities. These results demonstrate that the trienoic fatty-acids of the lipids in thylakoid membranes do not affect thermal properties of the photosynthetic activities.

To investigate the effect of dienoic fatty-acids on thermal properties of photosynthetic membranes a transformant of *Synechocystis PCC6803* was used (Gombos et al., 1994a). Fad^6/desA::Km^r strain completely lacks dienoic fatty-acid. The experimental results indicate that elimination of dienoic lipid molecules decreases, to a small but distinct extent, the heat tolerance of photosynthetic oxygen evolution. This conclusion contrasts with the previous hypothesis that the heat tolerance enhanced upon an increase in the level of saturation of membrane lipids. It is also shown that light does not affect the nature of the effect of lipid unsaturation on the heat tolerance of photosynthesis.

The contribution of the enhanced level of unsaturation in membrane lipids to the ability of the photosynthetic processes to tolerate heat stress was studied in a transgenic cyanobacterium, *A. nidulans R2-Spc/desA* (Gombos et al., 1994b). The introduction a second double bond at delta^{12} position had no detectable effect on the heat-stress tolerance. These results imply that an increase in unsaturation of membrane lipids does not affect the tolerance of photosynthetic processes toward heat stress.

Conclusion

The role of unsaturation of glycerolipids was studied in transgenic cyanobacteria. Our results demonstrate that an increase in the unsaturation of fatty-acids enhanced the tolerance of photosynthetic machinery to chilling stress but not to heat stress and that such an increase does not affect photosynthesis within the range of physiological temperatures.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 93–97 (1995)

Effects of Nitrite and Nitrate on Potassium Uptake of Rice and Wheat Seedlings at Different pH Values

F. ZSOLDOS, Á. VASHEGYI, A. PÉCSVÁRADI, E. HAUNOLD* and P. HERGER*

Attila József University, Department of Plant Physiology, P.O. Box 654, H-6701 Szeged, Hungary
* Forschungszentrum Seibersdorf, Hauptabteilung Agrarforschung und Biotechnologie, Austria

Rice (*Oryza sativa* L. cv. Oryzella) and wheat *Triticum aestivum* L. cv. GK Öthalom) seedlings were grown in nutrient solutions with nitrite and nitrate supplies. Potassium uptake at different pH values was followed by tracer technique. Results showed that in the physiological pH range plants can assimilate nitrite taken up by roots but at low pH the toxic HNO_2 is formed. Potassium influx and efflux rates indicate plasma membrane damage at low pH. When nitrate is present in the outer medium, no marked changes were observed in the flux parameters.

In most soils, the level of nitrite (NO_2^-) is usually negligible (ca.1 $\mu g\ g^{-1}$), but certain environmental stress conditions, e.g. waterlogging, some soil-applied herbicides, heavy metals, etc., may lead to an accumulation of NO_2^- in the soil solution to concentrations which are toxic to plants (Haynes, 1986; Haynes and Sherlock, 1986; Marschner, 1986).

NO_2^- is an obligatory intermediate in the assimilation of NO_3 and usually present at low concentrations in NO_3 grown plants (Beevers and Hageman, 1969; Breteler and Luczak, 1982). However, it may accumulate in plant tissues under certain stress conditions and be toxic to plants (Peirson and Elliot, 1981).

Nitrite toxicity is believed to be due to undissociated HNO_2 rather than NO_2^- (Breteler and Luczak, 1982; Lee, 1979; Zsoldos et al., 1993). Detailed investigations of the effects of NO_2^- on plants are therefore of importance.

Materials and Methods

Rice (*Oryza sativa* L. cv. Oryzella) and wheat (*Triticum aestivum* L. cv. GK Öthalom) seedlings were selected for our investigations because these plants are varyingly sensitive to environmental stress factors (Zsoldos and Karvaly, 1979). The seedlings were cultivated in either 0.5 mM $CaSO_4$, full nutrient or nutrient deficient solutions. The composition of the nutrient solution was as follows: $NaNO_2$ or $NaNO_3$ from 0.1 to 10 mM, KH_2PO_4 1 mM, Na_2HPO_4 0.5 mM, $CaCl_2$ 0.5 mM, $MgSO_4$ 0.5 mM and micronutrients as described earlier (Zsoldos et al., 1986). The pH of the absorption solutions was initially adjusted to the appropriate values, with 0.1 M HCl or 0.1 M NaOH as needed. All nutrient solutions were renewed every second day.

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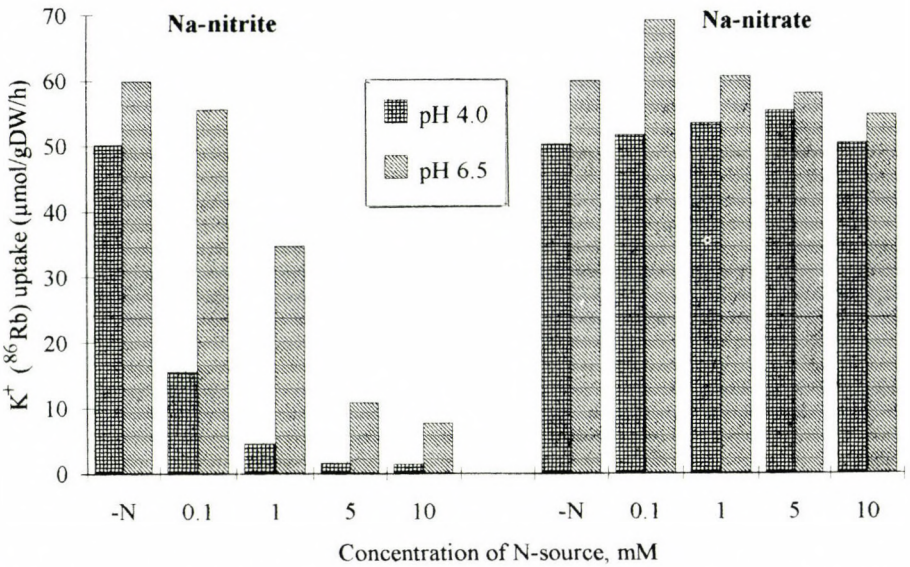

Fig. 1. Eects of varying the $NaNO_2$ and $NaNO_3$ supply on K^+(^{86}Rb) uptake of roots of 7-day-old rice seedlings at different pH values. Plants were grown in 0.5 mM $CaSO_4$ solution at pH 6.5 in the absence of $NaNO_2$ and $NaNO_3$. Uptake solution: 1 mM K^+(^{86}Rb)Cl + 0.5 mM $CaCl_2$+ $NaNO_2$ or $NaNO_3$ as indicated. SD ≤ 10

The ion inlux and eftlux experiments were followed via tracer techniques under controlled conditions. For technical reasons ^{86}Rb was used to monitor the K^+ movement. A series of results from three independent experiments were carried out with three parallel samples, and the data given are averages.

Results and Discussion

When applied without pretreatment, increasing concentrations of $NaNO_2$ caused decreases in the K^+ uptake of rice roots (Fig. 1, left). The retarding effect of NO_2^- on the K^+ uptake of roots was markedly influenced by the H^+ concentration of the uptake solution. A decrease of the pH of the outer medium led to an increased inhibitory effect of NO_2^- on the ion uptake of the roots. When $NaNO_3$ was present in the uptake solution, marked changes were not observed in the K^+ uptake of rice roots (Fig. 1, right).

Figure 2 shows the effects of varying the $NaNO_2$ and $NaNO_3$ supply on the K^+ uptake of roots of wheat seedlings at different pH values. The data clearly show that increasing concentrations of $NaNO_3$ caused decreases in the inhibitory effect of $NaNO_2$ even at low pH too. The retarding effect of $NaNO_2$ on the K^+ uptake of roots was less expressed or disappeared at higher pH.

Parallel efflux studies, illustrated in Fig. 3 left, clearly show that no signifcant change was found in the K^+ efflux of rice roots at pH 6.5. In contrast, at pH 4 higher

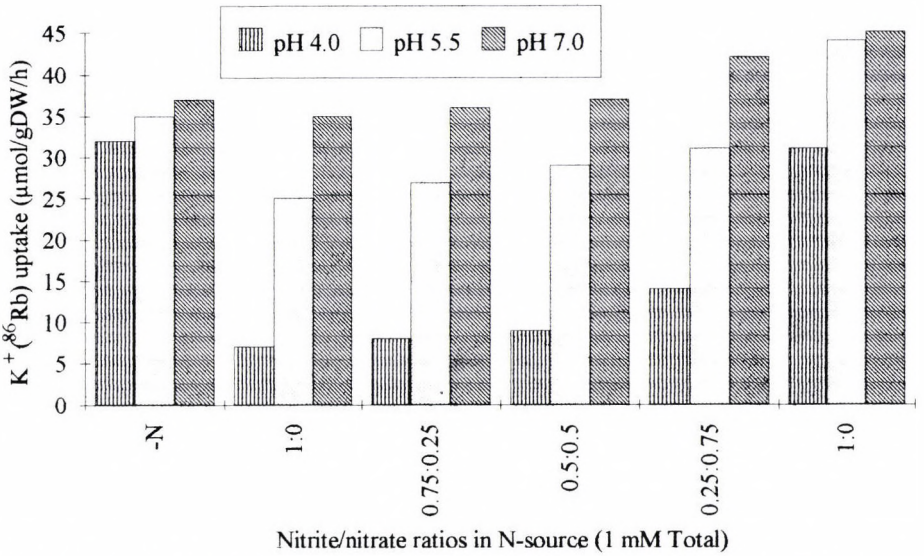

Fig. 2. Effects of varying the $NaNO_2$ and $NaNO_3$ supply on K^+(^{86}Rb) uptake of roots of 6-day-old wheat seedlings at different pH values. Plants were grown in 0.5 mM $CaSO_4$ solution at pH 6 in the absence of $NaNO_2$ and $NaNO_3$. Uptake solution: 1 mM K^+(^{86}Rb)Cl + 0.5 mM $CaCl_2$+ NO_2 or $NaNO_3$ as indicated. SD ≤ 9

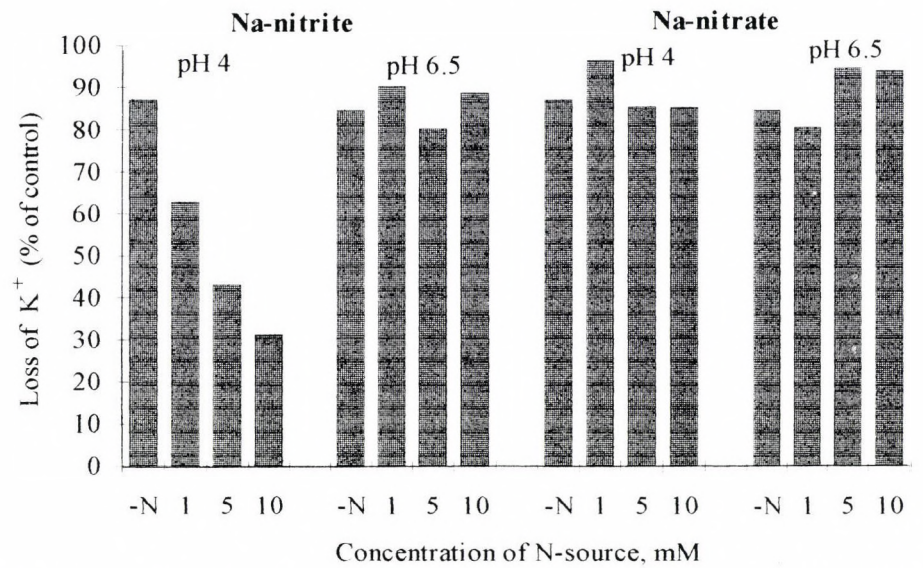

Fig. 3. Effects of varying the $NaNO_2$ and $NaNO_3$ supply on K^+(^{86}Rb) efflux of roots of 7-day-old rice seedlings at different pH values. Plants were grown in 0.5 mM $CaSO_4$ solution at pH 6 in the absence of $NaNO_2$ and $NaNO_3$. Uptake solution: 1 mM K^+(^{86}Rb)Cl + 0.5 mM $CaCl_2$. Efflux medium: 0.5 mM $CaCl_2$+ $NaNO_2$ or $NaNO_3$ as indicated. Efflux time: 1 h. Control: K^+(^{86}Rb) uptake during 1 h. SD ≤ 9

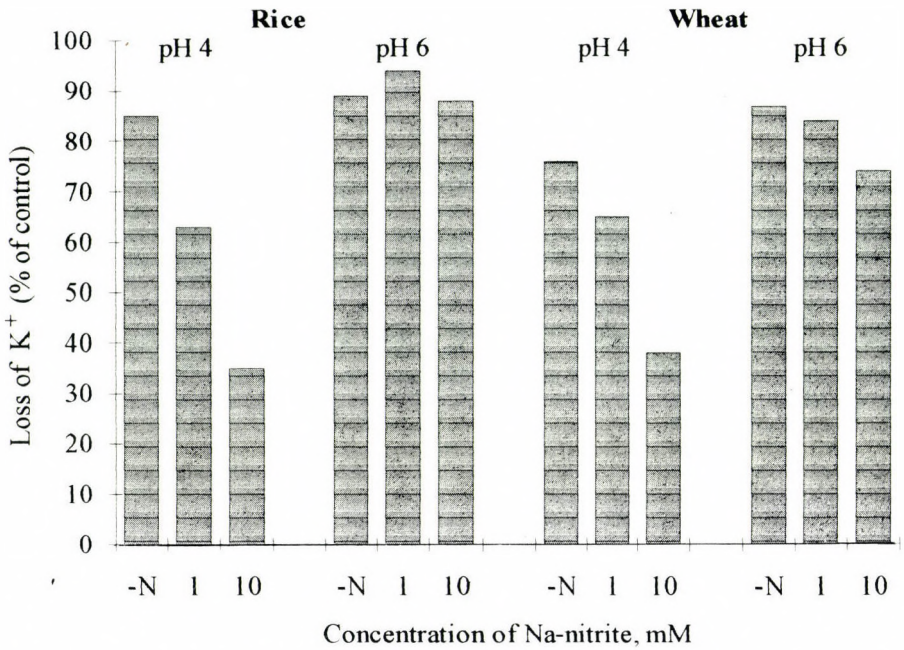

Fig. 4. Effects of varying the $NaNO_2$ supply on K^+(^{86}Rb) efflux of roots of 7-day-old rice wheat seedlings at different pH values. Otherwise as in Fig. 3

concentrations of $NaNO_2$ caused significant increase in the loss of K^+ of the roots. When $NaNO_3$ was present in the efflux medium no significant change was observed in the K^+ efflux of roots (Fig. 3. right).

Figure 4 shows the effects of varying the $NaNO_2$ supply on the K^+ efflux of roots of rice and wheat seedlings at different pH values. From the data it can be stated that treatment with $NaNO_2$ of seedlings the K^+ efflux of the roots was about the same in both seedlings. It means that the plasma membrane damage of roots could also be the same in both species during a short time NO_2^- treatment.

To summarize, our results show that in the physiological pH range plants can assimilate NO_2^- taken up by roots, but at low pH HNO_2 is formed, usually considered as strong oxidant, and toxic substance. The results of K^+ efflux and K^+ influx into the roots strongly suggest a distinct role of $NO_2^-(HNO_2)$ in plasma membrane damage at low pH. It is noteworthy, when NO_3^- was present in the outer medium marked changes were neither observed in the K^+ influx nor in the K^+ efflux of the roots.

Acknowledgements

This work was supported in part by a grant from the PHARE ACCORD PROGRAMME (Contract No. 9112-0124) and by the Hungarian National Science Research Foundation (Project No. I/3. 462).

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 99–104 (1995)

Effect of Nitrite and Nitrate Nutrition on Ethylene Production by Wheat Seedlings

IRMA TARI and L. SZÉN

Department of Plant Physiology, Attila József University, H-6701 Szeged, P.O. Box 654, Hungary

The nature of nitrogen (N) source in the nutrient solution determines the ethylene production of plants. This effect depends on the pH of the medium.

Ethylene release by shoots of 5-day-old wheat seedlings increased compared to the N-deficient status of seedlings when 1 mM nitrate had been applied as N source in the nutrient solution, 1 mM nitrite treatment however resulted in an insignificant increase. No differences were found in ethylene production of shoots growing at different pH-s (pH 4; 5.5 and 7.0) in the same nutrient solution.

In contrast, the ethylene production of the root system exhibited about twofold increase at pH 4.0 in N-deficient and nitrite-treated plants compared to those growing at pH 5.5 and 7.0. Nitrate as a sole N source resulted in significantly lower ethylene release by roots than N-deficiency and nitrite treatment at pH 4.0 but there were no differences in ethylene production by nitrate-treated roots at different pH-s. Roots contained more 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene than shoots and the ACC level – with the exception of the N-deficient samples at pH 4.0 – positively correlated to the ethylene production.

The increased ethylene production by the root system at pH 4.0 coincided with the inhibition of root elongation by nitrite nutrition and the early period of N deficiency.

The nature of N (nitrogen) source in the nutrient solution determines the ethylene production of plants.

Substituting ammonia for nitrate resulted in chlorosis and an increased ethylene evolution by tomato plants (Barker and Corey, 1988). Urea or nitrate nutrition did not affect ethylene production of plants and the growth rate was normal (Barker and Corey, 1990).

Nutrient deficiencies may also modulate ethylene production. Corn seedlings deficient in nitrogen produced less ethylene than control (Rengel and Kordan, 1988). Drew et al. (1989) found decreased ethylene production, 1-aminocyclopropane-1-carboxylic acid (ACC) content, ACC synthase and ACC oxidase activities in nitrogen deficient corn seedlings.

Nitrogen source affected the growth rate and dry matter accumulation of plants, but the effect was dependent on pH (Zsoldos et al., 1993).

Exogenous ethylene inhibits the longitudinal expansion of pea epicotyls (Apelbaum and Burg, 1972), stem and leaf growth of seedlings in Gramineae family (van Andel and Verkerke, 1978) and the elongation of roots (Chadwick and Burg, 1970). Endogenous increases in ethylene production resulted in reduced root length in pea (Eliasson and Bollmark, 1988).

The aim of the present paper was to examine the effect of different nitrogen sources (nitrite, nitrate) on ethylene production, ACC and 1-(malonylamino)cyclopropare-

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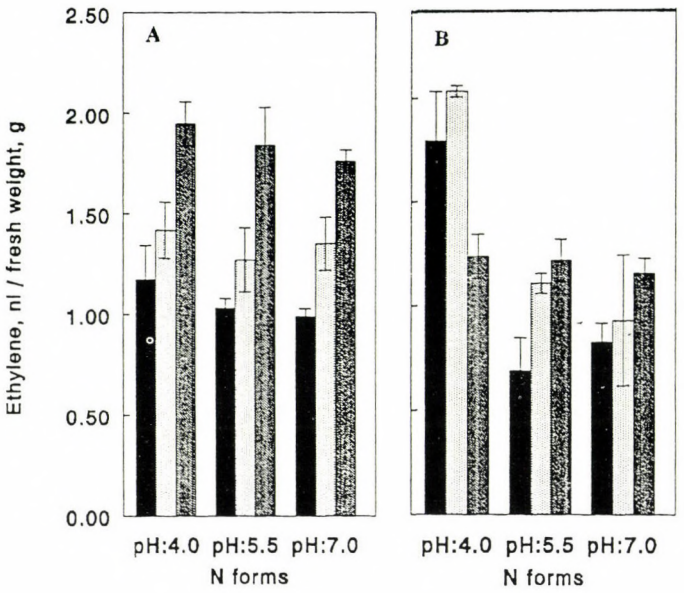

Fig. 1. Effect of N supply on ethylene production by 5-day-old wheat shoots (A) and roots (B) at different pH-s

pane-1-carboxylic acid (MACC) levels in leaves and roots of wheat seedlings and to reveal the possible physiological significance of ethylene in the growth inhibition caused by nitrite at pH = 4 as a sole N source.

Materials and Methods

Plant material. Seeds of winter wheat (*Triticum aestivum* L. cv. GK Öthalom) were germinated in thermostat in Petri dishes at 25 °C for 24 hours. The seedlings were cultivated in hydroponics for 5 days under controlled conditions. The composition of the N deficient solution was as follows: 1 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$ and micronutrients as described earlier (Zsoldos et al., 1993). Concentrations of nitrite or nitrate in the form of natrium salt as a sole N source were 1 mM. The measurement of ethylene production occurred at 5 days of age.

Ethylene determination. Ethylene was measured by a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and an alumina column. Flow rates were adjusted to 35 ml min^{-1} for He, 30 ml min^{-1} for H_2 and 300 ml min^{-1}

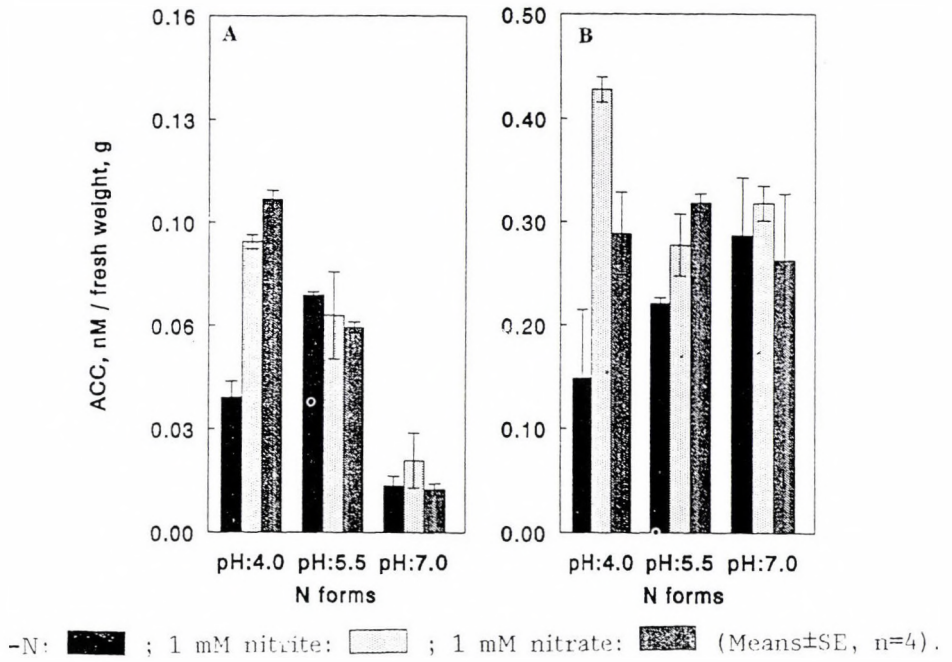

Fig. 2. Effect of N supply on ACC content of 5-day-old wheat shoots (A) and roots (B) at different pH-s

for air. The oven, injector and detector temperatures were 100, 120 and 160 °C, respectively. The tubes with plant material were incubated in darkness for 1 hour when 2.5 ml samples were withdrawn from the gas phase and measured by GC. All experiments were run in five parallels and were repeated two times.

Determination of ACC and MACC. The ACC content was measured by the method of Lizada and Yang (1979) after chemical conversion to ethylene, and MACC was determined as ACC after hydrolysis in 2 M HCl.

Results and Discussion

Nitrate as a sole nitrogen source increased the ethylene production by shoots of wheat plants compared to the nitrite nutrition and N-deficient status of seedlings (Fig. 1A). We did not find significant differences in ethylene production of shoots grown in the same nutrient solution at pH = 4, 5.5 or 7.

In contrast, the ethylene production of the root system exhibited considerable increases at pH = 4 in N-deficient and nitrite-treated plants in comparison with the nitrate

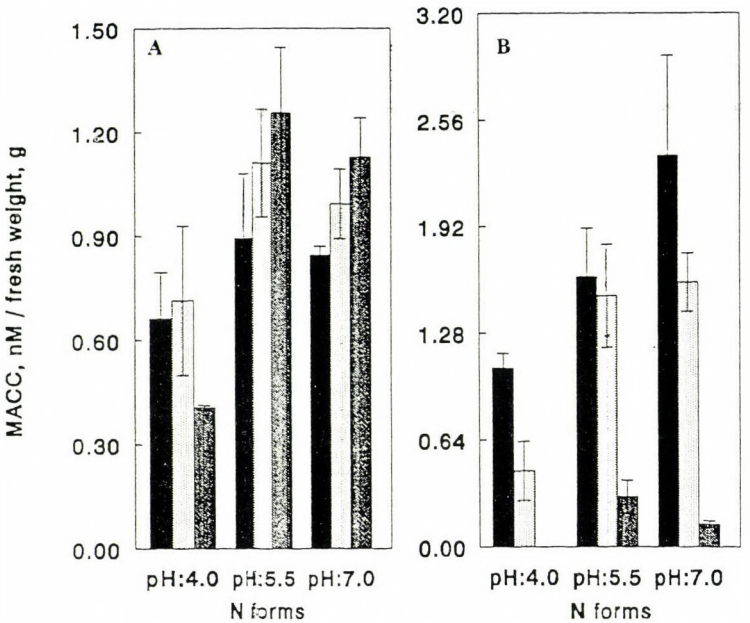

Fig. 3. Effect of N supply on MACC content of 5-day-old wheat shoots (A) and roots (B) at different pH-s

nutrition at the same pH or in the same nutrient solution at higher pH-s. Nitrate as a sole N source did not change ethylene production of the root system at the pH-s used (Fig. lB).

The level of ACC, the immediate precursor of ethylene, followed the pattern for ethylene in the shoots of seedlings growing at pH = 4 (Fig. 2A) with the exception of nitrite-treated shoots. In these tissues the high ACC content was not correlated with the ethylene release, no correlation could be found at higher pH values, too. This can be explained by different efficiency of ACC conversion to ethylene at different pH-s.

Roots contained more ACC than shoots and the ACC level was positively correlated with ethylene production with the exception of the N-deficient roots at pH = 4 (Fig. 2B).

MACC, a major nonvolatile metabolite of ACC accumulates in stressed as well as in non-stressed tissues and can readily be transported from leaves to roots (Machaèkova et al., 1992).

MACC could be detected both in shoots and roots (Fig. 3A and B), and irrespective of pH, nitrate nutrition prevented the accumulation of the conjugate in the root system.

Ethylene production of shoots was decreased by nitrite nutrition and N-deficiency but the treatments did not affect the shoot height of plants (Fig. 4A).

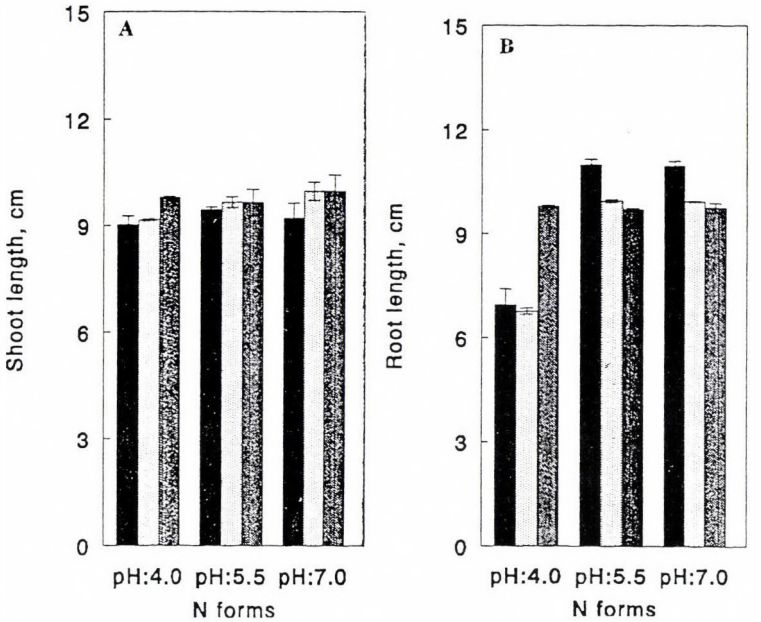

Fig. 4. Effect of N supply on shoot (A) and root length (B) of 5-day-old wheat seedlings at different pH-s

Increases in ethylene production by the root system at pH = 4 in N-deficient plants or in the presence of nitrite coincided with the inhibition of root elongation (Fig. 4B).

In present work, earlier informations on the effect of different nitrogen forms on ethylene production were extended to nitrite.

Nitrite nutrition decreased the ethylene production of wheat shoots at pH-s used but increased the ethylene evolution by roots at pH = 4 in comparison with nitrate. Although ACC and MACC patterns are different, the effect of nitrite on ethylene release does not differ significantly from the effect of nitrogen deficiency in 5-day-old wheat seedlings.

The increased ethylene production of the root system at pH = 4 coincides with the inhibition of root elongation by nitrite nutrition.

Acknowledgement

This work was supported by PHARE ACCORD Funds. Contract No. H 9112-0124.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 105–118 (1995)

The Influence of Enriched Root-zone CO_2 Concentrations on Growth, Nitrogen Metabolism and Root HCO_3^- Incorporation in Salinity Stressed *Lycopersicon esculentum*

M. D. CRAMER and S. H. LIPS

Plant Adaptation Research, J. Blaustein Institute for Desert Research, Sede Boqer 84990, Israel

Tomato plants grown with salinity reduce/assimilate a larger proportion of NO_3^- taken up in the roots than do non-salinized plants. We investigated whether enriched CO_2 in the root solution could increase anaplerotic provision of carbon for root nitrogen assimilation in salinity stressed plants. Tomato seedlings were grown in hydroponic culture with and without 100 mM NaCl and with aeration of the root solution with either ambient or CO_2 enriched air (5000 μmol mol^{-1}). The salinity treated plants accumulated more dry weight and higher total N when the roots were supplied with CO_2 enriched aeration than when aerated with ambient air. Concentrations of K^+ in the leaves and roots were higher in plants treated with enriched CO_2. Enriched root-zone CO_2 increased root incorporation of dissolved inorganic carbon (DIC). In salinity stressed plants the products of $DI^{14}C$ were diverted into amino acid synthesis to a greater extent than in non-salinized plants. It was concluded that enriched root-zone DIC could provide an increased anaplerotic source of carbon for amino acid synthesis in roots, partially ameliorating the influence of salinity on plant growth.

Salinity stress results in a complex suite of changes in the physiology of plants depending on the severity of the stress, environmental factors and the sensitivity of the plant to salinity. One of the symptoms of salinity stress in tomato plants is an alteration in the site of NO_3^- reduction/assimilation within the plant (Cramer et al., unpublished). In tomato plants ca 80% of NRA is located within the shoots of non-salinized plants (Lorenz, 1976), although the proportion of root reduction of NO_3^- varies with environmental factors and the concentration of NO_3^- supplied to the roots (Andrews et al., 1992). This alteration in the site of NO_3^- reduction/assimilation brought about by salinity may be related to the inhibition of NO_3 uptake in the presence of salinity (Aslam et al., 1984) and the interference of salinity with the loading of NO_3^- onto the xylem. Root reduction/assimilation of NO_3 is energetically more costly to the plant than shoot reduction and may result in increased efflux of the NO_3^- from the root due to higher root tissue NO_3^- concentrations (Poorter et al., 1991).

The translocation of NO_3^- may occur through the operation of a K^+-shuttle described by Lips et al. (1970). In this proposed shuttle K^+-malate is translocated from the shoot to the root and malate decarboxylated in the root to yield HCO_3^- which may exchange directly or indirectly for further NO_3 and the K^+-NO_3^- translocated to the shoot.

Abbreviations – DIC, dissolved inorganic carbon; H/LRZC, high/low root zone CO_2 concentrations; LWR, leaf weight ratio; NR(A), nitrate reductase (activity); PEP(c), phosphoenolpyruvate (carboxylase).

0238–1249/95/$ 5.00

This shuttle may serve to maintain the pH balance in the shoot by balancing the OH^- produced during shoot NO_3^- reduction (Raven and Smith, 1976) through export of malate to the root. The operation of the K^+-shuttle provides a link between the influence of salinity on K^+ concentrations and the translocation of NO_3^- to the shoot. It is well known that Na^+ displaces other cations from the membrane surface (Cramer et al., 1987). This interference with K^+ uptake may therefore inhibit NO_3^- translocation to the shoot.

Salinity stress in tomato plants results in a decrease in the ability of the roots to incorporate dissolved inorganic carbon (DIC) and a shift in the products of this incorporation towards the synthesis of amino acids instead of organic acids (Cramer et al., unpublished). Increased synthesis of amino acids utilizing the products of root DIC incorporation has been observed previously with plants supplied with NH_4^+ nutrition in comparison to those supplied with NO_3^- nutrition (Cramer et al., 1993). Since NH_4^+ taken up by the root is almost exclusively assimilated within the roots, it is possible that increased anaplerotic provision of carbon from root DIC incorporation for amino acid synthesis in the root is a feature of root assimilation of nitrogen.

The nutrient solutions used for hydroponic culture of plants are usually aerated with ambient air. This is an artificial situation since the DIC of soils is usually higher than that of ambient air due to root and microbial respiratory activity. Only in situations where the soil is very porous, dry, or lacking in organic material is the DIC concentration likely to be low. In this investigation we examined the possibility that elevated root-zone DIC concentrations could stimulate the provision of anaplerotic carbon for root nitrogen assimilation in plants grown with and without salinity. We determined whether this could influence the growth of the plants, uptake of NO_3^-, the site of NO_3^- reduction/assimilation, tissue concentrations of nitrogen, Na^+ and K^+ and the products formed from root HCO_3^- incorporation.

Materials and Methods

Plant Material

Fourteen day-old seedlings of *Lycopersicon esculentum* L cv. F144 grown on a mixture of vermiculite and compost, were transferred into hydroponic culture after carefully rinsing the roots with deionized water. The hypocotyls of the plants were wrapped with foam rubber and the plants inserted through holes in the lids of 140 l hydroponic tanks (24 plants per tank) containing 130 l half strength Long Ashton nutrient medium (Hewitt, 1966) which was modified to contain 1 mM $NaNO_3$and 60 mg l^{-1} Fe-sequestrene 138 (Ciba-Geigy, Switzerland) as the iron source. Salinity was supplied at 0 and 100 mM NaCl. After transfer of the plants into hydroponics the concentration of NaCl was increased at a rate of 50 mM d^{-1}. The plants were utilized 10 to 15 days after transfer into hydroponics.

The pH of the medium (6.5) was adjusted on a daily basis with HCl, and the nutrient solution was replaced weekly. The concentration of NO_3^- in the nutrient solution was

maintained at 1 mM NO_3 by additions of NO_3 every third day. The nutrient solution was strongly aerated and the temperature was maintained at 20 ± 1 °C with aquarium heaters. The plants were grown in a greenhouse in April with a midday irradiance of ca 700 µmol m^{-2} s^{-1} and midday temperature ranging between 28 and 30 °C with minimum night temperatures of 15 °C. Relative humidity varied between 40% and 60% in the day and 70% to 90% at night.

Carbon dioxide was supplied at elevated levels (5000 µmol mol^{-1}) by enriching ambient air with pure CO_2 using a peristaltic pump. The CO_2 concentration was monitored continuously with an APPA-3 IRGA (Anarad Inc., Santa Barbara, CA). Work with enriched root-zone CO_2 concentrations has been criticised because the possibility exists that the CO_2 may be released from the root-zone and may be assimilated by photosynthesis (Enoch and Olesen, 1993). In these experiments the CO_2 was flushed from below the lids of the containers with a high flow rate of ambient air. A powerful fan was used to maintain air movement around the plant shoots. The air sampled routinely from above the lids of the hydroponic containers with an ADC LCA2 IRGA (Analytical Development Corporation, Hoddesdon, England) was found not to differ from ambient CO_2 concentrations.

Biomass Parameters

Ten replicate plants of each treatment were harvested after 15 days in hydroponics. The roots of the plants were rinsed in running deionized water and the plants divided into leaf, stem and root components. The roots were blotted dry, the components weighed and the plant material dried at 80 °C for 48 hours and re-weighed. The moisture (g H_2O g^{-1} dry weight), shoot:root ratios and leaf weight ratios (LWR) were calculated.

Tissue NO_3^-, total N, K^+ and Na^+ concentrations

The oven dried (48 hours, 80 °C) plant material of each treatment (n = 10) was milled in a Wiley mill (A. H. Thomas, Philadelphia, PA) using a 60 mesh screen and 0.05 g material digested in 35 cm long tubes with 4 ml 3.4% (w/v) salicylic acid in 13.5 M sulphuric acid and 0.2 g selenium. The samples were digested at room temperature for 2 h, 200 °C for 1 h, 270 °C for 1 h and 370 °C for 1 h. The digest was diluted and assayed for NH_4^+ according to Solorzano (1969). Concentrations of K^+ and Na^+ in the digested samples were determined using a Corning 410 Flame, Photometer (Halstead, England).

For determination of tissue NO_3^- concentrations a homogenous sample of tissue of ca 0.3 g was quenched in liquid N_2, suspended in 10 ml distilled water and placed in a water bath at 80 °C for 2 h. The extract was mixed, sub-samples of 1 ml centrifuged in 1.5 ml Eppendorf tubes on a Kubota KM15200 microfuge (Tokyo, Japan) at 6000 rpm and the NO_3^-, concentration of the supernatant determined according to Cataldo et al. (1975).

Nitrate reductase activity (NRA)

Four plants of each treatment were harvested between 10 h and 12 h and the roots washed in deionised water. The plants were separated into root, stem and leaf components. After weighing, the components were sliced into 1 to 2 mm sections and duplicate homogenous samples of ca 0.3 g of tissue vacuum infiltrated in 10 ml 0.1 M $NaHPO_4$ with 1% isopropanol and 100 mM KNO_3 (pH 7.2) until degassing occurred (1 min). One of the pair of samples was killed and extracted by immersing the vials in boiling water for 2 h immediately after vacuum infiltration to provide a measure of the initial NO_2^- concentrations. The remaining samples were incubated anaerobically for 30 min in a shaking water bath at 30 °C in the dark prior to killing and extraction. The samples were assayed for NO_2^- according to Snel and Snel (1949).

Nitrate uptake and transpiration

Four plants of each treatment were transferred into 250 ml fresh, aerated (1.2 l h^{-1} either 360 or 5000 µmol mol^{-1} CO_2) nutrient solution containing 10 mM MES (pH 6.5) and 2 mM $NaNO_3$ 9 h prior to the beginning of the experiment. Sub-samples (1 ml) of nutrient solution were taken from 6 h onwards at regular intervals for 24 h. At each sampling time the volume of the nutrient solution remaining in the cylinders was determined gravimetrically for calculation of the transpiration rates. The NO_3 concentration in the nutrient solutions was determined according to Cataldo et al. (1975). The K^+ concentrations were determined using a Corning 410 Flame Photometer.

$NaH^{14}CO_3$ Feeding and fractionation

The nutrient solutions were renewed 12 h prior to the experiment. A stock of fresh nutrient solution was aerated with low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2 concentrations. At the commencement of the experiment (10 h 30´), 4 plants of each treatment were transferred to sealed 250 ml containers with fresh nutrient medium (20 °C). To each container 1.4 µmol $NaHCO_3$ containing 2.8 MBq $NaH^{14}CO_3$ was added. After 1 h the plants were removed from the nutrient solutions, the roots rinsed in deionized water and blotted dry. The plants were divided into leaf, stem and root components, weighed, quenched in liquid N_2 and stored at −18 °C. Extraction of the tissue, fractionation into 80% (v/v) ethanol insoluble (structural and bound components), water insoluble (non-polar compounds), neutral (carbohydrates), amino acid and organic acid component was performed according to Cramer et al. (1993).

Results

Biomass accumulation and plant water relations

Plants grown with salinity accumulated less biomass than non-salinized plants (Table 1). Enriched ("high") root-zone CO_2 concentrations (HRZC) resulted in greater biomass accumulation in leaves and roots of salinized plants than ambient ("low") root-zone CO_2 concentrations (LRZC) but reduced biomass accumulation in leaves and roots of non-salinized plants. There were no large differences between the leaf weight ratios of the treatments (Table 1) Plants grown with salinity had lower moisture contents in the stems and roots than non-salinized plants. There was a small, but significant, increase in the moisture content of HRZC-salinity compared to LRZC-salinity grown plants (Table 1). Daily transpiration rates, expressed on the basis of leaf fresh weight, were ca 2-fold lower in salinized than in non-salinized plants, but RZC had only a slight influence on transpiration (data not shown).

Tissue NO_3^-, total N, K^+ and Na^+

There was a lower concentration of NO_3^- in the salinized than in the non-salinized plants but RZC had no significant influence on the tissue NO_3^- concentrations (Fig. 1).

Table 1

The dry weights, moisture contents and leaf weight ratios of tomato plants grown for 15 d hydroponic culture with either 0 or 100 mM NaCl and aeration with either low (360 μmol mol^{-1}) or high (5000 μmol mol^{-1}) CO_2. The means±SE are followed letters indicating whether the salinity had a significant ($P<0.05$, Fisher's protected LSD) influence (n=10)

	0 mM NaCl		100 mM NaCl	
	Low CO_2	High CO_2	Low CO_2	High CO_2
		Dry weight (g)		
Leaf	0.73 ±0.03d	0.65 ±0.02c	0.40 ±0.20a	0.50 ±0.20b
Stem	0.31 ±0.01b	0.30 ±0.01b	0.18 ±0.01a	0.21 ±0.01a
Root	0.26 ±0.01c	0.21 ±0.01b	0.17 ±0.01a	0.21 ±0.01b
		Moisture (g H_2O g^{-1} DW)		
Leaf	6.83 ±0.14ab	6.81 ±0.09ab	7.21 ±0.25b	6.40 ±0.09a
Stem	15.7 ±0.03b	15.5 ±0.3b	11.6 ±0.3a	12.1 ±0.2a
Root	17.0 ±0.03c	16.7 ±0.3c	13.4 ±0.2a	14.7 ±0.3b
		Leaf weight ratio		
	0.56 ±0.1b	0.56 ±0.01b	0.53 ±0.01a	0.55 ±0.01b

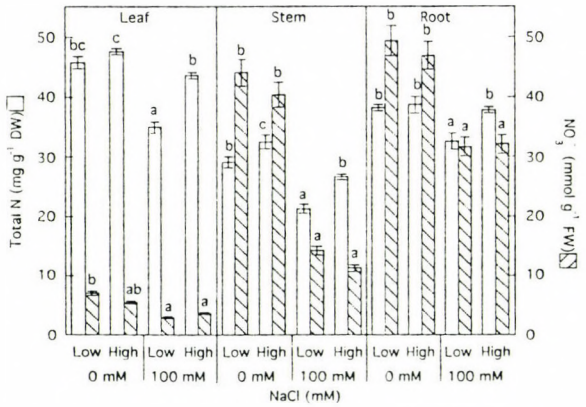

Fig. 1. The influence of either 0 or 100 mM NaCl and aeration with either low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2 on the total N (n=10) and NO_3^- (n=4) concentrations in the tissue of tomato plants. The error bars represent the SE of the means. Dissimilar letters above of bars indicate significant (P>0.05) differences between means determined from analysis of variance followed by Fisher's protected LSD tests. The leaf, stem and root components were tested separately

Total N was lower in salinized than in non-salinized plants (Fig. 1) and was significantly higher in HRZC-salinity grown plants than in LRZC-salinity grown plants.

Plants grown with salinity had significantly lower K^+ and higher Na^+ concentrations than non-salinized plants (Fig. 2). The concentration of K^+ was significantly increased in leaf and root tissue of HRZC-salinity grown plants in comparison with LRZC-

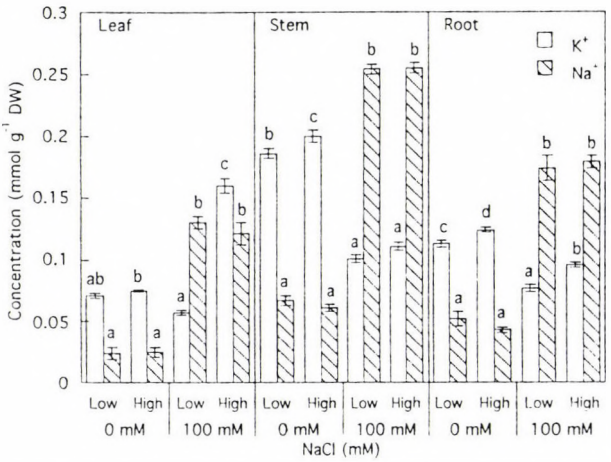

Fig. 2. The influence of either 0 or 100 mM NaCl and aeration with either low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2 on the K^+ and Na^+ concentrations in the tissue of tomato plants 15 d after transfer into hydroponic culture (n=10). Statistics as in Fig. 1

salinity grown plants. There were only small increases in the concentration of K^+ in stem and root tissue of non-salinized plants gro wn with HRZC compared to those grown with LRZC.

Nitrate and K^+ uptake rates

Nitrate and K^+ uptake rates were higher in non-salinized than in salinized plants (Table 2). In addition HRZC compared with LRZC increased the uptake of NO_3^-, in salinized plants.

Nitrate reductase activity (NRA)

Stem and root NRA, measured *in vivo* as the dark anaerobic accumulation of NO_2, were unchanged by RZC or salinity treatments (Fig. 3). Plants grown with salinity had higher NRA in leaves than non-salinized plants and HRZC-salinity grown plants had higher NRA in comparison to LRZC-salinity grown plants.

Table 2

Nitrate and K^+ uptake and rates of HCO_3^- incorporation (nmol g^{-1} root DW s^{-1}) in tomato plants 14 d after transfer into hydroponic culture with 0 or 100 mM NaCl and aeration with either low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2. Different letters following the means±SE indicate that salinity had a significant ($P<0.05$, Fisher's protected LSD) influence (n=4)

Root CO_2 level	Salinity (mM)	
	0	100
	NO_3^- uptake	
Low	31.3 ±3.2b	17.0 ±2.7a
High	34.1 ±4.4b	26.5 ±1.3b
	K^+ uptake	
Low	5.9 ±0.4b	0.0 ±0.5a
High	7.0 ±0.7b	1.2 ±0.2a
	HCO_3^- incorporation	
Low	2.9 ±0.2a	1.0 ±0.1a
High	26.4 ±4.0c	11.0 ±0.7a

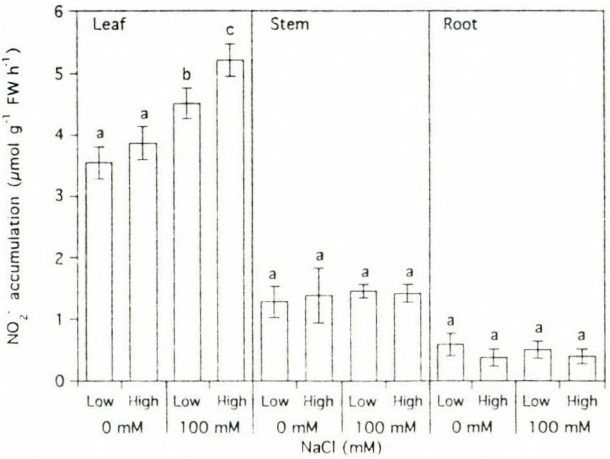

Fig. 3. The influence of either 0 or 100 mM NaCl and aeration with either low (360 μmol mol^{-1}) or high (5000 μmol mol^{-1}) CO_2 on the NRA in the tissue of tomato plants 13 d after transfer into hydroponic culture. NRA was measured *in vivo* as the accumulation of NO_2^- (n=4). Statistics as in Fig. 1

Root incorporation of $H^{14}CO_3^-$

The rate of HCO_3^- incorporation was higher in salinized than in non-salinized plants and higher in HRZC grown plants than LRZC grown plants (Table 2). After the 1 h pulse of $H^{14}CO_3^-$ supplied to the root the label was largely restricted to the roots, as can be seen from the low ^{14}C shoot:root ratios (Fig. 4). The ^{14}C shoot:root ratios were highest in the LRZC-salinity grown plants indicating that these plants translocated a greater proportion of the label to the shoots. Of the fractions separated, the neutral fraction had high ^{14}C shoot:root ratios, especially in the salinized plants. The ^{14}C shoot:root ratios of the amino acid and organic acid fractions were also high in the non-salinized HRZC treated plants.

Differences between ^{14}C-C:^{14}C-N ratios (ratio of ^{14}C labelled organic acid + neutral : amino acid fractions) of the LRZC grown salinized and non-salinized plants were small. In the leaves of plants grown HRZC and salinity the ^{14}C-C:^{14}C-N ratios were ca 2-fold higher than in plants grown with HRZC and without salinity, as a result of the large component of neutral compounds in the leaves of the salinized HRZC grown plants (Fig. 5). In the roots of HRZC grown plants salinity resulted in a greater allocation of ^{14}C to nitrogenous compounds than in the corresponding non-salinized plants. This was attributable to greater allocation of ^{14}C to amino acids in the roots of salinized HRZC grown plants than in the other treatments (Fig. 6). The absolute incorporation of all fractions was increased by HRZC relative to LRZC (Fig. 7), but the biggest increases occurred in the organic acid and insoluble fractions.

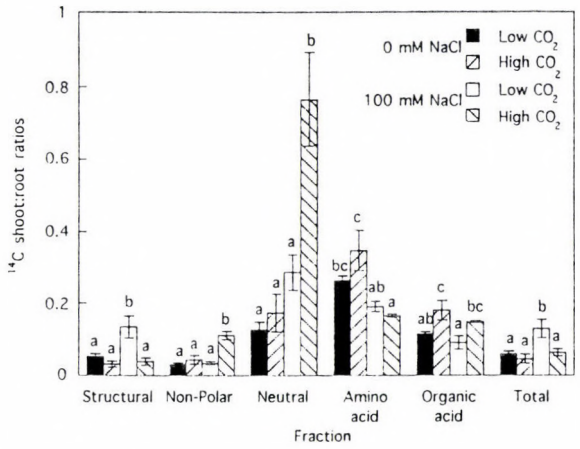

Fig. 4. The influence of either 0 or 100 mM NaCl and aeration with either low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2 on the shoot:root ratios of the ^{14}C labelled products of root incorporation of DI^{14}C by tomato plants 14 d after transfer into hydroponic culture (n=4). Dissimilar letters above of bars indicate significant (P>0.05) differences between means determined from analysis of variance followed by Fisher's protected LSD tests. The various fractions were tested separately

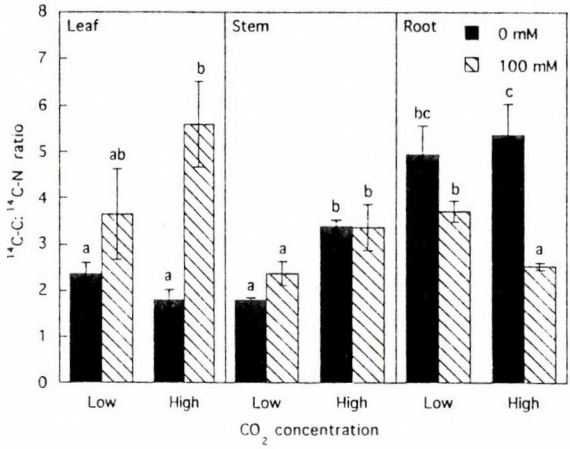

Fig. 5. The influence either 0 or 100 mM NaCl and aeration with either low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2 on the ^{14}C-C: ^{14}C-N ratios of the soluble ^{14}C labelled products of root incorporation of DI^{14}C by tomato plants 14 d after transfer into hydroponic culture (n=4). Statistics as in Fig. 1

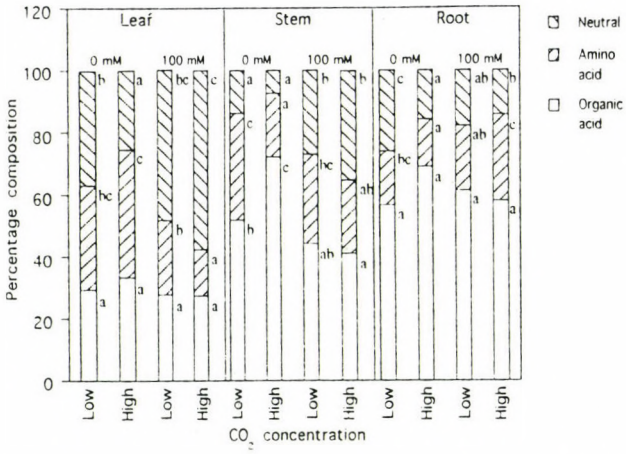

Fig. 6. The influence of either 0 or 100 mM NaCl and aeration with either low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2 on the distribution of ^{14}C between neutral, amino acid and organic acid fractions of acid stable 80% (v/v) ethanol soluble components of the leaves, stems and roots tomato plants 14 d after transfer into hydroponic culture. Dissimilar letters to right of bars indicate significant (P>0.05) differences between means determined from analysis of variance on arcsine transformed data (Zar 1984) followed by Fisher's protected LSD tests. The leaves, stems, roots and each fraction were tested separately (n=4)

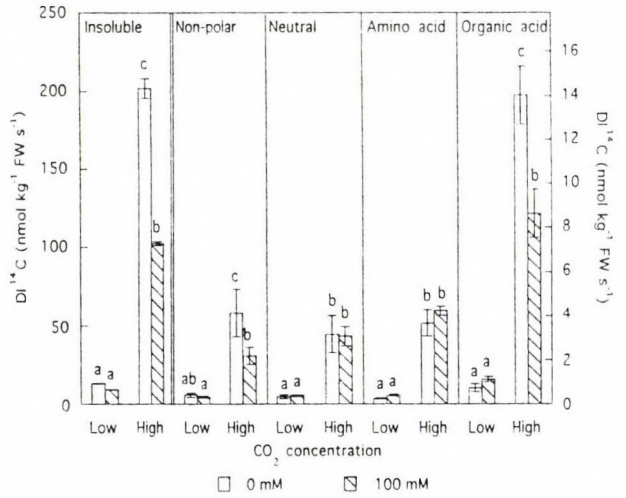

Fig. 7. The influence of either 0 or 100 mM NaCl and aeration with either low (360 µmol mol^{-1}) or high (5000 µmol mol^{-1}) CO_2 on the incorporation of DI^{14}C into labelled products by tomato plants 14 d after transfer into hydroponic culture (n=4). The insoluble fraction is plotted against the left vertical axis and the others against the right. Statistics as in Fig. 1

Discussion

Research on the response of plants to enriched atmospheric CO_2 concentrations has focussed on the shoot environment to the neglect of the root environment. Respiration of shoots has been reported to be inhibited by high CO_2 concentrations (Bunce, 1994; Thomas and Griffin, 1994; Wullschleger et al., 1994; Ziska and Bunce, 1994). Inhibition of respiration by high CO_2 has, however, been measured using CO_2 flux. Dark incorporation of HCO_3^- is likely to occur in most plant tissues due to the presence of PEPc which, in combination with carbonic anhydrase, has a high affinity for CO_2 (Edwards and Walker, 1983) and may thus explain the apparent response of respiration to CO_2 concentrations. The quantity of HCO_3^- incorporated by PEPc in roots is small in comparison to photosynthesis (Cramer and Lewis, 1993; Enoch and Olesen, 1993), but may serve an important anaplerotic role in root nitrogen assimilation (Cramer et al., 1993). It is possible that the transpiration stream translocated DIC taken up by the root to the shoot where it was photosynthetically assimilated (Enoch and Olesen, 1993). In the experiments reported here, however, the proportion of ^{14}C label from the 1 h pulse of $NaH^{14}CO_3$ located in organic products in the shoot was small (Fig. 4).

Changes in leaf weight ratios with altered RZC were small (Table 1). The dry weight was, however, significantly greater in HRZC-salinity treated plants than in LRZC-salinity treated plants. The results indicate that root incorporation of HCO_3^- can have a significant influence on plant growth, especially under salinity stress conditions. The alteration in growth occurred despite a lack of differences between the RZC treatments in the proportion of the plant comprised of photosynthetically active tissue. Since the actual amount of HCO_3^- incorporated was small relative to photosynthesis (Table 2), the influence of HRZC on plant growth must be mediated through the anaplerotic provision of carbon to the root or through some secondary affect (e.g. pH or nutrient availability) of CO_2 on root physiology (Enoch and Olesen, 1993).

Lower uptake of NO_3 (Table 2) and correspondingly lower tissue NO_3 concentrations (Fig. 1), are symptoms of salinity stress in tomato plants. Although RZC had a significant influence on NO_3^- uptake by salinized plants, the tissue NO_3^- concentrations were unaffected by RZC. The influence of salinity on NO_3^- uptake has been associated with anionic interference in NO_3^- translocation across the plasmalemma (Aslam et al., 1984). Salinity inhibited K^+ uptake almost completely (Table 2) and thus the ratio of NO_3^- :K^+ was higher in the salinized plants than in the nonsalinized plants where it was ca 5, showing that there was no direct link between K^+ and NO_3^- uptake. The low uptake of K^+ in salinized plants compared to non-salinized plants was associated with lower stem and root K^+ concentrations (Fig. 2). This may have resulted in a lack of availability of K^+ for loading of NO_3^- onto the xylem (Rufty et al., 1981) and inhibition of the translocation of NO_3^- to the shoot. In salinity treated plants the K^+-shuttle is likely to be less operative than in the non-salinized plants due to the lack of K^+. In the HRZC-salinity treated plants the need for the K^+-shuttle may be further reduced because the root has additional capacity to reduce/assimilate NO_3^- as a consequence of the greater supply of anaplerotic carbon. This

may explain why plants grown with HRZC-salinity had much higher K^+ concentrations in the leaves than in LRZC-salinity grown plants.

Although NRA was higher in the leaves of HRZC-salinity grown plants than LRZC-salinity grown plants (Fig. 3), this does not necessarily mean that the *in situ* NRA was different between these treatments as *in vivo* NRA has been shown to be poorly correlated with *in situ* activity (Cramer et al., unpublished). Despite this uncertainty concerning the *in situ* rate of NRA, total N was significantly higher in HRZC-salinity grown plants than in LRZC-salinity grown plants (Fig. 1). This corresponds with the increased anaplerotic provision of carbon for the assimilation of N in the roots of the HRZC-salinity grown plants.

The incorporation of HCO_3^- by the roots was ca 10-fold higher in the plants supplied with HRZC compared to those supplied with LRZC. This corresponds with the finding that CO_2 enriched atmosphere results in lower respiratory CO_2 release (Bunce, 1994; Thomas and Griffin, 1994; Wullschleger et al., 1994; Ziska and Bunce, 1994). Respiration rates of tomato plants grown at LRZC with 0 and 100 mM salinity were previously found to be 85 ± 10 and 109 ± 18 nmol g^{-1} root DW s^{-1} respectively (Cramer et al., unpublished). Thus at LRZC, the incorporation of HCO_3^- accounted for 3.4 and 0.9% of respiratory carbon in the 0 and 100 mM salinity treated plants respectively. The corresponding figures for the HRZC plants were 30 and 10% in the 0 and 100 mM salinity treated plants respectively. The rates of root incorporation do not therefore represent a major source of carbon for the plant, in agreement with the conclusions of Enoch and Olesen (1993), but the rates are significant with respect to respiration. The fact that the HRZC nonsalinized plants grew less well than the LRZC non-salinized plants provides further evidence that the carbon associated with root dark incorporation is not a significant contribution to the carbon budget of the plant. The presence of salinity was apparently required to enable the plants to utilize the enhanced supply of carbon for anaplerotic purposes which benefited growth. The HRZC treatment may have perturbed the carbon allocation of the non-salinized plants by diverting carbon into organic acid synthesis resulting in reduced growth.

The high ^{14}C shoot:root ratios of the neutral fraction in salinized plants (Fig. 4) may be due to two factors. It is possible that some $DI^{14}C$ was translocated to the shoot through the xylem and photosynthetically assimilated into neutral compounds within the leaves. This, however, seems unlikely since the transpiration rates of the plants grown with salinity were ca 2-fold lower than those of plants grown without salinity (data not shown). It is more likely that labelled organic acids or amino acids produced in the root were translocated to the shoot and there converted into neutral compounds.

The ca 10-fold higher $DI^{14}C$ incorporation by HRZC than LRZC plants was associated with increases in the ^{14}C label associated with all the fractions separated. Of the soluble components, the organic acid fraction was most strongly affected by RZC, particularly in the absence of salinity. The low ^{14}C-C:^{14}C-N ratios of the roots of the HRZC-salinity grown plants compared to the LRZC-salinity grown plants (Fig. 5) indicates a shift in the proportion of ^{14}C allocated to organic acids and neutral compounds towards amino acids. Thus in salinized plants, which reduce/assimilate NO_3^- in the roots,

HRZC increased PEPc activity which could provide carbon skeletons for amino acid synthesis.

It is attractive to speculate that enriched root-zone DIC may improve fruit quality of tomato crops. In initial experiments tomatoes were grown in a mixture of perlite and compost with drip irrigation without salinity on 5 mM NO_3^- with and without 0.1 mM $NaHCO_3$. Fruit titratable acidity, a measure of fruit quality, was 7.2 meq 100 ml^{-1} fruit sap without supplemented DIC and 9.3 meq 100 ml^{-1} with supplemented DIC ($P < 0.05$). Total solids (g DW g^{-1} FW) in the fruit and soluble sugar content of the fruit was also higher in HRZC than LRZC grown plants. This corresponds with the increased allocation of anaplerotic carbon to organic acids in non-salinized HRZC compared to LRZC grown plants.

Acknowledgements

We thank G. Shickman and T. Popova for technical assistance. Z. Gao conducted the fruit quality experiments. We are grateful to AID for a research grant and to the Blaustein International Center for a post-doctoral fellowship for M.D.C.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 119–122 (1995)

Involvement of Stress Related and Cell Cycle Genes in Transition from Somatic to Embryogenic Cells of Alfalfa

MÁRIA DEÁK, MÁRTA SUTUS, J. GYÖRGYEY and D. DUDITS

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, H–6701 Szeged, P.O. Box 521, Hungary

Recent molecular studies emphasise the functional role of stress-related genes during development. We have cloned and sequenced the cDNAs of several classic heat shock proteins, a heat shock transcription factor and a stress regulated calcium dependent protein kinase. Northern analysis experiments showed, that the mRNA of these genes is increased not only during stress, but also during induction of somatic embryos. To understand the role of these genes in the developmental process requires further investigation.

Uniquely, among the higher eukaryotes, the flexibility of differentiation program in flowering plants allows the reset of the ontogenesis and initiation of embryo development from somatic cells. Like in fertilized egg, after exposition of plant cells to stress factors or hormonal treatment, a defined signal transduction chain is activated, and through several cell division cycles somatic embryos can be formed in tissue cultures (for review see D. Dudits et al., 1991).

Somatic embryogenesis first was observed in carrot suspension cultures, and that experimental system has been extensively used for molecular studies (for review see L. Zimmerman, 1993). The alfalfa culture system can be especially useful for studying the embryo induction phase. The microcallus suspension culture originated from Medicago sativa RA3 genotype (described by Dudits et al., 1991) offers a possibility for the production of a large number of embryos through the following phases:

1. Micro callus suspension (MCS): The dedifferentiated callus tissues are propagated in liquid culture, where the cells are continuously dividing in the presence of naphtyl-acetic acid and kinetine. For a long period (several years) MCS keeps its potential for embryo differentiation.

2. Induction phase: application of aphysiologically high concentration (10 mg/l) strong auxin analogue (2,4-D) for a short period (0.5–3 hours).

3. Division phase: after the 2,4-D treatment intensive cell division takes place on hormone free medium (from several hours to some days).

4. Differentiation phase: as the result of division well characterized structures – globular, heart and torpedo shaped embryos – appear between the non-differentiated calli (2–4 weeks).

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At these different stages certain characteristic genes have been identified showing enhanced expression, or being crucial for that stage:

Induction: – auxin regulated (Conner et al., 1990; Oeller et al., 1993) and stress related (Deák, et al., unpublished) genes.

Division: – cell cycle genes (cdc2, cyclins, cdc25 etc.)

Embryo differentiation: – abscissic acid inducable genes, (for review see Zimmerman, 1993) small heat shock protein (Györgyey et al., 1991, Marrs et al., 1993, DeRocher and Vierling, 1993).

Induction Phase

Based on extensive Northern analysis of RNA samples from 2,4-D treated micro callus suspension we can see transcriptional activation of a large set of genes coding for structural, or stress related genes (Györgyey et al., 1991, Deák et al., unpublished).

Our observation in tissue culture experiments indicated a significant influence of wounding on embryogenic response. On primary explants the somatic embryos frequently emerge from the cut surface. The number of embryos can be significantly increased by chopping the inocula into small pieces.

Experiments searching for 2,4-D induced messages resulted the cloning the cDNAs of several heat shock proteins (Györgyey et al., 1991, Deák et al., unpublished). These indications suggested, that the stress system may be activated by this treatment.

To investigate this problem we have cloned the cDNAs of several heat shock protein (HSP) genes, between them the key regulator of this process, a heat shock transcription factor (HSF) as well (Deák et al., unpublished).

The cDNA library used for screening was constructed from the RNA of 2,4-D induced alfalfa tissue. We could identify many heat inducable clones in this library, suggesting the abundant presence of such RNAs in studied tissues. This observation was further confirmed by Northern analysis.

The Heat Shock System

Cells induce the heat shock system in response to heat or many other extreme environmental factors. The expression of active genes is stopped, and abundant HSP mRNAs and proteins appear. As a result of this process the cell stability is increased, repair activity and regeneration are stimulated (for review, see Alexandrov, 1994). Further experiments are needed to investigate, if such process can be beneficial, or even necessary for the induction of somatic embryos.

Another important question is, what can be the signal for HSF induction, generated by 2,4-D treatment. There are indications that abnormal proteins are responsible for the activation of heat shock response (Parsell and Lindquist, 1993). High concentration of 2,4-D treatment may produce such proteins. Other possibility can be, that the HSF promoter directly would respond to auxins. Also, a second messenger

system, like calcium could play a role in transmitting the inductive effect of 2,4-D. These questions are presently addressed in our experiments.

Hopefully these studies will help to learn more both about the transition from somatic to embryonic cells, and the nature of stress response as well.

Induction of Cell Division as Basic Prerequisite for Embryo Development from Somatic Cells

As the consequence of hormonal (auxin) treatment of cells, the division cycle is reactivated in the differentiated cells, with a simultaneous reprogramming of the gene expression pattern. Depending on the hormone sensitivity of the alfalfa genotype, different cell structures and different division pattern can be observed in alfalfa cells derived from protoplasts (Bögre et al., 1990). The molecular changes in hormone stimulated cells can be monitored with the help of cell cycle control genes. Previously we have cloned the homologues of yeast cdc2/cdc28 and cyclin genes from alfalfa (Hirt et al., 1991, 1992). Northern analysis experiments showed the increase of cdc2 message 1–3 days after the 2,4-D treatment. This expression peak coincided with the elevated mRNA level of S-phase specific histone H3-1 gene variant (Magyar et al., 1993). The hormonal activation could be detected on the expression of cyclin genes as well (Dedeoglu et al., unpublished).

Molecular Markers for Embryo Differentiation

As a result of the cell division, characteristic structures, as globular, heart and torpedo shaped embryos start to develop. In this phase of development the cell-cell interactions become more important.

Several genes, showing high expression in zygotic and somatic embryos have already been cloned. The so-called Late Embryogenesis Abundant (LEA) cDNAS have been found in many plant species. They respond to abscissic acid treatment (see the review from Zimmerman 1993). We have cloned the cDNA of another ABA regulated gene (ferritin), showing embryo specific expression (Deák et al., in prep.). Small HSPs have also been proved to be activated in zygotic and somatic embryos, during microsporogenesis, without any heat shock treatment (Györgyey et al., 1991; Atkinson et al., 1993; Marrs et al., 1993; DeRocher and Vierling, 1994).

The studies on the expression of these genes have further confirmed the similarity between somatic and zygotic embryos. They provide a useful molecular tool to study and manipulate this development stage in plants.

The process of embryogenesis is a very basic problem of plant biology. Considering the potentials of the use of somatic embryos in plant propagation technologies, the results in understanding the molecular basis of this process may stimulate biotechnical application.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 123–124 (1995)

Short Communications

Regulation of Freezing Tolerance by Abscisic Acid in Wheat

G. GALIBA[1], S. A. QUARRIE[2], R. TUBEROSA[3], O. VEISZ[1] and J. SUTKA[1]

[1] Agricultural Research Institute of the Hungarian Academy of Sciences, H-2462 Martonvásár, Hungary
[2] Cambridge Laboratory, John Innes Centre for Plant Science Research, Colney Lane Norwich NR4 7UJ, U.K.
[3] Istituto di Agronomia Generale e Coltivazioni Erbacee, Facolta di Agraria, Universita di Bologna, Via Filippo Re 6-8, I-40126 Bologna, Italy

The physiological changes associated with tolerance to different environmental stresses can be very similar. Several studies indicate that drought stress (limited desiccation) can induce increased frost tolerance in some plants, including cabbage, wheat and rye. Levels of frost tolerance induced by desiccation were similar to those induced by cold acclimation in rye and wheat (Cloutier and Andrews, 1984). As a consequence of the similarity between extracellular freezing and desiccation (i.e. the removal of water from the cell), plants may have developed responses to both stresses that involve common adaptive mechanisms like osmotic adjustment. Comparisons of the proteins synthesized or accumulated during induction of freezing tolerance at low temperature, in response to desiccation or abscisic acid (ABA) treatment at room temperature, have revealed a common subset of proteins (Guy 1990). Hajela et al. (1990) reported at least some of the *Arabidopsis cor* genes are responsive to water stress. The striking similarities between the cold-regulated proteins (COR) and RAB, LEA and dehydrin proteins also suggest that freezing tolerance might include tolerance to water stress.

The level of ABA has been shown to increase during environmental stresses, particularly, as a result of drought. The elevated level of ABA is thought to mediate a number of physiological, morphological and molecular changes in response to water stress. These changes are necessary for plants to survive during drought stress (Quarrie, 1989). Since ABA levels have also been shown to increase during low temperature stress, it is possible that ABA might be involved in regulation of the adaptive response of the plant during cold acclimatization. Exogenously applied ABA increased freezing tolerance in plants and in cell cultures previously grown in non-cold-acclimatization conditions (Chen and Gusta, 1983). In wheat, it has been observed that, following cold-hardening treatment, the levels of endogenous ABA increase to a greater extent in frost-tolerant varieties than in frost sensitive ones (Dörffling et al., 1990).

The frost resistance can be evaluated under controlled experimental conditions and genes influencing frost resistance have been located on group 5 chromosomes of wheat (Sutka and Snape, 1989). We localised a gene responsible for frost resistance (*Fr1*)

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on the long arm of 5A chromosome by RFLP techniques (Galiba et al., 1994). The RFLP-data show close genetic linkage (7.5 cM) between the *Fr1* and the gene responsible for vernalization requirement (*Vrn1*).

We will show here that the 5A chromosome regulates the ABA accumulation during cold hardening (Galiba et al., 1993b). The 5A chromosome has been also associated with both non-ionic stress induced free amino acid accumulation (Galiba et al., 1992) and polyamine accumulation (Galiba et al., 1993a). These results suggest that the 5A chromosome carries gene(s) involved in the regulation of osmotic adjustment as well as the regulation of ABA content. To determine the position of the gene responsible for osmotic stress induced ABA accumulation single chromosome recombinant lines have been used. We present a genetic map, based on RFLP data, about the possible position of a gene on 5A chromosome of wheat, which affects the ABA content during environmental stresses. We also show, there is a close genetic linkage between the gene involved in ABA accumulation and frost tolerance (*Fr1*).

Acknowledgements

The studies involved in this presentation were supported by the Hungarian National Research Fund No. 150, No 151 and No 5474, and by a Royal Society Hungarian Postdoctoral Fellowship to G. GALIBA.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 125–126 (1995)

Acridones: A Chemically New Group of Protonophores

MAGDOLNA DROPPA[1], G. HORVÁTH[1], I. FODORPATAKI[1], A. ISTÓKOVICS[1], GY. GARAB[1], and W. OETTMEIER[2]

[1] Institute of Plant Biology, Biological Research Center, Szeged, Hungary
[2] Lehrstuhl Biochemie der Pflanzen, Ruhr-Univesität, Bochum, Germany

Although the interaction of protonophores with photosynthetic electron transport has been extensively studied during the past decade, the mechanism of their mode of action remained uncertain. It has been demonstrated that they interfere with the oxidizing side of photosystem II (PSII) via accelerating the S_2 and S_3 oxidation states of the water splitting enzyme system [1]. Protonophores also facilitate oxidation of cytochrome (cyt) b_{559HP} [2] which can undergo fast flash induced re-reduction via Q_B^- [3]. Now, it is generally accepted that protonophores accelerate a cyclic, proton-conducting pathway around PSII [4].

The basic structures of the recently known protonophores (CCCP, FCCP, SF6847, ANT-2p) chemically are very similar because all of these molecules are polysubstituted derivates of the phenol ring. For a better understanding of the molecular mechanism of the action of protonophores, introduction of chemically new type of molecules is required. In this work, we demonstrate that acridones, 9-azaacridin-10-ones [5] completely fulfill this requirement.

At low concentrations of acridones, the thermoluminescence band at + 20 °C (B band) and + 10 °C (Q band) were strongly inhibited, while the normal electron transport activity was retained. This indicates that the concentrations of S_2 and S_3 states involved in the generation of the Q and B bands are reduced [1]. The relative resistance of the A (–20 °C) and C (+ 45 °C) bands against acridone suggests the moderate sensitivity of a S_4 and S_0, S_1 states to acridone treatment [6].

At higher acridone concentrations, an increased activity of PSI and whole chain electron transport could be observed, which is due to the typical uncoupler effect of protonophores. This is in a good agreement with the observation that acridones accelerate the decay of the electrochromic absorbance change at 520 nm. This uncoupler effect of acridones was light intensity dependent similarly to that of CCCP and SF6847 [4]. The variable part of fluorescence induction, measured in the absence of electron acceptors, was quenched even at low concentrations of acridones, but it could be restored by either a long term illumination or high light intensity [4]. This fact indicates that acridones interact with the acceptor side of PSII as well.

The FeCy oxidizable amount of $cytb_{559HP}$ decreased considerably in the presence of acridones and only part of it could be re-reduced by hydroquinone (HQ). The HQ insensitive part of the $cytb_{559}$ was, however, completely reducible by ascorbate indicating that part of the HP form was converted by acridones to IP or LP form of $cytb_{559}$.

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These results suggest that acridone acts as a typical protonophore which uncouples the electron transport, accelerates the deactivation of the S_2 and S_3 states on the donor side and facilitates the oxidation of $cytb_{559HP}$ on the acceptor side of PSII.

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Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 127–129 (1995)

Effects of Heavy Metal Induced Stress on the Photosynthetic Membrane Characteristics

G. HORVÁTH[1], MAGDOLNA DROPPA[1], L. I. HORVÁTH[2] and B. SZALONTAI[2]

[1] Institute of Plant Biology and
[2] Institute of Biophysics, Biological Research Center, Szeged, Hungary

One of the main targets of heavy metals is the chloroplast, the energy producing machinery of plants. As environmental pollutants, many heavy metals (Cu, Cd, Ni, Pb, Zn etc.) directly or indirectly interact with both the light and dark reactions of photosynthesis. Most of these cations have a repressive effect on photosynthetic electron transport and photosystem II seems to be more susceptible than photosystem I. In some cases, however, the precise action site and the underlying inhibitory mechanisms are still not clearly understood in all details. In order to understand these effects of heavy metals in plants, one cannot leave out of consideration the background processes which occur at various levels of the photosynthetic membrane. In the present work, therefore, parallel with the functional investigation of photosynthetic electron transport, we studied the heavy metal induced structural modification of proteins as well as their interaction with membrane lipids to obtain further information about the mechanism of heavy metal induced damages within thylakoids.

The heavy metal induced activity changes were characterized by fluorescence induction kinetics of isolated chloroplasts measured at various metal concentrations. As shown in Fig. 1 Cu strongly inhibits the electron transport activity, while Cd does not affect significantly the F_{max} in the concentration range used. Similarly to Cu, Pb and Zn also diminished F_{var} but Ni was completely ineffective. Since most of these cations can interact with proteins, it was interesting to look at the heavy metal induced conformational changes of membrane proteins within chloroplast thylakoids.

FTIR spectra of thylakoids can provide information about both membrane structure and protein conformation in the region of amide I and amide II vibrations of the constituent proteins (1750–1350 cm^{-1}). To avoid interference from water we have recorded thylakoid FTIR spectra in D_2O. All metal ions caused changes in the amide regions of the FTIR spectra when measured around 5 °C. These changes, however, were not decisive enough in respect to the differences of various metal induced alterations in the protein conformation. These small metal induced differences in the amide region of the FTIR spectra can be studied by perturbing the membrane structure, e.g. by increasing temperature. Figure 2 shows the temperature induced changes in untreated thylakoid membrane between 5 and 65 °C. Arrow indicate the band around 1620 cm^{-1} which could be used as monitor of the effect of the heavy metals. This band appeared similarly to the control membrane in the presence of Ni and Cd, while it was absent in the case of Cu, Zn and Pb indicating that these metals caused serious alterations in the protein organization.

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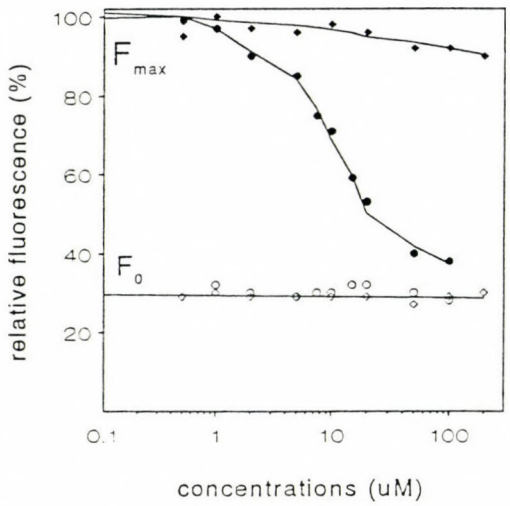

Figure 1. Effect of Cu (●, ○) and Cd (◆, ◇) on fluorescence induction of isolated thylakoids

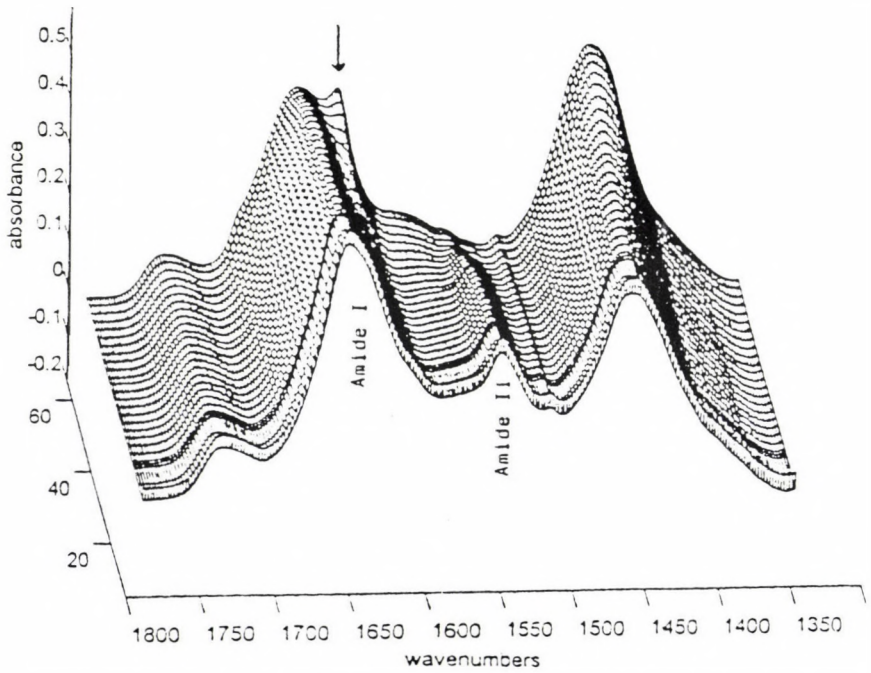

Figure 2. FTIR spectra of control chloroplasts measured at various temperarures

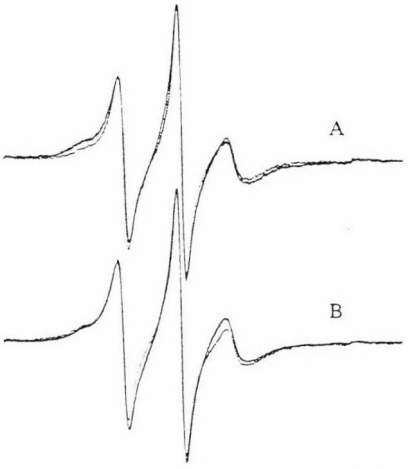

Figure 3. ESR spectra of spin labeled PG in thylakoids in the presence of Cu (A) and Cd (B); the controls are given as dashed lines

It can be expected that these structural alterations of proteins resulted in changes in the lipid-protein interaction in the heavy metal treated thylakoids.

Spin label ESR is a powerful spectroscopic technique for studying lipid-protein interactions in the thylakoid membrane. Using spin labeled phospholipids two populations of unlabeled lipids, namely motionally restricted solvation lipids and freely tumbling bulk lipids could be identified in the ESR spectra the relative weight of which depended on the lipid/protein ratio and the lipid selectivity of membrane proteins. On adding Cu, Pb and Zn ions to the thylakoids, the relative amount of solvation lipids significantly increased in a concentration dependent manner, while the lipid selectivity pattern remained unchanged. The other divalent cations (Cd, Ni) were less effective (Fig. 3).

Our results indicate that under heavy metal stress conditions, the inhibition of photosynthetic electron transport is related to the stress induced alterations of the protein conformation as well as the modifications of the lipid-protein interaction within the chloroplast thylakoid.

Acta Phytopathologica et Entomologica Hungarica 30 (1–2), pp. 131–132 (1995)

Recovery of Photosynthetic Activity of the Desiccation Tolerant Plant, *Xerophyta scabrida*

L. FODORPATAKI[1,2], MAGDOLNA DROPPA[1], G. HORVÁTH[1] and Z. TUBA[3]

[1] Biological Research Center, Institute of Plant Biology, Szeged, Hungary
[2] Babes-Bolyai University, Department of Plant Biology, Cluj, M. Kogalniceanu 1, Ro-3400, Romania
[3] University of Agricultural Sciences, Department of Botany and Plant Physiology, Gödöllő, Hungary

There is a very limited group of vascular plants which is capable of tolerating drastic water loss approaching desiccation. These species are termed resurrection plants. They endure in the state of anabiosis with their metabolism arrested, and when water becomes available, they rehydrate and their physiological functions are quickly reactivated.

Xerophyta scabrida (Pax) Th. Dur. et Schinz (Liliatae, Velloziaceae) is one of the very few poikilochlorophyllous monocotyledonous angiosperms which exhibits the specific behavior of a resurrection plant regarding the recovery of its photosynthetic activity with the onset of wet season. In contact with water, rehydration of leaves is completed in approximately 24 hours. Upon rehydration, leaves which survived in anabiosis during the long dry season, unfold quickly and start to rebuild the photosynthetic apparatus. Synthesis of a new set of photosynthetic pigments starts immediately after rehydration of leaves if they are illuminated. In conditions of continuous illumination with the incident photon flux density of 600 μmole photons $m^{-2}s^{-1}$ and under constant 27 ± 1 °C temperature, the amount of chlorophylls was only 0.24 μg cm^{-2} leaf area after 12 hours of rehydration. The pigment content increased four times in the next 12 hours (0.97 μg cm^{-2}) and it reached 4.17 μg cm^{-2} in the 60 th hour of illumination in presence of water. Chlorophyll synthesis is followed by the recovery of the photosynthetic electron transport activity. The progressively increasing ground chlorophyll fluorescence (F_0) during regreening of leaves reflected that more light harvesting antenna complexes are synthesized and assembled in the developing thylakoid system of chloroplasts, but at the beginning they cannot transfer the absorbed energy to the reaction center which are only partially maturated and are, therefore, not completely functional at this stage of development. This is the reason why more photochemically unused light energy is emitted as ground fluorescence from the expanding light harvesting pigment-protein complexes.

The F_{var}/F_{max} ratio of the fast (1.5 second) fluorescence induction is usually considered as a measure of the PS II effectiveness in primary photochemical reactions. This ratio increased very obviously during the first two days after contact of desiccated leaves with water and reached progressively a level which is constantly maintained from the third day after rehydration.

Dynamics of $^{14}CO_2$ fixation after rehydration revealed that the photosynthetic carbon reduction cycle started its function only after the stroma of chloroplasts regained its

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normal hydration status. Biochemical reactions of the dark phase of photosynthesis are recovered when the light harvesting pigment complex is already built up and photochemical reactions of the light phase are restored, being able to supply the chemical energy and the reducing power requested for synthesis of organic compounds. The establishment of a constantly high rate of $^{14}CO_2$ assimilation in the third day after rehydration indicated that the plant started its efficient active metabolism.

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PRINTED IN HUNGARY

Akadémiai Kiadó és Nyomda, Budapest

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HU ISSN 0238–1249

VOLUME 30 • NUMBERS 3–4 • 1995

acta

Phytopathologica et Entomologica Hungarica

AKADÉMIAI KIADÓ
BUDAPEST

Acta Phytopathologica et Entomologica Hungarica

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Acta Phytopathologica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

H-1117 Budapest, Prielle Kornélia u. 19–35

Manuscripts and editorial correspondence should be sent to the Editorial Office

H-1525 Budapest, P.O. Box 102
Phone: (36–1) 155–8722
Fax: (36–1) 156–3698

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Orders should be addressed to

AKADÉMIAI KIADÓ

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Subscription price for Volume 30 (1995) in 4 issues US$ 104.00, including normal postage, airmail delivery US$ 20.00.

Acta Phytopathologica is indexed in Current Contents

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STRESS PHYSIOLOGY

INSECT PESTS

Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 133–143 (1995)

Membrane Potentials of Virus-infected Cells in Cowpea Leaves Infected with Tobacco Ringspot Virus or Southern Bean Mosaic Virus

FATHI I. BELEID EL-MOSHATY[1], SHARON M. PIKE, ANTON J. NOVACKY and O. P. SEHGAL

Department of Plant Pathology, University of Missouri, Columbia, MO 65211, USA

Membrane potential responses of primary cowpea leaves to infection by tobacco ringspot virus (TRSV, local lesion reaction) and the cowpea strain of southern bean mosaic virus (SBMV, systemic infection) were investigated with glass microelectrodes. The means of initial membrane potentials in light (E_mL) of SBMV-infected cells and control cells did not change throughout the experimental period and were not significantly different from one another. The mean E_mL values of TRSV-infected cells significantly depolarized in comparison with both control and SBMV-infected cells throughout the experimental period. Coincident with lesion appearance, mean E_mL's of TRSV-infected cells were even more depolarized. Depolarization was attributed equally to the active and diffusion membrane potential components. The absence of response following treatment with the fungal toxin fusicoccin suggests that alteration in potassium channels or the H^+-ATPase itself are responsible for the decline in active potential. Darkness-induced changes in response patterns of cells in lesions may reflect alterations in the cellular pH stat or single ion channels. A higher level of lipid peroxidation and superoxide production in cowpea leaves infected with TRSV as compared to SBMV precedes lesion development (Beleid et al., 1993) and could be responsible for the changes in membrane potential.

Permeability changes during the interaction of plant cells and leaf-spot pathogens have been observed in numerous host-pathogen combinations and are commonly identified as the earliest physiological change in the hypersensitive reaction (HR). A few laboratories have employed a more sensitive technique to detect membrane alterations, the measurement of membrane potentials (E_m) using microelectrodes (9, 10, 16). This technique permits a more thorough examination of membrane status during a plant/pathogen interaction than the commonly used method of electrolyte efflux measurements.

Electrophysiology is widely used in animal and plant membranology to monitor cell membrane activities *in situ* with minimal disturbance of cell activities (15). The E_m value is a characteristic feature of each species of higher plants and reliably identifies the state of the membrane and its transport properties. Passive (diffusion) and energy-dependent (electrogenic pump, H^+-ATPase) components of the membrane potential have been experimentally characterized (14). Our laboratory previously reported pathogen-related alterations in membrane polarization during bacterial pathogenesis (9) and HR (4, 10), and after treatments with host-selective toxins (13, 18). Both membrane components

[1] First author's present address: University of Garyounis, Benghazi, Libya.

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were depolarized in the bacterial HR as ion leakage increased. The dramatic depolarization of the diffusion E_m component (10) suggested aberrations in the lipid part of the membrane. Indeed, a later investigation demonstrated that lipid peroxidation occurred in cells early during bacterial HR development (4).

As in bacteria-induced HR, active oxygen production and lipid peroxidation have been detected during virus-induced HR (1, 2). Since the electrophysiological study of viral HR by Stack and Tattar (16) indicated a possible alteration in the energy-dependent membrane potential, we examined the membrane function in two plant/virus interactions. We used the primary leaves of cowpea infected with tobacco ringspot virus (TRSV), an HR-causing viral pathogen, or the cowpea strain of southern bean mosaic virus (SBMV), a viral pathogen causing systemic infection. Our objective was to identify differences in membrane responses to viral HR and compatible viral reactions in order to relate these differences to our previously reported study of active oxygen production and lipid peroxidation in the same plant/pathogen interactions (1).

Materials and Methods

Plants and viruses

Cowpea seedlings [*Vigna unguiculata* (L.) Walp.] cv. California Blackeye, were raised in autoclaved soil in a greenhouse, or in the laboratory (23–27 °C) under fluorescent light (120 μE $m^{-2}s^{-1}$, 14 h photoperiod). TRSV and SBMV cultures were maintained by sap-inoculation on cowpea. Uniformly growing plants were selected for electrophysiological experiments and shaded for 12 h to increase susceptibility to viral infection. Twelve-day-old cowpea seedlings were used because solid brown lesions formed after TRSV inoculation in these plants. Cowpea primary leaves were rub inoculated with clarified leaf homogenates from infected plants plus Celite, buffer plus Celite, or received no treatment.

Electrophysiology

At 24-h intervals post-inoculation, leaf segments (10 × 5 mm) were cut parallel to and adjoining the midrib with a razor blade. This region was selected specifically because abundant lesion development ensured that measurements made prior to lesion appearance in TRSV-infected leaves were in cells likely undergoing the HR. After lesions developed, lesions on the edges of segments were bisected during excision. Measurements were made at the center of these bisected lesions. Segments were floated immediately on a bathing solution under fluorescent light for 2–5 h, to allow recovery of the membrane potential following wounding (aging).

Leaf segments were mounted in a 4-ml plexiglass chamber which was constantly perfused with 1X bathing solution [1 mM KCl, 1 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$,and 1 mM NaH_2PO_4, pH 5.7 (3)] at a flow rate of approximately 10 ml min^{-1}. One-mm, fiber-

filled borosilicate capillaries [W.P. Instruments, Inc. (WPI); New Haven, CT] were pulled with a vertical pipette puller (Model 700 C, D. Kopf Instruments, Tujunga, CA) to a < 0.5-μm tip diameter and were filled with 3 M KCl (salt bridge). The tip potential of the microelectrode was –5 to –15 mV and the resistance was 10–15 MΩ. The reference electrode was a 2- to 3-cm long piece of 1.57-mm diameter polyethylene tubing filled with 3 M KCl solidified with 2% agar. Ag/AgCl electrodes connected the two salt bridges to an electrometer amplifier (Model M4-A, WPI).The electrometer output was continuously recorded on a Fisher 5000 chart recorder. The micropipette was inserted through a cut edge into a cell in the spongy parenchyma tissue with a Leitz micromanipulator and a horizontally-mounted microscope. A quartz halogen (200 μE cm^{-2} s^{-1}) goose-neck fiber optics lamp was used to illuminate the upper surface of the tissue segment.

The bathing solution was saturated by bubbling with N_2 gas to remove O_2. In darkness, the energy-dependent E_m component was thus inhibited so that the diffusion potential could be measured. Returning to N_2-free 1X solution restored oxygenated conditions. Only cells that maintained stable values of E_m for at least 20 min were used for measurements.

Fusicoccin (FC), a gift from Prof. E. Marrè (Milan, Italy), was dissolved in ethanol and diluted with 1X solution to a final concentration of 15 μM. Aged segments of control and TRSV-inoculated primary leaves with lesions were treated with FC only after stable Em values had been maintained for at least 15 min.

Statistics

Numbers presented are least square means ± SE derived using the general linear models procedure (12). The probability level of mean comparisons by LSD test is 0.05 unless otherwise noted, and the numbers of observations are listed together with results.

Results

Symptoms

Lesions on cowpea primary leaves inoculated with TRSV first became apparent as circular, 0.5–1.0 mm diameter, brown or purple spots. They were first seen at approximately 4 days postinoculation in both greenhouse- and laboratory-grown plants. On greenhouse-grown plants, these spots enlarged gradually with time attaining a maximum diameter of 3.5–4.0 mm at 7–8 days postinoculation. TRSV spread to the trifoliate leaves 12–14 days after inoculation, causing occasional necrosis of the apical meristem. In laboratory-grown plants lesions were smaller, with a maximum diameter of 1–2 mm.

SBMV induced distinctive, pale chlorotic spots on primary cowpea leaves at 8–10 days postinoculation. This was followed by a systemic mottle of the trifoliate leaves at 12–15 days postinoculation. During the chronic stage of infection, the primary leaves became totally chlorotic and sometimes abscised.

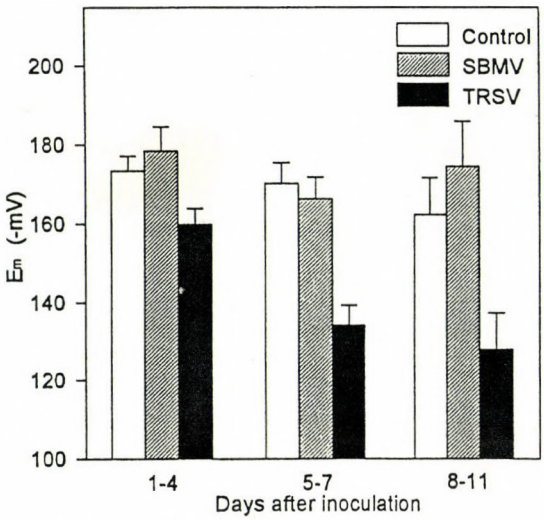

Fig. 1. Least square means of initial membrane potentials (± SE) in light for cells of cowpea primary leaves inoculated with TRSV or SBMV or controls. Time periods approximately correspond to latent period and lesion initiation (1–4 days), lesion growth and expansion (5–7 days), and mature lesions and tissue collapse (8–11 days) in TRSV-infected leaves. Numbers of measurements are as follows: days 1–4, control = 19, SBMV = 7, TRSV = 17; days 5–7, control = 10, SBMV = 9, TRSV = 10; days 8–11, control = 3, SBMV = 2, TRSV = 3

Electrophysiology, greenhouse-grown plant

E_m measurements were performed at 24-h intervals up to 7 d after inoculation. Measurements became increasingly more difficult as TRSV-infected tissue became flaccid but a few were performed up to 10 days after inoculation. Cells in SBMV-infected leaves were monitored until 11 days postinoculation. There were no apparent differences between the E_m values of cells from primary leaves which had been rubbed with phosphate buffer containing Celite and that of cells in the untreated tissue.

The means of the initial cell membrane potentials in light (the highest initial stable value, E_mL) were compared by treatment and day after inoculation. There was no significant change over time in the E_mL values of control and SBMV-infected cells (data not shown). The E_mL values of TRSV-infected cells depolarized significantly ($P = 0.01$) from -155 ± 7 mV ($n = 6$) on day 4 to -130 ± 6 mV ($n = 7$) on day 5 after inoculation. Day 5 is the day after lesions were first observed on some TRSV-infected leaves.

The means of E_mL values were also compared by treatment at 9 three time periods (Fig. 1). Days 1–4, days 5–7 and days 8+ after inoculation approximately correspond to the following symptoms in TRSV-infected leaves: latent infection and lesion initiation, lesion growth and expansion, and mature lesions and tissue collapse, respectively. There were no statistically significant differences in the means of E_mL values measured in con-

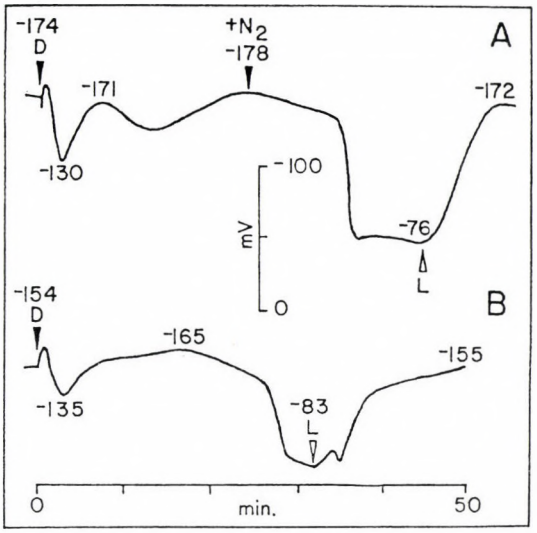

Fig. 2. Representative recordings of cells in primary leaves of greenhouse-grown cowpea control plants. The numbers on curves are E_m in mV. A. Expected transient changes in response to darkness (D), depolarization resulting from ATP depletion after exchanging the bathing solution for one saturated with N_2 gas ($+N_2$), and repolarization again in light (L). B. Response to dark and light in control plant

trol and SBMV-infected cells at any time within or between treatments. The greater variability in mean E_mL values of cells measured more than 8 days after all treatments probably results from the smaller number of measurements at that time. The means of cells in TRSV-infected tissue, however, were significantly less negative ($P = 0.0001$ to 0.02) than either means of control or SBMV-infected cells throughout the experimental period. The mean E_mL value of cells in TRSV-infected tissue became significantly less negative ($P = 0.0002$) when lesions developed (5–7 days after inoculation with TRSV) but did not change further as tissue collapsed.

Diffusion potential and response to light and dark, greenhouse-grown plants

The application of N_2 in light caused no apparent changes in E_mL values of control, SBMV-, or TRSV-infected cells. Applying N_2, in the dark or turning the light off in the presence of N_2, depolarized E_m, to the apparent diffusion potential in all cells (Fig. 2A). Measurements that continuously declined were excluded from tabulation as possibly those of dying cells. The mean diffusion potential of TRSV-infected cells for days 1–4 after inoculation was more negative (-81 ± 3 mV, $n = 11$) than on days 5–7 (-72 ± 4 mV, $n = 10$), approaching significance ($P = 0.07$). Small numbers of observations for several times and treatments made most comparisons by time and treatment impossible.

Table 1

Least square means ± SE of diffusion potentials[a] and E_mD[b] values in leaves of greenhouse-grown cowpea plants inoculated with TRSV (days 1–10 postinoculation) and corresponding control leaves

Treatment	Diffusion	E_mD
Control	–88±4 mV (n=15)	–94±5 mV (n=9)
TRSV	[c]–77±3 mV (n=25)	–86±3 mV (n=12)

[a] ATP production was inhibited by saturating the bathing solution with N_2 gas and measuring in darkness to obtain the diffusion potential.

[b] E_mD is the depolarized membrane potential value resulting from darkness alone in all TRSV lesion cells and some control cells.

[c] TRSV mean diffusion potential is significantly different from control mean diffusion potential (P=0.01), control E_mD (P=0.004), and TRSV mean E_mD (P=0.03). Other differences are nonsignificant.

However, from Table 1 it is clear that over the entire experimental period, the means of diffusion potentials were significantly lower in TRSV-inoculated leaves than controls (Table 1). About half of this difference (7 mV, not significantly different from control) was seen during the first 4 days after inoculation.

The E_m of control cowpea leaves transiently hyperpolarized and depolarized before recovering to the initial value upon switching the light off; this is the case in leaves of most higher plant species (6). Unexpectedly, the E_m in dark often depolarized again to a low stable value after 10–30 min (E_mD) (Fig. 2B). Recovery only occurred when light was restored. This depolarization was indistinguishable in shape of the curve and timing from that in response to anoxic conditions induced by saturating the bathing solution with N_2 gas. Indeed, some measurements were excluded from tabulation because it was impossible to distinguish whether the depolarization was a result of darkness or exposure to N_2 gas. The mean E_mD of control cells was not statistically different from the mean diffusion potential of control cells (Table 1). However, the E_m usually depolarized an additional 5–10 mV when N_2 was applied following the depolarization caused by darkness.

SBMV- (up to 11 d postinoculation) and TRSV-infected cells (before lesion appearance) responded to darkness similarly to the controls (recordings not shown). However, after lesion appearance in TRSV-infected plants, the darkness-induced hyperpolarization of cells changed dramatically (Fig. 3A). The first phase of hyperpolarization, immediately after turning the light off was 5–10 mV and lasted for 3–5 min. It was followed by a second hyperpolarization of an additional 5–10 mV. After reaching the maximum hyperpolarization, the E_m sharply depolarized to a low stable potential. As in the green-house-grown control, recovery to the previous level of E_mL occurred only when light was restored. The pattern of response remained constant up to 10 days after

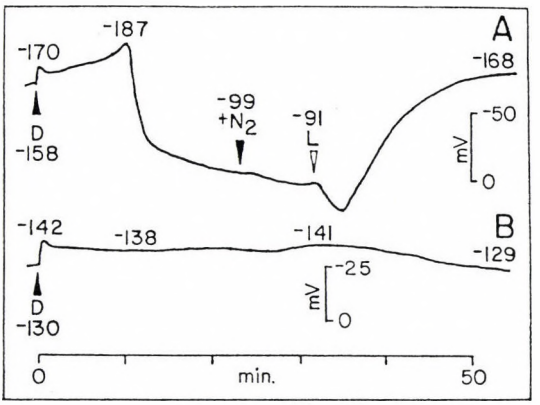

Fig. 3. Representative recordings of cells in TRSV-induced lesions. The numbers on curves are E_m in mV. A. Response of greenhouse-grown plant to darkness (D), exchanging the bathing solution for one saturated with N_2 gas (+N_2), and light (L). B. Response of laboratory-grown plant to darkness

inoculation. The mean E_mD of TRSV-infected cells was not significantly different from the mean control E_mD, but it was significantly more negative than the mean diffusion potential of cells treated with TRSV (Table 1).

Response to light and lark, laboratory-grown plants

In control leaves, the transient hyperpolarization and depolarization after turning off the light was followed by recovery to the initial E_mL values without further depolarization (not shown). In TRSV-infected plants, darkness caused the Em of cells within lesions to immediately hyperpolarize similarly to the control; however, the transient depolarization was reduced or absent in 14 of 15 measurements. The E_m remained hyperpolarized in darkness for 6 to 70 min (Fig. 3B). An abrupt depolarization to the diffusion potential level was never measured in these plants.

Response to fusicoccin (FC)

Fusicoccin is known to cause hyperpolarization of the plasma membrane potential in higher plant cells (7); however, as we recently reported, cowpea and a few other taxonomically related species are exceptions (19). Only a transient hyperpolarization was observed in healthy cowpea mesophyll cells treated with FC in either dark or light (Fig. 4A, B). Subsequently, the membrane potential depolarized in both greenhouse- and laboratory-grown plants. In darkness E_mL was slightly less negative than the diffusion potential (Fig. 4A). In light the depolarization reached a value approximately half-way

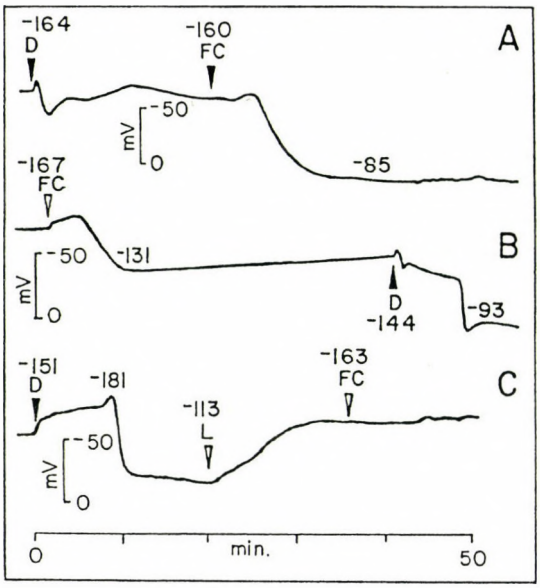

Fig. 4. Representative recordings of cells in control and TRSV-infected primary cowpea leaves when the 1X bathing solution is exchanged for solution containing the fungal toxin, fusicoccin (FC, 15 μM). The numbers on curves are E_m in mV. A. Control segment treated with FC in dark (D). B. Control segment exposed to FC in light (L), then dark. C. Response of cell in TRSV-induced lesion to FC applied in light

between the E_mL and the diffusion potential (Fig. 4B). The E_mL slowly recovered but the potential further depolarized when the light was turned off (Fig. 4B).

In TRSV-infected plants, treatment with FC in light did not affect the membrane potential (Fig. 4C). However, membrane potentials became extremely unstable and were lost in darkness (not shown).

Discussion

We previously reported that increased lipid peroxidation caused by free radical production, in particular superoxide, was a likely basis for membrane damage in the cowpea/TRSV system (1). Potassium efflux, a sensitive indicator of electrolyte loss, began at the time of lesion appearance and markedly increased until the time of tissue collapse in TRSV-infected plants. Lipid peroxidation and superoxide production began before both lesion appearance and potassium efflux. Lipid peroxidation continued at a high level until tissue collapse.

Free radical damage is thought to cause both lipid peroxidation and damage to membrane protein components, such as ion channels (17). The present study suggests

damage to both. Membrane potentials in light in TRSV-infected cowpea leaf cells were approximately 15 mV less negative than controls or SBMV-infected cells during the first 4 days after inoculation and depolarized another 15 mV as lesions developed. About one half of this deficit at each time period corresponded to depolarized diffusion potential and the other half to depolarized active potential.

Depolarized potentials also have been reported for cotton and cucumber cotyledons undergoing HR following infiltration with incompatible bacteria (4, 10). However, in bacterial HR both the energy-dependent and diffusion components declined to a much greater extent than was measured in this study of viral HR. The primary damage was considered to be in the lipid portion of the membrane in bacterial HR, because the diffusion potential continuously depolarized, reaching the level of the cell wall potential (about –40 mV) (10) and lipid peroxidation was found (4). We suggest that this difference in magnitude reflects the differences in inoculation technique, symptom development, and infection process in bacterial and viral HR. In experiments with bacteria, a high concentration of cells was infiltrated into the intercellular spaces with a hypodermic needle. The bacteria remain in the intercellular space, and their products diffuse to surrounding cells, so that the possibility of measuring a bacteria-affected cell is much greater than that of measuring a cell harboring virus particles. Tissue collapse occurred much faster in these bacterial HR experiments (within 10 hours). Undoubtedly tissue collapse severely affected most cells by the final measurements. Another obvious difference was that, in contrast to viral lesions, bacteria-inoculated tissue remains green even as tissue collapses and begins to dry. Brown pigmentation occurs a day or two after inoculation with a lower concentration of bacteria than that used in these studies.

The most striking electrophysiological change resulting from hypersensitivity caused by TRSV infection was in the light/dark response. All higher plants undergo changes in E_m as light is imposed and removed. The pattern of response varies by species and growth conditions (6). These responses may stem from pH changes resulting from photosynthetic processes such as the transport of K^+ and 3-phosphoglycerate into chloroplasts (20). Control cowpea leaves under our two growth conditions exhibited large changes in E_m upon switching the light off. Within one minute there was typically a hyperpolarization followed by an immediate transient depolarization and slower recovery. TRSV-inoculated plants lost the ability to depolarize following the hyperpolarization. The E_m either remained hyperpolarized or hyperpolarized to even more negative values. A similar pattern of response to darkness, together with partially recovered E_mL values, was obtained in cells of cotyledonary tissue 24 h after inoculation with HR-inducing bacteria when segments remained in the aerated 1X solution over night (8). An altered light/dark response may reflect a change in the function of voltage-related single ion channels. Alternatively, the cellular pH stat (5) may function differently as a result of cytosolic acidification or virus-altered metabolic processes.

The absence of hyperpolarization or depolarization in measurements following the application of FC suggests that potassium channels or the H^+-ATPase itself may have been affected. Fusicoccin permanently modifies the regulatory part of the H^+-ATPase, as well as potassium and calcium channels, which, in the healthy cowpea control cells, re-

sulted in slight hyperpolarization followed by deep depolarization. The response of healthy cowpea to FC is unusual among higher plants. It has been further investigated; however, the cause of the depolarization is yet unknown (19). The fact remains that this pattern was markedly altered in TRSV-infected plants in light, and this alteration was apparently lethal in darkness. The brief hyperpolarization in healthy cowpea cells is accompanied by an influx of potassium as in most higher plants (19). Correspondingly, potassium efflux accompanies the depolarization (19). The absence of E_m change in the light indicates that FC did not stimulate the H^+-ATPase even briefly and did not affect the level of potassium accumulation.

As reported in the present study, Stack and Tattar (16), observed depolarized membrane potentials after lesion appearance in cowpeas infected with TRSV. However, they measured potentials of –30 to –80 mV in about 45% of TRSV-infected cells, though some cells had potentials of –200 mV. We measured potentials ranging from –110 mV to –189 mV in the TRSV-infected cells. The absence of very depolarized potentials in our measurements probably reflects a difference in technique. In the Stack and Tattar study potentials were measured in the center of lesions by inserting electrodes through the cuticle. Potentials were recorded after 5 minutes of stable measurement. These two features favor recording of potentials from cells that are barely functional. Our measurements were performed by inserting electrodes through the cut edge of bisected lesions and usually lasted for more than one hour. The cell layers actually measured were not immediately adjacent to the cut surface and thus were closer to the margin of the lesion where Stack and Tattar also measured somewhat higher potentials. We suggest that in our study, severely compromised cells could not have been successfully impaled and those cells that sustained measurable potential, but did not live long enough to exhibit light/dark/N_2 responses, were excluded from tabulations.

Systemic infection with SBMV caused no measurable effect on the plasma membrane potential during the 11 days of measurement. Dark and light E_m responses and the mean E_m of mesophyll cells in cowpea primary leaves infected with SBMV and measured in light were similar to those of the control throughout the monitoring period. These results correspond to those reported in our study of lipid peroxidation (1). In that study, SBMV-infected leaves were barely different from those of the controls in K^+ leakage and exhibited about one-third the level of lipid peroxidation recorded in TRSV-infected leaves.

The depolarization in response to darkness in both virus-infected and control plants probably results from summer greenhouse growth conditions with little period of acclimation to the lower laboratory light conditions in which E_m measurements were performed. We occasionally have recorded similar patterns in other plant species. This depolarization in response to darkness may indicate an increased sensitivity to anoxia since the tracing curves following N_2 application were similar and the mean control diffusion potentials were not different from the mean depolarization levels of control cells in dark. Cowpea has been reported to be unusually sensitive to anoxia (11).

Acnowledgements

The authors thank Dr. Gary Krause for statistical computation and advice. This study was supported in part by a grant from the University of Garyounis, Faculty of Science, Benghazi, Libya. Contribution from Missouri Agricultural Experiment Station Journal Series No. 12,319. Project No.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 145–151 (1995)

Natural Occurrence of Cucumber Mosaic *Cucumovirus* on *Malva sylvestris* Plants in Hungary*

J. HORVÁTH[1], P. SALAMON[2] and D.-E. LESEMANN[3]

[1] Pannon University of Agricultural Sciences, Institute for Plant Protection, H-8361 Keszthely, P.O. Box 71, Hungary
[2] University of Horticulture and Food Industry, H-1502 Budapest, P.O. Box 53, Hungary
[3] Federal Biological Research Centre for Agriculture and Forestry, Institute of Biochemistry and Virology, D-3300 Braunschweig, Germany

Cucumber mosaic *Cucumovirus* (CMV) was isolated from *Malva sylvestris* plants showing severe vein clearing and interveinal mosaic symptoms. The Ma isolate was identified as CMV by host range, serology, electron microscopy, ways of transmission, and cross protection. This is the first report on the presence of CMV in the common perennial weed *Malva sylvestris* plants in Hungary.

Some of the plants belonging to the family Malvaceae (e.g. *Malva borealis, M. neglecta, M. nicaeensis, M. parviflora, M. rotundifolia, M. sylvestris, Lavatera assurgentiflora, L. cretica, L. trimestris*) play an important role as natural or experimental virus hosts. Out of the viruses having occurred so far in malvaceous plants the Malva vein clearing virus (MVCV) (Hein, 1956; Costa and Duffus, 1957; Kitajima et al., 1962; Schmidt and Schmelzer, 1964; Majorana and Silberschmidt, 1967; Marco, 1975; Horváth et al., 1979), the beet mosaic *Potyvirus* (BtMV) (Martelli et al., 1969), the alfalfa mosaic *Alfamovirus* (AMV) (Marco, 1975), the Abutilon mosaic *Geminivirus* (AbMV) (Jeske and Schuchalter-Eicke, 1984; Abouzid and Jeske, 1986) and the tomato spotted wilt *Tospovirus* (TSWV) (Cho et al., 1989) are known. According to the data of Tomlinson et al. (1970) a few plants of *Malva sylvestris*, a headland weed at the sandy Bertforshire site in England, showed leaf chlorosis symptoms associated with cucumber mosaic *Cucumovirus* (CMV). In past years we observed in *Malva sylvestris* plants serious vein clearing and interveinal mosaic symptoms partly differing from earlier observed symptoms caused by MVCV (see Horváth et al., 1979). With an etiological study of the disease in view we carried out experiments to identify the virus pathogen.

Materials and Methods

The *Malva sylvestris* plants to be found in the natural weed flora of Hungary showed severe symptoms of vein clearing and interveinal mosaic (Fig. 1). The tissue sap of the diseased plants diluted with 0.05 M phosphate buffer (pH 7.0) at a ratio of 1 : 1 was

* Dedicated to Prof. Dr. Dr. h.c. Z. Király academician on the occasion of his 70th birthday.

0238–1249/95/$ 5.00

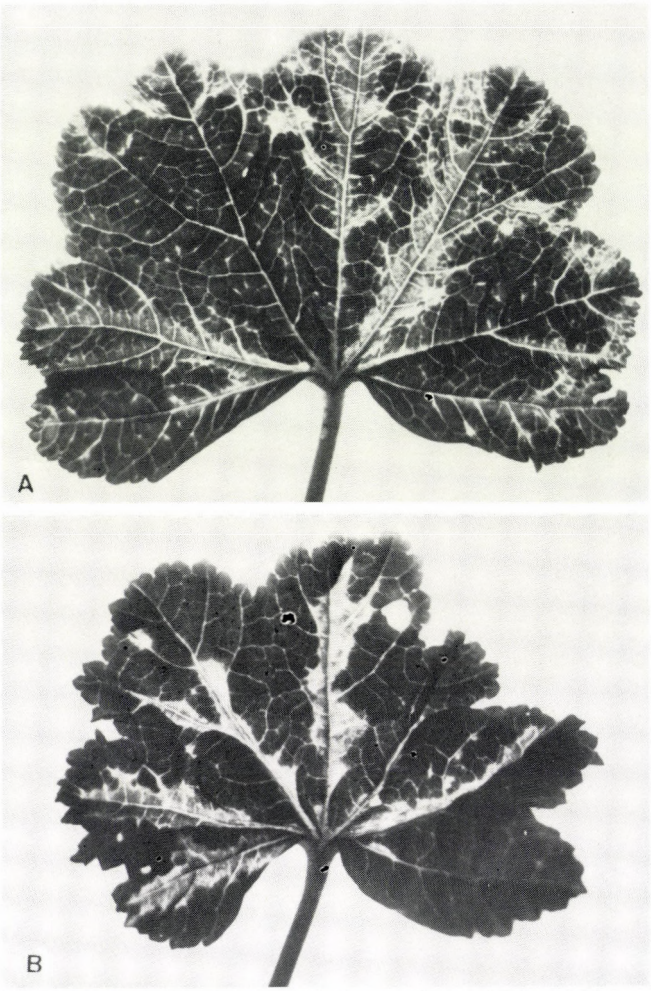

Fig. 1. Mosaic and interveinal mosaic symptoms on the leaves of natural infected *Malva sylvestris* plant (A and B). Photograph courtesy of Cs. Pintér, PUAS-IPP Keszthely, Hungary

transferred mechanically to various test plants (*Chenopodium quinoa, Cucumis sativus*, *Nicotiana* spp., *Vigna sinensis* etc.). In the course of identifying the virus agar gel-diffusion serological and electron microscope examinations were performed. Serological experiments were done in 1 per cent agarose gel (Reanal) prepared in 5 mM borate buffer (pH 9.0) containing 5 mM EDTA and 0.02 per cent NaN_3. Leaves of healthy and infected tobacco (*Nicotiana tabacum* cv. Xanthi-nc) and cucumber (*Cucumis sativus*) test plants were homogenized in either 5 mM borate-EDTA or 0.05 M Na_3-citrate (pH 6.5) and centrifuged at low speed (6000/min). The supernatants were used as antigens. Serological

tests were performed using a rabbit polyclonal anti-CMV antiserum prepared to an *Echinocystis lobata* isolate of CMV (CMV-El; Salamon, 1993). The isolates CMV-Sz 2645 and CMV-SnT22 were the same as characterized earlier (Salamon, 1989). By electron microscopy experiment a drop of virus extract was put on the carbon face of a carbon-Formvar filmed grid. The grid was then rinsed with water and negatively stained in 2 per cent aqueous uranyl acetate (UA). Virus particles were visualized at an instrument magnification of 40 000 in a ZEISS EM 10C electron microscope. Vector transmission of the virus was achieved by means of *Myzus persicae*. The virus free aphids were starved for 3 hours, then left for virus acquisition for 10 minutes. Aphids were then transferred onto young *Malva sylvestris* and *Nicotiana tabacum* cv. Xanthi-nc plants for 5 minutes and destroyed afterwards with an insecticide. We studied also the cross protection between the Northern European W strain of CMV (CMV-W, see Skiebe and Schmelzer, 1967) and the Ma Isolate on *Nicotiana tabacum* cv. Xanthi-nc plants.

Results and Conclusions

1. Host plants of the Ma isolate

The isolate Ma obtained from *Malva sylvestris* proved to be readily mechanically transmissible to several test plants (Table 1). On the basis of the examined host-virus relations of the Ma isolate it is considered identical with the common strain of CMV and different from MVCV earlier isolated in Hungary (Horváth et al., 1979). Some species from the family Malvaceae (e.g. *Alcea rosea, A. sulphurea, Malva parviflora, M. sylvestris, Lavatera trimestris*) reacted by systemic vein clearing and interveinal mosaic symptoms. *Beta vulgaris* is a non-host of the Ma isolate.

2. Serology

In the Ouchterlony's agar gel-double diffusion tests antigens prepared from tobacco or cucumber leaves infected by the Ma isolate reacted with antiserum to CMV-El. Similarly, leaf extracts of tobacco plants infected either by CMV-SnT22 or by CMV-TrK7 (Beczner et al., 1978) gave positive reactions. The antiserum, however, did not react with clarified sap of healthy tobacco (Fig. 2).

The appearance and position of precipitin bands strongly depended on the test conditions. To obtain visible bands it was essential to prepare the antigens from tobacco leaves with clear symptoms harvested 6–10 days after inoculation. When borate-EDTA buffer was used for extracting the antigens, a single, but weak precipitin line near the peripheral antigen wells appeared. This line was probably specific for the intact virions of CMV. When 0.5 M Na_3-citrate was used as extracting buffer, besides the virus band (V-band) several stronger line near the antiserum well usually appeared. The presence of the soluble antigen (which gave this latter band, S-band) showed that the virions were degraded in the presence of Na_3-citrate without additives. When the antigens of Ma iso-

Table 1

Test plant reactions of Ma isolate of cucumber mosaic *Cucumovirus* from *Malva sylvestris*

Test plants with the code of botanical name[1]	Reaction[2]
Alcea rosea, ALGRO	–/Vc, IvM
A. sulphurea, ALGSU	–/Vc, IvM
Beta vulgaris, BEAVA	–/–
Capsicum annuum, CPSAN	NL/Vc, M
Chenopodium amaranticolor, CHEGI	NL/–
C. quinoa, CHEQU	NL/–
Cucumis sativus, CUMSA	ChL/Vc, M
Cucurbita pepo, CUUPE	–/Vc, M
Datura metel, DATME	–/Vc, M
Datura stramonium, DATST	–/M
Lycopersicon esculentum, LYPES	–/M
Malva parviflora, MALPA	–/Vc, M
M. sylvestris, MALSI	–/Vc, IvM
Nicotiana benthamiana, NIOBE	–/M, Led, Gr
N. glutinosa, NIOGT	–/Vc, M, Led, Gr
N. tabacum cv. Xanthi-nc, NIOTA-XNC	–/Vc, M
Lavatera trimestris, LAVTR	–/Vc, M
Petunia hybrida, PEUHY	–/Vc, M
Phaseolus lunatus, PHSLU	NL/–
Tetragonia expansa, TEATE	ChL/ChL, M[3]
Vicia faba, VICFX	NL/–
Vigna sinensis, VIGSI	NL/–
Zinnia elegans, ZIIEL	–/Vc, M

[1] See the reviewed paper of Anonymous (1992) and Horváth (1993a, b, c).

[2] Local/systemic symptoms. ChL, chlorotic lesions; Gr, growth reduction; IvM, interveinal mosaic; Led, leaf deformation; M, mosaic; NL, necrotic lesions; Vc, vein clearing.

[3] Symptoms occurred on the axillary shoots.

late and isolates CMV-SnT22 and CMV-Sz 2645 were placed in adjacent wells, the S-bands of the different isolates fused. Clear results concerning the fusions or spur formations between the V-bands of these isolates have not yet been obtained. Further studies are needed to determine the serotype (To or D) of the CMV-Ma.

3. Electron microscopy

Isometric particles about 28–30 nm in diameter were observed in electron micrographs (Fig. 3).

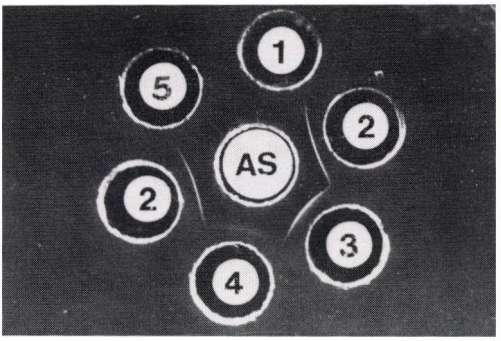

Fig. 2. Agar gel-double diffusion test with antiserum of cucumber mosaic *Cucumovirus*, CMV (AS, central well) and the antigens of *Malva sylvestris* strain (Ma strain) of CMV (peripheral wells 2), TrK7 isolate of CMV (well 3), SnT22 isolate of CMV (well 4). The other wells (1 and 5) were filled with clarified leaf saps from healthy tobacco plants

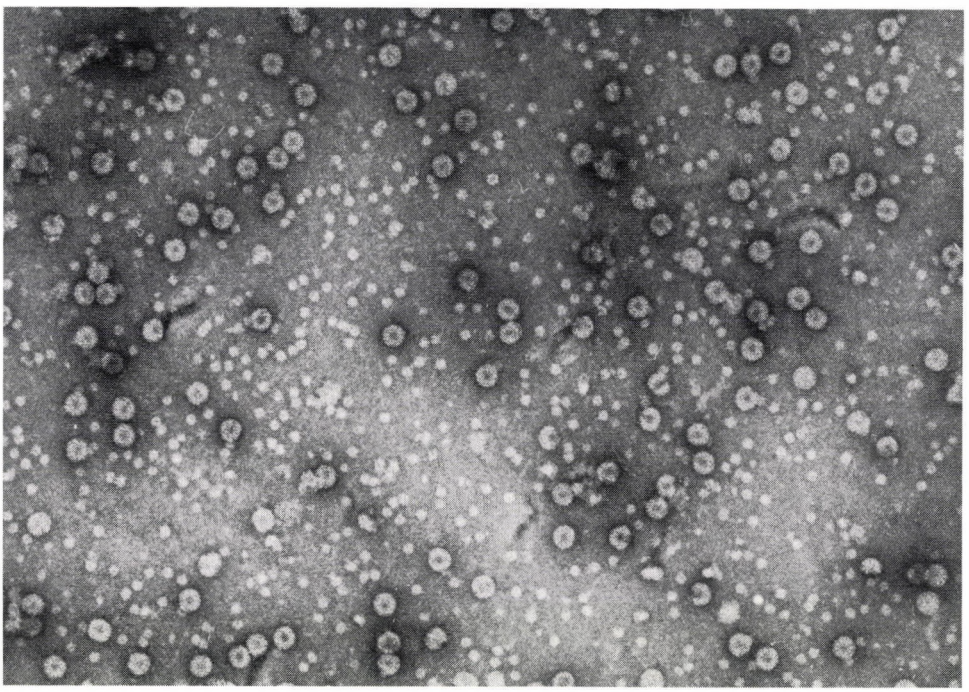

Fig. 3. Electron micrograph of the *Malva sylvestris* isolate of cucumber mosaic *Cucumovirus* (CMV-Ma) stained with uranyl acetate

4. Vector transmission

By *Myzus persicae* the virus isolate Ma was readity transmitted by non-persistent manner from experimentally infected *Nicotiana tabacum* cv. Xanthi-nc to young *Malva sylvestris, Nicotiana tabacum* cv. Xanthi-nc and *Cucumis sativus* plants. These plants showed vein clearing and mosaic symptoms as well as interveinal mosaic on the leaves of *Malva sylvestris*.

5. Cross protection

In cross protection experiments the tobacco plants inoculated with the Ma isolate, similar to other 17 isolates of CMV (see Horváth, 1994), were protected against a subsequent infection by the CMV-W. The results of the cross protection test give evidence of a relationship between Ma isolate and CMV.

The results presented here indicate that *Malva sylvestris* is an important reservoir of CMV. This is apparently the first report on the presence of CMV in *Malva sylvestris*.

Acknowledgement

Thanks are due to Miss K. Molnár for her technical assistance.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 153–156 (1995)

Influence of Soybean Mosaic Virus Infection on Total Nitrogen Content in Nodules of Soybean (*Glycine max* (L) Merr.)

U. P. GUPTA

Botany Department, Harish Chandra Post Graduate College, Varanasi (U. P.), India

The gradual increase in total nitrogen content was observed through out the experimental period in nodules of both healthy and diseased plants. However, the nodules of diseased plants contained more total nitrogen content than nodules of healthy plants.

Since replication of a virus inside a host system requires abnormal proteins, an obvious shift in the nitrogen metabolism of the infected tissue is discernible. Changes both towards the positive and negative levels in comparison to healthy tissue has been reported depending upon virus host combination. In general yellows type viruses reduce the total nitrogen while mosaic type viruses increase it. An experiment was, therefore, set up to study the nature of shift in the total nitrogen content of nodules in soybean mosaic virus (SMV) infected soybean plants.

Materials and Methods

In this study two lots each of 30 plants were taken. The first lot of 30 plants was inoculated with the SMV and the second lot was kept as control. The seedlings were of the first trifoliate stage at the time of inoculation. The plants used for this experiments were grown in 25 cm earthen pots. These pots were filled with sterilized gardensoil. The rhizobium peat culture (Obtained from Microbiology Dept., of G. B. Pant University of Agriculture & Technology, Pantnagar) was mixed in the soil of these pots before sowing the soybean var. Bragg seeds. The plants were raised in an insect proof glass house, which was fumigated regularly to keep it free from insects. The environmental conditions, of glasshouse were photoperiod 11L : 13D; temperature 25–30 °C during day and 20–25 °C during night throughout the experimental period. Nodules of healthy and diseased plants were subjected separately to estimate total nitrogen contents which were harvested after 20, 30, 40, 50, 60, and 90 days of inoculation. Total nitrogen was estimated by the method described by Snell and Snell (1949). In each treatment the nodule material to be treated was taken from five plants. This material was dried at 65 °C for 72 h.

0.1 g of dried material was transferred to micro-kjeldahl flask which 3 ml of digestion mixture (32 g of salicylic acid/litre of conc. H_2SO_4) was added. It was mixed well and allowed to stand for 30 minutes. Then 4 drops of 50% sodium thiosulphate solution

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Table 1

Effect of soybean mosaic virus infection on total nitrogen content in nodules of soybean

Days after inoculation	Total nitrogen content (% dry weight) Healthy	Diseased
20	4.1	4.3
30	4.2	4.5
40	4.7	5.2
50	5.1	5.7
60	5.4	6.1
90	5.6	6.4
Average	4.85	5.36

Analysis of variance

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	F calculated value
Treatment	1	0.80	0.80.	40.0*
Interval	5	5.44	1.09	54.5*
Error	5	0.12	0.02	
Total	11	6.36		

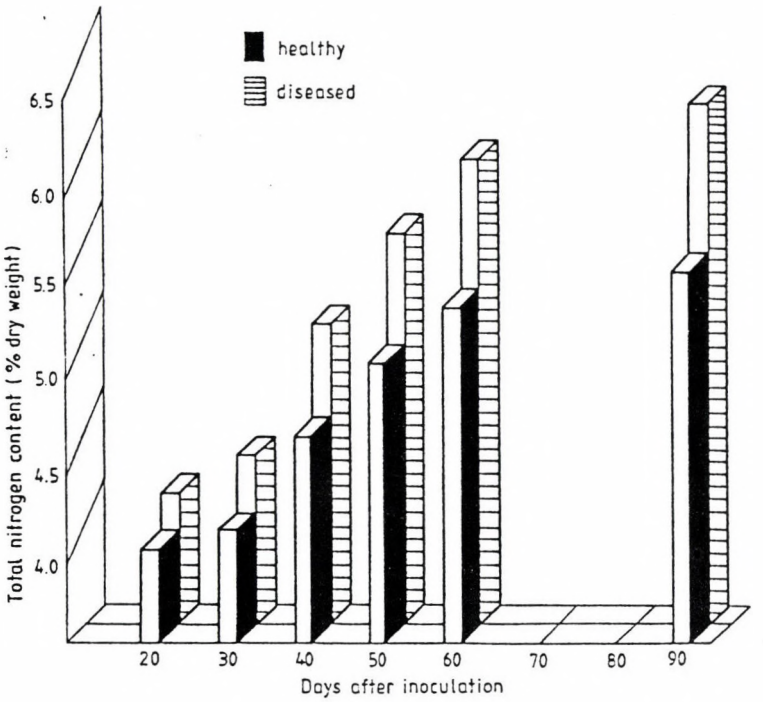
* Significant at 5% level

followed by 5 ml of an other digestion mixture (2.5 g of selenium/litre conc. H_2SO_4) were added. The contents were heated slowly to boiling till the digestion of nodule material was completed. After the digestion was complete, about 5 drops of 10% perchloric acid were added and the contents were gently heated till the solution became clear. The entire solution was then made up to 100 ml with addition of distilled water. In a colorimetric tube 1 ml of this solution, 8.5 ml of Nessler's reagent (BDH) and 0.5 ml of gum-ghati solution were added. The contents were mixed thoroughly and thus ammonia evolved during nesslerization was estimated colorimetrically with 'AIMIL' Biochem Absorptiometer, using filter No. 42. A standard solution of ammonium sulphate (AR quality of BDH) was prepared. A portion of this solution was taken and treated with Nessler's reagent and gum-ghati solution similarly and readings were noted in absorptiometer using the same filter. Total nitrogen was calculated by the following formula using $(NH_4)_2SO_4$ standard:

$$\text{Reading of unknown} \times \text{factor} = \text{Concentration of unknown},$$

$$\text{and factor} = \frac{\text{Conc. of standard}}{\text{Reading of standard}}$$

The results are presented in Table 1 and Fig. 1.



Results

The results presented in Table 1 and Fig. 1 show that the total nitrogen content in nodules of diseased plants was more than in nodules from healthy ones. This increase was gradual in increasing order throughout the experimental period in both healthy as well as in diseased samples. These observations are significant.

Discussion

The present studies have revealed a striking disparity in total nitrogen content in nodules of healthy and SMV-infected soybean plants. The nodules of SMV-infected soybean contained higher total nitrogen than did their healthy counterparts. Investigations with several viruses have shown increased nitrogen content of infected hosts (John, 1963; Narayanasamy and Ramakrishnan, 1966; Harman et al., 1970; Tu, et al., 1970; Jeyarajan and Ramakrishan, 1972; Joshi and Dubey, 1974; Singh et al., 1978; Shukla et al., 1984). The main components of total nitrogen were probably insoluble proteins, free

amino acid, nucleic acids, amines and amides. Alteration in any one of the components may disturb the nitrogen metabolism. A few viruses such as TMV and potato virus X multiply to such an extent that virus protein contributes significantly to the total protein of the host and as a consequence there is an increase in total nitrogen. This might be one of the possibilities in the present case, too. The increased concentrations of free amino acids in nodules of infected plants (Gupta and Joshi, 1976) may also appear one ot tbe possible reason for this increase of total nitrogen. An other and rather more possible eplanation is that the nodules of diseased plants were so inefficient that the previously fixed nitrogen by Rhizobium (symbiosis) in nodules was not effectively used and (or) transported elsewhere which might also cause this increase of total nitrogen content.

Acknowledgements

The author is greatly indebted to Prof. R. D. Joshi, Botany Department, University of Gorakhpur for his kind advice, and also thanklto Dr. B. B. Singh Bisen, Principal, Harish Chandra P. G. College, Varanasi for their constant inspiration and encouragement.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 157–160 (1995)

Ds-RNA Investigation from Grapevine Affected by Different Viruses

B. S. FELD, N. A. MULYUKINA and B. N. MILKUS

Tairov Research Institute of Viticulture and Enology, Odessa-103, Ukraine

Ds-RNA was extracted from leaves of healthy grapevine and infected by different virus diseases. Electrophoresis was carried out in 1.5 % agarose gel for 3.5 hours at 100 V. The bands of ds-RNA were notable for molecular weight estimation. The data obtained in this study show that the method of extraction of ds-RNA may be useful for diagnosis of RNA viruses.

Ds-RNA is the replicative form of single-stranded (ss) RNA of many plant viruses. The presense of such ds-RNA in plants is an evidence of infection by RNA viruses (Hansen et al., 1986).

Some articles contained data concerning the extraction of ds-RNA from tobacco (Heick, personal communication), fruit trees (Hansen et al., 1986; Kurppa and Martin, 1986; Spiegel, 1987) and grapevine (Mossop et al., 1985) affected by virus diseases. Ds-RNA may be purified by different methods: The 2 column method of Morris and Dodds (Dodds, personal communication), the extraction of nucleic acids in buffers with different additions with further treatment by nucleases (Kurppa and Martin, 1986).

Ds-RNA extraction from grapevine tissues has some difficulties in consecuence with low pH of cellular juice and high activity of polyphenoloxydase. These difficulties can be elyminated with the help of buffers with high pH and compounds preventing the oxidative processes or by extraction the nucleic acid from tissues of lower activity of oxidative enzymes (phloem tissue or dormant canes) (Mossop et al., 1985).

The method of ds-RNA extraction from infected grapevine tissues can be useful for testing of small groups of plants (Dodds, personal communication, Spiegel, 1987).

Materials and Methods

Extraction of ds-RNA

The leaves of grapevine, infected with fahleaf, leafroll, stem pitting, vein mosaic were used. The leaves of healthy plants were taken as control.

All the solutions were prepared with bidistilled water. Redistilled phenol and recrystallised NaCl and distilled ethanol were used. Flasks and tubes were sterilized.

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Washed and dried grapevine leaves (3 g) were powdered in liquid nitrogen and mixed for 5 min with 4 volume of 0.2 M tris-HCl buffer, pH 7.5, with additions (Resain and Krake, 1987).

Samples were centrifugated for 10 min (6–7000 g). Nucleic acids in the aqueous phase were recovered by precipitation with 2–2.5 volumes of ethanol (30 min, –20 °C). Pellet was collected by centrifugation (6 min, 4–5000 g) and resuspended in TE-buffer, pH 7.5 (10 mM tris-HCl, 0,1 mM EDTA). One volume of a water-saturated phenol was added to the samples. After 5 min extraction samples were centrifugated for 5 min (6–7000 g). Nucleic acids in aqueous phase were precipitated with 2–2.5 volumes of ethanol. After centrifugation nucleic acids pellet was resuspended in a small volume of TE-buffer for electroforesis.

Electroforesis

Electroforesis was carried out in 1.5% agarose gel containing 1×10^{-4}% ethidium bromide for 3.5 hours at 100 V. Tris-borate buffer, pH 8.3 (90 mM tris, 90 mM borate, 2.5 mM EDTA) was used for electroforesis.

Starting buffer contained 30% glycerol, 0.025% brom-phenol blue. Samples for loading into the gel were prepared in proportion 50 µl of sample and 10 µl of starting buffer.

Nucleases treatment

The pellet was resuspended in 0.05 M tris-HCl buffer, pH 7.6, containing 0.1 M NaCl, 0.002 M $MgCl_2$ and 10 µg/ml DNA-ase, free of RNA-ase. Treatment was carried out at 37 °C for 30 min. Then 2–2.5 volumes of ethanol was added.

RNA-ase treatment was carried out in 2 × SSC buffer (0.15 M NaCl, 0.015 M sodium acetate, pH 7.4) at 37 °C for 30 min at concentration of RNA-ase A 1 µg/ml. Then samples were treated by phenol to eliminate the enzyme. Ds-RNA was precipitated by adding of 2–2.5 volumes of ethanol. The nature of extracting nucleic acids was examined by digestion using RNA-ase A in 0.1 × SSC buffer.

Samples were kept at –20 °C till electroforesis.

Results and Discussion

Samples of ds-RNA were compared after electroforesis. The definite amounts of ds-RNA can be extracted from the samples weighted less than 3 g because from 1/3 to 1/6 share of samples was taken for the analysis.

The bands of ds-RNA extracted from different sources were notable for molecular weight estimation. The DNA-restricts EcoR 1 and EcoR 2 were used as molecular weight markers.

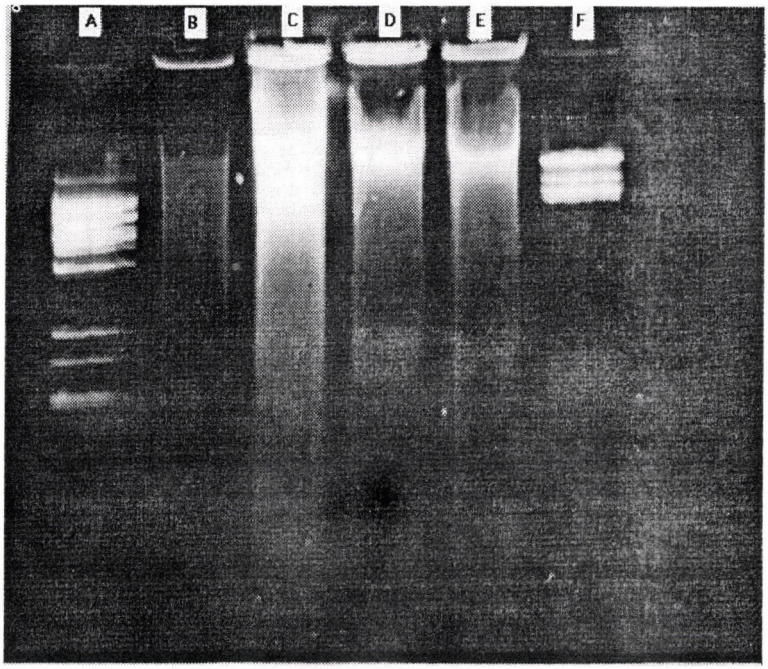

Fig. 1. Agarose gel electroforesis of ds-RNA isolated from grapevines affected with different virus diseases. Lanes A, F – DNA-restricts EcoR1 and EcoR2; B – ds-RNA isolated from grapevine affected with latent virus infection; C, D, F – ds-RNA isolated from grapevine affected by grapevine leafroll, grapevine stem pitting and grapevine vein mosaic diseases, respectively; G – extract from a healthy grapevine

The molecular weight of ds-RNA from grapevine affected by leafroll (about 8–8.5 × 10^6 D) was corresponded to data presented by Mossop et al. (1985), the relative molecular weight of ds-RNA from other sources was estimated at first. The molecular weight of ds-RNA estimated in the samples of grapevine affected by stem pitting and vein mosaic diseases was very close to those found in samples of leafroll diseased grapevine (Fig. 1).

Also we found the traces of ds-RNA in some samples of healthy plants. This fact indicates that some latent viruses may present in this plants. This fact corresponds to data presented by Hansen et al. (1986) and Spiegel (1987). But in this case the amounts of ds-RNA are significantly less than those extracted from the same quantity of leaves with visual symptoms of virus diseases.

Ds-RNA was not extracted from samples examined after the short period of maximum symptomes appearance.

The data received in this investigations are evidence of the fact that the method of ds-RNA study may be useful for the preliminary diagnosis of some grapevine viruses. It application will allow to reduce the tine of testing from several months or years to several days.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 161–168 (1995)

In vitro Differences in Extracellular Polygalacturonase (EC-PG) Activities of Six Isolates of *Botrytis cinerea* (Pers.): A Possible Factor in their Differential Pathogenicity in Cabbage Leaves

M. M. EL-NAGGAR[1] and A. L. ÁDÁM

Department of Pathophysiology, Plant Protection Institute, Hungarian Academy of Sciences, P.O. Box 102, 1525 Budapest, Hungary

A positive correlation was found between *in vitro* extracellular polygalacturonase (EC-PG) activity and pathogenicity on cabbage leaves of six different isolates of polyphagous gray mould fungus, *Botrytis cinerea* (Pers.). The possible *in planta* significance of increased PG activity in pathogenicity of gray mould fungus is discussed.

The plant cell wall represents a physical defence barrier to the attacking pathogenic fungi. Therefore it is not a surprising phenomenon, that a large variety of hemibiotrophic and necrotrophic fungi produce cell wall degrading enzymes such as pectinesterase, pectinase, pectinlyase, polygalacturonase, cellulase and cutinase. Some of them have been implicated in the pathogenesis (Cooper, 1984). The presence and differentiation-specific expression of cellulolytic enzymes as well as pectinesterase and pectinlyase in an obligate biotrophic rust fungus, *Uromyces viciae fabae* have been reported quite recently (Frittrang et al., 1992; Heiler et al., 1993; Deising et al., 1995). These comparative studies also called the attention to the completely different way of regulation of polymer-degrading enzymes in sapro- and necrotrophic fungi as compared to an obligate biotrophic one (Mendgen and Deising, 1993). On the other hand, the outcome of plant-pathogen interactions is also influenced by proteinaceous pectic enzyme inhibitors of host plant origin, found in numerous plant species (Bugbee, 1992; Walton, 1994).

The pectic enzymes (endo- and exo-pectate lyase, PL; endo- and exo-polygalacturonase, PG and pectin methyl esterase, PME) were also characterized as important factors in pathogenesis of gray mould (*Botrytis cinerea*) by many authors (Barash et al., 1964; Sherwood, 1966; Van der Berg and Yang, 1969; Urbanek and Zalewska-Sobczak, 1975; Wasfy et al., 1978; Di Lenna et al., 1981; Marcus and Schejter, 1983). Polygalacturonase (PG) isoforms of *B. cinerea* has been also described (Zalewska-Sobczak and Urbanek, 1975a, b; Drawert and Krefft, 1978).

Botrytis cinerea isolates were distinguished on the basis of their different pectolytic activity by Gorlenko and Manturovskaya (1971), although they did not differ much

[1] Present address: Department of Agricultural Botany, Faculty of Agriculture, Kafr El-Sheikh, Egypt

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in their cultural and morphological characteristics. Magro et al. (1980) also demonstrated that three *B. cinerea* isolates differed markedly in their extracellular protein and PG patterns.

The aim of the present work was to study the connection between *in vitro* PG activity and pathogenicity of *B. cinerea* isolates, originating from different host plants in Hungary.

Materials and Methods

Isolates of Botrytis cinerea *(Pers.)*

Six isolates of *Botrytis cinerea* (Pers.) isolated from different host plants (Table 1) were used for pathological and enzymological studies. The isolates were identified on the basis of their morphological characteristics in culture: formation of conidiophores, size and shape of conidia.

Cultivation conditions of the fungus for pectic enzyme production

For extracellular enzyme production, isolates of the fungus were grown in McCarthy bottles, containing 3 ml culture medium of $(NH_4)_2HPO_4$ (0.1 g/l), $MgSO_4.7H_2O$ (0.5 g/l), KCl (10 g/l), citrus pectin (10 g/l) (Serva, Germany), and sodium polypectate (10 g/l) (Sigma, USA). The media was buffered with a non-metabolizable buffer, 2-(N-morpholino) ethanesulphonic acid (MES), at pH 5.0. The above culture media were inoculated with an agar plug (5 mm in diameter) taken from the marginal region of seven days old colonies of isolates maintained on potato dextrose agar (PDA). After 10 days, culture fluids were sampled, filtered and stored at –20 °C until assay.

Native polyacrilamide gel electrophoresis (PAGE) of pectic enzymes

PAGE was carried out according to Cruickshank and Wade (1980). Improved resolution was obtained when the enzyme samples were loaded onto the acrilamide gel slabs as a moist, pipettable slurry, prepared with Sephadex G-150 superfine gel (Pharmacia, Sweden). After electrophoresis the gels were incubated in 0.1 M DL-malic acid for 1.5 h at 25 °C and then stained overnight in 0.01% solution of ruthenium red at 5 °C. The stained gels were washed for 24 h with distilled water at 5 °C.

Densitometric analysis of gels was performed at 540 nm using a Shimadzu CS-930 scanner (Japan). Photographs of gels were prepared by direct printing onto high contrast photographic paper.

Table 1

List of *Botrytis cinerea* (Pers.) isolates collected in Hungary (1992–1993)

Isolates	Host plant	Source of the host plant
1	*Vitis vinifera*	Gödöllő (1992)
2	*Capsicum annuum*	Hévízgyörk (1993)
3	*Lycopersicum esculentum*	Tura (1992)
4	*Helianthus annuus*	Eger (1993)
5	*Lactuca sativa*	Budapest (1992)
6	*Cucumis sativus*	Galgahévíz (1993)

Pathogenicity test

Mature leaves from field-grown cabbage were washed, dried gently on a tissue paper. Ninety discs (2.5 cm in diameter) from the leaves were cut and placed surface uppermost on moist polysterol balls floating on sterilized water in Petri dishes. Each Petri dish contained 3 discs. For mycelial inocula, 5 mm discs in diameter were taken from the edge of young cultures on PDA medium. Discs were placed on the leaf surface. After incubation at 20 °C in a moist chamber for 10 days, virulence between *Botrytis cinerea* isolates were compared according to Wellman (1943). Fourteen categories of graduation showing degrees of the disease development were established according to the colonization of the fungus (0 = no infection; 14 = the whole surface of cabbage leaf disc is colonized). The pathogenicity degree (P) was evaluated according to Townsend and Heuberger (cited by Gartner, 1971):

$$P = \frac{\Sigma(n \times v) \times 100}{Z \times N}$$

where, n = Number of leaves under graduation (number of replicates);
v = Categories number;
Z = The highest number of graduation;
N = The total number of leaves.

Results

PG profile of Botrytis cinerea *isolates in PAGE system*

The native PAGE technique developed by Cruickshank and Wade (1980) is suitable for studying simultaneously the isozyme pattern of different pectic enzymes (PME, PL and PG).

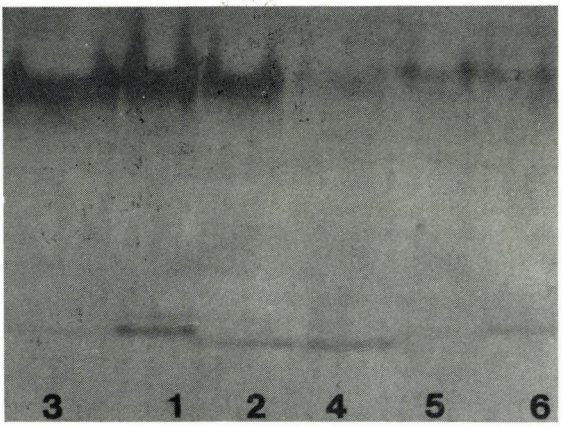

Fig. 1. Extracellular polygalacturonase (EC-PG) zymograms of *Botrytis cinerea* (Pers.) isolates after native PAGE and specific staining. Isolates originate from: (1), *Vitis vinifera*; (2), *Capsicum annuum*; (3), *Lycopersicum esculentum*; (4), *Helianthus annuus*; (5), *Lactuca sativa*; (6), *Cucumis sativus*. In each lane the same amount of culture filtrate was applied to the gel

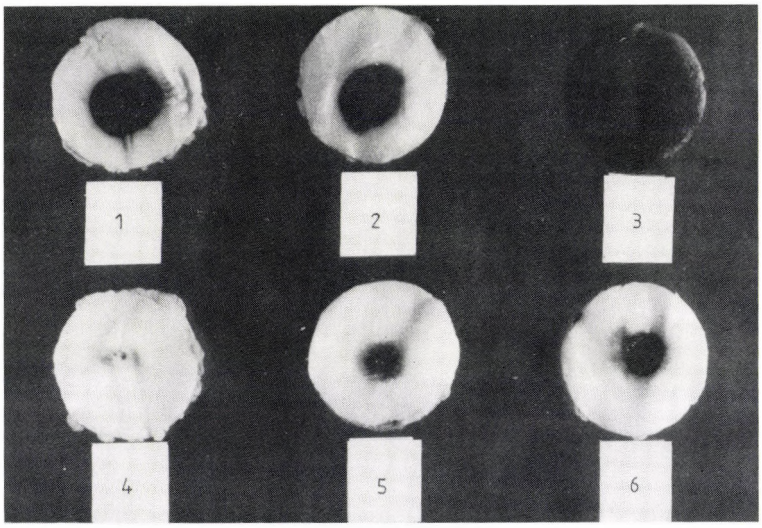

Fig. 2. Pathogenicity test of *Botrytis cinerea* (Pers.) isolates on cabbage leaf discs. Numbers indicate the same isolates as in Table 1

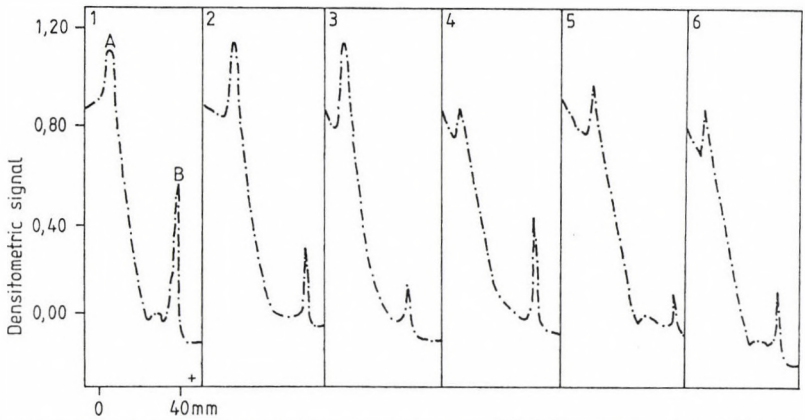

Fig. 3. Densitometric patterns of extracellular polygalacturonase (EC-PG) activities of six different isolates of *Botrytis cinerea* (Pers.) after native PAGE and specific staining. A and B refer to cathodic and anodic isoforms of the enzyme, respectively. Numbers indicate the same isolates as in Table 1

In this study, PME activities were not detectable in the *Botritys* isolates investigated (Figs 1 and 3). All isolates of *B. cinerea* tested showed only two PG bands. The anodic isoform in most cases showed much higher activity than the cathodic one (isoenzymeas A and B in Fig. 3). Especially isolates 1, 2 and 3 (isolateld from grape, pepper and tomato, respectively) showed a greater amount of the anodic isoform (isoenzyme A in Fig. 3) while isolates l, 2 and 4 showed a somewhat higher activity of the cathodic isoenzyme than the other isolates (Figs 1 and 3). The growth rates of different isolates on a Czapek-Dox agar suggested no connection with the differences in PG-production, mentioned above (data not shown).

Pathogenicity test in cabbage leaves

Botrytis cinerea isolated from tomato, lettuce grape, cucumber, pepper and sunflower showed variability in the virulance on cabbage leaves. The strain isolated from tomato showed the highest pathogenicity degree (100%) followed by grape, pepper, cucumber, lettuce and sunflower isolates with pathogenicity degree of 69.2, 61.5, 46.1, 38.8 and 12.3%, respectively (Table 2 and Fig. 2).

Discussion

Isoenzyme analysis of pectic enzymes from *Botrytis cinerea* an other plant pathogenic fungi were applied formerly both in taxonomical and pathological studies (Drawert and Krefft, 1978; Scala et al., 1981; Cruickshank and Wade 1980; Cruickshank, 1983; Cruickshank and Pitt, 1987). Pectic enzyme zymograms have provided more reliable

Table 2

Results of pathogenicity test of *Botrytis cinerea* (Pers.) isolates on cabbage leaf discs

Isolates	Pathogenicity degree (%)	Diameter of infected area[1]
1	69.2	12.5 ± 0.50
2	61.5	10.4 ± 0.38
3	100	24.3 ± 0.58
4	12.3	4.5 ± 0.30
5	38.8	7.3 ± 0.58
6	46.1	7.9 ± 0.80

[1]Data presented, means±SD of three replicates in two experiments

basis for differentiation between *Botrytis* species than other characteristics. Our present results indicated no qualitative differences between isolates of *Botrytis cinerea* in their PG pattern (Figs 1 and 3). However, there were considerable quantitative differences among PG-activities of different isolates (Figs 1 and 3). The comparison of PG-activities and pathogenicity of different isolates on cabbage leaves (Figs 1 and 3, Table 2) suggests that PG-activity could be characterized as an important factor of virulence of this fungus. The relation between PG pattern in different isolates *of B. cinerea* and pathogenicity on cabbage leaves clearly showed that the most virulent isolates produced *in vitro* the greatest amounts of the anodic PG isoform. Tomato, grape and pepper isolates showing high amounts of the anodic isoform, were highly pathogenic, while the others, isolated from lettuce, cucumber and sunflower showing low activity were weakly pathogenic. These results may explain at least partly the differences found in virulence between six *B. cinerea* isolates. Generally, cell wall degrading enzymes are considered as "basic compatibility factors" (Walton, 1994) but not as determinants of race or cultivar specificity. Di Lenna et al. (1981) compared also the virulence of three *B. cinerea* isolates on a wide range of hosts. They also found a positive correlation between the virulence of isolates and the *in vitro* pectic enzyme production: The most virulent isolate produced the greatest amounts of enzymes.

However, some other factors also influence the *in planta* situation. Microbial cell wall depolymerases are generally subjected to catabolite repression and substrate induction (Walton, 1994). Reignault et al. (1993) reported that PME produced by *B. cinerea* is regulated by catabolite repression of mono- and disaccharides. In addition, it is also possible, that new isoforms of pectic enzymes expressed only *in planta*, are also important in the plant-pathogen interaction as it was found in *Erwinia chrysanthemi* (Kelemu and Collmer, 1993). These facts indicate that *in vitro* data for PG-activity of *B. cinerea* isolates should be accepted carefully if conclusions are related to the *in planta* role of this

enzyme in pathogenicity. This question (i.e. the role of pectin degradation in pathogenicity of *B. cinerea*) could not be exactly answered until a mutant strain which is unable to grow on pectin will be constructed and tested *in planta*.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 169–173 (1995)

Hyperparasitic Activity of Some Saprophytic Fungi against *Curvularia lunata* a Leaf Spot Pathogen of *Setaria italica*

M. L. UPADHYAYA and R. C. GUPTA

Department of Botany, Kumaun University Campus, Almora 263 601 (U. P.), India

Colony interaction among some selected leaf surface fungi and the test pathogen were studied by dual culture technique. Hyphal interference among these fungal species were also observed. The test fungus *Curvularia lunata* was found to be attacked by *Fusarium moniliforme*.

Antagonism commonly means a relation between organisms in which one organism, the antagonist creates adverse circumstances for the other (Fokkema, 1976). The practical importance of antagonistic fungi in disease control appears to be a great challenge for plant pathologists. In recent years studies on hyperparasitic interactions have received great attention due to its application in biological control (Van den Heuvel, 1971; Blakeman, 1972; Skidmore and Dickinson, 1976; Fokkema, 1976; Rai and Singh, 1981; Singh, 1985). The present piece of work presents the results of mycoparasitic activities of saprophytic phylloplane fungi against *Curvularia lunata* through colony interaction and hyphal interference.

Materials and Methods

The following fungi were isolated from the leaf surface of *Setaria italica* (L.) Beauv. in order to study their interaction with the test fungi: *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Rhizopus nigricans*, *Mortierella subtilissima*, *Fusarium moniliforme* and the test fungus *Curvularia lunata*. Interactions among these fungal species were studied by employing the following two methods:

(i) Colony interactions

The selected fungal species were isolated from the test plants and maintained in pure cultures. The influence of fungal species among each other was examined by all possible paired combinations by placings agar blocks 3.5 cm apart on PDA (pH 5.6) in five replicate Petri dishes (Skidmore and Dickinson, 1976). Controls were single and dual inoculated cultures of the same fungus. The inoculated Petri dishes were incubated at 25 ± 1 °C in polythene bags to prevent drying. Interactions were assessed using a key based on the observations of Porter (1924) and Dickinson and Boardman (1971). Porter recognized five separate modes of interacting colony growth:

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(a) Mutually intermingling growth where both fungi grew into one another without any rnacroscopic sign of interaction (1).

(b) Intermingling growth where the fungus being observed is growing into the apposed fungus either above or below or above and below its colony (2).

(c) Intermingling growth where the fungus under observation has caesed growth and is being overgrown by another colony (3).

(d) Slight inhibition where the fungi approached each other until almost in contact and a narrow demarcation line, 1–2 mm, between the two colonies was clearly visible (4).

(e) Mutual inhibition at a distance of 2 mm (5).

Assessment were made when the fungi had achieved an equilibrium after which there was no further alteration in growth pattern.

(ii) Hyphal interference

Slide coverslip technique (Gupta and Tandon, 1976) was employed for this study. Two small blocks of sterilized and cooled Czapek's agar medium were placed on a sterilized glass slide at two points approximately 1 cm apart and were inoculated with the opposing fungal species separately, then a sterilized coverslip of 22 × 40 cm was placed over the inoculated agar and pressed a little so that some space was left between the glass-slide and the coverslip, where the fungal hyphae could easily grow. The whole set in triplicate was kept in moist chamber and incubated at 25 ± 1 °C. The two colonies met or came in close proximity within 2–3 days. Direct microscopic observations were made at room temperature after the hyphae were stained with a mixture of cotton blue and lactophenol.

Results and Discussion

(i) Colony interactions

As it is evident from the results (Table 1) *Aspergillus niger* was rated grade 1, which showed mutual intermingling growth with the test fungi, whereas *A. flavus*, *A. fumigatus*, *Rhizopus nigricans*, *Mortierella subtilissima* and *Trichoderma album* were rated grade 3, where the test fungi had caesed growth and was being overgrown by these fungal species, while *Fusariurn moniliforme* grew both below and above the colony of *Curvularia lunata*.

Different types of interactions were encountered between paired fungi on agar medium. Porter (1924) made a detailed study of various types of inhibition by more than 100 fungal and bacterial isolates on agar medium for selecting one isolate for testing *in vivo*.

Table 1

Antagonists	Colony interaction
Aspergillus niger	1
A. flavus	3
A. fumigatus	3
Rhizopus nigricans	3
Mortierella subtilissima	3
Fusarium moniliforme	3

The observations were based on Porter (1924)

It may be concluded from the observations that the intermingling growth of interacting fungi is possible only when both the fungal species showed equal growth rate, equal competitive and resistance capacity. A fungal species shows overdominating capacity when it has higher growth rate, or produces antibiotic substances and has tolerance capacity. Diffusion of active growth substances in nutrient agar by the antagonistic fungal species may be inhibitory or lethal for the test fungus (grades 5 or 4). Skidmore and Dickinson (1976) and Dickinson and Boardman (1970) also observed different types of interactions between paired fungi on agar media.

(ii) Hyphal interference

Different types of interference reactions were observed, of which penetration was more pronounced (Fig. 1a–d). The test fungus *Curvularia lunata*, was attacked by the mycelia of *Fusarium moniliforme* and affected penetration and vacuolation. The counteraction of the test hyphae with parasitic hyphae resulted in distortion and vacuolation in the infected hyphae (Dennis and Webster, 1971).

Dennis and Webster (1971) opined that the size of interacting hyphae might be a possible factor for penetration inside and around the host hyphae by antagonists. Wider hyphae are more susceptible and are penetrated by narrower hyphae (Durell, 1966; Rai et. al., 1977; Upadhyaya et al., 1979; Gupta and Tandon, 1976).

Acknowledgements

The authors are thankful to the Head, Department of Botany, Kumaun University Campus, Almora for providing laboratory facilities. The financial assistance by CSIR, New Delhi is gratefully acknowledged.

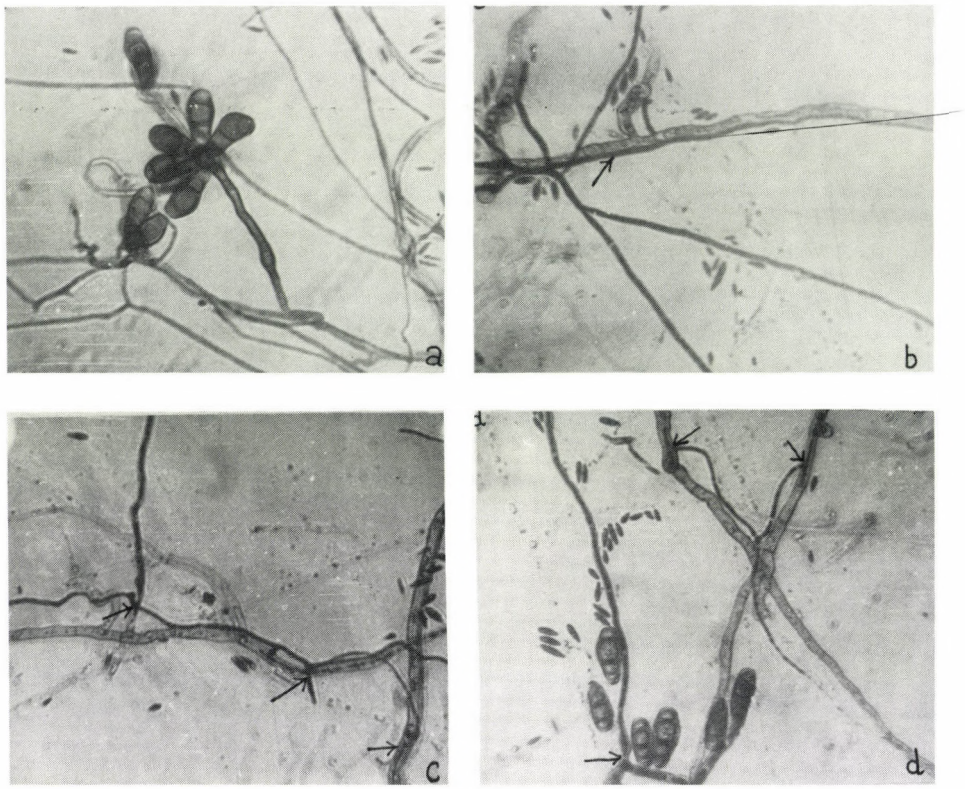

Fig. 1. (a) *Curvularia lunata* showing attachment of conidia; (b) *Fusarium moniliforme* hyphae running along the hot hyphae; (c) *Fusarium moniliforme* penetrating the host hyphae; (d) *Fusarium moniliforme* hyphae penetrating and growing inside the host hyphae

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 175–180 (1995)

Influence of Soil Temperature on Wilt of Muskmelon Incited by *Fusarium oxysporum* f. sp. *melonis* and *Fusarium solani*

PIOUS THOMAS[1] *, TUKARAM A. MORE and BINEETA SEN[2]

[1] Division of Vegetable Crops, [2] Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012, India

The influence of soil temperature on muskmelon wilt incited by *Fusarium oxysporum* f. sp. *melonis* and *Fusarium solani* was studied using different genotypes of the crop. The disease caused by *F. o. melonis* was favoured by a low soil temperature. The genotypes Kakri and Chittidar which exhibited resistance to this pathogen at average soil temperatures of 26.5 °C showed breakdown of resistance at 17.5 °C. The disease also appeared early and in more intense form at this temperature. On the other hand, the disease induced by *F. solani* was favoured by higher soil temperature. At an average soil temperature of 17.5 °C, there was no disease but when the plants were transferred to higher soil temperature of 23–24 °C, symptoms appeared immediately.

Considerable loss to muskmelon crop has been caused by the two pathogens, *Fusarium oxysporum* f. sp. *melonis* (Leach and Currence, 1938) Snyder and Hansen and *Fusarium solani* (Mart.) Sacc. in the riverbeds and garden lands of North India (Waraitch et al., 1976; Palodhi and Sen, 1981). *F. solani* was found to be the major pathogen in the vegetable research fields of the Institute (IARI) whereas Jamuna riverbed showed the prevalence of both the pathogens (Radhakrishnan and Sen, 1981).

Contrary to most vascular *Fusarium* wilts, muskmelon wilt by *F. o. melonis* is more common in cool soils (Mas et al., 1981). The pathogen grows well at 27 °C but the optimum temperature for pathogenesis is lower than that for growth (Leach and Currence, 1938). An increase in disease severity at temperatures below 30 °C was observed (Miller, 1945) and resistant varieties were found to become more resistant as the temperature increased (Molot and Mas, 1975). However, the influence of temperature on *F. solani* disease reaction is only poorly understood. *F. solani* isolates from melon were found to grow best at 24–30 °C in *in vitro* studies but only limited growth was observed at lower temperatures (Joffe and Palti, 1972). The present study examines the response of some genotypes of muskmelon to *F. o. melonis* and *F. solani* under different temperature conditions and discusses the possible role of this factor in governing wilt incidence in the Jamuna riverbed and garden lands of North India.

* Present address for correspondence: Tissue Culture Laboratory, Indian Institute of Horticultural Research, Hessarghatta Lake, Bangalore 560 089, India

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Materials and Methods

Screening against F. o. melonis

Five genotypes of melon viz. Kakri, Chittidar, Phoot, M 3 and Lucknow Safeda were used in this study. Of these, Kakri and Chittidar had been classified as resistant (R), Phoot as moderately resistant (MR) and M 3 and Lucknow Safeda as susceptible (S) to *F. o. melonis* based on earlier observations (Thomas et al., in press). These genotypes were screened against MM 4 isolate of the pathogen (Radhakrishnan and Sen, 1981) using a seed inoculation technique as described earlier (Thomas et al., in press). The inoculated seeds were sown in a steam sterilized medium containing garden soil, sand and farm yard manure (1:1:1), in aluminium trays and the trays were incubated under plastic-house conditions. Three such screening trials were conducted between the months of September and December. Soil temperature was recorded twice or thrice a week once in the morning (8.30–9.0 h) and once afternoon (15.00–15.30 h). The extent of mortality was recorded on days 14, 21 or 28 from sowing and, the genotypes were classified into three categories (R = 0–35.0, MR = 35.1–70.0 and S = 0.1–100%) at different temperature regimes.

Screening against F. solani

Seven genotypes (Pusa Madhuras, Lucknow Safeda, Kakri, Chittidar, Phoot, M 3 and M 4) were screened against *F. solani* using two isolates (MM 7 and MM 21) employing a seed inoculation technigue as in the case of other pathogen. There were 10–15 seeedling per genotype per isolate and an equal number in control.

Results

Soil temperature and seedling vigour

There was a gradual decline in soil temperature during the screenings against *F. o. melonis* (Table 1). At higher temperature (T1), the seedlings emerged in two-three days but as the temperature came down (T3) seedlings took more time (up to 7 days) for germination. At lower temperature the vigour of the seedlings also declined.

Disease development with F. o. melonis *inoculation*

At higher temperatures (T1), the disease symptoms appeared in susceptible genotypes three to four days after emergence. However, at a lower temperature (T3) it appeared just after seedling emergence. Pre-emergence mortality was also observed at this temperature.

Table 1

Soil temperature regimes during the screening trials against *F. o. melonis*

Exp. No.	Temperature (°C)		
	8.30–9.00	15.00–15.30	Average
1	22–24	30–32	26.5 (T1)
2	17–18	26–28	22.8 (T2)
3	12–14	22–24	17.5 (T3)

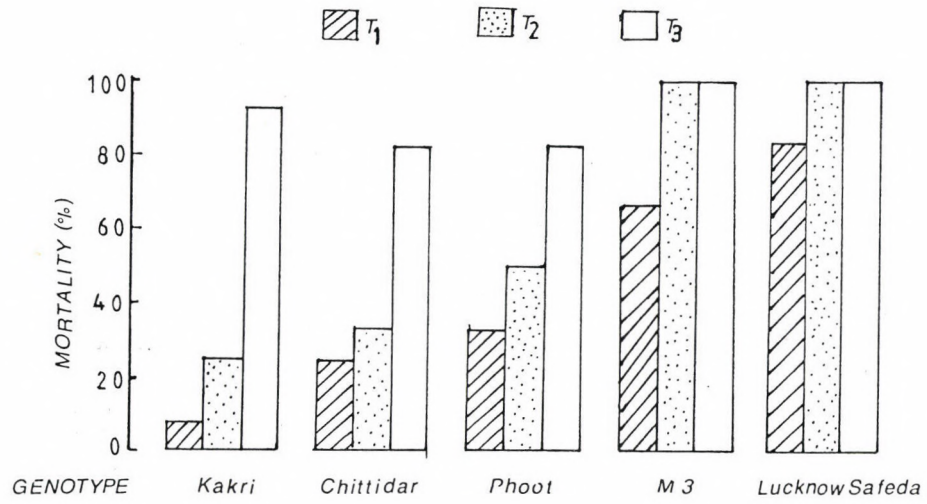

Fig. 1. Seedling mortality in different genotypes at three temperature regimes, following inoculation with *F. o. melonis*

Kakri and Chittidar which showed resistant reaction at higher temperatures became less resistant or susceptible as the temperature came down (Fig. 1). M 3 and Lucknow Safeda showed susceptible reaction at the three temperature regimes. However, the extent of disease incidence was higher at lower temperatures. Phoot behaved as moderately resistant at T1 but was susceptible at T2 and T3.

Temperature had an influence on symptom development, too (Fig. 2). In Kakri, Chittidar and Phoot, there was not much mortality after 14 days at average temperatures of 26.5 °C but at average temperatures of 17.5 °C they continued to show mortality.

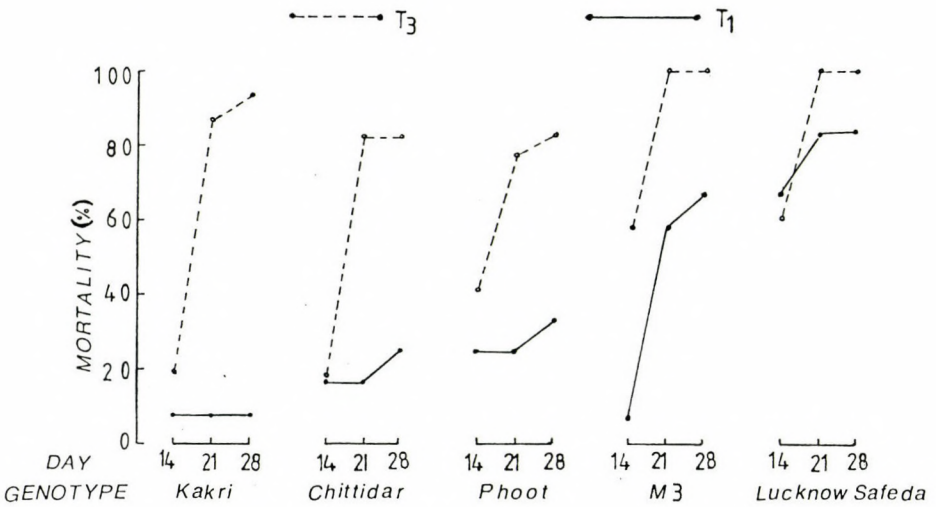

Fig. 2. Relative seedling mortality on different daye at two temperature regimes in screenings against *F. o. melonis*

Disease development in F. solani *inoculation*

The soil temperature in the plastic-house during the 45 days experiment ranged from 12–24 °C with an overall mean of 17.5 °C. However, there was no disease symptoms except for collar rot in one seedling of M 3, inoculated with MM 7 isolate. The trays were subsequently shifted to controlled conditions where a constant temperature of 23–24 °C was maintained. Disease appeared within three days of shifting to higher temperature. The behaviour of genotypes to the two isolates was similar (Table 2). The disease incidence was the highest in Pusa Madhuras and Lucknow Safeda and the lowest in M 4.

Table 2

Per cent mortality in different genotypes at 60 days from sowing following seed inoculation with *F. solani* isolates MM 7 and MM 21

Genotype	Mortality (%)	
	MM 7	MM 21
Pusa Madhuras	54.5	57.1
Lucknow Safeda	50.0	61.5
M 3	50.0	50.0
Kakri	50.0	41.7
Chittidar	45.5	41.7
Phoot	28.6	28.6
M 4	0	14.3

Discussion

Temperature has been highlighted as a factor capable of altering the disease reaction against *F. o. melonis* in crops like cabbage, peas (Walker, 1965), watermelon (Shimotsuma et al., 1972), tomato (Alon et al., 1974) and coriander (Srivastava, 1972). In these crops, the resistance either broke down or symptoms appeared earlier and in more severe form at higher temperatures. Contrary to this, muskmelon wilt by *F. o. melonis* is more common in cool soils (Leach and Currence, 1938; Mas et al., 1981). In this study, it was observed that the resistance in the genotypes Kakri and Chittidar was temperature dependent; the resistance property was lost at low temperature. This might be due to increased virulence of the pathogen at low tempearture or reduced vigour of seedling or both.

On the other hand, the disease caused by *F. solani* was favoured by a higher temperature. Disease did not appear when incubated at average soil temperature of 17.5 °C but the shifting of the plants to higher temperature (23–24 °C) induced sudden symptom expression.

Under North Indian conditions, *F. o. melonis* has been found to be a common wilt pathogen in the Jamuna riverbed whereas at the IARI experimental fields, *F. solani* is more damaging (Radhakrishnan and Sen, 1981; Thomas, 1989). In a recent study at IARI experimental fields, 78.0% of the isolations from diseased muskmelon plants were found to be *F. solani* and the rest showed a mixture of the two pathogens. Temperature prevalent during the crop period may be one of the factors which govern the relative incidence of disease by the two pathogens at these two locations. In the Jamuna riverbed, muskmelon is sown during November–December (Seshadri, 1986) and the seedlings are exposed to low temperature for quite a long time, whereas at the IARI fields, sowing is done in mid-February and the crop is exposed to higher temperature soon. Thus the crop at Jamuna riverbed becomes vulnerable to *F. o. melonis* whereas the crop at the IARI fields escapes the infection from it. The two pathogens induce similar symptoms but they are opposite in their temperature requirements.

Acknowledgements

The authors are grateful to Dr. V. S. Seshadri, former Head, Division of Vegetable Crops, for providing the facilities for the study. The Senior author acknowledges the Sr. Research Fellowship offered by P. G. School, IARI, New Delhi for his Doctoral research.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 181–182 (1995)

Occurrence of Seed Borne *Fusarium oxysporum* Schlecht and *Stemphylium botryosum* Wallr. on Sainfoin (*Onobrychis viciifolia* Scop.) in Hungary

ENDRE I. SIMAY

I. A. Q. Research Centre for Agrobotany, Tápiószele*

In course of seed health tests of Hungarian gene bank *Fusarium oxysporum* and *Stemphylium botryosum* were observed on seeds of sainfoin (*Onobrychis viciifolia* Scop.) in Hungary at first. The fungi were identified from sporulations developed on seeds and from pure cultures. The contamination of seeds caused seed and seedling rot on the host investigated.

Sainfoin (*Onobrychis viciifolia* Scop.) is one of the rather traditional forage crop in Hungary (Villax, 1933) and numerous fungi are reported from this host (Bánhegyi et al., 1985). However occurrence of *Ascochyta onobrychidis* Bondarz.-Montev. was known only from the gene bank works in village Tápiószele (Holly and Walcz, 1974). *A. onobrychidis* could infect the germinating seeds or seedlings, but other seed-transmissible pathogens were not reported from the gene bank occurring on sainfoin.

Fusarium oxysporum Schlechtendahl and *Stemphylium botryosum* Wall-roth were observed on molded seeds in our tests made during 1986–90. The fungus species were identified from the sporulation developed on the molded surface and pure cultures were initialized from these sporulations, too. The pure cultures were maintained on potato dextrose agar (PDA) and 2% malt extract agar (MEA) media. The identifications based on Booth (1971) and Corlett et al. (1982).

Fusarium oxysporum developed as whitish mold on contaminated seeds, and numerous conidia were observable in the mycelium. Both microconidia and macroconidia were observable. Microconidia were aseptated or with one Septum while macroconidia were mostly four celled. The measurements of micro- and macroconidia were 5.2–9.6 × 2.4–3.6 μm and 26.4–48 × 3.2–4.8 μm respectively. The colonies of fungi were fast growing, reaching 4.8–6.5 cm diameter in four days. The aerial mycelium were pale, but intensive violet discolouration of stromatic agar surface were observed. Chlamydospores and small sclerotia were developed, too.

Stemphylium botryosum was observed on seeds developing black mold and stromatic protoperithecia on seed surface, too. The conidiophores of fungus were flexous, unbranched, yellow brown. The conidia were medium to dark yellow brown, oblong or ellipsoidal, with 2–4 transverse and 2–3 vertical septa. The measurements of conidia

* Present adress: Enterprise for Extension and Research in Fruit Growing and Ornamentals, Budapest, Hungary

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were 24–36 × 15.4–24.8 μm from seeds, but were slightly smaller from cultures. The cultures of fungus were variable by dense of aerial mycelium even of sporulation. Protoperithecia of fungus were often observed on agar media, but maturation of them were not.

Both identified fungi are well known from different seeds (Neergaard, 1979), and the seed transmission of *Fusarium oxysporum* could have an epidemic role, too (Gambogi, 1983). However the fungi mentioned above are known from different substrates from Hungary, to the author's best knowledge this is the first report on their occurrence on seeds of sainfoin from this area.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 183–190 (1995)

Effect of Growth Stage of the Rice Plant on the Severity of Sheath Blight Infection

CHONG SONG JONG[1] and A. PREMALATHA DATH[2]

[1] Rice Research Institute, Academy of Agricultural Sciences, Pyang Yang D. P. R., Korea
[2] Central Rice Research Institute, Cuttack-753 006, Orissa, India

When 12 rice cultivars were inoculated with 3 isolates of *Rhizoctonia solani* at active tillering, maximum tillering and flowering stages, it was found that the interaction between *R. solani* isolates, rice cultivars and stages of the plant growth was significant thereby indicating that everyone of the combination of these factors influenced sheath blight severity. But the disease severity, extent of leaf infection and sclerotial production in many cultivars, in general, was more at the flowering stage an less at the active tillering stage.

Of late, sheath blight disease of rice (*Rhizoctonia solani* Kuhn) is gaining importance in many rice growing countries. Though the sheath blight fungus can parasitise rice at any growth stage (Tu, 1968; Kamjaipai and Giatgong, 1971), the damage on young plants was reported to be more severe (Valdez, 1955). Therefore, the growth stage of rice plant is one of the important factors that influence the severity of sheath blight. But incongruous results have been reported in literature (Premalatha Dath, 1990) on the stage of the rice plant in relation to sheath blight severity. Therefore, this aspect was re-investigated during the wet season of 1990 utilizing three isolates of the causal fungus.

Materials and Methods

Seven susceptible rice cultivars such as Annada, Annapurna, DV 85, HPU 2801, HR 12, IR 50, Neela and five cultivars such as Dular, Ratna, Tadukan, Tetep, Taichung (Native)1 (T(N)1) that recorded a variable reaction of resistant-susceptible and *vice versa* by different workers (Premalatha Dath, 1990) were included in the study. Plants were grown in 30 cm dia earthen pots @ one seedling per pot under a fertility level of $N_{120}P_{60}K_{60}$ kg/ha. They were sown and transplanted at different times in order to get three growth stages of the plants such as active tillering, maximum tillering and flowering stages on the same day of inoculation.

Plants at the 3 stages were inoculated with 3 isolates of *R. solani* that were collected from different states of India such as Punjab (U), Nagaland (N_1) and Orissa (BV).

Correspondence send to: A Premalatha Dath, Division of Plant Pathology, Central Rice Research Institute, Cuttack-753 006, Orissa, India

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Table 1

Percentage of infection produced by 3 isolates of *Rhizoctonia solani* at different growth stages of rice plants

Isolate	Growth stage			Mean
	Active tillering	Maximum tillering	Flowering	
U	8.33	16.92	31.67	18.98
N1	15.83	26.93	48.26	30.34
BV	11.84	22.16	37.53	23.84
Mean	12.00	22.00	39.15	
			5%	1%
C.D. for isolate mean			2.47	3.25
C.D. for growth stage mean			2.47	3.25
C.D. for interaction (growth stages × isolates)			4.27	5.61

Matured sclerotia of uniform size were picked from 10-day-old growth of *R. solani* isoplates on PDA plates and the plants were inoculated at second leaf sheath from top in each of the 3 tillers per plant by inserting single sclerotium between sheath and stem with the help of a sterilized forceps. Three replicated pots were maintained in each case.

Sheath blitht severity was assessed by recording the percentage of infection (disease build-up) in each plant on the 21st day after inoculation. On the same day, observations were also recorded on the extent of leaf infection and the number of sclerotia produced in each cultivar.

Results

The mean percentage of infection was highest at flowering stage and lowest at active tillering stage (Table 1). All the 3 isolates of the causal fungus significantly differed with each other in aggressiveness (percentage of infection). Isolates N_1 and U were high and least aggressive respectively.

At active tillering stage, the infection produced by U and BV and BV and N_1 did not differ. But at the other two stages, N_1 produced significantly more infection while U produced less infection. All the 3 isolates produced significantly more infection at flowering and less at active tillering stage.

The mean percentage of infection in different rice cultivars varied (Table 2). IR 50 and Annapurna recorded maximum and Tetep and Tadukan recorded least infection which was on par with DV 85.

The interaction between rice cultivars and growth stages of plants in relation to the percentage of infection was also significant. In Annapurna, Dular, HPU 2801, IR 50, Neela, Ratna and T(N)1, the percentage of infection was maximum at flowering and less at active tillering stage. Though Annada also showed more infection at flowering, the infection on this cultivar at active tillering and maximum tillering stages did not differ significantly. HR 12 exhibited a different trend. The infection at active tillering and flowering stages failed to differ. There was also no difference between active tillering and maximum tillering stages. DV 85, on the other hand, exhibited no difference in infection between active tillering and flowering and maximum tillering and flowering stages. On the contrary, none of the stages differed in Tadukan and Tetep.

At active tillering stage, the percentage of infection was significantly more in HR 12. At this stage most of the cultivars except IR 50 and Annapurna showed less disease. At maximum tillering stage, the infection was more in IR 50 and less in Tetep, Tadukan and Annada. At flowering stage, Annapurna recorded highest percentage of infection. Less infection at this stage was recorded in Tetep, Tadukan and DV 85.

The 3 isoletes of *R. solani* also varied in their aggressiveness on different rice cultivars (Table 3). Though isolate N_1 produced more percentage of infection than U and BV in Annada and IR 50, the infection produced by the 3 isolates failed to differ in DV 85, HPU 2801, Ratna, Tadukan and Tetep. But in Annapurna and T(N)1, the infection produced by U and BV and BV and N_1 did not differ. Similarly, on Dular and Neela, U and BV failed to differ while N_1 produced more infection on these two cultivars. On HR 12, BV and N_1 were at par with each other.

The infection produced by U was more on Annapurna followed by Neela. Least infection was recorded in Tetep that was on par with that of Tadukan, Annada and DV 85. Isolate N_1 produced more infection on IR 50 followed by HR 12 and least infection was recorded in Tetep that was on par with that of Tadukan and DV 85. Isolate BV also produced more infection on IR 50 that was on par with that of HR 12 and Annapurna. It produced significantly less infection in Tadukan and Tetep.

The interaction between rice cultivars, plant growth stages and isolates of *R. solani* in relation to the percentage of infection was significant (Table 4). The differences in disease build-up at different stages of plant growth is more clearly seen from the data. The maximum infection recorded at flowering stage was 70–80% while maximum infection recorded at the other two stages was less than 55%.

Infection at different plant growth stages was influenced by the cultivar × isolate combination. For instance, isolate U on Annada produced high infection at active tillering and flowering stages and less at maximum tillering stage. On the same cultivar, N_1 produced more infection at flowering and less at the other two stages. All the 3 stages significantly differed when inoculated with BV. This isolate produced more infection at flowering and less at active tillering stage.

Similarly, on HR 12, isolate BV produced significantly more infection at active tillering. The same isolate on Ratna produced highest infection at maximum tillering. On the conrary, the plant growth stages did not influence the amount of infection in DV 85 irrespective of the degree of aggressiveness of the isolates.

Table 2

Interaction between rice cultivars and plant growth stages in relation to the percentage of infection produced by *Rhizoctania solani*

Cultivar/ Growth stage	Percentage of infection in												Mean
	Annada	Annapurna	Dular	DV 85	HPU 2801	HR 12	IR 50	Neela	Ratna	Tadukan	Tetep	T(N)1	
Active tillering	12.66	19.33	5.79	10.00	10.08	37.97	21.53	4.80	4.24	4.24	4.43	8.98	12.00
Maximum tillering	13.34	28.12	21.79	19.06	27.59	29.46	38.45	28.32	19.53	10.23	8.75	19.44	22.00
Flowering	45.91	73.91	32.19	15.36	42.50	39.73	64.69	68.10	30.46	11.81	11.06	34.11	39.15
Mean	23.97	40.45	19.92	14.81	26.72	35.72	41.56	33.74	18.07	8.76	8.08	20.84	

	5%	1%
C. D. for growth stage mean	2.47	3.25
C. D. for cultivar mean	4.94	6.49
C. D. for interaction (cultivars ×growth stages)	8.57	11.26

Table 3

Interaction between rice cultivars isolates of *Rhizoctania solani* in relation to the percentage of infection

Cultivar/ Isolate stage	Percentage of infection in												Mean
	Annada	Annapurna	Dular	DV 85	HPU 2801	HR 12	IR 50	Neela	Ratna	Tadukan	Tetep	Z(N)1	
U	11.72	36.56	16.71	12.54	26.70	19.57	27.94	32.31	14.47	7.39	5.36	16.44	18.98
N1	37.50	45.94	26.36	15.26	25.02	47.60	54.53	39.19	21.36	13.27	11.11	26.93	30.34
BV	22.69	38.86	16.69	16.61	28.46	39.99	42.19	29.71	18.39	5.63	7.76	19.16	23.84
Mean	23.97	40.45	19.92	14.81	26.72	35.72	41.56	33.74	18.07	8.76	8.08	20.84	

	5%	1%
C. D. for isolate mean	2.47	3.25
C. D. for interaction (cultivars × isolates)	8.57	11.26

Table 4

Interaction between rice cultivars, plant growth stages and isolates of *Rhizoctania solani* in relation to the percentage of infection

Cultivar	Active tillering Isolates			Maximum tillering Isolates			Flowering Isolates		
	U	N_1	BV	U	N_1	BV	U	N_1	BV
Annada	5.18	28.74	4.05	5.74	12.37	21.90	24.23	71.39	42.12
Annapurna	32.60	21.34	4.05	12.92	30.29	41.13	64.17	86.17	71.39
Dular	4.05	8.13	5.18	19.62	40.00	5.74	26.45	30.96	39.16
DV 85	5.74	10.53	13.73	18.06	22.90	16.22	13.82	12.37	19.89
HPU 2801	5.18	9.97	7.01	32.71	15.14	35.00	34.12	50.00	44.04
HR 12	21.90	45.82	54.84	23.16	39.06	26.15	22.29	57.91	38.98
IR 50	4.05	29.22	30.76	23.74	54.83	36.76	55.47	79.55	59.06
Neela	5.18	5.74	4.05	25.19	41.15	18.61	66.47	70.69	66.47
Ratna	4.05	4.61	4.05	8.13	18.05	33.07	31.23	42.09	18.05
Tadukan	4.05	4.05	4.61	9.97	15.66	5.74	8.13	20.76	6.54
Tetep	4.61	4.05	4.61	8.13	9.97	10.53	5.74	19.31	8.13
T(N)1	4.05	17.70	5.18	18.05	25.19	15.07	27.22	37.91	37.22

	5%	1%
C. D. for interaction (cultivars × growth stages × isolates)	14.84	19.50

Leaf infection and sclerotial production

The extent of leaf infection and the number of sclerotia produced were more at flowering stage and very less at active tillering stage (Table 5). At all the stages, isolate N_1, in general, produced more leaf infection and sclerotia than U and BV.

At active tillering stage, all the 3 isolates produced more leaf infection on HR 12 and at maximum tillering stage, they produced more infection on HPU 2801 and IR 50. At flowering stage, it was more on Annapurna, HPU 2801, IR 50 and Neela. But in general, the extent of leaf infection and sclerotial production was less in Dular, DV 85, Tadukan and Tetep.

Discussion

Maximum infection of sheath blight, according to Roy (1979), was caused when plants were inoculated at active tillering stage, less at early tillering and least at the booting stage. Yoshimura and Nishizawa (1954) found that maximum tillering stage was the optimum for varietal testing. But according to others (Lee and Rush, 1983; Manian, 1984), rice plants showed greatest susceptibility at panicle emergence. Reasons for such discrepancies could be better understood by the data gleaned in the present study.

The cultivar used seemed to be one of the factors responsible for getting such variable results as reported by different workers. For instance, at active tillering stage, cultivar HR 12 showed highest percentage of infection whereas IR 5U and Annapurna recorded highest infection at maximum tillering and flowering stages, respectively. Similarly, the disease build-up at active tillering and maximum tillering stages did not differ significantly in some cultivars such as Annada while in other cultivars such as HR 12 and DV 85, infection at active tillering and flowering stages did not differ. On the contrary, none of the stages differed in Tadukan and Tetep. Therefore, the interaction between rice cultivars and stages of rice plant reflected in a different way with different varieties.

Infection at the plant growth stages was also found to be influenced by the cultivar × isolate combination. For instance, isolate BV produced more infection at flowering and less at active tillering stage on the cultivar Annada. But on the same cultivar, isolate U produced highest infection at active tillering and flowering stages and less at maximum tillering stage. On the other hand, N_1 produced more infection at flowering and less at the other two stages.

In the present study, the percentage of infection was used as a parameter to score the disease severity because in this parameter both horizontal spread of the disease to adjacent tillers and also the vertical spread of infection to upper plant parts including infection on leaves, extent of sclerotial production were all taken into account for assessing the disease severity. In other words, the quantum of disease build-up in a plant as a whole was assessed rather than measuring the lesion length or scoring infection based on 0–9 scale IRRI Standard Evaluation System for Rice (1980) on the inoculated tiller. This

Table 5

Extent of leaf infection and the number of sclerotia produced by *Rhizoctania solani* isolates at 3 stages of plant growth in different rice cultivars

Rice Cultivar	Active tillering stage			Maximum tillering stage			Flowering stage		
	U	N_1	BV	U	N_1	BV	U	N_1	BV
Annada	+(0)	++(0)	+(0)	++(0)	++(0)	**	+++(2.0)	+++(12.0)	++(1.3)
Annapurna	**	+(0)	+(0)	+++(2.0)	+++(1.0)	++(0)	+++(3.0)	+++(11.3)	+++(5.0)
Dular	**	**	**	+(0)	++(0)	+(0)	++(1.7)	++(0.3)	+(0)
DV 85	**	**	**	+(0)	++(0)	++(0)	+(0)	+(1.7)	++(0)
HPU 2801	+(0)	++(0)	**	+++(2.3)	+++(2.0)	+++(0)	+++(0)	+++(7.7)	+++(0)
HR 12	++(2.0)	+++(2.7)	++(0)	++(1.7)	+++(4.0)	++(0)	+++(8.3)	+++(6.7)	++(0)
IR 50	+(0.7)	++(0)	**	+++(0)	+++(3.3)	+++(0)	+++(2.3)	+++(5.0)	+++(5.0)
Neela	**	**	+(0)	++(0)	+++(0)	+++(0)	+++(8.7)	+++(2.7)	+++(4.0)
Ratna	**	**	**	+++(0.7)	++(0.7)	++(0)	++(0.3)	+++(5.3)	+++(2.0)
Tadukan	**	**	**	+(0)	+(0)	**	**	+++(1.7)	+(0)
Tetep	**	**	**	+(0)	++(0.3)	**	**	+++(1.0)	+(0)
T(N)1	**	**	**	+(0)	++(0)	+(0)	+++(0)	+++(3.0)	++(0)

+ = Very less leaf infection
++ = Moderate leaf infection
+++ = Severe leaf infection
( ) = Average number of sclerotia
** = No leaf infection and no sclerotia

might be the reason why clear-cut differences could be seen between highly susceptible and less susceptible cultivars by this parameter.

The interaction between rice cultivars, plant growth stages and isolates of *R. solani* in relation to disease build-up was significant thereby indicating that the permutation and combination of these factors was responsible for obtaining different trends in the disease. However, distinct differences in the amount of disease among susceptible and less susceptible rice cultivars, in general, was seen at flowering than at active tillering and maximum tillering stages. Even the leaf infection and sclerotial production in different cultivars was maximum at flowering stage and least at the active tillering stage. In other words, plants became more susceptible as they grew older. This observation was in consonance with that of Hashioka (1951) and Kozaka (1961).

Kozaka (1961) reported that rice plants as they approach heading stage become more compact and more subject to contact infection. This might be the reason for severe occurrence of leaf infection on most of the susceptible cultivars at flowering stage than at the other two stages. Thus sheath blight severity is better differentiated on mature plants than young plants.

It is important to note that amongst the 5 cultivars that gave a variable reaction of resistant – susceptible and *vice versa* by different workers (Premalatha Dath, 1990), only Tetep showed less infection at all the 3 growth stages against all the 3 isolates of *R. solani* thus indicating its remarkable tolerance to sheath blight disease.

Acknowledgements

Grateful thanks are expressed to Dr. J. P. Sharma of ICAR Research Complex for N. E. H. Region, Medziphema, Nagaland and Dr. Raina, Rice Research Station, Kapurthala, Punjab for kindly providing the isolates of *Rhizoctonia solani*.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 191–203 (1995)

Changes in the Phenol Metabolism of Rice Cultivars with Reference to Nitrogen Fertilization and Sheath Blight Disease

K. MANIBHUSHANRAO[1] and S. MANIAN[2]

[1] Centre for Advanced Studies in Botany, University of Madras, Madras 600 025, India
[2] Department of Botany, Bharathiar University, Coimbatore, India

Various physiological changes accompanying sheath blight (ShB) pathogen *Rhizoctonia solani* Kuhn in rice was studied in two resistant (ARC 59443 and B1757d Sm-6-1) and two susceptible cvs cultures (IR1487-14d-5-3-2 and B1749d-Sm-50-50) under unfertilized and N-fertilized conditions. There was a general increase in the levels of orthodihydroxy and total phenols and flavonoids of rice tissue upon N-fertilization. *R. solani* infection significantly increased these cell constituents in both resistant and susceptible cvs. Interestingly enough, these increases were more pronounced in cvs resistant than ausceptible.

Changes in enzyme activities of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) were consistant with phenolic increase in the infected under N-fertilized and unfertilized conditions. Further, the oxidizing enzymes peroxidase (PO) and polyphenoloxidase (PPO) registered higher activities in the diseased plants.

In nature 'resistance is the rule rather than an exception' (Yarwood, 1967). Plants have some mechanism(s) to defend from most of the microorganisms in their environment. Pathogenesis results only when the pathogen overcomes the host defence reactions. Thus, the pathogens trigger host metabolism and the expression of symptoms is the reflection of these alterations.

Quantitative changes in phenolic compounds due to infection have been observed by many researches in various host-pathogen interactions. Many phenols and their oxidative products (quinones) are highly fungitoxic (Byrde et al., 1960; Farkas and Király, 1962; Patil and Dimond, 1967; Rohringer and Samborski, 1967; Hampton, 1970; Alfenas et al., 1982) and hence phenol have been asigned a role in disease resistance. It has been found that increased N-fertilization resulted in a decrease in phenol level of host tissues (Matsuyama and Dimond, 1973; Vidhyasekaran, 1974; Naidu et al., 1979). Phenylalanine ammonia lysae (PAL) and tyrosine ammonia lyase (TAL) catalyse the deamination of the respective amino acids (Neish, 1961) which in turn serve as major substrates for eventual biosynthesis of a variety of phenolics. A close association of these enzymes with increased aromatic biosynthesis in diseased plant tissue is well documented (Young et al., 1966; Farkas and Szirmai, 1969; Novacky and Acedo, 1970; Camm and Towers, 1973; Green et al. 1975; Glazener, 1982). The oxidative enzymes, in particular peroxidase (PO) and polyphenol oxidase (PPO) are known to bring about the oxidation of phenols and related compounds increasing their toxicity (Tomiyama and Stahmann, 1964; Webb, 1966; Kosuge, 1969; Moustafa and Whittenbury, 1970; Frič, 1976). Recently,

0238–1249/95/$ 5.00

Manibhushanrao et al. (1988) critically evaluated the role of phenolics on disease resistance of higher plants.

Rice ShB is considered one of the major diseases as it causes heavy yield losses and becomes potential threat in rice cultivation in recent years. The disease occurs in all stages of the rice plant viz. seedlings, tillering and boot leaf stages. Earlier, Zuber and Manibhushanrao (1984) and Manibhushanrao et al. (1986) reported the changes in phenol contents in resistant (IR 20) and susceptible (TKM 9) rice seedlings during different stages of ShB disease progress. Further, the effect of phenyl acetic acid (FAA), the phytotoxin produced by *R. solani* on the phenolics and enzymes of phenol metabolism was studied in both the resistant (IR 50) and susceptible (TKM 9) cvs to ShB. Interestingly, the impetus of PAA in general was more susceptible on cv. TKM 9 and the magnitude of increase of total OD phenols and the activities of PAL, TAL and PO were greater than the resistant cv. IR 50. However, no information is available on N-fertilization application on the levels of phenolics as well as of activities oxidative enzymes viz. PO and PPO in *R. solani* infected rice plants.

In the present study the changes in phenolic and enzyme activities of pheno biosynthesis and oxidation products in ShB pathogen infected resistant and susceptible rice cvs have been reported.

Materials and Methods

Method of raising the plants and inoculation

Two resistant (ARC 59443 and B 1757d-Sm-6-1) and two susceptible (IR 1487-194-5-3-2 and B 1749d-Sm-50-5) rice cvs were selected from the varietal screening trials (Manian and Manibhushanrao, 1982) as they showed consistantly the respective reactions through successive trials *kharif* (July to October) 1978; *rabi* (December to March) 1978–79; *rabi* (1979–80). Each cv was raised in 1 m^2 micro plots arranged in a randomized block design with the following treatments: unfertilized uninoculated, unfertilized *R. solani* inoculated, N-fertilized uninoculated, and N-fertilized *R. solani* inoculated. Rice seeds were sown directly in 1 meter rows, 25 cm apart from each other. Approximately 5 g of seeds were used per row. Over crowded seedlings were thinned to maintain 10–15 cm between them. The plots were irrigated daily for 4 weeks after which they were flooded with water.

To N-fertilized plots, N in the form of urea was applied in four split doses at 25 g N/ha/application, 15-d before each sampling. The total N applied was 100 kg/N/ha at the time of final sampling. Fresh rice plants were artificially inoculated ten days before each sampling with a virulent R5 isolate of *R. solani* (ATCC 48505) using stem-tape inoculation technique (Amin, 1975). Samplings were made from 45, 60, 90 and 120-d rice plants.

Ethanol extraction

Samples (5 g healthy and infected shoot portions) were comminuted, extracted in boiling 80% ethanol, for 5 min and homogenized. The homoginate was filtered through four layes of cheese cloth are the residue was reextracted. The extracts were pooled, filtered through Whatman No. 41 filter paper and the final volume was adjusted to 25 ml with 80% ethanol to represent 5 ml/g of the tissue.

Estimation of phenolic compounds

OD-phenols in the ethanol extracts of plant material were determined colorimetrically using Arnow's reagent (Johnson and Schall, 1952). Total phenols were estimated by following Bray and Thorpe's (1954) folin-ciocalteau reagent method. Flavonoids were estimated following the method of Shinoda (1928) with catechin hydrate as the standard.

Preparation of acetone powders and extraction enzyme(s)

Acetone powder of the samples (5 g) was prepared according to the method of Umbreit et al. (1972). Acetone powder (150 mg) was suspended in 3 ml buffer (specified for the enzyme) and extracted overnight at 4 °C. The extract was squeezed through cheese cloth and the filtrate centrifuged at 25.000 g for 30 min. The clear aupernatant was used as enzyme preparation (Neisch, 1961).

Estimation of protein

The protein content of the crude enzyme extract was estimated using the method of Lowry et al. (1951).

Assay of PAL and TAL

Using phenylalanine or L-tyrosine as the substrates, PAL, TAL activities were assayed spectrophotometrically at 268 and 333 nm respectively, following method of Higuchi (1966). The enzyme activity was expressed as μg cinnamic or coumaric acid/h/ml of enzyme preparation.

Assay of PPO and PO

Using DL – DOPA and pyrogallol as the substrates, PPO and PO activities were estimated following the methods of Palmer (1963) and Loberstein and Linsely (1961), respectively. PPO activity is expressed as increase in OD/h/ml enzyme preparation. Taking 0.001 increase in OD/min at a unit, PO activity is expressed in terms of specific units.

Results

Changes in OD phenols

There was a general increase in OD phenols in the N-fertilized susceptible rice plants, however, in N-fertilization of resistant plants, no such trend was observed (Fig. 1). *R. solani* infection resulted in the accumulation of OD phenols in both the resistant and susceptible cvs under N-fertilized and unfertilized conditions with an exception of the resistant B 1757d-Sm-6-1 in N-fertilized condition. The four rice cvs studied could not be differentiated into resistant and susceptible to *R. solani* on the basis of their OD phenol content (Fig. 1). The OD phenols in the plant tissue decreased as the plants grew to maturity. The response of younger plants was slightly higher upon infection than older ones in the accumulation of OD phenols.

Changes in total phenols

Figure 2 gives the changes in the total phenol content of resistant and susceptibly rice cvs. All the four cvs studied showed slight increase of total phenols as a response to N-fertilization in split doses with an exception of ARC 5943 on 45th day. Such an increase was pronounced in the *R. solani* infected plants of N-fertilized condition. The resistant ARC 5943 and B 1757d-Sm-6-1 responded more to sheath blight infection by accumulation of phenolics. However, neither resistance nor susceptibility of uninoculated cvs were marked by their total phenol content. The older plants generally showed a decrease in total phenols and such a decrease was high in the cv. ARC 5943 of unfertilized plants.

Changes in flavonoids

N-fertilization significantly increased the flavonoid content in both resistant and susceptible rice cvs, and this increase was more pronounced in the 90-d plants (Fig. 3). There was a general accumulation of flavonoids in the ShB infected plants over the healthy ones. Further, the unfertilized plants accumulated more flavonoids upon infection than the N-fertilized plants. The response of resistant cvs was more to *R. solani* infection than the susceptible ones in the accumulation of flavonoids.

Changes in the activities of PAL and TAL

It has been well documented that N-fertilization decreases the host phenol content, which are increasingly related to host defence system. However, the results showed an increased OD and the total phenol content upon N-fertilization in split doses. Hence, with a view to understand this phenomenon, an assay of four key enzymes involved in the phenol metabolism was carried out using 90-d plants of resistant ARC 5943 and susceptible IR 1487-194-5-3-2 cvs.

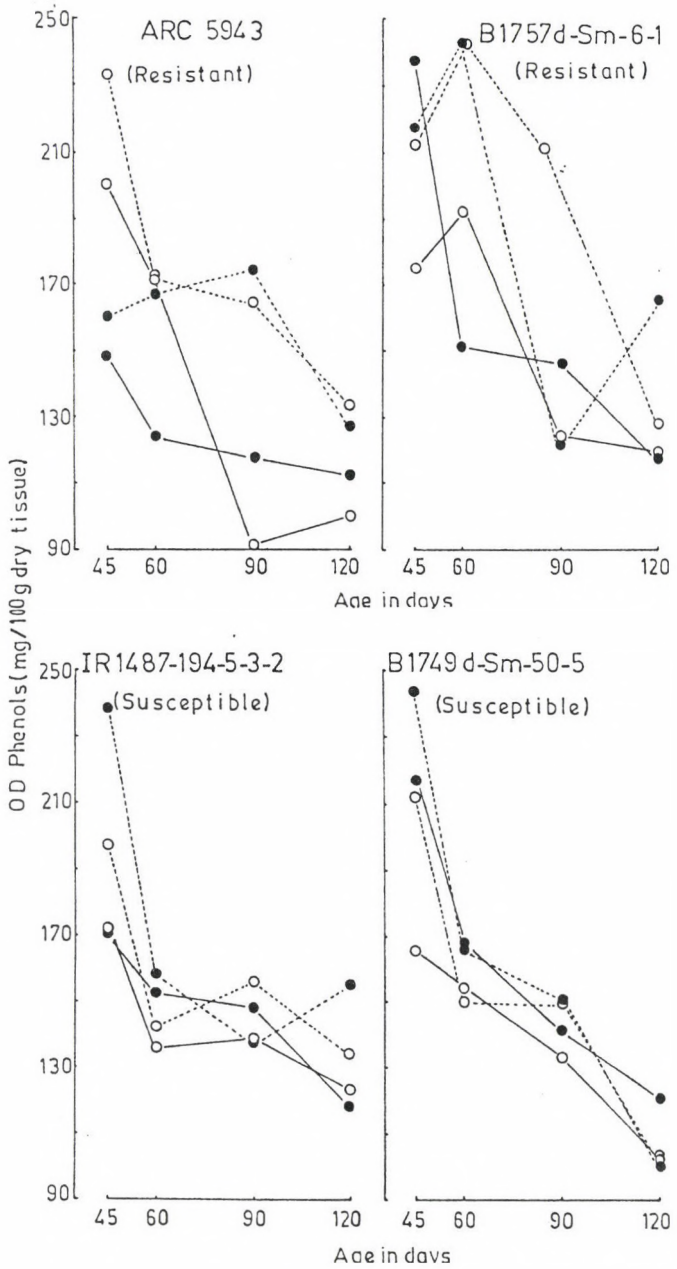

Fig. 1. Changes in OD phenols in resistant and susceptible rice cvs following *R. solani* infection

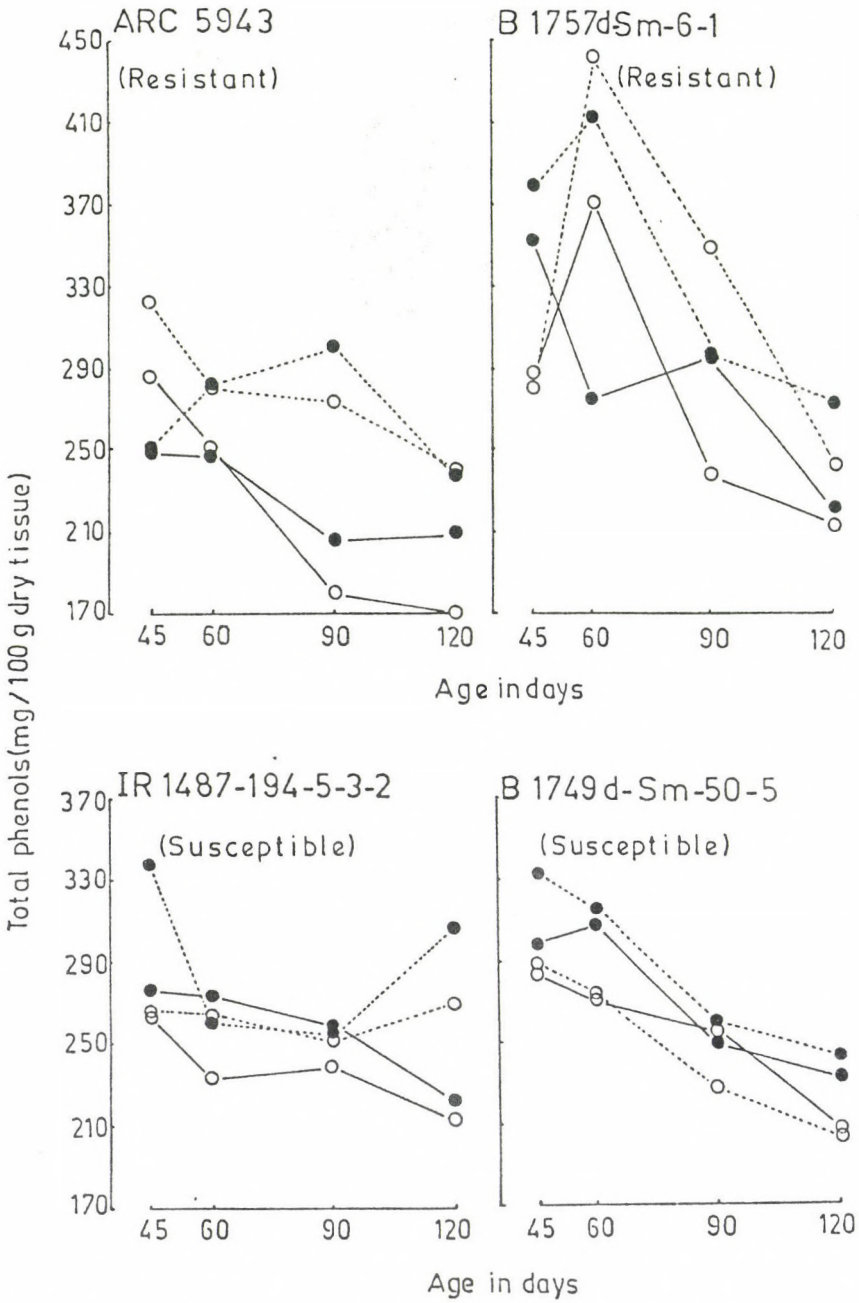

Fig. 2. Changes in total phenols in resistant and susceptible rice cvs following R. *solani* infection

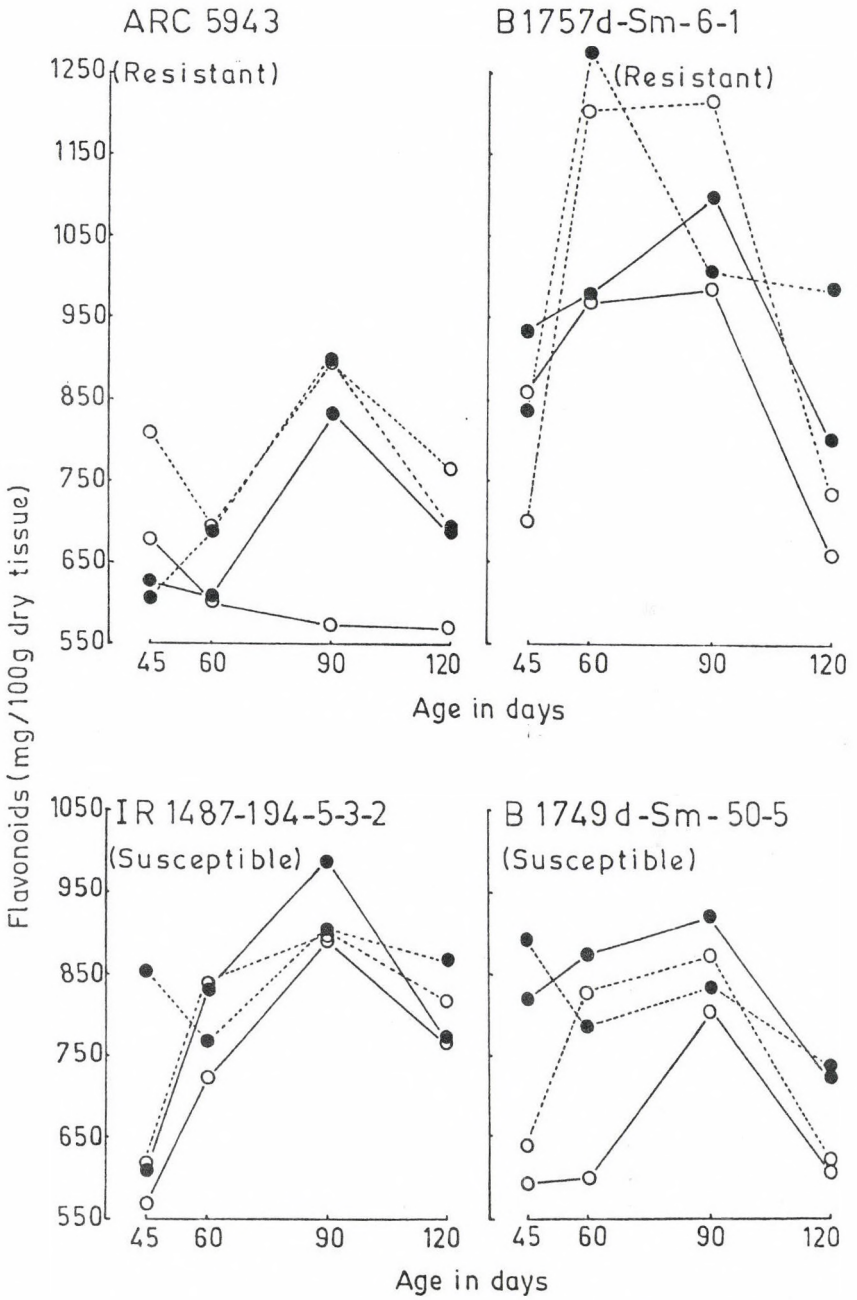

Fig. 3. Changes in flavonoids in resistant and susceptible rice cvs following *R. solani* infection

In general, PAL activity increased in response to N-fertilization and ShB infection. Among the unfertilized, *R. solani* infected plants, the resitant ARC 5943 registered more (nearly twofold over uninoculated) PAL activity compared to susceptible IR1487-194-5-3-2, while the reverse was true under N-fertilization (Fig. 4a). Similar to PAL activity, the rice plants responded to N-fertilization and *R. solani* infection with increased activity of TAL (Fig. 4b). There was a twofold increase in TAL activity in the susceptible, infected plants under fertilized condition while this was not so in the resistant cv. However, among unfertilized plants the resistant cv showed twofold increase in activity over the susceptible one.

Both N-fertilization and ShB infection increased PO activity in the two rice cvs studied (Fig. 5a). The resistant plants registered more activity than the susceptible ones upon infection in both unfertilized and N-fertilized conditions. However, the uninoculated resistant and susceptible cvs did not show any marked difference in their PO activity. Figure 5b shows that unlike PO, PPO activity slightly decreased in response to N-fertilization. However, ShB infection resulted in an increased PPO activity in both the cvs. No obvious difference was recorded between the resistant and susceptible cvs in their PPO activity irrespective of N-fertilization.

Discussion

N-fertilization in split doses (25 kgN/ha/application) has generally resulted in an increase, though not significantly, on the levels of phenolics of rice plants (Figs 1, 2 and 3). This increase ia also noticed with the activities of PAL (Fig. 4a) TAL (Fig. 4b) and PO (Fig. 5a), the enzymes involved in phenol metabolism. These results are contradictory to the results obtained in various host-pathogen systems with heavy application of N-fertilization (Sridhar, 1969; Matsuyama and Dimond, 1973; Vidhyasekaran 1974; Naidu et al. 1979). The fact that the plants were exposed to low N level by applying the fertilizer in split doses when compared with normal N application (100 to 150 kgN/h single dose) may explain this anomaly. Sridhar (1969) has reported that the optimum N (42.7 kg N/ha) increased the total and OD phenol and flavonoid contents while the heavy dose (85.4 kB N/ha) decreased their level considerably. The accumulation of soil N in the final sampling (90 and 120-d) may be ruled out, as there had been enormous loss of N through leaching and denitrification (Sharma and Rajat, 1979).

It is explicit from Figs 1 and 2 that infection increased the soluble phenolic pool in all the four cvs but this accumulation was more in the resistant than in the susceptible ones. Soluble phenols should have accumulated at a rapid rate in the infected resistant cvs compared to susceptible plants. This phenomenon was even more evident in the accumulation of flavonoids in the infected tissues (Fig. 3). In line with the above observation, an increased phenolic content in diseased rice plants has been reported by many workers (Ramakrishnan, 1966; Sridhar, 1972; Sridhar and Ou, 1974; Sridhar et al., 1976; Naidu et al., 1979; Sridhar et al. 1979). Further, Ramakrishnan (1966) found that the blast resistant cvs accumulated more phenolics in the early stages of infection than the

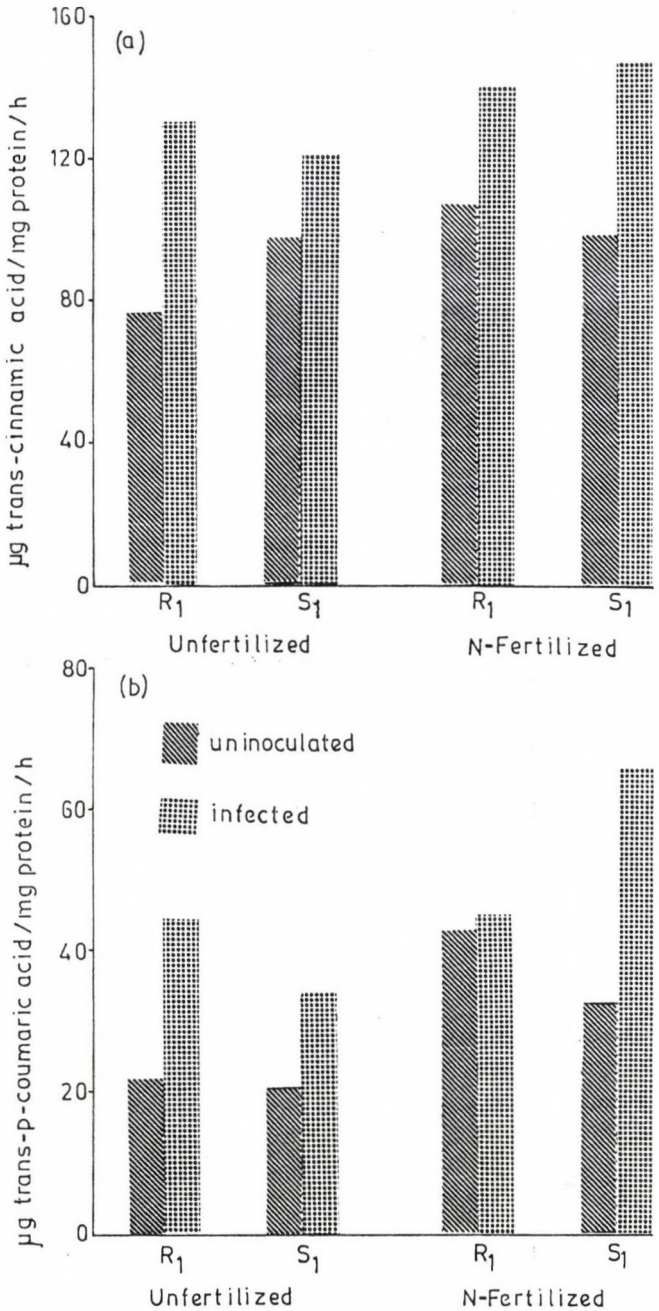

Fig. 4(a). PAL and (b) TAL activities in uninoculated and *R. solani* infected resistant ARC 5943 (R_1) and susceptible IR 1487-194-5-3-2 (S_1) rice cvs under unfertilized and N-fertilized conditions

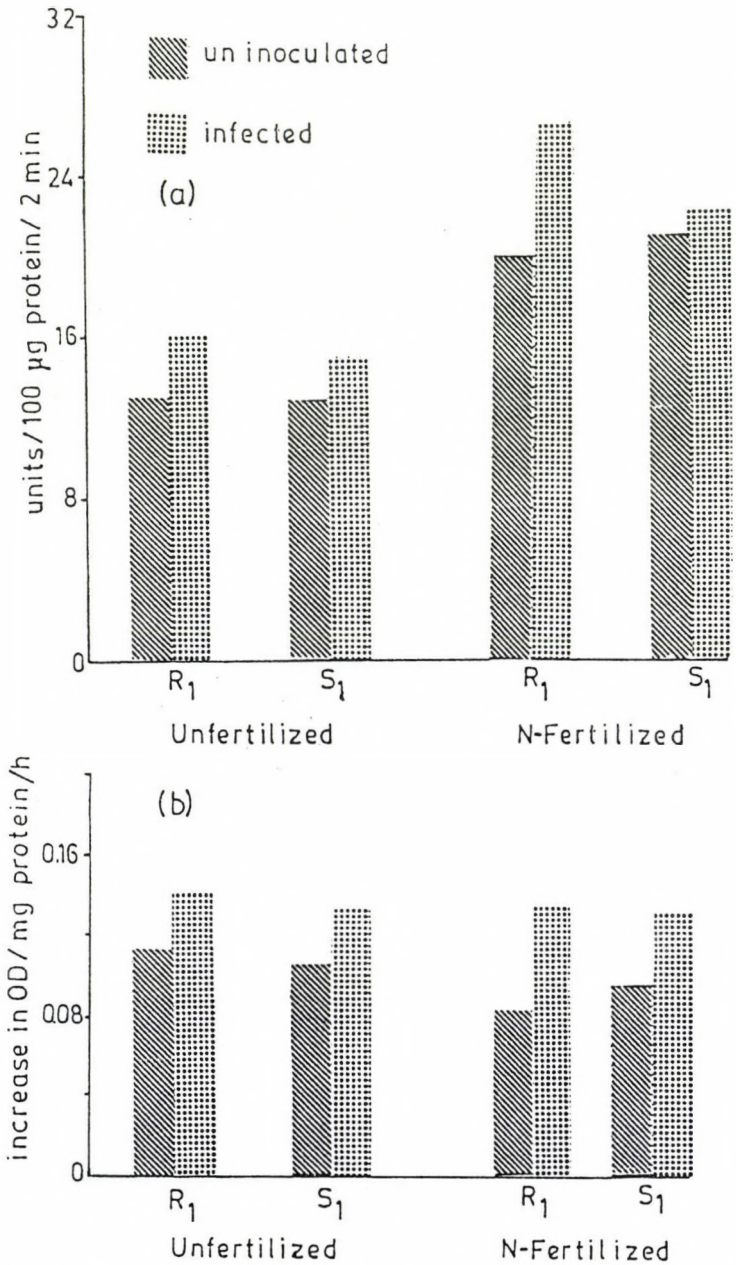

Fig. 5(a). Peroxidase and (b) polyphenol oxidase activities in uninoculated and *R. solani* infected resistant ARC 5943 (R_1) and susceptible IR 1487-199-5-3-2 (S_1) rice cvs under unfertilized and N-fertilized conditions

susceptible ones, a result which is confirmed in the present work with rice *R. solani* system.

The increased accumulation of phenols in infected tissue may be due to their release from glycosides or glycosidic esters by enzymatic activity of the pathogen and/or the host tissue (Noveroske et al., 1964), enhanced synthesis by the host through shikimic acid pathway (Neish, 1964), production by pathogen (Farkas and Ladingham, 1959) or by migration of phenols from healthy tissue (Rubin and Artsikhovakaya, 1964). It is observed that the pathogenic isolates of *R. solani* are capable of releasing a large number of phenolic compounds into the medium during *in vitro* growth (Reddy et al., 1975) and the same could be expected in the host although the nutritional status of the host plants differ from synthetic medium.

Almost all higher plants investigated so far, convert phenylalanine to cinnamic acid and many of them are also capable of deaminating tyrosine to p-coumaric acid (Rohringer and Samboraki, 1967). In the present study the close association of PAL and TAL with increase in phenolics suggests that these are synthesized at least in part by the shikimic acid pathway. Manian (1981) observed a decrease in the content of total amino acids in the infected plants. The deamination of aromatic amino acids (Figs 4a and 4b) could be the primary cause for this decrease. Sadasivan (1968) suggested the role of phenylalanine and tyrosine, the respective substrates of these enzymes, in disease resistance mechanism of rice against *Drechslera oryzae* and *Pyricularia oryzae*.

PAL activity in the extracts of healthy/infected tissue of both resistant and susceptible cvs greatly exceeded that of TAL activity (Figs 4a and 4b). Possibly, TAL pathway may have only a secondary role in synthesizing shikimic acid derived phenolics (Green et al., 1975).

N-fertilization increased the activity of the oxidative enzymes PO and PPO (Figs 5a and 5b). This may be due to the increased availability of the substrates (phenols) in N-fertilized plants (Figs 1 and 2). Further PO activity increased in rice plants at the application of single optimum dose (43 kg N/ha) of N-fertilizer (Sridhar, 1969).

PO and PPO bring about the oxidation of phenolics and related compounds increasing their toxicity (Tomiyama and Stahmann, 1964; Kosuge, 1969). As these oxidative enzymes increased in the diseased plants (Figs 5a and 5b), they are implicated with a role in the defence reactions of plants (Rautela and Payne, 1970; Stahmann and Demorest, 1973; Macri et al., 1974; Sridhar and Ou, 1974; Clark and Lorbeer, 1975; van Lellyveld and Brodrick, 1975; Hammerschmidt et al., 1982; Kratka and Kudela, 1982). The increased activity of these enzymes involved in the phenol oxidation may be due to the activation of latent enzymes or the *de novo* synthesis in the host tissue or their production by the pathogen.

To sum up, the forgoing results of physiology of host-pathogen interaction indicate a role for phenolics in the disease resistance as evidenced by their accumulation. This accumulation, however, seems to be earlier and faster in the resistant than in the susceptible cvs, commensurate with earlier restriction of lesions in the former.

Acknowledgements

We thank Prof. A. Mahadevan Director, C. A. S. in Botany for facilities. One of us (SM) thank the University Grants Commission, New Delhi for financial assistance.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 205–213 (1995)

Effect of Phenyl Acetic Acid on the Phenolics and Enzymes of Phenol Metabolism in Rice

K. MANIBHUSHANRAO[1], D. CHELLADORAI[1] and S. SREENIVASAPRASAD[2]

[1] Centre for Advanced Study in Botany, University of Madras, Madras 600 025, India
[2] Department of Applied Plant Science, The Queen's University of Belfast, Belfast BT9, 5PX, UK

Effect of phenyl acetic acid (PAA), produced by *Rhizoctonia solani*, on the phenolics and enzymes of phenol metabolism was studied in rice cvs IR 50 and TKM 9, moderately resistant and susceptible to sheath blight (ShB), respectively. The PAA treatment even at low concentrations (0.1 and 1.0 ppm) resulted in rapid accumulation of phenolic compounds in both the rice cvs and imparted resistance especially in susceptible cv. TKM9 against ShB disease.

Accrual of total and OD phenols was clearly greater in TKM 9 and the enhancement of flavonoids was higher in IR 50 but for certain minor fluctuations. Concomitant to the increases caused in phenolic compounds, PAA elicited the activities of phenol biosynthetic (PAL, TAL) and oxidative (PPO and PO) enzymes in both IR 50 and TKM 9 cvs.

Interestingly, the impetus of the toxin PAA in general, was more on the susceptible cv. TKM 9 and the magnitude of increase of total and OD phenols and the activity of PAL, TAL and PO was greater than in the moderately resistant cv. IR 50. The physiological alternations triggered by PAA appears to lead to the induction of resistance in rice.

Sheath blight disease (ShB) of rice incited by *Rhizoctonia solani* has gained importance with the recently introduced high yielding cvs favouring the disease incidence under cultural conditions like close planting and heavy nitrogen application. Phenyl acetic acid (PAA) and its hydroxy derivatives (*m*-PAA, *o*-HPAA and *p*-HPAA) are certain phytotoxins produced by *R. solani* (Manibhushanrao et al., 1981). These metabolites at concentrations above 100 ppm induce lesions on rice tissue, comparable to those caused by the pathogen.

Recently, induction of moderate resistance in rice to ShB was reported (Waheeta et al., 1987) through PAA (1.0 ppm) and its derivatives (0.1 ppm) treatment. Incidently, PAA up to 100 ppm is neither phototoxic nor fungitoxic. Chemical activation of host defence mechanism has been reported in several systems, namely, tomato to *Fusarium* wilt (Davies and Dimond, 1953; Chet et al., 1978; Carrasco et al., 1978) and verticillosis (Russel, 1975; Emmanouil and Wood, 1981), egg plants to *Fusarium oxysporum* f. sp. *melongenae* (Chet et al., 1978) and *Verticillium dahliae* (Emmanouil and Wood, 1981), pepper to *V. dahliae* (Emmanouil and Wood, 1981) and rice to *Helminthosporium oryzae* (Sinha and Hait, 1982). Accumulation of phenols (Retig and Chet, 1974), increase of lignified cells and the generation of fungitoxic effects (Carrasco et al., 1978) were the factors associated with the catechol and quinic acid induced resistance in tomato plants, respectively.

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Secondary metabolites of plant origin particularly phenolics and related substances confer protection against pathogenic microorganisms (Mahadevan, 1969; Schönbeck and Schlösser, 1976) and the quinones are known for their toxicity to microbes (Misaghi, 1982). Enhanced PPO and PO activity was associated with induction of resistance in potato (Weber et al., 1966), sweet potato (Stahmann et al., 1966) and tomato plants (Retig, 1974). Further, PAL and TAL have also been implicated in plant defense mechanisms (Young et al., 1966). In the present work, which is aimed at elucidating the mechanism(s) involved in the PAA induced resistance of rice to *R. solani*, the physiological alterations triggered in the host with respect to phenolic compounds and certain enzymes of the phenol metabolism were studied and their relevance in the manifestation of resistance is discussed.

Materials and Methods

Hydroponic culture of rice seedlings

Rice seeds (IR 50 and TKM 9) were surface sterilized (0.1% $HgCl_2$) and allowed to sprout on moist blotting paper. After 24 h the sprouting seeds were spread on an nylon net stretched over and held tightly by plastic clips near the brim of a plastic trough (25 g/trough) containing 660 ml of complete culture solution (Yoshida et al., 1976). The pH of the solution was maintained at 5.0 using a mixed indicator. The culture solution was changed once in five days to avoid depletion of nutrients.

Phytotoxin treatment and collection of samples

Rice seedlings (14 d) were treated with PAA by replacing the nutrient solution with 660 ml of the necessary concentration (0.1, 1.0, 10 and 100 ppm). Control seedlings were kept in distilled water. Treated and untreated rice tissue (10 g) for analyses were collected 24, 48 and 72 h after treating with PAA. Samples (5 g shoot and root) were comminuted, extracted in boiling 80 ethanol for 5 min and homogenized. The homogenate was filtered through four layers of cheese cloth and the residue was reextracted. The extracts were combined, filtered through Whatman No. 41 filter paper and the final volume was adjusted to 25 ml with 80% ethanol to represent 5 ml/g of the tissue.

Ethanol extraction estimation of total OD phenols and flavonoids

Preparation of acetone powder, acid extraction of enzymes, assay of PAL, TAL, PPO and PO were estimated as per the standard methods, for which the details are given elsewhere (Manibhushanrao and Manian, 1993).

Results

Total OD phenols and flavonoids

The PAA treatment resulted significantly in rapid accumulation of total phenols in both cultivars (Table 1). In the moderately resistant cv. IR 50, the initial (24 h) turnover of total phenols was lower at 0.1 and 1.0 ppm (23 and 80%) compared to 10 and 100 ppm (96.7 and 116.7). However, at the end of the 72 h toxin treatment, the magnitude of increase was higher with 0.1 and 1.0 ppm (185.7 and 160.0%) than with 10 and 100 ppm (142.9 and 122.9%) respectively. The trend in increase was different in susceptible cv. and PAA at all concentrations induced accrual of large quantities of total phenols initially (24 h). The impetus of PAA was optimal by 48 h after which a general decreasing trend was observed in the accumulation of total phenols with 0.1 ppm being an exception. The PAA considerably enhanced significantly the OD phenol content of both the rice cvs (Table 2). In IR 50 the different concentrations of *o*-PAA did not show much variation in their stimulatory effect on OD phenols at 24 h (23–32%). However, further enhancement was higher with 0.1 and 1.0 ppm (56.4 and 82,1%) than with 10 and 100 ppm (45.2 and 51.3%). On the other hand, a consistent pattern was recorded in TKM 9, where the increasing concentration and prolonged exposure of PAA resulted in greater accrual of OD phenols. Flavonoid content escalated significantly upon PAA treatment in IR 50 as well as TKM 9 (Table 3). In both cvs. elicitation of flavonoids content was significant with higher concentration of PAA and on prolonged treatment. The overall increase of flavonoids caused by PAA treatment was slightly more in IR 50 than TKM 9.

PAL, TAL, PPO and PO activities

Attendent with accumulation of phenolic compounds the activity of PAL was greatly elicited by PAA treatment in both rice cvs (Fig. 1a). In IR 50, optimal enhancements was at 100 ppm, followed by 10 ppm. In TKM 9, though the maximal amplification of PAL activity was at 10 ppm, 0.1 and 1.0 ppm also showed enormous increases (337.5 and 303.1%) by 72 h. Escalation of TAL activity by PAA was observed in both IR 50 and TKM 9 (Fig. 1b). In IR 50 10 ppm PAA showed maximum stimulatory effect on TAL (45, 75 and 60 at 24, 48 and 72 h, respectively). At 100 ppm, despite marked initial (24 h) increase elicitation of TAL activity decreased on further exposure. In cv. TKM 9, however, amplification of TAL activity followed a consistent pattern and the enzyme activity was greater with increasing concentration as well as prolonged treatment of PAA. Effect of PAA on the two oxidative enzymes was almost similar (Figs 2a and b). Longer the exposure to PAA, higher the concentration, greater was the accelaration of PPO and PO activity. Comparing the two cvs, stimulatory effect of PAA on PO activity was greater in TKM 9. Fluctuations were observed in the enhancement of PPO activity by PAA and no specific trend was recorded.

Table 1

Total phenols* in untreated and PAA treated rice seedlings

Cultivar	PAA conc. in ppm	PAA treatment (h)		
		24	48	72
IR 50 (Moderately resistant)	control	0.30	0.32	0.35
	0.1	0.40	0.85	1.00
	1.0	0.54	0.70	0.91
	10.0	0.59	0.75	0.85
	100.0	0.85	0.76	0.78
TKM 9 (Susceptible)	control	0.20	0.25	0.30
	0.1	0.35	0.55	0.70
	1.0	0.45	0.60	0.70
	10.0	0.53	0.75	0.85
	100.0	0.55	0.75	0.83

* mg catechol-equivalents/g fresh weight. (Values are the mean of the three replicates)
Data subjected to ANOVA (Three-way).
Cultivars: F(1.58) = 4.01 at 5% level. (Significant difference between the cvs.)
Hours: F(2.58) = 3.16 at 5% level. (Significant difference among the h.)
PAA conc.: F(4.58) = 2.54 at 5% level. (Significant difference among the PAA conc.)

Table 2

Ortho-dihydric phenols* (OD) in untreated and PAA treated rice seedlings

Cultivar	PAA conc. in ppm	PAA treatment (h)		
		24	48	72
IR 50 (Moderately resistant)	control	0.38	0.36	0.39
	0.1	0.47	0.57	0.61
	1.0	0.50	0.64	0.71
	10.0	0.48	0.49	0.57
	100.0	0.49	0.52	0.59
TKM 9 (Susceptible)	control	0.37	0.31	0.28
	0.1	0.41	0.49	0.53
	1.0	0.48	0.61	0.69
	10.0	0.49	0.52	0.56
	100.0	0.51	0.56	0.64

* mg catechol-equivalents/g fresh weight. (Values are the mean of the three replicates)
Data subjected to ANOVA (Three-way).
Cultivars: F(1.58) = 4.01 at 5% level. (Significant difference between the cvs.)
Hours: F(2.58) = 3.16 at 5% level. (Significant difference among the h.)
PAA conc.: F(4.58) = 2.54 at 5% level. (Significant difference among the PAA conc.)

Table 3

Flavonoid content* in untreated and PAA treated rice seedlings

Cultivar	PAA conc. in ppm	PAA treatment (h)		
		24	48	72
IR 50	control	2.01	2.04	2.05
(Moderately	0.1	3.01	3.41	3.56
resistant)	1.0	3.13	3.56	3.74
	10.0	3.21	3.51	3.77
	100.0	3.35	3.60	3.81
TKM 9	control	1.83	1.86	1.84
(Susceptible)	0.1	2.26	2.64	2.96
	1.0	2.63	2.91	3.13
	10.0	2.82	3.16	3.41
	100.0	2.86	3.21	3.51

* mg catechol-equivalents/g fresh weight. (Values are the mean of the three replicates)
Data subjected to ANOVA (Three-way).
Cultivars: $F(1.58) = 4.01$ at 5% level. (Significant difference between the cvs.)
Hours: $F(2.58) = 3.16$ at 5% level. (Significant difference among the h.)
PAA conc.: $F(4.58) = 2.54$ at 5% level. (Significant difference among the PAA conc.)

Discussion

High turnover of phenolic compounds and enhanced activity of phenol biosynthetic and oxidative enzymes was observed in tissues of PAA treated rice seedlings, particularly at lower concentrations (0.1 and 1.0 ppm). However, corresponding increases were not observed with the higher concentration (10 and 100 ppm) and the magnitude of increase was at the maximum threefold. There is a strong possibility of the higher quantities of the toxic metabolite(s) interferring with other metabolic processes of the host and the concentrations of PAA is vital in determining the resistance susceptible reactions. The stimulatory effect of PAA on the total and OD phenols was higher in TKM 9 and the enhancement of flavonoids by PAA was greater in IR 50 except for some minor fluctuations. It is pertinant to note that culture filtrates (Daniels and Hadwiger, 1976), fungal extracts (Trivedi and Sinha, 1976), fungicides and phytotoxic metabolites (Oku et al., 1973) induced the production of phytoalexins, the antimicrobial substances often implicated in host defence reactions. Moreover, Retig and Chet (1974) Carrasco (1978) repored the accumulation of phenols, increase of lignified cells and the generation of fungitoxic effects as the factors associated with the induction of resistance in tomato plants upon catechol, quinic acid and phenylalanine treatment. Phenols and their oxidized forms have been ascribed a striking role in host defence strategies (Kosuge, 1969;

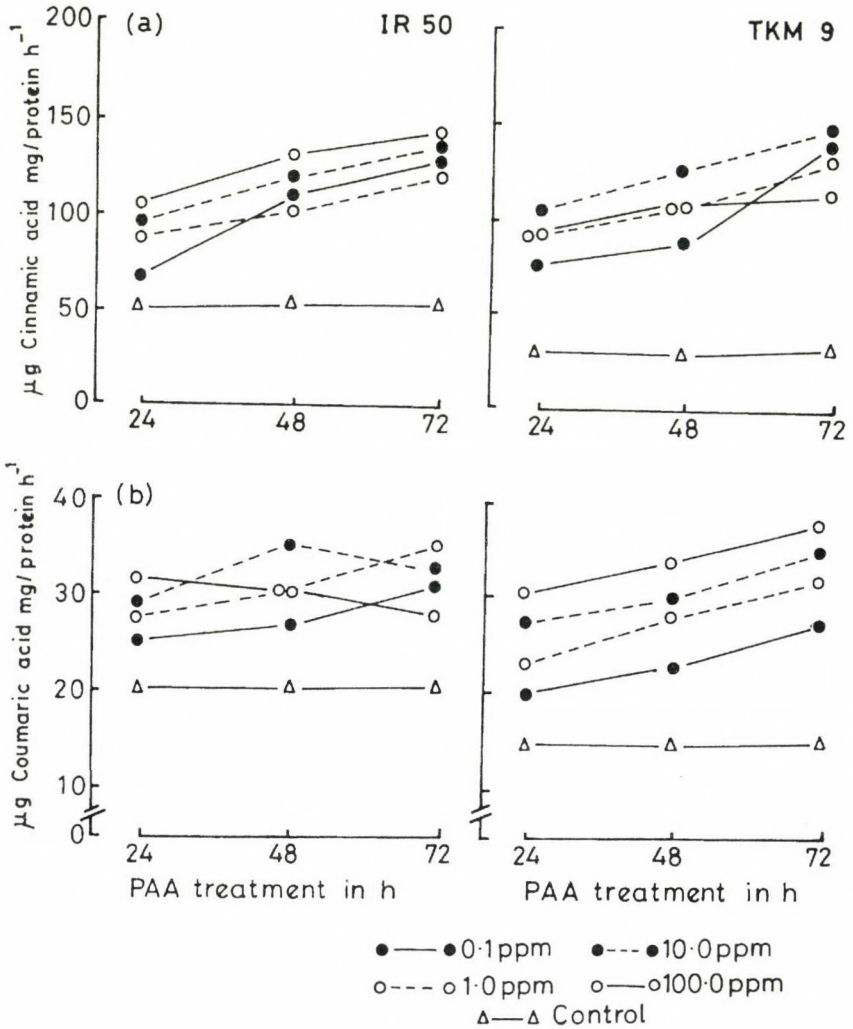

Fig. 1. PAL (a) and TAL (b) activities in untreated and PAA treated rice seedlings

Misaghi, 1982). Quinones and free radicals inactivate certain biologically important substances such as proteins, nucleic acids and enzymes (Stahmann et al., 1966; Leatham et al., 1980; Manibhushanrao and Sreenivasaprasad, 1985). In this context, accumulation of phenolic compounds in the PAA treated rice tissue appears to be significant and might well be associated with the induction of resistance to *R. solani*.

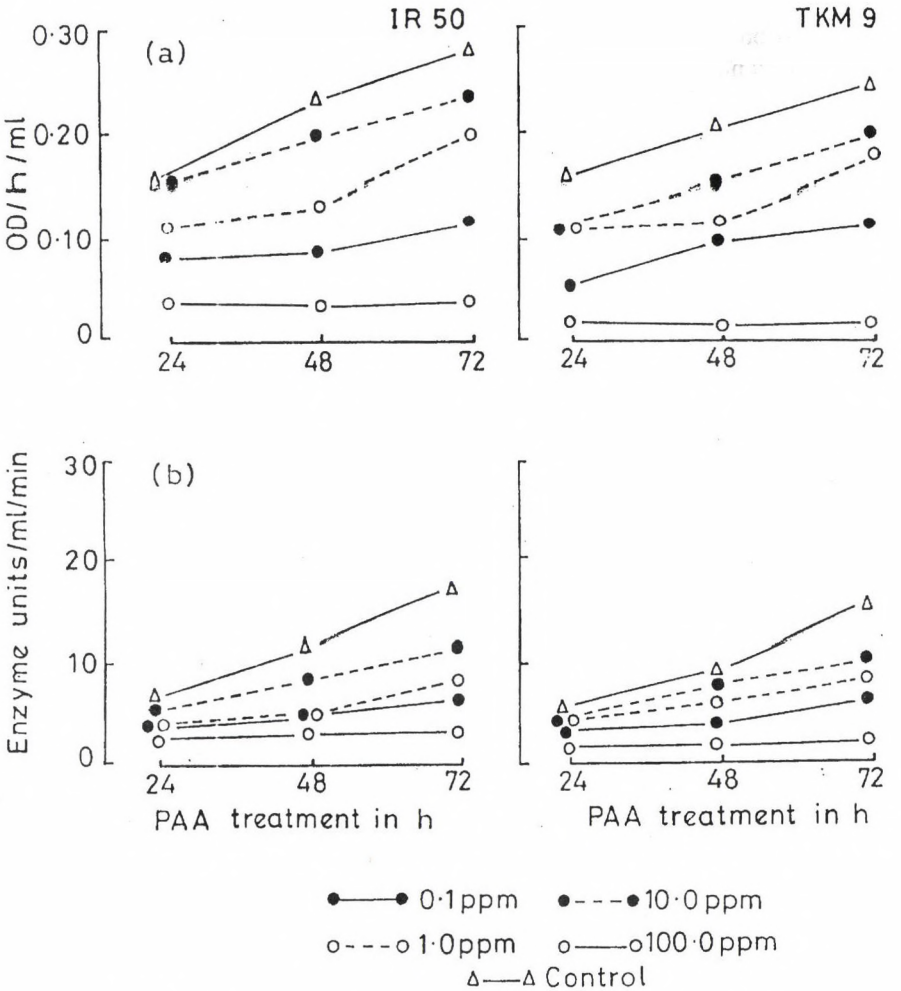

Fig. 2. PO (a) and PPO (b) activities in untreated and PAA treated rice seedlings

Concomitant to the increases caused in the total, OD phenol and flavonoid content of the rice tissue PAA on PAL, TAL and PO activity was greater (Figs 1a, b and 2a) in the susceptible cv. TKM 9, though fluctuations were recorded in the enhancement of PPO activity. Correlation between increased PAL activity and enhanced production of phenols (Camm and Towers, 1973), close association of PAL activity with the development of hypersensitive reactions (Vegetti et al., 1975) and rapid lignifications (Friend et al., 1973) are well known. Enhanced activity of PAL, TAL, PPO and PO would lead to accelerated biosynthesis resulting in the formation of physical and chemical barriers.

Papilla formation and lignification are the two most conspicuous respones of plant cells to fungal invasion (Ouchi, 1983); PAL and PO play vital role in lignin biosynthesis and the polymerization of lignin precursors, respectively (Vance et al., 1980). Lignification of the host tissue and the accumulation of fungitoxic lignin precursors may generate an environment hostile for the pathogen (Kuć and Preisig, 1984).

Thus, the physiological alterations triggered by PAA, namely accumulation of phenolic compounds, enhanced activity of both phenol biosynthetic and oxidative enzymes in concert may lead to the induction of resistance in rice to ShB as plant resistance is often a function of a number of mechanism(s) operating in concert, each contributing somewhat to the overall defence (Misaghi, 1982). This can be further substantiated by monitoring the metabolic changes in the protected system.

Acknowledgements

We thank Professor A. Mahadevan, Director, CAS in Botany for facility. The junior authors thank the University Grants Commission, New Delhi for financial assistance.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 215–220 (1995)

Effects of Chemical Stress Caused by Heavy Metals on the Glutathione S-Transferase Enzyme Activity in Maize (*Zea mays* L.)

T. KŐMÍVES, A. A. A. AIOUB and G. GULLNER

Plant Protection Institute, Hungarian Academy of Sciences; H-1525 Budapest, P.O.B. 102, Hungary

Treatment of maize seedlings with different concentrations of micronutrient [copper(II) and zinc(II)] and environmental toxicant heavy metals [cadmium(II), lead(II), mercury(II), and nickel(II)] in nutrient solution culture led to markedly changed activities of the enzyme glutathione S-transferase (GST, E.C. 2.5.1.18.) depending on the heavy metal, its dose, the tissue of the treated plant and the duration of exposure. The observed responses correlated with heavy metal phytotoxicity, as indicated by their time course and dose-dependence.

Environmental pollutant heavy metals such as cadmium, lead and mercury, are powerful inhibitors of important life processes in mammalian and plant tissues (Almar and Dierickx, 1990, Steffens, 1990). Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is known to participate in different plant stress reactions (Smith et al., 1990). GSH and cysteine-rich oligopeptides, mostly the GSH-derived phytochelatins [(γ-L-glutamyl-L-cysteinyl)$_n$-glycine] play significant roles in heavy metal detoxification processes in plants (Grill et al., 1989; Rüegsegger et al., 1990; Meuwly et al., 1995). We reported earlier that the activity of the enzyme glutathione S-transferase (GST, E.C. 2.5.1.18.), which has an important function in detoxification processes (Kőmíves and Dutka, 1989; Gullner et al., 1991), was strongly influenced by cadmium(II) in pea and wheat (Uotila et al., 1994), and by mercury(II) in maize (Kőmíves et al., 1994) seedlings.

As a result of a continuing study of the basic stress response of plants to heavy metal exposure at the enzymatic level (Kőmíves et al., 1994; Uotila et al., 1994), in this paper we present data of a comparative study on the effects of two micronutrient (copper and zinc) and four toxic heavy metals (cadmium, lead, mercury and nickel) on the activity of GST in maize seedlings.

Materials and Methods

Plant material

Maize seeds (Favea hybrid, obtained from Dr. Endre Széll of the Cereal Research Institute, Szeged, Hungary) were germinated in Petri dishes, and after 72 hours (when root lengths were ca. 25 mm) transferred to nutrient solutions containing different concentrations of heavy metals as described previously (Kőmíves et al., 1994). Plants were

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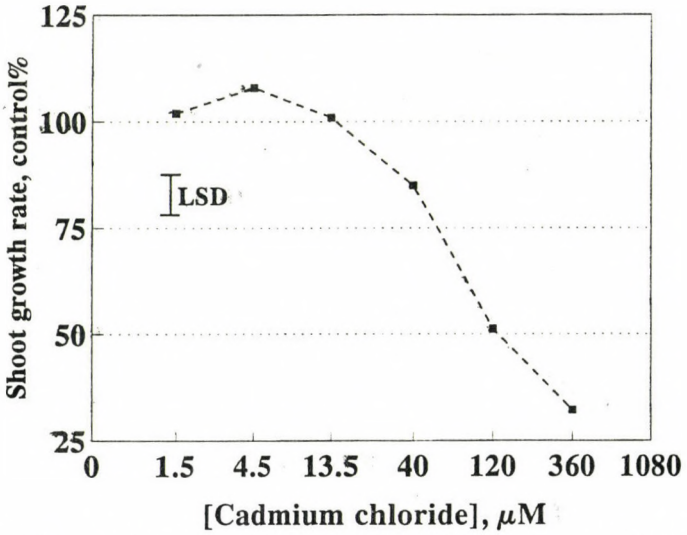

Fig. 1. Effects of different cadmium chloride concentrations on the shoot growth rate of maize seedlings after 48 h of exposure. Control value: 19.1 mm/day

grown in greenhouse under normal conditions (18–23 °C; supplemental light: 160 μE $m^{-2} s^{-1}$ for 8 h per day; relative humidity: 75–80%). Plant shoot lengths were measured daily.

Chemicals

Solvents, acetate (Pb, Ni) and chloride (Cd, Cu, Hg and Zn) salts of heavy metals, enzyme substrates and other chemicals were obtained from Reanal Fine Chemicals (Budapest, Hungary).

Enzyme assay

Preparations of cell-free homogenates from shoot and root tissues, and measurement of GST activities followed previous lines (Uotila et al., 1995).

Statistical analysis

Results are expressed as the means of three independent experiments. Significance of differences was evaluated by Student's *t*-test.

Table 1

ED_{50} values characterizing the ability of heavy metals to reduce the shoot growth rate of maize seedlings (Favea hybrid) cultivated in nutrient solution

Heavy metal	ED_{50} (μM)
Cd(II)	121
Cu(II)	247
Hg(II)	13.6
Ni(II)	265
Pb(II)	194
Zn(II)	9580

Results

Effects of heavy metals on the growth of maize seedlings

Heavy metal toxicity to plants was manifested by a rapid reduction of growth rate, followed by loss of tissue turgor, yellowing of leaves, and finally by necrosis of plant tissue. A typical dose-response curve on the effects of cadmium in the shoots is shown in Fig. 1. Mercury(II) was the most toxic to maize seedlings, followed by cadmium(II), lead(II), copper(II), and nickel(II): ED_{50} values on shoot growth reduction by these heavy metals are listed in Table 1.

Effects of heavy metals on gluthatione S-transferase activity

GST enzyme activities of shoots and roots of untreated maize seedlings were 0.78 ± 0.11 and 1.01 ± 0.10 μmol g^{-1} fresh weight min^{-1}, respectively. Treatments with heavy metals resulted in significantly altered enzyme activities. As illustrated by data on effects of cadmium in Fig. 2, treatments with low, non-toxic or slightly toxic heavy metal concentrations led to GST enzyme induction in the shoots. The enzyme activity reached a maximum at 48 h, and ultimately declined to the control value. However, enzyme induction by high and strongly toxic heavy metal doses occurred less rapidly, and resulted in higher enzyme activities only after longer times of exposure. In the roots, GST induction appeared only at the highest heavy metal concentration, while at lower concentrations the activities were reduced. Thus, Cd(II) caused a strong enzyme induction after 48 h in the shoots, with the largest changes taking place following a 96 h treatment with 360 μM heavy metal (241% of the control) (Table 2). In contrast, GST levels in the roots of the Cd(II)-treated maize seedling were reduced (by more than 50% following a 24 h treatment with 120 μM heavy metal), and an increase in the enzyme activity occurred only after long (96 h) incubation with the highest cadmium concentration (360 μM) (Fig.

2). Treatments with other heavy metals resulted in similar dose-response relationships: characteristic data on changes of GST activity in heavy metal-treated maize plants are listed in Table 2.

Table 2

Effects of heavy metals on the glutathione S-transferase (GST) activity in shoots and roots of maize (Favea hybrid) grown in nutrient culture. Heavy metals were applied at 1/3 (48 h data) and 3-fold (96 h data) rates of their ED_{50} concentrations (see Table 1). For control values see text in Results

Heavy metal	GST control %			
	Root		Shoot	
	48 h	96 h	48 h	96 h
Cd(II)	77	157	189	241
Cu(II)	81	138	175	322
Hg(II)	82	143	242	303
Ni(II)	95	159	137	178
Pb(II)	74	125	144	169
Zn(II)	111	146	196	195

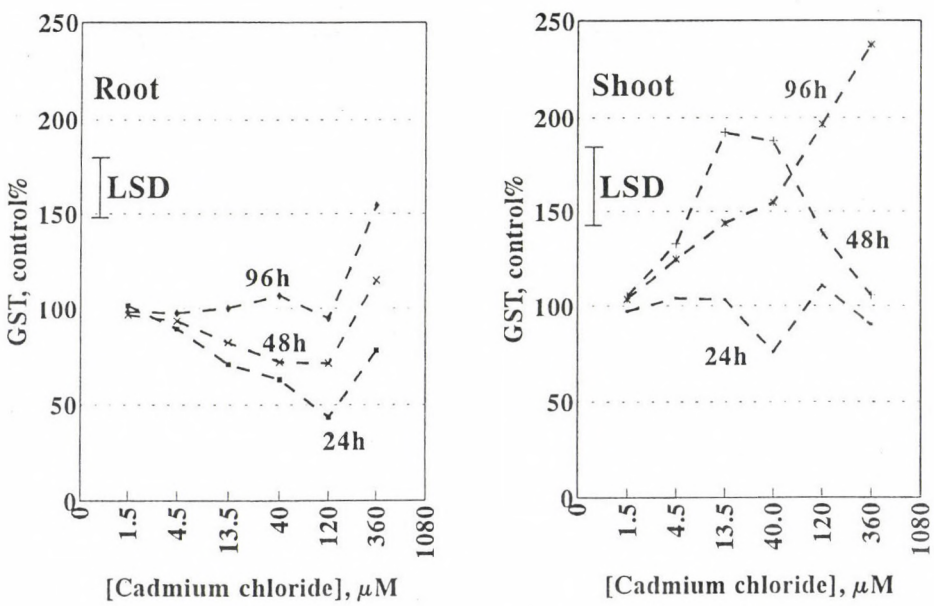

Fig. 2. Effects of cadmium chloride on the glutathione S-transferase (GST) activity in shoots and roots of maize seedlings after different exposure periods. For control values see text in Results

Discussion

Chemical stress of plants by heavy metals and herbicides may result in enhanced activities of enzymes that participate in the biosynthesis of phytotoxic natural products (Kőmíves and Casida, 1982; Kőmíves and Casida, 1983), thereby promoting plant tissue damage. Increased activities of GST in maize seedlings (Fig. 2) treated with subtoxic or moderately phytotoxic doses of the heavy metals cadmium and mercury, however, represent a different type of response: in this case the plant's detoxication potency is elevated (Kőmíves et al., 1994; Uotila et al., 1994). In preliminary experiments (Kőmíves and Brunold, unpublished, 1991) cadmium was found to be an inhibitor of GST from shoots and roots of pea and maize *in vitro*. *In vivo*, however, Cd(II) caused massive induction of GST in maize (Fig. 2), similarly to effects by herbicides and herbicide safeners (Kőmíves and Hatzios, 1991; Holt et al., 1995; Walton and Casida, 1995). In addition to Cd(II) and Hg(II), all the tested heavy metals had the ability to influence levels of GST. The observed responses seem to be heavy metal-specific, as indicated by their nearly identical time-course and the similarity of their dose-dependence.

GST isoenzymes play roles in the detoxication of a number of xenobiotics not only as catalysts of GSH conjugation, but also as ligandins (xenobiotic-binding proteins) (Kőmíves and Dutka, 1989). Inhibition of maize and pea GSTs by Cd(II) and Hg(II) (Kőmíves and Brunold, unpublished, 1991) raises the question, whether GST proteins may be involved in heavy metal detoxication in plants as ligandins. Although relatively high cytosolic concentrations and superb stress-inducibilities of GST proteins (Kőmíves and Dutka, 1989) seem to support this hypothesis, further research is needed for its evaluation.

Acknowledgements

This work was supported by grants from the Hungarian Scientific Research Fund (OTKA T6314 and F6321). The authors thank Prof. Christian Brunold (University of Bern, Switzerland) for helpful discussions.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 221–245 (1995)

Species Composition of *Coleoptera* Assemblages in the Canopies of Hungarian Apple and Pear Orchards

V. MARKÓ[1], O. MERKL[2], A. PODLUSSÁNY[2], K. VIG[3], CS. KUTASI[4] and S. BOGYA[1]

[1] Department of Entomology, University of Horticulture and Food Industry, H-1502 Budapest, P. O. Box 53, Hungary [2] Department of Zoology, Hungarian Natural History Museum, Budapest, Hungary, [3] Savaria Museum, Szombathely, Hungary, [4] Bakony Natural History Museum, Zirc, Hungary

The species richness and species composition of *Coleoptera* assemblages were investigated in the canopies of differently treated Hungarian apple and pear orchards during 1990–94. Altogether 324 species belonging to 41 families and 203 genera were found; 253 species in apple orchards and 188 species in pear orchards. As a result of our and other reviewed investigations it was found that 371 species, almost 4% of the Hungarian beetle fauna were represented in the canopies of the apple and pear orchards. In each orchard the species richness varied between 29 and 123.

Most of the species belonged to the families Curculionidae, Chrysomelidae and Coccinellidae. Higher numbers of beetles of the families Rhynchitidae, Buprestidae and Cerambycidae were found only in untreated orchards. The species diversity of Cantharidae depended on the forests surroundings. Predators were represented by 20%, phytophages by 75%, fungivores by 2% and scavengers by 3% of entire fauna. Among the herbivorous species the ratio of fruit tree-feeders was 10%.

In orchards where integrated pest management was practiced (treated with selective insecticides) the diversity of predator species was not significantly higher than in conventionally treated (treated with broad-spectrum insecticides) orchards.

The most widely occurring species in apple orchards in the decreasing order were *Coccinella septempunctata, Stethorus punctillum, Sitona callosus, Propylea quatuordecimpunctata, Adalia bipunctata, Meligethes aeneus, Hippodamia variegata, Anthonomus pomorum, Peritelus familiaris, Notoxus monoceros, Cortinicara gibbosa* and *Phyllotreta vittula*, in pear orchards *Stethorus punctillum, Propylea quatuordecimpunctata, Adalia bipunctata, Coccinella septempunctata, Calvia quatuordecimpustulata, Phyllobius oblongus, Rhagonycha fulva, Byctiscus betulae, Phyllotreta vittula.*

During the development of different environmentally friendly and integrated plant protection methods the apple and pear orchards were placed in the main focus. A number of scientific papers [e.g. Balázs (1992), Blommers (1994), Herard (1986)] included the biology of some key pests and key predators. But the species composition and the diversity of the arthropod assemblages in the apple and pear orchards are still little known. However, the "species composition" might contain essential information in relation to the understanding of the organization of arthropod communities in the orchards, the analysis of different crop protection technologies in the environmental protection, the detection of long-term changes in the field of agriculture and the general understanding of biodiversity relations of the plantations as was discussed by Zilahi-Sebess (1955), Steiner (1962), Oatman et al. (1964), Steiner et al. (1970), Mészáros et al. (1984), Brown et al. (1988) Szentkirályi and Kozár (1991) and Kozár (1992).

The order Coleoptera has the largest variety of the species among arthropods, thus beetles represent a considerable portion of species diversity in apple and pear orchards. Since there are different species belonging to different guilds, the composition changes in

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Coleoptera assemblages might also be properly characterized by the composition of the entire arthropod fauna in the orchards.

Our main goal is, to consider the previous faunistic studies in apple orchards in Hungary, and to prepare a list of the beetles occurring in the canopy of apple and pear trees, as well as to describe the biodiversity of the beetle assemblages of differently treated orchards.

Materials and Methods

Our investigations were carried out in three diferent Hungarian geographical regions which are located in woodland areas of medium height mountains and in agricultural lowland environment. The samples were collected at the following localities: Nagykovácsi (3 plots), Sárospatak (4 plots), Kecskemét (5 plots). However, in case of Kecskemét, two different methods were simultaneously applied in three plots. Some selected plots were untreated, others treated with broad-spectrum insecticides (mainly with organophosphorus insecticides and piretroids), also treated with selective insecticides (mainly IGR pesticides), in IPM orchards. The samples were collected between 1990 and 1994, from April till November by beating methods. The collecting was made by using the Winkler-type umbrella (d = 0.7m) and 0.25 m^2 plastic sheet. The Table below shows the parameters of each area (Table 1).

The identifications were made by the following persons: S. Bogya (SB), Zs. Czető (ZSC), R. Holynski (RH), F. Kádár (FK), Cs. Kutasi (CSK), V. Markó (VM), O. Merkl (OM), A. Podlussány (AP) and K. Vig (KV) in the given distribution by families: Carabidae – FK, Hydrophilidae – OM, Silphidae – VM, Staphylinidae – OM, Histeridae – OM, Cantharidae – CSK, Melyridae – CSK, Cleridae – CSK, Trogossitidae – OM, Elateridae – SB, Buprestidae – RH, Helodidae – OM, Demestidae – OM, Cybocephalidae – OM, Silvanidae – OM, Nitidulidae – OM, Cryptophagidae – OM, Phalacridae – OM, Latridiidae – OM, Coccinellidae – VM, OM, Anobiidae – OM, Ptinidae – OM, Oedemeridae – OM, Pyrocroidae – OM, Anthicidae – VM, OM, Mordellidae – ZSC, Scraptiidae – ZSC, Lagriidae – VM, Alleculidae – OM, Tenebrionidae – OM, Melolonthidae – OM, VM, Scarabaeidae – OM, Cerambycidae – CSK, VM, Chrysomelidae – KV, Bruchidae – OM, Anthribidae – AP, Nemonychidae – VM, Rhynchitidae – VM, Curculionidae – AP, VM, Apionidae – AP, Scolytidae – AP.

Results and Discussion

Tables 2, 3 show the composition of Coleoptera assemblages by regions and treatments in the canopies of the Hungarian apple and pear orchards. There were 253 species belonging to 37 families and 136 genera in the canopies of apple trees. On the pear trees there were 188 species found belonging to 32 families and 119 genera. The above two cases represented altogether 324 species, belonging to 41 families and 203 genera.

Table 1

Description of the investigated orchards

Environment Locality	Woodland in mountain of medium height			Agricultural lowland			
	Nagykovácsi			Kecskemét			Sárospatak
Fruit species	apple	pear	pear	apple		apple	pear
Age of plantation	M	M	M	Y		M	M
Size of plantation	5.8 ha	1.1 ha	51 ha	3×2 ha		5–6 ha	6–50.6 ha
Untreated	+	+					
Conventionally treated			+	+	+	+	+
IPM applied				+	+	+	+
No. of treatments	–	–	3–6	7–8	7–8	7–8	7–8
Method	U	U	U	U	S	U	U
Years	1990–92	1990–94	1992–94	1992–93	1992–94	1992–94	1993–94
Sampling per year	12	12	12	12	23–24	21–22	4/1993 7/1994
Trees per sampling	10	10	12	10	30 branches	10	10
Code	ANTR	PNTR	PTR	ACOM AIPM/1 AIPM/2	ACOMs AIPM/1s AIPM/2s	OACOM OAIPM	PCOM/1 PCOM/2 PCOM/3 PIPM

M – mature tree, more than 13 years old, Y – younger than 13 years; U – umbrella, S – plastic sheet

The faunistical studies which were previously carried out by Zilahi-Sebess (1955) in Hungary registered 38 additional species that were not collected by us. These are as follows: *Brachynus crepitans* Linnaeus, 1758; *Hapalaraea ioptera* (Stephens, 1832); *Hapalaraea nigra* (Gravenhorst, 1806); *Tachyporus nitidulus* (Fabricius, 1781); *Cantharis pulicaria* Fabricius, 1781; *Troscus exul* (Bonvouloir, 1859); *Adrastus rachifer* (Fourcroy, 1783); *Cidnopus pilosus* (Leske, 1785); *Attagenus pellio* Linnaeus, 1758; *A. unicolor* (Brahm, 1791); *Epuraea pallescens* (Stephens, 1832); *Meligethes coracinus* Sturm, 1845; *M. lepidii* Miller, 1852; *Cryptophagus acutangulus* Gyllenhal, 1828; *C. dentatus* (Herbst, 1793); *C. scanicus* (Linnaeus, 1758), *Telmatophilus brevicollis* Aubé, 1862; *Olibrus bisignatus* (Ménétries, 1849); *Phalacrus coruscus* (Panzer, 1797); *Stilbus oblongus* (Erichson, 1845); *Latridius minutus* (Linnaeus, 1767); *Melanophtalma transversalis* (Gyllenhal, 1827); *Vibidia duodecimguttata* Poda, 1761; *Dorcatoma serra* Panzer, 1796; *Anaspis flava* (Linnaeus, 1758); *Grammoptera ustulata* (Schaller, 1783); *Rhaphitropis marchicus* (Herbst, 1797); *Acanephodus onopordi* (Kirby, 1808); *Aspidapion radiolus* (Marsham, 1802); *Cyanapion columbinum* (Germar, 1817); *Protapion fulvipes* (Fourcroy, 1758); *P. trifolii* (Linnaeus, 1768); *Curculio nucum* Linnaeus, 1758; *Mecinus pyraster* (Herbst, 1795).

Table 2

Species composition of *Coleoptera* assemblages in the canopies of differently treated apple orchards in Hungary
(Beating method, 1990–1994; see the codes in Table 1)

Locality		Kecskemét			Kecskemét		Kecskemét		Nagykovácsi
Code	ACOM	AIPM/1	AIPM/2	ACOMs	AIPM/1s	AIPM/2s	OACOM	OAIPM	ANTR
Method	umbrella	umbrella	umbrella	sheet	sheet	sheet	umbrella	umbrella	umbrella
Years	1992–93	1992–93	1992–93	1992–94	1992–94	1992–94	1992–94	1992–94	1990–92
Carabidae									
Amara aenea (De Geer, 1774)							92		
Amara fulva (O. F. Müller, 1776)		92	92						
Calathus ambiguus (Paykull, 1790)	92, 93								
Dromius linearis (Olivier, 1795)						93			90, 91*
Harpalus froelichi Sturm, 1818	92		92						
Lebia humeralis Dejean, 1825				94					
Microlestes fissuralis (Reitter, 1900)								94	
Hydrophilidae									
Enochrus quadripunctatus (Herbst, 1797)							92		
Staphylinidae									
Stenus sp.			92	93					
Tachinus sp.					93				
Tachyporus sp.							93		91
Histeridae									
Saprinus aeneus (Fabricius, 1775)		92							
Cantharidae									
Cantharis livida Linnaeus, 1758									90, 92
Cantharis nigricans (O. F. Müller, 1776)									90
Cantharis obscura Linnaeus, 1758									91
Cantharis rustica Fallén, 1807								92	90, 91, 92

Metacantharis haemorrhoidalis Fabricius, 1792									90, 91, 92
Rhagonycha fulva Scopoli, 1763									91, 92
Rhagonycha lignosa (O. F. Müller, 1764)									91
Rhagonycha limbata C. G. Thomson, 1864									91, 92
Rhagonycha rorida Kiesenwetter, 1867									90
Melyridae									
Charopus concolor (Fabricius, 1801)									91
Charopus flavipes (Paykull, 1798)					92				
Clanoptilus geniculatus Germar, 1824	93		93	93			92, 93	92, 93	90, 91
Dasytes aerosus Kiesenwetter, 1867									90, 92
Dasytes plumbeus (O. F. Müller, 1776)					92				
Cleridae									
Clerus mutillarius Fabricius, 1775		93							
Trogossitidae									
Tenebrodeis fuscus (Goeze, 1777)								94	
Omophlus lividipes Mulsant, 1856							93	93, 94	
Elateridae									
Adrastus limbatus (Fabricius, 1776)				93					
Agriotes lineatus (Linnaeus, 1767)							92		
Agrypnus murinus (Linnaeus, 1758)							93	92, 94	90, 91, 92
Ampedus sanguinolentus (Schrank, 1776)	93								90
Athous haemorrhoidalis (Fabricius, 1801)									90, 91, 92
Cardiophorus cinereus (Herbst, 1784)		93	93			93	93	93, 94	91
Cardiophorus discicollis (Herbst, 1806)				93			94	93	
Cardiophorus equiseti (Herbst, 1784)	93	92, 93	93			92, 94	93	93	
Cardiophorus nigerrimus (Erichson, 1840)							94		
Cardiophorus rubripes (Germar, 1824)	93	93	92, 93		92	93	93	94	
Cardiophorus vestigialis (Erichson, 1840)				93	92, 93, 94	93, 94	92	92, 93, 94	
Dalopius marginatus (Linnaeus, 1758)								94	
Melanotus crassicollis (Erichson, 1841)								93	
Melanotus niger (Fabricius, 1772)			93					94	

Table 2 cont.

Locality		Kecskemét			Kecskemét			Kecskemét		Nagykovácsi
Code	ACOM	AIPM/1	AIPM/2	ACOMs	AIPM/1s	AIPM/2s	OACOM	OAIPM	ANTR
Method	umbrella	umbrella	umbrella	sheet	sheet	sheet	umbrella	umbrella	umbrella
Years	1992–93	1992–93	1992–93	1992–94	1992–94	1992–94	1992–94	1992–94	1990–92
Buprestidae									
Agrilus roscidus Kiesenwetter, 1857								94	
Anthaxia fulgurans (Schrank, 1789)									92
Anthaxia nitidula (Linnaeus, 1758)									92
Coroebus elatus (Fabricius, 1787)								93	
Dicerca berolinensis (Herbst)									92
Helodidae									
Cyphon padi (Linnaeus , 1758)						93			
Cyphon sp.	93				93, 94			94	
Dermestidae									
Trogoderma versicolor (Creutzer, 1799)								92	
Nitidulidae									
Meligethes aeneus (Fabricius, 1775)	92, 93	92, 93	93	92, 93	92, 93, 94	92, 93, 94	92, 94	92, 93, 94	90, 91, 92
Meligethes sp.				93	93				
Soronia grisea (Linnaeus, 1758)							92	92, 94	
Cryptophagidae									
Cryptophagus sp.				92	93				
Phalacridae									
Olibrus sp.		93	93	92	92, 93, 94	93	93	92, 93, 94	90, 91, 92
Phalacrus sp.		93	92	93, 94	92, 93	92, 93	92, 93	92, 94	
Stilbus sp.	92	92		92, 94	92	92	92, 93	93	92

Lathridiidae									
Cortinicara gibbosa (Herbst, 1793)		92	92	92, 93, 94	92, 93, 94	92, 93, 94	92	92, 94	90
Corticaria sp.					93				
Coccinellidae									
Adalia bipunctata (Linnaeus , 1758)	92, 93	93	92, 93	93, 94	92, 93	92, 93, 94	92, 93, 94	92, 93, 94	90, 91, 92
Adalia decempunctata (Linnaeus , 1758)							92, 93, 94	92	90, 91, 92
Anatis ocellata (Linnaeus , 1758)						92		94	
Calvia quatuordecimguttata (Linnaeus , 1758)	92	93	93		94	93	92, 94	92, 93, 94	90, 91, 92
Chilocorus bipustulatus (Linnaeus , 1758)									91, 92
Coccinella septempunctata (Linnaeus, 1758)	92, 93	92, 93	92, 93	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94	90, 91, 92
Coccinula quatuordecimpustulata (Linnaeus , 1758)	93	93	93	93	93	92, 93	93, 94	93, 94	91*
Exochumus quadripustulatus (Linnaeus , 1758)			93			93			90, 91, 92
Harmonia quadripunctata (Pontoppidan, 1763)								94	
Hippodamia tredecimpunctata (Linnaeus , 1758)								94*	
Hippodamia variegata (Goeze, 1777)	93	92, 93	92	92, 93, 94	92, 93	92, 93, 94	92, 93, 94	92, 93, 94	90, 92
Nephus redtenbacheri (Mulsant, 1846)						93			
Oenopia conglobata (Linnaeus , 1758)			93	93, 94		93	92, 94		91, 92
Oenopia lyncea (Olivier, 1808) ssp. agnata (Rhosenhauer, 1847)									92
Propylea quatuordecimpunctata (Linnaeus , 1758)	92, 93	92, 93	92, 93	92, 93		92, 93, 94	92, 93, 94	92, 93, 94	90, 91, 92
Psyllobara vigintiduopunctata (Linnaeus , 1758)	92			92		92, 93, 94	92	92, 93	90, 91, 92
Scymnus frontalis (Fabricius, 1787)							93		
Scymnus mediterraneus (Khnzorian, 1972)				93		93		93	
Scymnus mimulus (Capra & Fürsch, 1967)					93				
Scymnus rubromaculatus (Goeze, 1777)	92								
Scymnus sp.				94	92	92	92		
Stethorus punctillum (Weise, 1861)	92, 93	92, 93	92, 93	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94	90, 91, 92
Subcoccinella vigintiquatuorpunctata (Linnaeus , 1758)		92*	92*	93		93	93, 94	93, 94	
Anobiidae									
Xyletinus laticollis (Duftschmidt, 1825)						93			
Oedemeridae									
Oedemera lurida (Marsham, 1802)									92

Table 2 cont.

Locality		Kecskemét			Kecskemét		Kecskemét		Nagykovácsi
Code	ACOM	AIPM/1	AIPM/2	ACOMs	AIPM/1s	AIPM/2s	OACOM	OAIPM	ANTR
Method	umbrella	umbrella	umbrella	sheet	sheet	sheet	umbrella	umbrella	umbrella
Years	1992–93	1992–93	1992–93	1992–94	1992–94	1992–94	1992–94	1992–94	1990–92
Pyrochroidae									
Pyrochroa coccinea (Linnaeus , 1761)							94		
Silvanidae									
Psammoecus bipunctatus (Fabricius, 1792)							92		
Anthicidae									
Anthicus antherinus (Linnaeus , 1761)				93		93	92, 93		
Notoxus monoceros (Linnaeus , 1761)	92	92, 93	92	92, 93	92, 93, 94	92, 93, 94	93, 94	92, 93, 94	
Notoxus trifasciatus (Rossi, 1794)		93	92, 93	93, 94	93	92, 93, 94		93	
Mordellidae									
Mordellistena consobrina Ermisch, 1977							93		
Mordellistena parvula Gyllenhal, 1827							94		
Scraptiidae									
Anaspis frontalis (Linnaeus , 1758)					94		92		90, 92
Anaspis rufilabris Gyllenhal, 1827								94	90, 91
Lagriidae									
Lagria hirta (Linnaeus , 1758)						94	92	94	90, 91, 92
Alleculidae									
Hymenalia rufipes (Fabricius, 1792)			92				92		
Tenebrioidae									
Leichenum pictum (Fabricius, 1801)		92	92						
Melanimon tibiale (Fabricius, 1781)				94	92, 94	93	94	93, 94	

Melolonthidae									
Phyllopertha horticola (Linnaeus , 1758)	92	92						94	
Cetonia aurata aurata (Linnaeus , 1758)									91
Epicometis hirta (Poda, 1761)	93		93	93	92, 93	93	92	92	90
Anomala dubia (Scopoli, 1763)		92			93	93			
Anomala vitis (Fabricius, 1775)	92, 93	92, 93	93	92	93	93		92, 93, 94	
Melolontha melolontha (Linnaeus , 1758)						92		93, 94	
Netocia cuprea obscura (Andresch, 1797)								93, 94	
Omaloplia sp.	93				93				
Anoxia orientalis (Krynicki, 1832)								93	
Valgus hemipterus (Linnaeus , 1758)							92	92	
Scarabaeidae									
Aphodius distinctus (O. F. Müller, 1776)							94		
Aphodius sp.							94		
Cerambycidae									
Chlorophorus varius (Müller, 1766)	92						92, 94	93	
Dinoptera collaris (Linnaeus , 1758)									90, 92
Plagionotus floralis (Pallas, 1767)							94		
Pogonochaerus hispidus (Linnaeus , 1758)									92
Stenopterus rufus (Linnaeus , 1767)									91
Tetrops praeusta (Linnaeus , 1758)				94		94		94	90, 91, 92
Chrysomelidae									
Altica sp.									90
Aphtona euphorbiae (Schrank, 1781)					92, 93	92	92	92, 93, 94	92
Aphtona franzi Heikertinger, 1944					92				
Aphtona lacertosa (Rosenhauer, 1847)						93			
Aphtona semicyanea Allard, 1859								94	
Cassida nebulosa Linnaeus, 1758							92, 94	92	92
Cassida stigmatica Suffrian, 1844				94					
Cassida subreticulata Suffrian, 1844					93, 94		94	94	
Chaetocnema arenacea (Allard, 1860)				93					

Table 2 cont.

Locality		Kecskemét			Kecskemét			Kecskemét		Nagykovácsi
Code	ACOM	AIPM/1	AIPM/2	ACOMs	AIPM/1s	AIPM/2s	OACOM	OAIPM	ANTR
Method	umbrella	umbrella	umbrella	sheet	sheet	sheet	umbrella	umbrella	umbrella
Years	1992–93	1992–93	1992–93	1992–94	1992–94	1992–94	1992–94	1992–94	1990–92
Chaetocnema breviuscula (Faldermann 1857)						92			
Chaetocnema concinna (Marsham, 1802)	93	93		92	92		94	93, 94	
Chaetocnema conducta (Motschulsky, 1838)							92		
Chaetocnema semicoerulea (Koch, 1803)						92			
Chaetocnema tibialis (Illiger, 1807)	93	93	93	92, 94		92, 94	93	94	
Cryptocephalus chrysopus Gmelin, 1788									91
Cryptocephalus hypochoeridis hypochoeridis (Linnaeus , 1758)									91, 92
Cryptocephalus imperialis Laicharting, 1781									90, 91
Cryptocephalus sp.					93				
Chrysolina marginata (Linnaeus , 1758)								94	
Epitrix pubescens (Koch, 1803)				94					
Galeruca tanaceti (Linnaeus , 1758)								94	
Lachnaia sexpunctata (Scopoli, 1763)									90, 91, 92
Leptinotarsa decemlineata (Say, 1824)		93							
Longitarsus atricillus (Linnaeus , 1761)			93						
Longitarsus parvulus (Paykull, 1799)				92	92, 93	94	93	92, 93	
Longitarsus pellucidus (Foudras, 1860)									90, 91, 92
Oulema melanopus (Linnaeus , 1758)		92, 93		92	94	93			90
Pachybrachis tesselatus (Olivier, 1791)									91
Phyllotreta atra (Fabricius, 1775)	93		93				93	94	91
Phyllotreta cruciferae (Goeze, 1777)	93	93		93			93, 94	93, 94	91
Phyllotreta nemorum (Linnaeus , 1758)									90, 91
Phyllotreta nigripes (Fabricius, 1775)		93					93	93	90, 92
Phyllotreta nodicornis (Marsham, 1802)									92
Phyllotreta vittula (Redtenbacher, 1849)	93	93	93	92, 93, 94	92, 93, 94	92, 93	93, 94	93, 94	
Psylliodes attenuata (Koch, 1803)						93		92	
Psylliodes dulcamare (Koch, 1803)									90*
Psylliodes sophiae Heikertinger, 1914			93			94			

Psylliodes toelgi Heikertinger, 1914								92*
Smaragdina affinis (Illiger, 1794)								90
Smaragdina cyanea (Fabricius, 1775)								90, 92
Bruchidae								
Bruchidius sp.			93	93			92	92
Bruchus affinis Frölich, 1799				92, 93	92, 93			92
Bruchus brachialis Fåhraeus, 1839	92			93				
Bruchus luteicornis Illiger,1794			92	93	92		92	
Bruchus rufimanus Boheman, 1833		92				92		
Spermophagus sericeus (Fourcroy, 1785)			93, 94	92, 94	93, 94			
Anthribidae								
Brachytarsus nebulosus (Forster, 1771)								92*
Rhaphitropis marchicus (Herbst, 1797)								92
Rhynchitidae								
Caenorhinus aequatus (Linnaeus , 1767)								91, 92*
Caenorhinus aeneovirens (Marsham, 1802)								92
Caenorhinus germanicus (Herbst, 1797)								92*
Caenorhinus pauxillus (Germar, 1824)								90, 91, 92
Involvulus caeruleus (De Geer, 1775)								90, 91, 92
Involvulus pubescens (Fabricius, 1775)						92		
Pselaphorhynchites tomentosus (Gyllenhal, 1839)								90*
Rhynchites bacchus (Linnaeus , 1758)		93	94					90
Apinoidae								
Apion sp.				92				
Catapion seniculus (Kirby, 1808)								91
Ceratapion gibbirostre (Gyllenhal, 1813)			93					
Dieckmanniellus helvetius (Tournier, 1867)								91*
Diplapion confluens (Kirby, 1808)	93	92	93	92, 93	92, 93		93	
Diplapion stolidum (Germar, 1817)	92		94	92	92, 93	92	92, 93	
Eutrichapion viciae (Paykull, 1800)					93			
Holotrichapion aestimatum (Faust, 1891)			94	94				

Table 2 cont.

Locality		Kecskemét			Kecskemét		Kecskemét		Nagykovácsi
Code	ACOM	AIPM/1	AIPM/2	ACOMs	AIPM/1s	AIPM/2s	OACOM	OAIPM	ANTR
Method	umbrella	umbrella	umbrella	sheet	sheet	sheet	umbrella	umbrella	umbrella
Years	1992–93	1992–93	1992–93	1992–94	1992–94	1992–94	1992–94	1992–94	1990–92
Holotrichapion pisi (Fabricius, 1801)					92			92	
Protapion ononidis (Gyllenhal, 1827)					94				
Stenopterapion tenue (Kirby, 1808)					93	92			
Curculionidae									
Anthonomus pomorum (Linnaeus , 1758)	93		93	93, 94	93, 94	92, 93, 94	92, 93, 94	92, 93, 94	90, 91, 92
Baris coerulescens (Scopoli, 1763)								92	
Baris scolopacea Germar, 1824		93	93			93		93	
Ceutorhynchus erysimi (Fabricius, 1787)							94	92, 94	
Ceutorhynchus floralis (Paykull, 1792)						92	92		92
Ceutorhynchus napi Gyllenhal, 1837							94		
Ceutorhynchus picitarsis Gyllenhal, 1837							92		
Ceutorhynchus posthumus Germar, 1824						93			
Ceutorhynchus sophiae (Steven, 1829)					92	92			
Ceutorhynchus sp.				94	93, 94	94			
Coeliastes lamii (Fabricius, 1792)						94	92	94	
Cryptorhynchus lapathi (Linnaeus , 1758)	93								
Curculio glandium Marsham, 1802									91
Dorytomus filirostris (Gyllenhal, 1836)								94	
Dorytomus longimanus (Forster, 1771)			92	92					
Dorytomus rufatus (Bedel, 1888)				93		92			
Dorytomus schoenherri Faust, 1882				94					
Dorytomus suratus (Gyllenhal, 1836)				93			92		
Dorytomus tremulae (Paykull, 1800)						92		92	
Gymnetron antirrhini (Paykull, 1800)				92					
Gymnetron rostellum (Herbst, 1795)		92, 93	93	92, 94	94	93		94	
Gymnetron tetrum (Fabricius, 1792)				93, 94	93, 94		94	93	91
Hypera fuscocinerea (Marsham, 1802)					92				

Hypera dauci (Olivier, 1807)									94	
Hypera viciae (Gyllenhal, 1813)			92		92					
Larinus sturnus (Schaller, 1783)						93				
Larinus turbinatus (Gyllenhal, 1836)	92									92
Lignyodes enucleator (Panzer, 1798)										92
Lixus albomarginatus Boheman, 1843	92	92	93	92, 94	93	93, 94		92, 94		
Lixus elegantulus Boheman, 1843					94					
Lixus rubicundus Zoubkow, 1833						93				
Lixus subtilis Boheman, 1836	93	92, 93		92, 93	92, 93		92, 93, 94	92, 93, 94		
Lixus vilis (Rossi, 1790)		93	93	93, 94	93, 94	92, 93, 94	92, 93, 94	93, 94		
Magdalis barbicornis (Latreille, 1804)										90, 91, 92
Magdalis cerasi (Linnaeus , 1758)										90
Magdalis ruficornis (Linnaeus , 1758)			92	94		93		92, 94		90, 91, 92
Notaris acridulus (Linnaeus , 1758)	93									
Otiorhynchus fullo (Schrank, 1781)										90, 91, 92
Otiorhynchus ovatus (Linnaeus , 1758)							92	92		
Peritelus familiaris Boheman, 1834	92, 93	92, 93	92, 93	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94		
Phyllobius argentatus (Linnaeus , 1758)										90, 91
Phyllobius betulinus (Bechstein & Scharfenberg, 1805)										90, 91, 92
Phyllobius maculicornis (Germar, 1824)										92*
Phyllobius oblongus (Linnaeus , 1758)							92	92, 94		90, 91, 92
Phyllobius pyri (Linnaeus , 1758)					93					90, 91, 92
Phyllobius scutellaris L. Redtenbacher, 1849				94						
Phyllobius virideaeris (Laicharting, 1781)	92	92		92			94	92, 94		
Polydrusus cervinus (Linnaeus , 1758)										90
Polydrusus flavipes (De Geer, 1775)					92, 93	92, 93, 94		94		
Polydrusus sericeus (Schaller, 1783)										91
Polydrusus pterygomalis Boheman, 1840						93				
Rhamphus pulicarius (Herbst, 1795)						93, 94				90, 91
Rhynchaenus pilosus (Fabricius, 1781)										92*
Sibinia pellucens (Scopoli, 1772)		93	93	92, 93		92, 93		92		
Sibinia phalerata (Gyllenhal, 1836)	92			93, 94	93, 94	92, 93, 94		93		
Sitona callosus (Gyllenhal, 1834)	92, 93	92, 93	92, 93	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94	92, 93, 94		90, 91, 92
Sitona cylindricollis (Fåhraeus, 1840)	92									

Table 2 cont.

Locality		Kecskemét			Kecskemét			Kecskemét		Nagykovács
Code	ACOM	AIPM/1	AIPM/2	ACOMs	AIPM/1s	AIPM/2s	OACOM	OAIPM	ANTR
Method	umbrella	umbrella	umbrella	sheet	sheet	sheet	umbrella	umbrella	umbrella
Years	1992–93	1992–93	1992–93	1992–94	1992–94	1992–94	1992–94	1992–94	1990–92
Sitona griseus (Fabricius, 1775)					94	94		94	
Sitona humeralis Stephens, 1831	92			92		92, 93		92, 93, 94	91
Sitona lineatus (Linnaeus , 1758)								92	92
Sitona macularius (Marsham, 1802)					92, 94	93		92, 93	91, 92
Sitona sulcifrons (Thunberg, 1798)					94	93		94	
Stenocarus cardui (Herbst, 1784)								93	
Tychius breviusculus Dresbrochers des Loges, 1873				93	93, 94			94	
Tychius flavus Becker, 1864							94		
Tychius meliloti Stephens, 1831	92	92		93			93		
Tychius sp.								94	
Tychius stephensi Schönherr, 1836				92					
Scolytidae									
Hylesinus varius (Fabricius, 1775)								92	
Scolytus rugulosus (Müller, 1818)				92, 93	92, 93, 94	92, 93, 94	93	92, 93, 94	

During the years marked with * the given species occurred exceeding the number of samples given in Table 1.

In the apple orchards there were 9 additional species found by Mészáros et al. (1984): *Synchita humeralis* Fabricius, 1792; *Stilbus testaceus* (Panzer, 1797); *Halyzia sedecimguttata* (Linnaeus, 1758); *Semiadalia undecimnotata* (Schneider, 1792); *Cassida nobilis* Linnaeus, 1758; *Galerucella calmariensis* (Linnaeus, 1767); *Curculio salicivorus* (Paykull, 1792); *Otiorhynchus ligustici* (Linnaeus, 1758); *Tanymecus palliatus* (Fabricius, 1787).

Considering the above species 300 beetle species occur altogether in the canopies of the Hungarian apple orchards. Zilahi-Sebess (1955) found 4 further Coleoptera species in the canopies of pear trees which were not registered by us: *Enicmus transversus* (Olivier, 1790); *Dorcatoma chrysomelina* Sturm, 1837; *Cassida vibex* Linnaeus, 1767; *Protapion aprians* (Herbst, 1790).

Considering that there were not found only apple-feeding phytophagous beetle species in apple orchards, and there were only two specialized pear feeder species (*Rhynchites lenaeus, Anthonomus pyri*) occurred in pear orchards and supposing that the non-specialist Coleoptera species could be found in both orchards we can estimate 371 beetle species occurring in the canopies of Hungarian apple and pear orchards. This number represents 4% of the total Hungarian beetle fauna. In comparison there were the following number of Coleoptera species registered in the canopies of apple orchards 95 by Zilahi-Sebess (1955), 95 by Steiner (1962), Steiner et al. (1970), 89 by Oatman et al. (1964), 63 by Brown et al. (1988), 42 by Mészáros et al. (1984).

In our investigations the majority of the species both in apple and pear orchards belonged to the families Curculionidae (apple: 27%, pear: 18%), Chrysomelidae (apple: 16%, pear: 21%) and Coccinellidae (apple: 9%, pear: 12%). Higher number of beetles of the families Rhynchitidae, Buprestidae and Cerambycidae were found only in untreated orchards. The species diversity of the soldier beetles (Cantharidae) were independent of the treatments and depended only on the forest surroundings. Predators represented 20%, phytophagous beetles 75%, fungivores 2% and scavengers represented 3% of entire fauna. Among the herbivorous species the ratio of fruit tree-feeders was 10%. In the investigated orchards the total number of species varied between 29 and 122 but the number of predatory species were registered between 10 and 29. We are emphasizing the fact that there was no significant difference between the number of predators in conventionally treated (sprayed mainly with high toxic chemicals) and IPM plots (sprayed with IGR) i.e. usage of the toxic chemicals does not result in the decrease the species richness of predatory beetles. We estimated the distribution of each Coleoptera species and found out which species were present in the highest frequency each year in the different orchards. The species wich were found with a frequency more than 60% – in the investigated orchards and years – are described below in the following decreasing order: *Coccinella septempunctata, Stethorus punctillum, Sitona callosus, Propylea quatuordecimpunctata, Adalia bipunctata, Meligethes aeneus, Hippodamia variegata, Anthonomus pomorum, Peritelus. familiaris, Notoxus monoceros, Cortinicara gibbosa, Phyllotreta vittula* and in the pear orchards *Stethorus punctillum, Propylea quatuordecimpunctata, Adalia bipunctata, Coccinella septempunctata, Calvia quatuordecimpustulata, Phyllobius oblongus, Rhagonycha fulva, Byctiscus betulae, Phyllotreta vittula.*

Table 3

Species composition of *Coleoptera* assemblages in the canopy of differently treated pear orchards in Hungary
(Beating method, 1990–1994; see the codes in Table 1)

Locality	Nagykovácsi		Sárospatak			
Code	PNTR	PTR	PIPM	PCOM/1	PCOM/2	PCOM/3
Method	umbrella	umbrella	umbrella	umbrella	umbrella	umbrella
Years	1990–94	1992–94	1993–94	1993–94	1993–94	1993–94
Silphidae						
Xylodrepa quadrimaculata (Linnaeus, 1758)	94	94				
Staphylinidae						
Omalium rivulare (Paykull, 1789)				94		
Staphylinidae sp.		92			93	
Tachinus sp.		92				
Histeridae						
Saprinus aeneus (Fabricius, 1775)		94				
Cantharidae						
Cantharis bicolor Herbst, 1784		94				
Cantharis fusca Linnaeus, 1758		92				
Cantharis lateralis Linnaeus, 1758		92				
Cantharis livida Linnaeus, 1758	90, 91, 92, 93, 94	92, 93, 94				
Cantharis nigricans (O. F. Müller, 1776)	94					
Cantharis obscura Linnaeus, 1758	92					
Cantharis rufa Linnaeus, 1758		92, 94	94	94	94	94
Cantharis rustica Fallén, 1807	90, 92, 93, 94	92, 93, 94				
Metacantharis haemorrhoidalis (Fabricius, 1792)	90, 91, 92, 93, 94					
Rhagonycha fulva (Scopoli, 1763)	92, 93, 94	92, 93, 94	94	94	93	93, 94
Rhagonycha lignosa (O. F. Müller, 1764)	94					
Rhagonycha limbata C. G. Thomson, 1864	91, 92					
Rhagonycha rorida Kiesenwetter, 1867	91, 92, 94					

Melyridae						
Clanoptilus geniculatus Germar, 1824	91	94				
Dasytes aerosus Kiesenwetter, 1867	91, 92*	93				
Dasytes plumbeus (O. F. Müller, 1776)	90	92				
Cleridae						
Opio mollis (Linnaeus, 1758)			93			
Elateridae						
Adrastus limbatus (Fabricius, 1776)		92, 93			93	93
Agriotes pilosellus (Schönherr, 1817)			93, 94	93		94
Agriotes sputator (Linnaeus, 1758)	91					
Agrypnus murinus (Linnaeus, 1758)	90, 91, 92, 93	93, 94				94
Athous haemorrhoidalis (Fabricius, 1801)	90, 93, 94	92, 93, 94				
Cardiophorus cinereus (Herbst, 1784)	91	93	93	93	93	
Cidnopus minutus (Linnaeus, 1758)	93	93, 94				
Melanotus rufipes (Herbst, 1784)		94				
Prosternon tessclatum (Linnaeus, 1758)	93					
Pseudathous hirtus (Herbst, 1784)		92, 93				
Buprestidae						
Agrilus fuscosericeus (Daniel, 1899)	91, 93					
Agrilus laticornis (Illiger, 1803)	90, 94					
Agrilus roscidus Kiesenwetter, 1857	93, 94					
Agrilus sinuatus (Olivier, 1790)	93					
Agrilus sulcicollis Lacordaire, 1835	91					
Anthaxia nitidula (Linnaeus, 1758)	90, 91, 92, 94					
Trachys minuta (Linnaeus, 1758)	93, 94					
Helodidae						
Microcara testacea (Linnaeus, 1767)				94		
Nitidulidae						
Brachypterolus pulicarius (Linnaeus, 1758)	94			94		

Table 3 cont.

Locality	Nagykovácsi		Sárospatak			
Code	PNTR	PTR	PIPM	PCOM/1	PCOM/2	PCOM/3
Method	umbrella	umbrella	umbrella	umbrella	umbrella	umbrella
Years	1990–94	1992–94	1993–94	1993–94	1993–94	1993–94
Meligethes aeneus (Fabricius, 1775)	90, 91, 92, 93	92, 93	94		94	94
Meligethes sp.	91					
Soronia grisea (Linnaeus, 1758)						94
Cybocephalidae						
Cybocephalus fodori Endrődi-Younga, 1965	93, 94					
Cryptophagidae						
Cryptophagus sp.			94			
Phalacridae						
Olibrus sp.	90, 91, 92, 93, 94	93, 94			93	
Phalacrus sp.					94	
Stilbus sp.		94				94
Lathridiidae						
Cortinicara gibbosa (Herbst, 1793)	90	93	94	94	93	93, 94
Corticaria sp.		92				
Coccinellidae						
Adalia bipunctata (Linnaeus, 1758)	90, 91, 92, 93, 94	92, 93, 94	94	93, 94	93, 94	93, 94
Adalia decempunctata (Linnaeus, 1758)	90, 91, 92, 94	92, 94			93	93
Anatis ocellata (Linnaeus, 1758)		92				
Calvia quatuordecimguttata (Linnaeus, 1758)	90, 91, 92, 94	92, 93, 94	93, 94	94	93, 94	94
Chilocorus bipustulatus (Linnaeus, 1758)	90, 91, 92, 93, 94					
Coccinella septempunctata Linnaeus, 1758	90, 91, 92, 93, 94	92, 93, 94	93	93	93, 94	93
Coccinula quatuordecimpustulata (Linnaeus, 1758)	91					
Exochomus quadripustulatus (Linnaeus, 1758)	90, 91, 92, 93, 94					

Hippodamia undecimnottata (Schneider, 1792)		92				
Hippodamia variegata (Goeze, 1777)	90, 91*. 94	94	93	94	93, 94	93
Hyperaspis reppensis (Herbst, 1783)	90, 92					
Oenopia conglobata (Linnaeus, 1758)	91, 92, 93		93	94	93, 94	93, 94
Oenopia lyncea (Olivier, 1808) ssp. agnata (Rhosenhauer, 1847)	90, 91, 92					
Platynaspis luteorubra (Goeze, 1777)					94	
Propylea quatuordecimpunctata (Linnaeus, 1758)	90, 91, 92, 93, 94	92, 93, 94	93, 94	93, 94	93, 94	93, 94
Scymnus auritus Thunberg, 1795					93	93
Scymnus frontalis (Fabricius, 1787)	90					
Scymnus subvillosus (Goeze, 1777)					93	
Scymnus sp.	90, 93				94	
Psyllobora vigintiduopunctata (Linnaeus, 1758)	90, 91, 92	92, 94	94	94	94	
Stethorus punctillum Weise, 1861	90, 91, 92, 93, 94	92, 93, 94	93, 94	93, 94	93, 94	93, 94
Subcoccinella vigintiquatuorpunctata (Linnaeus, 1758)		94				
Tytthaspis sedecimpunctata (Linnaeus, 1758)		94				
Ptinidae						
Ptinus rufipes (Olivier, 1790)	92					
Anthicidae						
Anthicus antherinus (Linnaeus, 1761)					94	
Notoxus monoceros (Linnaeus, 1761)		93			93	
Mordellidae						
Mordella brachyura Mulsant, 1856	91					
Scraptiidae						
Anaspis frontalis (Linnaeus, 1758)	90, 91, 94					
Lagriidae						
Lagria hirta (Linnaeus, 1758)	90, 91, 92, 93, 94	92, 94				

Table 3 cont.

Locality	Nagykovácsi		Sárospatak			
Code	PNTR	PTR	PIPM	PCOM/1	PCOM/2	PCOM/3
Method	umbrella	umbrella	umbrella	umbrella	umbrella	umbrella
Years	1990–94	1992–94	1993–94	1993–94	1993–94	1993–94
Alleculidae						
Gonodera luperus (Herbst, 1783)	92, 93					
Podonta nigrata (Fabricius, 1794)	93, 94					
Tenebrionidae						
Stenomax aeneus (Scopoli, 1763)	90					
Melolonthidae						
Cetonia aurata aurata (Linnaeus, 1758)	90, 92, 94					
Epicometis hirta (Poda, 1761)	94					
Melolontha melolontha (Linnaeus, 1758)		92				
Netocia cuprea obscura (Andresch, 1797)	92, 94		94			
Valgus hemiphterus (Linnaeus, 1758)		93				94
Scarabaeidae						
Aphodius sp.		93				
Cerambycidae						
Agapanthia violacea (Fabricius, 1775)	91					
Cortodera humeralis (Schaller, 1783)	90, 91					
Dinoptera collaris (Linnaeus, 1758)	92					
Leiopus nebulosus (Linnaeus, 1758)	91					
Pachytodes erraticus (Dalman, 1817)	93					
Tetrops praeusta (Linnaeus, 1758)	90, 91, 92, 94	94	94			
Chrysomelidae						
Altica oleracea (Linnaeus, 1758)		93, 94				
Altica sp.		92				

Aphtona euphorbiae (Schrank, 1781)	90, 92	92, 93				
Cassida flaveola Thunberg, 1794	90					
Cassida nebulosa Linnaeus, 1758	92, 93, 94	94				
Chaetocnema breviuscula (Faldermann, 1857)						94
Chaetocnema concinna (Marsham, 1802)		93	93, 94	93	93	94
Chaetocnema laevicollis (Thomson, 1866)			93			
Chaetocnema tibialis (Illiger, 1807)	91, 92					
Clytra laeviuscuala Ratzeburg, 1837	91, 92, 94					
Crepidodera aurata (Marsham, 1802)		94			93	
Cryptocephalus chrysopus Gmelin, 1788	94					
Cryptocephalus hypochoeridis hypochoeridis (Linnaeus, 1758)	93					
Cryptocephalus imperialis Laicharting, 1781	91, 94					
Cryptocephalus nitidus (Linnaeus, 1758)	91, 93, 94					
Gastrophysa polygoni (Linnaeus, 1758)	91					
Labidostomis longimana (Linnaeus, 1761)	90					
Lachnaia sexpunctata (Scopoli, 1763)	92, 94					
Leptinotarsa decemlineata (Say, 1824)				93		
Longitarsus atricillus (Linnaeus, 1761)				93		
Longitarsus luridus (Scopoli, 1763)				94		
Longitarsus pellucidus (Foudras, 1860)					93	94
Luperus xantopoda (Schrank, 1781)	90, 91, 92, 93, 94	93, 94				
Orsodacne lineola (Panzer, 1795)	90, 91, 92, 93, 94	93				
Oulema melanopus (Linnaeus, 1758)	91	92, 93, 94	94			94
Pachybrachis tesselatus (Olivier, 1791)	93					
Phratora vulgatissima (Linnaeus, 1758)		93				
Phyllotreta atra (Fabricius, 1775)		92, 93	93	93	93	93
Phyllotreta cruciferae (Goeze, 1777)	91	93			93	93
Phyllotreta diademata (Foudras, 1860)		93				
Phyllotreta nemorum (Linnaeus, 1758)	91	92, 93				
Phyllotreta nigripes (Fabricius, 1775)	90, 91, 93	92, 93			93	93
Phyllotreta nodicornis (Marsham, 1802)	91					
Phyllotreta undulata Kutschera, 1860		93				
Phyllotreta vittula (Redtenbacher, 1849)	93	92, 93	93, 94	93, 94	93	93, 94
Psylliodes sophiae Heikertinger, 1914		93				

Table 3 cont.

Locality	Nagykovácsi		Sárospatak			
Code	PNTR	PTR	PIPM	PCOM/1	PCOM/2	PCOM/3
Method	umbrella	umbrella	umbrella	umbrella	umbrella	umbrella
Years	1990–94	1992–94	1993–94	1993–94	1993–94	1993–94
Psylliodes sp.	91					
Smaragdina aurita (Linnaeus, 1767)	94					
Smaragdina cyanea (Fabricius, 1775)	90, 91, 92, 93, 94	93				
Sphaeroderma testaceum (Fabricius, 1775)		93				
Bruchidae						
Bruchidae sp.					94	94
Bruchidius sp.						94
Bruchus affinis Frölich, 1799	90, 92, 93					
Bruchus luteicornis Illiger, 1794	91					
Bruchus rufimanus Boheman, 1833	92					
Spermophagus sericeus (Fourcroy, 1785)				94	94	94
Nemonychidae						
Nemonyx lepturoides (Fabricius, 1801)	91					
Rhyncitidae						
Byctiscus betulae (Linnaeus, 1758)	91*, 92	93, 94	94	93, 94	93, 94	94
Caenorrhinus aequatus (Linnaeus, 1767)	90, 91, 93, 94					
Caenorrhinus pauxillus (Germar, 1824)	90, 91, 92, 93, 94					
Involvulus caeruleus (De Geer, 1775)	90, 91, 92, 93, 94					
Rhynchites auratus (Scopoli, 1763)	92*					
Rhynchites bacchus (Linnaeus, 1758)	91					
Rhynchites lenaeus (Faust, 1891)	92					
Apionidae						
Apion sp.				93		
Catapion seniculus (Kirby, 1808)						94

Dieckmanniellus helvetius (Tournier, 1867)						94
Holotrichapion aestimatum (Faust, 1891)	91					
Curculionidae						
Anthonomus piri Kollar, 1837	90, 91, 92, 93, 94					
Anthonomus pomorum (Linnaeus, 1758)	90, 91, 92, 93, 94					
Ceutorhynchus erysimi (Fabricius, 1787)	91*					
Ceutorhynchus floralis (Paykull, 1792)		92		94		
Ceutorhynchus sp.	92					
Cleonis pigra (Scopoli, 1763)						93
Curculio glandium Marsham, 1802	90, 91, 92*, 93, 94	92				
Curculio pyrrhoceras (Marsham, 1802)	90					
Curculio venosus (Gravenhorst, 1807)	91		94	94		94
Eusomus ovulum Germar, 1824		94				
Gymnetron antirrhini (Paykull, 1800)					94	94
Magdalis barbicornis (Latrielle, 1804)	90, 91, 92, 93					
Magdalis cerasi (Linnaeus, 1758)	90, 91, 92, 93, 94					
Magdalis nitidipennis (Boheman, 1843)	90	94				
Magdalis ruficornis (Linnaeus, 1758)	90, 91, 92, 93, 94		94	94		
Microplontus figuratus (Gyllenhal, 1837)	92					
Otiorhynchus fullo (Schrank, 1781)	90, 91, 92, 93, 94					
Phyllobius argentatus (Linnaeus, 1758)	90, 91, 94					
Phyllobius betulinus (Bechstein & Scharfenberg, 1805)	90, 91, 92, 93, 94	92, 93, 94				
Phyllobius incanus Gyllenhal, 1834	94					
Phyllobius maculicornis (Germar, 1824)	91, 94	93, 94				
Phyllobius oblongus (Linnaeus, 1758)	90, 91, 92, 93, 94	92, 93, 94	94	94	94	94
Phyllobius pyri (Linnaeus, 1758)	90, 91, 92, 93, 94	92, 93, 94	94	94	94	94
Polydrusus cervinus (Linnaeus, 1758)	90, 91, 92, 93					
Polydrusus sericeus (Schaller, 1783)	90, 91, 94					
Rhamphus pulicarius (Herbst, 1795)	90, 91, 92*					
Sirocalodes nigrinus (Marsham, 1802)	92					
Sitona callosus Gyllenhal, 1834	90, 91, 93, 94	92, 93, 94				
Sitona humeralis Stephens, 1831	91, 92	92, 94				
Sitona languidus Gyllenhal, 1834	91*					

Table 3 cont.

Locality	Nagykovácsi			Sárospatak		
Code	PNTR	PTR	PIPM	PCOM/1	PCOM/2	PCOM/3
Method	umbrella	umbrella	umbrella	umbrella	umbrella	umbrella
Years	1990–94	1992–94	1993–94	1993–94	1993–94	1993–94
Sitona lineatus (Linnaeus, 1758)	91, 93	92			94	94
Sitona macularius (Marsham, 1802)	91, 92, 94					
Sitona sulcifrons (Thunberg, 1798)					93	93
Scolytidae						
Scolytus mali (Bechstein, 1805)	94*					
Scolytus rugulosus (Müller, 1818)			93			

During the years marked with * the given species occurred exceeding the number of samples given in Table 1.

We could conclude on the basis of the fairly low number of widely distributed species in the orchards that there is a considerable difference in the species composition of Coleoptera assemblages. In the case of other arthropod groups Szentkirályi and Kozár (1988) and Brown et al. (1989) arrived to the same conclusions.

Acknowledgements

Professor Z. Mészáros, Dr. Klára Balázs and Professor G. Jenser have helped and supported the study in various aspects. We thanks Mr. Zs. Czető, Mr. R. Holynski, Mr. F. Kádár for identification of some groups, and Professor M. Kosztarab for his helpful comments and language corrections to the manuscript. This research was supported by a grant (no. 006392) from the Hungarian Scientific Research Fund (OTKA).

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 247–254 (1995)

Incidence of Nuclear Polyhedrosis Virus in Different Populations of the Beet Armyworm *Spodoptera exigua* (Hb.) (Lepidoptera: Noctuidae)

M. A. ALI[1], A. S. EL-KHOULY[1], G. M. MOAWAD[2] and M. G. M. RAGAB[2]

[1] Department of Plant Protection, Faculty of Agriculture, Al-Azhar University Nasr City, Cairo, Egypt
[2] Institute of Plant Protection, ARC, Dokki, Cairo, Egypt

The natural incidence of the nuclear polyhedrosis virus (NPV) in different populations of the beet armyworm *Spodoptera exigua* (Hb.) collected from maize fields (*Zea mays* L.) at El-Behiera, El-Fayoum and El-Gharbia governorates (Egypt) was assessed during maize seasons of 1985 and 1986. Maize fields of the different localities were infested by the insect larvae from mid-April to early August. Two peaks of larva populations were recorded during the maize season, however, the occurrence date of these peaks varied from locality to the other due to the environmental factors prevailing in each locality.

Virus infected larvae could be detected in the field from May first to the end of July. The maximum rate of virus infection in larval populations averaged 25.96%. El-Gharbia-larval population had the highest rate of virus infection (25.8%), followed by El-Fayoum and El-Behiera populations, respectively. A positive correlation between virus infection on one side and the larval density and air temperature and relative humidity on the other side have been proved. However, virus infected larvae, in El-Gharbia governorate was more correlated to the prevailing environmental factors rather than to larval density.

In Egypt, maize fields suffer greatly from the invasion of different insect pests particularly the corn borers (El-Saadany, 1965). However the beet armyworm *Spodoptera exigua* (Hb.) have only recently attracted the attention as an important pest of maize. Larvae attack young plants (20 days old) and feed on leaves causing serious damage. Although the beet armyworm populations are abundant in early maize season, most larvae could not be eliminated by their natural parasites (El-Heneidy and El-Dakroury, 1984). The vast increase in the use of chemical insecticides in maize fields is met by a groving concern to develop other control agents that leaves no hazardous residue in the environment. Baculoviruses are likely to offer such an advantage. The nuclear polyhedrosis virus (NPV) has been long recognized as an important natural mortality factor atfecting populations of several lepidopterous species (Abul Nasr, 1956; Hunter and Hall, 1968; Klein and Podlier, 1978; Ali et al., 1991).

This paper reports the nuclear polyhedrosis virus (NPV) observed in the beet armyworm larval populations in different governorates of Egypt during the years 1985 and 1986 as correlated to host density and certain environmental factors.

0238–1249/95/$ 5.00

Materials and Methods

The beet armyworm larval density and the incidence of nuclear polyhedrosis virus were monitored after the first occurrence of the disease in the field at two weeks intervals during maize seesons 1985 and 1986. Host density and disease incidence were estimated by randomly selecting 100 maize plants of about 20 days old. Plants were placed in plastic bags and returned to the laboratory for inspection. Larvae were collected from the infested plants, counted and reared individually in plastic tube (7 cm length and 3.5 cm diameter) with perforated lids. Larvae were supplied with fresh leaves of castor oil (*Ricinus communis* L.): daily serving as larval food. Larvae were kept under labory (26 + 1 °C and 70 + 5% R.H.) for further observations.

A daily observation of insect larvae for the occurrence of the disease continued till the larvae had either died or pupated. Disease mortality factor was established by marked symptoms. Microscopical examination was carried out to assure the viral death. Examining tissue smears under phase contrast microscope gave an evidence of NPV-death when polyhedral inclusion Hatrp bodies (PIBs) were detected inside the tissue cells (Harrap et al., 1977). The number of larvae exhibited viral symptoms was counted and the percentages of larval mortality were estimated based upon the proportion of diseased larvae of all those accounted for in the sample.

Records on daily mean temperature (°C) and daily mean of relative humidity (R.H.%) were supplied by the metereological station of each governorate.

Results and Discussion

Seasonal trend of virus occurrence

El-Fayoum governorate

Results in Table 1 clearify the occurrence of few numbers of *S. exigua* larvae on mid-April during the years 1985 and 1986. A gradual increase in larval population was noticed in the successive sarnples which peaked once on mid-June, 1985 and twice in 1986 (mid-June and mid-July). Following each population peak, the number of collected larvae markedly declined reaching the lowest density on early August. The first occurrence of virus diseased larvae was recorded on the first of May and the rate of virus infection increased in the successive samples during the two maize seasons. The highest percentages of virus infection (29.5%) were recorded in larval populations occurring throughout June at a temperature range of 26.7–28 °C and 47–51% rel. humidity. At the same time, it was found that the rate of virus infection markedly correlated to the density of larvae. On mid July few individuals of the beet armyworm larvae were infected by the virus, later samples of field collected larvae exerted no evidence of virus incidence. The overall percentage of virus infection in *S. exigua* larval populations in El-Fayoum governorate averaged 18.7–19.3%.

El-Gharbia govenorate

Field studies on infestation rate of maize fields in El-Gharbia governorate by the beet armyworm larva revealed heavy infestation as indicated by the presence of high larval density in maize plants (Table 1). Counts of sampled larvae during the two maize seasons (1985, 1986) showed two distinct peaks for each season on first of June and mid-July. In general, the infestation of maize plants by the concerned pest was higher in 1985 season than in 1986.

Infection by virus was detected for the first time in larval population sampled on May 1st, 1985 where the percentage of infection averaged 11.1, while samples of larvae collected from the field at the same date of the next season (1986) were quite virus free larvae (Table 1). In E1-Gharbia region, not similar to the other two regions, spread of virus-infected larvae was more correlated to the prevailing air temperature and relative humidity rather than to the population density of larvae existing in maize fields. Peaks of larvae infected by virus were denoted at mid-July and early August when air temperature and relative humidity raised to about 30 °C and 70–77%. On the other hand, the percent of virus infection showed considerable increase through the entire season with the simultaneous raising of temperature and relative humidity. However, the population density of larvae was higher during the first maize season (235 larvae) than in the second season (188 larvae), the rate of virus infection in the two seasons was nearly equal.

El-Behiera governorate

Counts of the biweekly field collected *S. exigua* larvae during 1985-maize season showed low population on mid-May. Two weeks later, the larval population considerably increased to more than twofolds showing the first peak (29 larvae). Another higher peak (50 larvae) was recorded by mid July. Field observations during 1986-maize season showed two conspicious peaks of insect larva, the first occurred on mid June and the second by early August (Table 1).

The first virus-diseased larva was observed in the first sample of larvae collected on May 15, 1986 when the virus infection rate in the population averaged 9.52%. Lavae collected at the same of date of the preceding season were completely healthy and virus free. The nuclear polyhedrosis virus of *S. exigua* was so widespread and virulent in nature that further larvae were infected by the gradual increase of virus infection in the successive samples and reached the maximum (24.0%) on mid-July, 1985 and by early August 1986 (20.96%). It is evident as shown in Table 1 that a high rate of virus infection was associated with higher density of larvae infesting maize plants. It was also noticed that the rate of maize fields infestation by the beet armyworm was quite similar during the two seasons, consequently the percentages of virus infected larvae were insignificantly differed. Air temperature and relative humidity prevailed through the highly spread of virus infection were 28 °C and 62–67%, respectively.

Table 1

Biweekly numbers of collected and rates of virus infection of *Spodoptera exigua* infesting maize in relation to some weather factors in different governorates – Egypt (seasons, 1985 and 1986)

Date of inspection		Governorate	1985					1986				
			Total no. of collected larvae	No. of infected larvae	% Infection	Weather factors		Total no. of collected larvae	No. of infected larvae	% Infection	Weather factors	
						Temp. °C	R. H.%				Temp. °C	R. H.%
April	15		5	0	0.00	22.6	49	10	0	0.00	23.1	54
May	1		13	1	7.69	24.5	51	22	5	22.72	22.6	53
	15		32	6	18.75	27.1	49	13	3	23.07	24.2	51
June	1	Fayoum	38	9	23.68	26.7	47	45	12	26.66	26.7	47
	15		44	13	29.54	28.4	51	32	5	15.62	28.4	51
July	1		30	7	23.30	28.3	52	39	6	15.38	28.3	49
	15		25	3	12.00	28.3	53	40	9	22.50	28.3	59
August	1		15	0	0.00	27.3	53	13	0	0.00	30.2	56
		Total	202	39	19.31			214	40	18.59		
		Mean	25.3	4.88				26.75	5			
May	1		27	3	11.11	22.7	63	13	0	0.00	20.1	63
	15		21	4	19.04	25.6	66	24	34	16.66	22.9	58
June	1		46	8	20.00	27.3	63	47	9	19.14	24.7	56
	15	Gharbia	37	8	21.62	26.6	67	23	8	34.78	26.8	63

July	1		21	6	28.50	26.5	66	30	12	40.00	26.5	62
	15		50	15	30.00	27.9	74	39	15	38.46	28.7	70
August	1		39	17	43.58	26.2	77	12	0	0.00	26.6	70
		Total	235	61	25.96			188	48	25.53		
		Mean	33.57	8.71				26.86	6.86			
May	15		12	0	0.	23.3	60	21	2	9.52	21.7	60
June	1		29	4	13.79	26.2	61	28	5	17.85	24.3	61
	15	Behreia	20	3	15.00	29.1	65	43	8	18.60	26.9	68
July	1		47	9	19.14	28.0	62	39	6	15.38	27.6	60
	15		50	12	24.00	27.0	67	10	2	20.00	28.3	66
August	1		43	10	23.25	27.7	62	62	13	20.96	28.2	62
		Total	201	38	18.91			203	36	17.73		
		Mean	33.5	6.33				33.83	5			

Values followed by the same letter are not significantly different (Duncan's multiple range test).

Table 2

Correlation coefficient and partial regression values on the relationship between infection rates of NPV with larval density of the beet armyworm *S. exigua* and certain meteorological factors for sampling periods in 1985 through 1986

Locality	Season	Larval density			Daily mean of temperature			Daily mean of rel. humidity			Analysis of variance		
		r	P	b	r	P	b	r	P	b	F	P	E.V.
El-Fayoum	1985	0.907	1	1.262	0.624	5	−2.865	−0.212	NS	1.500	7.15	5	84.3
	1986	0.654	5	0.641	−0.108	NS	−1.793	−0.690	5	0.810	2.19	5	62.2
El-Gharbia	1985	0.392	NS	−0.211	0.581	1	1.673	0.878	1	1.138	7.85	1	88.7
	1986	0.688	5	0.260	0.600	1	4.656	−0.177	NS	−1.630	2.33	NS	70.0
El-Behreia	1985	0.824	5	0.381	−0.044	NS	2.983	0.376	NS	0.194	8.89	1	92.3
	1986	0.352	NS	0.022	0.829	5	0.862	0.547	NS	0.024	1.81	NS	73.0

r = Correlation coefficient; P = Probability (%); b = Regression coefficient; E.V. = Explained variance (%); NS = Not significant

Effects of environment

Density of larval populations, averages of daily air temperature and relative humidity as ecological factors played a great role on virus spread and infectivity. Values of data analysis as presented in Table 2 elucidate this important role. The incidence of virus is considerably correlated to larval density in maize fields. This relation is quite evident in maize fields located in the various governorates under investigation. It means that NPV spread eventually increases when high population of *S. exigua* larvae occurs than in low population. Jacques (1974) and El-Husseini (1980) reported that NPV-diseased larvae widespread when the natural population was enumerated for the beet armyworm. Similar results which greatly concide with our present data on the natural incidence of *S. exigua*-NPV were discussed by Semel (1956), Hofmaster (1961), Tanada and Omi (1974) which generally concluded that the association of virus incidence with the development of tleavy natural infestation of insects and air temperature.

The effect of temperature and relative humidity on the rate of virus infection were more different than the aforementioned factor and seems to be seasonal and locality dependent. As shown in Table 2, temperature had positive and significant effect on virus spread and infectivity in El-Gharbia governorate while this effect was positive in one maize season and negative for the second season in El-Behiera and El-Fayoum governorates. The same trend was denoted regarding relative humidity effect in El-Fayoum and El-Gharbia, while in El-Behiera this factor had strong positive relation to virus spread and virulent.

In nature, it is hard to evaluate the role played by each factor singly and usually the environmental factors always work together. Ecological factors under taken in the present study pronouncedly affected in harmony on the spread of epizootic virus which is reflected by the values of explained variance as shown in Table 2. These values ranged 73–93% 62–84% and 70–88% for El-Behiera, El-Fayoum and El-Gharbia governorates, respectively. These results suggest that NPV coula be a useful biological control agent in an integrated pest management (IPM) program of the beet armyworm in Egypt.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 255–263 (1995)

Further Data on the Food-choice of Wheat-sawflies (*Dolerus* spp., Hymenoptera, Tenthredinidae)

A. HARIS

Plant Health and Soil Conservation Station, P.O. Box 340, H-1519 Budapest, Hungary

Four *Dolerus* species, namely *Dolerus nigratus* Müller, *Dolerus puncticollis* Thomson, *Dolerus gonager* Fabricius, *Dolerus haematodes* Schrank, and 13 host-plants – as follows: *Agrostis alba, Agrostis alba ssp. gigantea, Bromus inermis, Dactylis glomerata, Festuca arudinacea, Festuca pseudovina, Festuca valesiaca, Lolium perenne, Poa pratensis variety Keszthelyi-58, Poa pratensis variety Mervel, Phleum pratense, Typhoides arudinacea* were examined. The two *Poa pratensis* varieties are studied first. *Poa pratensis* variety Keszthelyi-58 is a primary host plant, *Poa pratensis* variety Mervel is an alternative host-plant. Host-plant ability to compensate the negative effect of moisture was also observed. *Agrostis alba, Lolium perenne, Bromus inermis* have a good compensation ability. *Phleum pratense, Dactylis glomerata* and *Festuca rubra* only slightly compensate. Dominancy of the species was also observed. *Dolerus gonager* was the dominant species on *Typhoides arudinacea* while on the other plants *Dolerus nigratus* and *Dolerus puncticollis* were dominant. *Triticum aestivum* has not own sawfly fauna. Its fauna is derived from the surrounding grass-cultures. Mainly the females move to the wheat. Copulation happens on grasses only. Wheat development has a significant inftuence on the settlement of the females.

In the last quarter of this century, the wheat-sawflies have become more and more important insect pest not only on wheat but also on seed-grass crops. The four most important species: *Dolerus nigratus* (Müller, 1776), *Dolerus puncticollis* (Thomson, 1871), *Dolerus gonager* (Fabricius, 1781) and *Dolerus haematodes* (Schrank, 1781) are known relatively little. Zombori (1994) mentions *Dolerus niger* (Linnaeus, 1767) as an insect pest of wheat but this species occurs only occasionally on this plant.

These species are oligophagous, attached mainly to the different grass species and also to *Juncus*, *Scirpus* and *Carex* species.

There are different records on their host-plants in the literature, as follows:

Zombori (1982): *Dolerus puncticollis*: Poaceae; *Dolerus gonager*: Poaceae; *Dolerus haematodes*: *Carex* spp., *Scirpus* spp., *Juncus* spp., *Avena*, spp., *Poa* spp., *Triticum* spp.; *Dolerus nigratus*: Poaceae.

Aichorn (1978): *Dolerus haematodes*: *Alopecurus pratensis*, *Avena sativa*, *Bromus inermis, Dactylis glomerata, Festuca ovina, Hordeum hexastichon, Phleum pratense, Poa pratensis, Secale cereale, Triticum aestivum, Zea mays.*

Buhl (1975): *Dolerus nigratus*: *Triticum aesfivum, Hordeum hexastichon, Secale cereale*; *Dolerus gonager*: *Triticum aestivum, Hordeum hexastichon, Secale cereale; Dolerus haematodes*: *Avena sativa.*

Lorenz and Kraus (1957): *Dolerus gonager*: *Festuca* spp., *Poa* spp., *Triticum* spp.; *Dolerus puncticollis*: Gramineae, *Triticum* spp.; *Dolerus nigratus*: *Festuca* spp.,

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Holcus spp., *Triticum* spp.; *Dolerus haematodes*: *Avena* spp., *Carex* spp., Gramineae, *Juncus* spp., *Poa* spp., *Scirpus* spp.

Wetzel (1971): *Dolerus haematodes*: *Alopecurus pratensis, Bromus inermis, Festuca ovina, Phleum pratense, Poa pratense*; *Dolerus nigratus*: *Alopecurus pratensis, Bromus inermis; Festuca ovina Phleum pratense, Arrhenatherum elatius, Dactylis glomerata, Festuca pratensis, Phalaris arudinacea.*

Our aim is to continue the previous examination (Haris, 1994). The recent study is a special controll of the previous one. We are searching for the answers to the following questions: How large the abundance alternation of pests on different host-plants and in what measure could the plants compensate the negative effects of the weather? In addition to we estimate the effect of host-plants for the occurrence of their sawfly fauna and study the movement of the adults into the wheat.

Methods and Materials

Research started in 1990. Till 1994 11 host-plants were observed. In 1995, the following host-plants were studied: *Agrostis alba, Bromus inermis, Dactylis glomerata, Festuca arudinacea, Festuca rubra, Festuca valesiaca, Lolium perenne, Phleum pratense, Poa pratensis variety Keszthelyi-58, Poa pratensis variety Mervel, Typhoides arudinacea. Agrostis alba, Dactylis glomerata, Festuca arudinacea, Festuca valesiaca, Lolium perenne, Phleum pratense* were superelite crops, *Poa pratensis* races are primary elite. All of them had been on the same patch, while elite crops, namely: *Bromus inermis, Festuca rubra* and *Typhoides arudinacea* on the other patch.

Collections were done on the experimental farm of Pannon University of Agriculture in Keszthely. Only the culmination period was studied. This is the part of the Normal curve where differences are most expressed. To estimate the pests abundance we swept the cultures by net. One netsweeping was measured as 0.14 sqm.

Larvae were not sorted into species because their identification has not been solved well (Lorenz and Kraus, 1957).

By identifying adults, populations were composed mainly by *Dolerus puncticollis* and *Dolerus nigratus*. *Dolerus gonager* was a dominant species only on *Typoides arudinacea. Dolerus haematodes* presented in a negligible amount.

Results

Average population densities on the different host plants can be seen in Tables 1 and 2.

Largest population densities were measured on *Arostis alba* (4.86 specimens/sqm.). Abundance was also high on *Typhoides arudinacea* (2.82 specimens/sqm.), *Poa pratensis Keszthelyi-58* (2.50 specimens/sqm.), *Lolium perenne* (1.93 specimens/sqm.) and *Phleum pratense* (1.86 specimens/sqm.). Smallest density was observed

Table 1

Abundances of *Dolerus* larvae
(specimen/sqm.)

Date	A	B	C	D	E	F	G	H
1. 24. 05.	2.00	3.21	1.43	2.00	0.86	0.36	0.00	0.00
2. 24. 05.	3.43	1.07	1.71	2.57	1.14	0.36	0.00	0.00
3. 30. 05.	2.00	2.14	3.71	1.71	0.29	0.36	0.29	0.00
4. 30. 05.	4.86	3.57	0.86	1.14	0.29	0.00	0.29	0.00
Average	3.07	2.50	1.93	1.86	0.65	0.27	0.15	0.00
s	1.37	1.13	1.24	0.60	0.43	0.18	0.17	0.00
Sign. diff. (P=10%)	A-E, F, G, H;		B-E, F, G, H;		C-E, F, G, H;		D-F, G, H	

A: *Agrostis alba*, B: *Poa pratensis Keszthelyi-58*, C: *Lolium perenne*, D: *Phleum pratense*, E: *Festuca arudinacea*, F: *Poa pratensis* Mervel, G: *Festuca valesiaca*, H: *Dactylis glomerata*

Table 2

Abundances of *Dolerus* larvae
(specimen/sqm.)

Date	A	B	C
1. 16. 05.	1.57	0.29	1.00
2. 24. 05.	2.57	2.00	0.57
3. 24. 05.	4.00	3.43	0.57
4. 30. 05.	3.14	1.14	0.57
Average	2.82	1.72	0.68
s	1.02	1.34	0.22
Sign. diff. (P=10%)	A-C		

A: *Typhoides arudinacea*, B: *Bromus inermis*, C: *Festuca rubra*

Table 3

Mean abundances of *Dolerus* species from 1994 to 1995
(specimen/sqm.)

	A	B	C	D	E	F	G
Adults 1994	0.57	0.46	0.14	–	–	–	–
Adults 1995	2.07	1.14	1.57	–	–	–	–
Tendency	+	+	+				
Larvae 1994	0.50	1.19	0.98	2.71	2.31	1.29	0.14
Larvae 1995	1.72	0.68	2.82	3.01	1.86	1.93	0.00
Tendency	+	–	+	+	–	+	–

A: *Bromus inermis*, B: *Festuca rubra*, C: *Typhoides arudinacea*, D: *Agrostis alba*, E: *Phleum pratense*, F: *Lolium perenne*, G: *Dactylis glomerata*

Table 4

Specific distribution of *Dolerus* spp. on *Bromus inermis* in 1994–95
(specimen/100 netsweepings)

Date	*D. nigratus*		*D. puncticollis*		*D. gonager*	
	m	fm	m	fm	m	fm
1994						
18. 03.	0	1	2	0	0	0
24. 03.	2	1	0	0	0	0
25. 03.	0	0	0.67	0	0	0
01. 04.	6	2	0	0	0	0
19. 04.	0	0	0	0	0	0
1995						
19. 03.	1	0	2	0	0	0
06. 04.	6	0	8	0	0	0
06. 04.	10	0	15	1	0	3
Sex ratio %	86	14	97	3	20	80
Spec. ratio %	45.5		46.5		8.0	

m: males, fm: females

Table 5

Specific distribution of *Dolerus* sp. on *Agrostis alba* ssp. *stolonifera* in 1990–91 (specimen/100 netsweepings)

Date	*D. nigratus*		*D. puncticollis*		*D. gonager*		*D haematodes*	
	m	fm	m	fm	m	fm	m	fm
1990								
16. 03.	2.5	0	0	0	0	0	0	0
21. 03.	0	0	0	2.5	0	0	0	0
02. 04.	1.25	0	0	0	0	0	0	0
13. 04.	0	0	0	0	0	0	0	0
25. 04.	1	0	0	0	0	0	0	0
1991								
21. 03.	0	0	0	0	0	0	0	1
03. 04.	7	1	0	1	4	0	0	0
07. 04.	8	1	0	2	1	0	0	0
09. 04.	6	1	0	1	1	0	0	0
24. 04.	0	0.67	0	0	0	0	0	0
29. 04.	0	0.67	0	0	0	0	0	0
Sex ratio %	85.5	14.5	0	100	100	0	0	100
Specific ratio %	68.0		15.0		14.0		2.0	

m: males, fm: females

on *Festuca valesiaca* (0.15 specimens/sqm.) and on *Poa pratensis variety Mervel* (0.27 specimens/sqm.).

Significant differences (P = 10%) were found between *Agrostis alba-Festuca arudinacea, Festuca valesiaca, Poa pratensis Mervel*; *Lolium perenne-Festuca arudinacea, Festuca valesiaca, Poa pratensis Mervel*; *Poa pratensis Keszthelyi-58 – Festuca arudinacea, Festuca valesiaca, Poa pratensis Mervel*; *Phleum pratense – Festuca valesiaca, Poa pratensis Mervel*. Critical value: 1.27. *Typhoides arudinacea–Festuca rubra*. Critical value: 1.56.

Specific distribution on different host-plants can be seen from Tables 4 to 8. *Dolerus nigratus* and *Dole puncticollis* were the dominant species on the most grasses. *Dolerus nigratus* were in relatively higher rate on *Agrostis alba* ssp. *gigantea*. *Dolerus gonager* has reached larger amount on *Typhoides arudinacea*. *D. nigratus* and *D. puncticollis* were the dominant species on wheat, too.

Table 6

Specifc distribution of *Dolerus* species on *Festuca rubra* in 1994–95
(specimen/100 netsweepings)

Date	*D. nigratus*		*D. puncticollis*		*D. gonager*		*D. haematodes*	
	m	fm	m	fm	m	fm	m	fm
1994								
18. 03.	1	1	0	0	0	0	0	0
24. 03.	2	0	2	2.67	0	0	0	0
25. 03.	0	1.60	0	2	0.80	0	0	0
01. 04.	1	0	0	0	0	1	0	0
19. 04.	0	1	0	0	0	0	0	0
1995								
19. 03.	0	0	0	0	0	0	1	0
06. 04.	6	0	9	0	1	0	0	0
06. 04.	3	0	5	0	0	1	0	0
Sex ratio %	78	22	77	23	47	53	100	0
Specific ratio %	39.5		49.0		9.0		5.25	

m: males, fm: females

Sexual distribution on *Agrostis alba* ssp. *gigantea*: 72.8% males, 27.2% females, *Festuca rubra*: 75.6% males, 24.4% females, *Bromus inermis*: 85.9% males, 14.1% females, *Thypoides arudinacea* 88.9% males and 11.1% females. On wheat, 12.2% males, 87.8% females.

Discussion

The results of the year of 1995 support the ones of 1994 (Haris, 1994) but some changes were perceived. These were caused by the extremely high moisture in the first two decades of May. Larvae are very sensitive for moisture in this time. Heavy rains destroy young larvae very easy by rolling them down into the wet soil.

Larvae's chance to stay alive, as we have experienced, depends on the host-plants.

From the year of 94 to 95 adult density were increased while larvaes one just grown on certain plants and reduced on the others (Table 3). *Agrostis alba, Lolium perenne, Bromus inermis* compensate well. *Phleum pratense, Dactylis glomerata* and *Festuca rubra* just slightly compensate.

Table 7

Specific distribution of *Dolerus* spp. on *Typhoides arudinacea* in 1994–95 (specimen/100 netsweepings)

Date	*D. nigratus*		*D. punctricollis*		*D. gonager*	
	m	fm	m	fm	m	fm
1994						
18. 03.	0	0	0	0	0	0
24. 03.	0	0	0	0	2	0
25. 03.	1	0	0	0	0	1
01. 04.	0	0	0	0	0	0
19. 04.	0	0	0	0	0	0
1995						
19. 03.	0	0	2	0	2	0
06. 04.	8	0	6	1	19	1
06. 04.	2	0	5	0	9	4
Sex ratio %	100	0	93	7	84	16
Specific ratio %	17.5		22.0		60.5	

m: males, fm: females

Poa pratensis is introduced into the experiments first time in 1995. This species differs from the others by having many variations (Soó, 1973). Two varieties were examined: Keszthelyi-58 and Marvel.

Typhoides arudinacea was considered earlier as a secondary host plant (Haris, 1994). Population density has grown in this year on this plant. Now, it is a primary host-plant of *Dolerus gonager* only. This is a relatively new appearance of this sawfly species. This is why its abundance was relatively small last year.

Population density of the larvae has grown also on *Festuca arudinacea* in this year. This increasing has caused only weeds (mainly *Agrostis alba*) as on *Festuca valesiaca.*

Primary host-plants: *Agrostis alba, Agrostis alba* ssp. *stolonifera, Poa pratensis Keszthelyi-58, Phleum pratense, Typhoides arudinacea.*

Secondary host-plants: *Lolium perenne* (relatively high but very fluctuating population density), *Bromus inermis, Festuca rubra.*

Alternative host-plants: *Festuca arudinacea, Dactylis glomerata, Poa pratensis Mervel.*

Host-plants with zero population density: *Festuca pseudovina, Festuca valesiaca.*

Table 8

Specific distribution of *Dolerus* species on *Triticum aestivum* in 1990, 1991, 1995 (specimen/100 netsweepings)

Date	*D. nigratus*		*D. puncticollis*		*D. gonager*		*D. haematodes*	
	m	fm	m	fm	m	fm	m	fm
1990								
16. 03.	1	0	0	0	0	0	0	0
21. 03.	0	5	0	1	0	0	0	0
02. 04.	0	1	0	0	0	1	0	0
13. 04.	0	2	0	1	0	0	0	0
25. 04.	0	0	0	0	0	0	0	0
1991								
21. 03.	0	0	0	0	0	0	0	0
03. 04.	0	3	1	6	0	0	0	0
07. 04.	0	0	0	2	0	0	0	0
09. 04.	1	0	0	2	0	0	0	0
1995								
19. 03.	0	1	0	0	0	0	0	0
04. 06.	0	6	1	2	1	0	0	1
04. 06.	0	8	1	1	0	0	0	0
Sex ratio %	7	93	17	83	50	50	0	100
Specific ratio %	57.0		37.0		4.0		2.0	

m: males, fm: females

Their specific distribution depends on two factors, namely the host-plants and the climatic circumstances. Now, we are studing only the effects derived from host-plants. The other one, the climatic changes caused the decrease of the *Dolerus haematodes* population. This species is very sensible for drough. This is the explanation of its very low population density. In the years of examination, parasitoids and natural enemies had no selective effect to influence our results. Their abundances were approximately equal on each cultures.

Dolerus nigratus and *Dolerus puncticollis* were dominant species on three of the examined grasses, namely *Festuca rubra, Bromus inermis, Agrostis alba* ssp. *gigantea.* Altough, on this latest grass the abundance of *Dolerus nigratus* was relatively higher. *Dolerus gonager* reached its maximum population density on *Typhoides arudinacea.*

Specific distribution on winter wheat depends on the sawfly fauna of the surrounding seed-grasses. *Dolerus nigratus* and *Dolerus puncticollis* has settled into the

wheat-field in the largest number, while *Dolerus gonager* preferred the grasses. Only one female was observed on wheat.

Mainly females moved to the wheat. Female dominance on wheat was between 86–89%, while the ratio of males is restricted to 11–14%. It means the copulation happens mainly in grass cultures. After copulation females move to the wheat laying their eggs partly on grasses, partly on wheat. Wheat is a good host-plant for *Dolerus* species, but their abundance depend on the neighbouring grasses. The neighbourhood of primary hostplants show larger population density on wheat, too.

The development of wheat also has influence for the movement of females. Higher crops are more attractive for sawflies. We examined two patch of wheat, cultures A and B. Height of culture A was 30.1 cm (3rd of April), 33.0 cm (9th of April), culture B 19.0 and 25.0 cm. Maximum population density (specimen/100 netsweeping) on A 10, on B2.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 265–272 (1995)

Investigations Related to Timothy Fly (*Nanna flavipes* Fallen; *Diptera, Scathophagidae*) in Hungary

KORNÉLIA CZENCZ

Department of Entomology, Pannonian University of Agricultural Sciences, Institute for Plant Protection, P.O. Box 71, H-8361 Keszthely, Hungary

Out of the members of genus *Nanna*, the species *Nanna flavipes* Fallen turned out in our investigations set about in several timothy-improving and seed-producing zones of Hungary from 1991 to 1993 to be damageous.

In the cooler parts of the country, 7.8–8.4% of seedstalks were damaged when no control has been done. In addition to swarm observations, the full knowledge of the phenological state of plants is also very important for determining the moment suitable of intervention. In order to pursue the process of swarming, larvae can easily be collected from the stalk parts under the panicles of the damaged plants. In our observations, the panicle fundaments were in the peaktime of swarming only of 4–6 mn long and to found deeply in the bottom of seedstalks. At this time, an insecticide of strong gassy effect is suggested. At the time of a large-number appearence of larvae when the initial inflorescences already are 10–15 mn long, some systemic or deep-effective substances are to be used.

It is known out of experiences obtained in several North-European countries producing timothy seed that *Phelum pratense* L. as host plant strongly attracts two fly species such as *Amaurosoma armillatum* Zett. and *Amaurosoma flavipes* Fall. Degree and number of their appearance are very different in each region (Newbold, 1963; Wetzel, 1963; Mühle and Wetzel 1966; Ricou, 1967).

The manner of life of both fly species has been discussed thoroughly by Fröhlich (1960) and Ricou (1967). The works of Ahnert (1969, 1970) and Ayres (1986) deal with forecasting and controlling them. The timothy flies have been classed among the Nanna species by the latter author. In faunistic works of Dely-Draskovits (1981) the name of the genus *Amaurosoma* also occurs as a synonymous name of *Nanna*.

In Hungary there are three geographically well separable zones where timothy seeds are produced or improved such as the south-eastern country part (Szarvas), the western Transdanubia (Keszthely) and the north-western part (Mosonmagyaróvár). These regions are permanently damaged by timothy flies. Up to the present, nobody has thought it necessary to determine and control these damaging species in home relations.

As a result of our investigations set about in 1991, it has been stated that the timothy seed production was threatened in the western Transdanubian part of Hungary by the species *Nanna flavipes* Fallen (Czencz et al., 1993).

Some biological and etological results of our observations to be used in forecasting species *N. flavipes* will be explained in this study such as control tests with insecticides in the years 1991–1992 as well as methods for evaluating damages or estimating effectiveness of substances. The results of our investigations obtained in all three seed breeding and producing regions in 1993 will also be presented.

0238–1249/95/$ 5.00

Materials and Methods

Our open-air observations and control tests were done on 3 hectares in the western Transdanubia (Keszthely), in a 2 and 3 years old timothy seed field of "elite" class, respectively. We intended to trace the timothy fly swarming in 1991 by using nets and coloured sticky traps. At the middle of June, for being able to ascertain the initial swarming time in the following year with more certainty, we have collected seed stalks containing larvae. The developed larvae taken out of these seed stalks were then placed on the soil of the laboratory garden inside of several bottomless pails. The small (50 cm high) black linen tentiso lators have then got to these places of pupation in the following year (8 April, 1992).

Damages were evaluated and larvae were also collected in both of other seed producing areas in order to clear up occasional differences between the spectra of *Nanna* species at the middle of June 1993. The pupation of larvae took place as described above. In view of the warm springtime in 1994, the tentisolators already were put out on the places of pupation at the middle of March.

Plant dissections were systematically carried out on the spot in order to pursue individual development processes of host plants and pests as well as larva damages.

In our big-plot control tests (1991 and 1992) treatments with insecticides were done on 1 hectare each, having let free one hectare as control plot. Substances with phosphamidon (0.13%) and phosalon (0.26%) and those with phosphamidon (0.2%) and methylparation (0.2%) were applied in the years 1991 and 1992, respectively. Spraying was done on the 12 May, 1992 when the timothy was in a 5–6 mm initial sympodium stage.

The effects of the substances were estimated at the beginning of July by passing from the eastern and western ends towards the inside of the field, in patch depths of 5, 10, 20, 50 and 100 meters, respectively. In each sampling place, the plants were examined within a 5 × 20 cm section (1 running m).

Two indices in each sampling position were used to compare damage degrees statistically:

1) Number of damaged plants pro running metre (D.p./m)

2) Damage degree in % (D%). – This is a weighed index calculated by taking all plants and their damage grade into account within a running metre examined. In calculating this index, the pseudo-spikelets were divided into 5 groups such as healthy spikelets, ones damaged in 1/3, 1/2, 2/3 part, and small, totally damaged spikes, respectively. The 20 cm sample units, as repetitions, made us possible to estimate and compare the errors of the calculated indices statistically. The numbers of damaged plants pro running metre were compared by a *t*-test while the damage grades (%), were controlled by an *u*-test.

Table 1

Damage indices of *Nanna flavipes* Fall. in the timothy seed producing zones of Hungary (1991–1993)

Annual moisture Average temperature	W-Transdanubia (Keszthely) 700 mm 10.5 °C		NW-Hungary (Mosonmagyaróvár) 594 mm 10.1 °C		SE-Hungary (Szarvas) 528 mm 10.8 °C	
	D. p./m	D%	D. p./m	D%	D. p./m	D%
1991	25.4	3.75	–	–	–	–
1992	25.1	4.92	–	–	–	–
1993	22.2	4.23	16.3	3.70	2.30	0.70

D. p./m = Number of damaged plants pro running metre
D% = Weighed damage

Results and Discussion

a) Specific composition and importance of the timothy fly population living in the timothy seed producing zones of Hungary

It has by identificating imagoes bred out of the plants damaged by timothy flies been stated that only *Nanna flavipes* Fallen existed on the three areas in Hungary were timothy seed was improved and produced. The "other" timothy fly species (*N. armillata* Zett.) was not to be found at all.

In the three seed producing areas of different climatic conditions, the density indices of *N. flavipes* were shaped as presented in Table 1. The annual moisture amounts and average temperatures based on the many years' data as measured on the areas examined are also registered in the Table.

By estimating data in Table 1 while reckoning with 300 timothy seed stalks pro running metre, it can be stated that the zone of Keszthely and that of Mosonmagyaróvár and the dry-warm country part were touched by the damagings of *Nanna flavipes* in 7.9 and 5.4 and 0.77%, respectively when no control measures have been done.

This coincides with some statements of literature according to which the cooler and damper temperatures would be preferred by the timothy flies.

b) Biological observations for a successful control

A schematic pattern of the annual development of timothy fly has been designed based on our open-air phenological observations, tent-trap swarm examinations and laboratory breeding tests (Fig. 1). On the figure there are shown the phenological points where a successful chemical control against the imagoes or larvae could be expected as

Table 2

Effects of control measures set about with insecticides against timothy flies (Hungary, Keszthely, 1992)

Sampling place		Control (Ø)	Damage indices Wofatox 50 EC (methylparathion)	Dimecron 50 (phosphamidon)	Stat	Statistical test Ø–W	Ø–E	W–E
5 m	D. p./m	34.5	17.0	13.5	t_{16}	3.27***	3.73**	1.19ns
	D%	6.72	2.73	2.92	u	4.16***	3.71***	0.25ns
10 m	D. p./m	23.5	5.5	7.0	t_{16}	5.09***	4.97***	0.65ns
	D%	4.70	1.00	1.33	u	4.73***	4.16***	0.64ns
20 m	D. p./m	23.0	6.0	2.5	t_{16}	3.42**	6.14***	1.29ns
	D%	4.20	0.81	0.48	u	4.96***	5.47***	0.99ns
50 m	D. p./m	23.5	4.0	6.0	t_{16}	6.61***	4.55***	0.93ns
	D%	4.66	0.64	1.14	u	5.36***	4.42***	1.16ns
100 m	D. p./m	21.0	4.5	5.5	t_{16}	4.59***	4.06***	0.44ns
	D%	4.27	0.59	1.14	u	5.12***	4.00***	1.31ns
Average	D. p./m	25.1	7.4	6.9	t_{80}	9.07***	10.11***	0.41ns
	D%	4.92±0.32	1.15±0.14	1.45±0.18	u	10.79***	9.45***	1.32ns

D. p./m = Number of damaged plants pro running metre
D% = Weighed damage
***p < 0.1%;
**p < 1%;
ns = not significant
$t_{(16)}$, $t_{(80)}$ = 16, 80 degree of freedom
t = t statistics;
u = u statistics

Ø = control
W = western end of the field
E = eastern end of the field

well as the period when still larvae can with a good deal of certainty be collected from the conspiciously damaged seed stalks for a next year swarm observation.

The figure and the foregoing of the swarm examinations are to be interpreted as follows:

In spite of the fact that 24.5 plant individuals pro running metre were found to be damaged in average after headling up in the first year of examination (1991), no sign of swarming imagoes has been shown by nettings or stick-trappings previously. This phenomenon could be explained by the experience that the timothy flies do not fly high or far. Activity of flying could certainly be continued to decrease during the windy and rainy weather conditions in the test period, and the imagoes emerging out of the wintering places (from beside of stems) and looking for egg-laying nooks would fly to the plant parts sheltered from the wind.

In the second year of examination (1992), the first timothy fly imagoes came out of the isolators which have operated with definite numbers of larvae (21 April). Previously, a 23–26 °C maximum in temperature had been reached under the black tent-isolators at the middle of April. This was 5–6 degress higher than the outdoor temperature and able to induce a swarming a couple days earlier by speeding up the development of the insects.

According to the previous notices given by the isolators, the swarming peak in the nature was by the beginning of May to be expected. It is to be noted that at this moment suited for control measures against the imagoes, the inflorescence fundaments still were 4–6 mm long and hidden deeply inside of the seed stalks.

Based on the data of laboratory breedings, the large-scale hatching of larvae took place at the middle of May 1991. At that time optimal for controlling larvae, the inflorescence fundaments were 10–15 mm long.

In the course of our regular plant examinations we found some mucuous and rotten surrounding surfaces about the worms on the damaged generative parts unfolded from the leaf pods as if there were a saprophageous feeding form.

On the base of the length of these parts drying up after heading, the degree of damage can also be estimated.

As observed in the course of plant dissections, only 25–30% of larval population leaves the plant by the time of heading. The most serious damages are caused by these larvae because of feeding on the inflorescence fundaments throughout the whole time of growth. The larvae which did not develope by the heading time retire to the leaf pod that holds the seed stalk and hide 5–10 cm under the panicle. Their damage caused by feeding will already be insignificant by that time. At the middle of June these "late" larvae can easily be collected out of the seed stalks showing suspicious damages, for a next-year swarming observation. The whole population left the plants by 30 June in 1991 when the springtime and the early summer were cool and did so by 18 June in the warmer year of 1992.

c) Results of the timothy fly control with insecticides

The results of our control test carried out on the base of a more precise forecast in the year 1992 will be explained as follows. The damage indices calculated on the control fields and on the treated timothy seed producing areas in different depths within one hectare each as well as a statistical comparison between these data are summarized in Table 2.

Paying attention to the damage degrees in different field depths, it can be seen that a 5 m strip on the edge of the field proves to be most damaged. In the other depths, the differences among their damage conditions are insignificant. This phenomenon can be explained by the fact that the density of timothy flies became gradually uniform on the relatively small (3 ha) seed producing area planted in the year 1989. The stronger contamination of the field edges results from some new individuals arrived in the springtime of 1992.

By analyzing both insecticide substances it came out that the number of the damaged plants pro running metre decreased significantly and the degree of damage also became inconsiderable in both cases. The difference of both substances to the control could be proved at a significance level 0,1% while no statistically reliable differences between their to be valid for each field depths.

Conclusions

Out of the *Nanna* species known by the literature as "timothy flies", only *N. flavipes* Fallen is living and damaging in the timothy seed producing zones of Hungary. As for density of this species, a chemical control is only required on the cooler part of the country. In the regions, in a direct proportion to the aging of grasses, 22–25 pseudospikes pro running metre in average are touched by this damaging species, when no control has been done. The damage degree of panicles has also to be considered when estimating.

In addition to swarm observing, a knowledge of the phenological state of the plant (i.e. the size of the inflorescence fundaments within the seed stalks) is also very important. The process of swarming can be observed most effectively by means of black linen tent-traps. Developed larvae may easily be collected to observations at the mid-June of the previous year. At the peaktime of swarming (in Hungary, at the beginning of May) the inflorescence fundaments still are 4–6 mm long. At this time, substances of strong gassy effect are to be used. The most damaging population of larvae hatch out of the early eggs in large numbers at the middle of May. Use of some systemic or deep-effective substances for destroying young larva masses is suggested.

In 1992, on the plots treated with substances containing methylparathion and phosphamidon, respectively, there were 70.6 and 72.6% fewer panicles damaged by timothy flies than on the untreated control plot.

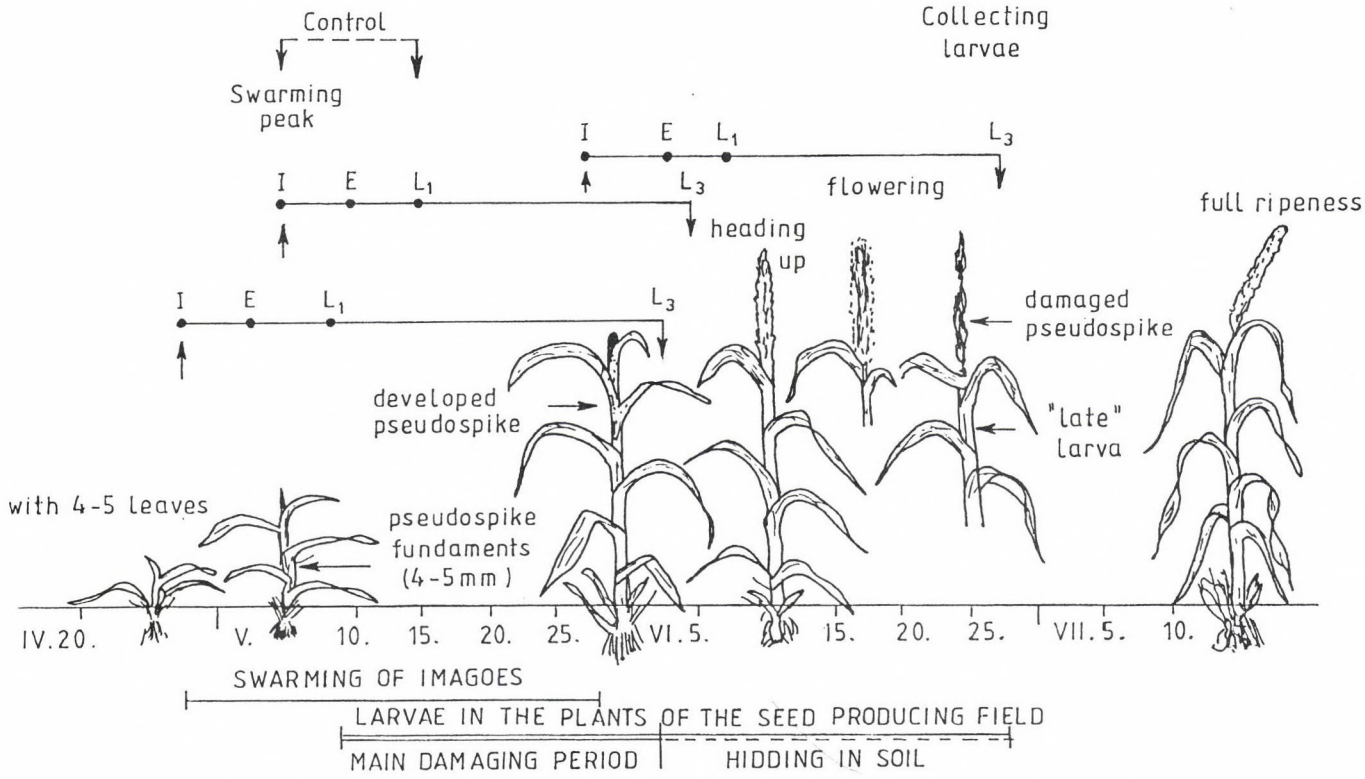

Fig. 1. Annual development of timothy fly (*Nanna flavipes* Fall.) (Hungary, Keszthely, 1991–1992)

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 273–277 (1995)

Use of Colour Traps for Monitoring Males of *Pseudaulacaspis pentagona* (Homoptera, Coccoidea) and its Parasitoid *Encarsia berlesei* (Hymenoptera, Aphelinidae)

DIAA A. F. SHEBLE and F. KOZÁR

Plant Protection Institute, Hungarian Academy of Sciences, P.O. Box 102, Budapest, H-1525, Hungary

Various colours were tested for their attractivity of the male flight of white peach scale *Pseudaulacaspis pentagona* (Targioni-Tozzetti). Three colours were chosen: yellow, white and light brown with sticky material (Tanglefoot and Vaseline) on both sides of the traps. The traps were hanged between the infested branches of *Sophora japonica* trees in the parks of Budapest.

The yellow colour had the highest attractivity, especially in 2nd generation (1217 males per week), white colour attracted 384, and there were only 57 males in the light brown traps.

Tanglefoot collected more males than Vaseline (190 males, comparing to 37).

The number of parasitoids collected by yellow traps was twice than light brown and white. The parasitoids were collected much better by Tanglefoot than by Vaseline.

Coloured traps have been used worldwide for monitoring the population of various insects. The sensitivity of insects to colours and the attraction by colours were utilised in collecting various insects and studying flight by many authors (Southwood, 1966).

The males of *Quadraspidiotus pernicious* (Comstock) were found to fly actively towards white and blue colours (Kozár, 1972/73). On the other hand, the males of *Aonidiella aurantii* (Maskell) were attracted by white and yellow colours much more frequently than by others (Rice and Moreno, 1969).

The response to colour of some aphelinid parasitoids has been studied (Dowell and Cherry, 1981; Neuenschwander, 1982; Schultz, 1985), too. The majority of the parasitoids were from genera known to be endoparasitoid, including three species from the genus Encarsia (Viggiani, 1988). Many insects and parasitoids were attracted to yellow colour (Brach and Trimble, 1985). However, insects not particularly associated with foliage do not show positive response to yellow colour alone (Kirk, 1984). Information about flight of pests and parasitoids is very important for pest management program for monitoring of flight and population levels and for timing insecticide treatment to get maximal effect of pest control with minimal damage to beneficial insects.

The white peach scale is one of the principal armoured scale pest of the world (Kozár, 1991). It is a polyphagous pest with very wide host plant range especially on fruit trees and ornamental plants (Kosztarab and Kozár, 1988). Growers very often have to spray against pests by insecticides, and for this they need methods to monitor the appearance of different stages. This pest has also an effective parasitoid (*Encarsia berlesei*),

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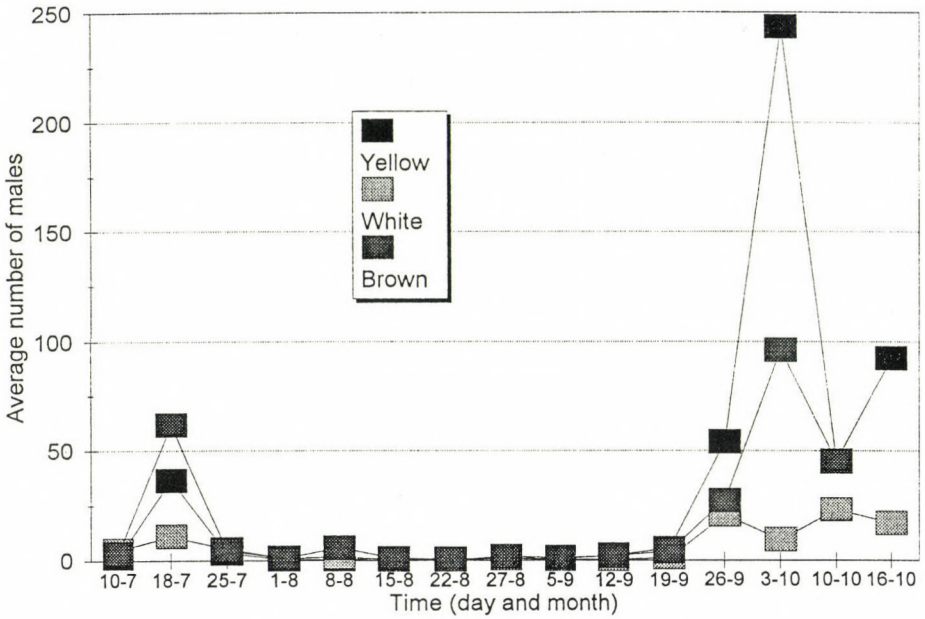

Fig. 1. Average number of males in colour traps (1991)

which should be also considered in spraying programs (Kosztarab and Kozár, 1988). Our aim was to study the possibility of using the colour traps to follow the flight of males of this pest and its parasitoids.

Materials and Methods

To find out the role of colours we employed plastic circle (diameter 12.6 cm), plates with a size of 10 × 5 cm yellow white (milky) and brown (light) traps with size 10 × 5 cm. All traps were used with either of the sticky materials Vaseline or Tanglefoot (Tanglefoot Company, U.S.A.) applied on both sides.

The traps were hanged between the infested branches of *Sophora japonica* trees in parks of Budapest, about 2 meters high. The traps were regularly changed every week starting 23 May, 1991 until 16 October, and 27 July, 1992 until 26 October. The numbers of traps were among 3–5 for each colour. The number of males and parasitoids were counted under the microscope.

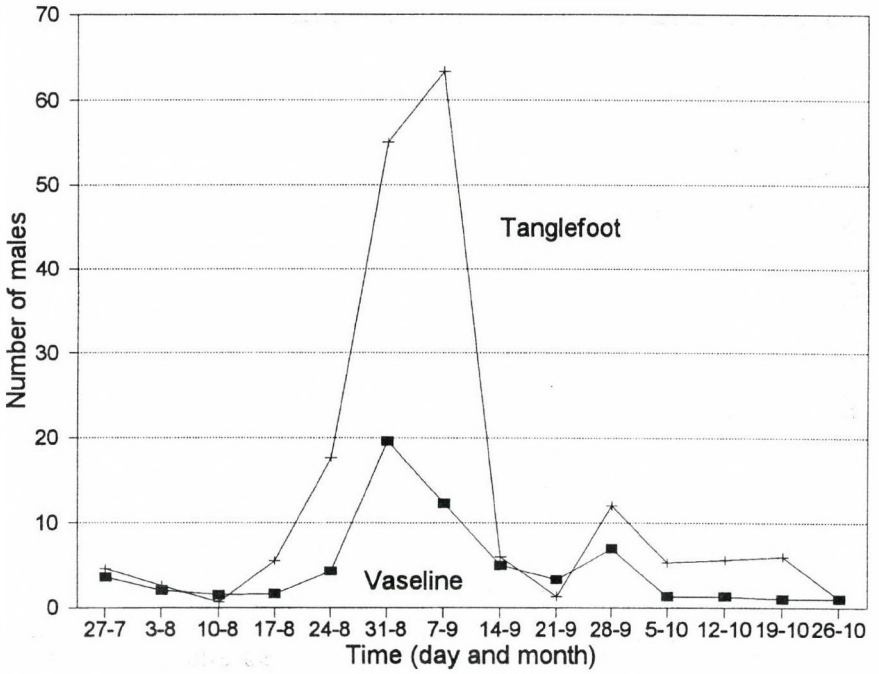

Fig. 2. Average number of males caught by yellow traps using Tanglefoot and Vaseline (1992)

Results and Discussion

In our study we found that the highest numbers of males were collected by yellow traps. The yellow traps collected at the second flight more than twice more males than the other colours (white and light brown), (Fig. 1). The average number of males in the first flight shows that there is no great difference between the three colours, but usually the number of males was very low in the first flight. In the second part of our experiment we tested the effect of Vaseline and Tanglefoot sticky materials. We found that the number of males caught by traps with Tanglefoot was about 3 times more than with Vaseline (Fig. 2). The number of parasitoids (*Encarsia berlesei*) collected by yellow traps during whole season was 37 but the number collectedby light brown and milk white traps was only 14. The parasitoids were collected by yellow traps with Tanglefoot also much better than with Vaseline (Fig. 3).

Our results were similar to the other literature data; for example Rice and Moreno (1969) found that the males of *Aonidiella aurantii* were also attracted by yellow and white colours approximately in similar numbers. The *Encarsia berlesei* parasitoid also prefer the yellow colour like several other species of parasitoids. Neuenschwander (1982) found that many parasitoids and predators were caught by yellow sticky traps, also

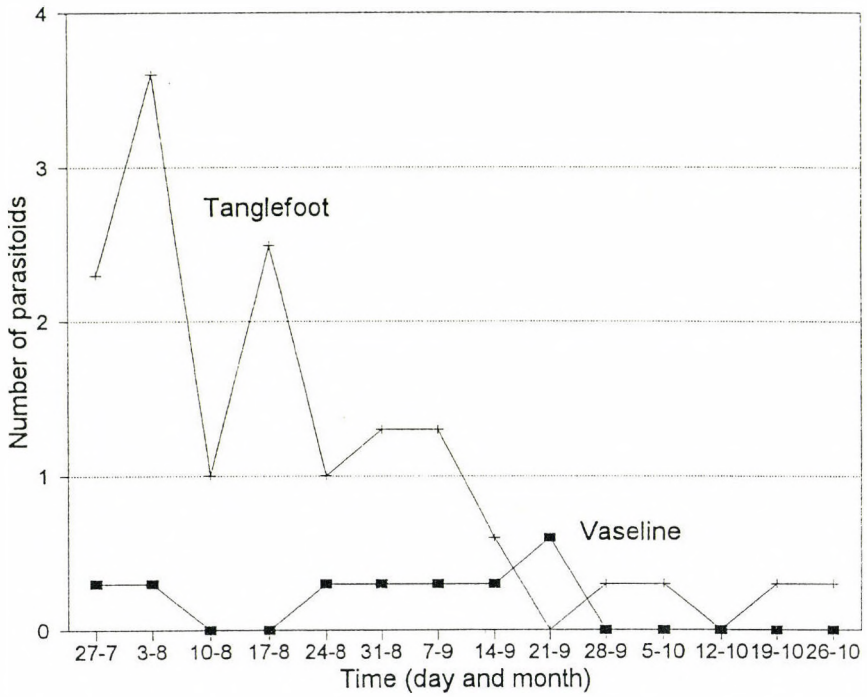

Fig. 3. Average number of parasitoids caught by yellow traps using Tanglefoot and Vaseline (1992)

Dowell and Cherry (1981) studied the attractiveness of sticky traps of eight colours for two parasitoids *Amitus hesperidum* Silvestri and *Prospaltella opulenta* Silvestri. The authors investigated seven species of Coccinellid predators of citrus blackfly, *Aleurocanthus woglumi* Ashly (Homoptera, Aleyrodidae) in insectary and field tests. They found that the yellow traps captured significantly more parasitoids and coccinellids than other colours. We found that for practical application colour traps are cheaper and simpler than pheromone traps, but they are less efficient, so they could be used at higher population densities. At low density, for survey of the possible infestation, and monitoring only the use pheromone traps are suggested to be used (Sheble and Kozár, 1994). For parasitoids the yellow colour traps only with Tanglefoot were enough attractive.

Acknowledgements

We express our thanks for the financial help to OTKA (No. 12980), and for the English corrections to Dr. F. Samu.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 279–282 (1995)

Additional Data for the Expansion and Biology of Thuja Aphid (*Cinara tujafilina* Del Guercio) in Hungary

J. VAS, A. HALTRICH and Z. MÉSZÁROS

Department of Entomology, University of Horticulture and Food Industry,
H-1502 Budapest, P.O. Box 53, Hungary

Biological observation and population-dynamics distribution studies were carried out to the biology of thuja aphid (*Cinara tujafilina*). It was recognised that these species are able to survive during the winter in Hungary. It seems that the thuja aphid is a standard member of our aphid fauna. It was determined some natural enemies of the above-mentioned taxon.

In the period 1991–1994 biological observation and population dynamic studies have been carried out in Budapest, Nyíregyháza and their surroundings. Faunistical research has been done in the whole country in the same period. We have supposed that it is a widely spread species in Hungary, that can be found wherever its food-plants live. Systematically faunistical observations have been done (Fig. 1).

It's presence could be detected in more than 50 localities of the country. It can be found wherever *Thuja orientalis* and *Chamaecyparis lawsoniana* are present. We can only presume how and when it got into the country. The extent of spreading can be explained by the natural migration ability of the species (winged form), but human activities cannot be excluded either (nursery multiplication material; Van Rossem et al., 1979) mentions the appearance of thuja aphid in Holland and remarks, that the *Thuja* example on which he has found came from Italy. Presumably it did not recently gets into Hungary. Only last years' mild winters have provided favourable conditions for their mass propagation.

Mode of life

It has been observed that population-dynamics of the species is hard to define. Assuming to the results of observation done in several places, a general picture has been drawn, to which, of course, there are deviations due to local condition. They are specifically to the biology of the species. A specifically population-peak of the species could be observed in Hungary in spring (IV–VI) contrary to the relevant literature (Colombo and Parisini 1985). No significant population-peak was experienced in autumn.

In Italy, it was distinguisled two population-peaks: one in springtime (IV–VI) and the other in autumn (X–XI). According to our observations, the numbers of individuals decreased by end of June, July. However, they could not be found anywhere after the

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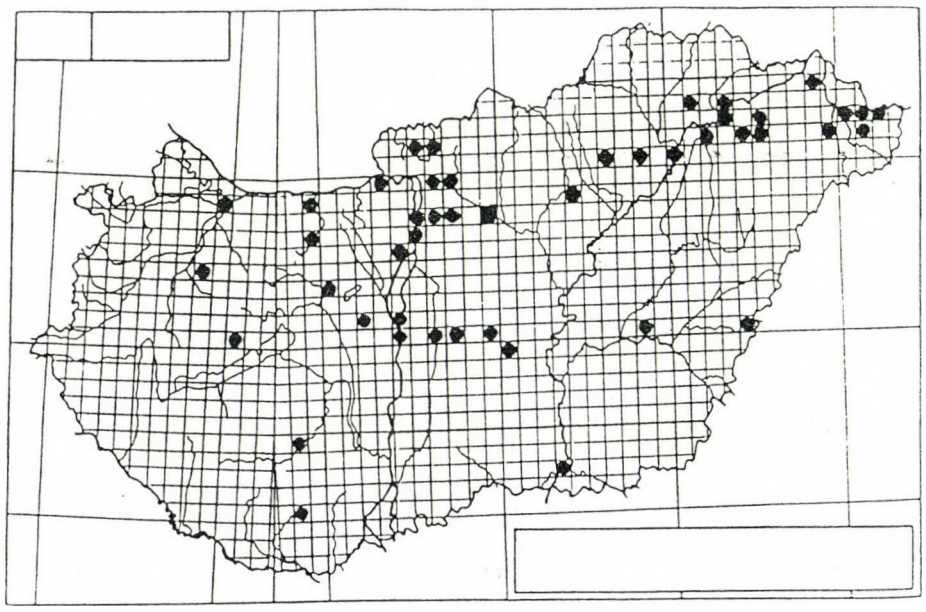

Fig. 1. The distribution map of thuja aphid (*Cinara tujafilina* Del Guercio) in Hungary (1991–1994). UTM 10 km grid

August heat. The fall of individual numbers in the population coincides with dryness and the summers and early autumns heat. With the diminishing of heat, the number of winged individuals have been increased. The colonies disappeared from the sunny side of the trees. In mid-summer we found aphid colonies on plants, living in adequate circumstances as far as water-supply is concerned. From spring onwards a great deal of honeydew extraction can be observed. Particularly was remarkable in places less visited by ants. Their honeydew covered thickly the old branches. Afterwards sooty-coloured fungus (*Apiosporium spp*) showing up coloured the foliage black.

As far as the species of thuja aphid concerned Szelegiewicz remarks in the first volume of "Aphididae, Fauna Hungariae", that it is hardly or rarely visited by ants. According to our experience smaller colonies (not more than 10–20 individuals) was regularly visited by ants.

Overwintering

There are a few data available about thuja aphid living through the winter in Hungary. According to our observation, the species are able to survive through the winter on underground parts of plants, especially in protected places on mild winters, that characterise our clime in the last few years.

On *Thuja orientalis* in 10 January, 1994, colonies of thuja aphid 7–10 individuals was found in Nyíregyháza sucking the leaves. They were found in Budapest too on 19th in the same month. Mild weather in January was favourable for *Lachnidae*, consequently by mid-February colonies of 40–50 individuals could already be observed at the some places. Beside wingless females winged females have been observed. It is also remarkable, because there is no hint about their such an early appearance in central European literature. On basis of our estimates cooling down to –7 °C caused a mortality rate of at least 50%, however a part of the colonies still could survived cooling down so suddenly and to such an extent.

In March 1994 discovering the root-zone of an about 2.5 m tall *Thuja orientalis* the root-living form of thuja aphid accompanied by *Lasius niger* L. was found. As far as we know it has been indicated from two places: from the USA (Bray,1953) and Italy (Colombo and Parisini, 1985). On basis of this observation it has become evident, that thuja aphid is able to live through the winter in domestic circumstances both in underground and overground parts. Thus it can be regarded as a permanent member of our fauna.

Natural enemies

In course of our assembly predators feeding on thuja aphid have often been observed. They have been collected and further raised. On basis of our assembly the following list of species has been obtained (Table 1).

Sunny weather at early spring (end of March, beginning of April) already cajoles ladybirds from the places they had lived through the winter and start feeding on colonies of thuja aphid. At the population peak in May, lacewings join ladybirds. As far as predators are concerned activities of lacewings and ladybirds have been found significant (particularly that of *Adalia bipunctata* L.). Other authors (Furuta, 1988) emphasised species of hoverflies and *Ophiobolus*. There are quite a few data available about the importance of predators, but presumably not only summer heat but also natural enemies take part in the fall of population of thuja aphid.

Conclusion

– after our observations we can conclude that the thuja aphid is widespread in Hungary where its host plants (*Thuja orientalis* and *Chamaecyparis lawsoniana*) occur;

– in contradiction to the Italian observations it was manage to find only one population peak until now;

– we obtained that the species is able to overwinter in our climatic conditions in the root–zone of the host plants.

– it was determine many natural enemies of thuja aphid such as: predatory bugs, ladybirds, lacewings, hoverflies which prey on the aphid-colonies.

Table 1

Natural enemies of thuja aphid (Cinara tujafilina) in Hungary

Ladybirds (Coleoptera, Coccinellidae)
Adalia bipunctata*
Adalia decempunctata*
Exocomus quadripustulatus*
Calvia quatourdecimguttata*
Coccinella septempunctata*
Hippodamia variegata*
Oenopia conglobata
Scymnus subvillosus
Lacewings (Neuroptera, Chrysopidae)
Chrysopa formosa*
Chrysoperla carnea*
Raphidia flavipes
Hoverflies (Diptera, Syrphidae)
Scaeva pyrastri,*
Syrphus torbus*
Bugs (Heteroptera)
Deleocoris ruber
Gonocerus juniperi

* Predators feeded on thuja aphid, it has become evident that thuja aphid could serve as essential food for these species.

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Acta Phytopathologica et Entomologica Hungarica 30 (3–4), pp. 283–289 (1995)

Nutrition Inhibition by *Ajuga* sp. Plants Studied with two Major Insect Pests (*Sitona humeralis* Steph., *Pieris brassicae* L.)

M. NÁDASY and CS. GÁL

Department of Entomology, Pannon University of Agricultural Sciences, Institute for Plant Protection, H-8361 Keszthely, P.O. Box 71, Hungary

Experiments with *Sitona humeralis* adults and *Pieris brassicae* larvae prove that the *Ajuga* species possess considerable inhibitory effect on nutrition.

In both cases we found that from leaves treated with *Ajuga* extracts the animals consumed small, if any quantities. This was established from the values of the consumption index. The relative growth rates and gross efficiencies calculated for the *Pieris brassicae* larvae showed that the consumption of most of the extract treated leaves was not sufficient for the growth of the larvae, they lost weight, while in the control the larvae developed undisturbedly.

On plants treated with *Ajuga* extracts a large proportion of the insects stay for a short time, according to observations they even leave the plant.

With the *Sitona humeralis* adults all the 5 extracts gave similar results. In the case of *Pieris brassicae* larvae the best result was obtained with *Ajuga reptans* var. reptans extract (ARR-BT33/i/MT/2) prepared at the time of flowering.

From the results we have drawn the conclusion that the plant produces the largest amount of nutrition inhibiting substance of "highest efficiency" at the time of flowering, so in the course of further investigations it would be necessary to study the plants on flowering. It may be important to separate such substances of the plants, select the most efficient compound or compounds, and examine them for usability. Research work with the *Ajuga* species has left many questions unanswered, but we hope that the investigations will go on and contribute to the realization of a "purer" plant protection.

During the past several decades of intensive chemical insect pest control few papers dealt with the problems of selectivity and environment protection. Recently investigations have started with compounds that fulfil these requirements, too. A group of such compounds is composed of nutrition inhibitory substances (antifeedings). Namely, a large group of plants produces high amounts of secondary plant materials that inhibit the nutrition of insects. These may prevent the insects from feeding in different ways. The effect may be repellent (the insect does not consume the plant) or deterrent (the insect tries the plant but because of the nutrition inhibitory substance contained in it refuses it). With a proper research background the use of such compounds in the practice of plant protection would be desirable. By themselves they naturally can seldom be used, their combined application can mostly be conceived (Szentesi, 1990).

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Literary Survey

The *Ajuga species* produce a considerable amount of secondary plant material. A part of the compounds (iridoids and the "*Ajuga* specific" neo-clerodanes) inhibit the nutrition of non-adapted Articulate (e.g. *Spodoptera* spp., *Periplaneta maericana, Leptinotarsa decemlineata*), while with other compounds (phytosectysteroids) a growth regulation effect was observed (Darvas et al., 1994a).

Wide investigations are being carried on to determine the allelochemicals of the *Ajuga* species.

Out of the diterpenoids ajugarin I–V was isolated from *Ajuga bracetosa* (Kubo et al., 1976, 1982, 1983), ajugareptansin from *Ajuga reptans* (Camps et al., 1981a, b) as well as ajugareptanson A and B, ivain 1–4 from *Ajuga iva* (Camps et al., 1981a), and ajugapitin from *Ajuga chamaepitys* (Hernandez et al., 1982) and later its derivatives and chamaepitin too (Camps et al., 1987).

Many of the growth regulator phytoecdisteroids were also separated. From several *Ajuga* species *(A. decumbens, A. incisa, A. japonica, A. nipponensis*) Korreeda et al. (1972) separated 20–OH ecdizon, cyasterone, polypodin B, ajugasterone B and C and ajugalactone. Tomás et al. (1992) isolated macisterone A, polypodin B, sengosterone, 29-norsengosterone and other phytoecdisteroids from *Ajuga reptans*. Camps et al. (1985) when analysing *Ajuga chamaepitys* plants found ajugalactone, macisterone A and 20–OH ecdizone (Darvas, 1991).

The specialized pest of the *Ajuga* species is not known, the number of species able to develop on them is low. The *Liriomyza* species develop on a number of *Ajuga* species undisturbed (Darvas et al., 1994b). *Chromatomyia horticola* only lays eggs to young *Ajuga chamaepitys* leaves (Darvas, 1991) but even there a 10–20% destruction of larvae was oberved.

Materials and Methods

The experiments were set up in the Entomological Laboratory of the Plant Protection Institute of the Pannon University of Agricultural Sciences. The examinations were performed with the method of Sáringer (1967).

The consumption index (CI) shows how much the insects consumed of the feed plant in question.

$$CI = \frac{\text{fresh- or dry weight of the feed consumed}}{\text{period of feeding in days} \times \text{fresh- or dry average weight of the animal during the experiment}}$$

The relative growth rate (GR) expresses the extent of the animals' development as a consequence of consumption.

$$GR = \frac{\text{dry weight increase}}{\text{period of feeding in days} \times \text{average dry weight of the animal during the period of feeding}}$$

Efficiency of conversion of ingested food to body matter (ECI) expresses the efficiency that reflects the conversion of the food consumed to body weight.

$$ECI = \frac{\text{dry weight increase}}{\text{dry weight of food consumed}}$$

The extracts used were obtained from the Plant Protection Research Institute of the Hungarian Academy of Sciences, Budapest. They were:

AC-HHJ/MT/3: *Ajuga chamaepitys*, HHJ strain, methanol extract, collected on flowering (1 July)

ARR-BT33/i/MT/1: *Ajuga reptans* var. reptans, BT33 strain, this year's leaf, methanol extract, collected before flowering (28 April)

ARR-BT33/i/MT/2: *Ajuga reptans* var. reptans, BT33 strain, this year's leaf, methanol extract, collected on flowering (14 May)

ARR-BT33/i/MT/3: *Ajuga reptans* var. reptans, BT33 strain, this year's leaf, methanol extract, collected after flowering (4 June)

ARR-BT33/i/MT/4: *Ajuga reptans* var. reptans, BT33 strain, this year's leaf, methanol extract, collected on seed production (22 July)

Nutrition inhibition of Sitona humeralis (Steph.) adults by Ajuga extracts

The experiment was started in spring 1994 (15 April). The adults were collected from an outdoor lucerne field. The period of the experiment was 24 hours. We worked with a photoperiod of 17/7 LD at a temperature of 22 °C, and used lucerne leaves as feed plant.

The adults collected were starved for 48 hours then placed in a hygrostat. Considering the low consumption four adults were used for a replication. To 1.0 ml *Ajuga* extract 4.0 ml distilled water was pipetted, the lucerne leaves serving as feed were treated with the diluted *Ajuga* extracts, then the extracts were left to dry. The dry, treated leaves were cut in half, then the half-leaves weighed with analytical exactness. One half of the leaf was used again for dry matter determination, the other half was placed in hygrostat. In the experiment outside the replications treated with the 5 *Ajuga* extracts *Ajuga* leaves as well as leaves treated with the solvent were used as control. In every case 10 replications were made. In the end of the experiment the leaf remnants were also dried (for 24 hours at 105 °C). The dry half-leaves and leaf remnants were weighed back. The results of the experiment are contained in Table 1.

Nutrition inhibition in Pieris brassicae (L.) larvae by Ajuga extracts

The experiment started in autumn 1994 (26 October). The larvae were obtained from the Plant Protection Research Institute of the Hungarian Academy of Sciences. At the time of the experiment the larvae were in L_4 stage. The priod of the experiment was again 24 hours, the photoperiod 17/7 LD, the temperature 22 °C.

Table 1

Nutrient consumption (CI) by *Sitona humeralis* adults (average of 10 replications)

Treatment	CI
Ajuga chamaepitys (on flowering)	0.0
Ajuga reptans var. reptans (before flowering)	0.0
Ajuga reptans var. reptans (on flowering)	0.0003
Ajuga reptans var. reptans (after flowering)	0.0
Ajuga reptans var. reptans (on seed production)	0.0
Ajuga reptans var. reptans (fresh leaf)	0.0
Control	0.0016

Table 2

Nutrient consumption (CI) by *Pieris brassicae* larvae (average of 10 replications)

Treatment	CI
Ajuga chamaepitys (on flowering)	0.9628
Ajuga reptans var. reptans (before flowering)	0.0370
Ajuga reptans var. reptans (on flowering)	0.0
Ajuga reptans var. reptans (after flowering)	0.6634
Ajuga reptans var. reptans (on seed production)	0.5052
Ajuga reptans var. reptans (fresh leaf)	0.1146
Control	0.3900

The larvae were weighed with analytical exactness, then placed in hygrostat. From the cabbage leaves serving for feed leaf-disc pairs were prepared. The discs possibly were of uniform tissue, free from thicker veins. One of the disc pairs was dried after weighing in exsiccator with the same purpose as described for the previous experiment. The leaf-discs serving for feed were treated with the *Ajuga* extracts, then when dried up weighed and placed in hygrostat. In the end of the experiment we took the weights of the larvae, then weighed the dried leaf-discs, leaf-disc remnants and larvae. In the course of the examinations, outside the replications treated with the 5 *Ajuga* extracts, control (treated with ethanol) and *Ajuga* leaves in 10 replications each were used.

The results are shown in Tables 2 to 4.

Table 3

Growth (GR) of *Sitona humeralis* larvae (average of 10 replications)

Treatment	GR
Ajuga chamaepitys (on flowering)	0.0721
Ajuga reptans var. reptans (before flowering)	–0.1065
Ajuga reptans var. reptans (on flowering)	–0.0003
Ajuga reptans var. reptans (after flowering)	0.0112
Ajuga reptans var. reptans (on seed production)	–0.1058
Ajuga reptans var. reptans (fresh leaf)	–0.2399
Control	0.2603

Table 4

Feed conversion (ECI) by *Pieris brassicae* larvae (average of 10 replications)

Treatment	ECI
Ajuga chamaepitys (on flowering)	7.48
Ajuga reptans var. reptans (before flowering)	–287.50
Ajuga reptans var. reptans (on flowering)	–85.88
Ajuga reptans var. reptans (after flowering)	1.69
Ajuga reptans var. reptans (on seed production)	–20.94
Ajuga reptans var. reptans (fresh leaf)	–209.25
Control	18.73

Results

Nutrition inhibition of Sitona humeralis (Steph.) adults by Ajuga extracts

According to the data of Table 1 consumption was only observed from the sample of *Ajuga reptans* var. reptans collected at the time of flowering (CI = 0.0003). The consumption index (0.0016) obtained for the control shows that the adults consumed more than that from the control plants.

The animals did not consume from the fresh *Ajuga* leaves, neither did they from leaves treated with extracts prepared from the following samples: *Ajuga chamaepitys* (collected on flowering), *Ajuga reptans* var. reptans (collected before and after flowering and on seed production). According to the results the extracts prepared from the *Ajuga* species had a considerable inhibitory effect on the nutrition of *Sitona humeralis* adults.

Nutrition inhibition of Pieris brassicae L. larvae by Ajuga extracts

From the data of Tables 1 to 4 the following facts can be established. According to the values of the consumption index (CI) the larvae consumed from the ajuga leaves in every case except the replications treated with extracts prepared from the sample of *Ajuga reptans* var. reptans collected at the time of flowering. Compared to the control (1.39) consumption was the lowest in the following two cases: *Ajuga reptans* var. reptans before flowering and on seed production, that is, the highest nutrition inhibition was observed in these cases. The larvae consumed small quantities from the fresh *Ajuga* leaves as well.

The data of the relative growth rate (GR) show that while the larvae of the control replications grew well (0.2603), on the treated leaves the growth was of low rate, or even a weight loss was observed (on the flowering of *Ajuga reptans* var. reptans (0.1175). The weight loss was caused by the insufficient feeding explained by the inhibitory effect of the extracts on nutrition.

From the gross efficiency (CI) values too we drew the conclusion that the efficieny was low or of negative value compared to the control (18.73%). This too can be explained with the small amount that the larvae consumed from the treated leaves; they could not convert this small amount of feed, and could not develop.

All in all, the experiment proves that a part of the allelochemicals of the *Ajuga* species possess a considerable inhibitory effect on nutrition.

Acknowledgement

We should like to express our thanks to Dr. Bela Darvas, senior member of the Research Institute for Plant Protection of the Hungarian Academy of Sciences, Budapest, for his professional assistance in our work and for placing the *Ajuga* extracts at our disposal.

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Ingestion, Gut Emptying and Respiration Rates of Clubionid Spiders (Araneae: Clubionidae) Occurring in Orchards

S. BOGYA[1] and P. J. M. MOLS[2]

[1] Department of Entomology, University of Horticulture and Food Industry, Budapest, Hungary
[2] Wageningen Agricultural University, Department of Entomology, Wageningen, The Netherlands

To assess the potential role of spiders in orchards experiments were executed to estimate the potential food consumption of two Clubionid spider species (*Clubiona pallidula* (Clerck) and *C. phragmitis* C. L. Koch, each of ±12 mg in weight) by measuring mealsize, the relative gut emptying rate, assimilation rate and respiration at three different temperatures by gravimetric methods. The relative rate of gut emptying varied from 1.9/day at 10 °C to 3. 7/day at 20 °C. Mealsize was 2.5 mg for both species. Assimilation efficiency based on fresh weight was approximately 35% and respiration depended on temperature ranging from 0.07 mg at 10 °C to 0.17 mg at 20 °C. There were only small differences between the two species. With a small computer model the potential daily consumption was estimated and ranged between 3.3 mg/day at 10 °C to 5.7 mg/day at 20 °C.

To quantify the role of spiders in orchards infoxmation is needed on their searching and feeding behaviour. This paper concentrates on the amounts of prey that can be ingested potentially by these predators.

The role of spiders in orchards has been discussed in a qualitative way by Mansour et al. (1980a, 1980c, 1981). From all those spider species occurring in orchards the Clubionidae take an important part. Clubionid spiders are well known predators of important pest species in orchards e.g. feeding on small caterpillars (Baker, 1983; Corrigan and Bennett, 1987; Mansour et al., 1977, 1980b, 1981), Psyllids (Berg et al., 1992; Selivanov, 1991), mites (Chant, 1956) and scale insects (Mansour and Whitecomb, 1986). They are hunting during the night and hide themselves during daytime in small sack like tubes made of webbing.

We wanted to investigate whether it was possible to get more insight in the feeding potentials of these predators by using the same method of research which showed profitable in measuring the potential intake of food by the carabid beetle *Pterostichus coerulescens* L. (Mols, 1988).

Factors that influence the feeding behaviour of predators can be divided into internal and external factors. The internal factors, originating from the physiological condition or state of the predator, compose the 'motivation' of the animal. This 'motivational' state may be the result of the states of different organs like the filling of the gut, the size of the ovaries, the fatbody and the concentration of carbonhydrates and aminoacids in the haemolymph. The rates of change of these internal states are influenced by external factors like: food quality, temperature, day length (inducing reproductive activity or the opposite diapause) and sometimes humidity.

0238–1249/95/$ 5.00

To be able to estimate the daily potential consumption at variable field temperatures the ingestion, gut emptying and respiration rate of the spiders had to be measured at a range of constant temperatures.

Materials and Methods

Experiments

The spiders were collected at the experimental orchard "De Schuilenburg" (Lienden, the Netherlands) by means of treebands. After capture they were stored in an outdoor insectary and fed twice a week with young caterpillars of the leafroller *Adoxophyes orana* until the start of the experiments. At the moment of the start of the experiments the subadult spiders could not be identified, therefore, similar sized and coloured spiders were selected. When they reached adulthood after the experiments they were identified. The experiments were carried out in climate rooms at 3 constant temperatures 10, 15 and 20 °C. In the experiments 20 spiders (6 *Clubiona phragmitis* and 12 *Clubiona pallidula*, 2 died before reaching adulthood) at 10 °C, 15 spiders (5 *Cl. phragmitis* and 10 *Cl. pallidula*) at 15 °C and 15 spiders (4 *Cl. phragmitis* and 10 *Cl. pallidula*, 1 died) at 20 °C (one/Petri dish) were used. The day-length was set at 18/6 (L:D). The humidity was kept very high (R.H. = ± 95%) by using moist filter paper as substrate to prevent exication. The predators were starved at the experimental temperatures for a week. Before the starvation period the weight of these animals was measured by means of a microbalance. Therefore, they were placed into a gelatine capsule and weighed. The weight of the capsule was subtracted. After starvation the spiders got 6 caterpillars (*Adoxophyes orana* L_2 instar larvae) each and were allowed to feed for 2 hours in dark. Preliminary experiments indicated that for spiders 2 hours were sufficient to get satiated.

After the feeding period the remaining food was removed and the predators were weighed again. Firstly the weighing was repeated twice with an interval of 1.5 hours and later with 2 hours interval, in total 7 weighings in the first day. The following day two additional measurements were done. For each temperature the experiment was repeated three times with the same animals.

Calculation method of ingestion, assimilation, respiration and relative gut emptying rate

Gut content decreases by the assimilation of food into the haemolymph and by defaecation. For some arthropods this process can be described in general by an exponential decay (Fransz, 1974; Mols, 1988). The general equation of this process is:

$$A_1 = A_0 e^{r \times t}$$

where A_0 and A_1 are the gut contents respectively before and after the time period 't'. The relative rate of gut emptying 'r' is independent of the amount of food in the gut and

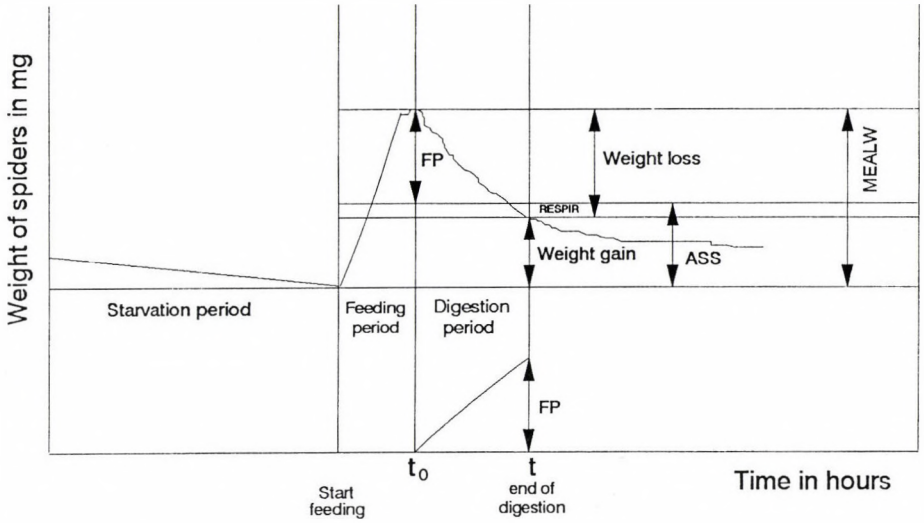

Fig. 1. A schematic representation of the changes in weight of the spiders and of faeces produced during and after ingestion of a prey caused by digestion (after Mols, 1988)

mainly determined by temperature and food quality. Using the same type of food the quality of it is assumed to be the same for all the spiders. Knowing the assimilation efficiency, the relative rate of gut emptying can be derived by measuring the decline in weight after satiation, which is the combined result of faeces excretion (FP), respiration (RESPIR) and sometimes dehydration. When the gut is empty the decline in weight equals the weight loss caused by respiration, because from that moment onwards the predator stops producing faecal pellets. The amount of food assimilated is the weight gain of the spider at the moment the weightloss after feeding equals the respiration rate plus the weight used for respiration during the starvation afer feeding.

In Fig. 1 the process of ingestion and egestion is shown.

FP: faecal production (quantity of faeces produced)

ASS: assimilation (quantity of food assimilated from the gut into the haemolymph)

MEALW: meal weight (quantity of food ingested)

The assimilation efficiency (EFF) is defined as:

EFF = ASS/MEALV

FP = weight loss –RESPIR

ASS = weight gain + RESPIR

MEALW = FP + ASS

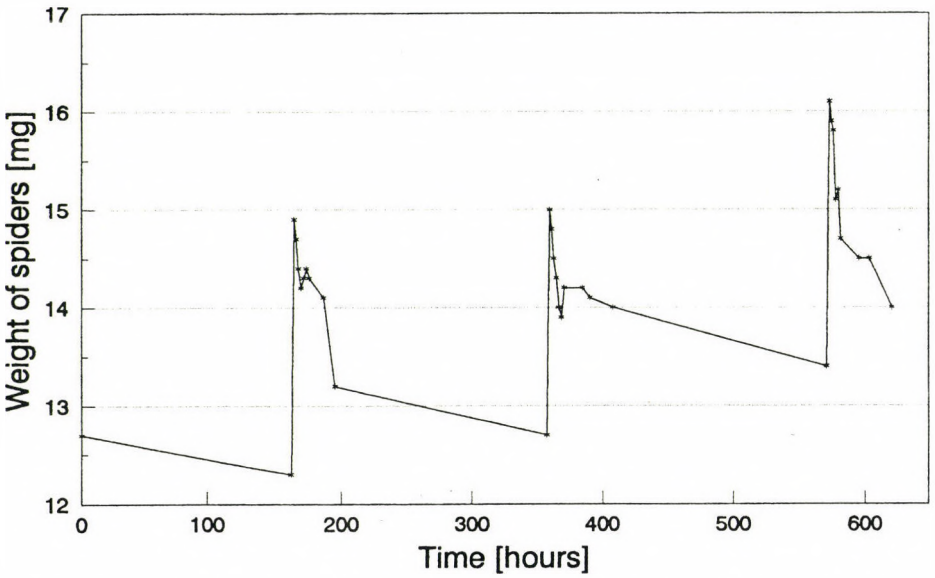

Fig. 2. Change in weight by ingestion, egestion and respiration for three repetition in sequence

Results

The general course of the change in weight of the spiders is shown in Fig. 2. Firstly, it starts with a decrease caused by respiration and digestion when the spiders come from storage conditions. Thereafter, an increase of weight caused by ingestion is followed by a steep decrease which is mainly caused by gut emptying. This is repeated three times in sequence. The results for the two spider species are given in Table 1.

The course of decrease does not follow a smooth exponential decay but shows some fluctuates. This can be explained by the drinking behaviour of the spiders. In the first two experiments it was difficult to keep humidy at such a high level that dehydration was prevented. Adding water during the observation resulted in drinking by some of the spiders causing a sudden increase of body weight. In the last experiment putting the Petri dishes with the spiders under a controlled humidity regime above a salt solution prevented this drinking behaviour and made calculation of egestion parameters easier.

Respiration

Weightloss caused by respiration was measured over the first starvation period and over the periods between end of the second and the end of the third starvation period (Fig. 3). Respiration increases significantly with temperature. At 10 and 15 °C the respiration values for the two species do not differ significantly, but for 20 °C a significant difference is observed.

Table 1

Summarized table about the calculated characteristics

	Cl. pallidula			*Cl. phragmitis*		
	average	std. error	N	average	std. error	N
10 °C						
Respir	0.096	0.0093	32	0.072	0.012	13
RRGE	1.93	0.114	26	1.98	0.169	8
ASS%	29.4	5.7	17	33	3.6	8
Mealsize	2.39	0.15	34	2.2	0.251	15
15 °C						
Respir	0.11	0.0085	26	0.11	0.022	11
RRGE	2.33	0.067	17	2.2	0.063	6
ASS%	31.6	3.8	12	32.6	4.6	5
Mealsize	2.52	0.18	26	2.47	0.375	10
20 °C						
Respir	0.168	0.001	21	0.144	0.001	11
RRGE	3.77	0.232	18	2.69	0.197	9
ASS%	37.4	4.2	13	44	9.3	5
Mealsize	2.66	0.36	20	2.49	0.302	11

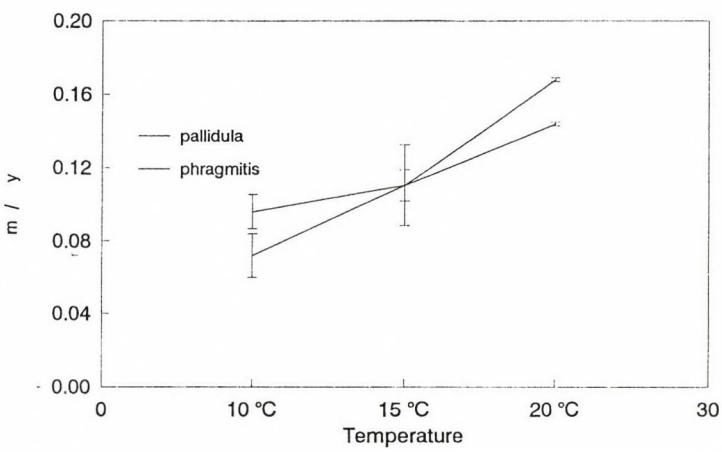

Fig. 3. Respiration rate

Table 2

Estimated daily potential food consumption (mg) by the two spider species, using RRGE, Mealsize (2.5 mg), Ingestion rate (2.5 mg/h) and an Ingestion threshold of 85% (calculated from feeding interval times observed by videocamera)

Temperature	*Cl. pallidula* consumption	*Cl. phragmitis* consumption
10 °C	3.3	3.4
15 °C	4.0	3.6
20 °C	5.7	5.0

Mealsize

The mealsize of the two clubionid species was not different significantly (Student's *t*-test, $p < 0.05$) for all temperatures (Table 1) approximately 2.5 mg, but it was sometimes influenced by drinking behaviour. Those spiders were not included in the results.

Assimilation

For both species assimilation efficiency shows a high variation and a tendency to increase with temperature, but this is not significant (Fig. 4). The average is approximately 35%.

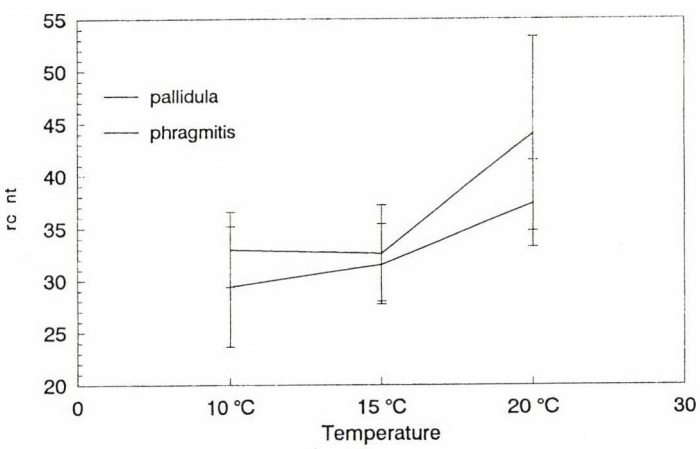

Fig. 4. Assimilation efficiency

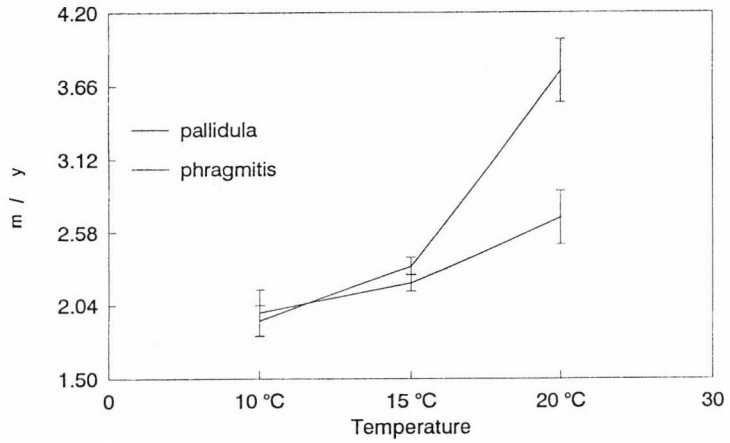

Fig. 5. Relative rate of gut emptying

Relative rate of gut emptying

For the two spider species RRGE shows a positive relationship with temperature (Fig. 5). In *C. phragmitis* the relationship is weak, but *C. pallidula* on the contrary shows a strong positive relationship with temperature.

Discussion and Conclusions

A problem for the proper calculation of the weightloss characteristics occurred by the drinking behaviour of the spiders. This caused fluctuation in weight. This is probably a normal behaviour of these type of spiders because they have thin cuticula and therefore are sensitive to exication. It makes the use of gravimetric methods for assessing respiration and RRGE rather cumbersome.

Although fluctuations occurred it could be partly cured by putting the spiders under very high humidity. The general process could be described by an exponential decay. A similar process was found in the cockroaches *Periplaneta americana* L. (Davey and Treherne, 1963) and *Leucophaea maderae* Jam. (Engelmann, 1968), the blowfly *Phorbia regina* L. (Gelperin, 1966), the preying mantis *Hierodula crassa* F. (Holling, 1966), the predatory mite *Amblyseius potentillae* Chant. (Rabbinge, 1976) and the wolf spider *Lycosa pseudoannulata* Clerc (Nakamura, 1968). The value that Nakamura (1972) obtained for RRGE in *Pardosa laura* was 5.46/day at 25 °C. This is higher than our results, but the temperature was also higher. In the carabid beetle *P. coerulescens* the RRGE depends both on reproductive state, temperature and daily rhythmicity (Mols, 1988). When reproductive the average RRGE ranged from 0.7/day at 12 °C to 3.3/day at 27 °C. Non-repro-

ductive beetles reach half of these values. The values for the Clubionidae are higher than for the carabid beetles, but these spiders are already active at low temperatures. For *P. coerulescens* the threshold of activity is 8 °C. while Clubionids are locomotory moving above 1.5 °C and even feed around 0 °C.

The weight of the meal size to satiate the spiders offers a good estimate for the gut capacity. In the spiders observed the meal weight of 2.5 mg is about 20% of the fresh body weight. For the wolf spider *L. pseudoannulata* this is about 34% of the body weight (Nakamura, 1968) and this equals the value found in the Clubionids.

The respiration or metabolic rate is generally estimated by oxigen consumption or carbondioxide production. As we assessed respiration by fresh weight it is difficult to compare our results with those from literature. Fresh weightloss measured in the spider *Lycosa lenta* Hentz was 0.0055 mg/mg bodyweight/day (Anderson, 1974). As the Clubionid have an average weight of about 12.5 mg we calculated the respiration to be 0.0052 mg/mg bodyweight/day which agrees well with the previous value. Other spiders may show values that are two to three times higher (Matsura, 1981). Respiration depends also on the duration of the starvation period. Spiders are able to decrease respiration and thus are able to survive long periods without food (Anderson, 1974).

At 20 °C Carabids of about 40–60 mg use about 0.6 mg fresh weight per day, which is 0.012 mg/mg/day (Mols, 1988). This is twice the amount of the spiders. Assimilation efflciency of the spiders is lower compared to Carabids (average of about 35% for spiders to 50% for Carabids), because spiders ingest only liquid food containing more water.

The potential food consumption can be calculated with help of a small computer model using the ingestion and egestion parameters, the gut capacity and the threshold for feeding. The latter was found by measuring the interval time between prey captures. This time was 50 ± 30 minutes. By using the formula for gut emptying the threshold appeared to be between 80–90%. For the two spider species the results are given in Table 2. The spider species can consume about the same amount of food each day. Varying between 3.3 mg at 10 °C and 5.7 mg at 20 °C.

One L_2 caterpillar weight about 2 mg. If the spiders ingest only liquid food half of it will be consumed. This indicates a potential daily killing rate of 3–6 small caterpillars depending on temperature. This agrees rather well with some preliminary observations done in our laboratory. We also found that only the small L_1–L_3 stages of leafroller caterpillars were accepted by the spiders. The results of this table shows only the potential food consumption and whether these potentials will be fullfilled will mainly depend on the prey density and on the searching behaviour of the spiders.

Acknowledgements

We thank Filippo Mazzanti for his help with the experiments and Dr L. Blommers and all the members of the experimental orchard "The Schuilenburg" for their help and encouraging hospitality.

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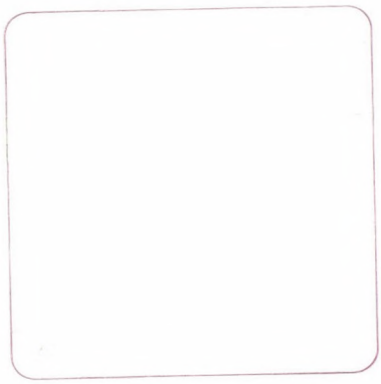

HU ISSN 0238–1249