OPTIMIZATION OF THIOUREA LEVEL AT CELLULAR AND WHOLE PLANT LEVEL FOR MAIZE HYBRIDS (*ZEA MAYS* L.)

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Abstract. Thiourea is a potential plant growth regulator that improves stress tolerance potential in a variety of plants. Exogenous application of thiourea enhanced the growth and yield of plants, but different concentrations are required by different crops therefore optimization of thiourea is needed for each crop plant. These experiments were arranged to screen out optimum level of thiourea for maize hybrids at both whole plant and cellular levels. Two experiments were done, first at whole plant level; two hybrids DK6789 and 33M15 were used. Thirteen levels of thiourea (0, 200, and 400, to 2400 μM) were applied through sand application to plants after 15 days of germination, and in second experiment same hybrids were used to investigate optimum level of thiourea for callusing attributes. Immature embryo were cultured on N6 (Chu et al) medium supplemented with 3.99g/L salt, 3% sucrose, 2.5 g/L proline, 0.2g/L casein hydrolysate, 2,4-D 2g/L, $AgNO_3$ 0.015 g/L, and agar 8g/L. Above applied levels of thiourea were supplemented to callus induction medium. Among all applied thiourea levels, 400 μM thiourea level was found to be most effective. Thiourea significantly improved *in vivo* attributes like shoot and root length, fresh and dry weights of shoot and root, leaf area and shoot/root mass ratio. Thiourea was also found very effective in improving *in vitro* attributes especially improved callus quantity and quality. This could be a great achievement towards better maize genetic transformations for development of resistant crops.
Keywords: *soil application; immature embryo; embryogenic callus; plant regeneration; genetic transformation*

Abbreviations: *2, 4-D (2, 4-Diholorophenoxyacetic acid)*

Introduction

Any factor that is negatively affecting the plant growth and developing and declining plant biomass and yield is referred to as stress. Stress may be due to biotic and abiotic factors. Substantial reductions in crops have been observed under abiotic stresses (Hassine et al., 2010; Ayari, 2014). Abiotic stresses such as salt stress causes great reduction in growth by acting as the most limiting factor for plants (Misra and Saxena, 2009; Hayat et al., 2012; Tripathi et al., 2007). The salinity toxicity is associated with (1) water stress (low osmotic potential in soil), (2) nutritional imbalance, (3) specific ion effect or (4) combination of all these factors (Tripathi et al., 2007; Hayat et al., 2012). By virtue of these factors, the salt stress deteriorates the proliferation (quantity) and color and texture (quality) attributes of callus (Sharma *et al*., 2013). During past few decades, considerable improvements have been made in field of crop sciences through conventional breeding methods, using selected hybrids and by employing the tissue culture techniques (Ashraf and Haris, 2004).

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It is a hard fact that conventional breeding methods are unable to keep pace with the rapidly growing population due to being long time consuming in performing wide crosses (Ombori et al., 2008). It is believed that selection of tolerant crops at whole plant and cellular levels is quite reliable to get better crop yields (Ikram et al., 2014; Ikram and Javed, 2015). The biotechnological approach is additional compliments to conventional selection and breeding approaches to achieve desired levels of maize production in relatively shorter period of time with great agricultural value (Chen et al., 2008; Cheng-Hao et al., 2008; Joshi et al., 2009, 2010; Hakeem et al., 2012).

Exploitation of prompt and inexpensive means being employed these days (Oduor et al., 2006). Tissue culture techniques can be used for efficient propagation of stress tolerant lines, and to study the whole plant mechanism of stress tolerance (Ahmad et al., 2013; Joshi et al., 2010). Among the most effective approaches, the exogenous application of eco-friendly stress alleviating growth bioregulators as seed priming, foliar spray or soil application are in use (Asthir et al., 2013). In this respect many growth bio-regulators, many nitrogenous compounds, sulfur compounds, inorganic salts, natural or synthetic growth promoters such as kinetin, glycinebetain, proline, trehalose, thiourea and nitrates are well known (Khan and Unger et al., 2001).

Thiourea is a nitrogen and sulfur containing compound and is being widely used for crop yield improvement studies. Use of thiourea significantly improved plant growth in terms of root and shoot weight, height and number and leaf area (Perveen et al., 2013, 2015). Thiourea not only improved growth at whole plant level but it also enhances growth at cellular level under stress (Ikram et al., 2014, 2015). It significantly improved fresh and dry weights under stressed condition (Siddiqui et al., 2006). Salt stress causes oxidative damage to plants at whole plant in barely (Yonova et al., 2009) and at cellular level in maize (Abdelkader et al., 2012).

The objective of this research was to assess the best level of thiourea at which it gives best amelioration of salt stress by two maize hybrids. Growth parameters are the best indicators of plant response to any type of stress (Bhardwaj et al., 2009; Ikram et al., 2015). That's why all results were assessed on the basis of morphological parameters at both whole plant and cellular level, to determine whether thiourea is effective at cellular level as well as at whole plant level.

Material and Methods

Whole plant level

This experiment was conducted in autumn season 2013 in Old Botanical Garden University of Agriculture Faisalabad. Seeds of two selected maize genotypes (DK6789 and 33M15) were sown in plastic pots containing river sand. After complete germination, the seedlings were irrigated with half strength Hoagland's solution with three days interval (Hoagland and Arnon, 1950). The experiment was completely randomized with three replicates. After fifteen days of germination half of the pots of each maize hybrid were subjected to 0 and 120 mM (NaCl) in combination with different concentrations of thiourea (*Table 1*) in two planting seasons for morphological and growth studies and half of pots without any treatment were left for yield and cobs (immature embryos) were utilized for *in vitro* studies. After fifteen days of salt application, data was taken for growth parameters such as shoot and root length (cm), shoot and root fresh weights (g), shoot and root dry weights (g), leaf area (cm^2), number of roots and leaves.

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Table 1. *Different combinations of thiourea applied with NaCl for screening in two maize hybrids*

NaCl (mM)	Thiourea (µM)
0	0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400
120	0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400

Callus level

This part of experiment was carried out in Somatic Cell Genetics Laboratory, Centre of Agricultural Biotechnology and Biochemistry, University of Agriculture Faisalabad.

Plant material

Cobs of all the six maize hybrids P1543, 34N43, 31P41, DK6789, 33M15, 32B33 were collected 14-20 days after anthesis from the plants grown in Old Botanical Garden, University of Agriculture Faisalabad.

Immature embryo dissection

Cobs were sterilized and dehusked using Songstad et al. (1996) method. In large autoclaved Petri plate kernel crown was cut off with a sharp scalpel blade (top 1-2mm). Embryos were excised according to Songstad et al. (1996) method of embryo dissection. The embryos were generally coaxed on the spatula tips and plated with the embryo axis side down and scutellum side up on the medium. 10 to 12 embryos were cultured on each plate as presented in *Picture 1* (all steps of immature embryo culture; harvesting to dissection of immature embryo are illustrated in the picture).

Callus induction and proliferation

Immature embryo were cultured on N6 (Chu et al., 1975) medium supplemented with 3.99g/L salt, 3% sucrose, 2.5 g/L proline, 0.2g/L casein hydrolysate, 2,4-D 2g/L, $AgNO_3$ 0.015 g/L, agar 8g/L. Above levels of thiourea were supplemented to callus induction medium (Danson et al., 2006). The cultures were incubated in dark for callus induction and proliferation. The callusing explants were sub cultured on fresh medium with same composition devoid of $AgNO_3$ for further proliferation (Jakubekova et al., 2011). The data was recorded for callogenesis and its various traits after 1 week of explants culture on callus induction medium. The cultures with proliferated calli were transferred to regeneration media, comprised of MS salts with different growth regulators in different combinations for enhanced regeneration.

Selection of thiourea level

Immature embryo cultures of two selected maize hybrids that performed well for callus proliferation and regeneration were screened for thiourea treatments alone. Immature embryos were directly cultured on Media containing different combinations of thiourea (0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200 and 2400 µM).

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Direct shoot emergence percentage (%)

Percentage of callus induction for best hybrid assessment was recorded by using the percentage formula:
% direct emerging shoot = (No of direct emerging shoot/total no of embryos cultured) ×100 (Eq.1)

Direct root emergence percentage (%)

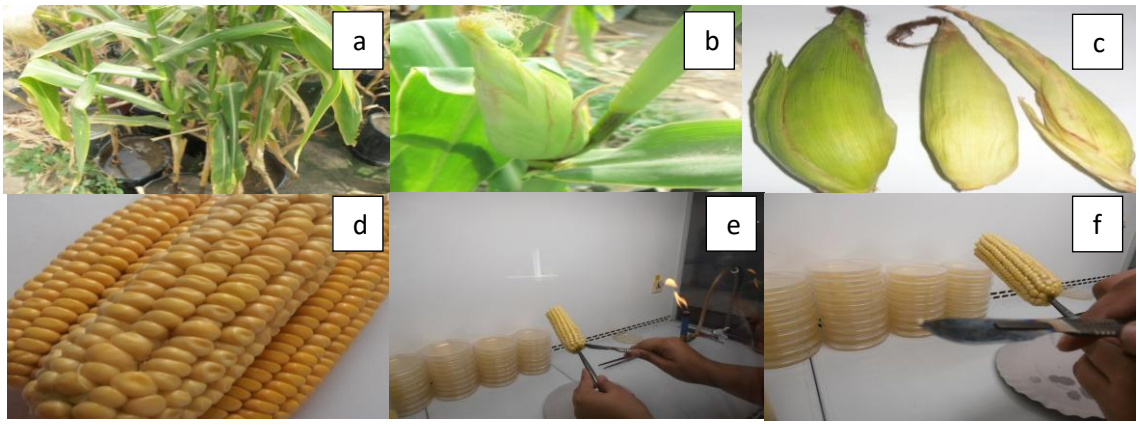
Percentage of callus induction for best hybrid assessment was recorded by using the percentage formula:
% direct emerging root = (No of direct emerging root/total no of embryos cultured) ×100 (Eq.2)

Percent callus induction for hybrids

Percentage of callus induction for best hybrid assessment was recorded by using the percentage formula:
%callus induction= (No of embryos showing callus induction/total no of embryos cultured) ×100 (Eq.3)

Callus Morphology

Callus texture, callus color, callus type, callus amount were recorded for different treatments of thiourea. All the cultures mentioned above were transferred to new fresh media at 2 week intervals. Visual scoring data was recorded as (++++ = Best callus Induction, +++ = Good callus, ++ = Low callus formation, + = very low callus formation, --- = No callus)



Picture 1. *Procedure of immature embryo culture (a. plants in garden, b. cob of 14 days old, c. cob removed from plant, d. dehusked cob, e. embryo dissection, f. dissected immature embryo)*

Statistical Analysis

A three way analysis of variance was applied by computer software COSTAT was used for all statistical analysis and MS-Excel was used for graphical presentation of data. The means and standard errors were computed from each treatment. The data collected were analyzed statistically through ANOVA using LSD test at 5% of

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significance for comparing the difference among treatments. Analyses of variance were performed separately for each analysis. Treatment means were marked with alphabets when H×S×TU interactions were significant.

Results

At whole plant level

Shoot length

Results revealed significant (P<0.01) differences in the maize hybrids, salinity and different concentrations of thiourea. A detailed comparison of different treatments showed that increasing shoot length behavior was observed with thiourea supplementation in control as well as under salt stress. Maximum increment was observed at 400 µM and up to 12% more in non-stressed condition. After 600 µM supplementation further increase in the concentration of thiourea, shoot length of both the hybrids decreased, the lowest shoot length was found at 2400 µM of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~3 and 9% respectively). The comparison of two hybrids showed the reduction of 6% and 12% in shoot length of DK6789 more than hybrid 33M15 at 0 and 120 mM NaCl *(Table 3*; *Fig. 1A).*

Table 3. *Mean squares from analyses of variance of data for growth attributes, when 15-days old Zea mays plants were subjected to varying levels of thiourea for 15 days under control or saline conditions.*

Source of Variance	df	Shoot length	Root length	Shoot f. wt.	Root f. wt.	Shoot d. wt.
Hybrids (H)	1	1902.61***	485.36***	2403.801***	271.47***	20.64***
Salinity (S)	1	1322.03***	328.47***	10935.23***	585.22***	185.34***
Thiourea (TU)	12	5981.94***	1223.26***	8251.51***	213.59***	92.48***
H × S	1	470.54***	30.90**	82.86**	98.54***	1.86***
H × TU	12	106.41**	12.39***	134.61***	6.502***	1.24***
S × TU	12	121.24**	10.65***	975.21***	27.23***	12.53***
H × S × TU	12	51.43 **ns**	13.44***	69.802***	10.52***	0.79***
Error	104	40.44	3.003	10.05	1.48	0.04

Source of Variance	df	Root d. wt.	Leaf area	Shoot/Root ratio
Hybrids (H)	1	20.64***	17065.38***	12.72 ***
Salinity (S)	1	185.34***	121033.32***	5.91**
Thiourea (TU)	12	92.48***	67979.76***	5.602***
H × S	1	1.86***	2653.32 **ns**	8.206 ***
H × TU	12	1.24***	1104.96 **ns**	1.22*
S × TU	12	12.53** *	5285.13***	0.95 **ns**
H × S × TU	12	0.79***	1296.98 **ns**	1.36 *
Error	104	0.04	800.15	0.59

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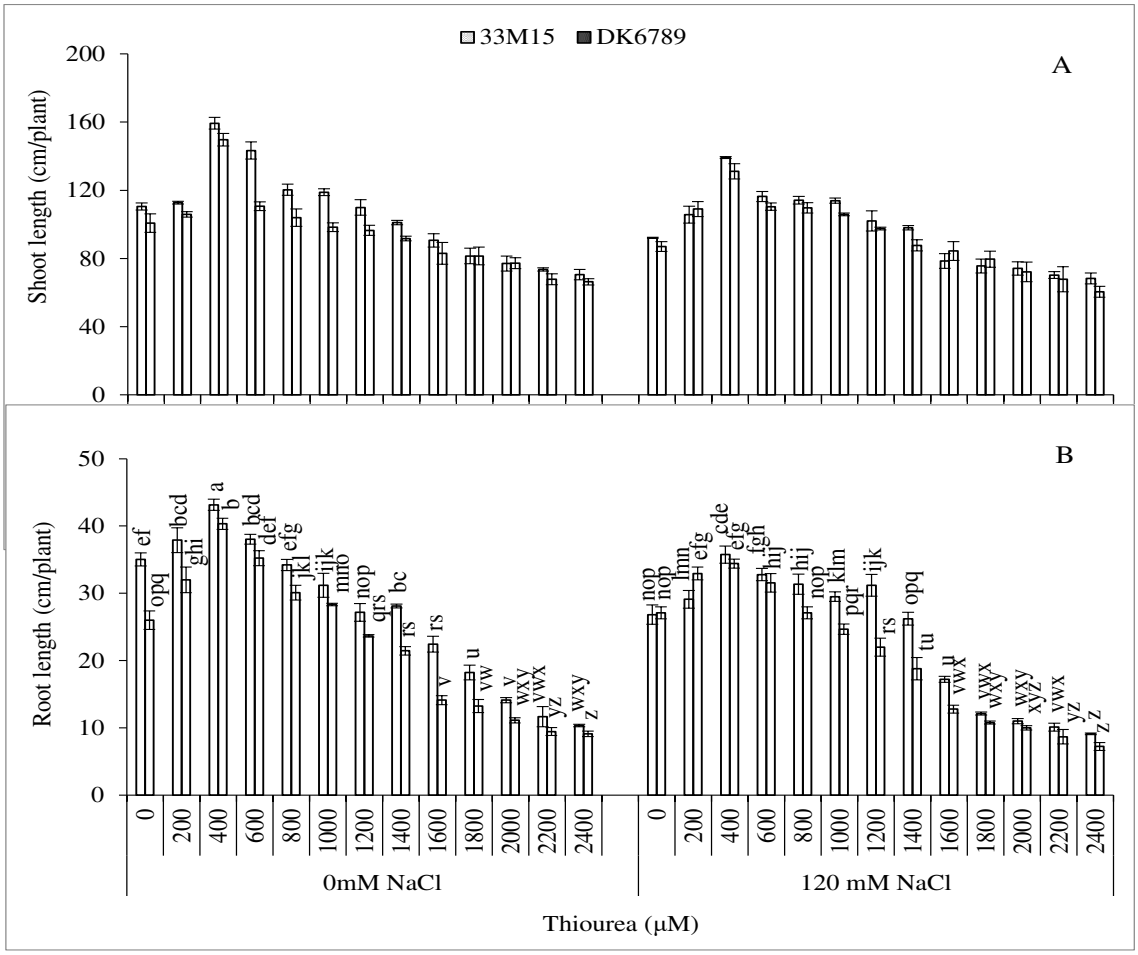

Figure 1. *Changes in shoot length and root length of two maize hybrids at different concentrations of thiourea under non-saline and saline conditions*

Root length

Analysis of variance showed significant (P<0.01) difference in the maize hybrids, salinity and thiourea levels. Maize hybrids when treated with different treatments of thiourea under non-saline and saline conditions behaved differently. Thiourea treatments when compared showed that 400 µM increased root length of 33M15 and DK6789 up to 7% more than non-treated plants. After 600 µM treatment as the concentration of thiourea increased, root length of both the hybrids decreased, the lowest root length was found at 2400 µM of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~12 and 21% respectively). The comparison of two hybrids showed the reduction of 12% and 21% in root length of DK6789 more than hybrid 33M15 at 0 and 120 mM NaCl *(Table 3*; *Fig. 1B).*

Shoot fresh weight

Results showed thiourea significantly affected shoot fresh weight of maize hybrids under salinity (P<0.01). When different levels of thiourea compared it was found that 400 µM increased shoot fresh weight of 33M15 and DK6789 up to 41 and 43% more

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than non-treated plants. After 600 µM as the thiourea concentration further increased, shoot fresh weight of both the hybrids started to decrease; the lowest shoot fresh weight was found at 2400 µM of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~6 and 13% respectively). The comparison of two hybrids showed the reduction of 6% and 13% in shoot fresh weight of DK6789 more than hybrid 33M15 at 0 and 120 mM NaCl *(Table 3*; *Fig. 2A).*

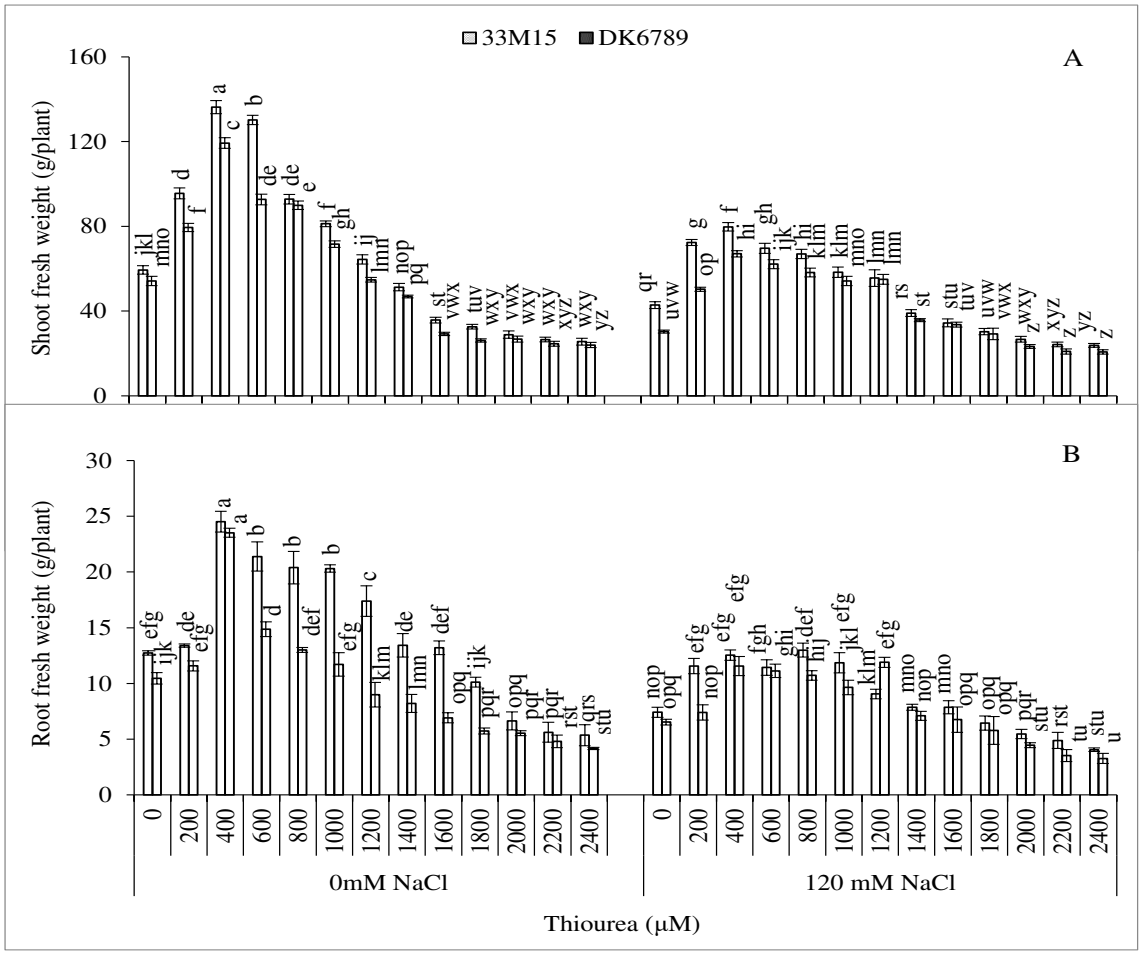

Figure 2. *Changes in shoot fresh weight and root fresh weight of two maize hybrids at different concentrations of thiourea under non-saline and saline conditions*

Root fresh weight

Data revealed significant (P<0.01) difference in maize hybrids salinity and thiourea. Data showed that 400 µM thiourea increased root fresh weight of 33M15 and DK6789 up to 49 and 51% more in control. As the concentration of thiourea increased after 600 µM, root fresh weight of both the hybrids decreased, maximum reduction was observed at 2400 µM thiourea that decreased root fresh weight of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~22 and 19% respectively). The comparison of two hybrids showed the reduction of 24% and 21% in root fresh weight of DK6789 more than hybrid 33M15 at 0 and 120 mM NaCl *(Table 3*; *Fig. 2B).*

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Shoot dry weight

The data indicated significant (P<0.01) differences in maize hybrids, salinity and thiourea levels. Results revealed that thiourea increased shoot dry weights of both the hybrids under non-saline and saline conditions. 400 µM thiourea increased shoot dry weight of 33M15 and DK6789 up to 42 and 51% more in control. As the concentration of thiourea increased, shoot dry weight of both the hybrids started to decrease, 2400 µM thiourea decreased shoot dry weight of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~27 and 38% respectively). The comparison of two hybrids showed the reduction of 28% and 40% in shoot dry weight of DK6789 more than hybrid 33M15 at 0 and 120 mM NaCl *(Table 3*; *Fig. 3A).*

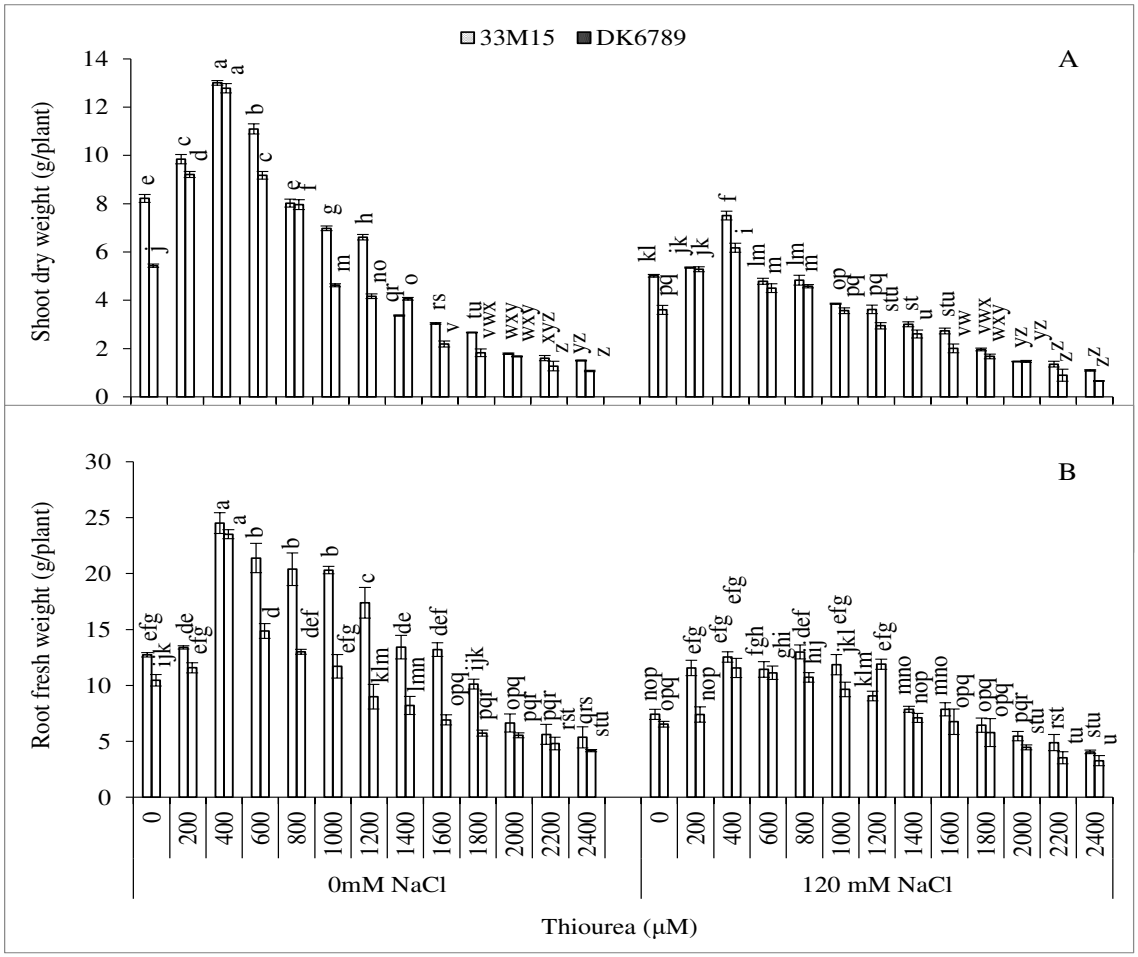

Figure 3. *Changes in shoot dry weight and root dry weight of two maize hybrids at different concentrations of thiourea under non-saline and saline conditions*

Root dry weight

It is noted from the data that significant difference existed in the maize hybrids (P<0.01), salinity and different concentrations of thiourea used for root fresh weight. The concentrations of thiourea when compared showed that thiourea with 200, 400, 600, 800 and 1000 µM improved root dry weights by 45, 60, 62, 62, 60% in 33M15 while by 28, 62, 72, 63, 48% in DK6789 at the respective levels in non-treated plants.

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As the concentration of thiourea increased, root dry weight of both the hybrids decreased, 2400 µM thiourea decreased shoot dry weight of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~35 and 24% respectively). 33M15 hybrid showed 27 and 16% more root dry weights as compared to hybrid DK6789 at 0 and 120 mM NaCl treatments *(Table 3*; *Fig. 3B).*

Leaf area

Results regarding leaf area per plant indicated that maize hybrids, salinity and thiourea levels exerted significant (P<0.01) effects. As regards the concentrations of thiourea, treatments with 200, 400, 600, 800and 1000 µM improved leaf area by 28, 15, 31, 24, 20% in 33M15 while by 39, 28, 23, 32, 27% in DK6789 at the respective levels in control. As the concentration of thiourea increased, leaf area of both the hybrids decreased, 2400 µM thiourea decreased leaf area of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~9% respectively). 33M15 hybrid showed 17 and 8% more leaf area as compared to hybrid DK6789 at 0 and 120 mM NaCl treatments *(Table 3*; *Fig. 4A).*

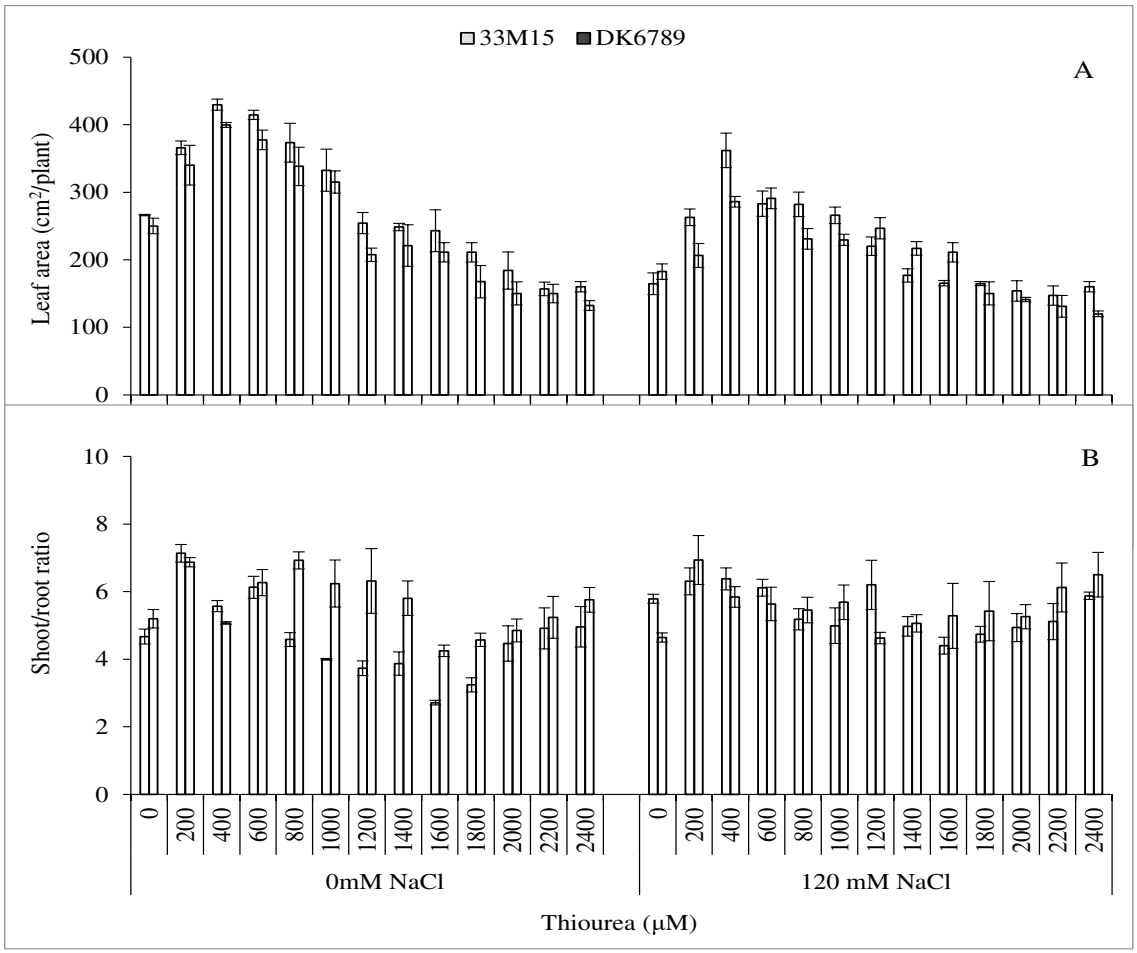

Figure 4. *Changes in leaf area and shoot root ratio of two maize hybrids at different concentrations of thiourea under non-saline and saline conditions*

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Shoot/root ratio

Data suggested that there was significant (P<0.01) difference in the maize hybrids salinity and different concentrations of thiourea used for shoot/root ratio. Comparison of concentrations of thiourea showed reduced shoot/root ratio of 33M15 hybrids under non-saline and saline conditions. 200 µM thiourea increased shoot/root ratio of 33M15 and DK6789 up to 12 and 0.8% more in control. As the concentration of thiourea increased, shoot/root ratio of both the hybrids increased, 2400 µM thiourea increased shoot/root ratio of hybrid 33M15 and DK6789 especially at 120 mM NaCl (~19 and 12% respectively). The comparison of two hybrids showed improvement of 16% and 11% in shoot/root ratio of DK6789 more than hybrid 33M15 at 0 and 120 mM NaCl *(Table 3*; *Fig. 4B).*

Although all the concentrations of thiourea were effective in enhancing shoot and root length, shoot and root fresh and dry weights, leaf area and shoot/ root ratio, the 400 µM thiourea was found most effective in both the maize hybrids under control as well as salt stress. As far as hybrids are concerned 33M15 was much better responsive to thiourea and all of its concentrations than DK6789. As regards the use of different concentrations of thiourea, the 400 µM thiourea level was found most effective.

At callus level

Morphological parameters

A comparison of thiourea treatments for direct emerging shoot and root and callus induction among two maize hybrids for thirteen varying levels of thiourea was measured on a 7 day interval for up to 42 days of immature embryo culture on N6 2 medium.

Direct emerging shoot percentage

Statistical analysis of data revealed significant (P<0.01) differences in the hybrids and thiourea. 33M15 showed more direct shoot emergence response at 400 µM thiourea. 80-95 percent immature embryos produced shoot before callus induction started. Least value for direct emerging shoot percentage was recorded for 33M15 at 2400 µM *(Table 4*; *Fig. 5A).*

Direct emerging root percentage

Hybrids exerted non-significant (*P*>0.05) effects and thiourea levels exerted significant (P<0.01) effects *(Table 4*; *Fig. 5B).* 33M15 showed maximum value at 400 µM thiourea. 85-95% immature embryo produced root before callus induction. No direct rooting was recorded for 33M15 at 2400 µM.

Callus induction percentage

Analysis of data indicated non-significant (*P*>0.05) differences among hybrids and significant (P<0.01) differences among thiourea levels. Maximum value for both hybrids was found at 400 µM thiourea (*Picture 2*). 33M15 showed more callus induction % at 400 µM thiourea. 90-95% immature embryos produced callus. Almost zero callus induction % was recorded at 2400 µM *(Table 4*; *Fig. 5C).*

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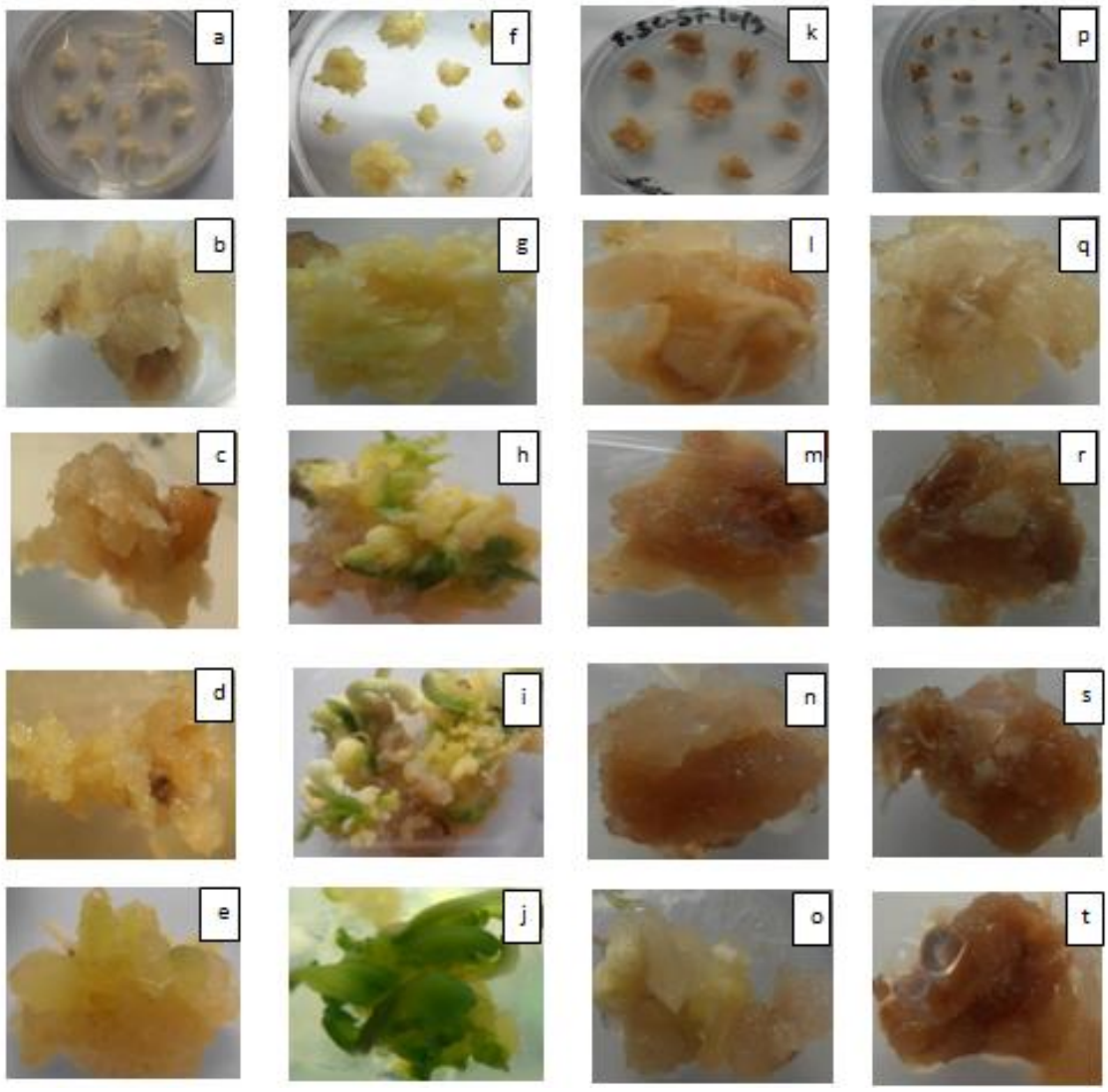

0µM Thiourea (a,b,c,d and e are 2nd, 4th, 6th, 8th, 10th week respectively)	400µM Thiourea (f,g,h,I and j are 2nd, 4th, 6th, 8th, 10th week respectively)	800µM Thiourea (k,l,m,n and o are 2nd, 4th, 6th, 8th, 10th week respectively)	1200µM Thiourea (p,q,r,s and t are 2nd, 4th, 6th, 8th, 10th week respectively)

Picture 2. *Data recorded at 2 weeks interval after 1st culture. Plate is arranged according to two week interval data for 33M15 hybrid.*

Table 4. *Mean squares from analyses of variance of data for callus attributes of Zea mays immature embryo when subjected to varying levels of thiourea for 8-weeks under control or saline conditions.*

Source of Variance	df	Direct emerging shoot %	Direct emerging root %	Callus induction %
Thiourea (TU)	12	4123.58***	7420.24***	10421.15***
Hybrid (H)	1	984.61***	10.15 **ns**	69.471154 **ns**
H × TU	12	105.83**	60.54**	58.794071**
Error	78	31.49	19.97	21.43

*, ** and *** = significant at 0.05, 0.01 and 0.001 levels respectively; ns = non-significant

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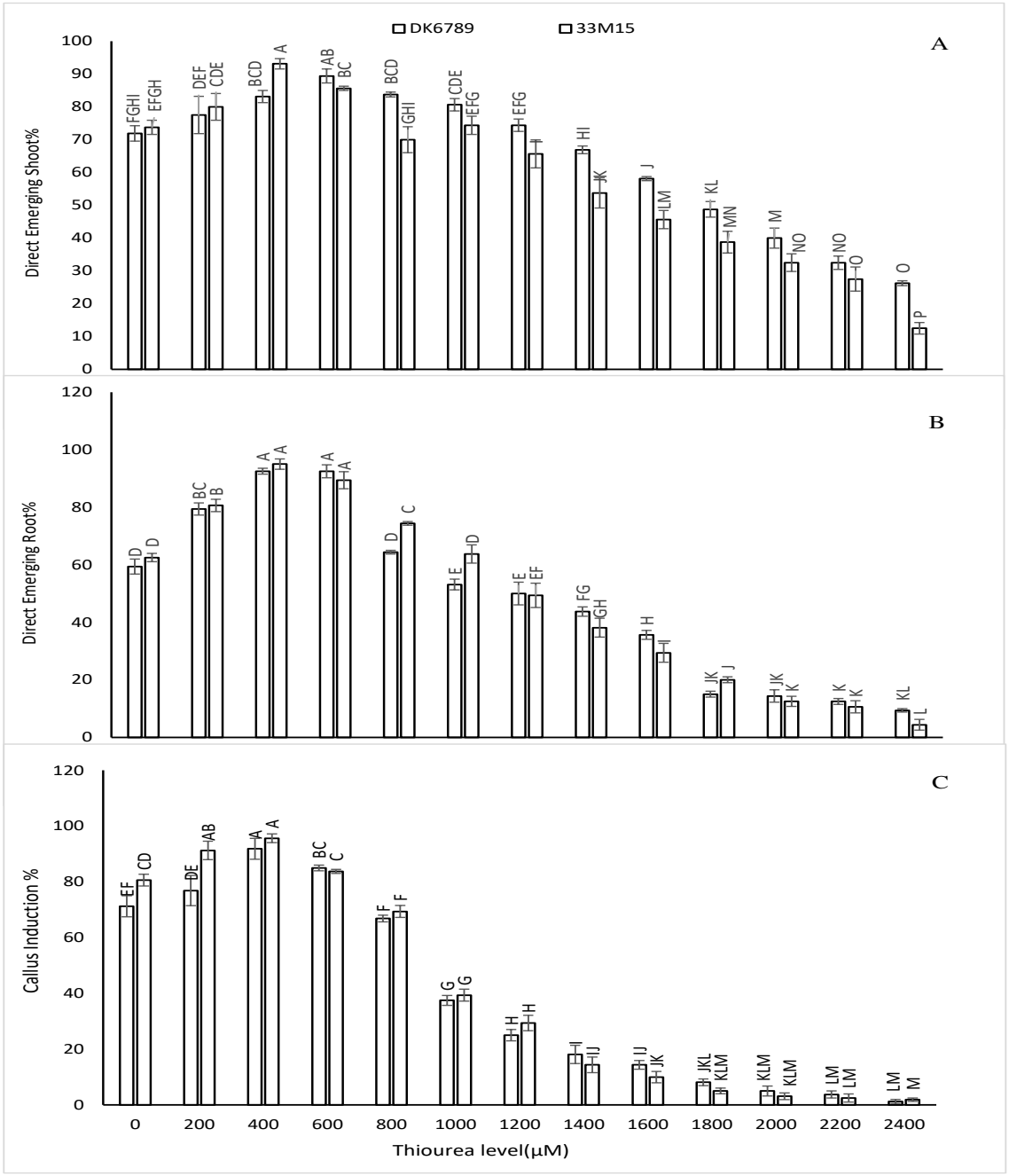

Figure 5. *Changes in direct emerging shoot and root and callus induction of two maize hybrids at different concentrations of thiourea*

Morphological characterization of calli

Callus-related traits like callus color, texture and proliferation rate were assessed for immature embryo-derived callus for different thiourea levels. These variations are marked in *Table 2*. Callus cultures induced from immature embryo of maize hybrids showed variation in callus proliferation, color and type under the influence of different thiourea levels. Hybrid 33M15 embryo-derived calli showed cream yellow and friable callus with maximum proliferation (++++) on 400-600µM thiourea level. While at

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higher levels at 1200 µM and above thiourea acted as a toxic agent towards callus growth and proliferation. While DK6789 showed maximum callus proliferation (++++) on 400-600µM thiourea level at 1000 µM and above thiourea started to hinder callus growth and proliferation. The lowest or zero potential for callus proliferation (-----) was observed at 2000 µM thiourea and totally stopped at 2400 µM thiourea. *Picture 2* is a plate of visual recordings of different thiourea treatments. Photographs were taken at 2 weeks interval for twelve consecutive weeks. Maximum callus proliferation with highest regeneration capacity was observed at 400 µM thiourea (*Picture 2 f,g,h,I,j*).

Table 2. *Morphological characterization of callus at different levels of thiourea*

Hybrids	Thiourea Tretments	Amount	Colour	Texture	Quality
DK6789	**0µ M**	+,++	Cream	Watery	Soft
	100 µM	+,++,+++	Cream Yellow	Watery	Soft
	200 µM	++,+++	Cream Yellow	Crystalline	Compact
	400 µM	+++,++++	Yellow	Crystalline	Compact
	600 µM	++++	Yellow	Crystalline	Compact
	800 µM	+++	Yellow	Crystalline	Compact
	1000 µM	+++	Yellow Brown	Crystalline	Compact
	1200 µM	++	Yellow Brown	Crystalline	Compact
	1600 µM	++	Brown	Crystalline	Compact
	1400 µM	+	Brown	Crystalline	Compact
	1800 µM	+	Brown	Watery	Soft
	2000 µM	------	Brown	Watery	------
	2200 µM	------	------	Watery	------
	2400 µM	------	------	Watery	------
33M15	**0 µM**	+,++,+++	Cream	Watery	Soft
	100 µM	+,++,+++	Cream Yellow	Watery	Compact
	200 µM	++,+++,++++	Cream Yellow	Crystalline	Compact
	400 µM	+++,++++	Yellow	Crystalline	Compact
	600 µM	+++,++++	Yellow	Crystalline	Compact
	800 µM	+++	Yellow	Crystalline	Compact
	1000 µM	++,+++	Yellow Brown	Crystalline	Compact
	1200 µM	++,+++	Yellow Brown	Crystalline	Compact
	1400 µM	+,++,+++	Brown	Crystalline	Compact
	1600 µM	+,++	Brown	Crystalline	Compact
	1800 µM	+,++	Brown	Watery	Soft
	2000 µM	+	Brown	Watery	Soft
	2200 µM	+	Brown	Watery	Soft
	2400 µM	**------**	Brown	Watery	Soft

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Discussion

Elevated levels of soluble salts in the soils as well as water are a pervasive threat in arid and semi-arid regions resulting in restricted growth and production of most of the crops. So, enhanced crop yield is of dire need to overcome food insecurity prevalent these days in many countries of the world. Despite the substantial importance of maize because of its multiple uses, the optimum yield of the crop is not being achieved due to a number of environmental factors including salinity stress. Salinity causes oxidative damage (Wahid et al., 2007; Farooq et al., 2008) but exogenous application of thiourea (it has imino and thiol functional groups) provides a ready source of nitrogen and thiol which has great role in alleviating oxidative damage in plants. Improved growth parameters in maize observed with thiourea application (Hassanein et al., 2015). Thiourea is a potential plant growth regulator (Flowers, 2004; Wahid, 2007; Farooq et al., 2009). Clear action or mode of action of thiourea is not understood yet but may be involved in chelation process or involved in storage in vacuole, mobilization of nutrients which indirectly enhances the biomass and accelerate growth by reducing oxidative damage (Ikram et al., 2014). Primary action of thiourea is to improve net assimilation efficiency of plant by alleviating salinity damage to photosynthetic area which is crucial for stress tolerance (Anjum et al., 2011). Thiourea application in root promotes development of roots, which has been identified as a major trait in giving salinity tolerance to sugarcane (Wahid et al., 1997), wheat (Mane et al., 2010), barley (Zaltauskaite and Sliumpaite, 2013) and in maize hybrids (Perveen *et al*., 2015).

Role of thiourea, in salinity tolerance with respect to growth was observed in selected maize hybrids. Salt stress caused a marked suppression in growth. However, exogenously medium supplemented varying levels of thiourea significantly promoted growth at both whole plant and cellular level. Of 13 varying levels (0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400 μM) of thiourea 400 μM was found most effective in improving growth of maize plants under saline regimes for 33M15. That thiourea application promoted development of roots which has been identified as a major trait in giving stress tolerance. Findings of this research indicated that under salinity stress, shoot and root length *(Fig. 1A, 1B),* fresh *(Fig. 2A, 2B),* and dry weights *(Fig. 3A, 3B),* leaf area *(Fig. 4A)* and shoot/root mass ratio *(Fig. 4B)* reduced and their damages were greatly improved by application of thiourea, better growth was observed in 33M15. Increased plant height, leaf area, dry matter is the result of thiourea application (Jharia, 2002). Thiourea is involved in chlorophyll biosynthesis, ultimately improving growth (Akram and Ashraf 2011a, b). Exogenous application of thiourea proved to be very effective in improving salinity tolerance in a variety of plants (Youssef and Awad, 2008). Increased fresh and dry weights with thiourea medium supplementation were observed due to accelerated cell multiplication (Mayer, 1956; Gibberd et al., 2002; Wahid, 2004).

Because of increasing population classical methods are not enough to meet a world food demand, that's why tissue culture and genetic engineering methods are, required (Jedidah et al., 2006). Genetic engineering is one of the options for the improvement of maize genetic manipulation techniques and it holds great promise in improving productivity percentage (Frame et al., 2002). In our research work, a highly reproducible tissue culture system has been established for selected maize genotypes. During this research hybrids performed differently. The study indicated that maize genotypes 33M15 was found best responsive genotype (*picture 2*). So 33M15 hybrid have the potential for future exploitation in transformation approaches for improved

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maize crop production. Exogenously applied inorganic salts and nutrient solutions are helpful in improving callus quality by enhancing tolerance and accelerating cell divisions (Sairam and Tyagi, 2004; James et al., 2006; Wahid, 2007; Ikram et al., 2014). In our findings 400 µM thiourea level was indicated as best among all levels *(Fig. 1,2 and 3).* Thiourea treatment significantly improved direct emerging shoot and root percentage and improved callus induction up to 90 percent *(Fig. 5A, 5B, 5C).*

Overall, thiourea application was highly effective for both *In Vivo* and *In Vitro* studies in improving the growth and physiological attributes under salinity. In case of tissue culture studies, it was found that medium supplemented thiourea improved the callus attributes, which were primarily linked to accelerated cell divisions. This suggested that thiourea has a specific mechanism for alleviating the adversaries of salinity.

Conclusions

Hybrid performed good at whole plant level also performed better at cellular level and optimum level of thiourea is same for *In Vivo* and *In Vitro* studies. Furthermore, as noted from the changes in most of the maize attributes in this research there is a need to explore the anticipated signaling properties of thiourea in gene expression of salt stressed maize.

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REFERENCES

[1] Abdelkader, A. F., Hassanein, R., Ali, H. (2012): Studies on effect of salicylic acid and thiourea on biochemical activities and yield production in wheat (*Triticum aestivum* var. Gimazaq) plant growth under drought stress. – African Journal of Biotechnology 11: 1728-12739.

[2] Ahmad, I., Akhtar, M. J., Asghar, H. N., Zahir, Z. A. (2013): Comparative efficacy of growth media in causing cadmium toxicity to wheat at seed germination stage. – International Journal of Agriculture and Biology 15: 517-522.

[3] Akram, N. A., Ashraf, M. (2011a): Pattern of accumulation of inorganic elements in sunflower (*Helianthus annuus* L.) plants subjected to salt stress and exogenous application of 5-aminolevulinic acid. – Pakistan Journal of Botany 43: 521-530.

[4] Akram, N. A., Ashraf, M. (2011b): Improvement in growth, chlorophyll pigments and photosynthetic performance in salt-stressed plants of sunflower (*Helianthus annuus* L.) by foliar application of 5-aminolevulinic acid. – Agrochimica 55: 94-104.

[5] Anjum, F., Wahid, A., Farooq M., Javed, F. (2011): Potential of foliar applied thiourea in improving salt and high temperature tolerance of bread wheat (*Triticum aestivum*). – International Journal of Agriculture and Biology 13: 251–256.

[6] Ashraf, M., Harris, P. J. C. (2004): Potential biochemical indicators of salinity tolerance in plants. – Plant Sciences 166: 3-16.

[7] Asthir, B., Thapar, R. Farooq M., Bains, N. S. (2013): Exogenous application of thiourea improves the performance of late sown wheat by inducing terminal heat resistance. – International journal of agriculture & biology 15: 1337–1342.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_001018

[8] Ayari, O. (2014): Effect of hydrogen peroxide and thiourea to the composition biochemical potato tubers (*Solanum tuberosum* L.). – Agriculture and Biological Sciences 11: 2-7.
[9] Bhardwaj, P., Chaturrvedi, A. K., Prasad, P. (2009): Effect of enhanced lead and cadmium in soil on physiological and biochemical attributes of *Phaseolus vulgaris* L. – Nature 7: 63-75.
[10] Chen, T. H., Murata, N. (2008): Glycinebetaine: an effective protectant against abiotic stress in plants. – Trends in Plant Sciences 13: 499-505.
[11] Cheng-Hao, Z., Li-Jun, Z., Chao, G. E., Kai, H. U. (2008): Establishment and optimization of the regeneration system of mature embryos of maize (*Zea mays* L.). – Agriculture and Sciences in China, 7: 1046 1051.
[12] Chu, H., Wang, P., Qu, J., Zhao L., Guan, S. (1975): Optimization of induction conditions of immature embryo callus for four maize inbred lines. – Journal of Henan and Agricultural Sciences 40: 26–29.
[13] Danson, J. W., Lagat, M., Mbogori, M. (2006): Screening tropical maize lines for the production and regeneration of friable and embryogenic type II callus. – African Journal of Biotechnology 5: 2367–2370.
[14] Farooq, M., Wahid, A., Kobayashi, N., Basra, S. M. A. (2008): Plant drought stress: effects, mechanisms and management. – Agronomy for Sustainable Development 29: 185-212.
[15] Farooq, M., Basra, S. M. A., Wahid, A., Ahmad, N., Saleem, B. A. (2009): Improving the drought tolerance in rice (*Oryza sativa* L.) by exogenous application of salicylic acid. – Journal of Agronomy and Crop Sciences 195: 237-246.
[16] Flowers, T. J. (2004): Improving crop salt tolerance. – Journal of Experimental Botany 55: 307-319.
[17] Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., Green, C. E., Phillips, R. L. (2002): Plant regeneration from tissue culture of maize. – Crop Sciences 15: 417-421.
[18] Gibberd, M. R., Turner, N. C., Storey, R. (2002): Influence of saline irrigation on growth, ion accumulation and partitioning and leaf gas exchange of carrot (*Daucus carota* L.). – Annals of Botany 9, 0: 715-724.
[19] Hakeem, K. R.,Ozturk, M., Memon, A. R. (2012): Biotechnology as an Aid for Crop Improvement to Overcome Food Shortage. – In: Ashraf et al. (eds.) Crop Production for Agricultural Improvement, pp. 239-261, Springer Verlag, NY,USA.
[20] Hassanein, R.A., Amin, A.A.E., Rashad, E.M., Ali, H. (2015): Effect of thiourea and salicylc acid on antioxidant defense of wheat plants under drought stress. – International Journal of Chemistry and Technology. 7: 346-354.
[21] Hassine, A. B., Bouzid, S., Lutts, S. (2010): Does habitat of Atriplex halimus L. affect plant strategy for osmotic adjustment? – Acta Physiologica Plantarum 32: 325-331.
[22] Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., Ahmad, A. (2012): Role of proline under changing environments: a review. – Plant Signaling and Behavior 7:1456–1466.
[23] Hoagland, D. R., Arnon, D. I. (1950): The Water-Culture Method for Growing Plants without Soil. – California Agriculture and Experiment Station Circular 347, 132.
[24] Ikram, S., Javed, F., Wahid, A., Ahmad, R. (2014): *In Vitro* relationship between cadmium stress and thiourea in two barley genotypes. – Pakistan Journal of Agriculture and Sciences 5:673-678.
[25] Ikram, S., Javed, F. (2015): Cadmium Stress Alleviation by Thiourea in Barley. – International Journal of Innovation and Applied Studies 12: 384-389.
[26] Jakubekova, M., Pretova, A., Obert, B. (2011): Somatic embryogenesis and plant regeneration from immature embryo induced callus of maize (*Zea mays* L.). – Journal of Microbiology, Biotechnology and Food Sciences 1: 478-487.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_001018

[27] James, J. J., Alder, N. N., Muhling, K. H., Lauchli, A. E., Shackel, K. A., Donovan, L. A., Richards, J. H. (2006): High apoplastic solute concentrations in leaves alter water relations of the halophytic shrub, *Sarcobatus vermiculatus*. – Journal of Experimental Botany 57: 139-147.
[28] Jedidah, W. D., Lagat, M. K., Mbogori, M. (2006): Screening tropical maize lines for the production and regeneration of friable and embryogenic type II callus. – African Journal of Biotechnology 5: 2367-2370.
[29] Jharia, A. (2002): Effect of iron fertilization and thiourea spray on the productivity of cluster bean [*Cyamopsis tetragonoloba* (L.) Taub]. – M. Sc. (Ag.) thesis, Rajasthan Agricultural University, Bikaner.
[30] Joshi, R., Shukla, A., Kumar, P. (2009): *In Vitro* flowering in hill maize: a novel technique for future. – Indian Journal of Plant Physiology 16: 299-302.
[31] Joshi, R., Shukla A., Kumar, P. (2010): Interactive effect of GA3 and Polyamines on *In Vitro* somatic embryogenesis from Immature Embryos in Maize (*Zea mays* L.). – Maydica 55: 111-119.
[32] Khan, M. A., Ungar, I. A. (2001): Role of dormancy regulating chemicals in release of innate and salinity induced dormancy in *Sporobolus arabicus*. – Seed Science Technology 28:29-37.
[33] Mane A. V., Karadge, B. A., Samant, J. S. (2010): Salinity induced changes in photosynthetic pigments and polyphenols of Cymbopogon Nardus. – Journal of Chemistry and Pharmacy Res. 2: 338-347.
[34] Mayer, A. M. (1956): The action of thiourea as a germination stimulator. – Journal of Experimental Botany 7: 93-96.
[35] Misra, N., Saxena, P. (2009): Effect of salicylic acid on proline metabolism in lentil grown under salinity stress. – Plant Sciences 177:181-189.
[36] Oduor, R. O., Njagi, E. N. M., Ndungu, S., Machuka, J. S. (2006): *In Vitro* regeneration of dry land Kenyan maize genotypes through somatic embryogenesis. – International Journal of Botany 2: 146–151.
[37] Ombori, O., Gitonga, N. M., Machuka, J. (2008): Somatic embryogenesis and plant regeneration from immature embryos of tropical maize (*Zea mays* L.) inbred lines. – Biotechnology 7: 224-232.
[38] Perveen, A., Wahid, A., Mahmood, S., Hussain, I., Rasheed, R. (2015): Possible mechanism of root-applied thiourea in improving growth, gas exchange and photosynthetic pigments in cadmium stressed maize (*Zea mays*). – Brazilian Journal of Botany 38: 71–79.
[39] Perveen, A., Wahid, A., Hussain, I., Rasheed, R., Mahmood, S. (2013): Growth bioregulatory role of root-applied thiourea: changes in growth, toxicity symptoms and photosynthetic pigments of maize. – Pakistan Journal of Agricultural Sciences 50: 455-462.
[40] Sairam, R. K., Tyagi, A. (2004): Physiology and molecular biology of salinity stress tolerance in plants. – Current Science 86: 407-421.
[41] Sharma, V., Ramawat, K. G. (2013): Salinity-induced modulation of growth and antioxidant activity in the callus cultures of miswak (*Salvadora persica*). – Biotechnology 3:11-17.
[42] Siddiqui, Z. S., Shaukat Zaman, S. S., A. U. (2006): Alleviation of salinity induced dormancy by growth regulators in wheat seeds. – Turkish Journal of Botany 30:321-330.
[43] Songstad, D. D., Armstrong, C. L., Petersen, W. L., Hairston, B., Hinchee, M. A. W. (1996): Production of transgenic maize plants and progeny by bombardment of Hi-II immature embryos. – *In Vitro* Cellular & Developmental Biology – Plant 32:179-184.
[44] Tripathi, R. D., Srivastava, S., Mishra, S., Singh, N., Tuli, R., Gupta D. K., Maathuis, F. J. M. (2007): Arsenic hazards: Strategies for tolerance and remediation by plants. – Trends in Biotechnology 25: 158-165.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_001018

[45] Wahid, A. (2004): Analysis of toxic and osmotic effects of sodium chloride on leaf growth and economic yield of sugarcane. – Botanical Bulletin-Academia Sinica 45: 133-141.
[46] Wahid, A. (2007): Physiological implications of metabolites biosynthesis in net assimilation and heat stress tolerance of sugarcane (*Saccharum officinarum*) sprouts. – Journal of Plant Research 120: 219-228.
[47] Wahid, A., Rao, A. R., Rasul, E. (1997): Identification of salt tolerance traits in sugarcane lines. – Field Crops Res. 54: 9-17.
[48] Wahid, A., Gelani, S., Ashraf, M., Foolad, M. R. (2007): Heat tolerance in plants: An overview. – Environmental and Experimental Botany 61: 199-223.
[49] Yonova, P., Gateva, S., Mincheva, N., Jovchev, G., Stergious, M., Kapchina-Toteva, V. (2009): Improvement of tolerance to paraquat in barley (*Hordium vulgare* L.) by a synthetic thiourea compound: effects on growth and biochemical responses. – General Applications of Plant Physiology 35:162-171.
[50] Youssef, T., Awad, M. A. (2008): Mechanisms of enhancing photosynthetic gas exchange in date palm seedlings (*Phoenix dactylifera* L.) under salinity stress by a 5-aminolevulinic acid-based fertilizer. – Journal of Plant Growth Regulations 27: 1-9.
[51] Zaltauskaite, J., Sliumpaite, I. (2013): Evaluation of Toxic Effects and Bioaccumulation of Cadmium band Copper in Spring Barley (Hordeum vulgare L.). – Environmental Research, Engineering and Management 64: 51-58.

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COMMUNITY ANALYSIS OF PLANT PARASITIC AND FREE LIVING NEMATODES ASSOCIATED WITH RICE AND SOYBEAN PLANTATION FROM PAKISTAN

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Abstract. Variety of nematode community indices have been proposed for purposes of environmental monitoring. During three phases of surveys (2005-08), thirty nine nematode genera other than root-knot nematodes were found associated with rice crop plants while 33 from soybean in the surveyed areas of rice and soybean. Nematodes were placed into five different groups (Yeates et al., 1993) viz., herbivores, fungivores, bacteriovores, omnivores and predators. In the present study highly abundant group is herbivores, followed by bacteriovores, fungivores, predators, and omnivores were encountered from rice and soybean fields. The occurrence percentage was observed in per 500 g soil for each nematode genus. All species of nematodes associated with soybean are first records of nematodes associated with soybean from Pakistan. Nematode communities were analyzed by mean abundance, prominence value, importance value and cluster analysis based on nematode numbers and presence or absence of nematode, respectively. Community relationship revealed the overall dominance of herbivores (*Hirschmanniella* and *Helicotylenchus*) and fungivores (*Aphelenchoides*) in rice and *Tylenchorhynchus* (herbivores), *Aphelenchoides* (fungivores), *Panagrolaimus* and *Acrobelus* (becteriovores) from soybean in all terms of community analysis. While the UPGMA cluster analysis showed that Multan and Dir localities exhibit close similarity coefficient in soybean and in rice Thatta and Sawat showed more similar coefficient or the nematode incidence
Keywords: *cluster analysis, glycine max, nematodes, survey, nematode community*

Introduction

Rice (*Oriza sativa* L.) is the staple food of more than two billion people, predominantly in Asia where more than 90% of the word' s rice is grown and consumed (Bridge etal., 1990). Worldwide, rice yield losses due to plant parasitic nematodes are estimated at 10% (Sasser and Freckman, 1987). The association of nematodes with rice in Punjab was reported by Anwar and Khan (1973) along with thirteen nematode genera including *Hirschmanniella oryza* and *Radopholus oryza*. Maqbool and Hashmi (1982) found plant parasitic nematodes in high frequency from rice growing areas of Pakistan. Maqbool (1983-84) reported *Tylenchorhynchus rassicae*, *T. clarus*, *T. mashoodi* and *Basiria graminicola*, respectively from soil around the roots of rice from Khyber Pakhtunkhawa (KP). Khan and Bilqees (1994) described a new species *Basiria bajorensis* from Bajore agency of paddy crops. Maqbool and Shahina (2001), was given biodiversity of nematode fauna of different Pakistani crops including rice. Musarrat et al. (2006) reported *M. incognita* from rice growing area of Sindh and KP, Pakistan. A review article on root knot nematode, Meloidogyne of Pakistan was given by Shahina et al. (2009).

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Soybean (*Glycine max* (L.) Merrill) is one of the most important oilseed crop in the world. The soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne* spp.) nematodes have received the greatest emphasis in breeding program, contributing up to 4% crop losses (Good, 1973). Maqbool (1981) in a general survey of crops reported the occurrence of root-knot nematode (*M. hapla*) on soybean. Severity of *M. incognita* on soybean was reported from Sindh and KP by Musarrat et al. (2006).More than 100 species of plant parasitic nematodes have been associated with soybean (Schmitt and Noel, 1984), the major pest species being *Heterodera glycines, Meloidogyne* spp., (*M.incognita, M. arenaria* and *M. javanica*) and *Rotylenchulus reniformis*. These nematodes are estimated to cause annual yield losses of over 10% to soybean on a worldwide basis (Sasser and Freckman, 1987).The aim of this study was to determine the percentage occurrence and community analysis of plant parasitic and free living soil nematodes.

Material and methods

Survey and sample collection

Extensive surveys were carried out from primary and secondary growing areas of rice and soybean from different agro ecological zones of Pakistan (except Balochistan) since 2005-2008. A total of 277 randomly chosen fields from 41 locations were visited and composite soil samples were taken from the rhizosphare of crop plants on each field (*Table 1, Fig.1A & B*). in KPK area, zone 1 includes northern mountainous areas of the country and irrigated rice is grown either in flat valleys.the climate is sub-humid monsoon with 750-1000 mm average rainfall,mostly concentrated in summer. While in Punjab area, zone 2 lies in the broad strip of land between rivers Ravi and Chenab where both canal and sub soil water are used for irrigation.the climate is sub humid, sub-tropical type with 400 to 700 mm of rainfall mostly in July-August. In Sindh area, Zone 4 includes Indus delta which consists of vast spill flats and basins. The climate is arid tropical marine with no marked season.

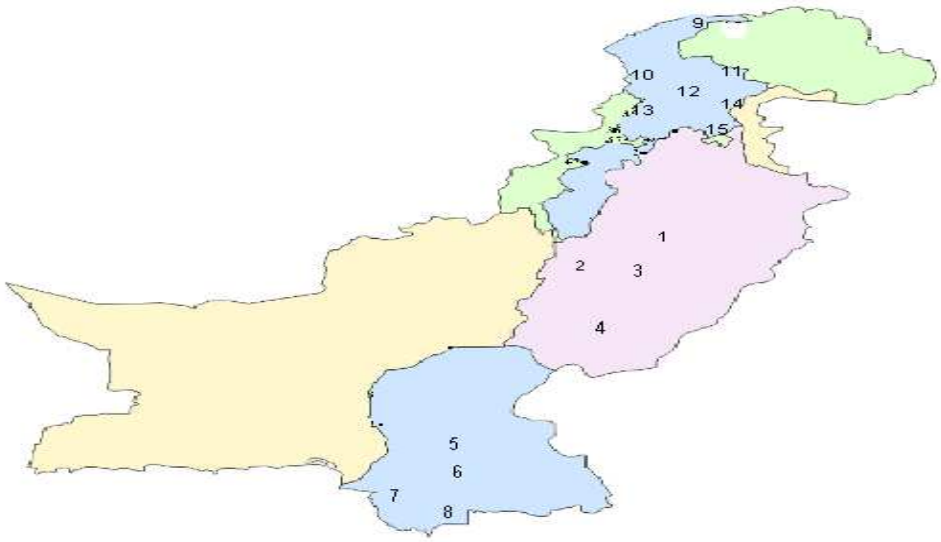

Figure 1A. *Geographic localities of soybean growing areas of Pakistan.*
Localities: 1-Faisalabad, 2-Multan, 3-Vehari, 4-Rahimyar Khan, 5-Sangharh, 6-Hyderabad, 7- Thatta, 8-Badin, 9-Kurram Agency, 10-Dir, 11-Swat, 12-Mansehra, 13-Malakand, 14- Abbottabad, 15-Hazara.

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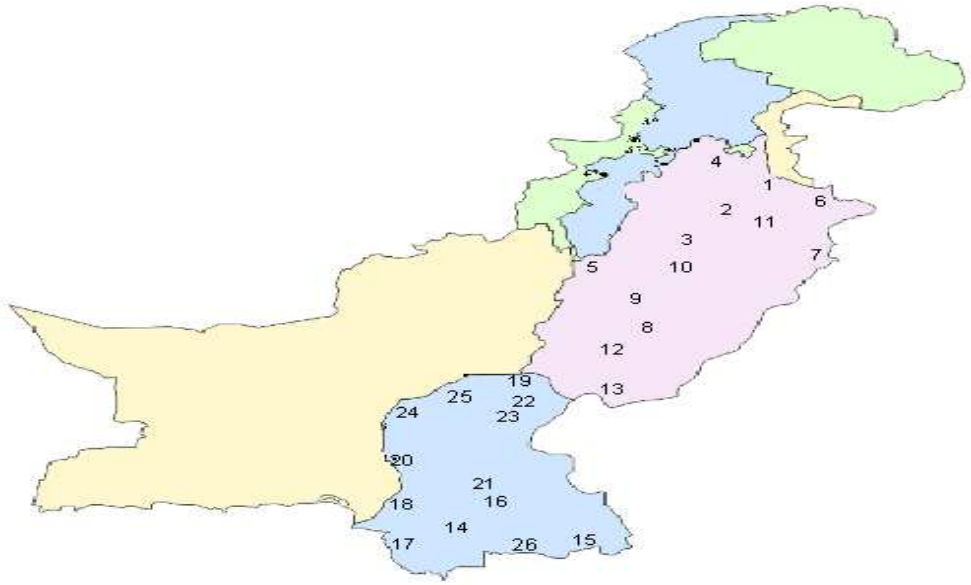

Figure 1B. *Geographic localities of rice growing areas of Pakistan.*
Localities: 1-Sialkot, 2-Gujranwala, 3-Sheikhupura, 4-Okara, 5-Jhang, 6-Narowal, 7-Kasur, 8-Pakpattan, 9-Sahiwal, 10-Faisalabad, 11-Hafizabad, 12-Vehari, 13-Rahimyar Khan, 14-Hyderabad, 15-Badin, 16-Sangharh, 17-Thatta, 18-Dadu, 19-Jaccobabad, 20-Larkana, 21-Nawabshah, 22-Sukkur, 23-Khairpur, 24-Shikarpur, 25-Nasirabad, 26- Gharo.

Extraction of nematodes

Extraction of plant parasitic and free living nematodes from soil was made by Cobb's sieving and Baermann funnel techniques (Baermann, 1917 and Cobb, 1918, respectively).

Quantitative analysis

For the community analysis nematodes were counted in an open counting chamber with only 5 ml extracted nematode suspension by a counter under binocular microscope. Process was repeated 3 times and average of three readings gave the number of nematodes per unit of soil.

Data analysis

Community analysis of phytoparasitic and free living nematodes in rice and soybean fields of Pakistan was done by the using of Norton techniques (1978).

The similarity matrix based on the quantitative analysis (presence (1)/absence (0) of nematodes, which used to establish the similarity between localities on the basis of Jacord's coefficient of similarity (Rohlf, 2005). Dandrogram constructed on the basis of data was related to the localities. All computations were carried out using the NTsys packages, version 2.2 (Rohlf, 2005).

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Table 1. *Surveyed localities of rice and soybean of Pakistan.*

S.#	Localities of rice	Latitude	Longitude	S.#	Localities of soybean	Latitude	Longitude
1	Rahimyar Khan	28°30`N	70°25`E	1	Multan	30°15`N	71°36`E
2	Sialkot	32°30`N	74°31`E	2	Vehari	29°15`N	71°30`E
3	Hafizabad	32°05`N	73°40`E	3	Faisalabad	31°25`N	73°09`E
4	Sialkot	32°15`N	74°52`E	4	Rahimyar Khan	28°30`N	70°25`E
5	Gujranwala	32°10`N	74°12`E	5	Hyderabad	25°23`N	68°24`E
6	Narowal	32°06`N	74°52`E	6	Badin	24°38`N	68°54`E
7	Jhang	31°15`N	74°22`E	7	Sangharh	26°20`N	68°57`E
8	Sheikhupura	30°32`N	71°80`E	8	Thatta	33°35`N	74°14`E
9	Sahiwal	30°45`N	73°80`E	9	Hazara	33°59`N	72°56`E
10	Okara	30°50`N	73°31`E	10	Swat	34°40`N	72°52`E
11	Vehari	29°15`N	71°30`E	11	Dir	35°12`N	71°53`E
12	Kasur	31°07`N	74°27`E	12	Kurram Agency	34°40`N	71°55`E
13	Pakpattan	31°21`N	73°24`E	13	Mansehra	34°25`N	71°50`E
14	Faisalabad	31°25`N	73°09`E	14	Malakand Agency	34°40`N	71°55`E
15	Nawabshah	26°15`N	68°25`E	15	Abbottabad	34°26`N	71°52`E
16	Sukkur	28°55`N	68°55`E				
17	Khairpur	27°06`N	87°44`E				
18	Sangharh	26°20`N	68°57`E				
19	Hyderabad	25°23`N	68°24`E				
20	Badin	24°38`N	68°54`E				
21	Thatta	33°35`N	74°14`E				
22	Dadu	26°06`N	67°45`E				
23	Jaccobabad	28°17`N	68°26`E				
24	Larkana	27°32`N	68°18`E				
25	Nasirabad	27°32`N	69°18`E				
26	Gharo	24°44`N	67°35`E				

Results

Percentage occurrence of nematode genera in rice plantations

The nematode genera encountered from rice plantations were identified and characterized into different groups as follows: Out of 39 nematode genera 15 belongs to harbivores viz., (*Basiria*, *Boleodorus*, *Criconemoides*, *Ditylenchus*, *Helicotylenchus*, *Heterodera*, *Hirschmanniella*, *Hoplolaimus*, *Longidorus*, *Merlinius*, *Paratylenchus*, *Pratylenchus*, *Psilenchus*, *Tylenchorhynchus* and *Xiphinema*); while four genera (*Aphelenchoides*, *Aphelenchus*, *Filenchus* and *Dorylaimus*) belong to fungivores. Six genera (*Clarkus*, *Ironus*, *Laimydorus*, *Mononchus*, *Mylonchulus* and *Oinchus*); and two genera (*Eudorylaimus* and *Mesodorylaimus*) of predators and omnivores were encountered respectively. Among these nematodes the second highest genera were bacteriovores (*Acrobelus*, *Cephalobus*, *Mesorhabditis*, *Diplogaster*, *Diploscapter*, *Discolaimium*, *Eucephalobus, Geomonhystera*, *Panagrolaimus*, *Plectus*, *Rhabditis* and *Zeldia*).

The most frequently occurring nematode genera were *Hirschmanniella*, *Aphelenchoides*, *Helicotylenchus* and *Tylenchorhynchus* from rice cultivated areas

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which were surveyed. These nematodes were found in relatively high densities as compare to other plant parasitic nematodes from surveyed areas of both provinces (*Table 2*).

Table 2. *Occurrence (percentage) of nematode genera regarding their diverse groups from rice fields of Pakistan during 2005-08.*

Trophic groups of nematode	Punjab			Sindh		
	2005-06	2006-07	2007-08	2005-06	2006-07	2007-08
Herbivores nematodes						
Basiria	3	4	1	2	3	0.99
Boleodorus	1	-	-	2.5	2	-
Criconemoides	1	0.9	-	-	-	1
Ditylenchus	10	12	9.8	5	3.9	6.3
Helicotylenchus	60	62	55	72	66	58
Heterodera	1	-	-	-	-	-
Hirschmanniella	66	58	62	90	95	88
Hoplolaimus	5	4	3	1	2.5	1.5
Longidorus	1	-	-	0.9	0.4	-
Merlinius	1	-	1.5	2	1.5	0.9
Paratylenchus	3.8	6	3	2	1	6.3
Pratylenchus	1	0.9	1	0.8	1.5	0.44
Psilenchus	1	-	-	1	0.9	1
Tylenchorhynchus	20	15	11	25	45	35
Xiphinema	2	1	3	1	-	-
Fungivores						
Aphelenchoides	60	45	73	70	71	62
Aphelenchus	32	30	18	25	40	20
Dorylaimus	2	1	3	3.3	4	2
Filenchus	1	0.9	0.8	-	-	1
Bacteriovores						
Acrobelus	25	20	18	40	39	33.2
Cephalobus	15	25	33	60	65	70
Mesorhabditis	20	15	23	35	38	33
Diplogaster	13	15	20	60	55	51
Diploscapter	8	10	7	15	13	11
Discolaimium	6	4	3	10	12	18
Eucephalobus	2	2.5	1	3	5	6.5
Geomonhystera	15	20	22	10	11	13
Panagrolaimus	18	11	19	23	42	40
Plectus	8	6	4	25	33	26
Rhabditis	15	13	10	8	6.5	10
Zeldia	15	22	23	11	13	20
Omnivores						
Eudorylaimus	1	-	-	3.5	4	2.5
Mesodorylaimus	3	1	1	1	2	1
Predators						
Clarkus	55	50	45	65	66	62
Ironus	1	1	-	0.8	0.6	1
Laimydorus	8	6	10	30	10	28
Mononchus	-	-	1	0.9	2	1
Mylonchulus	10	8	7	22	25	33
Oinchus	1	-	-	2	1	-

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The samples contained genera of diverse group with a range of 5 to 39 %. Studies on % occurrence of these groups revealed that herbivores dominated the entire nematode community in % occurrence (39%) followed by bacteriovores (31%), predators (15%), fungivores (10%) and omnivores (5%) (*Fig. 2A*).

In terms of overall % occurrence of nematode genera among five different groups, *Hirschmanniella* (herbivores) was most frequently occurred genus with the highest % occurrence (21%), followed by *Aphelenchoides* (fungivores) and *Helicotylenchus* (herbivores) sharing the same % occurrence (17%). The second highly abundance nematode genus was predator *Clarkus* (16%) followed by bacteriovores group with *Cephalobus* (16%), *Diplogaster* (10%) and *Panagrolaimus* (7%) in the surveyed areas of rice during three years (2005-2008) (*Fig. 2B*).

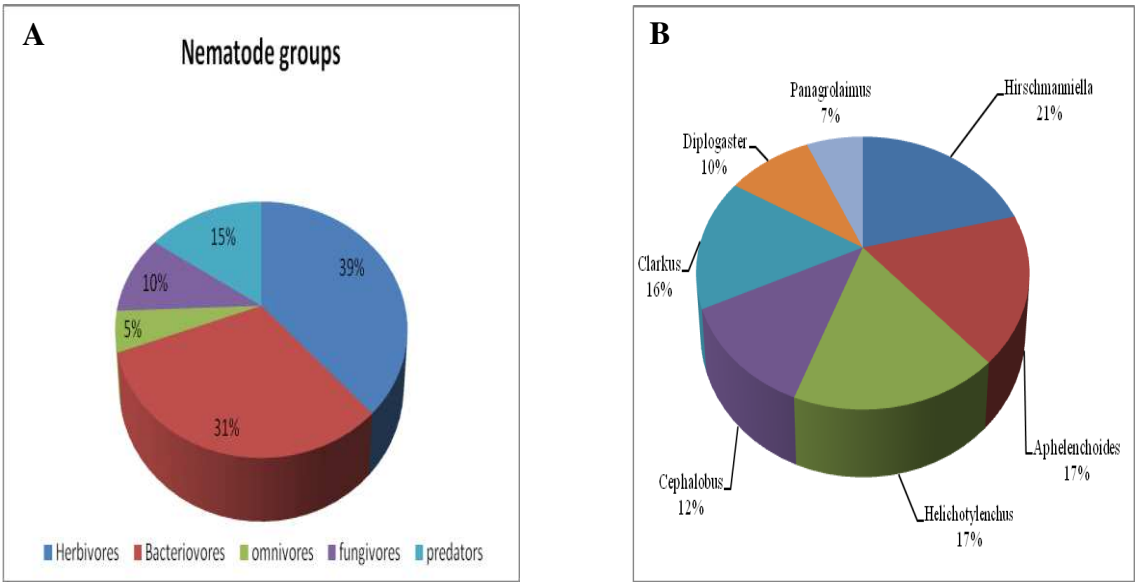

Figure 2. *A. Different groups of nematodes in rice; B. Occurrence (%) of nematode genera in rice surveyed areas.*

Percentage occurrence of nematode genera in soybean plantations

Soil analysis revealed the presence of 33 nematode genera encountered from soybean crop plantations during 2005 to 2008 surveys from Sindh, Punjab and KP (*Table. 3A-B; Fig. 3A-B*). These genera belonged to five diverse groups in which herbivores contain 15 nematode genera, fungivores with 6 genera; bacteriovores have 10 genera; predators contain 3 genera and only one genus representing the omnivores. Among these five groups herbivores dominated with the highest percentage occurrence (40%), followed by bacteriovores (30%), fungivores (18%), predators (9%) and omnivores (3%) in the nematode community structure (*Fig. 3A*).

In terms of overall % occurrence of nematode genera, *Helicotylenchus* (29%) was the most occurred (herbivores) genus whereas *Tylenchorhynchus* (herbivores, 17%) followed by *Aphelenchoides* (fungivores, 12%) were encountered in abundance from all fields of soybean crop .However other nematode groups such as bacteriovores: *Panagrolaimus* (18%), *Plectus* (14%) and *Acrobelus* (10%) frequently encountered from soybean soil samples (*Fig. 3B*).

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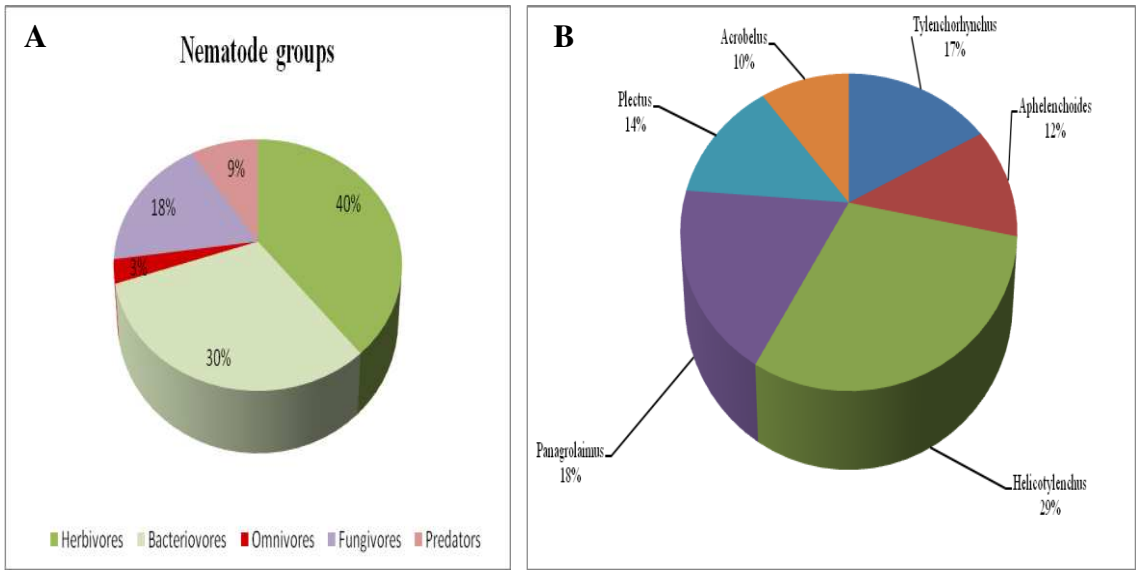

Figure 3. *A. Different groups of nematodes from soybean; B. % occurrence of nematode genera in soybean surveyed areas.*

Table 3A. *Percentage occurrence of nematode genera regarding their diverse groups from soybean fields of Pakistan during 2005-08.*

Nematode genera	**Punjab**			**Sindh**			**Khyber Pakhtunkhwa**		
Herbivores	2005-06	2006-07	2007-08	2005-06	2006-07	2007-08	2005-06	2006-07	2007-08
Boleodorus spp.	2	1	1	1	0.4	-	1	-	-
Criconemoides spp.	1	1	4	3.5	1	2	1	2.5	2
Ditylenchus spp.	7	10	10	8	5	3	-	2	-
Gracilacus spp.	-	-	-	1	0	2	-	-	-
Helicotylenchus spp.	36	60	53	42	55	63	41	33	52
Heterodera spp.	0.5	1	-	-	-	-	-	-	-
Hoplolaimus spp.	0.6	1	-	2	1	5	4	-	-
Longidorus spp.	0.2	1	-	-	-	1	0.4	0.2	-
Neodolichorhynchus spp.	-	-	-	3	5	-	2	5	3
Paratylenchus spp.	1	6	8	4	7	8	3	6	4
Psilenchus spp.	0.2	1	0.6	-	-	1	1	-	-
Tylenchorhynchus spp.	25	35	30	29	28	26	22	24	30
Xiphinema spp.	5	9	10	3	4	1	3	2	-
Fungivores									
Aphelenchoides spp.	25	30	15	20	22	28	15	16	-

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Aphelenchus spp.	15	20	30	23	34	37	20	10	10
Dorylaimus spp.	15	13	10	9	7	3	2	-	1
Discolaimus spp.	1	2	1	2	3	1	1	1.5	-
Filenchus spp.	4	3.6	5	3	2	1	1	3	1
Nygolaimus spp.	1	1.5	-	1	1	1	-	-	-

Table 3B. *Percentage occurrence of diverse groups of nematodes from soybean fields of Pakistan during 2005-08.*

Nematode genera	**Punjab**			**Sindh**			**Khyber Pakhtunkhwa**		
	2005-06	2006-07	2007-08	2005-06	2006-07	2007-08	2005-06	2006-07	2007-08
Bacteriovores									
Acrobelus spp.	12	10	19	25	36	30	8	6	5
Cephalobus spp.	30	25	20	15	19	20	15	13	12
Diplogaster spp.	5	8	20	15	14	18	6	8	3
Diploscapter spp.	1	0	1	0.4	0.5	-	1	-	-
Mesodorylaimus spp.	8	6	7	4	3	5	1	-	-
Panagrolaimus spp.	40	35	55	35	30	29	12	18	19
Plectus spp.	18	22	15	35	25	40	20	15	10
Rhabditids spp.	7	5	6.8	3	5	2	1	1.5	-
Rhabdolaimus spp.	3	2	1	1	3	4	1	-	-
Seleborca	1	2	1	0	0	1	2	1	1
Predators									
Laimydorus spp.	1	1	-	-	-	1	-	-	-
Mononchus spp.	5	6	8	2	4	6	1	3	5
Mylonchulus spp.	6	3	1	5	6	4	2	1	2
Omnivores									
Eudorylaimus spp.	-	5	2	8	10	15	4	2	-

Community analysis of nematode genera in rice fields

To exhibit the relationship of nematodes associated with host plants the community analysis was performed which provides information concerning frequency (absolute and relative frequency) and density (absolute and relative density) of nematodes on studied plant hosts.

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Community relationship revealed the overall dominance of herbivores (*Hirschmanniella* and *Helicotylenchus*) and fungivores (*Aphelenchoides*) in all terms of community analysis (*Table 4*).

Table 4. *Community analysis of plant parasitic and free-living soil nematodes in rice growing areas of Pakistan.*

Nematode genera	A.F	R.F	A.D	R.D	P.V
Plant parasitic nematodes					
Aphelenchoides spp.	27.9	6.2	233	5.9	16.7
Aphelenchus spp.	17.3	4.6	107	4.9	10.5
Basiria spp.	4.2	1.4	22	1.2	1.6
Boleodorus spp.	3.4	1.0	25	1.5	1.2
Criconemoides spp.	3.2	1.0	24	1.6	1.8
Ditylenchus spp.	3.8	1.8	66	3.3	3.2
Filenchus spp.	1.5	0.41	66	3.5	2.9
Helicotylenchus spp.	23	6.1	121	5.5	13.9
Heterodera spp.	3.6	2.0	8.0	2.1	2.0
Hirschmanniella spp.	66.2	12.8	253	9.8	33
Hoplolaimus spp.	15.3	5.1	110	4.9	1.2
Longidorous spp.	4.3	1.5	20	1.2	1.5
Merlinius spp.	1.6	0.51	39	1.6	1.14
Paratylenchus spp.	2.1	0.21	22	0.6	1.0
Pratylenchus	1.5	0.5	32	1.5	1.12
Psilenchus spp.	1.6	0.3	55	2.3	1.8
Tylenchorhynchus spp.	41.3	12.8	223	9.4	31
Xiphinema spp.	3.7	1.0	28	1.4	1.42
Free- living soil nematodes					
Acrobelus spp.	23.5	5.9	326	5.8	4.2
Cephalobus spp.	16.4	6.2	255	4.7	3.2
Cervidellus spp.	11.2	2.3	99	0.36	3.2
Clarkus	1.3	0.5	66	3.4	2.6
Diplogaster spp.	9.6	6.4	110	5.3	3.5
Diploscapter spp.	8.5	3.2	88	2.1	1.9
Discolaimium spp.	9.5	2.4	49	2.1	3.1
Dorylaimus spp.	1.9	0.34	26	1.3	2.8
Eucephalobus spp.	5.6	0.6	44	1.3	1.5
Eudorylaimus spp.	1.3	0.54	20	1.6	2.1
Geomonhystera spp.	9.8	1.2	33	2.3	2.3
Ironus spp.	8.5	2.4	22	1.6	1.3
Laimydorus spp.	1.1	0.5	15	1.32	2.4
Mesodorylaimus	8.3	3.1	79	2.0	1.8
Mononchus spp.	5.4	1.5	110	5.0	6.3
Mylonchulus	4.6	1.4	98.5	4.8	5.9
Oinchus spp.	3.2	0.4	9	1.0	1.1
Panagrolaimus spp.	14.2	5.6	99	2.3	2.1
Plectus spp.	25.3	5.8	206	5.6	7.3
Rhabditis spp.	11.3	2.5	76	3.3	2.6
Zeldia spp.	5.6	0.5	45	2.6	1.3

Absolute frequency (A.F) = (No of samples containing a species / Total samples collected) 100; Relative frequency (R.F) = (Frequency of a species in a sample/ Sum of frequencies of all species present) 100, Absolute density (A.D) = (No of individuals of a species in a sample/ volume of the sample) 100; Relative density (R.D) = No of individuals of a species in a sample / Total no. of individuals in the sample) 100; Prominence value (P.V) = (Absolute density/ Absolute frequency).

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Frequency

Hirschmanniella (AF= 66.2%) was the most prevalent genus in the entire plant parasitic nematode community, followed by *Tylenchorhynchus* (AF= 41.3%); *Aphelenchoides* (AF= 27.9%) and *Helicotylenchus* (AF= 23.0%), while the least prevalent genus was *Filenchus* (AF= 1.5%).

Among the free-living soil nematodes, *Plectus* (AF= 25.3%) and *Acrobelus* (AF= 23.5%) were the most prevalent genera. *Laimydorus* (AF= 1.1%) was the least prevalent genus in rice fields (*Table 4*).

Absolute density

Among all the nematode genera recorded, *Hirschmanniella* had maximum absolute density (AD= 253/ sample), followed by *Aphelenchoides* (AD= 233/ sample) and *Tylenchorhynchus* (AD= 223/ sample), while the genus *Longidorus* had lowest absolute density (AD= 20.0/ sample). In free-living soil nematode group studies, *Acrobelus* had the highest absolute density (AD= 326 / sample) followed by *Cephalobus* (AD= 255/ sample) and *Plectus* (AD= 206/ sample), while *Oinchus* had the lowest mean density 9 per sample.

Prominence value

The rice nematode genus *Hirschmanniella* had the highest prominence value (PV= 33.0), followed by *Tylenchorhynchus* (PV= 31.0), while the least PV was found in genus *Paratylenchus* (PV= 1.0). Among the free-living soil nematodes *Plectus* had maximum PV (7.3), followed by *Mononchus* (PV= 6.3), while the least prominence value was recorded in *Oinchus* (PV=1.1).

Community analysis of nematode genera in soybean plantations

Detailed analysis of different parameters for nematode community analysis has been provided in the *Table 5* for the soybean crop.

Frequency

Among the plant parasites, the most frequently encountered genus was *Tylenchorhynchus* (AF= 45.8%); followed by *Aphelenchoides* (AF= 45.3%), whereas *Boleodorus* was least frequent (AF= 1.3%) genus. Genus *Panagrolaimus* and *Acrobelus* were the most prevalent genera among the free-living soil nematodes with absolute frequencies (AF) 40% and 36%, respectively whereas the least frequent genus was *Diploscapter* (AF= 1.5%).

Absolute density

Genus *Tylenchorhynchus* had the highest absolute density (AD= 222), followed by *Aphelenchoides* (AD= 205) while the least density was of the genus *Gracilacus* (AD= 12). Among the free-living soil nematodes *Panagrolaimus* and *Acrobelus* were the most dominant genera in the entire nematode community in soybean fields with AD= 253 and AD=221, respectively. Whereas *Diploscapter* was the least dominant genus with AD= 22.

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Prominence value

Community analysis of plant parasitic nematodes associated with soybean crop revealed that highest prominence value of *Tylenchorhynchus* (PV= 32) and *Aphelenchoides* was noted as second most prevalent genus with PV= 31, while the least prominence values (PV= 1.1 and PV= 1.2) were recorded for *Filenchus* and *Longidorus* genera respectively.

In case of free-living soil nematodes maximum prominence value was recorded in genus *Acrobelus* (PV=33), while the least prominence value (PV=1.2) was recorded in the genus *Diploscapter* (*Table 5*).

Table 5. *Community analysis of plant parasitic and free-living soil nematodes in soybean growing areas of Pakistan.*

Nematode genera	A.F	R.F	A.D	R.D	P.V
Plant parasitic nematodes					
Aphelenchoides	45.3	12.4	205	9.4	31
Aphelenchus	15.3	5.5	106	4.8	10.5
Boleodorus	1.3	0.51	55	2.5	1.7
Criconemoides	3.8	1.3	38	1.6	1.6
Ditylenchus	3.7	1.0	33	1.4	1.4
Filenchus	1.9	0.5	35	1.5	1.1
Gracilacus	1.6	0.21	12	2.1	1.6
Helicotylenchus	20	7.3	109	5.5	9.8
Heterodera	5.5	1.5	44	5.0	6.2
Hoplolaimus	3.8	1.0	31	1.4	1.3
Longidorous	1.9	0.5	23	1.3	1.2
Neodolichorhynchus	2.9	1.0	30	4.3	1.4
Paratylenchus	3.7	1.5	36	1.5	1.5
Psilenchus	1.8	0.51	45	2.6	1.3
Tylenchorhynchus	45.8	11.8	222	9.5	32
Xiphinema	5.7	1.5	96	4.0	5.2
Free-living soil nematodes					
Acrobelus	36	2.5	221	8.9	33
Cephalobus	22	5.6	110	3.6	7.5
Diplogaster	1.9	2.5	33	2.4	2.1
Diploscapter	1.5	0.5	22	1.3	1.2
Discolaimus	1.8	0.4	39	2.6	1.7
Dorylaimus	5.7	1.5	110	5.0	6.2
Eudorylaimus	4.9	1.3	102	4.8	5.5
Laimydorus	1.9	2.6	59	2.6	2.1
Mesodorylaimus	7.6	2.0	93	4.2	6.1
Mononchus	3.8	1.9	35	1.5	1.5
Mylonchulus	2.2	1.5	40	2.0	1.6
Nygolaimus	3.6	1.2	69	2.8	2.9
Panagrolaimus	40	11.3	253	5.6	12
Plectus	18.3	4.3	196	4.6	10.7
Rhabditis	5.3	2.3	59	2.6	2.1
Rhabdolaimus	5.8	1.6	39	1.2	1.5
Seleborca	1.6	0.5	45	2.4	1.5

Absolute frequency (A.F) = (No of samples containing a species/ Total samples collected) 100; Relative frequency (R.F) = (Frequency of a species in a sample/ Sum of frequencies of all species present) 100; Absolute density (A.D) = (No of individuals of a species in a sample/ volume of the sample) 100; Relative density (R.D) = No of individuals of a species in a sample/ Total no. of individuals in the sample) 100; Prominence value (P.V) = (Absolute density/ Absolute frequency).

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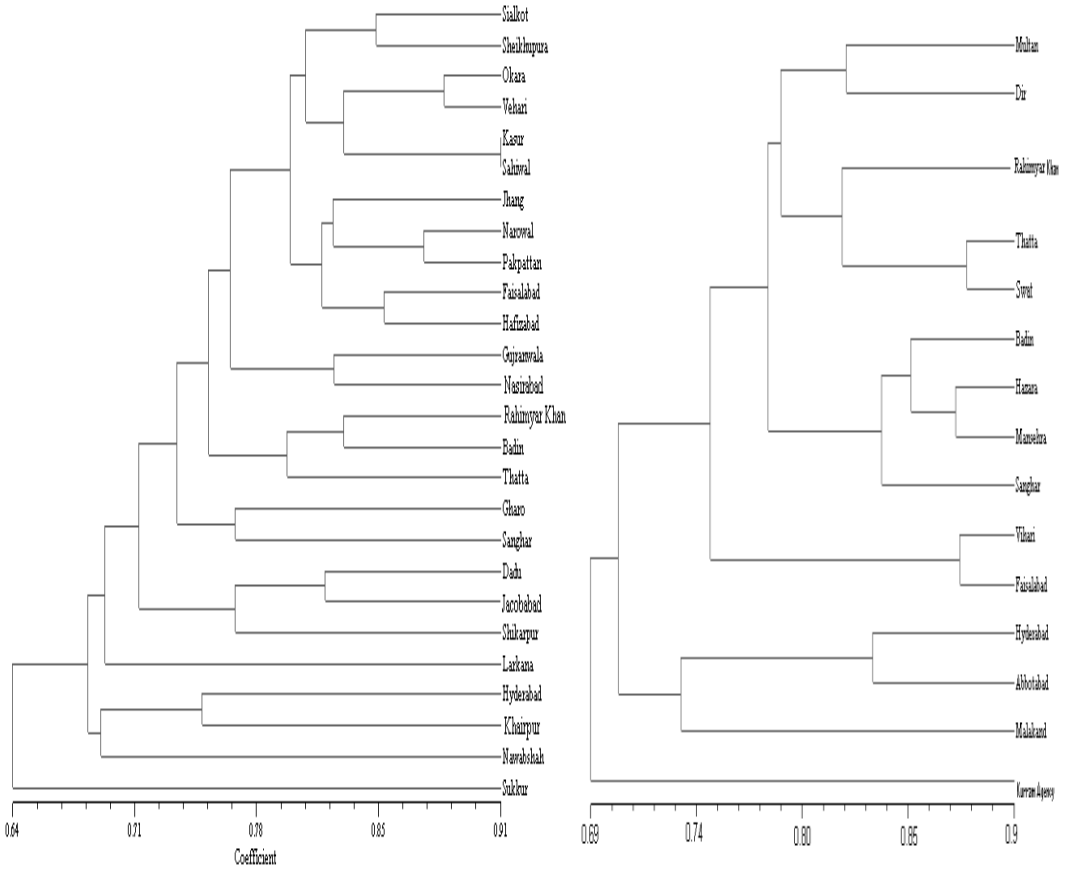
Cluster analysis of rice and soybean localities

A similarity matrix based on the proportion of shared nematode community was used to establish the level of relatedness between the different rice and soybean localities of Pakistan. Similarity of closest areas was estimated ranged from 0.64 to 0.91. Two localities of rice Kasoor and Sahiwal (Punjab) were the closest localities for the presence of plant parasitic nematodes with the other tested localities; whereas, Nasirabad and Sukkur (Sindh) showed very low similarities (64 %) for the all nematode genera, but both localities have same nematode genus i.e., *Hirschmanniella*.

Similarities of nematode density obtained from quantitative data were used to create a cluster diagram. Cluster analysis, based on Nei and Li's similarity coefficients using UPGMA dendrogram, showed that all 26 rice growing localities of Pakistan could be easily distinguished based on the information generated by the community analysis of nematodes.

As expected, localities were distinguished in 9 cluster groups corresponding to the Sindh and Punjab localities, with additional sub clusters and a few independent localities (*Fig. 4*). In cluster analysis those areas which soil samples having abundant density of nematodes genera showing a high frequency of nematode relatedness. While observing the clustering pattern, it is evident that in most of the cases the diversity level among the localities of the rice areas in close proximity is very narrow, forming small sub-clusters with very little linkage distances. Low difference within certain locations may be due to shorter history of cultivation.



Figures 4 and 5. *Dendrogram resulting from average linkage of 26 localities of rice and 15 localities of soybean growing areas of Pakistan based on nematode communities*

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A similarity matrix based on the presence/absence of nematodes data was used to construct the ratio of similarity between the different soybean growing localities of three provinces of Pakistan. Similarity coefficients among various soybean growing areas of Pakistan ranged from 0.69 to 0.90 were presented.

The UPGMA cluster analysis showed that all 15 soybean growing areas could be easily distinguished based on the information generated by the presence or absence of nematodes. As expected, areas were separated into 5 distinct groups. Group A lines comprised with the similarity coefficient of 0.82 (82 %). The localities of Multan and Dir showed close similarity coefficient. Thatta and Swat 0.87 (87 %) show more similar coefficient for the nematode incidence. Kurram Agency did not fall in any group, might be this area having dissimilar nematode fauna /or climate conditions and would have some difference of nematode density (*Fig. 5*).

Discussion

Nematodes co-exist together in different environment (Boag and Yeates, 1998), however, their frequency, density and diversity varies depending upon ecological and edaphic factors (Sohlenuis, 1979; Khatoon et al., 2001). Nematodes may form the most significant group for community indicator analysis because more information exists on their taxonomy and feeding groups (Gupta and Yeates, 1997) than for other macrofauna. Free-living nematodes are very important and beneficial in the decomposition of organic material and the recycling of nutrients in soil. Nematode bacterivores and fungivores do not feed directly on soil organic matter, but on the bacteria and fungi which decompose organic matter. The presence and feeding of these nematodes speed up the decomposition process. Their feeding recycles minerals and other nutrients from bacteria, fungi, and other substrates and returns them to the soil where they are accessible to plant roots.

Detailed study has revealed that host associated nematodes were more in rice than soybean cultivated areas of Pakistan. A total of 39 genera were identified from rice while 33 genera were recorded from soybean from 41 localities throughout Pakistan. Among these nematodes, *Hirschmanniella* spp., was predominant and reduced yield production Tiwari and Kumar (1996) reported *Hirschmanniella* spp., infection in irrigated rice. Many researchers described the *Hirschmanniella* spp., on rice fields from various parts of the country (Maqbool and Shahina, 2001). Rose et al. (1967) reported that *Tylenchorhynchus claytoni* suppressed yield of soybean by 21 % in microplots and Ahmad et al. (2001) studied the occurrence of plant parasitic nematodes in soybean fields of Bundel khand region. Also adding to the parasitic load were *Aphelenchoide*s spp., *Helicotylenchus* spp., and *Tylenchorhynchus* spp., which were found in large number of samples of rice and soybean examined. However, present findings are in confirmation of the results of previous surveys carried out by many researchers that worked on similar objectives viz., Khan and Shaukat (1999), Ahmad and Nadeem (1983), Sher (1963), Robbins et al. (1982).

On the basis of trophic composition nematodes were grouped in five categories: herbivores, bacteriovores, omnivores, fungivores and predators. Herbivores representing the highest number of genera and also the highest abundance in both rice and soybean crops. While within the taxonomic groups, *Hirschmanniella* was the most frequently encountered genus (21%) dominating the entire nematode community in rice and it could be related to its cosmopolitan nature of occurrence and specific host (rice). Similarly, *Helicotylenchus* was the most frequently occurring genus in soybean which may be due to its cosmopolitan and polyphagous nature. *Aphelenchoides*, *Clarkus* and *Cephalobus*

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showed comparatively high frequency and density than for other trophic group of nematodes due to mono-cropping. In rice and soybean herbivores showed highest abundance which was related to the abundance of other trophic groups. It may be concluded that a highly significant positive correlation exists between herbivores species of both rice and soybean samples. These relationships are broadly in line with findings of the Debabrata et al. (2007) and Yeates et al. (1993) who reported that free-living bacteriovorous, fungivorous and omnivorous species comprise 52 % of the total nematode genera and 26 % of the terrestrial genera. Higher population of herbivores may also be attributed to the fact that, the excessive use of fertilizers and other manures, add excessive nutrient to the soil. These findings are similar to the study of Tomar et al. (2006).

Cluster analysis was also performed based on nematode communities. The resulted dendogram showed that diversity level among the 26 localities of the rice areas in close proximity is very narrow, while the UPGMA cluster analysis showed that all 15 soybean growing areas could be easily distinguished. The localities of Multan and Dir showed close similarity coefficient. Thatta and Sawat showed more similar coefficient or the nematode incidence. Kurram Agency did not fall in any group and observed to be more distantly related to all groups.

REFERENCES

[1] Ahmad, R., Nadeem, A. (1983): Varietal susceptibility to rice plant nematodes. 294-302pp. - In: Proceeding of 4th National Seminar on Rice Research and Production. NARC, Islamabad.

[2] Ahmad, F., Srivastav, A.K., Pandey, R. (2001): Occurrence of plant parasitic nematodes in soybean field of Bundelkhand region. - Flora and Fauna Jhansi (7):88.

[3] Anwar, S.A., Khan, I.U. (1973): Association of nematodes with rice in Punjab. - Journal of Agricultural Research (11): 111-113.

[4] Baermann, G. (1917): Eine einfache method zur Auffindung Von Ankylostomum (Larven) in Erdproben Geneesk. - Tijdschr. Ned. Ind. 57: 131-137.

[5] Boag, B., Yeates, G.W (1998): Soil nematode biodiversity in terristerial ecosystems. - Biodiversity and Concervation 7:617-630.

[6] Bridge, J., Michel, L., Plowright, R.A. (1990): Nematodes parasites of rice. 69- 108 pp. - In: Luc, M., Sikora, R. A., Bridge, J. (eds.) Plant parasitic nematodes in subtropical and tropical agriculture, CAB International, Wallingford, UK.

[7] Cobb, N.A. (1918): Estimating the nema population of soil. Agricultural Technical Circular, US Department of Agriculture, 48 pp.

[8] Debabrata, S., Amalendu, C., Buddhadeb, M. (2007): A report on the relative abundance of Tylenchida, Dorylaimida and Mononchida population of guava orchards in 24-paraganas (South), West Bengal, India. - Indian Journal of Nematology (37):72-77.

[9] Ettema, C.H. (1998): Soil nematodes diversity: Species coexistence and ecosystem. - Journal of Nematology 30(2):159-169.

[10] Good, J.M. (1973): Nematodes. 527-543 pp. - In: Caldwell, B.E. (ed) Soybeans: Improvement, production and uses., American Society of Agronomy.

[11] Gupta, V.V.R., Yeates, G.W. (1997): Soil microfauna as bioindicators of soil health. 201-234 pp. - In: Doube, B.M., Gupta, V.V.R. (eds) Biological Indicators of Soil Health., CAB International, Wallingford, UK.

[12] Khan, A., Bilqees, F.M. (1994): Plant parasitic nematodes associated with paddy crops in Bajore Agency, Pakistan. 1-3 pp. - Proceeding of Pakistan Congress of Zoology, University of Karachi, Karachi.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_019033

[13] Khan, A., Shaukat, S.S. (1999): Survey of stylet bearing nematodes associated with rice (*Oriza Sativa* L.) in Thatta district, Sindh, Pakistan. - Sarhad Journal of Agriculture (15): 221-225.
[14] Khatoon, M., Sharma, S., Saxena, S.K. (2001): Community analysis of predatory nematodes of Rohilkhand division, UP. - Current Nematology (12): 11-14.
[15] Maqbool, M.A. (1981): Occurrence of root-knot and cyst nematodes in Pakistan. - Nematologia Mediterranea (9): 211-212.
[16] Maqbool, M.A. (1983): Description of Paktylenchus tuberosus gen. n., sp.n. (Nematoda: Tylenchinae) from Pakistan. - Journal of Nematology (15):76-79.
[17] Maqbool, M.A. (1984): Nematode diseases of rice in Pakistan and their control. - National Nematological Research Centre, University of Karachi, Urdu version.
[18] Maqbool, M.A., Hashmi, S. (1982): Nematode associated with *Oryza sativa* L. in Pakistan. - 21st Annual Meeting of Society of Nematologists, 26-30 July, 1982. Knoxuille, Tennesse, USA.42 pp.
[19] Maqbool, M.A., Shahina, F. (2001): Systematics and distribution : Biodiversity of nematode fauna in Pakistan. - National Nematological Research Centre, University of Karachi,Karachi-75270, Pakistan.179 pp.
[20] Musarrat, A.R., Firoza, K., Shahina, F. (2006): Study of root-knot nematodes in NWFP and Sindh, Pakistan. - Pakistan Journal of Nematology (24):1-7.
[21] Nei, N., Li, W. (1979): Mathematical model for studing genetics variation in term of restriction endonucleases. - Acta Hortica (262): 305-310.
[22] Norton, D.C. (1978): Ecology of plant parasitic nematodes. - John Wiley and Sons, New York, 268 pp.
[23] Robbins, R.T., Riggs, R.D., Von Steen, D. (1982): Phytoparasitic nematodes surveys of Arkansas cotton fields, 1986-1988. - Journal of Nematology (21): 619-623.
[24] Rohlf, F.J. (2005): NTSYS-pc. Numericaltaxonomy and multivariate analysis system. Exeter Software, New York.
[25] Sasser, J.N., Freckman, D.W. (1987): Aworld perspective of Nematology: The Role of the Society. 7-14 pp. - In: Veech, J.A., Dickson, D.W. (eds.) Vistas on Nematology, Society of Nematologists, Hyattsville, Maryland, USA.
[26] Schmitt, D.P., Noel,G.R. (1984): Nematode parasites of soybean. 13-59 pp. - In: Nickel, W.R. (ed.) Plant and Insect Nematodes. - Marcel Dekker, New York.
[27] Shahina, F., Zarina, B., Firoza, K., Soomro, M.H., Javed, N. (2009): Research on root-knot nematodes, Meloidogyne by Pakistani scientists: A review with description of species prevalent in Pakistan. - Pakistan Journal of Nematology (27):1-115.
[28] Sher, S.A. (1963): A revision of the *Hoplolaimus* (Nematoda) II. *Hoplolaimus* Daday, 1905 and *Aorolaimus* n.gen. - Nematologica (9):267-295.
[29] Sohlenuis, B. (1979): Acarbon budget for nematodes, rotifers and tardirades in a Swedish coniferous forest soil. - Holarctic Ecology (2):30-40.
[30] Timm, R.W. (1955): The occurrence of *Aphelenchoides besseyi* Christie, 1942 in deep water paddy of East Pakistan. - Pakistan Journal of Science (7):47-49.
[31] Timm, R.W. (1956): Nematode parasites of rice in East Pakistan. Pakistan Review of Agriculture (2): 115-118.
[32] Tiwari, S.P., Kumar, S. (1996): Root knot nematode *Hirschmanniella Oryza* (Sottwedel, 1889) Lucand Goodey, 1963 associated with *Cyperus* (L.)Species in some rice growing districts of Madhya Pradesh, India. - Weed News (3):9-14.
[33] Tomar, V.V.S., Baniyamuddin, M., Ahmad, W. (2006): Community structure of soil inhabiting nematodes in a mango orchard at Aligarh, India. - International Journal of Nematology (16): 89-101.
[34] Yeates, G.W., Bongers, R.G.M., Goede, R.G.M., Freckman, D.W., Georgieva, S.S. (1993): Feeding habits in soil nematodes families and genera an outline for soil ecologists. - Journal of Nematology 25: 315-331.

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ANTIOXIDANT ACTIVITY AND SECONDARY METABOLITES IN SELECTED VEGETABLES IRRIGATED WITH SEWAGE WATER

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Abstract. The study was conducted to investigate the impact of various concentration of domestic waste water on growth, physiological, and biochemical characteristics of tomato, okra and pumpkin. Sewage water was applied of 50% and 100% on vegetables grown under field conditions. Irrigation with tubewell water was considered as control. At maturity data for growth attributes was recorded. Maximum fresh weight of root, shoot and fruit was in tomato at 50% sewage water treatment followed by control. Dry weight also exhibited a considerable increase at 50% sewage water application. However, a decline in chlorophyll a, b and carotenoid contents was observed at 100% sewage water. Antioxidants and secondary metabolites increased and their maximum values were recorded at 100% sewage treatment in all the three vegetables.
Keywords: *domestic effluents, contamination, flavonoid, growth, MDA*

Introduction

Shortage of fresh water resources is a serious issue, due to which resource poor countries are using marginal quality water for irrigation purposes. Waste water may contain some nutrients useful for agriculture, however, its continuous application increases toxic metals in plants and soil (Rattan et al., 2005). Sewage water also contains industrial wastes and toxic metals, polluting soil and food chain (Khan et al., 2008). In Pakistan inadequacy of irrigation water comes up with the integrative use of ground water and industrial and sewage effluents for agriculture (Khan et al., 2013). Major cities of Pakistan produce sewage 116,590 million gallons/day irrigating 32,000 ha of land (FAO, 2002; Musa et al., 2013).

Tomato (*Solanum lycopersicum* L.), okra (*Abelmoschus esculentus* (L.) Moench) and pumpkin (*Cucurbita pepo* L.) are a vital part of human diet, because they are important

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source of nutrients like proteins, fiber, vitamins, iron and calcium (Ullah et al., 2009). Waste-water irrigation results in elevated metal uptake by most of the vegetables grown particularly in peri-urban areas thus metals become a vital part of food chain (Farooq et al., 2008). Waste water irrigation may cause growth stage dependent sensitivity in vegetables (Baksh, 2005). It has been estimated that approximately 1/10th of the global population is considered to eat food from plants irrigated with wastewater (Kouser and Samie, 2009).

Consequently, it is imperative to look deep into the prospects of sewage water irrigation for managing this nutrient rich resource. On the other hand, unveiling specific relationship between fertigation with waste-water and a crop is also important for appropriate application (Kumar et al., 2010). Therefore, the present study was executed to evaluate the role of waste-water fertigation for vegetable growth, development and sustainable production. Additionally, incorporation of the present knowledge into fertigation systems will likely to be a promising strategy for optimizing crop productivity in irrigation scarce areas.

Materials and methods

A field experiment was performed in Pakpattan district, Punjab, Pakistan to evaluate the effect of domestic effluents on morpho-physiological and biochemical attributes in tomato (*Solanum lycopersicum* L.), okra (*Abelmoschus esculentus* (L.) Moench) and pumpkin (*Cucurbita pepo* L.).

Field preparation

The field was prepared by dividing the main plot into three sub plots of 3x3 m^2 and digging the soil upto 1 foot. The three sub plots were separated by polythene sheet, filled with clay and sand in 1:1 ratio. The experimental design was split plot. The treatments applied were: control (tube well water), 50% (sewage water blended with tube well water) and 100 % sewage water.

Seeds were collected from AARI (Ayub Agricultural Research Institute) Faisalabad, Pakistan and sown in the appropriate growing season.

Water analysis

Physico-chemical analysis of waste water (*Table 1*) indicated that the EC values recorded were higher than the suitable limit for most of the crops (EPA, 1991). The pH was slightly alkaline (United State Salinity Laboratory staff, 1954). Analysis of waste water indicated the presence of toxic metals were more than the permissible limits. Cadmium was much higher than the recommended value. Similarly Cu, Pb, and Zn were in higher concentrations.

Table 1. *Physico-chemical characteristics of waste-water used for irrigation*

Parameters	Unit	Waste water	Recommended values
pH		7.3	6.0- 8.5
EC	$mScm^{-1}$	3.73	3
Carbonate	$meqL^{-1}$	2.4	
Bicarbonate	$meqL^{-1}$	5.1	
Heavy metals/ Ions	$mgkg^{-1}$	-	

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Zn	-	3.145	≤ 2.0
P	-	13.78	
Cd	-	0.625	< 0.01
K	-	115	
Fe	-	0.519	≤ 5.0
Pb	-	7.432	< 5.0
Cu	-	1.11	< 0.2

Physico-chemical characteristics of the soil

The pH of soil was 7.3, slightly alkaline and was in safe limit with reference to standard limit of 8.5 (*Table 2*). EC value of soil was 2.1 mS/cm, which was in the safe limit according to the standard permissible limit (Ilaco, 1985; MAAF, 1988; CCME, 2007; WHO, 2007). In soil, heavy metals were also in the safe limit.

Table 2. *Physico-chemical properties of the soil*

Parameters	Unit	Soil	Safe limits
Texture		Sandy loam	
pH		7.3	≤ 8.5
EC	$mScm^{-1}$	1.9	2-4
Heavy metals/ Ions	$mgKg^{-1}$	-	
Zn	-	3.01	300
K	-	1400	-
Fe	-	2.35	-
P	-	40.1	300
Cd	-	0.21	3
Cu	-	17	140
P	-	41.1	

Fresh and dry weight

The fresh weight and dry weight was measured in grams for shoot, root and fruit by using electrical balance. For dry weight samples were kept in an oven at 70 ºC for 72 hours.

Pigment analysis

The fresh leaves (0.5 g) were homogenized in chilled acetone (80%). Centrifuge (3000 rpm/10 mints) at 4 ºC. Separate the supernatant and measure absorbance at 663,645 and 480 nm respectively with spectrophotometer. Chlorophyll contents were determined by the method of Arnon (1949) and carotenoid contents were calculated as described by Kirk and Allen (1965).

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Antioxidant enzymes

Fresh leaves (0.5 g) of tomato, okra and pumpkin were ground in 8 ml of 50 m*M* phosphate buffer pH (7.8). The homogenate was centrifuged at 15000 x g for 20 min at 4 ºC. The supernatant was used for the assay of enzymes activity.

Catalase (CAT)

The activity was assayed in a 3 ml reaction solution containing phosphate buffer (50 m*M* ,7.0 pH), H_2O_2 (5.9 m*M*) and 0.1 ml of enzyme extract as described by Chance and Maehly (1955). A decline in activity of catalase enzyme due to H_2O_2 consumption was measured at 240nm absorbance after every 20 sec. A one unit catalase activity defined as a change in absorbance of 0.01 unit min^{-1}.

Peroxidase (POD)

The activity was determined as the peroxidation of H_2O_2 and guaiacol as an electron donor (Chance and Maehly, 1955). Add phosphate buffer (50 m*M*, pH 5), 20 m*M* of guaiacol, H_2O_2 (40 m*M*) and 0.1 mL enzyme extract in a reaction solution. Formation of tetra-guaiacol resulted in an increase in the absorbance at 470 nm measured after every 20 sec. One enzyme unit was the amount responsible for an increase in OD value of 0.01/ 1 min. The activity was expressed as unit min^{-1} g^{-1} fresh weight basis.

Superoxide dismutase (SOD)

It was determined by the method of Giannopolitis and Ries (1977) by measuring the inhibition rate of nitroblue-tetrazolium (NBT) reduction by xanthine oxidase acting as hydrogen peroxide generating agent. The absorbance was measured at 560 nm by using a UV-visible (IRMECO U2020) spectrophotometer. One unit activity reflected 50% photochemical inhibition of NBT.

Ascorbate peroxidase (APX)

The activity was monitored by a decrease in ascorbic acid absorbance at 290 nm (extinction coefficient 2.8 mM cm-1) in 1 ml reaction mixture containing phosphate buffer (50 m*M,* pH 7.6), 0.1 m*M* Na-EDTA, 12 m*M* H_2O_2, 0.25 mM ascorbic acid as described by Cakmak, (1994).

Malondialdehyde (MDA)

The estimation was done by using the method of Camak and Horst (1991). Ground fresh leaves (1g) in 20ml tri-chloroacetic acid (0.1%) and centrifuge at 12000g for 10 min. Take 1 ml of the supernatant and added 4 ml of 20 % TCA comprising 0.5% thiobarbituric acid and then it was heated for 30 min., at 95^{o}C in a water bath and then immediately cooled on ice. After centrifuge for 10 min., at 12000 g, the absorbance of the supernatant was read at 532 and 600 nm. The contents of MDA were calculated using extinction coefficient of 155/ (mM/cm) with the help of formula:

$$\text{MDA level (nmol)} = \Delta\ (\text{A 532nm-A 600nm})/1.56\times10^{5} \qquad \text{(Eq.1)}$$

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Total leaf phenolics content

To the 5 ml Folin-Ciocalteu reagent (formerly diluted with water 1:10 v/v) along with 4 ml (75 g/L) of Na_2CO_3, added the plant extract was added. The tubes were vortexed for 15 sec and allowed them to stay for 30 min at 40 °C for developing color. The absorbance read at 765 nm using Folin-Ciocalteu method (Wolfe et al., 2003). The absorbance was expressed as mg g^{-1} tannic acid and the amount worked out by using the equation based on the following calibration curve:

$y = 0.1216x$, $r^2 = 0.9365$, x was the absorbance, y the tannic acid equivalent (mg/g)

Total flavonoid contents

The contents were determined with the help of spectrophotometer by using the method of Park et al. (2008). Total flavonoid contents were expressed as mg of rutin equivalents per gram of dried fraction.

Statistical analysis

The results were statistically analyzed by using the software program Statistix 8.1 at $P \leq 0.05$. Means and standard errors were assessed on Microsoft Excel -2007 Version and the significance of means was tested at 5% probability level using Least Significant Difference test.

Results

Sewage water applied in 100% concentration reduced plant fresh weight in tomato (*Solanum lycopersicum* L.), okra (*Abelmoschus esculentus* (L.) Moench) and pumpkin (*Cucurbita pepo* L.) (*Fig. 1*). Application of 50% concentration of sewage water resulted in an increase in fresh weight in shoot of okra and pumpkin by 36% and 16%. In tomato 50 and 100% sewage reduced shoot fresh weight from control (773 g) to 630 and 352 g respectively. However, 50 % domestic sewage water increased fresh weight in tomato, okra and pumpkin (*Fig. 1*). Irrigation with 100% sewage water decreased root fresh weight by 4%, 8% and 43% in pumpkin, tomato and okra, respectively. Fruit fresh weight decreased in the order as 50% sewage water >control >100% sewage water.

Polluted water significantly affected shoot dry weight of all vegetables (*Table 3.*). A decrease in dry weight at 100% concentration of sewage water was observed in tomato, okra and pumpkin by 30%, 20% and 13%, respectively. At 100% sewage water dry weight of fruit was 99 g in tomato and 42g in okra. Different concentrations of sewage water also affected root dry weight (*Fig. 1*). An increase of 20, 28 and 60% in root dry weight was observed in tomato, okra and pumpkin, respectively as compared to that in control. In general tomato plants had the highest root dry weight 25g at 50% sewage water and pumpkin roots had minimum 5g at 100% sewage water. In okra and pumpkin, dry weight of fruit increased at 50% concentration of sewage water over control (*Fig. 1*). Dry weight of fruit in tomato, okra and pumpkin decreased by 38, 17 and 32% respectively at 100% sewage water application. Overall, maximum dry weight of fruit was in pumpkin (6 g) at 50 % sewage water application.

A decline in chlorophyll contents was observed at 50 and 100 % application of domestic sewage water in all the vegetables (*Fig. 1*). Reduction in tomato, okra and

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pumpkin at 50% sewage water was 12%, 30% and 32% respectively and at 100% sewage water it was 40%, 56% and 21% as compared to control.

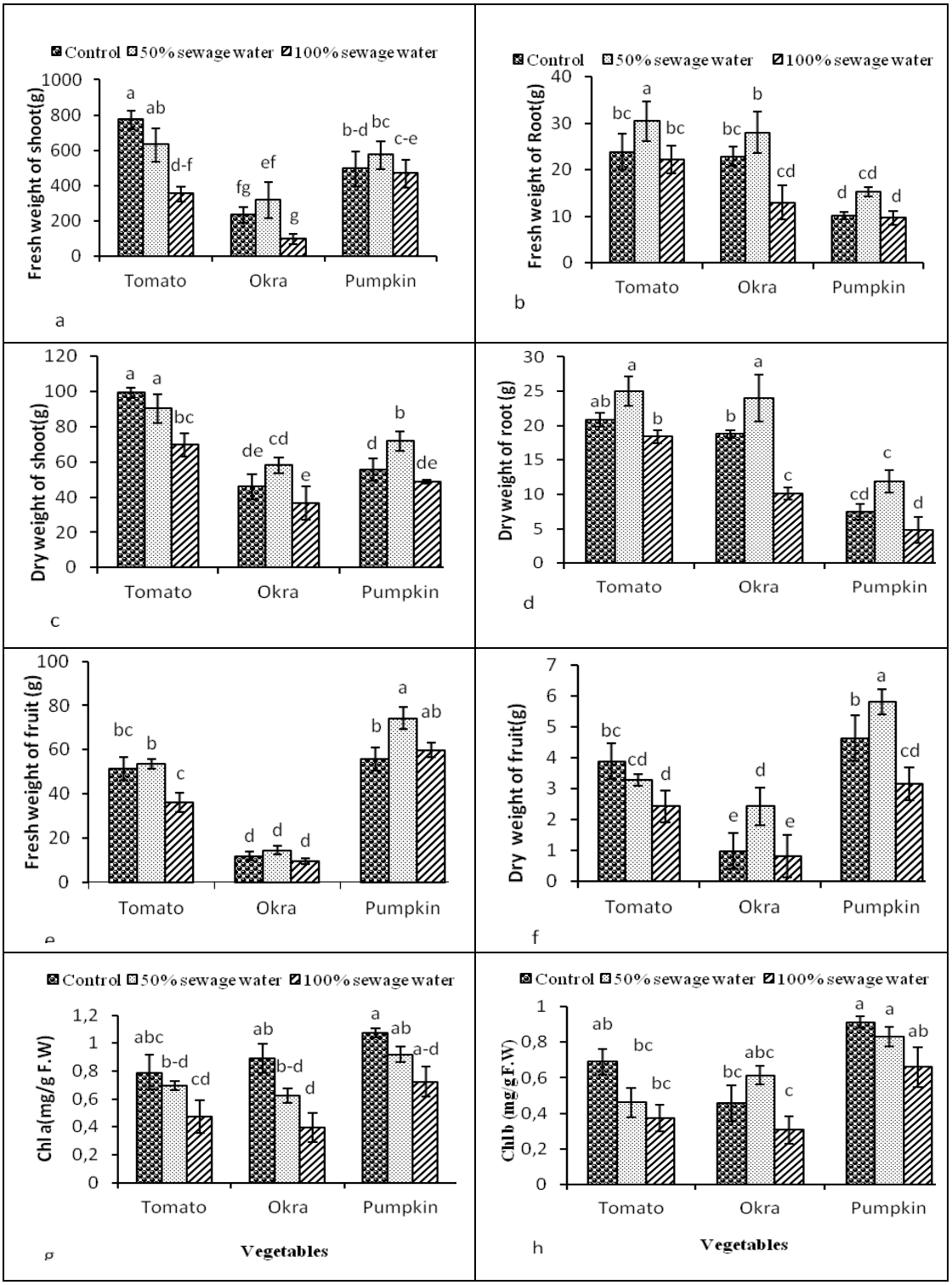

Figure1. *Effect of domestic sewage water on morpho-physiological attributes of tomato, okra and pumpkin. Bars with different letters in each group show significant difference at $p < 0.05$*

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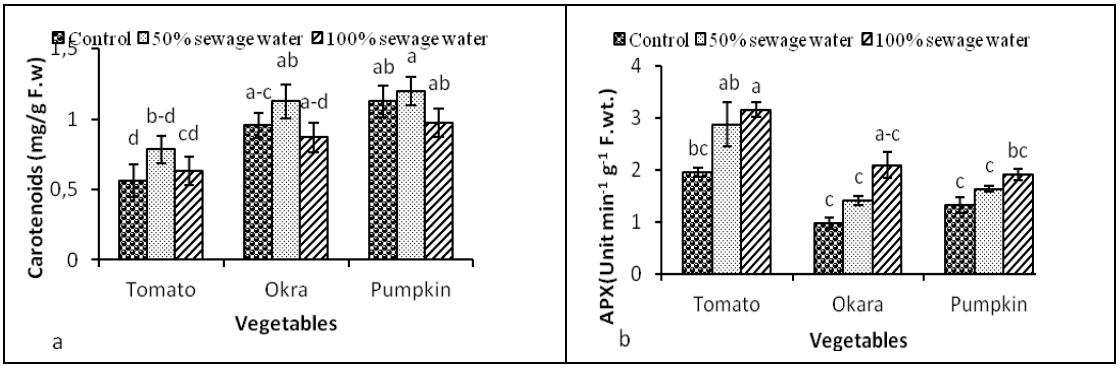
Reduction in chlorophyll *b* contents was 0.6 to 0.4 and 0.9 to 0.8 mg/g respectively at 50% waste-water treatment in tomato and pumpkin (*Fig. 1*). However, an increase in chlorophyll *b* contents from 0.4 to 0.6 mg/g was observed in okra at 50% waste-water treatment. At 100% irrigation of polluted water reduction in chlorophyll *b* was 0.6 to 0.37, 0.4 to 0.31 and 0.9 to 0.66 mgg^{-1} respectively in tomato, okra and pumpkin. An increase in the carotenoid contents at 50 and 100 % concentration of domestic sewage water was observed in all three vegetables. At 50% irrigation of polluted water, the carotenoid contents increased from 0.5 to 0.7, 0.9 to 1.1 and 1.12 to 1.2 mg ml^{-1} in tomato, okra and pumpkin, respectively.

Sewage water irrigation with 50 and 100% increased the ascorbate peroxidase activity in all three vegetables. Generally the APX activity at 100% sewage water in tomato was 3.15 (min^{-1} g^{-1} F. wt.) and 1.908 (min^{-1} g^{-1} F. wt.) in okra. A significant increase in the SOD activity 247 to 276 (min^{-1} g^{-1} F. wt.) was observed in tomato with irrigation of sewage water. At 100% sewage water the SOD activity was the highest in tomato 276(min^{-1} g^{-1} F. wt.) and the least in okra 176 (min^{-1} g^{-1} F. wt.).

Peroxidase activity increased with the irrigation of different concentrations of sewage water in all the vegetables (*Fig. 2*). Sewage water at 50% irrigation increased the POD activity in pumpkin, tomato, okra and from 57 to 68, 91 to 108 and 52 to 75 (min-1 g-1 F. wt.) respectively. The highest POD activity at 50% irrigation with domestic sewage water was 108 (min-1 g-1 F. wt.) in tomato and the least was in okra 68 (min-1 g-1 F. wt.). Irrigation with 50% domestic sewage water increased the catalase activity from182 to 199 and 69 to 101 (min^{-1} g^{-1} F. wt.) in tomato and pumpkin, respectively. An increase in MDA content was observed in pumpkin 43% and 49%, tomato 69%, 117% and okra 76%, 111% over control under 50% and 100%. The order of increase in flavonoid contents was 100% sewage water > 50 % sewage water >control. Irrigation of 50% and 100% sewage water increased the phenolic content in okra 27%, 33%, tomato 56%, 91% and pumpkin 46% and 96 % over control (*Fig. 2*). Overall, the highest concentration of phenolics was found in tomato followed by okra and pumpkin.



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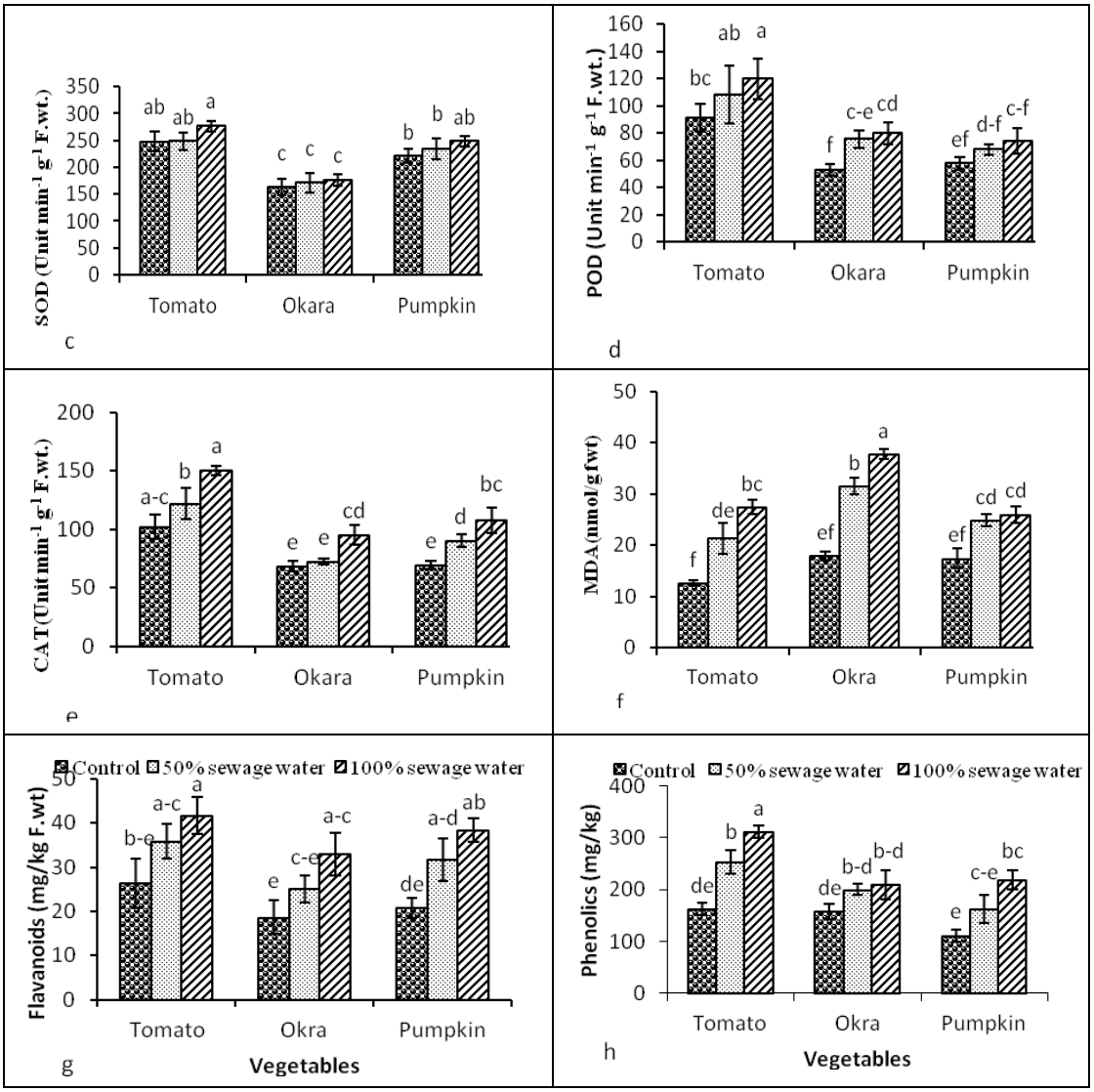

Figure 2. *Effect of domestic sewage water irrigation on antioxidant activities of tomato, okra and pumpkin irrigated with sewage water. Bars with different letters in each group show significant difference at $p < 0.05$.*

Discussion

Application of 100 % sewage water reduced the fresh and dry weights of tomato (*Solanum lycopersicum* L.), okra (*Abelmoschus esculentus* (L.) Moench) and pumpkin (*Cucurbita pepo* L.). Similar, decrease in biomass was observed with the application of 20 and 30 % polluted water in tomato plants (Saeed and Ahmed, 2009). A decrease in fresh biomass of shoot under sewage application was also investigated in wheat (Kakar et al., 2010). Irrigating *Leucaena leucocephala* with polluted water reduced growth and plant height (Hassan and Ali, 2013). Toxic metals may accumulate in the foliage parts of the plant, disturbing the physiological and biochemical activities in plant that ultimately reducing the growth (Sing and Agrwal, 2007). Physico-chemical analysis of waste water (*Table 2*) indicated that the EC value may cause increase in the soil salinity which may cause reduction of plant growth (Iqbal et al., 2013).

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A reduction in chlorophyll contents was recorded in tomato, okra and pumpkin. Chlorophyll *b* exhibited more decline than chlorophyll *a*. Similar, reductions in chlorophyll contents were exhibited by *Beta vulgaris* subjected to sewage water irrigation (Sing and Agrawal, 2010). According to Marwari and Khan (2012) irrigation of tomato plants with 20 and 30 % sewage water badly affected the chlorophyll contents, indicating that the decrease may be due to reduction of chlorophyll biosynthesis under stress (Bamniya et al., 2010).

Carotenoid contents increased in all vegetables due to sewage water irrigation and this increase was more pronounced at 100 % level of domestic sewage water irrigation. Increase of carotenoid contents is considered, a defense role in plants to alleviate metal stress (Sinha et al., 2007).

The antioxidants like SOD, APX, CAT and POD also showed an increment in activity (*Fig. 2*), responsible for scavenging of reactive oxygen species (ROS) (*Table 4*) (Noctor and Foyer, 1998). They play a defensive role against oxidative stress and indicators of metal uptake (Radotic et al., 2000). Similar results are observed in palak (*Beta vulgaris* var All Green) irrigated with different concentrations of sewage water (Singh and Agrawal, 2007). According to Ashraf (2009) antioxidants detoxify H_2O_2 (ROS) into H_2O (water) into O_2 (Sairam *et al*., 2005) and relieve the oxidative damage in plants caused by ROS. Total phenolics and flavonoids behave like antioxidants and also act in H_2O_2- scavenging system. Under multiple stresses, phenolic metabolism induction is observed in plants (Michalak, 2006). Flavonoids play an important role in plant-environment interactions under low concentrations (Fini et al., 2011). An increase in phenolic compounds was observed in *Albizia lebbek* under heavy metal stress (Tripathi and Tripathi, 1999). In the present work irrigation with waste-water increased phenolic contents and flavonoids with maximum values recorded at 100% concentration of sewage water (*Fig. 2*). Similarly, nickel and aluminum contents resulted increase in phenolic contents in wheat (Diáz et al., 2001) and maize (Winkel-Shirley, 2002). In biological systems, the presence of malondialdehyde as oxidation products is directly related to the peroxidation of unsaturated fatty acids constituting cellular membranes (Turton et al., 1997). Plants irrigated with waste-water exhibited higher MDA concentration, as compared to those irrigated with fresh water. Heavy metals cause peroxidation of lipid membranes due to the formation of ROS and free radicals, leading to enhanced permeability and oxidative stress to the plants (Nada et al., 2007; Zhang et al., 2007).

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Table 3. *Mean squares values from analysis of variance (ANOVA) of data for morpho-physiological attributes of tomato, okra and pumpkin treated with sewage water*

SOV	df	Shoot fresh weight	Root fresh weight	Shoot dry weight	Root dry weight	Fruit fresh weight	Fruit dry weight	Chla	Chlb
Variety (V)	2	571588.54****	1160.066***	6207.34***	711.846***	10425.6***	36.933***	0.342*	0.512**
Treatment (T)	2	196299.68***	1087.189***	1869.59***	315.384***	573.6**	11.044**	0.564**	0.238*
Interaction (VxT)	4	54865.411*	345.412**	277.57*	24.379ns	189.3ns	2.199**	0.0139ns	0.048ns
Error	36	15577.445	75.983	110.71	12.515	163.2	0.595	0.086	0.0622
Total	44								

*, **, ***= Significant at 0.05, 0.01, 0.001 levels, ns= non-significant

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Table 4. *Mean squares values from analysis of variance (ANOVA) of data for antioxidants of tomato, okra and pumpkin treated with waste water.*

SOV	df	Carotenoid contents	APX	SOD	POD	CAT	MDA	Flavanoids	Phenolics
Variety (V)	2	0.781***	3.701***	3.701***	1354.182**	3228.694***	160.267***	185.258*	14129.317***
Treatment (T)	2	0.177ns	2.119***	2.119***	4466.678***	5272.703***	502.708***	559.065***	23921.799***
VxT	4	0.017ns	0.133ns	0.239ns	48.058ns	122.394ns	30.536*	4.584	1871.005ns
Error	36	0.071	0.174	0.879	160.809	75.297	7.800	49.047	1061.001
Total	44								

*, **, ***= Significant at 0.05, 0.01, 0.001 levels, ns= non-significant

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Conclusion

Irrigation with waste-water is a common technique for cultivation of crops including vegetables in third world countries. It enhanced the biomass of tomato, okra and pumpkin on 50% dilution. However, its use as a nutrient source depends on the type of a crop grown, fertility level of soil and nutrients concentration. Waste water should be diluted with fresh water in areas having shortage of irrigation water. But still it should be checked for its heavy metal contents to avoid risk on human and animal lives.

REFERENCES

[1] Arnon, D I. (1949): Copper enzyme in isolated chloroplasts polyphenol oxidase in Beta vulgaris. - Plant Physiolgy 24: 1-15.

[2] Ashraf, M. (2009): Biotechnological approach of improving plant salt tolerance using antioxidants as markers. - Biotechnology advances 27(1): 84-93.

[3] Bakhsh, A., Kanwar, R.S., Karlen, D.L. (2005): Effects of liquid swine manure applications on NO 3–N leaching losses to subsurface drainage water from loamy soils in Iowa. - Agriculture, Ecosystems & Environment 109(1):118-128.

[4] Bamniya, B. R., Kapasya, V., Kapoor, C. S. (2010): Physiological and biochemical studies on the effect of waste water on selected crop plants.- Biological Forum International Journal 2 (2): 1-3.

[5] Cakmak, I. (1994): Activity of ascorbate-dependent H_2O_2-scavenging enzymes and leaf chlorosis are enhanced in magnesium-and potassium-deficient leaves, but not in phosphorus-deficient leaves.- Journal of Experimental Botany 45(9): 1259-1266.

[6] Camak, I., Horst, W. J. (1991): Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soyabean (*Glycine max*).- Physiologia Plantarum 83(3): 463-468.

[7] CCME. (2007): Canadian soil quality guidelines for the protection of environmental and human health. Canadian Council of Ministers of the Environment, distribution in plants of lactuca sp. Ca-Mn interaction.- Journal of Plant Science 162: 761-767.

[8] Chance, B., Maehly, A. C. (1955): Assay of catalase and peroxidase.- Methods in Enzymology 2: 764-775.

[9] Diaz, J., Bernal, A., Pomar, F., Merino, F. (2001): Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annum* L.) seedlings in response to copper stress and its relation to lignification. -Plant Science 161: 179.

[10] Environment Protection Authority.(1991): 'Guidelines for wastewater irrigation', Publication 168, Environment Protection Authority, Melbourne.

[11] FAO.(2002): Sewage flow in major cities of Pakistan. Food and Agricultural Organization, Islamabad, Pakistan.

[12] Farooq, M., Anwar, F., Rashid, U. (2008): Appraisal of heavy metal contents in different vegetables grown in the vicinity of an industrial area. - Pakistan Journal of Botany 40(5): 2099-2106.

[13] Fini, A., Brunetti, C., Di Ferdinando, M., Ferrini, F., Tattini, M. (2011): Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. - Plant Signaling & Behavior 6(5): 709-711.

[14] Giannopolitis, C. N., Ries, S. K. (1977): Superoxide dismutases occurrence in higher plants. - Plant Physiology 59: 309-314.

[15] Hassan, F. A., Ali, H. M. (2013): Impact of irrigation with Sewage effluent on the growth and wood properties of two forest tree seedlings. - Journal for Forest Products &Industries 2: 40-44.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_035048

[16] Ilaco, B. V. (1985): Agricultural compendium, for rural development in the tropics and subtropics, international land development consultants LTD., Netherlands; commissioned by the ministry of agriculture and fisheries. - The Netherlands Elsevier Scientific Publishing, The Hague.
[17] Iqbal, F., Ali, S., Tauqeer, H. M., Shakoor, M. B., Farid, M., Iftikhar, U., Nazir, M. M. (2013): Assessment of ground water contamination by various pollutants from sewage water in Chakera village, Faisalabad. - International Journal of Environmental Monitoring and Analysis 5: 182-187.
[18] Kakar, S. R., Wahid, A., Tareen, R. B., Kakar, S. A., Tariq, M., Kayani, S. A. (2010): Impact of municipal waste water of Quetta city on biomass, physiology and yield of canola (*Brassica napus* L.). - Pakistan Journal of Botany 42(1): 317-328.
[19] Marwari, R., Khan, T. I. (2012): Effect of textile waste water on tomato plant, *Lycopersicon esculentum*. - Journal of Environmental Biology 33(5): 849.
[20] Khan, A., Javid, S., Muhmood, A., Mjeed, T., Niaz, A., Majeed, A. (2013): Heavy metal status of soil and vegetables grown on peri-urban area of Lahore district. - Soil and Environment 32(1): 49-54.
[21] Khan, M.A., Shaukat, S.S., Khan, M.A. (2008). Economic benefits from irrigation of maize with treated effluents waste stabilization ponds. - Pakistan Journal of Botany 40:1091-1098.
[22] Kirk, J. T. O., Allen, R. L. (1965): Dependence of chloroplast pigment synthesis on protein synthesis: effect of actidione. - Biochemical and Biophysical Research Communications 21(6): 523-530.
[23] Kouser, S., Samie, A. (2009): Wastewater use in cauliflower production and farmer's health: an economic analysis. - The Pakistan Development Review 47-66.
[24] Kumar, V., Chopra, A. K., Pathak, C., Pathak, S. (2010): Agro-potentiality of paper mill effluent on the characteristics of *Trigonella foenum-graecum* L.(Fenugreek). - New York Science Journal 3(5): 68-77.
[25] MAAF. (1988): Fertilizer Recommendation. Reference Book 209. – HMSO, London
[26] Magen, H. (2005). Potential development of fertigation and its effect on fertilizer use.- Fertigation Proceedings: Selected papers presented at the joint 20:49.
[27] Marwari, R., Khan, T. I. (2012). Effect of textile waste water on tomato plant, *Lycopersicon esculentum*. - Journal of Environmental Biology 33(5): 849.
[28] Michalak, A. (2006): Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. - Polish Journal of Environmental Studies 15 (4): 523-530.
[29] Musa, J. J., Dauda, S. M., Abubakar, S. M., Fumen, G. (2013): Evaluation of Wastewater from a Refinery Treatment Plant for Agricultural Use.- International Journal of Science, Engineering and Technology Research (IJSETR) 2(1): 69-73.
[30] Nada, E., Ben, A.F., Rhouma, A., Ben, R.B., Mezghani, I., Boukhris, M.(2007): Cadmium-induced growth inhibition and alteration of biochemical parameters in almond seedlings grown in solution culture. - Acta Physiologiae Plantarum 29: 57-62.
[31] Noctor, G., Foyer, C. H. (1998): Ascorbate and glutathione: keeping active oxygen under control. - Annual review of plant biology 49(1): 249-279.
[32] Noctor, G., Foyer, C. H. (1998): Ascorbate and glutathione: keeping active oxygen under control. - Annual review of plant biology 49(1): 249-279.
[33] Park, Y. S., Jung, S. T., Kang, S. G., Heo, B. G., Arancibia-Avila, P., Toledo, F., Gorinstein, S. (2008): Antioxidants and proteins in ethylene-treated kiwifruits. - Food Chemistry 107(2): 640-648.
[34] Radotic, K., Ducic, T., Mutavdzic D. (2000): Changes in peroxidase activity and isozymes in spruce needles after exposure to different concentrations of cadmium. - Environmental and Experimental Botany 44: 105-113.
[35] Rattan, R. K., Datta, S. P., Chhonkar, P. K., Suribabu, K., Singh, A. K. (2005). Long-term impact of irrigation with sewage effluents on heavy metal content in soils, crops and groundwater–a case study. - Agriculture, Ecosystems & Environment 109(3): 310-322.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_035048

[36] Saeed, R., Ahmad, R. (2009): Vegetative growth and yield of tomato as affected by the application of organic mulch and gypsum under saline rhizosphere. - Pakistan Journal of Botany 41(6): 3093-3105.
[37] Sairam, R. K., Srivastava, G. C., Agarwal, S., Meena, R. C. (2005): Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. - Biologia Plantarum 49(1): 85-91.
[38] Singh, A., Agrwal, M. (2007): Effects of sewage sludge amendment on heavy metal accumulation and consequent responses of *Beta vulgaris* plants. - Chemosphere 69: 2229-2240.
[39] Singh, A., Agrwal, M. (2010): Effect of municipal waste water irrigation on availability heavy metals and morpho-physiological characteristics of *Beta vulgaris* L. - Journal of Environmental Biology 31(5): 727-736.
[40] Sinha, S., Gupta, A. K., Bhatt, K. (2007): Uptake and translocation of metals in fenugreek grown on soil amended with tannery sludge: involvement of antioxidants. - Ecotoxicology and Environmental Safety 67(2): 267-277.
[41] Tripathi, A.K., Tripathi, S. (1999): Changes in some physiological and biochemical characters in *Albizia lebbek* as bioindicators of heavy metal toxicity. - Journal of Environmental Biology 20: 93-98.
[42] Turton, H.E., Dawes, I.W., Grant, C.M. (1997): Saccharomyces cerevisiae exhibits a yAP-1- mediated adaptive response to malondialdehyde. - Journal of Bacteriology 179: 1096–1101.
[43] United States Salinity Laboratory Staff (USSL). (1954): Diagnosis and improvement of saline and alkali soils, Agriculture Handbook No. 60, U.S. Department of Agriculture, Washington, D.C.
[44] Ullah, R., Malik, R. N., Qadir, A. (2009): Assessment of groundwater contamination in an industrial city, Sialkot, Pakistan. - African Journal of Environmental Science and Technology 3(12).
[45] WHO. (2007): Joint FAO/WHO Expert standards program codex Alimentarius Commission. Geneva, Switzerland. - Available online http://www.who.int.
[46] Winkel-Shirley, B. (2002): Biosynthesis of flavonoids and effects of stress. - Current Opinion in Plant Biology 5: 218-223.
[47] Wolfe, K., Wu, X., Liu, RH. (2003): Antioxidant activity of apple peels. - Journal of Agricultural and Food Chemistry 51:609–614.
[48] Zhang, F.Q., Wang, Y.S., Lou, Z.P. Dong, J.D. (2007): Effect of heavy metal stress on antioxidative enzyme and lipid perdoxidation in leaves and root of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). - Chemosphere 61:44-50.

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ASSESSMENT OF WHEAT FOLIAR MYCOFLORA AND ITS MANAGEMENT STRATEGIES IN DISTRICT BHIMBER, AZAD KASHMIR, PAKISTAN

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Abstract. In this research, an analysis of wheat foliar mycoflora was explored with detection of 21 foliar fungal species from eight cultivated varieties in the wheat fields of District Bhimber of Azad Kashmir, Pakistan. Out of 21 species, 19 were isolated from three wheat varieties viz: V1 (Fareed-2006), V3 (Lasani-2008) and V6 (Aas 2011). Wheat variety V8 (Galaxy-2013) showed less number (28.5%) of fungal species invasion. *Mycosphaerella graminicola* was present ubiquitously on all eight wheat varieties with 100% prevalence while *Cephalosporium gramineum* was only found on three wheat varieties with 37.5%. The minimum occurrence was shown by fungus *Nigrospora sphaerica* (15%). In sub-division Samahni, wheat variety Fareed-2006 (V1) depicted the highest disease susceptibility with incidence of 63.3%. The variety Galaxy-2013 was found the best crop in Samahni having least incidence value of 22.3%. In sub-division Bhimber, wheat variety Seher-2006 was the most affected by mycoflora having highest disease incidence (60.0%) while least infection was measured in Galaxy-2013 (20.9%), being appropriate varietal crop for the area. In Barnala sub-division, Fareed-2006 indicated highest disease incidence (59.7%) while minimum disease incidence was measured in Galaxy-2013 (29.7%). As general conclusion Galaxy-2013 was proved as the best crop variety in the study area being nonth or least infected by fungal taxa. In second aspect of experiment comprising of optimization of management and control strategies, the parameter of grain yield was measured. As a general without any pre-treatment of seed crop, the variety V8 was the best of with yield of 1543 kg/ha, that might be due to its genetic resistance or better eco-climatic adaptability. Out of applied management strategies; use of fungicides (Quadris and Headline) spray on wheat leaves proved to be better having rise of yield i.e., 1550 kg/ha and 1560 kg/ha, respectively. The other strategy: use of biological products i.e. different plant extracts (*Acacia nilotica, Azadirachta indica, Curcuma longa, Eucaylptus citriodora, Ficus bengalensis*) spray proved that landmark rise was obtained in yield from variety V8 with 1739 kg/ha as compared to others. This rise was huge (1739 kg/ha) in comparison to the without treatment crop having 1543 kg/ha yield and it was proved pre-treatment produces better crop than control (blank) sample culminating the result that use of bio-products (plant extracts) are the best for control and management of mycoflora of wheat.
Keywords: *mycoflora, wheat varieties, foliar diseases, fungicides, biocontrol*

Introduction

Wheat (*Triticum aestivum* L.) is the largest crop present worldwide, which occupies about 217 m/hectors (FAO, 2012) and among cereal crops it is ranked third with annual production about 651 million tons in year 2010. It is likely that eco-climatic and frequently environmental changes have bad impacts on wheat yields and quality (Ortiz et al., 2008; Bender and Weigel, 2011). Climate change influenced wheat production. Therefore, better

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methods are applied for disease management in near future (Ewert, 2012). Wheat has high relation to environmental and climatic conditions (Rosada et al., 2010). Excessive moisture in the field and storage, humidity, drought and temperature extremes are principal environmental factors that determine the intensity of mycotoxins contaminations (Coulombe, 1993).

This crop is also very sensitive to pests, especially pathogenic fungi that cause leaf, head, and stem diseases (Jaczewska, 2010). These diseases contribute annually to a significant loss in wheat production (Korbas, 2004). A comprehensive survey of wheat pathogens including viruses, fungi and bacteria was carried out by Bockus et al., (2010). It is estimated that 10 % of net wheat yield losses were due to fungal and bacterial pathogens and only 2% yield was reduced by viruses (Oerke, 2006). The question arises that whether these overall estimated yield reduction will be higher or lower under globally fluctuating weather which will depend on the direct effects on different pathogens and the indirect effects through the host-plant interaction.

Current research shows fungal diseases because these are major components of yield losses in wheat crop at local level as well as worldwide. On the other hand, viral and bacterial pathogens are usually less important according to disease severity (Oerke, 2006). Another reason for this study is the extremely limited literature on viral and particularly bacterial wheat diseases with respect to climate change (Juroszek and von Tiedemann, 2011).

Changes of different wheat pathogens in India and Pakistan is a future risk (Kaur et al., 2008). For example, the importance of stripe rust and Kernel bunt in Punjab, India is assumed to be reduced in the future due to temperature and humidity changes. On the other hand, the importance of leaf rust, foliar blights, Fusarium head blight, and stem rust may increase in Punjab in the future, the latter disease particularly in the absence of resistance in wheat cultivars (Kaur et al., 2008).

Wheat varieties are affected by different diseases which reduce quantitative as well as qualitative yield losses in Pakistan. The foliar diseases like leaf and glumes blotch, powdery mildew, three types of wheat crop rusts, tan spot, head blight and smuts are the quickest spreading diseases in different wheat varieties. Yield losses reach upto 30% and depend on yield frequency according to environmental as well as wheat cultivar conditions.

Stripe rust is a more serious fungal disease of wheat crop. It infects susceptible host cultivars with symptoms occurring about one week after initial infection occurs. The disease often causes severe grain yield and quality loss as stripe rust can appear and spread rapidly when climatic conditions favor the disease. Stripe rust is reported in over 60 countries worldwide.

Fusarium culmorum, Fusarium graminearum and *Fusarium sporotrichioides* are the main producers of trichothecenes. These toxins are common fungal contaminants of cereals and occur naturally worldwide in the cultivation of wheat and other cereals (Brown et al., 2001; Champeil et al., 2004; Wagacha and Muthomi, 2007).

Therefore, management of fungal diseases is necessary for a sustainable agriculture. Crop management practices affect the development of the disease epidemics. In the humid subtropics of South Asia, there is evidence of stress conditions, which favor foliar blight (Dubin and Bimb, 1994). Factors such as minimum tillage or surface seeding, irrigation, late planting, or low soil fertility may be responsible for higher foliar blight severity in the wheat-based cropping systems of the Indo-Gangetic plains (Sharma and Duveiller, 2003). Lower disease severity with higher nitrogen application was reported by Chaurasia and Duveiller (2006) and Sharma and Duveiller (2004). However, Singh et al., (1998) reported more disease infection on higher nitrogen application. Selection and breeding for resistant cultivars is the

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main disease management strategy. Planting resistant cultivars is one of the least expensive and most effective management strategies to prevent diseases.

Fungicides are widely used to manage foliar wheat diseases in several countries of world (Carmona et al., 1999). The response to fungicide application depends on the severity of specific foliar diseases, cultivar disease resistance or tolerance, management practices and environmental conditions (Roth and Marshall, 1987; Varga et al., 2005; Carignano et al., 2008). Fungicides applied at flag leaf and spike emergence of winter wheat increased mean grain weight and grain yield when they extended canopy life (Gooding, 2006). Triazoles and Strobilurins are the most common systemic fungicides used to control foliar diseases on wheat. These types of fungicides generally move upward in the transpiration stream and may accumulate at the leaf margins (Arregui and Puricelli, 2008).

Introduction of the study area

The study area viz: District Bhimber (*Figure 1*) consists of three sub-divisions (tehsils). The environmental conditions of this district are ubiquitous in three tehsils. Geographcally,the district is located between latitude 32-48˚ to 33-34˚ and longitude 73-55˚ to 74-45˚ and has an area of 1516 km^2. The climate of this area is constant. Hot summer temperature is often over 38-46˚C from May to September and cold winters (4-10˚C). The total average rain fall is about 1233 mm per month. Hail occurs occasionally during the month of February and March. Humidity remains high in rainy season and winters. Topographically, the area is semi mountainous and plain. The southern part is plain while northern part consists of rough and precipitates steep sand stone hills and rugge. The plain areas of district Bhimber are very fertile for crops cultivation.

The objective of this research was to survey foliar diseases associated with wheat varieties; to measure the incidence, prevalence and severity of foliar diseases of selected eight wheat varieties; to improve wheat yields by reducing or eradicate foliar fungal diseases through fungicides treatments and biological treatment by use of plant extracts.

Figure 1. *Map of the study area (District Bhimber)*

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Materials and Methods

Sample collection

In this research study, all wheat growing areas of the district were surveyed. Fungal affected wheat leaves were collected in plastic bags and stored at room temperature for assessment and analyses of fungal diseases. The leaves were collected at seedling, young and mature stages. Morphological symptoms were recorded for presence or absence of foliar diseases in wheat growing areas of district Bhimber Azad Jammu and Kashmir (AJ&K). Leaves of four common plants were collected for biological treatments against different fungi. The disease free and fresh plants were selected for this investigation. About two gram (02 g) of fresh and healthy leaves were taken for each solvent. Then, surface sterilized with 1% Sodium hypochlorite and alcohol for 2-3 minutes and the plant materials were washed thoroughly with distilled water.

The following methods were applied for assessment of foliar diseases.

Assessment of disease prevalence by diagonal transect method

Affected leaves of wheat varieties were collected from different field trails from three tehsils. The samples were collected from five points along with a diagonal transect as X- pattern (Anonymous, 2003). At each sampling site, the collected foliar samples were put in sterile flasks and transferred to laboratory for further fungal analysis, identification and purification (Iftikhar et al., 2012). The prevalence of disease was measured according to presence or absence of foliar spots in each field trail. The prevalence and severity (% age) was calculated by applying following formulae:

$$\text{i. DiseasePrevalece}(\%) = \frac{\text{Locations with filiar disease}}{\text{Total locations}} \times 100 \qquad \text{(Eq.1)}$$

$$\text{ii. Severity} = \frac{\sum(a \times b)}{n \times z} \times 100 \qquad \text{(Eq.2)}$$

where $\sum$ (a×b) = Sum of the symptomatic plants and their corresponding scoring scales, n = Total number of sampled plants and z= Highest Score Scale.

The assessment of the disease was made in double digit system which comprises of two digits representing the vertical disease progress and percentage of the blight area covered (severity). The first digit gives the relative height of the disease in 0-9 scale as proposed by Saari and Prescot (1985) and second digit represents the percentage area covered by the blight pathogen on the flag leaf and one below it which is as under:

10%=1, 20%=2, 30%=3, 40%=4, 50%=5, 60%=6, 70%=7, 80%=8, 90%=9

As for example the disease score of 55 denotes that the first digit of 5 represents the height of the disease in' 0-9 scale whereas the second digit exhibits average disease severity in percent on the flag leaf and one below it.

Assessment of disease incidence (diagonal transect method)

A fixed plot survey was applied for wheat leaves collection. The plants within the fixed plot were assessed for foliar diseases and leaf samples were collected from

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selected localities by X pattern (Sharma and Duveiller, 2003) and brought to laboratory for further fungal study (Iftikhar et al., 2012). Five plants were selected by diagonal transect method for documentation of incidence of different types of foliar diseases (Teng and James, 2001) by using following formula:

$$\text{iii. Incidence (\%)} = \frac{\text{Number of leaves showing foliar spots}}{\text{Total No. of leaves of sample}} \times 100 \quad \text{(Eq.3)}$$

Identification of fungi by agar plate method

In agar plate method (APM), collected samples were placed on sterile PDA media. The PDA media prepared by mixing of prepared PDA media in 1000 ml distilled water. The media autoclaved at fifteen (15) psi pressure for twenty (20) minutes in autoclave. 15 ml media transferred in each petridish under aseptic circumstances. Foliar samples were surface sterilized in 1% $NaOCl_2$ for three to five minutes and then given three washing treatments with distilled water. The effected parts were transferred on PDA media after distillation. After 3-5 days, fungi were detected and identified by morphological characteristics (Domsch et al., 1980, Hussain et al., 2011).

Isolated fungi were purified by sub-culturing on PDA media (Usmani and Ghaffar, 1982). The plates were incubated at room temperature for 3-5 days. After the complete growth of fungi, the fungal cultures were sealed and preserved in refrigerator for future use.

Disease management strategies

There are different management strategies were applied to control foliar diseases of wheat varieties in district Bhimber Azad Kashmir.

Fungicide treatments (chemical control)

In field trails, each wheat variety was sprayed with five fungicides i.e., Tilt (0.29 l ha^{-1}), Proline (0.76 l ha^{-1}), Strobilurins (0.89 l ha^{-1}), Quadris (0.58 l ha^{-1}) and Headline at different rates. The fungicides were applied on leaves of each variety in selected fields separately. The timing of different fungicides was different for better result. After all treatments, wait for fruit ripe and harvested the crop and calculated the yield of each wheat variety (Fakir, 1999).

Biological treatments (biocontrol)

As fungicides were sprayed, similarly different plant extracts were sprayed on all selected eight wheat varieties for reduction of fungal contaminations and better yields. This treatment method is more reliable as compared to fungicides because this method has few/no side effects Yield (kg/ha) and 1000-grain weight were measured after harvesting all plots (Pathak and Razia, 2013).

Statistical analysis

The data were evaluated by help of computer software MSTAT-C. Some data was analyzed statistically by LSD and ANOVA. Significance was calculated at $p< 0.05$ and $p< 0.01$ levels of probability. Each value is mean of three replicates (Steel *et al.*, 1996; Pathak and Razia, 2013).

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Results

Wheat (*Triticum aestivum*) is being attacked by several fungal diseases many of them are foliar pathogens. In this research, 21 different foliar fungal species from 40 foliar samples of eight wheat varieties were collected from three tehsils (sub-divisions) of district Bhimber Azad Jammu and Kashmir during the year 2014 and 2015 for mycofloral analysis (Table 1). The foliar samples of eight wheat varieties were collected and preserved in Laboratory of Botany, Mirpur University of Science and Technology (MUST), Bhimber Campus Azad Jammu and Kashmir for further experimental works.

Highest number of fungal species were identified from wheat variety V1 (Fareed-2006), V3 (Lasani-2008) and V6 (Aas 2011). Out of 21 fungal species 19 (90.5%) appeared on these three wheat varieties. Less number of fungal species i.e., 6 (28.5%) were isolated from wheat variety V8 (Galaxy-2013). In the current analysis, occurrence of fungal species was found variable; *Mycosphaerella graminicola* was present on all wheat varieties (100%) while *Cephalosporium gramineum* was only found on three wheat varieties with 37.5% as indicated in Table 1. The identification of fungal pathogens was presented by field photography. *Figure 2* shows the attack of *Puccinia tritici-repentis, Figure 3* the attack of pathogen *Stagonospora nodorum.* Similarly, *Figure 4* presents attack of *Puccinia striiformis* on wheat crop, *Figure 5* the attack of pathogen *Drechslera tetramera*, while *Figure 6* shows attack of various fungal species on wheat crop and *Figure 7* the presence of *Alternaria* species on wheat.

Figure 2. *Attack of Puccinia tritici-repentis*

Figure 3. *Attack of Stagonospora nodorum*

Figure 4. *Attack of Puccinia striiformis*

Figure 5. *Attack of Drechslera tetramera*

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Figure 6. *Multiple fungal species attack on wheat*

Figure 7. *Attack of Alternaria species on leaf*

Table 1. *Survey/Isolation of Foliar mycoflora associated with eight wheat varieties from District Bhimber Azad Kashmir, Pakistan*

S/No	Fungi detected	Name of Wheat Varieties								Total	%
		V1	V2	V3	V4	V5	V6	V 7	V8	08	100
1	*Alternaria alternata*	+	-	+	+	+	+	-	-	5	62.5
2	*Aspergillus niger*	+	+	+	+	+	+	-	-	6	75.0
3	*Bipolaris sorokiniana*	+	+	+	+	+	+	-	+	7	87.5
4	*Blumeria graminis*	+	+	+	+	+	+	+	-	7	87.5
5	*Cephalosporium gramineum*	-	+	-	+	-	+	-	-	3	37.5
6	*Cladosporium herbarum*	+	-	+	-	+	+	-	+	5	62.5
7	*Drechslera tetramera*	+	+	+	-	+	+	-	+	6	75.0
8	*D. tritici-repentis*	+	-	+	-	+	+	-	-	4	50.0
9	*Helminthosporium sp.*	+	+	+	+	+	+	+	-	7	87.5
10	*Microdochium nivale*	+	+	+	+	+	+	-	-	6	75.0
11	*Mycosphaerella graminicola*	+	+	+	+	+	+	+	+	8	100
12	*Nigrospora sphaerica*	+	+	+	-	+	+	-	-	5	62.5
13	*Penicillium lilacinum*	+	+	+	+	+	+	-	-	6	75.0
14	*P. chrysogenum*	+	+	-	+	-	+	+	-	5	62.5
15	*Puccinia striiformis*	+	+	+	-	+	+	-	-	5	62.5
16	*P. triticina*	+	+	+	+	-	+	+	+	7	87.5
17	*P. recondite*	+	+	+	+	+	-	+	-	6	75.0
18	*Pyrenophora teres*	-	+	+	-	+	+	-	-	4	50.0
19	*P. tritici-repentis*	+	+	+	-	+	+	-	-	5	62.5
20	*Rhizoctonia cerealis*	+	-	+	-	+	-	+	+	5	62.5
21	*Stagonospora nodorum*	+	+	+	-	+	+	+	-	6	75.0
	Total Species Identified	19	17	19	12	18	19	8	6	118	
	%age of each variety	90.5	81	90.5	57	85.7	90.5	38	28.5	70.2	

Key: + = Present, - = Absent, V1= Fareed-2006, V2= Seher-2006, V3= Lasani-2008, V4= Faisalabad-2008, V5= Millat-2011, V6= Aas 2011, V7= Punjab-2011, V8= Galaxy-2013

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The present mycofloral picture of District Bhimber consists of phyto-geographical snapshot of three Sub-divisions i.e., Bhimber (Bm), Samahni (Sm) and Barnala (Br). The prevalence of identified fungal species was calculated by Equation 1 and presented in tabular form (*Table 2*). The highest (100%) prevalence of fungi was observed in three locations of Sm, two locations of Bm and four locations of Br. According to wheat varieties V1 (96%) showed more prevalent as compared to others. While minimum prevalence was shown by V8 (70.7%) as mentioned in *Table 2*.

Table 2. *Prevalence (%) of foliar diseases of eight wheat varieties from different locations of district Bhimber Azad Jammu and Kashmir, Pakistan*

Area	**Locations**	**Wheat varieties with disease prevalence (%)**								**Means**
		V1	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	
Samahni	5	100	100	100	100	100	100	100	100	100
Bandala	5	100	100	100	100	100	100	100	100	100
Chowki	5	100	100	100	100	100	100	100	100	100
Jandala	5	100	80	60	40	80	100	60	20	67.5
Poona	5	80	60	60	20	100	80	40	20	57.5
Bhimber	5	100	60	60	80	80	60	40	40	65
Bring	5	80	60	80	60	80	80	20	20	60
Sokasan	5	100	100	100	100	100	100	100	100	100
Punjairi	5	100	100	100	100	100	100	100	100	100
Kasgumma	5	80	100	80	20	60	80	40	40	62.5
Bernala	5	100	100	100	100	100	100	100	100	100
Chanb	5	100	100	100	100	100	100	100	100	100
Kadala	5	100	100	100	100	100	100	100	100	100
Tander	5	100	80	80	60	100	80	20	20	67.5
Thub	5	100	100	100	100	100	100	100	100	100
%age of each variety		96	89	88.3	78.7	93.3	92	74.7	70.7	

Key: + = Present, - = Absent, V1= Fareed-2006, V2= Seher-2006, V3= Lasani-2008, V4= Faisalabad-2008, V5= Millat-2011, V6= Aas 2011, V7= Punjab-2011, V8= Galaxy-2013

Disease incidence was measured by help of Equation 3. In tehsil Samahni, wheat variety (WV) Fareed-2006 (V1) prevailed highest disease incidence (63.3%) followed by Seher-2006 (60.3%), Aas-2011 (59.6%) and so on. The minimum disease incidence was measured in Galaxy-2013 (22.3%). These results indicated that wheat variety Galaxy-2013 was more suitable for high growth and better yields production of wheat crop in the study area. This WV showed low fungal infection rate (*Table 3*). In tehsil Bhimber, WV Seher-2006 showed highest disease incidence (60.0%) while minimum disease incidence was reflected in Galaxy-2013 (20.9%). In Bernala tehsil, variety (WV) Fareed-2006 (V1) indicated highest disease incidence (59.7%) while minimum disease incidence was measured in Galaxy-2013 (29.7%) (*Table 3*).

The comparative analysis according to disease severity among three tehsils of District Bhimber was also under investigation. The Severity was measured by use of Equation 2. According to 0-9 rating scales, foliar symptoms of diseases assessed through visual basis i.e., 0= no symptoms, 1= 10-20% spots on leaves, 2=20-30% spots, 3= 30-40% spots, 4= 40-50% and 5= 50-60% spots, 6= 60-70% spots, 7=70-80% spots, 8= 80-90% spots and 9= 90-100% (*Table 4*).

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Table 3. *Incidence of foliar diseases of eight wheat varieties in district Bhimber Azad Kashmir*

Tehseels	Locations	*Incidence (%)								Mean
		V1	V2	V3	V4	V5	V6	V7	V8	
Samahni	Samahni city	81.7	75.0	78.3	56.7	78.3	73.3	46.7	38.3	66.0
	Bandala	71.7	70.0	60.0	45.0	56.7	61.7	45.0	30.0	55.01
	Chowki	65.0	58.3	60.0	40.0	56.7	56.7	35.0	21.7	49.2
	Poona	48.3	46.7	45.0	20.0	48.3	48.3	18.3	8.3	35.4
	Jandala	50.0	51.7	48.3	26.7	48.3	58.3	23.3	13.3	39.9
	Mean	**63.3**	**60.3**	**58.3**	**37.7**	**57.6**	**59.6**	**33.7**	**22.3**	**49.2**
Bhimber	Bhimber city	70	65.0	66.7	56.7	66.7	60.0	51.7	33.3	58.7
	Bring	58.3	55.0	51.7	50.0	55	55.0	45.0	13.3	47.9
	Sokasan	35.0	50.0	55.0	35.0	53.3	45.0	30.0	16.7	40.0
	Punjairi	60.0	61.7	30.0	30.0	38.3	50.0	26.7	18.3	39.4
	Kasgumma	55.0	68.3	53.3	33.3	46.7	48.3	30.0	23.3	44.8
	Mean	**55.7**	**60.0**	**51.3**	**41.0**	**52.0**	**51.7**	**36.7**	**20.9**	**46.2**
Barnala	**Barnala city**	76.7	71.7	73.3	60.0	71.7	71.7	53.3	36.7	64.4
	Chanb	61.7	63.3	60.0	48.3	56.7	58.3	46.7	21.7	52.1
	Kadala	63.3	55.0	53.3	36.7	60.0	53.3	35	13.3	46.2
	Tander	48.3	45.0	40.0	23.3	26.7	33.3	18.3	67	37.7
	Thub	48.3	50.0	55.0	35.0	43.3	43.3	26.7	10.0	38.95
	Mean	**59.7**	**57.0**	**56.3**	**40.7**	**51.7**	**51.9**	**36.0**	**29.7**	**47.8**

Key: *Incidence=Percentage shows wheat plants infected with foliar diseases

Table 4. *Disease Severity of foliar diseases of eight wheat varieties in district Bhimber Azad Kashmir*

Tehseels	locations	*Severity (0-9)								Mean
		V1	V2	V3	V4	V5	V6	V7	V8	
Samahni	Samahni city	8.0	6.3	7.0	4.3	6.0	6.0	4.0	3.0	5.6
	Bandala	7.3	5.7	5.0	3.3	5.3	5.7	2.7	1.7	4.6
	Chowki	5.0	4.0	6.0	4.3	5.7	5.0	2.7	3.0	4.5
	Poona	5.0	3.3	4.0	4.0	3.7	3.7	3.3	1.3	3.5
	Jandala	5.3	4.0	5.3	3.0	5.3	4.3	2.3	1.7	3.9
	Mean	**6.1**	**4.6**	**5.5**	**3.9**	**5.2**	**4.9**	**3.0**	**2.1**	**4.4**
Bhimber	Bhimber city	8.0	6.0	6.0	3.7	5.0	6.0	4.0	2.7	5.2
	Bring	7.3	5.3	5.0	3.0	4.7	5.3	2.3	1.0	4.2
	Sokasan	6.7	6.3	6.7	4.7	5.3	4.0	2.3	2.0	4.7
	Punjairi	4.7	5.3	7.7	2.3	3.0	3.7	3.0	1.0	3.8
	Kasgumma	6.0	7.0	6.0	2.0	4.0	5.7	2.0	1.3	4.3
	Mean	**6.5**	**5.9**	**6.3**	**3.1**	**4.4**	**4.9**	**2.7**	**1.6**	**4.4**
Barnala	**Bernala city**	7.7	7.0	7.7	4.7	6.3	6.3	4.0	2.7	5.8
	Chanb	6.7	6.7	6.0	4.0	4.7	5.3	2.3	2.0	4.7
	Kadala	5.0	7.7	6.0	4.0	6.7	5.3	3.0	3.0	5.1
	Tander	5.3	4.0	3.3	1.7	3.3	3.3	1.7	1.7	3.0
	Thub	7.3	7.0	5.0	2.7	5.7	4.7	2.7	1.3	4.5
	Mean	**6.4**	**6.5**	**5.6**	**3.4**	**5.3**	**4.9**	**2.7**	**2.1**	**4.6**

***Severity= 0-9 rating scales show foliar symptoms of diseases assessed through visual basis i.e., 0= no symptoms, 1= 10-20% spots on leaves, 2=20-30% spots, 3= 30-40% spots, 4= 40-50% and 5= 50-60% spots, 6= 60-70% spots, 7=70-80% spots, 8= 80-90% spots, 9= 90-100%

In tehsil Samahni, highest severity was shown by WV Fareed-2006 (V1) as 6.1 on severity scale. The minimum severity was shown by Galaxy-2013 (2.1). Less severity on wheat variety produced better yields. In tehsil Bhimber, variety (WV) Seher-2006

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(V1) indicated highest disease severity (6.5) while minimum disease severity was measured in Galaxy-2013 (1.6). In Barnala tehsil, highest severity was shown by V1 and V2 (6.4, 6.5) while minimum disease severity was seen in Galaxy-2013 (2.1) as indicated in *Table 4*.

Management strategies

Foliar diseases of WV were managed by applying fungicides treatments through chemical spray and biological treatments by different plant extracts spray. In this research, five different fungicides were used for chemical treatment to wheat leaves at early growth stages/before appearance of fungal disease symptoms. These fungicides reduced fungal infections and improved wheat yields.

The total yields of eight WV were measured before and after treatment for comparative analysis. Without treatments, highest yield was obtained from V8 (1543 kg ha^{-1}). On the other hand, low yield was received from V1 (1210 kg ha^{-1}) and V2 (1245 kg ha^{-1}) as indicated in *Table 5*. After fungicides spray highest yield was obtained from V8 (1706 kg ha^{-1}) which is better than untreated samples. Similarly, 1602 kg ha^{-1} yield was produced by V4 while lowest yield was received from V1 (1362 kg ha^{-1}) that is also better from untreated samples as mentioned above (*Table 6*).

Table 5. *Total yields of untreated field trials of different wheat varieties in district Bhimber Azad Kashmir, Pakistan*

S/No	Field Trails	Wheat Varieties with their yield (kg ha^{-1}) before fungicide treatment								Means
		V1	V2	V3	V4	V5	V6	V7	V8	
1	T1	1150	1260	1300	1460	1430	1340	1480	1460	1360 ^{a}
2	T2	1200	1280	1250	1390	1340	1270	1420	1489	1330 ^{ab}
3	T3	1220	1190	1360	1450	1210	1340	1390	1570	1341 ^{a}
4	T4	1180	1298	1230	1270	1195	1290	1550	1620	1329 ^{ab}
5	T5	1300	1200	1175	1520	1290	1380	1500	1580	1368 ^{a}
Average		1210 ^{e}	1245^{e}	1263 ^{d}	1418 ^{b}	1293 ^{d}	1324 ^{c}	1468 ^{b}	1543 ^{a}	

Key: T1= Trail 1, T2= Trail 2, T3= Trail 3, T4= Trail 4, T5= Trail 5

Table 6. *Fungicides treatment on leaves of wheat varieties and measured total yield increase (kg ha^{-1}) from Bhimber district Azad Jammu and Kashmir, Pakistan*

S/No	Fungicide	Wheat Varieties with yield increase (kg ha^{-1}) after fungicides treatment								Means
		V1	V2	V3	V4	V5	V6	V7	V8	
1	F1	1300	1380	1490	1600	1580	1440	1610	1690	1511 ^{b}
2	F2	1350	1560	1385	1580	1500	1390	1580	1700	1506 ^{b}
3	F3	1410	1480	1520	1450	1450	1460	1550	1680	1500 ^{ab}
4	F4	1290	1500	1500	1685	1470	1400	1745	1810	1550 ^{a}
5	F5	1460	1520	1460	1695	1495	1530	1670	1650	1560 ^{a}
Average		1362 ^{e}	1488 ^{c}	1471 ^{c}	1602 ^{ab}	1499 ^{c}	1444 ^{cd}	1631 ^{b}	1706 ^{a}	

Key: F1= Tilt (0.29 l ha-1), F2= Proline (0.76 l ha-1), F3= Strobilurins (0.89 l ha-1), F4= Quadris (0.58 l ha-1), F5= Headline (0.58 l ha-1)

The impact of fungicides on yield rate of wheat varieties were also calculated and documented in *Table 6*. After spray of fungicide Quadris (0.58 l ha-1) and Headline

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(0.58 l ha-1) on wheat trails, maximum yield was produced as compared to other fungicides i.e., 1550 kg ha^{-1}, 1560 kg ha^{-1}. This means that the fungicide F4 and F5 are more effective to combat and reduce fungal diseases from surfaces of wheat leaves (*Table 6*).

On the other hand, after different plant extracts treatment/spray on wheat variety trails was showed maximum yields was obtained from V8 (1739 kg ha^{-1}) as compared to others WV. Lowest yield was obtained by WV Seher-2006 (1348 kg ha^{-1}) as mentioned in *Table 7*.

Table 7. *Treatment of leaves of wheat varieties with different crude plant extracts in Bhimber district Azad Jammu and Kashmir, Pakistan*

S/N	Plant Name	Wheat varieties spared with different plants crude extracts								Mean
		V1	V2	V3	V4	V5	V6	V7	V8	
1	*Acacia nilotica*	1350	1400	1490	1590	1570	1540	1650	1680	1534 [ab]
2	*Azadirachta indica*	1330	1500	1370	1570	1560	1400	1590	1750	1509 [b]
3	*Eucaylptus citriodora*	1420	1450	1550	1490	1400	1450	1565	1685	1501 [b]
4	*Ficus bengalensis*	1250	1470	1580	1630	1480	1460	1770	1870	1564 [a]
5	*Curcuma longa*	1390	1570	1420	1590	1500	1580	1685	1710	1556 [a]
	Average	1348 [f]	1478 [e]	1482 [e]	1574 [c]	1502 [d]	1486 [e]	1652 [b]	1739 [a]	

Discussion

Wheat (*Triticum aestivum*) is suffered from many diseases some of them are foliar pathogens. In this current research, 21 different foliar fungal species were identified from eight wheat varieties. The foliar samples of eight wheat varieties were collected and brought to Laboratory of Botany, Mirpur University of Science and Technology (MUST), Bhimber Campus Azad Jammu and Kashmir for experimental works.

Out of 21 fungal species 19 (90.5%) were isolated from V1 (Fareed-2006), V3 (Lasani-2008) and V6 (Aas-2011). these Highest number of fungal species were depicted that the ecoclimatic conditions of district Bhimber were more suitable for fungal growth and reproduction during wheat seasons. Secondly, these three wheat varieties have less resistance against fungal pathogens. Therefore fungal spores were easily attack on leaves of the varieties (Arregui and Puricelli, 2008). Low fungal frequencies (6 species) were identified from wheat variety V8 (Galaxy-2013). This means that V8 variety is more susceptible to fungi and more disease resistance as compared to other WV. Hence, it is more suitable for sowing in the study area. Similar observations were analyzed by Ortiz et al. (2008).

In current analysis, fungal pathogen *Mycosphaerella graminicola* was isolated from all wheat varieties. This may be due to high moisture contents and optimum temperature in District Bhimber Azad Kashmir. As previously same climatic study was conducted by Wagacha and Muthomi (2007). The fungi *Cephalosporium gramineum* was only present on three wheat varieties with 37.5% as indicated in *Table 1*. This fungal species did not spread more in the study area due to unfavorable environmental conditions.

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The present mycofloral picture of District Bhimber consists of a comparative study of phyto-geographical diversity and comparative mycological analysis of three sub-divisions (tehsils) of district Bhimber Azad Kashmir, i.e. Bhimber (Bm), Samahni (Sm) and Barnala (Br). Similar prevalence of fungi in the three areas depicts their ubiquity in all ecosystems of this climate. According to wheat varieties V1 is more vulnerable to fungal diseases while minimum dominancy was shown by V8 as mentioned in *Table 2*. These findings were similar to above described results as discussed previously by Singh et al., (1998).

These results indicated that wheat variety Galaxy-2013 was more suitable for high growth and better yields production of wheat crop in the study area. This WV showed low fungal infection rate and fewer incidences due to better and quicker adaptation in the area (Sharma and Duveiller, 2003).

The comparative analysis according to disease severity among the three sub-divisions of district Bhimber was also carried out. The Severity was measured according to 0-9 rating scales which showed foliar symptoms of diseases assessed through visual basis i.e., 0= no symptoms, 1= 10-20% spots on leaves, 2=20-30% spots, 3= 30-40% spots, 4= 40-50% and 5= 50-60% spots, 6= 60-70% spots, 7=70-80% spots, 8= 80-90% spots and 9= 90-100% (Bockus *et al.,* 2010). Highest severity was calculated from WV Fareed-2006 (V1) on severity scale. This is also due to low resistance. Secondly, it may be due to repetition every year in the study area. Therefore, it becomes more vulnerable to fungi (Chakraborty et al*.,* 2011).

The severity was also minimum on Galaxy-2013. Less severity on wheat variety produced good yields. Similar findings were obtained in other locations. This means that overall results can be explained by that WV Galaxy-2013 was first time introduced in the study area. Therefore, it was less effective less as compared to the other seven WVs. Therefore former should be preferred to this variety in the study area for better food and fodder yields (Fernandes et al., 2004).

Management strategies

The population on this planet increases day by day and agricultural lands have been reduced. This is an alarming horizon for future. Demand for food is increasing day by day. Therefore we should carefully think on this issue: what is a good way to increase food and fodder yields? We should control foliar diseases of WV by applying fungicides treatments through chemical spray and biological spray treatment of different plant extracts. In this research, five different fungicides were used as chemical treatment on wheat leaves at appearance of fungal disease symptoms. Spray of fungicides reduced fungal infections especially and improved wheat yields (Varga et al., 2005; Carmona et al., 1999).

The total yields of eight WVs were measured before and after treatment for comparative analysis. Without treatments low yield was received from V1 (1210 kg ha^{-1}) and V2 (1245 kg ha^{-1}) as indicated in *Table 5*. After fungicides spray the highest yield was obtained fromV8 which was better than untreated samples. Similar research was conducted by Gooding (2006).

The impact of fungicides on yield rate of wheat varieties were also calculated and documented. After spray of fungicides on wheat trails, maximum yield was produced as compared to other fungicides. This means that the fungicides F4 and F5 are more effective to combat and reduce fungal diseases from surfaces of wheat leaves (Oerke, 2006; Ortiz et al., 2008).

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On the other hand, after different plants extracts (*Acacia nilotica, Azadirachta indica, Curcuma longa, Eucaylptus citriodora, Ficus bengalensis*) treatment on wheat variety trails good yields were produced. This means that after biocontrol greater yields of wheat was obtained according to requirements. Similar biocontrol was applied by Benkeblia (2004). Finally, biological treatments are recommended to WV after sowing because this technique does not have fatal impact on health and environments. Similar work was done by Aqil et al., (2010). This biological method is cheap and easily manageable compared to every former. Previously, similar biological treatments were launched by Iftikhar et al., (2012) and Pathak and Razia (2013).

Fungicides treatments also resulted more net yields but they polluted the environment, produced harmful impacts on flora and fauna. Secondly, they also were costly applications. Therefore, we should prefer this new technique of plant extracts for fungal foliar disease management.

Conclusion

From these findings it is concluded that the control measures of fungal pathogens is a basic need to avoid crop failure. It is desirable that after sowing wheat crop, spreading of pathogens in wheat growing areas are treated with fungicides and biological treatments to attain maximum yield of wheat crops. In current research attention has been given to use biological techniques for foliar treatment to protect them against fungal pathogens. Chemical fungicides can control the crop diseases, but they have bad effects on plants, animals and human health and also on our environment. Since these fungicides are likely to have hazardous impacts on human life and environment, it is therefore necessary to search for better control measures that are cheap, ecologically sound and environmentally safe to eliminate or reduce the incidence of these pathogens and for the improvement of wheat quality and quantity, so as to obtain healthy and strong wheat plants for fodder as well as better yields of different varieties of wheat crop. Therefore biological treatment of wheat fungal diseases are recommended for obtaining better yields of wheat crop in future in the examined area.

REFERENCES

[1] Arregui, M.C., Puricelli, E. (2008): Mecanismos y modo de acción de fungicidas.- Dow Agroscience 208 .

[2] Aqil, F., Zahin, M., Ahmed, I., Owais, M., Khan, M.S.A, Bansal, S.S., Farooq, S. (2010): Antifungal Activity of Medical Plant Extracts and Phytocompounds: A Review - In: Ahmad, I., Owais, M., Shahid, M. (eds.) Combating Fungal Infections: Problem and Remedy, Springer-Verlag, Berlin, Heidelberg, Germany, pp. 449–485.

[3] Bender, J., Weigel, H. J. (2011): Changes in atmospheric chemistry and crop health: a review-Agronomy for Sustainable Development 31:81–89.

[4] Bockus, W.W., Bowden, R.L., Hunger, R.M., Morrill, W.C., Murray, T.D., Smiley, R.W. (2010): Compendium of wheat diseases and pests. - St. Paul: APS Press.

[5] Brown, K.H., Wuehler, S.E., Peerson, J.M. (2001): The importance of zinc in human nutrition and estimation of the global prevalence of zinc deficiency.- Food Nutr. Bull. 22:113–125.

[6] Benkeblia, N. (2004): Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*)- LWT- Food Sci. Technol. 37 (2): 263–268.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_049065

[7] Carmona, M., Cortese. P., Moschini, R., Pioli, R., Ferrazini, M., Reis, E. (1999): Economical damage threshold for fungicide control of leaf blotch and tan spot of wheat in Argentina.- XIV th International Plant Protection Congress, Jerusalem, Israel, 25-30 July 1999:119.
[8] Chakraborty, S., Luck, J., Hollaway, G., Fitzgerald, G., White, N. (2011): Rust-proofing wheat for a changing climate.- Euphytica 179:19–32.
[9] Chaurasia. P.C.P., Duveiller, E. (2006): Management of leaf blight (*Bipolaris sorokiniana*) disease of wheat with cultural practices.- Nepal Agric. Res. J. 7:63-69.
[10] Champeil, A., Dor´e, T., Fourbet, J.F. (2004): "Fusarium head blight: Epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by Fusarium in wheat grains.- Plant Science 166(6): 1389–1415.
[11] Carignano, M., Staggenborg, S.A., Shroyer, J.P. (2008): Management practices to minimize tan spot in a continuous wheat rotation.- Agronomy Journal. 100:145-153.
[12] Domsch, K.H., Gams, W., Anderson, T.H. (1980): Compendium of soil fungi.- Academic press. A subsidiary of Harcourt brace
[13] Dubin, H.J., Bimb, H.P. (1994): Studies of soil borne diseases and foliar blights of wheat at the National Wheat Research Experiment Station, Bhairahawa, Nepal.- Wheat special report no 36. CIMMYT, Mexico.
[14] Dumalasova, V., Bartos, P. (2009): Will climatic changes enhance the risk of Tilletia indica in Europe? - Plant Protection Science 45(Special Issue). 38–40.
[15] Ewert, F. (2012): Adaptation: opportunities in climate change?- Nature Climate Change 2: 153–154.
[16] FAO (2012). Food and Agriculture Organisation of the United Nations,- FAOSTAT, FAO Statistics Division.
[17] Fazal, M.J.R, Ihsan, I. (2005): Callus Induction and Regeneration in Seed Explants of Rice (*ORYZA SATIVA* CV.SWAT.2).- Pakikistan Journal of Botany (37): 829-836.
[18] Fernandes, J.M., Cunha, G.R., Del P.E., Pavan, W., Pires, J.L., Baethgen, W., (2004): Modeling fusarium ear blight in wheat under climate change using linked process-based models. - Proceedings of the 2nd International Symposium on Fusarium Head Blight, incorporating the 8th European FusariumSeminar, 11–15 December 2004, Orlando, pp. 441–443.
[19] Gilaman, J.C. (1945): A manual of soil fungi.- The Iowa State University Press, Ames, Iowa, U.S.A.
[20] Gooding, M.J. (2006): The effect of fungicides on the grain yield and quality of wheat.- Actas del Congreso "A Todo Trigo". 18 y 19 de Mayo de 2006, Mar del Plata, Argentina, pp. 45-52.
[21] Hussain, T., Ishtiaq, M., Hussain A., Sultana, K. (2011): Study of Drinking Water Fungi and its Pathogenic Effects on Human Beings From District Bhimber Azad Kashmir, Pakistan.-Pak. J. Bot. 43(5): 2581-2585.
[22] Iftikhar, S., Asad, S., Rattu, A., Anjum, M., Fayyaz, M. (2012): Screening of commercial wheat varieties to spot blotch under controlled and field conditions. - Pak. J. Bot. 44:361-363.
[23] Juroszek, P., Tiedemann, A. (2011): Potential strategies and future requirements for plant disease management under a changing climate.- Plant Pathology 60:100–112.
[24] Jaczewska, K.A. (2010): Efekt ekonomiczny zwalczania chorób grzybowych pszenicy ozimej latach.- Swiat Zbó. 14: 16-19.
[25] Korbas, M. (2004): Choroby podstawy zdzbła –mofliwosci i perspektywy zwalczania.- Plant Protection 44: 147-154.
[26] Kaur, S., Dhaliwal, L., Kaur, P. (2008): Impact of climate change on wheat disease scenario in Punjab.- Journal of Research 45:161–170.
[27] Madgwick, J.W., West, J.S., White, R.P., Semenov, M.A., Townsend, J.A., Turner, J.A. (2011): Impacts of climate change on wheat anthesis and fusarium ear blight in the UK.- European Journal of Plant Pathology 130: 117–131.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_049065

[28] Ortiz, R., Sayre, K. D., Govaerts, B., Gupta, R., Subbarao, G.V., Ban, T. (2008): Climate change: can wheat beat the heat?- Agriculture, Ecosystems & Environment 126: 46–58.
[29] Oerke, E.C. (2006): Crop losses to pests.- The Journal of Agricultural Science 144: 31–43.
[30] Pathak, N., Razia, K.Z. (2013): Comparative study of seed dressing fungicides and C*alotropis procera* latex for the control of seed-borne mycoflora of wheat.- Annals of Biological Research 4 (4):1-6.
[31] Pettitt, T.R., Parry, D.W. (1996): Effects of climate change on Fusarium foot rot of winter wheat in the United Kingdom.- In: Frankland, J. C., Magan, N., Gadd, G. M. (eds.) Fungi and environmental change., Cambridge: Cambridge University Press, pp. 20–31.
[32] Rosada, J., Dubas, A., Bubniewicz, P. (2010): Perspektywy agrotechnicznych metodochrony roslin.- Progress in Plant Protection 50: 1181-1187.
[33] Roth, G.W., Marshall, H.G. (1987): Effects of timing of nitrogen fertilization and a fungicide on soft red winter wheat.- Agronomy Journal 79:197-200.
[34] Sharma, R.C., Duveiller, E. (2003): Effect of stress on *Helminthosporium* leaf blight in wheat.- In: Rasmussen, J.B., Friesen, T. L., Ali, S. (eds) Proc. 4th int. wheat tan spot and spot blotch workshop, North Dakota State University, Fargo, pp. 140-144.
[35] Sharma, R.C., Duveiller, E. (2004): Effect of *Helminthosporium* leaf blight on performance of timely seeded wheat under optimal and stress levels of soil fertility and moisture.- Field Crop Research 89:205-218.
[36] Singh, R.V., Singh, A.K., Ahmad, R., Singh, S.P. (1998): Influence of agronomic practices on foliar blight and identification of alternate hosts in the rice-wheat cropping systems.-In: Duveiller, E., Dubin, H.J., Reeves, J., McNab, A. (eds) Helminthosporium leaf blight of wheat: Spot blotch and tan spot, CIMMYT, Mexico, pp. 346-348.
[37] Steel, R.G.D., Torrie, J.H., Dickey, D. (1996): Principles and Procedures of Statistics: A Biometrical Approach, 3rd edition. - Mc Graw-Hill, New York, U.S.A.
[38] Teng, P.S., James, W.C. (2001): Disease and yield loss assessment.- In: Waller, J.M., Lenne, J.M., Waller, S.J. (eds.): Plant Pathologist's Pocketbook, CABI Publishing Company Inc., Boston, Massachussetts, pp. 25-38.
[39] Usmani, S.M.H., Ghaffar, A. (1982): Polyethylene mulching of soil to reduce viability of sclerotia of *Sclerotium oryzae*.- Soil Biol. Biochem. 14: 203-206.
[40] Varga, B., Svecnjak, Z., Macesic, D., Uher, D. (2005): Winter wheat cultivar responses to fungicide application are affected by nitrogen fertilization rate.- Journal of Agronomy and Crop Science 191: 130-137.
[41] West, J.S., Holdgate, S., Townsend, J.A., Edwards, S.G., Jennings, P., Fitt, B.D.L. (2012): Impacts of changing climate and agronomic factors on fusarium ear blight of wheat in the UK.- Fungal Ecology 5: 53–61.
[42] Wagacha, J.M., Muthomi, J.W. (2007): *Fusarium culmorum*: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat.- Crop Protection 26: 877-885.
[43] Wolf, A., Schramm, U., Fahr, A., Aicher, L., Cordier, A., Trommer, W.E., Fricker, G. (1998): Hepatocellular effects of cyclosporine A and its derivative SDZ IMM 125 in vitro.- J. Pharmacol. Exp. Ther. 284: 817-825.

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APPENDIX

Appendix 1. *Wheat variety Fareed-2006 (V1)*

Appendix 2. *Wheat variety Seher-2006 (V2)*

Appendix 3. *Wheat variety Lasani-2008 (V3), Wheat variety Faisalabad- 2008 (V4)*

Appendix 4. *Wheat variety Millat-2011(V5)*

Appendix 5. *Wheat variety Aas 2011 (V6)*

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Appendix 6. *Wheat variety Punjab-2011 (V7)*

Appendix 7. *Wheat variety Galaxy-2013 (V8)*

Appendix 8. *Spray of Fungicides in field trails*

Appendix 9. *Spray of Plants extracts on wheat trails*

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NON-CHEMICAL WEED MANAGEMENT IN POTATO AT HIGHER ELEVATIONS

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Abstract. A field experiment was carried out at the Agriculture Research Station, Chitral, Pakistan in 2014 to determine the effect of sowing orientation, planting spacing and mulching on yield and weeds of potato crop, using a three factorial RCB design. The sowing orientation (Factor A) had two levels viz., north-south and east-west sowing, plant spacing (Factor B) with three levels of 15, 25 and 35 cm, and mulching (Factor C) of *Cannabis sativa* L. biomass as mulch, *Plantago lanceolata* L. biomass as mulch along with a hand weeded treatment and a weedy control. The experiment results revealed a significant effect of sowing orientation, planting spacing, mulching and their interactions on the crop and weeds. The east-west sowing resulted in an increased weed density and biomass, whereas the north-south direction produced greater plant heights, greater number of leaves, tuber weight plant^{-1} and tuber yield. Plant spacing of 35 cm showed a significant increase in weed density and biomass, number of leaves and tubers plant^{-1} and tuber weight plant^{-1}. Plant spacing of 15 cm resulted in increased plant height and tuber yield. On the other hand, lowest weed density and biomass, number of leaves and tubers plant^{-1} and tuber weight were noted at planting space of 15 cm. Among the treatments of factor C, weedy check gave highest weed density and biomass ha^{-1} and plant heights, while the number of leaves and tubers plant^{-1}, tuber weight plant^{-1} and yield ha^{-1} were the highest in hand weeding treatments. The weedy check showed lowest number of leaves and tubers plant^{-1}, tuber weight plant^{-1} and yield ha^{-1}. In conclusion, the N-S sowing orientation, planting spacing of 15 cm and the mulching of plant biomasses of *C. sativa* and *P. lanceolata* proved the best combination for an environment-friendly weed management in potato at the higher elevations.
Keywords: *Cannabis sativa, Plantago lanceolata, plant spacing, population, tuber yield, sowing orientation*

Introduction

Potato is among the four major food crops grown in the world and three major vegetable crops in Pakistan. This tuber crop contains all the essential ingredients required for the maintenance of proper health. Pakistan has extensive edaphic and ecological resources for ideal potato production, which is grown in Pakistan on a diversified area, starting from sea level to an altitude of almost 3650 m in the mountains of northern areas. Potato is mostly affected by the infesting weeds that trigger substantial tuber yield reduction. Weeds significantly reduce the tuber yield whereas weed control treatments substantially increase the tuber yield as well (Jaiswal and Lal, 1996a, 1996b; Hussain et al., 2013; Mehring et al., 2016). Chemical control is the least expensive weed control method in potato that gives highest marginal return (14.17%) as compared to other weed control methods (Jan et al., 2004). In case of no chemical use, hand weeding can help achieve highest potato yields (Hashim et al., 2003). Generally about Rs. (Pakistan rupees) 3 billions are annually lost due to weed competition, whereas 38% yield losses are there in potato only (Hassan and Marwat, 2001). Yield losses in potato increase exponentially with increase in weeds biomass growing in the

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crop (Banaras, 1993; Mondani et al., 2011). In addition, weeds obstruct the tubers' harvesting (Knezevic et al., 1995). An estimated number of 8000 plant species are suspected to act as weeds, however, just 250 species are considered important for agriculture in the world. Environmental factors such as soil, light, water, temperature and micro-organisms do influence the weed range, their growth and distribution (Barbour et al., 1980; Peng, 1984).

An experiment was planned to assess the effect of certain environment-friendly weed control techniques to minimize the reliance on chemical weed control and boost healthy weed management tools. As the sunlight falls on the leaves in different angles in various regions around the globe, the idea was to examine the effect of sunlight when the crop is sown in east-west as well as north-south directions. A similar research was conducted by Steele et al. (2006) in USA who reported no effect of sowing orientation on potato crop. A second way of environment friendly weed management was the planting spacing which can improve the competition potential of potato plants with the growing weeds (Berkowitz, 1988; Forcella et al., 1992). Decreasing row spacing may also limit the period of time that weeds can compete with crops (Conley et al., 2001). In addition, mulching is another method through which the growing weeds can be suppressed helping the crop get more competitive (Bhullar et al., 2015). As organic crop production is appreciated worldwide (Khan et al., 2012) therefore, non-chemical weed management in vegetables needs to be explored. In this regard, various kinds of mulches have been tested worldwide; however, very little study has been conducted on weeds biomass using as mulch before their seeding stage for the purpose of shading the growing weeds in potato crop. This way we get two benefits in one attempt. Firstly the existing biomass of the infesting weeds is reduced through manual weeding and secondly the pre-seeding stage of the weed biomass is utilized for mulching purpose which helps shade or suffocate the emerging weeds that will eventually become a source of organic matter for soil in addition to weed management. There is definitely a dire need of developing a weed management strategy in potatoes which is more effective, sustainable, enhancing crop yields, environment-friendly and economical.

Therefore, keeping in view the above mentioned eco-friendly weed management techniques in potato, a field experiment was planned to assess the various non chemical weed control strategies for improvement in potato production and reduction in weeds infestation through environment friendly means.

Materials and Methods

Experimental site and design

The coordinates of the experimental site the Agriculture Research Station (Sheen Lasht) Chitral Pakistan are 35.8523° N, 71.7871° E situated at an elevation of 1517 m. The area of Chitral is 14850 square kilometers which is the largest district of Pakistan on basis of area. Chitral is having 76% glaciers and mountains, 20% forests and grazing lands, and 4% agricultural land. A field experiment was conducted during 2014 for studying the effect of row sowing directions (north-south and east-west sowing), various plant to plant spacings (15, 25 and 35 cm) and varying weed control treatments (including *Cannabis sativa* plant biomass and *Plantago lanceolata* plant biomass used as mulch, a hand weeding and a weedy check treatment) on yield and yield components of potato. The trial was performed in a three-factorial randomized complete block design replicated three times.

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Agronomic practices

Soil was ploughed thoroughly and then leveled using a cutter. The soil was of calcareous type having pH about 7.5-8. Well rotten farm yard manure was mixed with the soil. NPK was applied at 250, 125, 125 kg ha^{-1}, respectively. Irrigation was carried out with an interval of seven days. There were four ridges of potato crop in each sub-plot, each ridge 4 m long and spaced 0.75 m apart. Measurements were made on individual plants present in the mid two ridges of the four ridge plots. A local potato variety 'Rako' was sown in a plot size of 3 m by 4 m having 4 ridges (rows) each 4 meters long having 0.75 m row to row distance, making a total treatment size of 4 m x 0.75 m x 4 = 12 m^2.

Statistical analysis

The collected data was analyzed through the statistical software Statistix 8.1 to generate ANOVA tables. The significant means were subjected to the LSD test. Graphs were generated using the MS-Excel in MS Office 2007. The mean factors are indicated in tables, whereas the significant interactions are presented in figures.

Results

Weed density m^{-2}

There was a significant effect of sowing orientation, plant spacing, and weed control treatments on the density of weeds per square meter (*Table 1*). The weed density was found significantly lower (106 weeds m^{-2}) in plots with potato plants sown in north-south direction as compared to the sowing in east-west direction (117 m^{-2}). Moreover, the number of weeds per unit area was significantly lowest (104 m^{-2}) in planting spacing of 15 cm, followed by plots in which potato plants were sown at a distance of 25 cm (111.5 weeds m^{-2}). The planting spacing of 35 cm exhibited the highest weed population (119.6 m^{-2}). For the factor C, the weed density was lowest (69.2 m^{-2}) in hand weeded treatments followed by plots where *Cannabis sativa* plant biomass was used as mulch (96.2 m^{-2}) and plots with plant biomass of *Plantago* spp. as mulch (110.5 m^{-2}) as compared to the significantly highest weed density (170.8 m^{-2}) in the weed check plots. The significant interaction for planting spacing and weed control treatments is given in *Fig. 1*.

Weed biomass (kg ha^{-1})

There was a significant influence of sowing orientation, planting spacing, and weed control treatments on weed biomass (*Table 1*). Plots of potato grown in north-south direction resulted in lower weed biomass (858 kg ha^{-1}) as compared to sowing in the east-west direction (940 kg ha^{-1}). Weed biomass was significantly lowest (728 kg ha^{-1}) in plant spacing of 15 cm, followed by plots in which potato plants were sown at a distance of 25 cm with weed biomass of 892 kg ha^{-1}. The highest weed biomass (1,076 kg ha^{-1}) was found in potato plant to plant distance of 35 cm. The weed biomass for the weed control treatments was significantly lowest (555 kg ha^{-1}) in hand weeded plots, followed by plots of *Cannabis sativa* plant biomass as mulch (773 kg ha^{-1}) and *Plantago* sp. plant biomass applied as mulch (882 kg ha^{-1}). The weed biomass was

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significantly highest (1386 kg ha^{-1}) in the control plots (weedy check). The significant interaction effect for P x T is given in *Fig. 2*.

Table 1. *Effect of sowing orientation, plant spacing and weed control treatments on weed density m^{-2} and weed biomass (kg ha^{-1}) in potato during 2014 at higher elevations of Pakistan*

Treatments	Weed density (m^{-2})	Weed biomass (kg ha^{-1})
Sowing orientation (S)		
East-west sowing (S1)	117.1 a	940.32 a
North-south sowing (S2)	106.2 b	857.68 b
Significance level	*	*
Plant spacing (P)		
15 cm (P1)	104.0 b	728.3 c
25 cm (P2)	111.5 ab	892.2 b
35 cm (P3)	119.6 a	1076.5 a
LSD (0.05)	12.3	96.70
Treatments (T)		
Cannabis sativa biomass as mulch (T1)	96.2 c	773.0 b
Plantago spp. biomass as mulch (T2)	110.5 b	881.7 b
Hand weeding (T3)	69.2 d	555.5 c
Weedy check (T4)	170.8 a	1385.6 a
LSD (0.05)	14.21	111.65
Interactions	Significance level	
S x P	Ns	ns
S x T	Ns	ns
P x T	*	*
S x P x T	Ns	ns

Means in the same column with different letters are significantly different at α = 0.05 using LSD test
* = Significant, NS = Non-Significant

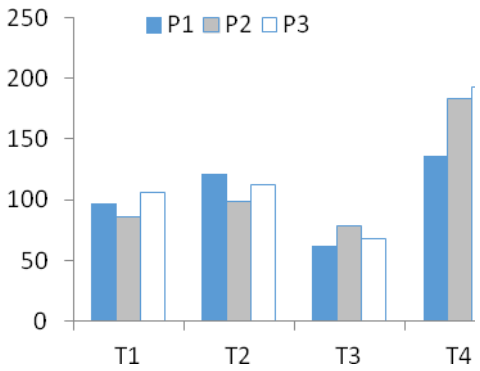

Figure 1. *Interaction effect of plant spacing and weed control treatments (P x T) for weed density m^{-2} in potato crop at higher elevation of Chitral during 2014*

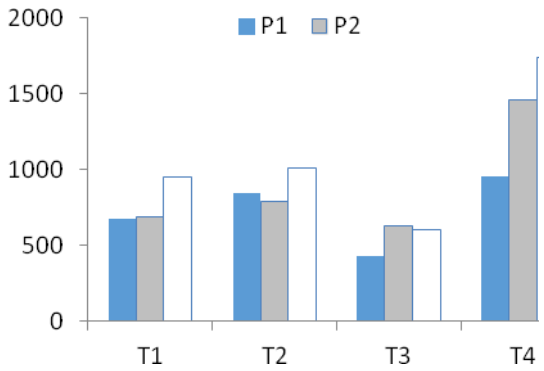

Figure 2. *Interaction effect of plant spacing and weed control treatments (P x T) for weed biomass kg ha^{-1} in potato crop at higher elevation of Chitral during 2014*

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Potato plant height (cm)

All the three factors and their interactions had a significant influence on potato plant height (*Table 2*). The plant height was lower (46.5 cm) in plots of east-west direction than in north-south (51.5 cm). As regards the plant spacing, potato plant height was lowest (47.8 cm) in 35 cm plant to plant spacing. It was followed by plots in which 25 cm spacing was maintained (48.6 cm). Highest potato plants (50.6 cm) were found at 15 cm plant-to-plant spacing. Among the weed control treatments, the plant height was significantly lowest (43.43 cm) in hand weeded plots followed by *Cannabis sativa* plant biomass used as mulch (48.16 cm) and plots with plant biomass of *Plantago* spp. applied as mulch (50.03cm) as compared to the significantly highest plant height (54.67 cm) in the weed check plots. The significant interactions are given in *Figs. 3a* and *3b* for interactions of S x P and P x T, respectively.

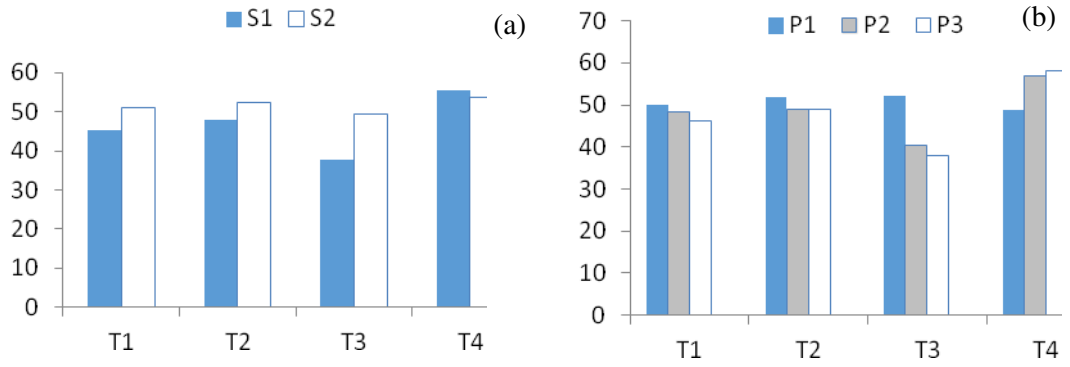

Figure 3. *Interaction effect of (a) sowing orientation and weed control treatments (S x T) (b) plant spacing and weed control treatments (P x T) for plant height (cm) of potato crop at higher elevation of Chitral Pakistan during 2014*

Number of leaves plant^{-1}

The analysis of the data showed that number of leaves plant^{-1} was significantly affected by the studied factors (*Table 2*). There was smaller number of leaves plant^{-1} (20.47) in plots of east-west sowing orientation as compared to sowing in the north-south (22.63). In plant spacing of 15 cm, the number of leaves plant^{-1} was significantly lowest (17.6), followed by plots planting spacing of 25 cm (22.3 leaves). The number of leaves plant^{-1} was highest (24.7 leaves) in plant spacing of 35 cm. The number of leaves plant^{-1} was significantly lowest (15.2) in weedy check plots among the weed control treatments. Weedy check was followed by mulching of *Cannabis sativa* plant biomass (23) and of *Plantago* spp. plant biomass applied as mulch (21.3) as compared to the significantly highest number of leaves plant^{-1} (26.5) in the hand weeded plots. The significant interaction for plant spacing x weed control treatments (P x T) is given in *Fig. 4*.

Tuber yield (t ha^{-1})

After analyzing the data, it was found that the sowing orientation, plant spacing, and weed control treatments all significantly affected the tuber yield of potato. *Table 2* indicated the mean values for potato tuber yield. The tuber yield was significantly

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higher (19.58 t ha^{-1}) in plots of north-south row sowing as compared to row orientation of east-west sowing (18.11 t ha^{-1}). In case of the plant spacing, the tuber yield was significantly highest (20.00 t ha^{-1}) in plots of 15 cm spacing between potato plants. The highest tuber yield was followed by 18.80 t ha^{-1} where there was 25 cm spacing between potato plants; while the lowest tuber yield (17.72 t ha^{-1}) was achieved in plant spacing of 35 cm. The tuber yield was also significantly highest (24.05 t ha^{-1}) in treatments of hand weeding which was followed by the mulching of *Cannabis sativa* plants (20.22 t ha^{-1}) and mulching of *Plantago* sp. whole plants (18.91 m^{-2}) as compared to the significantly lowest tuber yield of 12.18 t ha^{-1} in the control plots. *Fig. 5* shows the significant interaction for plant spacing x weed control treatments (P x T).

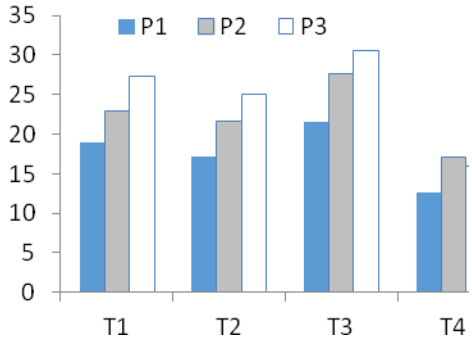

Figure 4. *Interaction effect of plant spacing and weed control treatments (P x T) for number of leaves plant^{-1} potato crop at higher elevation of Chitral during 2014*

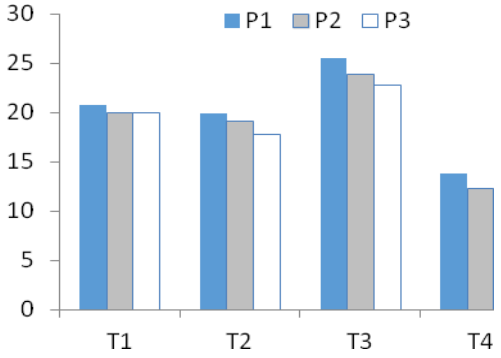

Figure 5. *Interaction effect of plant spacing and weed control treatments (P x T) for tuber yield (t ha^{-1}) of potato crop at higher elevation of Chitral during 2014*

Table 2. *Effect of sowing orientation, plant spacing and weed control treatments on number of potato leaves plant^{-1} and tuber yield (t ha^{-1}) of potato crop during 2014 at higher elevations of Pakistan*

Treatments	Potato plant height (cm)	Number of leaves plant^{-1}	Tuber yield (t ha^{-1})
Sowing orientation (S)			
East-west sowing (S1)	46.5 b	20.47 b	18.11 b
North-south sowing (S2)	51.5 a	22.63 a	19.58 a
Significance level	*	**	**
Plant spacing (P)			
15 cm (P1)	50.6 a	17.6 c	20.00 a
25 cm (P2)	48.6 b	22.3 b	18.80 b
35 cm (P3)	47.8 b	24.7 a	17.72 c
LSD (0.05)	1.39	0.98	0.43
Treatments (T)			
Cannabis sativa biomass as mulch (T1)	48.16 c	23.0 b	20.22 b
Plantago spp. biomass as mulch (T2)	50.03 b	21.3 c	18.91 c
Hand weeding (T3)	43.43 d	26.5 a	24.05 a
Weedy check (T4)	54.67 a	15.2 d	12.18 d
LSD (0.05)	1.61	1.13	0.50

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Interactions	Significance level		
S x P	ns	ns	ns
S x T	*	ns	ns
P x T	*	*	*
S x P x T	*	ns	ns

Means in the same column with different letters are significantly different at α = 0.05 using LSD test

* = Significant, NS = Non-Significant

Discussion

Weed density m^{-2}

The following weeds were recorded in the experimental field viz., *Plantago lanceolata, Convolvulus arvensis, Chenopodium album, Cannabis sativa, Sorghum halepense, Polygonum aviculare, Cynodon dactylon* and *Veronica didama*. The potato leaves received the solar radiation more effectively in the north-south sowing orientation as compared to the east-west sowing orientation due to which they became competent enough to suppress the emerging weeds. The canopy of the individual plants shades the adjacent plants in the east-west orientation of the potato rows (ridges) which may decrease the required solar radiation. Karanja et al. (2014) and Hozayn et al. (2012) reported significant effect of row sowing orientation on weed density and crop yield. The spaces between the potato plants significantly affected the weed density. Narrow spacing of 15 cm in potato plants suffocated weeds number per unit area whereas wider spacing (25 and 35 cm) gave room to the growing weeds with which the number and composition of weeds increased (Conley et al., 2001; Ara et al., 2007).

Mulching enhances the soil moisture retention and improves soil temperature (Dalorima et al., 2014; Khan et al., 2012), which helps boost crop performance making the crop more competitive against the associated weeds. In addition, regardless of what kind of mulch is used, mulching of the soil causes a decrease in the weed density in the beginning of the growing period of vegetables like tomato, potato and onion (Kosterna, 2014). In the field trial, mulching of *Cannabis sativa* resulted better in declining the weed density due to its bigger canopy which effectively shaded the emerging weeds as compared to the mulching of *Plantago* spp. Though hand weeding resulted best in reducing the number of weeds per unit area, it is not feasible in conditions of labor scarcity, or at a large scale. The interaction effect of all factors was however non-significant except that for interaction of plant spacing and weed control treatments (P x T). This indicated that P x T interaction is important for declining the weed density in potato crop of Chitral region of Pakistan.

Weed biomass (kg ha^{-1})

Weed biomass is directly proportional to weed density i.e. the biomass is generally higher in plots with higher weed density. Here again the relatively efficient reception of solar radiation by the crop plants in the north-south oriented sowing made them get more competent against weeds as compared to the east-west sowing orientation. The planting spacing of potato plants significantly affected the weed biomass. As the narrow spacing of 15 cm among potato plants suffocated the weeds number per unit area due to which the weed biomass was eventually reduced. The wider spacing of 25 and 30 cm

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provided room for the growing weeds with which the number and biomass of weeds increased (Ara et al., 2007).

The weed control treatments also were effective in declining the weed biomass. Mulching treatments were effective in reducing weed biomass. It boosted the crop performance because of suppression of the associated weeds. Mulching of *Cannabis sativa* in this experiment performed well in resulting in reduced weed biomass due to the bigger canopy and shading of the emerging weeds. The interaction effect for plant spacing and treatments (PxT) was significant which represented that P x T interaction has got a key role in minimizing the weed biomass in potato crop.

Potato plant height (cm)

Plant height has got a complex phenomenon. It increases in circumstances of competition, particularly for receiving the sunlight. With increase in height the plant stem diameter decreases which indicates that it is not necessary that crop yield may linearly increase or decrease with plant height (Thornley, 1999). The significant difference in plant height of north-south and east-west sowing orientations showed that the competition for light is greater in the north-south direction as compared to east-west direction, in Chitral region of Pakistan. Close spacing of 15 cm increased potato plant height whereas wider spacing of 25 and 35 cm resulted in decreased plant height. The results are in harmony with those of Papadopoulos and Ormrod (1991) who recorded increase in plant height with close spacing. The height will increase with decrease in competition with weeds. In this connection, Mochiah et al. (2012) reported an increase in plant height of pepper with mulching of the soil. The mulching of *Cannabis sativa* effectively reduced the weed competition which eventually helps increase the plant height as compared to weedy check. The interaction effects for sowing orientation and planting spacing (S x P) were significant. This indicated that S x P interaction improved the plant height of potato grown at the higher elevation of Chitral Pakistan.

Number of leaves plant^{-1}

The comparatively higher number of leaves plant^{-1} in the sowing orientation of north-south could be attributed to the more efficient reception of solar radiation as compared to that in the east-west sowing direction. The results for planting spacing were analogously reported by Zaag et al. (1990) who reported that wider spaces increase the branching of potato plants which enhances the number of leaves too. Regardless of what kind of mulch is used, mulching resulted in increased number of leaves plant^{-1} (Gudugi et al, 2012). The mulching of *Cannabis sativa* performed well in increasing the number of leaves plant^{-1} because of better plant height and less competition from weeds. The effect of plant spacing x treatments interaction was significant number of leaves plant^{-1} in potato crop.

Tuber yield (t ha^{-1})

Tuber yield of potato is the only parameter on which all the experiment is dependent upon. The north-south oriented crop resulted in better yield because of reduced weed density and biomass, and due to better plant height and number of leaves plant^{-1}. In contrary to our results, Karanja et al. (2014) reported 18.3% increase in cowpea grain yields in east to west row orientated crops over the north to south oriented crops. Tsubo et al. (2004) also mentioned that E-W row oriented crops received higher

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photosynthetically active radiation (PAR) than the N-S ones. These contrary results could be justified as that E-W oriented crops receive higher PAR in at higher latitudes which happened in the experiments of Karanja et al. (2014) and Tsubo et al. (2004) in South Africa whereas N-S oriented crops receive higher PAR at lower latitudinal areas like Pakistan. The other reason could be Chitral, a region of higher altitude of Pakistan where N-S orientation would be more successful in terms of enhancement in competitive ability against weeds. Moreover, the canopy of individual crop plants overlap with the adjacent plants in E-W, consequently rendering the situation favorable for PAR reception in plots of N-S orientation. The plant to plant spacing in potato crop had a significant effect on the tuber yield of potato. Increasing the plant spacing from 15 cm to 35 cm decreased the per plant yield because of intra- specific competition among the crop plants but the gross yield was highest in the same plant spacing. Per hectare yield however decreased with increasing the plant spacing from 15 to 35 cm. The results are in harmony with that of Ahmad and Singh (2005) who reported that even though the fruit size and the weight was higher in wider spaced rows, the total yield obtained was higher in the close spaced rows.

Among the weed control treatments, hand weeding provided best fruit yield as a result of efficient weed control. Hand weeding was however closely followed by mulching treatments of the selected weeds. The use of plant biomass of *Cannabis sativa* as mulch enhanced the yield of potato because of shading and eventual suppressing of the emerging weeds. Moreover, mulching of *Plantago* sp. was also better than the weedy check in significantly improving the potato tuber yield per hectare. The interaction between the plant spacing and weed control treatments was significant resulting in maximum tuber yield (t ha^{-1}) in potato crop grown at the higher elevation.

REFERENCES

[1] Ahmad, A., Singh, A. (2005): Effects of Staking and Row-Spacing on the Yield of Tomato Cultivar “Roma VF” in the SokotoFadama, Nigeria. - Nigerian Journal of Horticultural Sciences 10: 94-98.

[2] Ara, N., Bashar, M.K., Begum, S., Kakon, S.S. (2007): Effect of spacing and stem pruning on the growth and yield of tomato. - International Journal of Sustainable Crop Production 2: 35-39.

[3] Banaras, M. (1993): Impact of weed competition on potato production. - Pakistan Journal of Agricultural Research 14: 64-71.

[4] Barbour, M.G, Burk, J.H., Pitts, W.D. (1980): Terrestrial Plant Ecology. - Benjamin/Cummings Pub. Co., Menlo Park. California.

[5] Berkowitz, A.R. (1988): Chapter 7. - In: Weed Management in Agroecosysterns: Ecological Approaches. CRC Press, Boca Raton, FL. pp. 90-118.

[6] Bhullar, M.S., Kaur, S., Kaur, T., Jhala, A.J. (2015): Integrated weed management in potato using straw mulch and atrazine. - Horticultural Technology 25: 335-339.

[7] Conley S.P., Binning, L.I., Connell, T.R. (2001): Effect of cultivar, row spacing, and weed management on weed biomass, potato yield, and net crop value. - American Journal of Potato Research 78: 31-37.

[8] Dalorima, L.T., Bunu, A., Kyari, Z., Mohammed, T. (2014): Effects of different mulching materials on the growth performance of okra in Maiduguri. - International Research Journal of Agricultural and Soil Sciences 4: 145-149.

[9] Forcella, F., Westgate, M.E., Wames, D.D. (1992): Effects of row width on herbicide and cultivation requirements in row crops. - American Journal of Alternate Agriculture 7: 161-167.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_067076

[10] Gudugi, I.A.S., Odofin, A., Adeboye, M.K., Oladiran, J.A. (2012): Agronomic characteristics of tomato as influenced by irrigation and mulching. - Advances in Applied Science Research 3: 2539-2543.
[11] Hashim, S., Marwat, K.B., Hassan, G. (2003): Chemical weed control efficiency in potato (*Solanum tuberosum* L.) under agro-climatic conditions of Peshawar, Pakistan. - Pakistan Journal of Weed Science Research 9: 105-110.
[12] Hassan, G., Marwat, K.B. (2001): Integrated weed management in agricultural crops. - Proc. National Workshop on technologies for Sustainable Agriculture, Sep. 24-26, 2001, NIAB, Faisalabad, Pakistan, Pp. 27-34.
[13] Hozayn, M., El-Shahawy, T.A.E.G., Sharara, F.A. (2012): Implication of Crop Row Orientation and Row Spacing for Controlling Weeds and Increasing Yield in Wheat. - Australian Journal of Basic and Applied Sciences 6: 422-427.
[14] Hussain, Z., Munsif, F., Marwat, K.B., Ali, K., Afridi, R.A., Bibi, S. (2013): Studies on efficacy of different herbicides against weeds in potato crop in Peshawar. - Pakistan Journal of Botany 45: 487-491.
[15] Jaiswal, V.P., Lal, S.S. (1996a): Efficacy of cultural and chemical weed control methods in potato. - Journal of Indian Potato Association 23: 20-25.
[16] Jaiswal, V.P., Lal, S.S. (1996b): Efficacy of cultural and chemical weed-control methods in potato (*Solanum tuberosum*). - Indian Journal of Agronomy 41: 454-456.
[17] Jan, H., Muhammad, A., Ali, A. (2004): Studies on weed control in potato in Pakhal plains of Mansehra. - Pakistan Journal of Weed Sciences Research 10: 157-160.
[18] Karanja, S.M., Kibe1, A.M., Karogo, P.N., Mwangi, M. (2014): Effects of Intercrop Population Density and Row Orientation on Growth and Yields of Sorghum - Cowpea Cropping Systems in Semi Arid Rongai. - Kenyan Journal of Agricultural Sciences 6: 34-43.
[19] Khan, M.A., Marwat, K. B., Amin, A., Nawaz, A., Khan, H. (2012): Soil solarization: an organic weed management approach in cauliflower. - Communication in Soil Science and Plant Analysis 43 (13): 1847-1860.
[20] Kosterna, E. (2014): The effect of different types of straw mulches on weed-control in vegetables cultivation. - Journal of Ecological Engineering 15: 109–117.
[21] Knezevic, M., Durkic, M., Samota, D. (1995): Chemical and mechanical weed control in potatoes. - Fragmenta Phytomedica et Herbologica 23(2): 61-67.
[22] Mehring, G.H., Stenger, J.E., Hatterman-Valenti, H.M. (2016): Weed control with cover crops in irrigated potatoes. - Agronomy 6: 1-11. DOI 10.3390.
[23] Mochiah, M.B., Baidoo, P.K., Acheampong, G. (2012): Effect of mulching materials on agronomic characteristics, pests of pepper and their natural enemies' population. - Agricultural and Biological Journal of North America 3: 253-261.
[24] Mondani, F., Golzardi, F., Ahmadvand, G., Ghorbani, R., Moradi, R. (2011): Influence of weed competition on potato growth, production and radiation use efficiency. - Notulae Scientia Biologicae 3: 42-52.
[25] Papadopoulos, A.P., Ormrod, D.P. (1991): Plant spacing effects on growth and development of the greenhouse tomato. - Canadian Journal of Plant Sciences 71: 297-304.
[26] Peng, S.Y. (1984): The biology and control of weeds in sugarcane. – Eslevier, Amsterdam, p. 25.
[27] Steele, D.D., Greenlanff, R.G., Hatterman-Valenti, H.M. (2006): Furrow vs hill planting of sprinkler-irrigated russet burbank potatoes on coarse-textured soils. - American Journal of Potato Research 83: 249-257.
[28] Thornley, J.H.M. (1999): Modelling stem height and diameter growth in plants. - Annals of Botany 84: 195-205.
[29] Tsubo, M., Mukhala, E., Ogindo, H.O., Walker, S. (2004): Productivity of maize-bean intercropping in a semi-arid region of South Africa. - Water South Africa 29: 381-388.
[30] Zaag, P.V., Demagante, A.L., Ewing, E.E. (1990): Influence of plant spacing on potato (*Solanum tuberosum* L.) morphology, growth and yield under two contrasting. - Potato Research 33: 313-323.

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MORPHO-ANATOMICAL AND PHYSIOLOGICAL CHANGES IN GRAPEVINE LEAVES EXPOSED TO ATMOSPHERIC FLUORIDE AND SULFUR DIOXIDE POLLUTION

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Abstract. A comparative study on the effects of air fluoride and sulphur dioxide pollution on a local vine (*Vitis vinefera* L.) cultivar "Asli" growing in the vicinity of a factory producing phosphate fertilisers in the southern suburb of Sfax region was investigated. The chemical analysis of both peripheral and central necrotic leaf round slices reveals a preferential accumulation of fluoride, as well as calcium in leaf margins and tips. However, central leaf areas seem to accumulate both sulfur and magnesium. These findings confirm the involvement of these two elements not only in the detoxification of fluoride as CaF_2 and MgF_2, but also in regulating metabolic pathways. On the other hand, net photosynthesis is maintained at appropriate levels, while 30 to 40% of leaf surface are altered by necrosis. Furthermore, several structural changes are revealed in polluted leaves. Indeed, the decrease of leaf density is due probably to (i) a decrease in intercellular spaces, (ii) a decline of vascular bundles number and (iii) a gradual sclerotization of collenchyma cells occurring jointly with a thickening of their walls.
Keywords: *gaseous fluoride, fruit trees, ion accumulation, necrotic spots, collenchyma cells*

Introduction

Fluoride compounds and sulphur dioxide (SO_2) are among the most important phytotoxic air pollutants and prevalent near to industrial activity areas such as fertilizer factories, brick kilns, ceramic manufacturing etc (Bell and Treshow, 2002). Generally, industries are constructed in rural areas or outskirts of the cities near to agricultural activity areas. Air pollutants emitted from industrial activity areas become dispersed in surrounding and deposited on plant leaf surfaces in particulate form or gaseous form (Baillie et al., 2016; Baunthiyal et al., 2014). Despite grapevine (*Vitis vinefera* L.) sensitivity to air pollutants (Doley, 1986; Leece et al., 1986; Murray, 1984), some ecotypes of “Asli” local cultivars are still surviving in the area surrounding the SIAPE (factory producing phosphoric acid and phosphate fertilizers) located in the southern suburb of Sfax city (Tunisia) (*Fig. 1*). Air pollutants emitted by the SIAPE chimneys factory are mainly fluoride compounds, sulphur dioxide and particles (Azri et al., 2002; Ben-Abdallah and Boukhris, 1990). On the other hand,

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Ben Abdallah (2007) found that fluoride (F) concentration in the air surrounding the factory ranged from 0.3-0.68 μg m^{-3}. In some studies, plant responses of plants to air pollution were carried out on plants by exposing them to long or short term fumigations (Davieson et al., 1990; Doley, 1986; Murray and Wilson, 1988) or in hydroponics (Stevens et al., 1998) or by analyzing plants growing near to source of pollution industry (Abdallah et al., 2006; Elloumi et al., 2003; Jha et al., 2008; Zwiazek and Shay, 1987). It has been reported that plants can uptake fluoride through roots (Chakrabarti et al., 2013; Telesiński et al., 2011). However, fluoride mainly entered into plants via stomatal apparatus thus affecting transpiration and photosynthesis or caused necrosis in leaves (Jha et al., 2008). In addition to leaf injuries, air pollutants including F and SO_2 cause various physiological disorders including reduction in photosynthesis and photosynthetic pigments, carbohydrate metabolism, membrane dysfunction and anatomical cellular structures (Ali et al., 2008; Baunthiyal et al., 2014; Mesquita et al., 2010; Telesiński et al., 2011). However, detailed information about changes in anatomical and cellular structures with respect to their mechanisms of accumulation and tolerance is scarce. Such information can help us in using plant species for bio-monitoring (Festy, 2001) as such plant responses depend on type of species, type of cultivar used, plant age, climatic conditions and the distance from the pollution source (Elloumi et al., 2003). The major purpose of the present study was to appraise the combined effects of both fluoride and sulphur dioxide pollution on accumulation of mineral nutrients and anatomical traits of local Asli vine cultivar grown in Sfax city (Tunisia). A secondary objective of the present study was to assess the role of Ca and Mg in avoiding and / or mitigating pollutant toxicity. In addition, up to what extent different leaf anatomical characteristics were modulated or damaged by these air pollutants in an arid region of Tunisia.

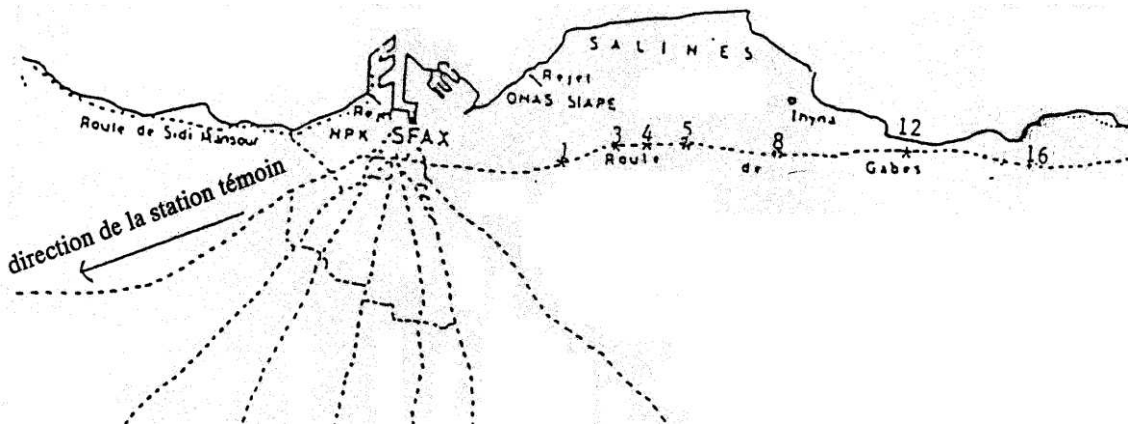

Figure 1. *Map of study area in Sfax, Tunisia*

Materials and Methods

Description of study area

The study area is 20 km wide Mediterranean coastal low land lined with 100-meter high peaks. It is submitted both to continental dry winds and to highly humid sea coastal winds. The prevailing winds from the southeastern sector have a frequency of (25.5%), and those of the southwestern sector appear with a frequency of about 16.25%. However, northwest and northeast winds occur with intermediate

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frequencies. The average total year rainfall is 210 mm, the average minimum temperature of the coldest month (January) is 6.5°C and the average maximum temperature of the hottest month (August) is 31°C. Most of the total annual rainfall is mostly occurring from October to December; the dry period is during June-September.

Sampling techniques

The 20-year old local vines (*Vitis vinefera* L.) growing in a loamy sand soil which were approximately 0.5 km and 30 km away from the polluting factory "SIAPE factory" were marked as polluted and control plants. Similarities between polluted and non polluted sites were verified by particle size analysis. Ninety (90) leaf samples from three plants of the cultivar were taken from branches located in the upper median and lower portions of the vine tree which were facing and exposed to the factory fume. Non-affected leaf samples were collected from non-polluted land plots situated 30 km away the factory. Aerial parts of vine that are located above the 12^{th} node of the shoot were taken. Samples were collected from polluted and non-polluted sites during the grapevine growing season (June, July and August 2009).

To determine leaf pollutant sites, we selected only grape leaves exhibiting at the same time central and marginal necroses. Owing to their available great leaf surfaces, 90 grapevine leaves, with 30 to 40% of necrotic leaf area, were cut into leaf marginal and leaf central necrotic pieces. The same leaf marginal and central pieces were also used to determine the concentration of F, Ca^{2+}, Mg^{2+} and P.

The leaf marginal necrosis was evaluated throughout the growing season (from May to August) of the leaves of "Asli" cultivar. Photosynthesis measurements were performed after the estimation of leaf necrose percentage according to Mabrouk and Carbonneau (1996) method. Necrotic peripheral slices of leaves were distributed into classes according to percent necrotic area (*Table 1*). Control or non-affected leaves were also cut into leaf marginal and central area and grouped into classes along with affected leaves.

Table 1. *Distribution of classes in function of percentage of leaf necroses.*

		Percentage of leaf necroses				
	Control	0	10-20	30-40	50-60	> 60
Classes	CT	C0	C1	C2	C3	C4

Photosynthesis measurements

Leaf net photosynthesis was measured in the field under ambient environmental conditions between 9:30 and 10:00 am using a portable gas exchange system (IRGA, CID 301 PS, Vancouver USA). The measurements were made on leaves that remain attached to the mother plant, occupying the $l0^{th}$ to the 12^{th} shoot node. All measurements for gas exchange were taken on sunny days with 1500 μmol m^{-2} s^{-1} light intensity. The PAR was measured directly by the infrared gas exchange analyzer system. The average leaf temperature during the experimental period was of 34 °C ± 2.55 °C. All measurements of gas exchange characteristics were measured on grapevine plants at both locations on with a difference of one day with same diurnal conditions. If there was any significant change in light or weather conditions, the measurements were taken on the next day but under similar diurnal conditions.

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Fluoride, sulphur, calcium and magnesium analyses

For fluoride analysis, different plant tissues (leaf blade, leaf stalk and internodes) were oven-dried at 70 °C for 72 hours. Oven dried leaf material were ground to make powder. Leaf powder was re-dried prior to weighing sub-samples for analysis. Fluoride concentrations were determined using the potentiometric technique described by Rhimi et al. (2011). Sulphur was determined by the turbidimetric method of (Blanchar et al., 1965), the detection limit of which is about 10 ppm with a RSD of 0.41%.

After digesting plant powder with nitric and perchloric acids (2 v/ 1 v), Ca^{2+} and Mg^{2+} were determined by the atomic absorption spectrophotometry technique with polarized Zeeman (HITACHI, Z-6100).

Anatomical sections

Fresh polluted and non polluted grape leaves were taken and fixed during 24 hours in Formalin - Acetic – Alcohol mixture (FAA) according to the fixation procedure described by Sass (1958). The fixed material was rinsed with water and then divided into two batches. The first one was dehydrated in alcohol and included in paraffin, cut and stained by the triple stain (Safranin, Heidenhain's and blue aniline). The second batch was cut by a freezing microtome and stained with aceto-carmin (Locquin and Langeron, 1978). Freezing and serial cross sections were observed under a light Reichert LKB microscope equipped with a camera.

Statistical analyses

Statistical analyses were performed with the SAS package (Statistical Analysis System, version 6.12, Cary, NC, USA) using both Duncan multiple range and Student Tests at the 5% significance level.

Results

Morphological responses

Our regular field observations in the polluted area allowed us to recognize various expressions of damage caused to the local vines growing in the vicinity of the factory. As shown in *Fig. 2*, two types of leaf necroses were observed: marginal necroses of brick red colour, occupying lobes extremities and central necroses occupying inter vein leaf blade spaces as few scattered circular red spots. Whereas the formers are very frequent and present in nearly all the vine leaves exposed to the factory smoke; the latter's are infrequent and appear especially on old leaves.

For the studied cultivar, necrotic tissues are separated from healthy ones by a dark violet borderline (*Fig. 2*). As time advances, necroses continue to invade the leaf surface. New necrotic tissues are limited by a new borderline less attenuated than the first one until obtaining concentric halos of necrotic zones giving the leaf margin a mosaic aspect (*Fig. 1*).

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Figure 2. *General pattern of necrotic areas in the leaves of grapevine cv. Asli affected by factory fumes*

Observation of anatomical sections

In comparison with control (*Fig. 3, A-D*), grapevine leaves from polluted area showed a decrease in number of upper and lower epidermal cells (*Fig. 4, E-M*), thickening in epidermal cuticle, thickening in cell walls of collenchyma cells at both sides of leaf midrib, an increase in sclerotisation of collenchyma cells.

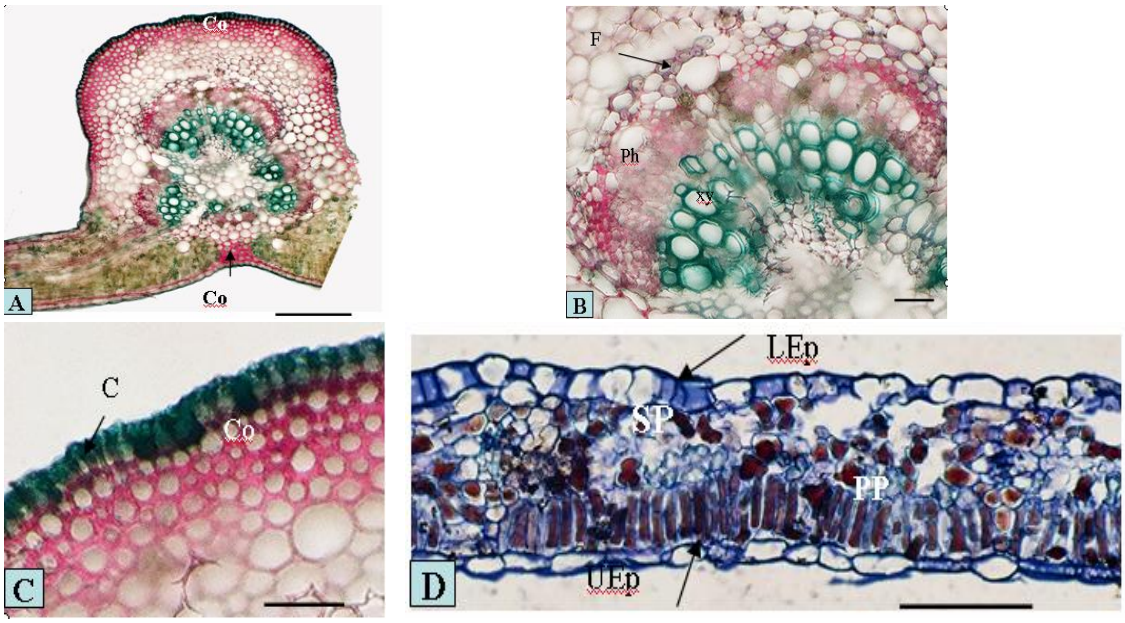

Figure 3. *(A) Transverse section, of control grape leaves, including the midrib, scale 1 bar = 100 µm. (B) Detail of the midrib vascular tissues, scale 1 bar = 10 µm. (C) Detail of the collenchyma, scale 1 bar = 20 µm. (D) Transverse section through the leaf blade. 1 bar = 100 µm. C = cuticle; Co = collenchyma ; LEp = lower epidermis; Ph = phloem; PP = palisade parenchyma; SP = spongy parenchyma; UEp = upper epidermis; Xy = xylem.*

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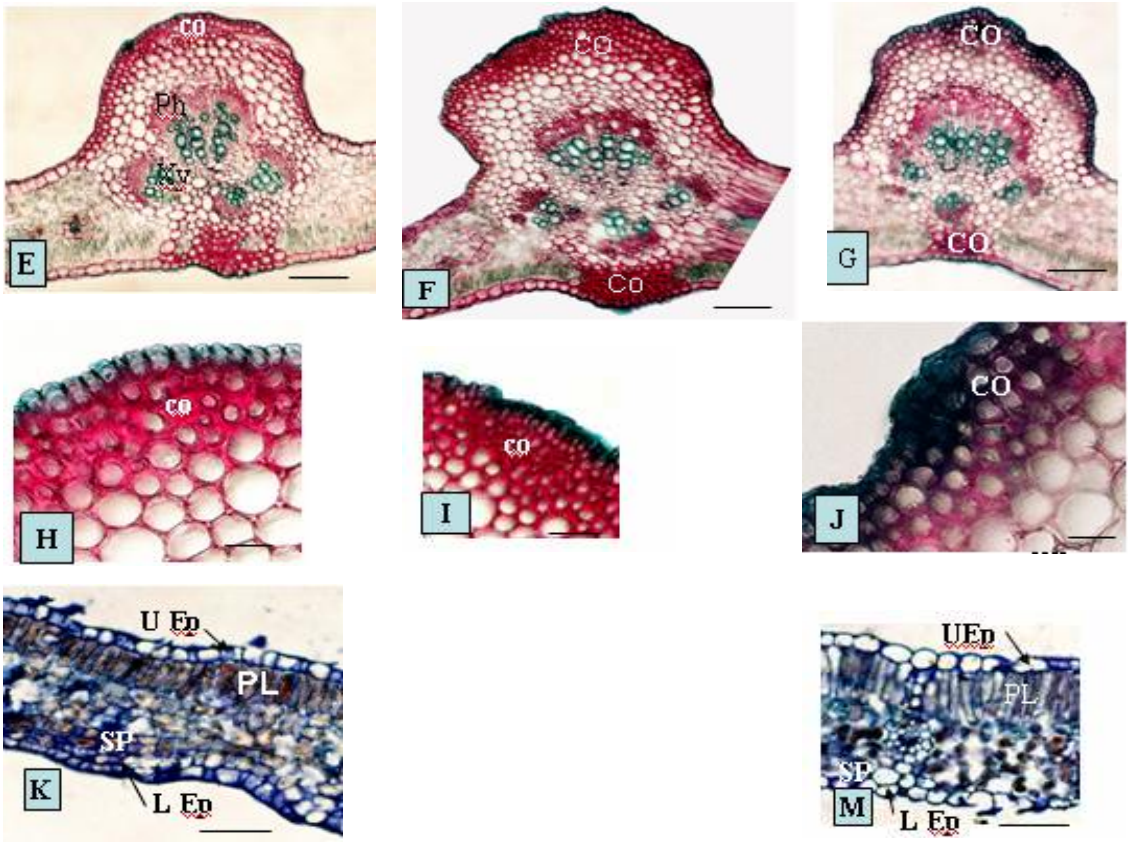

Figure 4. *Transverse sections of polluted grape leaves. (E-G) Detail of midribs, scale 1 bar = 100 µm.(H-J) Detail of the collenchyma. Note both its thickeness and sclerification with respect to the control, scale 1 bar = 20 µm. (K-M) Transverse section through the leaf blade, scale 1 bar = 100 µm. C = cuticle; Co = collenchyma ; LEp = lower epidermis; Ph = phloem; PP = palisade parenchyma; SP = spongy parenchyma; UEp = upper epidermis; Xy = xylem.*

Fluorine and sulphur distribution in different plant parts

Analyses of necrotic and central round leaf slices showed a significant increase in fluoride (F) accumulation in the necrotic leaf areas. On the other hand, the F content of the central leaf parts including the main veins were similar to that in control leaves (*Fig. 5*). Both leaf stalks and internodes accumulated lower F as compared to that in central or marginal areas of leaf from non-polluted and polluted area (*Table. 2; Fig. 5*).

Table 2. *Fluoride ($\mu g.g^{-1}DW$) content of leaf stalk and internodes of grapevine cv. Asli from polluted and non-polluted areas during two different periods.*

	May 15 th		**August 15 th**	
	F^-		**F^-**	
Area	**Control**	**Polluted**	**Control**	**Polluted**
Leaf stalk	5.7 ± 0.1	$6.1 \pm 0.6^{*}$	6.1 ± 0.4	6.5 ± 0.2^{NS}
Internode	0.71 ± 0.05	0.74 ± 0.07^{NS}	0.81 ± 0.05	$1.29 \pm 0.06^{***}$

Fluoride contents Vs controls *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ NS: non significant (n = 10).

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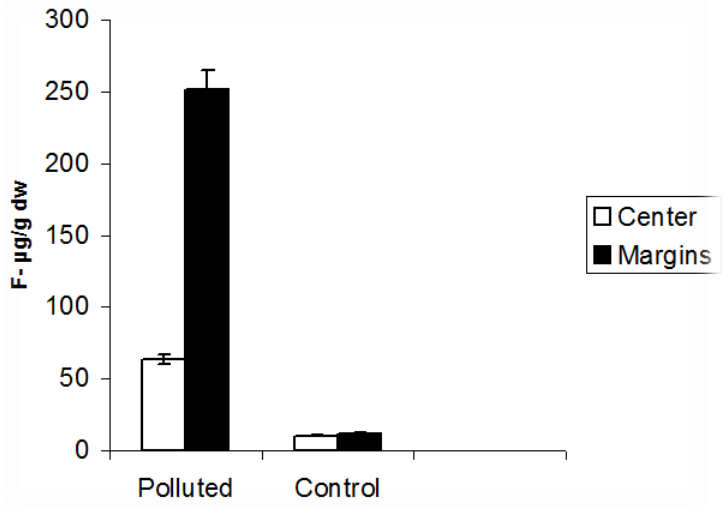

Figure 5. *Fluoride contents (µg F/g dw) in the center and in leaf margins of grapevine cv. Asli from non-polluted and polluted areas. Means of 10 replicates and confidence intervals at 5%.*

The chemical analyses of central leaf necrotic round slices showed high leaf sulphur content, as compared to control ones (*Fig. 6*). However, contents of sulphur in leaf margins and fluoride in the centre were not significantly higher than those of controls (*Figs. 5, 6*).

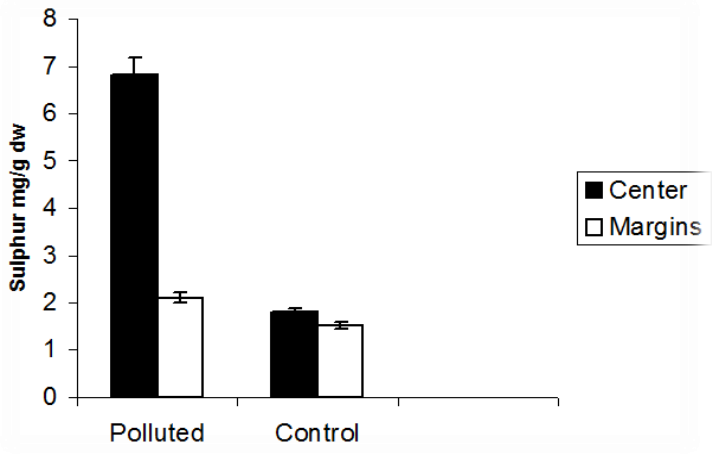

Figure 6. *Sulphur contents (mg S/g dw) in the center and in leaf margins of grapevine cv. Asli from non-polluted and polluted areas. Means of 10 replicates and confidence intervals at 5%.*

In the present study, a significant increase in fluoride accumulation was recorded in the necrotic peripheral round leaf slices. However, central parts of grapevine leaves including the main veins or midrib accumulated lower amount of fluoride (*Fig. 5*). The leaf stalks and internodes accumulated low fluoride (i.e. 1-6 µg/g dwt) throughout the growing season (*Table 2*) as compared to that in leaf lamina (50-250 µg/g dwt; *Fig. 5*). These results showed the preferential accumulation of fluorine in leaf tissues extremities.

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Besides, the accumulation of fluorine in coincidence with the appearance of peripheral grape leaf necroses suggest the existence of an external mechanism of fluoride sequestration allowing the exclusion of some elements as fluorine at leaf extremity level. Indeed, the restriction of damages to the leaf margins allows the plant to keep a great proportion of leaf surface intact and therefore, to maintain photosynthetic activity at appropriate levels. Such mechanism would explain the ability of this species to photosynthesize, even when the leaf surface was damaged up to 30 % (*Table 3*).

The chemical analyses of central leaf necrotic round slices showed high leaf sulphur content, compared to control (*Fig. 6*). For the same grape leaf exhibiting both marginal and inter-vein central necroses, contents of sulphur in margins and fluoride in the centre were not significantly higher than those of controls (*Fig. 5, 6*). These findings confirm that inter-vein central necroses are probably due to SO_2, whereas marginal leaf necroses are typical of fluoride compounds effects.

Leaf calcium and magnesium distribution and photosynthesis of healthy leaf areas

As also seen in *Fig. 7*, the mineral analyses of the central and necrotic grape leaf areas reveal high Ca and F concentrations in the leaf margins. The polluted grapevine seems to retain more Mg in the central leaf areas than in the leaf margins. As indicated in *Table 3*, photosynthesis of central (healthy) leaf areas decreased as the percentage of leaf necroses increased and that the plant could still photosynthesize while 30 to 40% of its leaf surface was necrotic. However, when 60% or more of the leaf surface was damaged, and there was less than 40% of healthy leaf area, low net photosynthesis of the central leaf area was observed (*Table 3*).

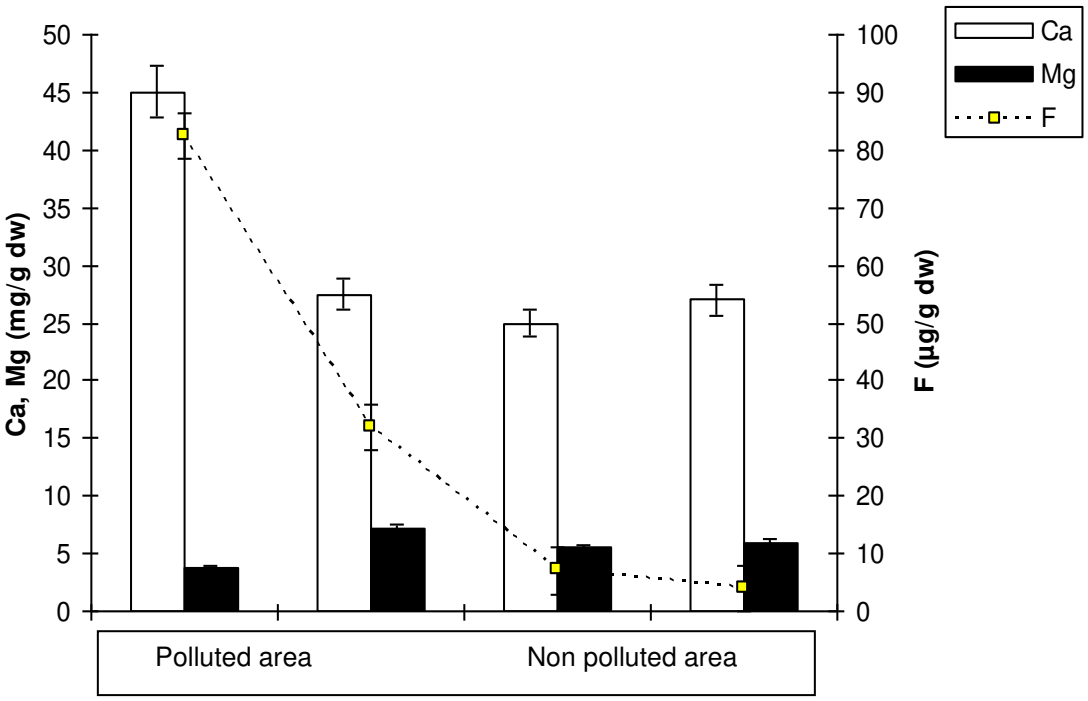

Figure 7. *Fluoride, calcium and magnesium concentrations in central and necrotic leaf area of grapevine cv. "Asli" from polluted and non polluted areas. Means of 10 replicates and confidence intervals at 5%.*

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Table 3. *Fluoride content ($\mu g.g^{-1}DW$) and net photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$) healthy and necrotic areas of grapevine leaves.*

	Percentage of leaf necroses (%)					
	Control	0	10-20	30-40	50-60	60
Classes	CT	C0	Cl	C2	C3	C4
F^-(n=10)	17.7 ± 1.7	24.0 ± 2**	36.5 ± 2.2***	64.7 ± 3. 4***	83.4 ±5,5***	117.4 ± 8.2***
Pn (n=19)	14.1 ± 1.1	13.8 ± 1.2 ^{NS}	12.16 ± 1.34^{+++}	10.24 ± 1.17^{+++}	8.3 ± 1.7^{+++}	6.0 ± 1.0^{+++}

Fluoride content vs controls***p < 0,001, **p < 0,01(n=10);net photosynthesis vs controls +++ p< 0.001
NS: non significant (n = 19).

Discussion

In the present study, regular field follow-up in the polluted area allowed us to recognize various expressions of damage caused to vine tree. For example, severe fluoride injuries to vine tree, in the form of necrosis of brick red color at leaf margins and tip burn were detected. In addition, necrosis in the centeral region of leaf was found as few scattered circular red spots. These findings are similar to those of Miller (1992) who reported that brick-red marginal necroses occupying lobes extremities of leaf grapevine represent the symptoms of fluorine pollution, whereas central necroses are the symptoms of SO_2 pollution. In the present study, necrotic tissues are separated from healthy ones by a dark violet borderline that consists of anthocyanin pigments. In an earlier study, it is suggested that anthocyanins accumulation in vegetative tissues of plants is an indicator of environmental stress (Fornasiero, 2001), which could be first line of defense to limit damage at leaf margins (Ben Abdallah, 2007). Our previous works demonstrated that the number of necrotic halos could be useful to estimate, within *Vitis vinifera* species, the degree of sensitivity to pollution (Ben Abdallah et al., 2006).

In some of earlier studies it has been shown that species resistant to air pollution are those accumulating fluoride without showing any symptom of F^- toxicity or growth restrictions (Baunthiyal et al., 2014; Ben-Abdallah and Boukhris, 1990; Fornasiero, 2001). In addition, some tolerant species are those exhibiting specific symptoms of F^- toxicity and still surviving in the polluted area. For example, our histochemical study shows that several structural changes were recorded such as (i) a decrease in the size of epidermal cells and the number of vascular bundles with time, (ii) an extension and tightening of palisade parenchyma cells. Decrease in epidermal cells in both upper and lower epidermis is one of the reasons of decrease in leaf thickening due to stress (Fornasiero, 2001). From the data for histochemical studies of fluoride injured plants from the present study, it is suggested that fluoride toxicity caused the damage to spongy mesophyll cells and lower epidermis, and then toxicity spread within the leaf. This argument can be supported by some of similar earlier studies on jack pine (*Pinus banksiana* L.) (Zwiazek and Shay, 1987), *Avena sativa* and *Lycopersicon esculentum* (Stevens et al., 1998). However, the gradual sclerotisation of collenchyma cells due to fluoride accumulation suggested that a wall thickening mechanism to protect leaf cells from injury and death is present in nearby cells. This argument can be supported by the fact that in angiospermic plants wall thickening, lignifications, and neo-synthesis of xylem cells are involved in plant defense mechanism against fungal invasion (Nicole et al., 1992). In the present study, fluoride become accumulated in the necrotic peripheral regions of leaf slices as compared to leaf central parts. These results can be explained in view of the findings of Rhimi et al. (2011) who found lower accumulation of fluoride in

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non-necrotic tissues and suggested that necroses would not appear unless certain concentrations were reached in leaf tissues. Thus, toxicity of fluoride depends on amount of fluoride accumulation.

While examining the fluorine pathway in plant tissues, Fornasiero (2001) reported that fluoride enters the leaf by diffusion through the stomata, and dissolves in the humid spaces of sub stomatal cavity. After that, the ions are translocated with the transpiration stream to sites of greatest evaporation, which are usually margins and tips where concentrated F^- amounts would cause the first signs of damage, such as necroses and burns (Eliftheriou and Tsekos, 1991). In view of these reports, we would suggest that apical and marginal necroses are specific and typical of fluoride compounds. In addition, a mechanism that limits fluorine at leaf margins allows the plant to keep photosynthetic activity at appropriate levels. Such mechanism would explain the ability of this species to photosynthesize, even when the leaf surface was damaged up to 30%. Under such conditions, grapevine plants can be used as bio-indicators of F pollution or used in mapping fluorine pollution as earlier suggested by Festy (2003).

The sulphur content was higher in central part of leaf than in control but lower at the margins than in control. These findings suggested that inter-vein central necroses are probably due to SO_2, whereas marginal leaf necroses are typical of fluoride compounds effects. Long ago, (Kaiser et al., 1993) fumigated green peas with SO2 or fluorine and found that sulphur is excreted in the root environment as sulphuric acid, while fluorine accumulated in leaf margins. Like other gases, SO_2 enters the leaf through the stomata by diffusion process and oxidizes to sulfite (SO_3^{2-}) and then in sulphate (SO_4^{2-}) in palisade cell walls (Cape et al., 2003; Eichert and Fernández, 2012). The lesser leaf central necroses in the study area might have been due to low concentration of SO_2 or the slow oxidization of SO_3^{2-} to SO_4^{2-} in leaves. These results are similar with some of earlier findings in which it was reported that in humid climate SO_2 molecules react with atmospheric water molecules to form more sulphuric acid and greater damages and injuries in leaves than those in arid climate (Ali et al., 2008; Fowler et al., 1989; Freer-Smith and Mansfield, 1987).

In the present study it was found that this local grapevine variety accumulated more calcium in leaf margins to balance fluoride accumulation. These results suggested that grapevine leaves lessen the toxic effects of fluoride by trapping fluoride as CaF_2. Long ago, it has been reported that accumulated fluoride as CaF_2 do not disturb plant metabolism (Abdallah et al., 2006; Machoy-Mokrzynska, 1995). Our findings confirm the non-translocation of fluoride, through phloem towards lower plant organs as demonstrated in previous works (Wiese et al., 1996). The interaction between fluoride and calcium has also been reported with other cations such as silicon and aluminium (Abdallah et al., 2006; Ben Abdallah et al., 2006; Rhimi et al., 2011). Since magnesium is a central component of the chlorophyll molecule as well as a compound of the cell wall pectin, and in view of its important role in enzyme balance and protein synthesis (Türk et al., 1993), the tendency to considerably increase the Ca content where F is present and to keep more Mg in the central leaf parts points to Mg being involved in a detoxification mechanism that consists in trapping fluoride in the form of MgF_2. Both mechanisms allow the plant to maintain its Ca and Mg concentrations at appropriate levels to survive under such harsh circumstances. Grapevine accumulated higher amound of phosphorus (P) and Mg in the central parts of the leaf as a strategy to minimize damage by F as much as possible.

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In conclusion, necrosis in peripheral regions of grapevine leaf is due to F toxicity where F cause damages to epidermal cells, and plants try to lessen the toxic effects of F by cell deaths at leaf margins while maintaining photosynthetic activity for survival. Moreover, grapevine tolerated F by increase in cell wall thickening of palisade or spongy cells and by detoxifying F by accumulating Ca and Mg as CaF_2 and MgF_2.

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REFERENCE

[1] Abdallah, F. B., Elloumi, N., Mezghani, I., Garrec, J., Boukhris, M. (2006): Industrial fluoride pollution of jerbi grape leaves and the distribution of F, Ca, Mg, and P in them. -Fluoride 39: 43.

[2] Ali, S. T., Mahmooduzzafar Abdin, M. Z., Iqbal, M. (2008): Ontogenic changes in foliar features and psoralen content of *Psoralea conrylifolia* L. exposed to SO2 stress. -Journal of Environmental Biology 29: 661-668.

[3] Azri, C., Tili, A., Serbaji, M. M., Medhioub, K. (2002): Etude des résidus de combustion des fuels liquide et solide et de traitement chimique du phosphate brut dans la ville de Sfax (Tunisie). -Pollution atmosphérique 44: 297-308.

[4] Baillie, C.-K., Kaufholdt, D., Karpinski, L. H., Schreiber, V., Hänsch, S., Evers, C., Bloem, E., Schnug, E., Kreuzwieser, J., Herschbach, C., Rennenberg, H., Mendel, R. R., Hänsch, R. (2016): Detoxification of volcanic sulfur surplus in planta: Three different strategies of survival. -Environmental and Experimental Botany 126: 44-54.

[5] Baunthiyal, M., Bhatt, A., Ranghar, S. (2014): Fluorides and its effects on plant metabolism. -Int J Agric Technol 10: 1-27.

[6] Bell, J. N. B., Treshow, M. (eds.) (2002): Air Pollution and Plant Life John Wiley and Sons. West Sussex, England.

[7] Ben-Abdallah, F., Boukhris, M. (1990): The effect of air pollutants on vegetation in the region of Sfax (Tunisia). -Pollution atmosphérique: 292-297.

[8] Ben Abdallah, F. (2007): *Les mécanismes de survie de certaines espèces fruitières dans une zone polluée par le fluor.* Université de Sfax, Tunisie.

[9] Ben Abdallah, F., Elloumi, N., Mezghani, I., Boukhris, M., Garrec, J.-P. (2006): Survival strategies of pomegranate and almond trees in a fluoride polluted area. -Comptes Rendus Biologies 329: 200-207.

[10] Blanchar, R., Rehm, G., Caldwell, A. (1965): Sulfur in plant materials by digestion with nitric and perchloric acid. -Soil Science Society of America Journal 29: 71-72.

[11] Cape, J. N., Fowler, D., Davison, A. (2003): Ecological effects of sulfur dioxide, fluorides, and minor air pollutants: recent trends and research needs. -Environment International 29: 201-211.

[12] Chakrabarti, S., Patra, P. K., Mondal, B. (2013): Uptake of fluoride by two paddy (Oryza sativa L.) varieties treated with fluoride-contaminated water. -Paddy and Water Environment 11: 619-623.

[13] Davieson, G., Murray, F., Wilson, S. (1990): Effects of sulphur dioxide and Hydrogen Fluoride singly and in combination on growth and Yield of wheat in open-top chambers. -Agriculture, Ecosystems & Environment 30: 317-325.

[14] Doley, D. (1986): Experimental analysis of fluoride susceptibility of Grapevine (Vitis vinifera L.): Leaf development during four successive seasons of fumigation. -New Phytologist 103: 325-340.

[15] Eichert, T., Fernández, V. (2012): Chapter 4 - Uptake and Release of Elements by Leaves

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_077089

and Other Aerial Plant Parts A2 - Marschner, Petra. Marschner's Mineral Nutrition of Higher Plants (Third Edition). Academic Press. San Diego. 71-84.
[16] Eliftheriou, E. P., Tsekos, L. (1991): Fluorides effects on leave cell ultra structure of olive Trees growing in the vicinity of Aluminum Factory in Greece. -Trees 5: 83-89.
[17] Elloumi, N., Ben Abdallah, F., Boukhris, M. (2003): Lead accumulation by a few crops grown in the vicinity of a lead smelter in Sfax. -Air Pollution 178: 285-295.
[18] Festy, B. (2001): About the assessment of human exposure to atmospheric: the need for a global and multi-stakeholder. -Air Pollution 169: 9-12.
[19] Festy, B. (2003): Air pollution phenomena and problems need a comprehensive environmental and health vision. -Air Pollution 178: 237-248.
[20] Fornasiero, R. B. (2001): Phytotoxic effects of fluorides. -Plant Science 161: 979-985.
[21] Fowler, D., Cape, J. N., Deans, J. D., Leith, I. D., Murray, M. B., Smith, R. I., Sheppard, L. J., Unsworth, M. H. (1989): Effects of acid mist on the frost hardiness of red spruce seedlings. -New Phytologist 113: 321-335.
[22] Freer-Smith, P., Mansfield, T. (1987): The combined effects of low temperature and SO2+ NO2 pollution on the new season's growth and water relations of Picea sitchensis. -New Phytologist 106: 237-250.
[23] Jha, S. K., Nayak, A. K., Sharma, Y. K., Mishra, V. K., Sharma, D. K. (2008): Fluoride Accumulation in Soil and Vegetation in the Vicinity of Brick Fields. -Bulletin of Environmental Contamination and Toxicology 80: 369-373.
[24] Kaiser, W., Höfler, M., Heber, U. (1993): Can plants exposed to SO2 excrete sulfuric acid through the roots? -Physiologia Plantarum 87: 61-67.
[25] Leece, D. R., Scheltema, J. H., Anttonen, T., Weir, R. G. (1986): Fluoride accumulation and toxicity in grapevines Vitis vinifera L. in New South Wales. -Environmental Pollution Series A, Ecological and Biological 40: 145-172.
[26] Locquin, M., Langeron, M. (1978): Manuel de Microscopie edition Masson. 352.
[27] Mabrouk, H., Carbonneau, A. (1996): Une méthode simple de détermination de la surface foliaire de la vigne (Vitis vinifera L.). -Progrès Agricole et Viticole 113: 392-398.
[28] Machoy-Mokrzynska, A. (1995): Fluoride–magnesium interaction. -Fluoride 28: 175-177.
[29] Mesquita, G. L., Tanaka, F. a. O., Cantarella, H., Mattos, D. (2010): Atmospheric Absorption of Fluoride by Cultivated Species. Leaf Structural Changes and Plant Growth. -Water, Air, & Soil Pollution 219: 143-156.
[30] Miller, G. W. (1992): The effect of fluoride on higher plants with special emphasis on early physiological and biochemical disorders. -Fluoride (USA) 26: 3-22.
[31] Murray, F. (1984): Effects of long term exposure to hydrogen fluoride on grapevines. -Environmental Pollution Series A, Ecological and Biological 36: 337-349.
[32] Murray, F., Wilson, S. (1988): The joint action of sulphur dioxide and hydrogen fluoride on the yield and quality of wheat and barley. -Environmental Pollution 55: 239-249.
[33] Nicole, M. R., Geiger, J. P., Nandris, D. (1992): Defense of Angiosperm Roots Against Fungal Invasion. -In: BLANCHETTE, R. A.BIGGS, A. R. (eds.) Defense Mechanisms of Woody Plants Against Fungi. Springer Berlin Heidelberg. Berlin, Heidelberg. 181-206.
[34] Rhimi, N., Nasri-Ayachi, M. B., Abdallah, F. B. (2011): The Grape Leaf: a Bioindicator of Fluoride and Sulphur Dioxide Pollution. -International Journal of Sustainable Water and Environmental Systems 3: 69-76.
[35] Sass, J. E. (1958): Botanical microtechnique. 3rd edition. The Iowa state University Press. Ames, lowa, USA.
[36] Stevens, D. P., Mclaughlin, M. J., Alston, A. M. (1998): Phytotoxicity of the fluoride ion and its uptake from solution culture by Avena sativa and Lycopersicon esculentum. -Plant and Soil 200: 119-129.
[37] Telesiński, A., Śnioszek, M., Smolik, B., Malinowska, K., Mikiciuk, M., Cichocka, J., Zakrzewskaa, H. (2011): Fluoride uptake in hydroponic culture by different clones of basket willow, *Salix viminalis* L. -Fluoride 44: 255-259.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_077089

[38] Türk, S., Gülpen, M., Fink, S. (1993): Aufnahme, Transport und Verbleib von Calcium und Magnesium in Fichten (Picea abies [L.] Karst.) und Kiefern (Pinus silvestris L.) bei unterschiedlicher Ernährung und Schadstoffbelastung. -Forstwiss Centralol 12: 191-208.
[39] Wiese, C., Slovik, S., Kaiser, W. (1996): Wirkungen hoher SO2-kon-zentratimen auf fichte (Picea abies L.karst). -Stechfichte, Waldkiefer und Latshe AFZ.: 570-572.
[40] Zwiazek, J. J., Shay, J. M. (1987): Fluoride-and drought-induced structural alterations of mesophyll and guard cells in cotyledons of jack pine (Pinus banksiana). -Canadian Journal of Botany 65: 2310-2317.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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NITRIC OXIDE REGULATED IMPROVEMENT IN GROWTH AND YIELD OF RICE PLANTS GROWN UNDER SALINITY STRESS: ANTIOXIDANT DEFENSE SYSTEM

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Abstract. The current work assessed the effects of exogenously applied sodium nitroprusside (SNP) on rice growth and yield under salt stress. Salt stress induced a significant reduction in biomass and grain yield while increased the plant proline, ascorbic acid, H_2O_2 and MDA contents in all studied cultivars. SOD, POD and CAT activities also significantly increased in salt stressed plants; however, the total phenolics content decreased. Application of SNP as seed priming reduced the adverse effects of salinity on plant biomass production and grain yield, while the accumulation of MDA and H_2O_2 decreased. Of different SNP levels, 0.1 mM regime was more effective in reducing the negative effects of salinity. Among fine rice cultivars, Shaheen basmati performed better, while among coarse rice cultivars the performance of IRRI-6 was better to exogenously applied 0.1 mM SNP. We concluded that exogenous application of SNP up-regulated the antioxidative defense mechanism in salt stressed rice plants which resulted in better yield.
Keywords: *antioxidative potential, biomass, grain yield, nitric oxide, Oryza sativa* L.

Introduction

Reactive oxygen species (ROS) induced lipid peroxidation under salt stressed environment is a major restricting factor for better plant growth and development (Ashraf and Foolad, 2007; Ashraf, 2009; Sian et al., 2015). Important ROS such as $^1O_2$, O_2^-, OH^-, and H_2O_2 being highly reactive in nature, can react with vital metabolites and macro-molecules in cells including photosynthetic pigments, lipids, proteins and DNA (Ashraf, 2009; Sian et al., 2015). Among various cell organelles, the major sites for ROS production are the chloroplast, mitochondria, vacuole and microbodies (Ashraf, 2009). The ROS can initiate a series of destructive processes including the inactivation of antioxidative enzymes (Tanou et al., 2009).

Plants possess an antioxidant defense mechanism to detoxify and scavenge the ROS. Among various antioxidants, flavonoids, tocopherols, phenolics, glutathione, carotenoids and ascorbic acid are non-enzymatic antioxidants; while peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX) are enzymatic ones (Ashraf, 2009; Ali and Ashraf, 2011). Of different ROS, production of hydrogen peroxide (H_2O_2) acts as a secondary messenger and helps the plants to rapidly adjust under stressful environmental conditions and mediates defense responses (Miller et al., 2010) but only at low concentrations. Hydrogen peroxide an important ROS also acts as a secondary messenger in low

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concentration, activates enzymatic antioxidative defense mechanism (Tanou et al., 2009; Hernandez et al., 2010), facilitates the stability of SOS1 mRNA and Na^+ detoxification under salinity (Chung et al., 2008). Under stressed conditions, such as NaCl salinity, over-production of H_2O_2 directly causes oxidative damages to cellular membranes (Miller et al., 2010).

Among various groups of plant secondary metabolites, phenolics and AsA are important with both biological and antioxidant properties (Posmyk et al., 2009; Ali et al., 2013). The activity of phenolics as an antioxidant metabolite is due to its greater radical stabilization and proton donating ability (Rice-Evans et al., 1996). Plant phenolic content has been reported to be affected under different abiotic stresses including salinity (Ashraf et al., 2010; Jamil et al., 2015). Similarly, AsA also plays its role not only as an important non-enzymatic antioxidant; also contribute efficiently in the completion of enzymatic antioxidation.

The content of MDA can be used as an effective indication to judge the salt tolerance ability of different cultivars (Sairam et al., 2005). Studies reveal that less accumulation of MDA is an indication for improved tolerance against salts stress (Liang et al., 2003; Brankova et al., 2005; Ruiz et al., 2005), because its increased content under salt stress resulted in stunted growth in the plants of sesame (Koca et al., 2007) and tomato (Li, 2009).

Alterations in plant water relations under salt stress are the major obstacle for normal plant growth (Habib et al., 2014). To overcome such problems, plants have adapted a special phenomenon for the accumulation of different osmolytes. It has been reported that among different organic compounds that take part in plant osmotic adjustment, proline is not only an osmoregulator but also an important ROS scavenger (Ashraf and Foolad, 2007). Increased accumulation of proline is a general phenomenon under salt stress, however more accumulation takes places in salt tolerant plants in comparison with the sensitive ones (Ashraf and Foolad, 2007; Habib et al., 2012). Therefore, the amount of proline accumulated could be taken as an important index of salinity tolerance (Ashraf and Foolad, 2007). Different plant species have different potential to tackle the adversative effects of salinity regarding the accumulation and/or synthesis of different organic compounds but several high yielding crop cultivars are not well investigated in this regard (Ali and Ashraf, 2011). So, different techniques are being used for the last two decades for the induction of salt tolerance in these crops and have been found effective to some extent (Ashraf and Foolad, 2007). Use of different chemicals exogenously such as foliar spray or their use as pre-sowing seed treatment is one such technique. Of these exogenously applied compounds, SNP is one such compound (Zhang et al., 2006; Habib et al., 2010). It acts as a free radical which is lipophilic and volatile in nature (Hayat et al., 2010). Apart from its regulatory roles in plants, such as in improving seed germination and seedling growth (Zhang et al., 2006; Habib et al., 2010), it also plays a protective role against different abiotic stresses including water stress (Gracia-Mata and Lamattina, 2001) and salinity (Zhao et al., 2007; Zheng et al., 2009; Habib et al., 2010). Under salinity the different protective role of nitric oxide include the improvement in chlorophyll pigments, gas exchange attributes, and PS-II efficiency (Habib et al., 2013). Its role as protective agent in salinity has been described in wheat (Zheng et al., 2009) and maize (Zhang et al., 2006), where it actively took part in increasing the antioxidant enzymes activity and the activities of proton pumps respectively.

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As there are reports available in literature about the protective role of NO_2 against the damages caused by salt stress in crop plants but very few is available about its role in seed yield increments especially in rice in relation with plant oxidative defense mechanism. Therefore the present study was considered with the objective to find out the effects of varying regimes of exogenously applied SNP on the yield of different rice cultivars in relation with its role in enhancing the plant antioxidative capacity with special reference to some enzymatic and non-enzymatic compounds under salinity.

Materials and Methods

The experiment was conducted under natural conditions (day length 13.8 h; relative humidity 45.2%; PPFD, 1275 μmol m^{-2} s^{-1}; the day and night temperatures were 36±3 ^{o}C and 27±2 ^{o}C respectively). The total four cultivars of rice were used in this study, two of these were fine rice (Basmati PB-95 and Bsmati Shaheen), while other two (IRRI-6 and KS-282) were coarse rice cultivars. Before treatment the seeds were surface sterilized with 1% solution of NaOCl for 5 min then washed the seeds thrice with ddH_2O. The seeds were subsequently soaked in varying levels (0, 0.1, and 0.2 mM) of SNP for 20 h. Thirty days old seedlings (prepared separately) were transplanted in tubs (each filled with 16 kg of sandy loam soil) arranged in CRD with four replicates of each treatment. The seedlings were established after one week of transplant and salt treatment (0 and 80 mM of NaCl) was applied by increasing the level gradually. The experiment was repeated to validate the results. Data for growth and biochemical attributes was recorded after 30 days of the start of NaCl treatment, while yield attributes were estimated at maturity.

Determination of the activity of different antioxidant enzymes

Enzyme extraction

To determine the activity of various antioxidant enzymes, enzyme extracts were prepared following the method described by Ali and Ashraf (2011).

Estimation of the activities of SOD, POD and CAT

The SOD activity was determined following the method of Giannopolitis and Ries (1977) with some modifications while for estimating the activity of CAT and POD the method of Chance and Maehly (1955) was followed with some modifications.

Leaf ascorbic acid contents

Leaf ascorbic acid content was determined by following a method described by Mukherjee and Choudhuri (1983). Varying levels of pure AsA were used to make the standard curves for measuring the AsA content in the mixture. While the leaf total phenolic contents was measured according to a method described by Julkenen-Titto (1985).

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) content was determined following the method described by Velikova et al. (2000).

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Malondialdehyde (MDA)

The method of Carmak and Horst (1991) was used to determine the MDA content.

Proline content

Leaf proline content was determined by following a method described by Bates et al. (1973).

Yield attributes

Different yield attributes such as number of tillers, weight of hundred grains and grain yield per plant were estimated at maturity after drying properly the spikes under natural sun light.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD) with four replicates and the data so generated for different attributes was analyzed using a software named CoSTAT V 6.3 (developed by, Cohort software, Berkeley, California, USA).

Results

Morphology and growth

Shoot and root fresh and dry biomass significantly decreased in studied rice cultivars grown under salt stress (*Fig. 1*; *Table 1*). Exogenous application of SNP as seed treatment was found effective in increasing the fresh and dry biomass of shoot and root in all rice cultivars under non-stressed and salt stressed conditions. For shoot fresh and dry weights, 0.1 mM SNP was more effective under both non-saline and saline conditions, while in relation with root fresh and dry biomass both SNP levels were found equally effective. Among rice cultivars, IRRI-6 and Shaheen Basmati exhibited higher shoot fresh and dry weights under salt stress, while higher root fresh and dry weights were observed in cvs. IRRI-6 and KS-282.

Imposition of saline stress also significantly reduced the shoot length of all rice cultivars (*Fig. 1*; *Table 1*). Exogenous application of different regimes of SNP as pre-sowing seed treatment significantly reduced the adverse effects of rooting medium salinity on shoot length in all rice cultivars. Of different SNP levels, more increase in shoot length was found in plants raised from seeds treated with 0.1 mM SNP under both non-saline and saline conditions. Maximum increase in shoot length was recorded in cv. Shaheen Basmati under saline conditions, however, under non-saline conditions, maximum increase in shoot length was observed in Basmati PB-95.

Enzymatic antioxidants

The activity of antioxidant enzyme superoxide dismutase (SOD) was increased significantly in all rice cultivars grown under salinity (*Fig. 2*; *Table 1*). Pre-sowing seed treatment with different SNP regimes further increased the SOD activity in all rice cultivars under non-saline and salt stressed conditions and the maximal increase in SOD activity due to SNP seed treatment was found in plants raised from seeds

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treated with 0.1 mM level of SNP. However in rice cultivar IRRI-6 both SNP regimes equally increased the SOD activity under non-saline and saline conditions. Comparatively SNP-induced more increase in SOD activity was found in cv. Shaheen Basmati under salinity stress.

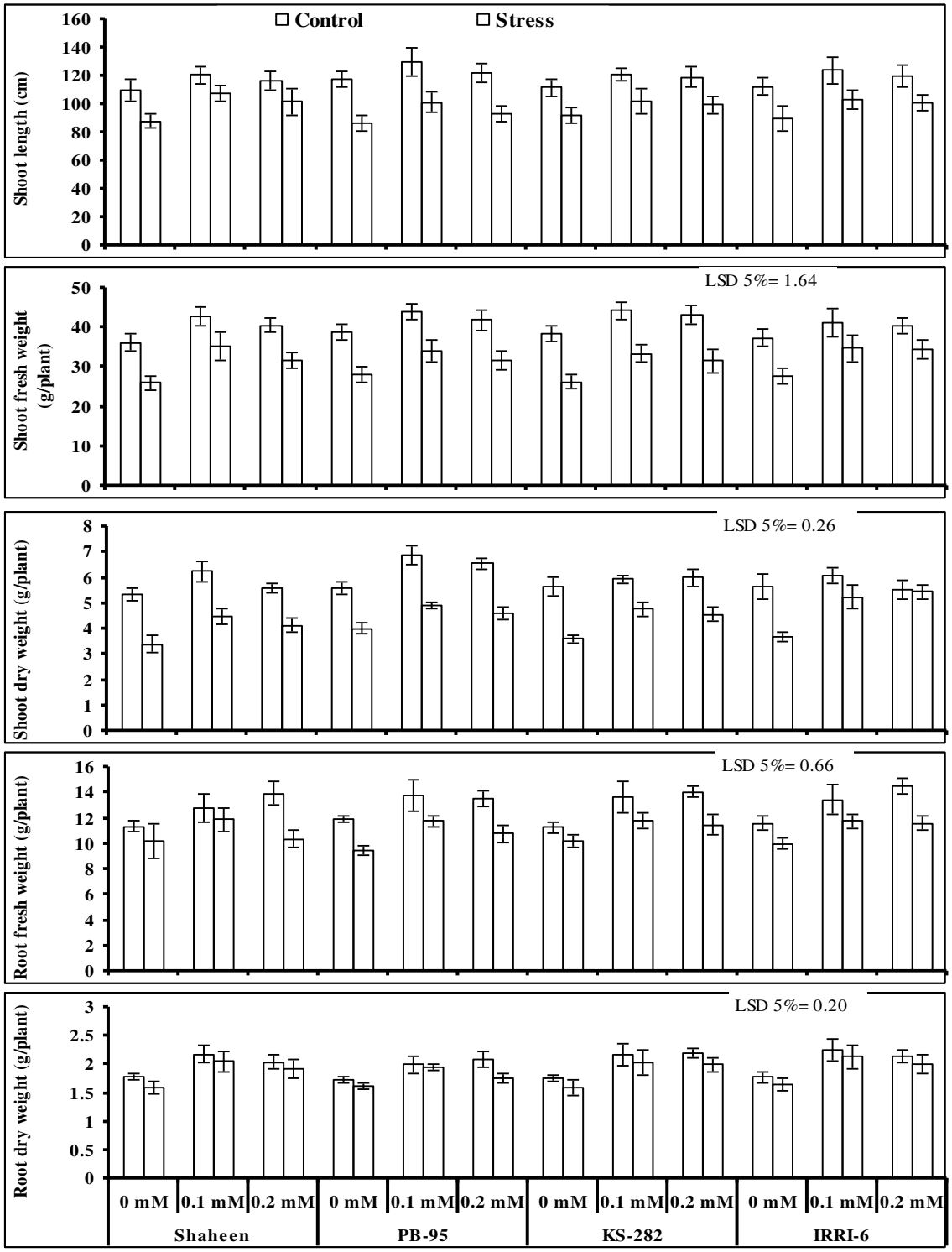

Figure 1. *Growth attributes of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)*

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Table 1. *Mean squares from statistical analysis of the data for studied attributes of rice plants of four rice cultivars influenced by exogenously-applied SNP under salt stress*

SOV	df	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Shoot Length	Number of tillers
Variety (V)	3	15.16 ns	1.74 ***	2.40 ns	0.208 ns	50.04 *	0.177 ns
Salinity (S)	1	2303.40 ***	65.50 ***	85.10 ***	0.432 *	10901.34 ***	29.260 ***
NO (N)	2	26.60 ***	5.64 ***	26.31 ***	2.346 ***	565.36 ***	5.281 **
V x S	3	23.90 ns	1.77 ***	1.10 ns	0.239 ns	31.76 ns	0.454 ns
V x N	6	4.31 ns	0.42 ns	0.09 ns	0.017 ns	7.63 ns	0.072 ns
S x N	2	3.91 ns	0.91 *	7.44 *	0.136 ns	132.01 *	0.947 ns
V x S x N	9	3.24 ns	0.50 ns	1.26 ns	0.036 ns	9.38 ns	0.434 ns
Error	72	10.77	0.26	1.75	0.159	35.72	0.802
SOV	**df**	**SOD**	**POD**	**CAT**	**H_2O_2**	**AsA**	**Proline**
Variety (V)	3	0.54 ns	0.490 ns	0.021ns	18.84 ns	7.957 ns	1.32 ns
Salinity (S)	1	6850.24 ***	7228.180 ***	1587.620 ***	10157.87 ***	1.227 ***	11961.73 ***
NO (N)	2	105.59 ***	100.730 ***	59.730 ***	274.21 ***	0.107 ***	126.41 ***
V x S	3	0.58 ns	0.013 ns	0.022 ns	17.54 ns	5.044 ns	4.11 ns
V x N	6	0.53 ns	0.033 ns	0.011 ns	1.86 ns	3.721 ns	0.59 ns
S x N	2	28.06 ***	19.576 ***	14.410 ***	22.51 ns	0.003 ns	31.56 *
V x S x N	9	0.28 ns	0.021 ns	0.006 ns	5.62 ns	0.001 ns	1.97 ns
Error	72	1.94	1.661	0.178	18.85	0.002	6.54
SOV	**df**	**MDA**	**Total phenolics**	**100 grain weight**	**Total grain weight/plant**		
Variety (V)	3	3.21ns	0.056 ns	7.957 ns	0.056 ns		
Salinity (S)	1	25734.95 ***	1229.801 ***	1.227 ***	1229.801 ***		
NO (N)	2	276.74 ***	16.510 ***	0.107 ***	16.510 ***		
V x S	3	19.64 ns	0.128 ns	5.044 ns	0.128 ns		
V x N	6	3.46 ns	0.055 ns	3.721 ns	0.055 ns		
S x N	2	32.43 ns	1.923 ***	0.003 ns	1.923 ***		
V x S x N	9	1.87 ns	0.012 ns	0.001 ns	0.012 ns		
Error	72	12.17	0.210	0.002	0.210		

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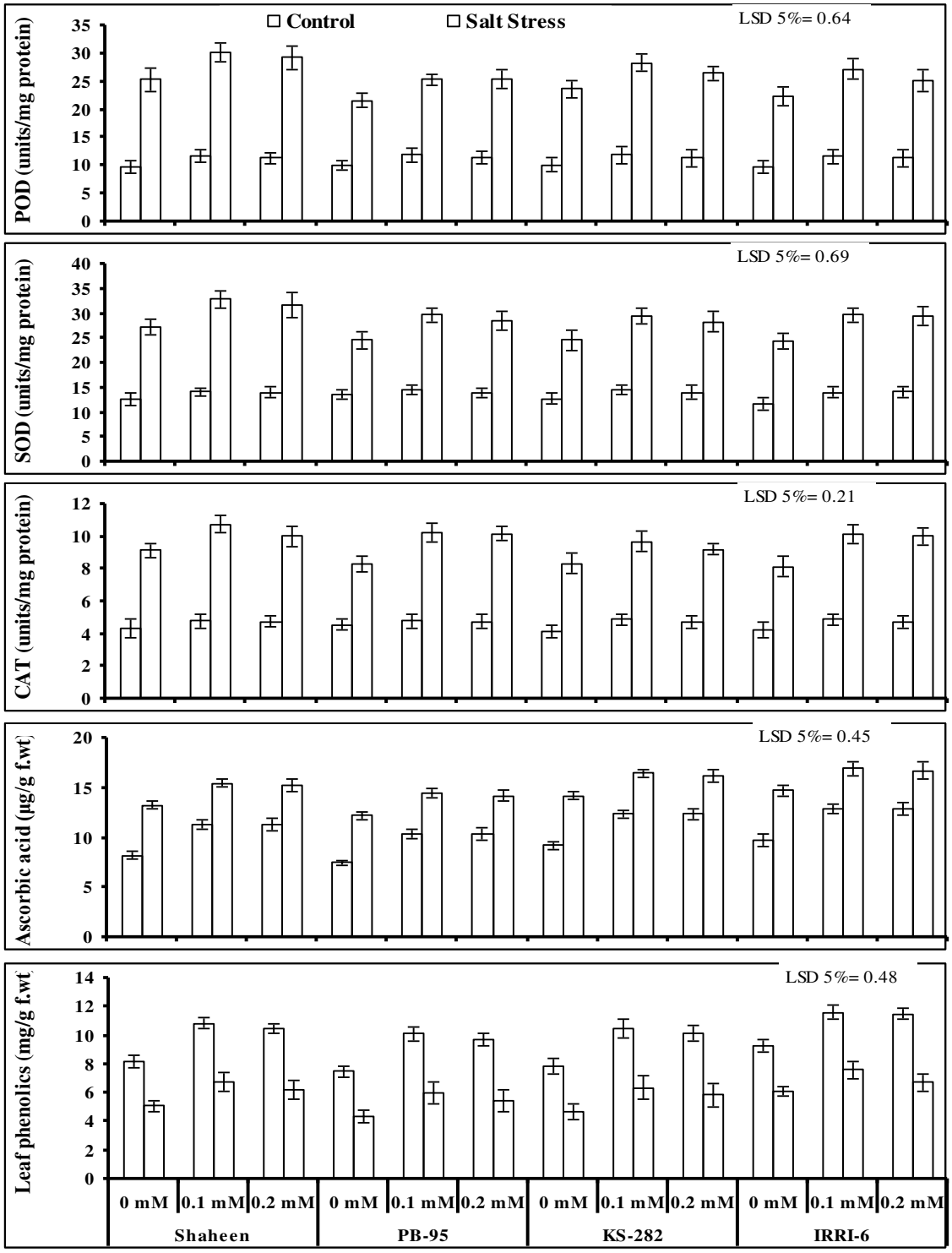

Figure 2. *Enzymatic and non-enzymatic antioxidants of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)*

Rooting medium salinity caused a significant increase in leaf POD activity in plants of all rice cultivars (*Fig. 2*; *Table 1*). Exogenous SNP treatment further increased the POD activity in plants of all rice cultivars grown under both saline and non-saline

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conditions and more increase in POD activity was recorded in plants raised from seeds treated with 0.1 mM SNP regime. Among all rice cultivars the maximal increase in POD activity due to SNP treatment was found in cv. Shaheen Basmati and IRRI-6 under salinity stress.

Like SOD and POD, the leaf CAT activity was also enhanced significantly in plants of all rice cultivars when grown under salt stress (*Fig. 2*; *Table 1*). The CAT activity was further increased due to SNP seed treatment under salinity. Both regimes of SNP were found equally effective in improving the activity of CAT. SNP-induced more increase in leaf CAT activity was recorded in cvs. Shaheen Basmati and IRRI-6 as compared with other rice cultivars under salinity.

Non-enzymatic antioxidants

Leaf ascorbic acid content in all rice cultivars was enhanced significantly when grown under salt stress (*Fig. 2*; *Table 1*). Sodium nitroprusside treatment as seed priming further enhanced the leaf ascorbic acid content under salt stress and both SNP levels were found equally effective in this regard. However, under non-saline conditions this increase in leaf ascorbic acid content was more in plants raised from seeds treated with 0.2 mM regime of SNP. More increase in leaf ascorbic acid due to SNP seed treatment was found in cvs. Shaheen Basmati and IRRI-6 under saline and non-saline conditions.

Leaf total phenolics content reduced significantly in all studied rice cultivars due to rooting medium salinity (*Fig. 2*; *Table 1*). Seed priming with SNP was effective in improving the total phenolic content in both saline and non-stressed conditions. Although seed priming with both SNP regimes (0.1 and 0.2 mM) found equally effective in increasing total phenolic content, however, under salinity 0.1 mM SNP regime as seed priming was found more effective Comparatively higher total phenolic content was found in cvs. IRRI-6 and KS-282 under non-saline and saline conditions.

Rooting medium salinity increased the leaf proline content in all studied rice cultivars (*Fig. 3*; *Table 1*). A further increase in leaf proline content was found in plants of all rice cultivars that grown from seeds pre-treated with both SNP regimes. This increase in leaf proline due to SNP priming was found at both SNP regimes in all rice cultivars under salt stress. Comparatively higher increase in proline content was observed in cvs. Shaheen Basmati and IRRI-6 as compared with other rice cultivars.

MDA and H_2O_2 content

A significant increase in leaf MDA was recorded in all rice cultivars due to rooting medium salinity (*Fig. 3*; *Table 1*). Comparatively less increase in MDA due to rooting medium salinity was recorded in cvs. Shaheen basmati and IRRI-6 as compared with other rice cultivars. A significant decrease in leaf MDA content was found in rice plants of all cultivars that were grown from seeds primed with SNP. However, this decrease was found only at 0.1 mM level of SNP.

Imposition of salt stress significantly increased the leaf H_2O_2 content (*Fig. 3*; *Table 1*). Exogenous SNP application was found effective in reducing the H_2O_2 content in all rice cultivars. Seed priming with both SNP levels (0.1 and 0.2 mM) were found equally effective in reducing the leaf H_2O_2 content in all rice cultivars.

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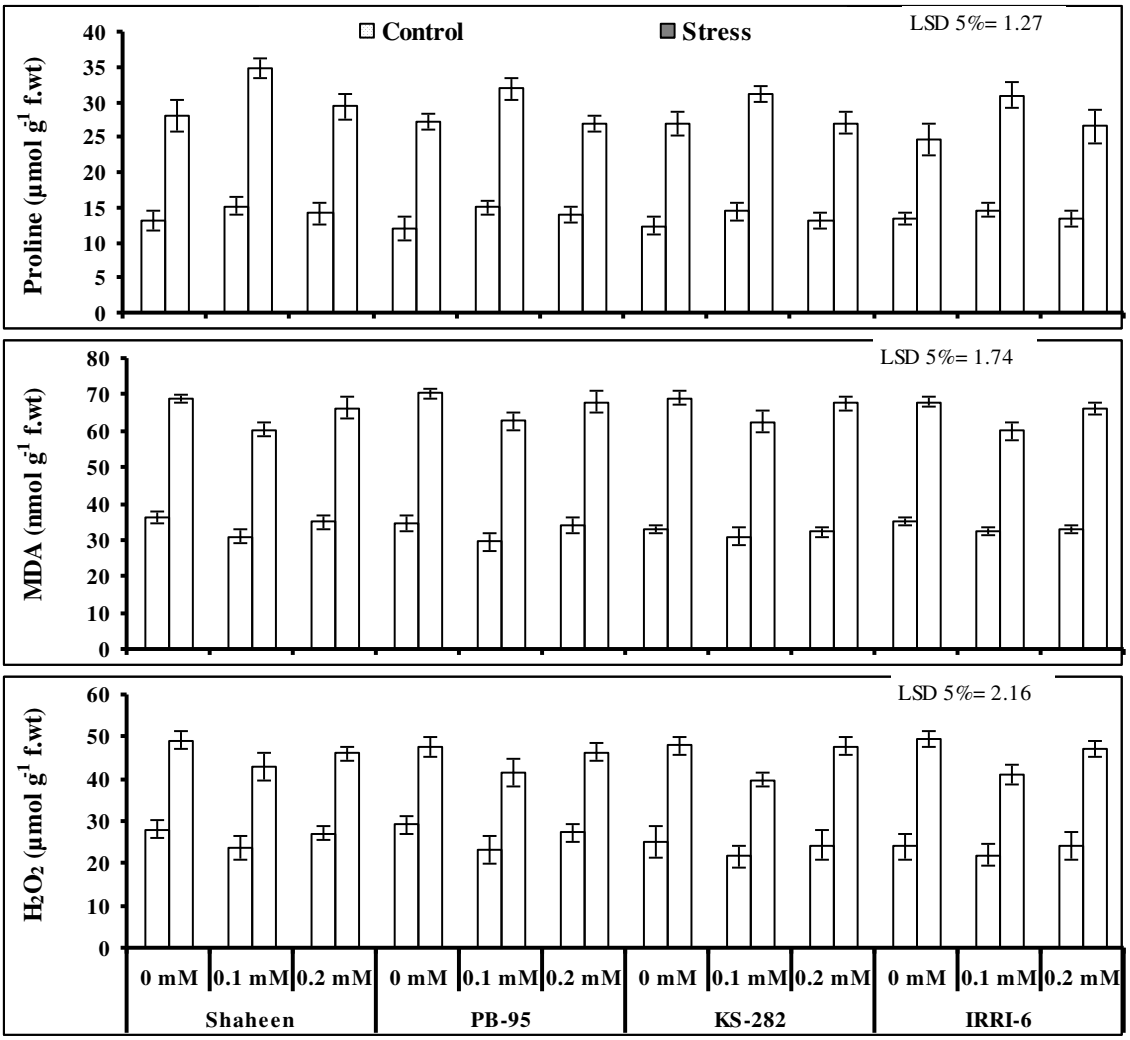

Figure 3. *Leaf proline, MDA and H_2O_2 content of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)*

Yield attributes

Like other growth and physico-chemical parameters, number of tillers was also significantly reduced due to rooting medium salinity in all rice cultivars (*Fig 4*; *Table 1*). A significant ameliorative effect of seed priming with SNP was found on number of tillers under salt stress in all rice cultivars and both SNP levels (0.1 and 0.2 mM) were found equally effective in this regard.

Imposition of salt stress decreased the hundred grain weight in all rice cultivars (*Fig. 4*; *Table 1*). Seed priming with only lower level (0.1 mM) of SNP was found effective in reducing the adverse effects of salt stress on hundred grain weight in all rice cultivars. Comparatively more amelioration on hundred grain weight due to SNP seed priming was recorded in cvs. KS-282 and IRRI-6

Grain yield per plant was also decreased significantly in all rice cultivars due to rooting medium salinity (*Fig. 4*; *Table 1*). Seed priming with SNP was found effective in reducing the adverse effects of salt stress on grain yield per plant in all rice cultivars. Of all SNP levels, 0.1mM was more effective in reducing the adverse effects of salt

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stress on grain yield per plant. Comparatively SNP-induced this amelioration on grain yield per plant was more in cvs. IRRI-6 and KS-282.

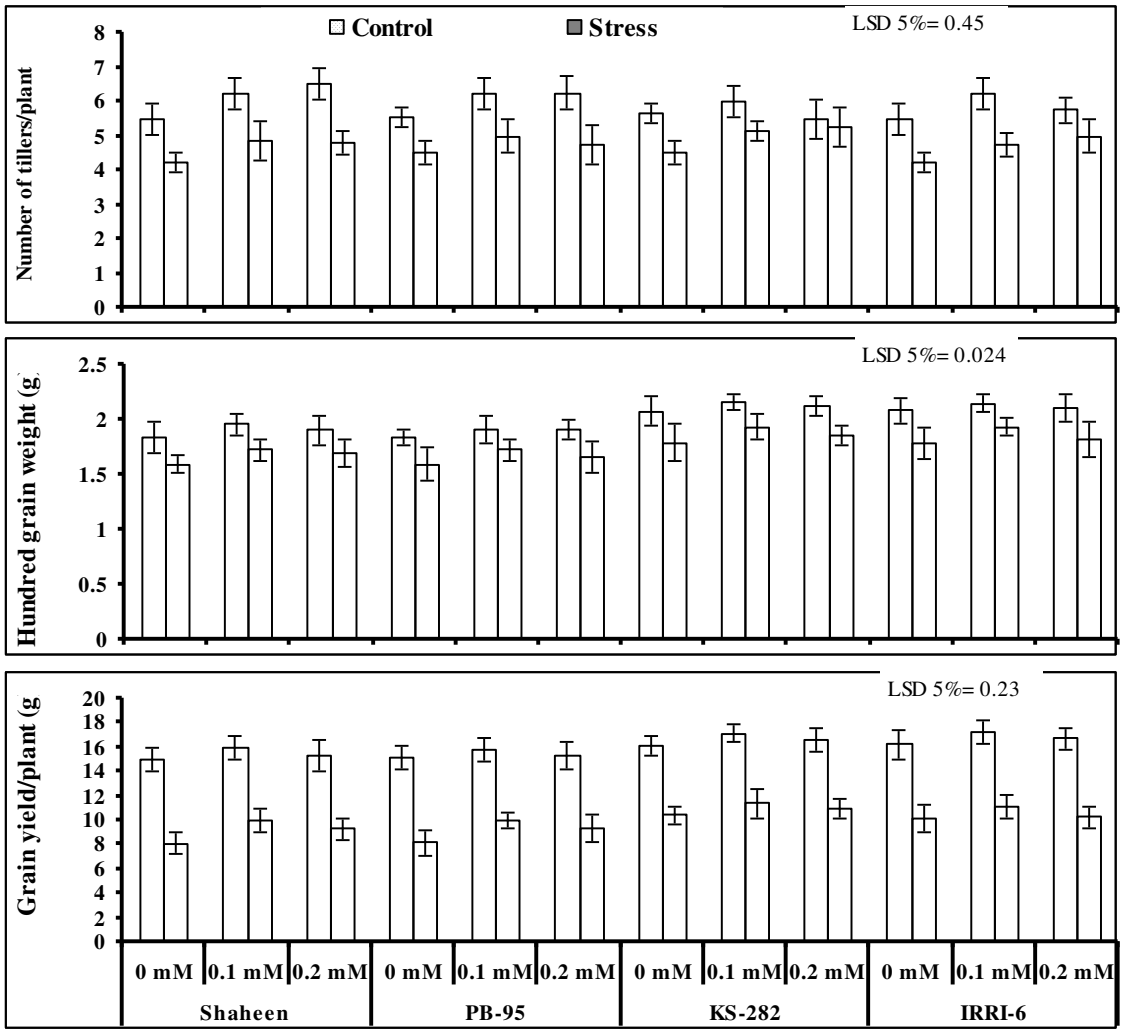

Figure 4. *Yield attributes of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)*

Discussion

The reduction of yield under adverse environmental conditions including salinity is the result of reduction in plant biomass production and has been the subject of many research studies. This reduction in biomass production under salt stress is the result of metabolic alterations in biochemical and physiological process. These include the disturbances in plant water relations, gas exchange attributes, photosynthetic machinery, as well as the production of ROS in excessive amount (known as oxidative stress). These ROS directly or indirectly damages the cellular components including the membranes, resulting in reduced biomass production (Ali and Ashraf, 2011). Of different ROS, production of H_2O_2 is also an important and common phenomenon which is beneficial as a signaling molecule at lower concentrations (Miller et al., 2010). It has been reported that H_2O_2 increases the activity of plasma membrane H^+-

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ATPase, which could in turn maintain the K^+/Na^+ homeostasis (Zhang et al., 2007). Moreover, H_2O_2 has also been reported to facilitate the SOS1 mRNA stability which helped in Na^+ detoxification in *Arabidopsis* (Chung et al., 2008). However, in high concentration H_2O_2 directly damages cellular membranes (Miller et al., 2010). Such damages to cellular membranes due to high production of ROS under salt stress results in accumulation of more MDA due to lipid peroxidation that determines the degree of damage to cellular membranes. Such salt-induced lipid peroxidation results in more leaky membrane (Ashraf and Ali, 2008; Sheokand et al., 2010) that results in lower biomass production and plant yield. Salt-induced increase in MDA content due to oxidative stress has been reported in plants such as canola (Ashraf and Ali, 2008), *Kosteletzkya virginica* (Guo et al., 2009) and wheat (Ashraf et al., 2010). The present findings also depicts that, rooting medium salinity increased the H_2O_2 and MDA contents with a reduction in plant biomass production and grain yield in all rice cultivars. Under such conditions, activity of antioxidative defense mechanism i.e. comprised of enzymatic and non-enzymatic antioxidants is effective to scavenge and detoxify the over produced ROS to reduce their damaging effects. However, the activity of these antioxidants differs among species/cultivars which are important in determining the salt tolerance ability among different cultivars of crop plants (Ashraf, 2009; Ashraf et al., 2012).

Apart from exploring inter-cultivar difference for salt tolerance, exogenous application of different compounds are also being extensively used and found effective in improving plant salt tolerance level (Ashraf and Foolad, 2005) by modifying the various physiological and biochemical processes. Similarly like other compounds, protective effects of exogenously applied SNP against salt induced damages in plants have been well documented (Zhang et al., 2006; Zheng et al., 2009; Habib et al., 2010, 2013). Number of reports has already shown the protective effect of SNP/NO_2 against salt-induced membrane lipid peroxidation in plants (Zhang et al., 2006; Shi et al., 2007). It has been found that NO_2 helps to prevent ROS-induced membrane damages by readily reacting with ROS as well as with lipid peroxyl (LOO^-) and alcoxyl (LO^-) radicals (Sheokand et al., 2010). In present study, exogenous SNP (source of NO_2) application as seed priming was found effective in decreasing the lipid peroxidation in rice plants grown under salt stress.

Normally H_2O_2 production takes places by a reaction of SOD with superoxide, radical while, ascorbate peroxidase (APX) and catalase (CAT) acts as H_2O_2 scavengers (Miller et al., 2010). In the present study, exogenous use of SNP was proved effective in improving the activity of enzymatic antioxidant (SOD, POD and CAT). The increase was also in accordance with previous reports in which increased antioxidant enzymes activity due to SNP treatment have been noted in seedlings of wheat (Zeng et al., 2009), mustard green (Zeng et al., 2011) and *Brassica campestris* (Chang-Li et al., 2011). Moreover, in present study this increase in antioxidative enzyme activities resulted in a decrease in H_2O_2 contents with a reduction in MDA accumulation. This decrease in MDA content with a reduction of H_2O_2 might be due to enhanced scavenging activity of antioxidant enzymes. Such SNP-applied decrease in leaf H_2O_2 under salinity in rice plants is in agreement with the studies has already been reported in *Arabidopsis* (Zhao et al., 2007) and chickpea (Sheokand et al., 2010) grown under salinity.

Leaf ascorbic acid and phenolic contents have significant roles in many metabolic processes in plants (Jamil et al., 2015). These antioxidative compounds play protective roles against salt-induced oxidative damages by scavenging ROS such as singlet

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oxygen, hydroxyl radical and superoxide (Ashraf and Ali, 2008). In the present study, exogenous SNP treatment was found effective in increasing the leaf ascorbic acid and phenolic content in rice plants grown under salt stress. Such SNP-induced enhanced synthesis of ascorbic acid and phenolic contents could be due to the involvement of NO2 in the activation of cellular antioxidative defence mechanism (Zeng et al., 2011). SNP-induced such increase in leaf ascorbic acid content has also been found in water stressed rice plants (Shehab et al., 2010). Similarly, it has been found that leaf total phenolic content also increased significantly due to exogenous SNP treatment under adverse environmental conditions (Shehab et al., 2010; Buss et al., 2011).

Leaf proline, a well-known secondary metabolite, plays a significant role as osmoprotectant as well as an antioxidant. As an osmotica, it not only actively takes part in cellular water relations under adverse conditions but also protects the cellular membranes by playing role as an antioxidant (Ali and Ashraf, 2011; Ashraf et al., 2012; Habib et al., 2012). In the present study, leaf proline content significantly increased due to exogenous SNP treatment in rice plants, showing its role as plant stress tolerant compound. SNP-induced accumulation in leaf proline has already been reported in *Kosteletzkya virginica* plants grown under salt stress (Guo et al., 2009).

Conclusions

It was concluded that the SNP seed priming increased the rice plant growth, grain yield and biomass production by the up-regulation of activities of antioxidant enzymes as well as the contents of non-enzymatic antioxidantive compounds which in turn resulted in enhanced ROS scavenging and reduced lipid peroxidation. The exogenous SNP seed treatment also modulated the proline synthesis/accumulation under salt stress there by reducing the salt-induced toxic effects. Regarding various SNP levels, it was concluded that the lower concentration of SNP (0.1 mM) can be used effectively for better yield in rice cultivated in salt rich areas.

REFERENCES

[1] Ali, Q., Ashraf, M. (2011a): Exogenously applied glycinebetaine enhances seed and seed oil quality of maize (*Zea mays* L.) under water deficit conditions. - Environmental and Experimental Botany 71(2): 249-259.

[2] Ali, Q., Ashraf, M. (2011b): Induction of drought tolerance in maize (*Zea mays* L.) due to exogenous application of trehalose: Growth, photosynthesis, water relations and oxidative defence mechanism. - Journal of Agronomy and Crop Science 197(4): 258-271.

[3] Ali, Q., Anwar, F., Ashraf, M., Saari, N. and Perveen, R. (2013): Ameliorating effects of exogenously applied proline on seed composition, seed oil quality and oil antioxidant activity of maize (*Zea mays* L.) under drought stress. - International Journal of Molecular Sciences 14: 818-835.

[4] Ashraf, M. (2009): Biotechnological approach of improving plant salt tolerance using antioxidants as markers. - Biotechnological Advances 27: 84-93.

[5] Ashraf, M. and Foolad, M.R. (2007): Roles of glycine betaine and proline in improving plant abiotic-stress resistance. - Environmental and Experimental Botany 59(2): 206-216.

[6] Ashraf, M. and Ali, Q. (2008): Relative membrane permeability and activities of some antioxidant enzymes as the key determinants of salt tolerance in canola (*Brassica napus* L.). - Environmental and Experimental Botany 63(1-3): 266-273.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_091105

[7] Ashraf, M.A., Ashraf, M. and Ali, Q. (2010): Response of two genetically diverse wheat cultivars to salt stress at different growth stages: leaf lipid peroxidation and phenolic contents. - Pakistan Journal of Botany 42(1): 559-565.
[8] Ashraf, M.A., Ashraf, M. and Shahbaz, M. (2012): Growth stage-based modulation in antioxidant defense system and proline accumulation in two hexaploid wheat (*Triticum aestivum* L.) cultivars differing in salinity tolerance. - Flora 207: 388-397.
[9] Bates, L.S., Waldren, R.P. and Teare, I.D. (1973): Rapid determination of free proline for water stress studies. - Plant and Soil 39: 205-207.
[10] Brankova, L., Ivanov, S., Alexieva, V. and Karanov, E. (2005): Salt induced alteration in the levels of some oxidative parameters and unspecific defence compounds in leaves of two plant species (cotton and bean) with different sensitivity to salinity. - Comptes rendus de l'Academie bulgare des Sciences 58(11): 1307-1312.
[11] Buss, D.S., Dias, G.B., Santos, M.P., Ventura, J.A. and Fernandes, P.M.B. (2011): Oxidative stress defence response of *Carica papaya* challenged by nitric oxide, papaya meleira virus and *Saccharomyces cerevisiae*. - Open Nitric Oxide Journal 3: 55-64.
[12] Carmak, I. and Horst, J.H. 1991: Effects of aluminum on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). - Physiologia Plantarum 83: 463-468.
[13] Chance, B. and Maehly, A.C. (1955): Assay of catalase and peroxidase. - Methods in Enzymology 2: 764-775.
[14] Chang-Li, Z., Man, L. and Min-chao, L. (2011): Physiological effects of exogenous nitric oxide on *Brassica campestris* ssp. Chinensis L. seedlings under salt stress. - In: Remote Sensing, Environment and Transportation Engineering (RSETE)5149-5152. IEEE.
[15] Chung, J.S., Zhu, J.K., Bressan, R.A., Hasegawa, P.M. and Shi, H. (2008): Reactive oxygen species mediate Na?-induced SOS1 mRNA stability in Arabidopsis. - Plant Journal (53): 554-565.
[16] García-Mata, C. and Lamattina, L. 2001. Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. - Plant Physiology 126: 1196-1204.
[17] Giannopolitis, C.N. and Ries, S.K. (1977): Superoxide occurrence in higher plants. - Plant Physiology 59: 309-314.
[18] Guo, Y., Tian, Z., Yan, D., Zhang, J. and Qin, P. (2009): Effects of nitric oxide on salt stress tolerance in *Kosteletzkya virginica*. - Life Science Journal 6: 67-75.
[19] Habib, N., Ashraf, M. and Shahbaz, M. (2013): Effect of exogenously applied nitric oxide on some key physiological attributes of rice (*Oryza sativa* L.) plants under salt stress. - Pakistan Journal of Botany 45(5): 1563-1569.
[20] Habib, N., Ashraf, M. and Ahmad, M.S.A. (2010). Enhancement in seed germinability of rice (*Oryza sativa* L.) by pre-sowing seed treatment with nitric oxide (NO) under salt stress. - Pakistan Journal of Botany 42(6): 4071-4078.
[21] Habib, N., Ashraf, M., Ali, Q. and Perveen, R. (2012): Response of salt stressed okra (*Abelmoschus esculentus* Moench) plants to foliar-applied glycine betaine and glycine betaine containing sugarbeet extract. - South African Journal of Botany 83: 151-158.
[22] Habib, N. and M. Ashraf. (2014): Effect of exogenously applied nitric oxide on water relations and ionic composition of rice (*Oryza sativa* L.) plants under salt stress. - Pakistan Journal of Botany 46(1): 111-116.
[23] Hayat, S.S.A., Hasan, M., Mori, Fariduddin, Q. and Ahmad, A. (2010): Nitric oxide: Chemistry, biosynthesis, and physiological role. - In: Hayat, S., Mori, M., Pichtel, J., Ahmad, A. (Eds.) Nitric Oxide in Plant Physiology. GmbH and Co. KGaA, Weinheim, Germany: WILEY-VCH Verlag, pp. 1-16.
[24] Hernandez, M., Fernandez-Garcia, N., Diaz-Vivancos, P. and Olmos, E. (2010): A different role for hydrogen peroxide and the antioxidative system under short and long salt stress in *Brassica oleracea* roots. - Journal of Experimental Botany 61: 521-535.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_091105

[25] Jamil, S., Ali, Q., Iqbal, M., Javed, M.T., Iftikhar, W., Shahzad, F. and Perveen, R. (2015): Modulations in plant water relations and tissue-specific osmoregulation by foliar-applied ascorbic acid and the induction of salt tolerance in maize plants. - Brazilian Journal of Botany 38(3): 527-538

[26] Julkenen-Titto, R. (1985): Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. - Agriculture and Food Chemistry 33: 213-217.

[27] Koca, H., Bor, M., Özdemir, F. and Türkan, I. (2007): The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. - Environmental and Experimental Botany 60: 344-351.

[28] Li, Y. (2009): Physiological responses of tomato seedlings (*Lycopersicon esculentum*) to salt stress. - Modern Applied Sciences 3: 171-176.

[29] Liang, Y., Chen, Q., Liu, Q., Zhang, W. and Ding, R. (2003): Exogenous silicon (Si) increases antioxidant enzyme activity and reduces lipid peroxidation in roots of salt-stressed barley (*Hordeum vulgare* L.). - Journal of Plant Physiology 160(10): 1157-1164.

[30] Miller, G., Suzuki, N., Ciftci-Yilmaz, S. and Mittler, R. (2010): Reactive oxygen species homeostasis and signaling during drought and salinity stresses. - Plant Cell and Environment 33: 566-589.

[31] Mukherjee, S.P. and Choudhuri, M.A. (1983): Implication of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. - Plant Physiology 58: 166-170.

[32] Navarro, J.M., Flores, P., Garrido, C. and Martinez, V. (2006): Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. - Food Chemistry 96: 66-73.

[33] Noreen, S., Ashraf, M., Hussain, M. and Jami, l.A. (2009): Exogenous application of salicylic acid enhances antioxidative capacity in salt stressed sunflower (*Helianthus annuus* L.) plants. - Pakistan Journal of Botany 41(1): 473-479.

[34] Pearson, G.A. and Ayers, A.D. (1960): Rice as a crop for salt-affected soil in process of reclamation. - USDA Production Research Report 43: 1-13.

[35] Posmyk, M.M., Kontek, R. and Janas, K.M. (2009): Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. - Ecotoxicology and Environmental Safety 72: 596-602.

[36] Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1996): Structure–antioxidant activity relationships of flavonoids and phenolic acids. - Free Radical Biology and Medicine 20: 933-956.

[37] Roshandel, P. and Flowers, T. (2009): The ionic effects of NaCl on physiology and gene expression in rice genotypes differing in salt tolerance. - Plant and Soil 315: 135-147.

[38] Ruiz, J.M., Blasco, B., Rivero, R.M. and Romero, L. (2005): Nicotine-free and salt-tolerant tobacco plants obtained by grafting to salinity-resistant rootstocks of tomato. - Physiologia Plantarum 124: 465-475.

[39] Sairam, R.K., Srivastava, G.C., Agarwal, S. and Meena, R.C. (2005): Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. - Biologiae Plantarum 49: 85-91.

[40] Shehab, G.G., Ahmed, O.K. and El-Beltagi, H.S. (2010): Effects of various chemical agents for alleviation of drought stress in rice plants (*Oryza sativa* L.). - Notulae Botanicae Horti Agrobotanici Cluj-Napoca 38(1): 139-148.

[41] Sheokand, S., Bhankar, V. and Sawhney, V. (2010). Ameliorative effect of exogenous nitric oxide on oxidative metabolism in NaCl treated chickpea plants. - Brazilian Journal of Plant Physiology 22: 81-90.

[42] Shi, Q., Ding, F., Wang, X. and Wei, M. (2007): Exogenous nitric oxide protects cucumber roots against oxidative stress induced by salt stress. - Plant Physiology and Biochemistry 45(8): 542-550.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_091105

[43] Siân L. Richards, Katie A. Wilkins, Stéphanie M. Swarbreck, Alexander A. Anderson, Noman Habib, Alison G. Smith, Martin McAinsh, and Julia M. Davies. (2015): The hydroxyl radical in plants: from seed to seed. - Journal of Experimental Botany 66(1): 37-46.

[44] Tanou, G., Molassiotis, A. and Diamantidis, G. (2009): Hydrogen peroxideand nitric oxide-induced systemic antioxidant prime-like activity under NaCl-stress and stress-free conditions in citrus plants. - Journal of Plant Physiology 166: 1904-1913.

[45] Velikova, V., Yordanov, I. and Edreva, A. (2000): Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Protective role of exogenous polyamines. - Plant Science 151: 59-66.

[46] Yuan, G., Wang, X., Guo, R. and Wang, Q. (2010): Effect of salt stress on phenolic compounds, glucosinolates, myrosinase and antioxidant activity in radish sprouts. - Food Chemistry 121: 1014-1019.

[47] Zeng, C.L., Liu, L., Wang, B.R., Wu, X.M. and Zhu, Y. (2011): Physiological effects of exogenous nitric oxide on *Brassica juncea* seedlings under NaCl stress. - Biologiae Plantarum 55(2): 345-348.

[48] Zeng, L., Lesch, S.M. and Grieve, C.M. (2003): Rice growth and yield respond to changes in water depth and salinity stress. - Agricultural Water Management 59: 67-75.

[49] Zhang, F., Wang, Y., Yang, Y.L., Wu, H., Wang, D. and Liu, J.Q. (2007): Involvement of hydrogen peroxide and nitric oxide in salt resistance in the calluses from *Populus euphratica*. - Plant Cell and Environment 30: 775-785.

[50] Zhang, Y.Y., Wang, L.L., Liu, Y.L., Zhang, Q., Wei, Q.P. and Zhang, W.H. (2006): Nitric oxide enhances salt tolerance in maize seedlings through increasing activities of proton-pump and Na^+/H^+ antiport in the tonoplast. - Planta 224: 545-555.

[51] Zhao, M.G., Tian, Q.Y. and Zhang, W.H. (2007): Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. - Plant Physiology 144: 206-217.

[52] Zheng, C., Jiang, D., Liu, F., Dai, T., Liu, W., Jing, Q. and Cao, W. (2009): Exogenous nitric oxide improves seed germination in wheat against mitochondrial oxidative damage induced by high salinity. - Environmental and Experimental Botany 67(1): 222-227.

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BIODEGRADATION OF PHENOL BY *STENOTROPHOMONAS* SP. AND *STAPHYLOCOCCUS* SP. ISOLATED FROM CONTAMINATED SITES

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Abstract. Phenol as environmental pollutant is detrimental to living organisms and needed to be eliminated for environmental safety. Among the various practiced approaches for its removal, bacterial utilization gets attraction due to its eco-friendly and cost effective nature. For this purpose, bacterial strains were isolated from bioremediation site and industrial waste through enrichment in phenol (250 mg L^{-1}) for 3 days at 28°C. After enrichment, morphologically distinct colonies were purified on phenol (200 mg L^{-1}) agar plates and the strains were identified through 16S rRNA gene sequence. Total of eight strains were identified, among them two strains, NCCP-310 and NCCP-405 had the best potential of phenol degradation which were identified as the members of the genera *Stenotrophomonas* and *Staphylococcus*. NCCP-310 and NCCP- 405 showed 98.85 and 98.9% sequence identity with *Stenotrophomonas maltophilia and Staphylococcus equorum* subsp. *equorum*, respectively. Both strains have ability to tolerate 1000 mg L^{-1} phenol. The isolated strains degraded 750 mg L^{-1} of phenol at pH 7 and 28+2°C. NCCP-310 and NCCP-405 showed degradation of such amount in 65 and 85 h with the average rate of 15.65 and 11.64 mg L^{-1} h^{-1}. Our work suggests that these strains are efficient in phenol removal and could be used for bioremediation.
Keywords: *phenol, biodegradation, Stenotrophomonas, Staphylococcus, bioremediation*

Introduction

The foremost challenge of environmental concern is the elimination of pollutant that is liberated at alarming rate in our ecosystem and food chain due to rapid industrialization. Untreated industrial wastes containing xenobiotics drained up and contaminate water resources which are used for domestic as well as in agricultural practices (Kwon and Yeom, 2009; Ahmed et al., 2012). Phenol is used most widely in many industries such as pharmaceutical, plastic, ceramics, oil refinery, resin manufacturing, coke plant, textile and steel industries etc. (Han et al., 2010; Zhu et al., 2012) and due to its toxicity, phenol is in the priority list of US Environmental Protection Agency (US EPA, 2007).

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Phenol ranging from 10 to 17500 mg L^{-1} is detected in industrial effluent (Carbajo et al., 2010) while, only 0.5 mg L^{-1} is permitted by the Environmental Protection Agencies in the effluent based on production, exposure and biological effects (Giti et al., 2005). The contamination of water by phenol generates polychlorinated phenols which at low concentration (2.0 µg L^{-1}) cause unpleasant smell in drinking water (Arutchelvan et al., 2006). Phenol is lethal for all form of life includes humans, animals, plants, aquatic life and microorganisms (Rocha et al., 2007). Proper hygienic techniques are obligatory to dispose bulk phenol containing effluents. To cope with this situation, many physical and chemical methods (adsorption, solvent extraction, activated carbon adsorption, chemical oxidation) are practiced which are no more desirable owing to high cost of production, hazardous to workers and nearby population (Idris and Saed, 2002). Therefore, biodegradation is the plausible approach for phenol removal because of low cost and eco-friendly nature (Saravanan et al., 2008).

Diverse groups of microorganisms including fungi, algae and bacteria are naturally endowed with the property of phenol degradation (Godjevargova et al., 2003; Fialova et al., 2004; Quan et al., 2004) and many bacterial isolates with high phenol degrading potential are evaluated belonging to various genera including *Rhodococcus* (Larkin et al., 2005), *Stenotrophomonas* (Urszula et al., 2009), *Pseudomonas* (Ahmad et al., 2014), etc.

The objectives of the current study were the isolation, identification of phenol degrading bacteria based on 16S rRNA gene and to determine their phenol degrading potential. We reported phenol degrading potential of *Stenotrophomonas* sp. NCCP-310 and *Staphylococcus* sp. NCCP-405 which was isolated from sludge of bioremediation site and industrial waste respectively.

Materials and methods

Isolation and enrichment

Samples (waste) were collected from two sites i.e. Bioremediation Garden, NARC and industrial area I-9 Islamabad, Pakistan. The enrichment of samples was conducted at ambient temperature for 3 days at 120 rpm in mineral salt medium (MSM) containing phenol (250 mg L^{-1}). Two to three drops of the enriched samples were spread on MSM plates containing only phenol (200 mg L^{-1}) for carbon need. Plates were placed in incubator at 28 ^{o}C till growth. Morphologically distinct colonies appeared was sub-cultured and purified on phenol (200 mg L^{-1}) containing agar plates again. Subculturing was perfumed many times to get pure culture. The isolated strains were preserved at -80^{o}C in 70 % glycerol solution.

Identification of bacterial strains

Identification of the isolated strains was performed on the bases of 16S rRNA gene followed by the method of Ahmed et al. (2007). For this purpose, pure culture of each strain was obtained by growing on Tryptic Soy Agar (TSA) plates incubated at 28^{o}C. After purification, single colony of each strain was dipped and stirred in TE buffer (20 µL) in PCR strips, homogenized and kept in thermal cycler at 95^{o}C for 10 min for extraction of DNA. Strips were then removed and centrifuged at 12000 rpm for 5 min.
Genes of 16S rRNA was amplified using 1 µL template bacterial DNA contained in supernatant. 49 µL master mix was prepared for each strain by mixing TAKARA pre-

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mix (25 µL), 2 µL of each following primers: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') and 20 µL PCR water. The final reaction volume was made to 50µL in PCR tube by adding the prepared 49 µL of master mix to 1 µL of the template DNA which was already added to each PCR tube. In centrifuge machine the samples were short spin for a min or two for homogenization. PCR strips were then placed in thermal cycler (Applied Biosystems, Veriti, USA), PCR program was set as described by Ahmed et al. (2007) to amplify the said gene. Amplified products were confirmed by gel electrophoresis using 0.8% agarose in which, bromo phenol blue and ethidium bromide were used as loading dye and staining dye, respectively. Images of the gel were taken on gel documentation system (UVIPro Platinum, England). Purification of the Amplified products were done following the manufacturer's protocol (Invitrogen, USA). The amplified 16S rRNA gene products were sequenced using universal forward 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse 1492R (5'-ACCTTGTTACGACTT-3') primers.

The obtained sequences were refined with BioEdit software and to retrieve closest matches, BLAST search was performed on Ez-Taxon Server. On the basis of maximum identity score sequences were selected which were aligned in Clustal W (V. 1.6) (Thompson et al., 1994). Phylogenetic trees were generated in MEGA-6 software using Neighbor-Joining algorithms (Tamura et al., 2011).

Biochemical characterization

Consumption of different carbon sources by the isolates were determined using API 20E kit (bioMerieux, France). Few pure colonies (16 to 18 h) of each strain were added in 0.85% saline solution and the microtubes of API 20E kit were filled with prepared inoculums. The kits were then placed in incubator at 28^{o}C for 24-48 h and after then, the results were recorded according to color change.

Phenol tolerance

Phenol resistance of isolated strains was determined by introducing the pre-culture of each strain in MS broth augmented with 0, 250, 500, 750 and 1000 mg L^{-1} phenol in 100 mL flasks. Flask were placed on shaker within incubator at 28^{o}C and incubated for 3-4 days. Blank without inoculum of each concentration was prepared in parallel. At different time gap growth was checked with the help of spectrophotometer (IMPLEN, Germany) at 600 nm wavelength. The growth of each strain at a given phenol concentration was determined with corresponding blank.

Phenol degrading potential and analysis

Phenol degrading efficiency of isolated strains was determined by adding the preculture of each strain in MSM broth augmented with 750 mg L^{-1} of phenol for 2-4 days at 200 rpm. Samples of the culture were collected at specific intervals for 3-5 days depending upon the growth of strain. Optical density (OD) of the samples was determined at 600 nm with spectrophotometer (IMPLEN, Germany) to observe the growth of cells over time.

One mL sample was taken from each flask at different time intervals and centrifuged at 12000 rpm for 7-10 min. Then 0.5 mL of centrifuged sample was diluted with an equal amount of acetonitrile. High Performance Liquid Chromatography (HPLC) (PerkinElmer, USA) consisting of C-18 column together with LC 295 UV/V detector.

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Mobile phase was comprised of acetonitrile and water at the rate of 60:40% (v/v) with flow rate of 0.8 mL/min. Detector wavelength was set to 280 nm (Ahmad et al., 2014). Identification of phenol was done on the basis of retention time and quantification on the basis of 6 points external standards calibration curve. The data obtained from bacterial growth versus time and phenol degradation versus time was analyze using regression analysis.

Results and discussion

Isolation and identification

Eight bacterial isolates were isolated through enrichment in 250 mg L^{-1} phenol but, here we focus on two strains i.e. NCCP-310 and NCCP-405 which showed high phenol degrading potential. Enrichment of culture was often practiced to isolate the desired microorganisms among the diverse microbial populations (Dunbar et al., 1997). After purification, the strains were characterized morphologically. Colony of the NCCP-310 was pale yellow in color with round shape and entire margin. The elevation was convex. Shape of NCCP-405 was circular with white color having smooth surface. Margin of the colony was entire with flat elevation.

Isolated strains were identified using 16S rRNA gene to avoid problems raised in identification merely on morphological bases (Roohi et al., 2012). Refined sequences (16S rRNA gene) of NCCP-310 and NCCP-405 were deposited to DNA Data Bank of Japan (DDBJ) with the accession numbers AB983341and AB983342, respectively. 16S rRNA gene sequence comparison of NCCP-310 showed that this strain shared 98.85% similarity with *Stenotrophomonas maltophilia* (AB008509) which was isolated by Hugh (1981) and assigned as *Pseudomonas maltophilia* but later on due to biochemical characterizations and 16S rRNA gene sequence affiliation, the strain was assigned as *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993). This strain shared 98.85, 98.75 and 98.66% sequence identity with *Pseudomonas geniculata* (AB021404), *Pseudomonas hibiscicola* (AB021405) and *Pseudomonas beteli* (AB021406), respectively. Phylogenetic analysis confirmed the affiliation of NCCP-310 (*Fig. 1*) with the above said strains but Anzai et al. (2000) performed phylogenetic analysis of γ-β subclasses of the *Proteobacteria* and reclassified the strains as the members of the genus *Stenotrophomonas*. However, we suggest the DNA-DNA hybridization of NCCP-310 with the closely related strains to determine the exact taxonomic position. NCCP-405 exhibit 98.9, 98.8 and 97.79% identity with *Staphylococcus equorum* subsp. *Equorum* (AB009939), *Staphylococcus equorum* subsp. *Linens* (AF527483) and *Staphylococcus xylosus* (D83374), respectively. Phylogenetic analysis (*Fig. 2*) affirmed the association of NCCP-405 with the genus *Staphylococcus*.

Biochemical characterizations

Using API 20E kit, the isolated strains were tested for various organic substrates utilization. *Table 1* shows biochemical characterizations of *Stenotrophomonas* sp. NCCP-310 and *Staphylococcus* sp. NCCP-405. Both strains showed positive results for β-galactosidase and NO_2 production. In addition, *Stenotrophomonas* sp. NCCP-310 showed positive results for lysine decarboxylase, citrate utilization, gelatinase while negative for all the other substrates used. Similarly, *Staphylococcus* sp. NCCP-405 showed positive results for urease while negative for all other substrates tested.

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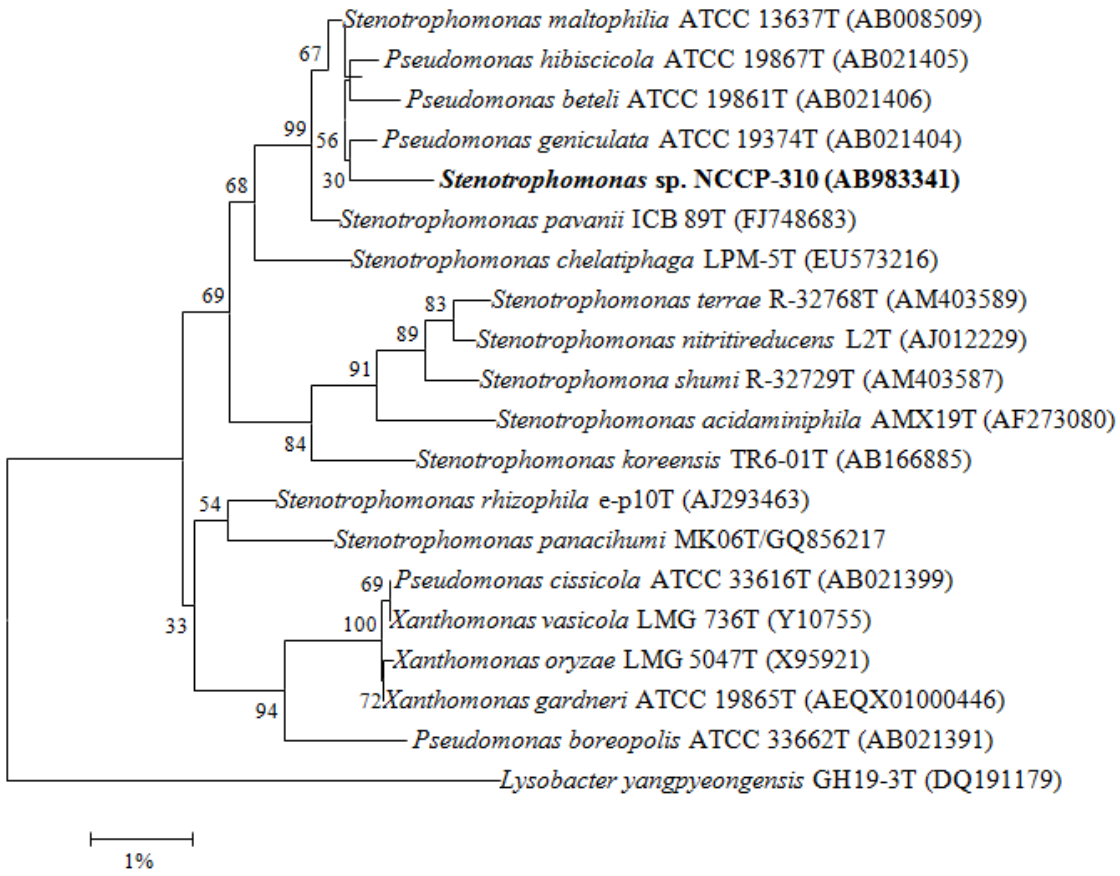

Figure 1. *Phylogenetic tree constructed in MEGA-5 with NJ method, showing the interelation of NCCP-310 with other closest matches using Lysobacter yangpyeongensis (DQ191179) as an out group.*

Table 1. *Biochemical characterization of Stenotrophomonas sp. NCCP-310 and Staphylococcus sp. NCCP-405.*

Biochemical tests	***Stenotrophomonas* sp. NCCP-310**	***Staphylococcus* sp. NCCP-405**
Arginine dihydrolase	–	–
Citrate utilization	+	–
Gelatinase	+	–
H_2S production	–	–
Indole production	–	–
Lysine decarboxylase	+	–
Ornithine dacarboxylase	–	–
Sodium pyruvate	–	–
Tryptophane deaminase	–	–
Urease	–	+
β-galactosidase	+	+
Fermentation/oxidation of:		
Amygdalin	–	–
Arabinose	–	–

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Glucose	–	–
Inositol	–	–
Mannitol	–	–
Melibiose	–	–
Rhamnose	–	–
Sacharose	–	–
Sorbitol	–	–
NO_2 production	+	+
Reduction to N_2 gas	–	–

+, positive reaction; –, negative reaction. These results are obtained after 48-72 h of incubation at 28°C.

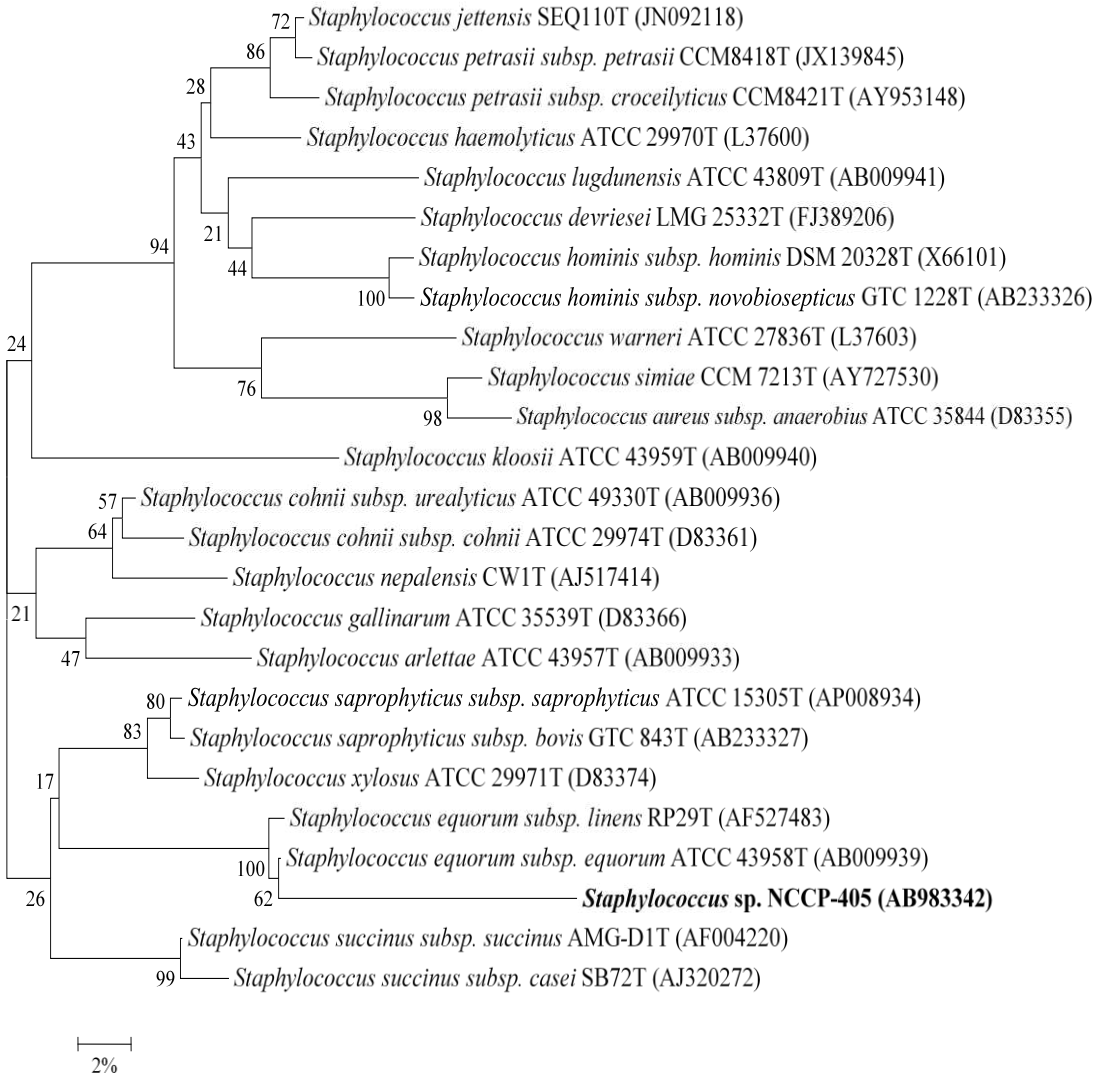

Figure 2. *Phylogenetic tree showing the inter-relationships of strain NCCP-405 with the most closely related type species inferred from sequences of 16S rRNA gene. The tree was generated using the NJ method. Bootstrap values were expressed as a percentage of 1000 replications. The bar shows 2% sequence divergence.*

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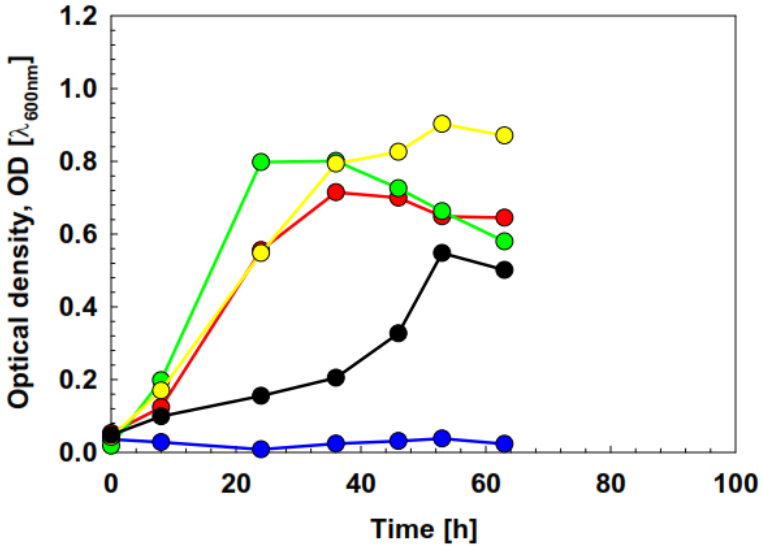
Phenol tolerance

Both stains were tested for their growth in MSM broth containing 0, 250, 500, 750 and 1000 mg L^{-1} phenol. The stain *Stenotrophomonas* sp. NCCP-310 was incubated for 63 h and growth was observed at all specified concentrations except 0 mg L^{-1}. Maximum growth was observed at 750 mg L^{-1} phenol after 53 h of incubation (*Fig. 3a*). Similarly, *Staphylococcus* sp. NCCP-405 was incubated for 86 h and showed growth at all concentrations except 0 mg L^{-1}. At all the given concentrations, the stain showed a very slow growth up to 12 h. Results indicated the extreme growth of NCCP-405 at 1000 mg L^{-1} phenol after 72 h of incubation (*Fig. 3b*).

(a)



(b)

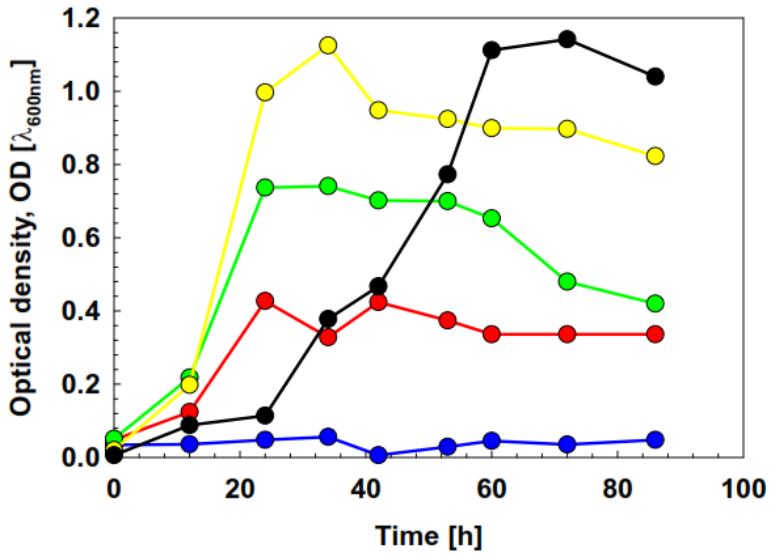


Figure 3. *Tolerance of phenol at different concentrations grown at 28°C in relation to time scale.* ***(a)*** *Stenotrophomonas sp. NCCP-310 and (b) Staphylococcus sp. NCCP-405. (●) denote 0 mg L^{-1}, (●)250 mg L^{-1}, (●)500 mg L^{-1}, (●)750 mg L^{-1}, and (●) denote 1000 mg L^{-1}.*

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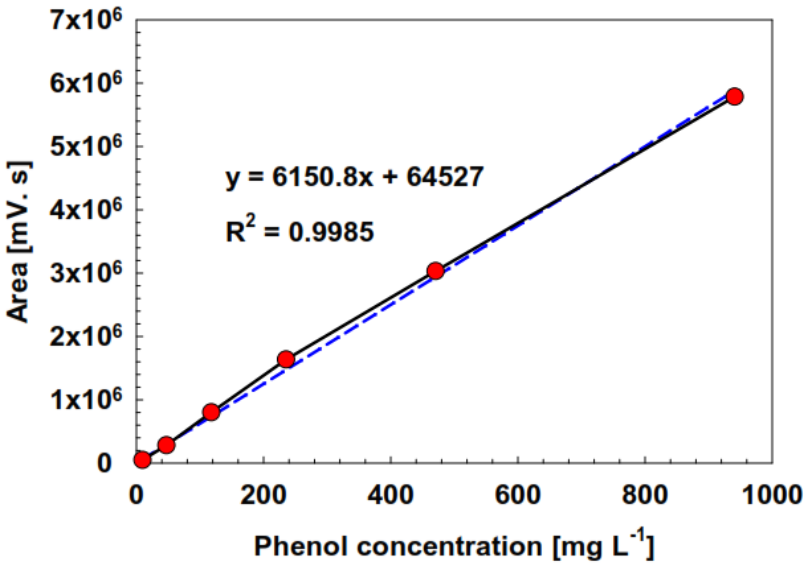
Tolerance of bacteria toward high concentration of phenol may be natural or due to some genetic changes particularly mutation in plasmid carrying gene for phenol degradation (Ajaz et al., 2004) or horizontal gene transfer. Tolerance of bacteria to a particular concentration of phenol is not related to the degradation of such amount. Nagamani et al. (2009) reported the tolerance of *Xanthobacter flavus* to 1100 mg L^{-1} of phenol but this strain could not degrade such amount and showed the ability to degrade only 650 mg L^{-1} phenol. This was because bacteria acquire different mechanism to withstand high concentration of phenol like increase in saturation of lipid membrane like in increase in fatty acid amount (Keweloh et al., 1991) and change in protein expression associated with efflux of phenol from cell (Randall et al., 2007).

Phenol degrading potential and analysis

Remaining phenol in cultural supernatant was quantified using the equation obtained from the regression analysis of external standards (*Fig. 4a and b*). The coefficient of determination (R^2) and adjusted R^2 values of standards were calculated as 99.09 and 98.9%, respectively. The closeness of both values indicates the accuracy of the model. Two types of controls were used for comparison of phenol degraded by the isolated strains, one control containing 750 mg L^{-1} phenol in MSM broth (uninoculated) and the other control with no phenol in MSM broth (inoculated). In both controls no growth observed nor observed any degradation of phenol. Similarly, no phenol degradation was detected in control without inoculation.

Stenotrophomonas sp. NCCP-310 was incubated for 74 h at 28±2°C in MSM broth augmented with 750 mg L^{-1} phenol. The strain degraded such amount in 65 h with average degradation rate of 15.65 mg L^{-1} h^{-1} for which the approximate doubling time was determined as 11.7 h^{-1}. After 49 h of incubation the strain showed maximum growth (*Fig. 5a*) as indicated by OD (0.95). Percentage removal of phenol from MSM by this strain was presented in *Fig. 5b*.

(a)



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(b)

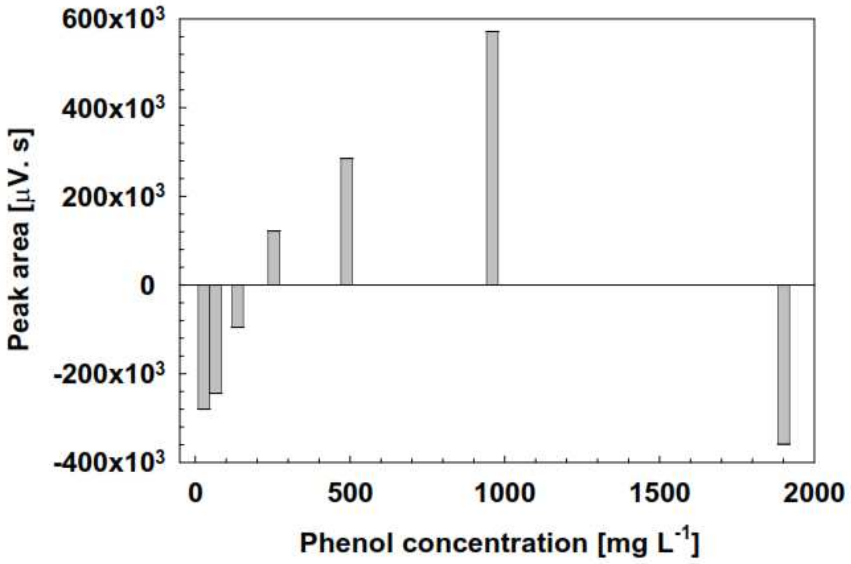

Figure 4*. (a) Standard curve and regression analysis presenting peak area and phenol concentration. (b) Residual plot of the external standards used in this study.*

The phylogenetic neighbor of *Stenotrophomonas* sp. NCCP-310 was *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993). This strain was well reported for phenol biodegradation (Gunasundari and Muthukumar, 2013). Phenol degradation pattern of *Stenotrophomonas* sp. NCCP-310 was found nearly similar to *Stenotrophomonas maltophilia* K279a (Han et al., 2010) which was able to degrade 805 mg L^{-1} of phenol and found that this strain degraded such amount in 48 h. Similarly, Urszula et al. (2009) reported the isolation of phenol degrading *Stenotrophomonas maltophilia* KB2 which degraded 12 mM phenol. Stains of this genus comprises a range of activities including plant growth promoting activity, human pathogenicity, role in nitrogen and sulphur cycles, production of secondary metabolites, metal tolerance and biodegradation of pollutants etc. (Ryan et al., 2009). Degradation of various organic compounds including p-nitrophenol and 4‑chlorophenol (Liu et al., 2007), polycyclic aromatic hydrocarbons (Juhasz et al., 2000), benzene, toluene (Lee et al., 2002), EDTA (Kaparullina et al., 2009) validates the natural biodegradation potential of the genus *Stenotrophomonas*.

Staphylococcus sp. NCCP-405 was incubated for 92 h at 28±22°C in MSM broth supplemented with 750 mg L^{-1} of phenol. The strain degraded such amount in 85 h with the average degradation rate of 11.64 mg L^{-1} h^{-1} for which the approximate doubling time was recorded as 12.95 h^{-1}. After 53 h of incubation the strain showed maximum growth (*Fig. 6a*) as indicated by OD (0.924). Percentage removal of phenol from MSM by this strain was presented in *Fig. 6b***.** Among the phylogenetic neighbors of *Staphylococcus* sp. NCCP-405, no strain is reported for phenol degradation. However, few strains of this genus are documented for bioremediation of phenol. Naresh et al. (2012) reported the isolation of *Staphylococcus aureus* from effluent which degraded 1000 mg L^{-1} phenol in 7 days. Prasanna et al. (2008) reported the degradation of 43.94% of 100 mg L^{-1} phenol in 120 h. The difference of time to degrade phenol by members of a genus is common and acceptable (Larkin et al., 2005). Similarly, few more strains showed the phenol degrading potential belonging to this genus. Phenol

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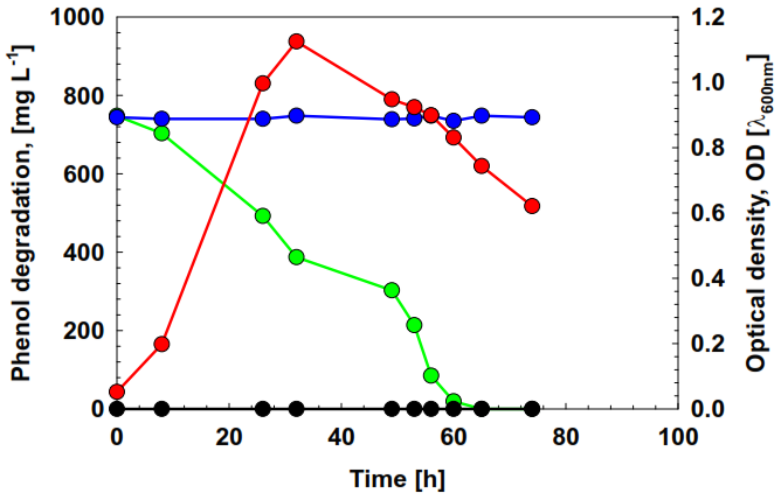
degrading pattern of *Staphylococcus* sp. NCCP-405 is somewhat similar to that of *Xanthobacter flavus* which was isolated from the soil near dye industry and showed complete degradation of 600 mg L^{-1} phenol in 120 h. In their work more than 97% of available phenol was degraded after 80 h of incubation but after that the process was prolonged (Nagamani et al., 2009). Comparatively, *Stenotrophomonas* sp. NCCP-310 degraded 750 mg L^{-1} phenol faster than *Staphylococcus* sp. NCCP-405. The presence of phenol degradation potential of phylogenetically diverse bacteria indicates the wide distribution of this trait. However, toxicity of phenol at high concentration showed growth with phenol by the strain *Staphylococcus* sp. NCCP-405 whose closely related strains don't have phenol degradation capacity indicates that horizontal gene transfer might have an important role in widely distribution of this trait.

(a)



(b)

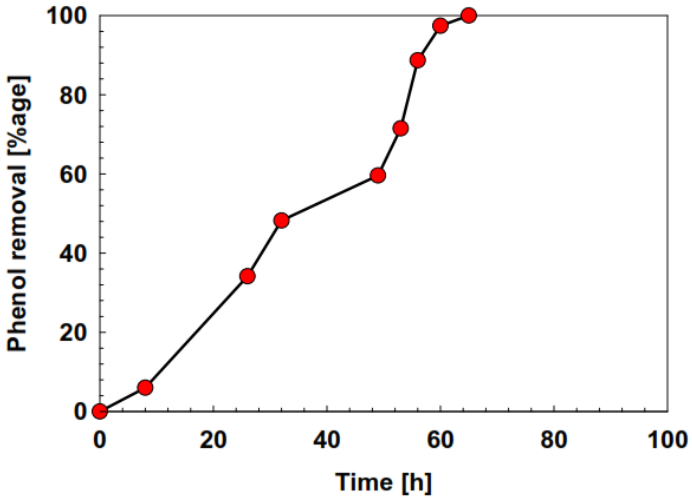


Figure 5. *(a) Degradation of phenol (750 mg L^{-1}) and growth as measured by optical density at 600 nm of NCCP-310. (—●—) denote phenol concentration in control (without inoculum), (—●—) denote phenol concentration in inoculum, (—●—) present observed OD in inoculum and (—●—) present OD in control. (b) Percentage phenol removal from MSM broth by NCCP-310.*

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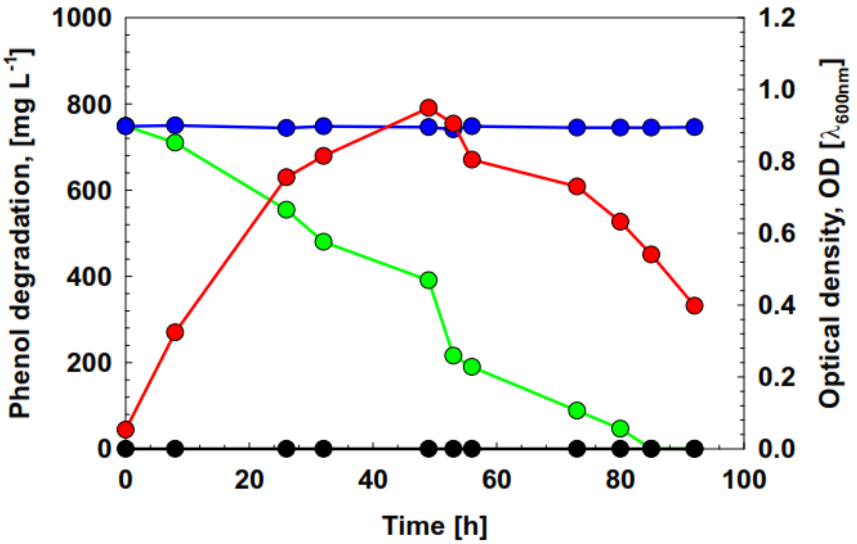
(a)



(b)

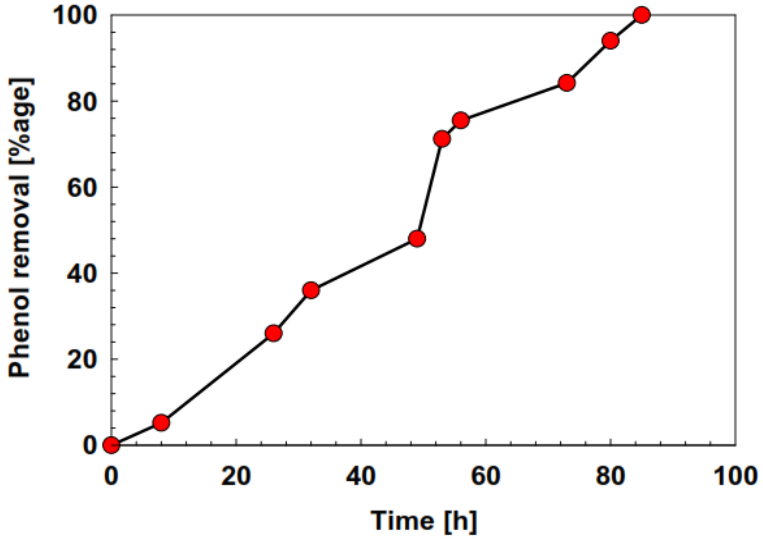


Figure 6. *(a) Degradation of phenol (750 mg L^{-1}) and growth as measured by optical density at 600 nm of NCCP-405. (—●—) denote phenol concentration in control (without inoculum), (—●—) denote phenol concentration in inoculum, (—●—) present observed OD in inoculum and (—●—) present OD in control. (b) Percentage phenol removal from MSM broth by NCCP-405.*

Conclusion

Present study delivers a useful guideline for identification of bacteria based on sequence analysis of 16S rRNA gene. In this study two bacteria strains NCCP-310 and NCCP-405 were isolated through enrichment process from two different contaminated sites. These strains showed growth up to 1000 mg L^{-1} of phenol. The isolated strains were found to degrade 750 mg L^{-1} phenol, given as sole source of carbon and energy. These degrade phenol in 65 and 85 h respectively.

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REFERENCES

[1] Ahmad, N., Ahmed, I., Shahzad, A., Khalid, N., Mehboob, F., Ahad, K. Ali, G. M. (2014): Molecular identification and characterization of *Pseudomonas* sp. NCCP-407 for phenol degradation isolated from industrial waste. – Journal of the Korean Society for Applied Biological Chemistry 57: 341-346.

[2] Ahmed, I., Yokota, A., Fujiwara, T. (2007): A novel highly boron tolerant bacterium, *Bacillus boroniphilus* sp. nov., isolated from soil, that requires boron for its growth. – Extremophiles 11, 217-224.

[3] Ahmed, W., Ahmed, A., Ahmad, A., Randhawa, M. A., Ahmad, R. Khalid, N. (2012): Heavy Metal Contamination in Vegetables Grown in Rawalpindi, Pakistan. – Journal of the Chemical Society of Pakistan 34: 914-919.

[4] Ajaz, M., Noor, N., Rasool, S. A. Khan, S. A. (2004): Phenol resistant bacteria from soil: identification-characterization and genetical studies. – Pakistan Journal of Botany 36: 415-424.

[5] Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H. Oyaizu, H. (2000): Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. –International Journal of Systematic and Evolutionary Microbiology 50: 1563-1589.

[6] Arutchelvan, V., Kanakasabai, V., Elangovan, R., Nagarajan, S. Muralikrishnan, V. (2006): Kinetics of high strength phenol degradation using *Bacillus brevis*. –Journal of Hazardous Materials 129: 216-222.

[7] Carbajo, J. B., Boltes, K. Leton, P. (2010): Treatment of phenol in an anaerobic fluidized bed reactor (AFBR): continuous and batch regime. –Biodegradation 21: 603-613.

[8] Dunbar, J., White, S. Forney, L. (1997): Genetic diversity through the looking glass: effect of enrichment bias. –Applied and Environmental Microbiology 63: 1326-1331.

[9] Fialova, A., Boschke, E. Bley, T. (2004): Rapid monitoring of the biodegradation of phenol-like compounds by the yeast Candida maltosa using BOD measurements. – International biodeterioration & biodegradation 54: 69-76.

[10] Giti, E., Mehdi, H. Nasser, G. (2005): Development of a microtitre plate method for determination of phenol utilization, biofilm formation and respiratory activity by environmental bacterial isolates.–International Journal of biodegradation and biodeterioration 56: 231-235.

[11] Godjevargova, T., Ivanova, D., Alexieva, Z. Dimova, N. (2003): Biodegradation of toxic organic components from industrial phenol production waste waters by free and immobilized *Trichosporon cutaneum* R57. – Process Biochemistry 38: 915-920.

[12] Gunasundari, D. Muthukumar, K. (2013): Simultaneous Cr (VI) reduction and phenol degradation using *Stenotrophomonas* sp. isolated from tannery effluent contaminated soil. –Environmental Science and Pollution Research 20: 6563-6573.

[13] Han, H.J., Fang, F., Ma, W.C., Xun, C.Y., Wang, W., Li, H.Q. (2010): Phenoloxidase activity of phenol-degrading bacteria. –Bioinformatics and Biomedical Technology, International Conference, IEEE, pp. 23-26.

[14] Hugh, R. (1981): Note: Pseudomonas maltophilia sp. nov., nom. rev. – International Journal of Systematic Bacteriology 31: 195-195.

[15] Idris, A. Saed, K. (2002): Degradation of phenol in wastewater using anolyte produced from electrochemical generation of brine solution. – Global Nest 4: 139-44.

[16] Juhasz, A. L., Stanley, G. A. Britz, M. L. (2000): Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_107120

Stenotrophomonas maltophilia strain VUN 10,003. – Letters in Applied Microbiology 30: 396-401.

[17] Kaparullina, E., Doronina, N., Chistyakova, T. Trotsenko, Y. (2009): *Stenotrophomonas chelatiphaga* sp. nov., a new aerobic EDTA-degrading bacterium. –Systematic and Applied Microbiology 32: 157-162.

[18] Keweloh, H., Diefenbach, R. Rehm, H.J. (1991): Increase of phenol tolerance of Escherichia coli by alterations of the fatty acid composition of the membrane lipids. – Archives of Microbiology 157: 49-53.

[19] Kwon, K. H. Yeom, S. H. (2009): Optimal microbial adaptation routes for the rapid degradation of high concentration of phenol. –Bioprocess and Biosystems Engineering 32: 435-442.

[20] Larkin, M. J., Kulakov, L. A. Allen, C. C. (2005): Biodegradation and *Rhodococcus*– masters of catabolic versatility. –Current Opinion in Biotechnology 16: 282-290.

[21] Lee, E. Y., Jun, Y. S., Cho, K.-S. & Ryu, H. W. (2002): Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia* T3-c. – Journal of the Air & Waste Management Association 52: 400-406.

[22] Liu, Z., Yang, C. & Qiao, C. (2007). Biodegradation of p-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp. –FEMS Microbiology Letters 277: 150-156.

[23] Nagamani, A., Soligalla, R. Lowry, M. (2009): Isolation and characterization of phenol degrading *Xanthobacter flavus*. –African Journal of Biotechnology 8: 5449-5453.

[24] Naresh, B., Honey, P. Vaishali, S. (2012): Biodegradation of phenol by a bacterial strain isolated from a phenol contaminated site in India. –Research Journal of Environment Sciences1: 46-49.

[25] Palleroni, N. J. Bradbury, J. F. (1993): *Stenotrophomonas*, a new bacterial genus for Xanthomonas maltophilia (Hugh 1980) Swings et al. 1983. –International Journal of Systematic Bacteriology 43: 606-609.

[26] Prasanna, N., Saravanan, N., Geetha, P., Shanmugaprakash, M., Rajasekaran, P. (2008): Biodegradation of Phenol and Toluene by *Bacillu*s sp., *Pseudomonas* sp., and *Staphylococcus* sp. Isolated from Pharmaceutical Industrial Effluent. 7: 20-24.

[27] Quan, X., Shi, H., Zhang, Y., Wang, J. Qian, Y. (2004). Biodegradation of 2, 4-dichlorophenol and phenol in an airlift inner-loop bioreactor immobilized with *Achromobacter* sp. –Separation and Purification Technology 34: 97-103.

[28] Randall, L., Cooles, S., Coldham, N., Penuela, E., Mott, A., Woodward, M., Piddock, L. Webber, M. (2007): Commonly used farm disinfectants can select for mutant Salmonella enterica serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. –Journal of Antimicrobial Chemotherapy 60: 1273-1280.

[29] Rocha, L. L., de Aguiar Cordeiro, R., Cavalcante, R. M., do Nascimento, R. F., Martins, S. C. S., Santaella, S. T. Melo, V. M. M. (2007): Isolation and characterization of phenol-degrading yeasts from an oil refinery wastewater in Brazil. –Mycopathologia 164: 183-188.

[30] Roohi, A., Ahmed, I., Iqbal, M. Jamil, M. (2012): Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. –Pakistan Jounal of Botany 44: 365-370.

[31] Ryan, R. P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M. B., Berg, G., van der Lelie, D. Dow, J. M. (2009): The versatility and adaptation of bacteria from the genus Stenotrophomonas. –Nature Reviews Microbiology 7: 514-525.

[32] Saravanan, P., Pakshirajan, K. Saha, P. (2008): Kinetics of phenol and m-cresol biodegradation by an indigenous mixed microbial culture isolated from a sewage treatment plant. –Journal of Environmental Sciences 20: 1508-1513.

[33] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. Kumar, S. (2011): MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_107120

distance, and maximum parsimony methods. –Molecular Biology and Evolution 28: 2731-2739.

[34] Thompson, J. D., Higgins, D. G. Gibson, T. J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. –Nucleic acids research 22: 4673-4680.

[35] Urszula, G., Izabela, G., Danuta, W. Sylwia, Ł. (2009): Isolation and characterization of a novel strain of Stenotrophomonas maltophilia possessing various dioxygenases for monocyclic hydrocarbon degradation. –Brazilian Journal of Microbiology 40: 285-291.

[36] US EPA (2007): National Recommended Water Quality Criteria. Washington, USA: U.S. Environmental Protection Agency Office of Water

[37] Zhu, X., Tian, J. Chen, L. (2012) : Phenol degradation by isolated bacterial strains: kinetics study and application in coking wastewater treatment. –Journal of Chemical Technology and Biotechnology 87: 123-129.

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INDUCTION OF CADMIUM STRESS TOLERANCE IN *TRITICUM AESTIVUM* L. BY ALFALFA LEAF EXTRACT

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Abstract. To assess the effect of foliar applications of alfalfa (*Medicago sativa* L.) leaf extract on three wheat (*Triticum aestivum* L.) varieties (Punjab-2011, Millat-2011 and Galaxy-2013) under cadmium (Cd) stress, two-week-old wheat plants were subjected to three levels of Cd (i.e. 0, 0.5 and 1.0 mM) stress and then foliarly applied with alfalfa leaf extract (0 and 2 % w/v). Data for 16-week-old wheat plants were collected for growth and physiochemical parameters, while yield were obtained at maturity. Cadmium stress significantly decreased growth, chlorophyll (chl.) *a*, flavonoids, total free amino acids, total soluble sugars and relative water contents (RWC %), while increased chl. *b*, relative membrane permeability (RMP %), malondialdehyde (MDA), total phenolics and free proline contents. Foliar treatment with alfalfa leaf extract (2%) significantly increased growth, yield, leaf ascorbic acid, free proline and total soluble sugars, while decreased RMP (%) (in Punjab-2011 and Galaxy-2013), flavonoids and H_2O_2 contents in all wheat varieties under cadmium stress. Wheat varieties showed significant difference as var. Punjab-2011 excelled in all measured attributes except RMP (%), MDA and activity of POD, however, negative effect of cadmium stress was more prominent in var. Punjab-2011 than Millat-2011 and Galaxy-2013.
Keywords: *chlorophyll, allelopathy, antioxidants, proline, ascorbic acid*

Introduction

Metals with densities greater than 5 g cm^{-3} are known as heavy metals. Cadmium (Cd) is one of the toxic heavy metals that decrease growth and development of plants due to its high mobility in phloem tissues and creates agricultural problems throughout the world (Page and Feller, 2015). Global estimated annual release of Cd is about 22,000 metric ton mainly through anthropogenic activities (Sing et al., 2003). Cadmium is highly soluble in water that can be easily taken up by plant roots and transported to upper parts of plant organs (Benavides et al., 2005; Amirjani, 2012). Metals transport from root to shoot through xylem driven by transpiration (Salt et al., 1995). Heavy metal transporters include legends such as phytochelatins, antioxidants, proline, heat shock proteins, polyamines, metallothioneins, nitric oxide and salicylic acid (Sunitha et al., 2012). Cadmium causes several physiological disorders and promotes inhibition of metabolism (Elobeid et al., 2012). Cadmium has negative effects on photosynthetic pigments (Parmer et al., 2013), electron transport chain in photosynthesis, and alter membrane structure due to phospholipids degradation in cell membrane (Dhir et al., 2004). Metals accumulate in different plant species such as mustard (*Brassica campestris* L.) (Khan et al., 2016), sunflower (*Helianthus annuus* L.) (Iqbal et al., 2015; Abd-Allah et al., 2015), soybean (*Glycine max* L.) (Abdo et al., 2012), cucumber (*Cucumis sativus* L.) (Sun et al., 2015) and exert health hazard effects on animals by entering in the food chain (Khan et al., 2016).

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For achieving high-quality products some appropriate and improved practices are required to reduce time, labor, investment and availability of resources. There is a need to devise strategies for new genotypes of plants with high metal accumulation and toxicity tolerance (Iqbal et al., 2015). Exogenous application of plant growth regulators (PGRs) can increase growth rate and yield of crops under both control and stress conditions (Perveen et al., 2016). However, these PGRs are sometimes very costly and most of the time not available for the farmers in different regions of the country and globally. Some alternative low cost and eco-friendly strategy is of the need that could be cost-effective and easy to apply for the farmers. Crops are known to extract heavy metals from soil and hyper accumulator. It is known to bioaccumulate metals and could be used in phytoremidiation to phytoextract heavy metals (Cd and Pb) in metal contaminated soils (Zhi-xin et al., 2007).

Alfalfa (*Medicago sativa* L.), a member of family Leguminosae (also Fabaceae) is an herbaceous, long-lived perennial legume (Ferreira et al., 2012). Alfalfa is used in rotation with other crops especially with the cereals crops to replenish the soil organic nitrogen reserves. Alfalfa has been reported to involve in uptake of about 40-50% heavy metals (Cd and Zn) from sludge contaminated soil (Miller et al., 1995). A species of alfalfa (*Medicago scutellata*) has been reported to accumulate more Cd in shoot than in roots and could be used in phytoremidiation of contaminated soils (Darvishi and Kamajian, 2014). Ries et al. (1977) has reported a plant growth regulator, triacontanol (TRIA) in alfalfa (*Medicago sativa* L.). Later on, TRIA has been found to be very effective in increasing dry weight, leaf area per plant, level of reducing sugar, total amino acids and soluble proteins (Rice, 1985). Alfalfa leaf extract is more effective on crop plants than that of the stem and root extract (Randhawa et al., 2002; Asgharipour and Armin, 2010). Leaves and stems of alfalfa contain different concentrations of crude proteins, fibres as well as profile of amino acid at different growth stages (Popovic et al., 2001; Mauries, 2003). Alfalfa can affect the seed germination of plants due to the ammonia and saponins which is released from alfalfa residue (Miller, 1983). For example, alfalfa plant extract of different plant parts such as root, stem and leaf at 10, 20 and 50 g/l has reduced the growth and germination rate of wheat plants (Mousavi et al., 2013). Alfalfa contains soluble chemicals that cause autotoxicity (Miller, 1996).

Wheat (*Triticum aestivum* L.) is a major cereal food crop for the people of Pakistan. Cadmium toxicity has been reported to reduce wheat growth and yield by causing adverse effects on physiological and biochemical attributes (Rady and Hemida, 2015). Exogenous application of organic fertilizers and use of allelopathic extract from crops as organic source is a promising approach for enhancing crop yield in agriculture sector (Oves et al., 2016). These plant-based biostimulants are thought to enhance plant growth, yield via improving the efficiency of plant metabolism, increasing tolerance to abiotic stresses, enhancing quality attributes of crops and facilitating uptake, translocation and assimilation of nutrients etc. (Ertani et al., 2014; Nardi et al., 2016). To examine the potential role of alfalfa leaf extract, it was hypothesized that foliar application of alfalfa leaf extract can reduce the adverse effects of cadmium stress on different growth and physiochemical attributes of different wheat varieties.

Materials and Methods

To explore the effect of alfalfa (*Medicago sativa* L.) leaf extract (2%) on wheat (*Triticum aestivum* L.) under varying levels of cadmium (Cd) (0, 0.5 and 1.0 mM)

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stress, seeds of three wheat varieties (Punjab-2011, Millat-2011 and Galaxy-2013) were obtained from Ayub Agricultural Research Institute (AARI) Faisalabad-Pakistan. Experiment was conducted during the years of 2014-2015 at Govt. College University, Faisalabad under natural climatic conditions. Ten seeds of each wheat variety were sown in plastic pots (12″ long and 10″ wide) containing thoroughly washed sand. There were total 72 pots and each variety contains 24 pots (6 treatments with 4 replicates). Two-week-old wheat plants were subjected to three levels of cadmium (0, 0.5 and 1.0 mM) stress. Fresh alfalfa leaf samples were collected from AARI, Faisalabad and air dried at room temperature. To 10 g crushed leaves added 100 ml of distilled water and kept at room temperature for 48 hours, filtered this mixture with Whitman's filter paper. The filtrate was diluted to various levels by applying molality formula. Two levels [0 and 2 % (optimized out of 0, 2, 4, 6, 8 and 10 v/v)] of alfalfa leaf extract were foliarly sprayed at the rate of 25 ml per pot to 4-week-old wheat plants. Data of 16-week-old wheat plants (nourished with full strength Hoagland's, nutrient solution) were collected for the determination of various parameters. Two plants from each pot were uprooted with proper care to avoid any root damage and measured fresh and dry biomass, lengths of shoot and root and total leaf area per plant. Then the same plant samples were air dried for two weeks, kept in an oven at 65^{o}C for 48 hours and determined shoot and root dry weights, while yield attributes were determined at maturity.

Determination of relative water contents (RWC %)

Fresh leaf samples were collected and determined their fresh weight (FW) with analytical balance. Then leaf samples were kept overnight in 10 ml distilled water and determined turgid weight (TW); oven-dried at 65^{o}C for 48 hours and determined dry weight (DW) according to Jones and Turner (1978) method.

Percentage relative membrane permeability (RMP %)

Fresh leaf (0.5 g) were chopped and added 10 ml distilled water in test tubes and calculated electrical conductivity (EC_o), vortexed and determined EC_1 and then autoclaved the samples for 1 h at 90^{o}C, cooled at room temperature and calculated EC_2. Membrane permeability (%) was calculated by using the following formulae
RMP (%) = $EC_1 - EC_o$ / $EC_2 - EC_o$ x 100.

Determination of chlorophyll contents

Fresh leaf (0.5 g) were extracted in 10 ml of 80% acetone and kept at 4^{o}C in refrigerator. Chlorophyll contents were determined at the wavelengths 663 and 645 nm on spectrophotometer (Hitachi-U-1800, Japan) according to the method of Arnon (1949).

Determination of hydrogen peroxide (H_2O_2) contents

Velikova et al. (2000) protocol was used for H_2O_2 contents determination. Fresh leaf (0.5 g) samples were finely homogenized in 10 ml of 0.1% (w/v) trichloroacetic acid (TCA) in an ice both. The samples were centrifuged at 12,000 × *g* for 15 min. at 4^{o}C. To 0.5 ml supernatant solution, added 0.5 ml of potassium buffer (50 mM; pH=7) and 1 ml of potassium iodide (KI) solution. Absorbance of supernatant was determined with

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spectrophotometer at 390 nm wavelength. The values for H_2O_2 were computed by using the standard calibration curve values at different concentrations of H_2O_2

Determination of malondialdehyde (MDA) contents

Carmak and Horst (1991) method was used for MDA determination. Fresh leaf (0.5 g) finely ground in 10 ml of 0.1 % trichloroacetic acid (TCA) andcentrifuged at 12,000 × *g* for 15 min. at 4°C. To 1 ml of supernatant added 0.5% of 4 ml thiobarbituric acid (TBA) (prepared in 20% TCA) and heated that mixture at 95°C for 25 min in a water bath. The samples were cooled in an ice bath and centrifuged at 7,500 × *g* for 5 min. and read the absorbance at two wave lengths 532 and 600 nm on spectrophotometer.

Determination of total phenolics

Total phenolic contents were determined by using the method of Julkenen-Titto (1985) method. Fresh leaf (0.1 g) was ground in 2 ml of 80% acetone andcentrifuged at 15,000 × *g* for 15 min. at 4°C. To 0.1 ml of supernatant, added 2 ml of distilled H_2O and 0.5 ml Folin-Ciocalteau's phenol reagent (FC- reagent) and shake. Then 2.5 ml of sodium carbonate (20%) was added to above mixture and made final volume up to 5 ml, vortexed for 5 s and incubated the sample at room temperature (20°C) for 15 minutes. The absorbance of prepared sample mixture was determined by using spectrophotometer at 750 nm.

Determination of ascorbic acid contents

Mukherjee and Choudhuri (1983) method was used for ascorbic acid contents determination. Fresh leaf (0.25 g) was finely homogenized in 10 ml of 6% TCA. Two 2 ml dinitrophenyl hydrazine (2%) was added in 2 ml of supernatant and one drop of 10 % thiourea and reaction mixture heated in a water bath for 20 min. After cooling 5 ml H_2SO_4 (80%) was added in a test tube and absorbance was taken at 520 nm with the help of spectrophotometer.

Determination of total free amino acid

Moore and Stein (1957) method was used for the determination of free amino acids. Fresh leaf (0.5 g) was homogenized in 10 ml of citrate buffer (pH 5.0). Then supernatant was centrifuged at 15,000 × *g* for 10 min. One ml of extract was added in 1 ml of 10% pyridine solution and 1% ninhydrin solution in test tubes. Then heat the solution at 95°C for 30 min. and read the optical density of the prepared solution at 570 nm by using spectrophotometer.

Determination of free proline contents

The method of Bates et al. (1973) was used for the determination of free proline contents. Fresh leaf (0.5 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid solution and filtered. To 2 ml of filtrate added 2 ml each of glacial acetic acid and acid ninhydrine in test tubes. Then the mixture was heated in water bath at 95°C, cooled in an ice bath. To reaction mixture added 4 ml of toluene and vortexed for 10-15 s. The optical density of mixture was obtained at 520 nm using spectrophotometer.

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Determination of total soluble sugars

Fresh leaf tissue (0.1 g) was ground in 5 ml of 0.2 % phosphate buffer. To 0.1 ml of supernatant added 3 ml of freshly prepared anthrone reagent, shake it well and heated in a water bath at a 95°C for 15 min. The absorbance was measured after cooling at 625 nm using spectrophotometer.

Determination of flavonoides

Flavonoides were determined according to the method of Zhishen et al. (1999). Fresh leaf (0.1 g) was extracted in acetone (80%). To 0.5 ml of supernatant added 2 ml of distilled water, 0.6 ml of $NaNO_2$ (5%), 0.5 ml of $AlCl_3$ (10 %) and 2 ml of NaOH (1 M) and reading of mixture was recorded at 510 nm with a spectrophotometer.

Statistical analysis of experiment

Analysis of variance (ANOVA) of data for completely randomized design was performed via Co-STAT computer program to compare least significant difference using method of Snedecor and Cochran (1980).

Results

Cadmium stress of varying levels (0.0, 0.5 and 1.0 mM) significantly ($P \leq 0.001$) decreased shoot fresh and dry weights of all wheat varieties (*Fig. 1a, b*). Foliar application of alfalfa leaf extract at the rate of 2% (v/v) significantly increased shoot fresh ($P \leq 0.05$) and dry ($P \leq 0.001$) weights in all three wheat varieties under both non-stress and Cd stress conditions. All wheat varieties exhibited significant ($P \leq 0.001$) difference under cadmium stress (*Table 1*). Galaxy-2013 showed more tolerance to cadmium stress as compared to Punjab-2011 and Millat-2011. For example, under 1.0 mM Cd Punjab-2011 showed more reduction in shoot fresh and dry weights (65.37, 57.53% and 71.73, 58.17%) as compared to Millat-2011 (57.43, 45.52% and 52.88, 40.25%) and Galaxy-2013 (39.58, 1.05% and 41.07, 1.30%) under non-spray or alfalfa spray conditions respectively.

Root fresh and dry weights significantly ($P \leq 0.001$) decreased in all three wheat varieties (Punjab-2011, Millat-2011 and Galaxy-13) (*Fig. 1c, d*). Varieties showed significant (($P \leq 0.001$) difference under cadmium stress as varieties Galaxy-2013 and Punjab-2011 showed more reduction in root fresh and dry weight as compared to Millat-2011 (*Table 1*). Foliar application of alfalfa leaf extract (2%) significantly increased root fresh ($P \leq 0.05$) and dry ($P \leq 0.01$) weights in Punjab-2011 under Cd-stressed or non-stressed conditions, while in Millat-2011 under Cd-stress conditions. Galaxy-2013 showed variable response in root fresh weight, while increased root dry weight under both Cd-stress regimes.

Cadmium stress significantly ($P \leq 0.001$) decreased root and shoot lengths in all wheat varieties (*Fig. 1e, f*). Foliar application of alfalfa leaf extract significantly ($P \leq 0.001$) increased shoot length in all wheat varieties (*Table 1*). All varieties showed significant ($P \leq 0.01$) difference under cadmium stress as Punjab-2011 showed more reduction in shoot (34.32, 29.55%) lengths as compared to Millat-2011 (21.29, 25.79%) and Galaxy-2013 (6.25, 15.65%) under non-spray and foliar application of alfalfa aqueous extract respectively. Galaxy-2013 showed more tolerance to cadmium stress and increased growth by foliar application of alfalfa leaf extract (*Table 1*). Furthermore, root length

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significantly ($P \leq 0.01$) decreased in Punjab-2011 and Millat-2011, while Galaxy-2013 showed uniform behavior under Cd-stress. All wheat varieties showed variable response in terms of root length. For example, root length decreased in Millat-2011 under Cd-stress or non-stress conditions, while increased in Punjab-2011 and Galaxy-2013 by foliar application of alfalfa leaf extract under 0.5 mM and 1.0 mM Cd levels respectively (*Table 1*).

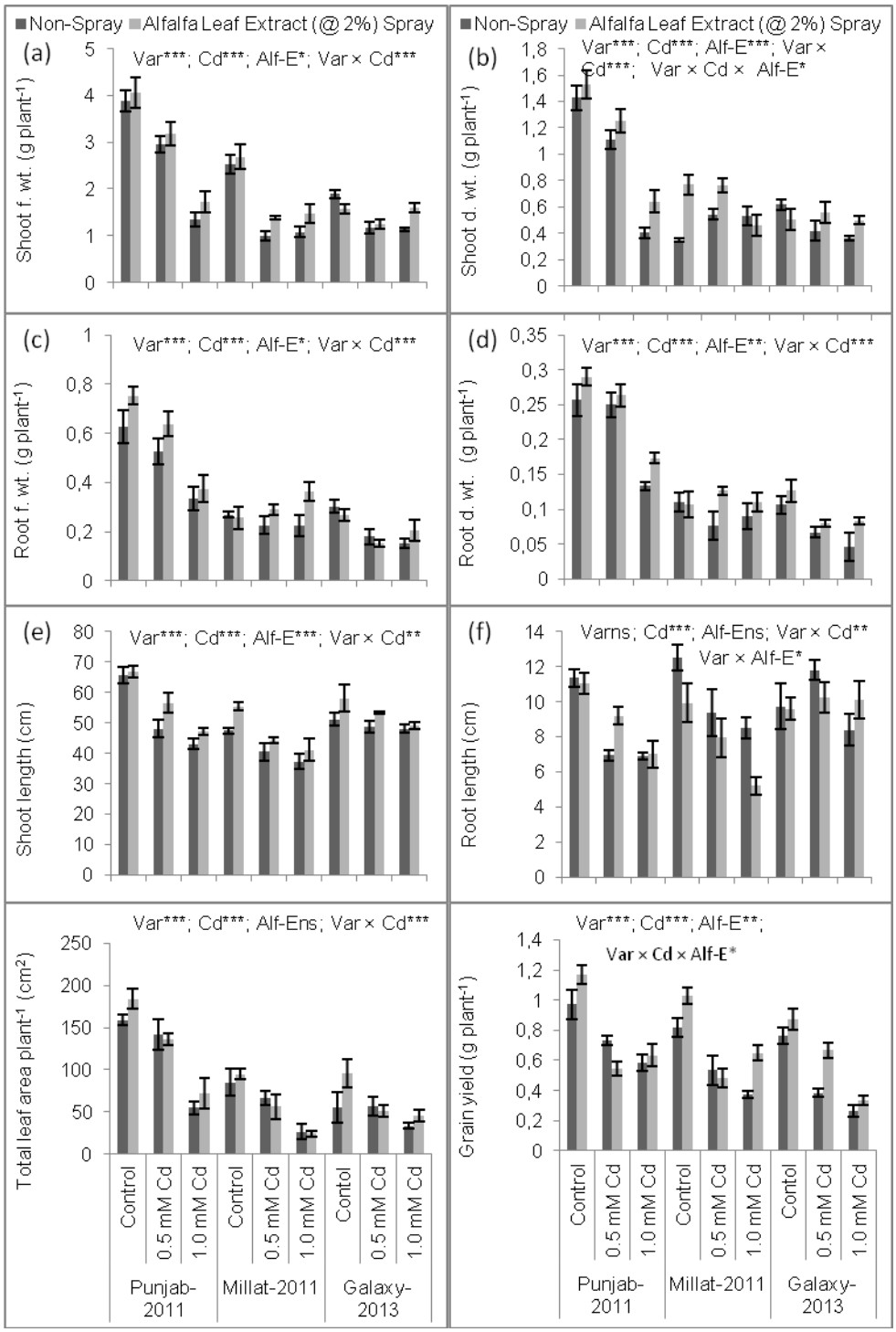

Figure 1. *Growth and yield parameters of 16-week-old wheat plants foliarly sprayed with 2% alfalfa leaf extract under cadmium stress and non-stress conditions.*

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Total leaf area per plant significantly ($P \leq 0.001$) decreased under varying levels of cadmium stress in all three wheat varieties (*Fig. 1g*). Foliar application of alfalfa leaf extract did not alter leaf area per plant under Cd stress or non-stress conditions. All three wheat varieties showed significant difference as Punjabb-2011 was higher in total leaf area per plant as compared to Millat-2011 and Galaxy-2013. However, cadmium-induced reduction in leaf area was more prominent in Millat-2011 than that of Punjab-2011 and Galaxy-2013 (*Table 1*).

Table 1. *Analysis of variance of the data for various growth, yield, yield components, relative water content (%) and membrane permeability of wheat plants foliarly-sprayed with 2% alfalfa leaf extract under cadmium stress and non-stress conditions.*

Source of Variations	df	Shoot f. wt.	Shoot dry wt.	Root f. wt.	Root dry wt.
Variety (Var)	2	10.37***	1.701***	0.558***	0.108***
Cadmium (Cd)	2	8.995***	0.720***	0.085***	0.016***
Alfalfa Extract (Alf-E)	1	0.614*	0.249***	0.035*	0.008**
Var × Cd	4	2.199***	0.390	0.059***	0.006***
Var × Alf-E	2	0.068ns	0.0233ns	0.0108ns	0.000ns
Cd × Alf-E	2	0.181ns	0.005ns	0.003ns	0.000ns
Var × Cd× Alf-E	4	0.040ns	0.062*	0.006ns	0.000ns
Error	36	0.122	0.018	0.006	0.000
Source of Variations	df	Shoot length (cm)	Root length (cm)	Total leaf area plant^{-1}	Grain yield (g plant^{-1})
Variety (Var)		492.5***	8.106ns	27163.2***	0.227***
Cadmium (Cd)	2	810.9***	40.82***	22126.2***	1.098***
Alfalfa Extract (Alf-E)	2	288.4***	4.564ns	1096.9ns	0.151**
Var × Cd	1	90.41**	11.14**	2351.9**	0.023ns
Var × Alf-E	4	1.22ns	11.95*	346.78ns	0.026ns
Cd × Alf-E	2	9.52ns	0.702ns	1180.01ns	0.030ns
Var × Cd× Alf-E	2	16.10ns	3.803ns	83.77ns	0.045*
Error	4	21.29	2.695	540.7	0.013
Source of Variations	36	Number of grains plant^{-1}	100-grain wt.	RWC (%)	RMP (%)
Variety (Var)	2	301.85***	6.907**	606.7***	211.71**
Cadmium (Cd)	2	435.3***	30.01***	294.7**	288.53**
Alfalfa Extract (Alf-E)	1	20.16ns	0.000ns	8.14ns	3.697ns
Var × Cd	4	39.68***	3.435*	130.8ns	48.66ns
Var × Alf-E	2	8.22ns	1.5ns	16.02ns	192.2**
Cd × Alf-E	2	7.166ns	0.5ns	19.65ns	40.09ns
Var × Cd× Alf-E	4	14.38ns	0.416ns	49.86ns	35.14ns
Error	36	6.129	0.962	53.98	35.58

MP (%) = membrane permeability in percentage; RWC (%) = relative water content; *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively; df = degrees of freedom; ns = non-significant.

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Yield and yield components significantly ($P \leq 0.001$) decreased under various cadmium regimes (*Fig. 1h* and *Fig. 2a, b*). Foliar application of alfalfa leaf extract significantly ($P \leq 0.01$) increased grain yield per plant in all three wheat varieties. In terms of grain yield per plant var. Galaxy-2013 showed more positive response to foliar application of alfalfa-leaf extract under cadmium stress as compared to other varieties. Varieties also showed significant ($P \leq 0.001$) difference under cadmium stress. For example, Punjab-2011 showed 57.60 and 56.31% reduction in number of grains/plant as compared to Millat-2011 (26.79, 33.33%) and Galaxy-11 (60.02, 34.11%). In hundred grain weight Punjab-2011 showed 48.01 and 38% reduction as compared to Millat-2011 (31.14, 33.33%) and Galaxy-11 (10.42, 11%) respectively under cadmium stress and non-stress conditions (*Table 1*).

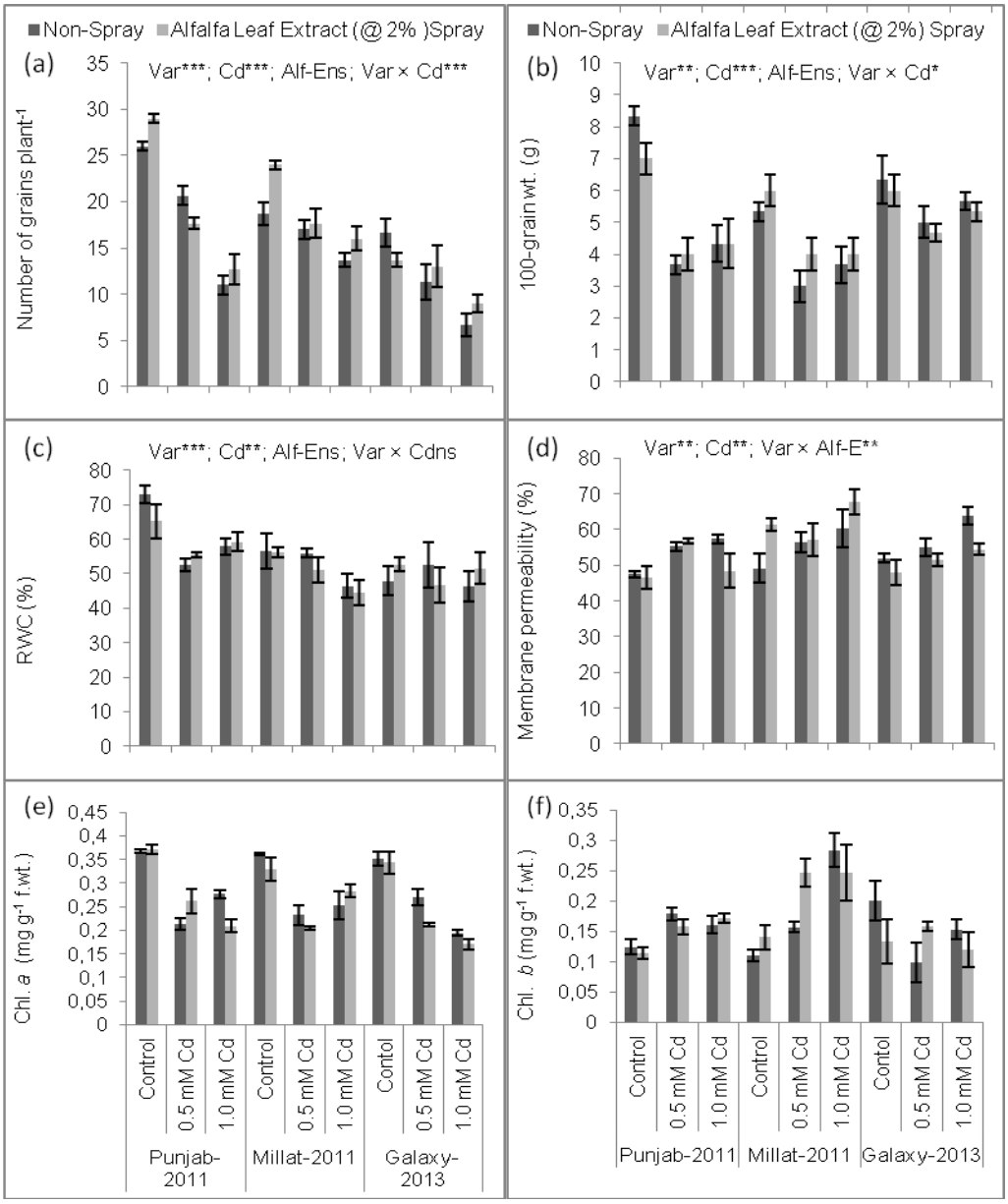

Figure 2. *Yield parameters, relative water content (%), membrane permeability (%) and chlorophyll contents of 16-week-old wheat plants foliarly sprayed with 2% alfalfa leaf extract under cadmium stress and non-stress conditions.*

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Relative water content (RWC%) markedly ($P \leq 0.01$) decreased in all wheat varieties under cadmium stress of varying levels (*Fig. 2c*). Reduction in RWC (%) was more in Punjab-2011 and Millat-2011 that that of Galaxy-2013 (*Table 1*). Imposition of foliar treatment with alfalfa extract did not alter RWC (%) significantly. Overall, under non-stress conditions wheat variety Punjab-2011 was higher in relative water content. However, under cadmium stress, reduction in relative water contents was more prominent in var. Punjab-2011 and var. Millat-2011 than that of var. Galaxy-2013.

Chlorophyll (chl.)'*a*' contents significantly ($P \leq 0.001$) decreased under cadmium stress in all wheat varieties. Varieties showed significant difference in chlorophyll contents (*Fig. 2e, f*). Overall, foliar treatment with alfalfa leaf extract did not modulate chlorophyll contents significantly under cadmium stress or non stress conditions (*Table 2*). However, under 0.1 mM Cd-stress wheat var. Millat-2011 showed higher chl.'*a*' contents than var. Punjab-2011 and var. Galaxy-2013 under both Cd-stress and by foliar application of alfalfa leaf extract. Similarly, under varying levels of Cd-stress var. Millat-2011 showed higher chl.'*b*' contents as compared to var. Punjab-2011 and Galaxy-2013.

Cadmium stress caused significant ($P \leq 0.05$) increase in H_2O_2 and MDA contents in all wheat varieties (*Fig. 2h, 3a*). Foliar treatment with alfalfa leaf extract significantly ($P \leq 0.05$) decreased H_2O_2 and MDA contents (*Table 2*). Of all wheat varieties, Galaxy-2013 was high in H_2O_2 and MDA contents as compared to Punjab-2011 and Millat-2011 (*Fig. 2h, 3a*).

Table 2. *Analysis of variance of the data for photosynthetic pigments, hydrogen peroxide, malondialdehyde, total phenolics, ascorbic acid, total free amino acids, free proline, ascorbic acid, soluble sugars and flavonoid contents of wheat plants foliarly-sprayed with 2% alfalfa leaf extract under cadmium stress and non-stress conditions.*

Source of Variations	df	Chl. *a*	Chl. *b*	H_2O_2	MDA
Variety (Var)	2	0.0034*	0.015**	0.378***	9.938*
Cadmium (Cd)	2	0.090***	0.012**	0.064ns	9.602*
Alfalfa Extract (Alf-E)	1	0.003ns	0.000ns	0.102*	7.31ns
Var × Cd	4	0.005**	0.012**	0.040ns	0.534ns
Var × Alf-E	2	0.000ns	0.002ns	0.019ns	0.045ns
Cd × Alf-E	2	0.000ns	0.005ns	0.082*	8.89*
Var × Cd× Alf-E	4	0.004**	0.004ns	0.006ns	0.157ns
Error	36	0.001	0.002	0.023	2.12
Source of Variations	df	Total phenolics	Ascorbic acid	Total free amino acids	Free proline
Variety (Var)	2	25.78ns	4.332***	7.660**	575.88***
Cadmium (Cd)	2	37.62*	0.613ns	5.59**	79.806*
Alfalfa Extract (Alf-E)	1	0.921ns	1.841*	0.124ns	229.7***
Var × Cd	4	8.320ns	9.009***	2.198ns	35.64ns
Var × Alf-E	2	1.632ns	0.993ns	0.359ns	6.761ns
Cd × Alf-E	2	3.464ns	0.006ns	3.029ns	6.881ns
Var × Cd× Alf-E	4	1.148ns	0.265ns	1.112ns	8.090ns
Error	36	10.26	0.360	1.065	17.72

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Source of Variations	df	Soluble sugars	Flavonoids
Variety (Var)	2	2870.2***	4.470***
Cadmium (Cd)	2	1463.7***	3.389***
Alfalfa Extract (Alf-E)	1	209.9*	1.243***
Var × Cd	4	429.9***	0.662***
Var × Alf-E	2	110.5*	0.239ns
Cd × Alf-E	2	113.7*	0.159ns
Var × Cd× Alf-E	4	126.9*	0.305*
Error	36	33.8	0.085

MDA = malondialdehyde; H_2O_2 = hydrogenperoxide; Chl. *a* = chlorophyll *a*; Chl. *b* = chlorophyll *b*; df = degrees of freedom; ns = non-significant; *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively.

Cadmium stress of varying levels significantly ($P \leq 0.05$) increased total phenolic (*Fig. 3c*) contents in all the three wheat varieties. Ascorbic acid contents increased in Punjab-2011 and Millat-2011, while decreased in Galaxy-2013 under cadmium stress (*Fig. 3d*). Foliar treatment with alfalfa extract did not alter total phenolics, while increased ascorbic acid contents in Punjab-2011 and Millat-2011 (*Table 2*). Accumulation of ascorbic acid contents was high in wheat var. Punjab-2011 and Millat-2011 than that of var. Galaxy-2013.

Cadmium stress of varying levels significantly ($P \leq 0.01$) decreased total free amino acids, while increased ($P \leq 0.05$) free proline in all wheat varieties (*Fig. 3e, f*). Foliar application of alfalfa leaf extract did not change total free amino acids, while increased free proline contents in all varieties under cadmium stress or non stress conditions (*Table 2*). Varieties showed significant ($P \leq 0.01$) difference as Galaxy-2013 was higher in total free amino acids contents as compared to Pujab-11 and Millat-2011, while reverse was true in the case of free proline contents. Effect of 1.0 mM cadmium was more intense as compared to 0.5 mM on all the three wheat varieties.

Current study revealed that cadmium stress significantly ($P \leq 0.001$) decreased total soluble sugars and flavonoid contents in all three wheat varieties (*Fig. 3g, h*). Varieties showed significant ($P \leq 0.001$) difference under Cd stress or non-stress conditions (*Table 2*). Overall, Punjab-2011 highly accumulated total soluble sugars and flavonoid contents than that of Millat-2011 and Galaxy-2013. Foliar application of alfalfa extract ($P \leq 0.05$) increased total soluble sugars in Millat-2011, while decreased in Galaxy-2013 under Cd stress conditions. However, flavonoid contents decreased by foliar treatment with 2% alfalfa leaf extract in all three wheat varieties under stressed or non-stressed conditions (*Fig. 3g, h*). Cadmium stress caused more reduction in both total soluble sugars and flavonoid contents in Galaxy-2013 as compared to other two wheat varieties.

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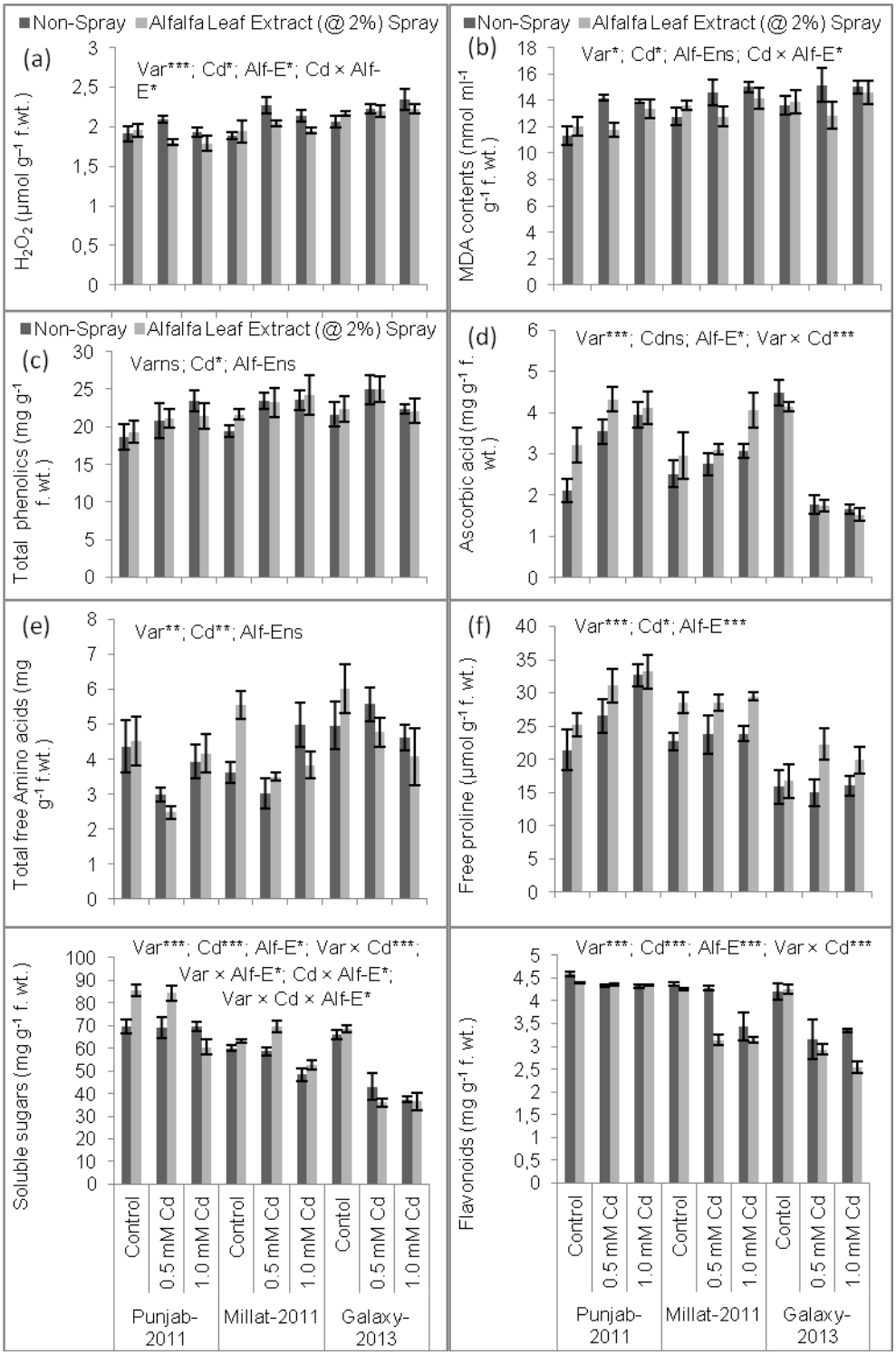

Figure 3. *Hydrogen peroxide, malondialdehyde, total phenolic, ascorbic acid, amino acids, free proline, soluble sugars and flavonoids of 16-week-old wheat plants foliarly sprayed with 2% alfalfa leaf extract under cadmium stress and non-stress conditions.*

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Discussion

Plants must have tolerance to heavy metal pollutants, ability to metabolize and immobilize metals and large biomass to remediate widespread chemicals in field. Plants tolerate to heavy metals through metal detoxification mechanism such as chelation of heavy metals with organic acids and high affinity ligands like metallothionein, phytochelatins, amino acids (malate, citrate, histidine, oxalate, nicotinamine) and phosphate derivatives in the cytosol and transforming them to nontoxic forms (Hall, 2002).

Mixture of biocommunicators/allelochemicals are more active than a single compound (Macias et al., 1998). Allelochemicals can be used to enhance abiotic stress tolerance in crop plants as concentration of allelochemicals is inversely related to the growth promoting effect of crop plants (Ul Subtain et al., 2014). These allelochemicals include phenolics, tannins, flavonoids, terpenoids cinnamic acid, quinines, long chain fatty acids, polyecetylenes, straight-chain alcohols, unsaturated lactones and various types of water-soluble organic acids (Li et al., 2010). Triacontanol (TRIA) is a plant growth regulator that has been isolated from chloroform-soluble extraction of alfalfa. TRIA is a constituent of plant waxes that has been shown to increase crop growth and yield both under normal (Ries et al., 1977) and abiotic stresses such as salinity (Shahbaz et al., 2013; Zulfiqar and Shahbaz, 2013; Aziz et al., 2015), acidic mist and cadmium stresses (Muthuchelian et al., 2001, 2003).

Rapid growth of crop species can reduce cost of field activities and enhance market value of field products. Among cheaper strategies mulching with alfalfa hay, watering plants with crude extract and foliar application of aqueous extract is very effective to mitigate environmental stresses (Kibatu, 2012). However, optimum method, time and rate of application to crops is necessary for positive results (Ries et al., 1977). It has been reported that alfalfa possesses both stimulatory and inhibitory effects. For example, aqueous extract (1 %) of alfalfa residues increase germination percentage, lengths of plumule and radical and nutrients uptake in maize (*Zea mays* L.), while at higher concentration (8 %) decreased seedling biomass (Ahmad et al., 2014).

Soil cadmium at 0-24 mg kg^{-1} concentration has been found to reduce dry weight of shoot and root, chlorophyll contents and photosynthesis in mustard and pakchoi (Chen et al., 2011). Cadmium at 0, 5, 20, 50 and 80 mg L^{-1} levels decreased seedling growth by decreasing shoot and root lengths and germination percentage of four wheat cultivars (Ahmad et al., 2012). Cadmium significantly decreased growth, relative water content (RWC) and chlorophyll contents, while increased hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and relative membrane permeability in maize seedling (Hussain et al., 2012). In this study, cadmium reduced growth and yield of all wheat varieties, however, alfalfa leaf extract at the rate of 2% (v/v) significantly increased growth of all three wheat varieties.

Foliar treatment with alfalfa extract did not alter RWC (%), chlorophyll contents, total phenolics and total free amino acids, while increased ascorbic acid contents in Punjab-2011 and Millat-2011, total soluble sugars in Millat-2011, free proline contents in all varieties, while decreased total soluble sugars in Galaxy-2013, H_2O_2, MDA and flavonoid contents in all wheat varieties under stressed or non-stressed conditions. Characterization of alfalfa crude extract has been found to contain phytotoxic compounds such as phenolics (Hall and Henderlong, 1989). Shikur (2015) reported that alfalfa crude aqueous extract spray could not alter growth and yield parameters of lettuce and pepper plant. However root length and yield of beetroot significantly

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changed alfalfa. Furthermore, due to reported phytotoxic effects one time application during early growth stage could be used for weed control and management practices. However, pot experiment is recommended with the objectives to explore time of application and methods for extraction. For, example, in addition to water extracts extraction in some chemicals for better research output for field experiments. Way of extract preparation and application time interval plays both stimulatory and inhibitory effects on different crop species. For, example cold and hot water extract of dried alfalfa leaf (15g) and their dilution (50%) has been reported different results on yield and yield components of lettuce (*Lactuca sativa*), beetroot (*Beta vulgaris*) and pepper (*Capsicum annum*) with no changes in pepper and lettuce, while significant effect on root length and yield in beetroot.

As it is evident from the current study that foliar application of 2% alfalfa leaf extract increased leaf ascorbic acid, free proline and total soluble sugars, while decreased RMP (%) (in Punjab-2011 and Galaxy-2013), flavonoids and H_2O_2 contents in all wheat varieties under cadmium stress. It is concluded from the present study that foliar application of 2% alfalfa leaf extract increased growth and yield of wheat plants that could be attributed to TRIA-induced metabolic changes in the major metabolic pathways in all wheat varieties.

REFERENCES

[1] Abd Allah, E.F., Hashem, H., Alqarawi, A.A., Alwathnani, H.A. (2015): Alleviation of adverse impact of cadmium stress in sunflower (*Helianthus annuus* L.) by arbuscular mycorrhizal fungi. − Pakistan Journal of Botany 47(2): 785-795.

[2] Abdo, F.A., Nassar, D.M.A., Gomaa, E.F., Nassar, R.M.A. (2012): Minimizing the harmful effects of cadmium on vegetative growth, leaf anatomy, yield and physiological characteristics of soybean plant [*Glycine max* (L.) Merrill] by foliar spray with active yeast extract or with garlic cloves extract.− Research Journal of Agriculture and Biological Sciences 8(1): 24-35.

[3] Ahmad, E., Zaidi, A., Khan, M.S., Oves, M. (2012): Heavy metal toxicity to symbiotic nitrogen-fixing microorganism and host legumes.− In: Zaidi, A., Wani, P.A., Khan, M.S. (eds) Toxicity of heavy metals to legumes and bioremediation. Springer, Wien, pp 29-44.

[4] Ahmed, H.A., Salama, M., El-Darier, Marwa, H., El-Dien, Z. (2014): Role of soil moisture in allelopathic activity of Medicago sativa L. residues in Zea mays L. fields.− Journal of Global Biosciences 3(5): 835-847.

[5] Amirjani, M.R. (2012): Effects of cadmium on wheat growth and some physiological factors. − International Journal of Forest, Soil and Erosion 2: 50-58.

[6] Arnon, D.I. (1949): Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*. − Plant physiology 24: 1-15.

[7] Asgharipour, M.R., Armin, M. (2010): Inhibitory effects of Sorghum halepens root and leaf extracts on germination and early seedling growth of widely used medicinal plants. − Advances in Environmental Biology 4: 316-324.

[8] Aziz, R., Shahbaz, M., Ashraf, M. (2015): Influence of foliar application of triacontanol on growth attributes, gas exchange and chlorophyll fluorescence in sunflower (*Helianthus annuus* L.) under saline stress. − Pakistan Journal of Botany 45: 1913-1918.

[9] Bates, L., Waldren, R.P., Teare, I.D. (1973): Rapid determination of free proline for water-stress studies. − Plant and Soil science 39: 205-207.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_121136

[10] Benavides, M.P., Susana, M., Tomaro, M. (2005): Cadmium toxicity in plants. − Brazilian Journal of Plant Physiology 17(1): 21-34.
[11] Carmak, I., Horst, J.H. (1991): Effects of aluminum on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). − Physiologia Plantarum 83: 463-468.
[12] Chance, B., Maehly, A. (1955). Assay of catalase and peroxidase. − Methods in Enzymology 2: 764-817.
[13] Chen, X., Wang, J., Shi, Y., Zhao, M.Q., Chi, G.Y. (2011): Effects of cadmium on growth and photosynthetic activities in pakchoi and mustard. − Botanical Studies 52: 41-46.
[14] Chon, S., Coutts, J.H., Nelson, C.J. (2000): Effect of light, growth media and seedling orientation on bioassays of alfalfa autotoxicity. − Agronomy Journal 92: 715-720.
[15] Darvishi, S.H, Kamajian, M. (2014): 'Effects Cd accumulation abilities of annual alfalfa (*Medicago scutellata* L.) by humic acid and growth promoting bacteria association'. − Iranian Journal of Plant Physiology 5 (1): 1243- 1247.
[16] Dhir, B., Sharmila, P., Saradhi, P. (2004): Hydrophytes lack potential to exhibit cadmium stress induced enhancement in lipid peroxidase and proline. − Aquatic Toxicology 66: 141-147.
[17] Elobeid, M., Göbel, C., Feussner, I., Polle, A. (2012): Cadmium interferes with auxin physiology and lignification in poplar. − Journal of Experimental Botany 63: 1413–1421.
[18] Ertani, A., Schiavon, M., Muscolo, A., Nardi, S. (2013): Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. − Plant and Soil 364: 145-158.
[19] Ferreira, M.P., Gendron, F., McClure, K.C., Kindscher, K. (2012): North American bioactive plants for human health & performance. − Global Journal of Research on Medicinal Plants & Indigenous Medicine 1(11): 568-582.
[20] Hall, J.L. (2002): Cellular mechanisms for heavy metal detoxification and tolerance. − Journal of Experimental Botany 53: 1-11.
[21] Hall, M.H., Henderlong, P.R. (1989): Alfalfa autotoxic fraction characterization and initial separation. − Crop Science 29: 425–428.
[22] Hussain, I., Iqbal, M., Qurat-Ul-Ain, S., Rasheed, R., Mahmood, S., Perveen, A., & Wahid, A. (2012): Cadmium dose and exposure-time dependent alterations in growth and physiology of maize (*Zea mays*). − International Journal of Agriculture and Biology 14: 959-964.
[23] Iqbal, M., Ahmad A., Ansari, M.K.A., Qureshi, M.I., Aref, I.M., Khan, P.R., Hegazy, S.S., El-Atta, H., Husen, A., Hakeem, K.R. (2015): − Environmental Reviews 23(1): 44-65.
[24] Jones, M.M., Turner. N.C. (1978): Osmotic adjustment in leaves of sorghum in response to water deficits. − Plant Physiology 61:122–126.
[25] Julkunen-Titto, R. (1985): Phenolic constituents in the leaves of northern willows methods for the analysis of certain phenolics. − Journal of Agriculture and Food Chemistry 33: 213–217.
[26] Khan, Z.I., Ahmad, K., muhammad Ashraf, M., sumaira Yasmeen, S., Ashfaq, A., Sher, M. (2016): Metal accumulation in a potential winter vegetable mustard (*Brassica campestris* L.) irrigated with different types of waters in Punjab, Pakistan. − Pakistan Journal of Botany 48(2): 535-541.
[27] Kibatu, T. (2012): Alfalfa as growth regulator: the effect of alfalfa (*Medicago sativa* L.) crude extract on the growth of troyer citrange seedlings. Lab publication, Germen.
[28] Li, Z.H., Wang, Q., Ruan, X., Pan, C.D., Jiang, D.A. (2010): Phenolics and plant allelopathy. − Molecules 15: 8933-8952.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_121136

[29] Macias, F.A., Oliva, R.M., Simonet, A.M., Galindo, J.C.G. (1998): What are allelochemicals? In Allelopathy in rice. Proceedings of the Workshop on Allelopathy in Rice, 25-27 Nov 1996. (ed. M. Olofsdotter), pp. 69-79. IRRI Press, *Manilla majus* L. fruit waxes. − Chemoecology 12: 107–111.
[30] Mauriès, M. (2003): Luzerne: culture, récolte, conservation, utilization.− France Agricole Editions.
[31] Miller, D.A. (1983): Allelopathic effects of alfalfa. − Journal of Chemical Ecology 9: 1059-1072.
[32] Miller, D.A. (1996): Allelopathy in forage crop systems. − Agronomy Journal 88: 854-859.
[33] Miller, R.W. Al-Khazraji, M.L., Sisson, D.R., Gardiner, D.T. (1995): Alfalfa growth and absorption of cadmium and zinc from soils amended with sewage sludge. − Agriculture Ecosystems and Environment 53: 179-184.
[34] Moore, S., Stein, W.H. (1957): A modified ninhydrine method for use in the chromatography of amino acids. − Journal of Biology and Chemistry 176: 367-388.
[35] Mousavi, S.H., Alami-Saeid, KH., Moshatati, A. (2013). Effect of leaf, stem and root extract of alfalfa (*Melilotus indicus*) on seed germination and seedling growth of wheat (*Triticum aestivum*). − International Journal of Agriculture and Crop Sciences 5(1): 44-49.
[36] Mukherjee, S.P., Choudhuri, M.A. (1983): Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. − Physiologia Plantarum 58: 166–170.
[37] Muthuchelian, K., Velayutham, M., Nedunchezhian, N. (2003): Ameliorating effect of triacontanol on acidic mistreated *Erythrina variegata* seedlings changes in growth and photosynthetic activities. − Plant Science 165: 1253-1257.
[38] Muthuchelian, K., Bertamini, M., Nedunchezhian, N. (2001): Triacontanol can protect *Erythrina variegata* from cadmium toxicity. − Plant Physiology 158: 1487-1490.
[39] Nardi, S., Pizzeghello, D., Schiavon, M., Ertani, A. (2016): Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. − Scientia Agricola 73(1): 18-23.
[40] Oves, M., Saghir Khan, M., Huda Qari, A., Nadeen Felemban, M., Almeelbi, T. (2016): Heavy Metals: Biological Importance and Detoxification Strategies. − Journal of Bioremediation and Biodegradation 7:334. doi: 10.4172/2155-6199.1000334
[41] Page, V., Feller, U. (2015). Heavy metals in crop plants: transport and redistribution processes on the whole plant level. − Agronomy 5(3): 447-463.
[42] Parmer, P., Kumari, N., Sharma, V. (2013): Structural and functional alterations in photosynthetic apparatus of plants under cadmium stress". − Botanical Studies 54: 45-50.
[43] Perveen, S., Farooq, R., Shahbaz, M. (2016): Thiourea-induced metabolic changes in two mung bean [*Vigna radiata* (L.) Wilczek] (Fabaceae) varieties under salt stress. − Brazilian Journal of Botany 39(1): 41-54.
[44] Popovic S., Grlju sic S., Cu pic T., Tu cak M., Stjepan ovic M. (2001): Protein and fiber contents in alfalfa leaves and stems. − In : Delgado, I., Lloveras J. (eds.) Quality in lucerne and medics for animal production. −Zaragoza-ciheam, pages 215-218.
[45] Rady, M.M., Hemida, K.A. (2015): Modulation ofcadmium toxicity and enhancing cadmium-tolerance in wheat seedlings by exogenous application of polyamines. − Ecotoxicology and Environmental Safety 119: 178–185.
[46] Randhawa, M.A., Cheema, Z.A., Ali, M.D.A. (2002): Allelopathic effect of sorghum water extract on germination and seedling growth of *Trianthema portulacastrum*. − International Journal of Agriculture and Biology 4: 383-384.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_121136

[47] Rascio, N., Navari-Izzo, F. (2011): Heavy metal hyper accumulating plants: How and why do they do it? And what makes them so interesting? − Plant Science 180: 169-181.
[48] Rice, E.L. (1985): Allelopathy- an overview. pp. 81-105.− In: Cooper-Driver, G. A., Swain, T., Conn, E. E. (eds.) Chemically mediated interactions between plants and other organisms., Springer US, doi: 10.1007/978-1-4757-9658-2_4
[49] Ries, S. (1977): Triacontanol and its second messenger adenosine as plant growth substances. − Plant Physiology 95: 986-989.
[50] Salt, D.E., Blaylock, L.M., Kumar, N.P.B.A., Dushenkov, V., Ensley, D., Chet, I., Raskin, I. (1995): Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. − Biotechnology 13: 468-474.
[51] Shahbaz, M., Noreen, N., Perveen, S. (2013): Triacontanol modulates photosynthesis and osmoprotectants in canola (*Brassica napus* L.) under saline stress. − Journal of Plant Interactions 8(4): 250-259.
[52] Shikur, T.K. (2015): Effect of alfalfa (*Medicago sativa*) extract on yield and yield components of lettuce (*Lactuca sativa*), beetroot (*Beta vulgaris*) and pepper (*Capsicum annum*). − World Journal of Agricultural Sciences 11 (2): 89-93.
[53] Singh, O.V., Labana, S., Pandey, G., Budhiraja, R., Jain, R.K. (2003): Phytoremediation: an overview of metallic ion decontamination from soil. − Applied Microbiology & Biotechnology 61: 405-412.
[54] Snedecor, G.W., Cochran, W.G. (1980): Statistical Methods. Seventh Edition.− Ames Iowa: The Iowa State University Press.
[55] Sun, S., Li, M., Zuo, J., Jiang, W., Liu, D. (2015): Cadmium effects on mineral accumulation, antioxidant defense system and gas exchange in cucumber. − Zemdirbyste-Agriculture 102(2): 193–200.
[56] Sunitha, M.S., Prashant, S., Kumar, S.A., Rao, S., Narasu, M.L., Kavi kishor, P.B. (2012): Cellular and molecular mechanisms of heavy metal tolerance in plants: a brief overview of transgenic plants over-expressing phytochelatin synthase and metallothionein genes. − Plant Cell Biotechnology and Molecular Biology (3&4): 99–104.
[57] Ul Subtain, M., Hussain, M., Tabassam, M.A.R., Ali, M.A., Ali, M., Mohsin, M., Mubushar, M. (2014): Role of allelopathy nn the growth promotion of plants. − Scientia Agriculture 6 (3): 141-145.
[58] Velikova, V., Yordanov, I., Edreva, A., (2000): Oxidative stress and some antioxidant system in acid rain treated bean plants: Protective role of exogenous polyamines. − Plant Science 151: 59-66.
[59] Zhi-xin, N., Li-na, S., Tie-heng, S., Yu-shuang, L., Hong, W. (2007): Evaluation of phytoextracting cadmium and lead by sunflower, ricinus, alfalfa and mustard in hydroponic culture. − Journal of Environmental Sciences 19: 961–967.
[60] Zulfiqar, S., Shahbaz, M. (2013). Modulation of gas exchange parameters and photosystem-II activity of canola (*Brassica napus* L.) by foliar-applied triacontanol under salt stress. − Agrochimica 57(3): 193-200.

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ALLEVIATION OF SALT STRESS BY K_2SO_4 IN TWO WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS

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Abstract. Salinity is a major abiotic stress which adversely affects productivity of all crops in the world specifically in cereals. Different strategies are being utilized to enhance the overall plant growth and productivity all over the world. The crop nutrients management is one the best options to increase the plant productivity in saline soils. The present study investigated the influence of potassium sulphate (K_2SO_4) in improving plant productivity and nutrient uptake in wheat grown under saline environment. Two wheat genotypes were subjected to different concentrations i.e., 0, 50, 100, 150 and 200 mM of K_2SO_4 grown at 0, 150 mM sodium chloride stress. The use of K_2SO_4 increased the fresh and dry plant biomass of both wheat genotypes with a maximum increase at 200 mM K_2SO_4 under saline and nonsaline conditions. It was observed that the uptake and accumulation of nutrients like calcium, magnesium, potassium and phosphorus increases in plants subjected with K fertilizer application under saline environments in both wheat genotypes.
Keywords: *potash fertilizer, biomass production, salinity, nutrients uptake*

Introduction

Wheat is the most important among the cereal grain crops in nutrition supply (Kausar and Gull, 2014a) of ever increasing world population. Its role has been increased exclusively in developing countries of the world where it is used as a staple food. The rain fall 30-90cm, 20-30C˚ temperature and clay loam soils are required to have optimum wheat grain yield (Kausar and Gull, 2014a). Adequate water supply and suitable potassium fertilization are mandatory for optimal shoot and proliferation of root for better uptake of nutrients (Ashraf et al., 2013; 2015).

Soil salinity is the major abiotic stress observed all over the world which causes severe crop productivity losses by affecting nutrient uptake to maintain proper metabolic activities (Ashraf et al., 2012; Kosova et al., 2013; Zafar et al., 2015). Due to

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soil salinity billions of dollars losses in crop productivity have been reported every year (Alam and Naqvi, 2003; Ashraf et al., 2003, 2009, 2011, 2015). Uptake of toxic ions by plants growing on saline soils disturb their metabolic activities and concentration of toxic ions like Na^+ and Cl^- increases inside the tissues consequently water potential decreases that reduces growth and productivity of plants (Akhtar et al., 2013; Kausar and Gull, 2014b). The increased concentrations of toxic ions decreases the absorption of essential nutrients like calcium, megnessium, phosphorus, potassium andiron (Marjan et al., 2012; Han et al., 2014) and as a result plants suffer from nutritional imbalance (Ashraf et al., 2013). The development of plants, mainly depends upon the rate of photosynthesis which is adversely affected by salt stress, especially sensitive genotypes of all crops (Kausar et al., 2012; 2015). Other investigators have also reported that salinity stress adversely affects the growth, physiological and biochemical attributes of plants (Ebrahimi et al., 2012; Ashraf et al., 2013; Hasanuzzaman et al., 2013; Zafar et al., 2015). The decrease in plant growth and yield depend on intensity of salt stress (Kausar and Gull, 2014b).The deficiencies in essential nutrient uptake and reduction in K^+/Na^+ is observed in plants growning on saline soils, however, nutrient management may be effective in achieving economical crop productivity from these soils (Ashraf et al., 2014). The potash fertilizer is best option among others particularly under saline conditions (Ashraf et al., 2013). Potassium is an essential element for plant growth because it plays a key role in regulation of plant metabolic activity as well as physiological and biochemical requirements of plants (Kausar and Gull, 2014a). Its role as macronutrient is very important in many enzymatic reactions and high concentrations are required for the best growth of plants (Ashraf et al., 2013). Activation of many enzymes, opening and closing of stomata, photosynthesis and tropism movements are controlled by potassium (Golldack et al., 2003; Kausar and Gull, 2014a). It also controls in certain cases the osmotic adjustment in plants under various stressed conditions (Kausar et al., 2012). It is well known fact that potassium deficiency closes the stomata openings which in turn reduces the rate of photosynthesis in many crops, so its adequate amount is necessary for better growth and more wheat production (Mesbah, 2009; Ashaf et al., 2015).

Keeping in view the significance of potassium in saline environments, the current study was planned to test hypotheses; if potassium alleviates the negative effects of salt stress in wheat cultivars as well as if application of K_2SO_4 enhances the wheat growth and nutrient uptake in wheat under saline conditions.

Materials and Methods

The study was completed in GC University Faisalabad and GC Women University Faisalabad Pakistan in collaboration with the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan in 2013-2014. The seeds of two cultivars of wheat i.e., MILLAT-11 and NAYAB-11 were obtained from Wheat Section, Ayub Agriculture Research Institute Faisalabad, Pakistan. Pots of 35 cm in diameter were filled with washed river sand (10 kg) and arranged in a completely randomized design (CRD) with four different treatments of K_2SO_4 having four replications. The seeds of two cultivars of wheat were subjected to 150mM NaCl stress in half strength of Hoagland nutrient solution except control. After two weeks of plant sowings, potassium sulphate at 0, 50,100,150 and 200 mM was applied (*Table 1)*. The watering was done as

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and when required. Physico-chemical characteristics of water used in this study are summarized in *Table 2*.

Table 1. *Levels of K_2SO_4 (Potassium sulphate) and NaCl (150mM) stress*

Treatments	NaCl Concentrations	K_2SO_4 Concentrations (mM)
T_0 (control)	0 mM	0
T_1	150 mM	0
T_2	150 mM	50
T_3	150 mM	100
T_4	150 mM	150
T_5	150 mM	200

Table 2. *Physiochemical characteristics of water used in the experiments*

Water characteristics	Readings	Water characteristics	Readings
PH	7.66	CO_3 (meq L^{-1})	-
EC(µS/cm)	681	Na^+ (mg Kg^{-1})	60
Mg (meq L^{-1})	5	Cl^- (meq L^{-1})	5-6
Ca(meqL^{-1})	4	K^+ (mg Kg^{-1})	3-4.0
HCO_3 (meq L^{-1})	4-6		

After 120 days, five plants were collected and their height, fresh and dry biomass were recorded and their means were calculated. Then the plants were oven dried at 65ºC and dry biomass was calculated. The dried ground plant material was used for nutrient analysis of potassium (K), sodium (Na), calcium (Ca), nitrogen (N) and phosphorus (P) of both cultivars.

The dried plant material was ground and digested according to the method of Wolf, (1982). Filtered aliquate was used to determine Na, K, Ca, K, N and P. Sodium (Na) and K were determined using Flame-photometer (Jenway PFP 7, UK); while Ca and Mg were determined titrametrically (Jackson et al., 1962). Chloride contents were analysed by chloride meter (920, Corning, UK), total phosphorus (P) was determined spectrophotometrically (Jackson, 1962). In the case of phosphorous Barton's reagent was used sepectrophotometerically (U2800, Hitachi, Japan) as described by Jackson (1962). Total nitrogen was measured by micro–Kjeldhal method (Bremner, 1965). The fresh material was taken from third plant leaf and total soluble proteins were determined by Lowery et al. (1951) method. Similarly total free aminoacids were found by the method of Hamilton and Van slyke (1943).

Data was collected and analysis of variance technique was used to determine the significant variations in treatments, varieties and their interactions. Means were compared using DMRT test at 5% probability level (Steel et al., 1997).

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Results

Two cultivars (MILLAT-11 and NAYAB-11) of wheat were used and it has been observed that salt stress has significantly affected growth of both the cultivars (*Fig. 1a*). Maximum plant height in both the cultivars was observed under normal conditions, however, salinity stress significantly reduced this parameter. Application of K_2SO_4 improved plant height of both cultivars of wheat under salt stress and the maximum improvement was noted at 200 mM K_2SO_4 level. Minimum plant height was recorded under NaCl salinity stress (*Fig. 1a*). The cultivar NAYAB-11 exhibited maximum plant height at 200 mM K_2SO_4 under salt stress followed by 150, 100 and 50 mM K_2SO_4. The fertilizer application of 200mM of K_2SO_4 proved better than other applications of potash fertilizer under salt stress conditions. Interaction between cultivar and treatment was also significant. Both wheat cultivars exhibited a decrease in plant height in salt stressed soils as compared to normal environments (*Fig. 1a*).

Wheat cultivar NAYAB-11 had higher fresh biomass than cultivar MILLAT-11 both under non saline and saline conditions. Salt stress negatively affected the fresh biomass production in both the cultivars of wheat, however, it varied subject to application of different levels of potassium (*Fig. 1b*). The maximum plant fresh biomass was observed under 200 mM K_2SO_4 treatment as compared to its other treatments under salt stress conditions. Cultivars and treatments interactions showed highly significant results for both wheat genotypes (*Table 3*).

Table 3. *Mean square values from analysis of variance (ANOVA) of data for plant height, fresh and dry weights and calcium contents of plants at different levels of K_2SO_4 of two cultivars of wheat under 0 and 150 mM of NaCl stress.*

Source of variation	df	Plant length	Plant fresh weight	Plant dry weight	Plant Ca^{2+} contents
Treament	5	201.27616***	1.0599983*	0.3070601 ns	37.035592***
Cultivars	1	301.659***	2.1364694*	0.2669444 ns	51.612251**
Treamentx Cultivars	5	280.86374***	3.3955494***	0.7095045 ns	96.639862***
Error	24	9.3421417	0.3406444	0.2909989	5.1284646
Total	35				

*** , **, * , ns = Significant at 0.05, 01, .001 %, non-significant respectively

Results indicated that maximum dry weight was attained by plants growing under normal conditions in both cultivars of wheat. Salt stress adversely affected the plant dry weights in both genotypes (*Fig. 1c*), however, MILLAT-11showed more reduction in dry biomass than NAYAB-11. The addition of K_2SO_4 increased plant dry weight under sodium chloride stress (*Fig. 1c*), with maximum increase at 200 mM K_2SO_4. Sodium chloride stress increased sodium and chloride contents in all cultivars of wheat. Plants grown in saline environments showed a noticeable increase in Na^+ uptake however, wheat cultivar MILLAT-11 had higher Na^+ than NIAB-11(*Fig.1d*). Minimum concentrations of Na^+ were present in plants grown under controlled conditions in both cultivars. The plants of both the wheat cultivars under salt stress, also exhibited higher

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Cl^- concentrations than those growing under normal conditions (*Fig. 2 c).* Application of K_2SO_4 reduced sodium contens in NAYAB-11 more adversely than MILLAT-11 (*Fig. 1d*). Interactions between treatments and cultivars presented highly significant performances in wheat cultivars (*Table 3*). Accumulation of Na^+ (*Fig. 1d*) and Cl^- (*Fig. 2c*) decreased with increasing K_2SO_4 levels in salt stress environments.

Salinity adversely affected the N contents in both wheat cultivars. Maximum N content was noted in plants grown in controlled environment, however, cultivar NAYAB-11 had higher N than culivar MILLAT-11 (Fig 1e). Application of K_2SO_4 significantly enhanced the uptake of N. Maximum N uptake was recoded at 200mM and the minimum at 50 mM K_2SO_4 in salt stressed plants of both cultivars of wheat (*Fig. 1e*). Maximum reduction in N (52%) was recorded in the plants growing at 150 mM sodium chloride level, while minimum reduction about 24% was in the plants treated with 200 mM K_2SO_4 in both the cultivars of wheat under NaCl stress (*Fig 1e*).

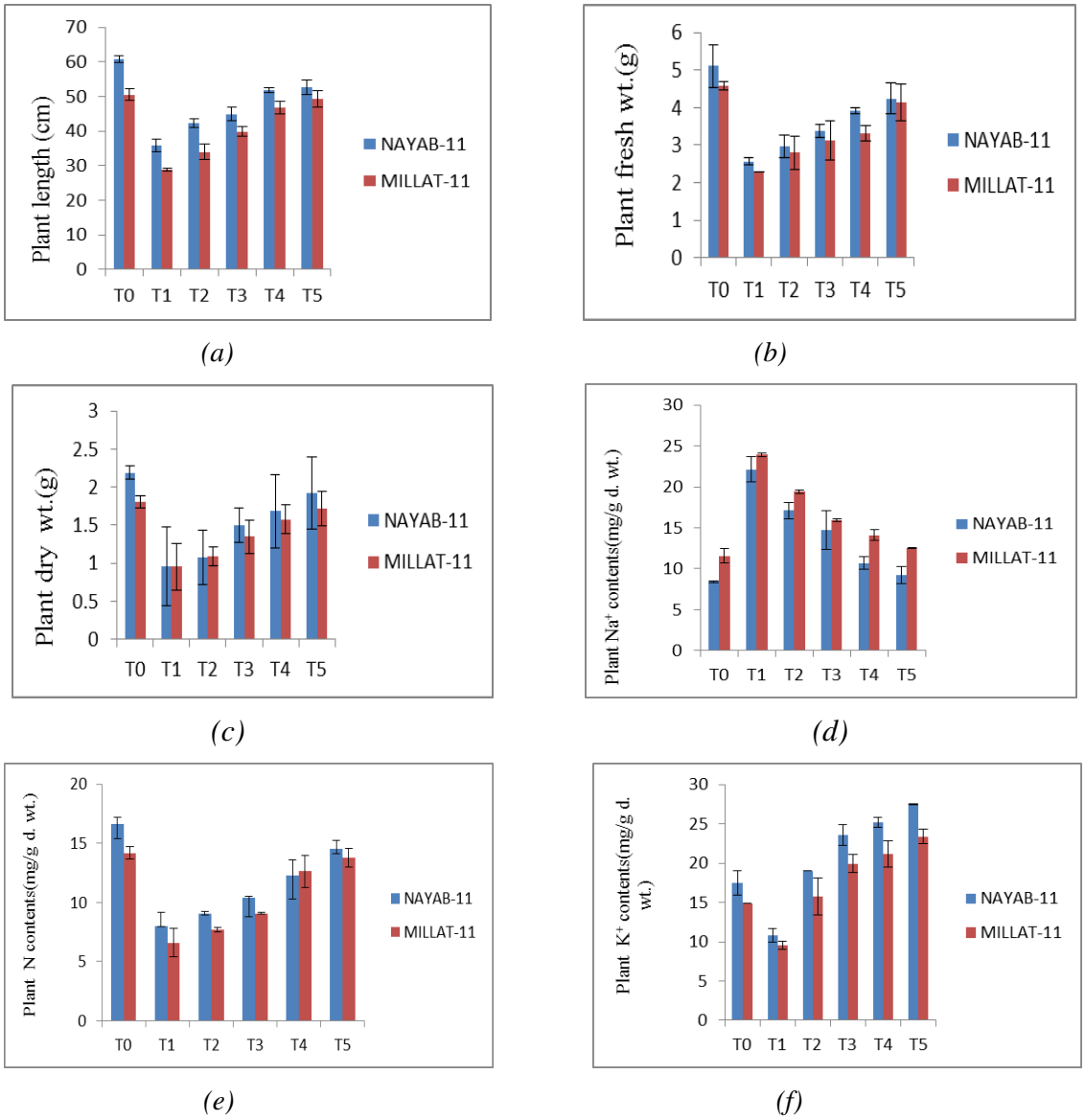

Figure 1. *Effect of different levels of K_2SO_4 on plant length (a), fresh weight(b), dry weight (c) Na^+ (d), N (e) and K^+ (f) contents in plants of two wheat cultivars at 0 and 150 mM of NaCl stress*

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Analysis of variance of the data indicated that K^+ contents significantly decreased in both wheat cultivars growing under saline environments. Maximum decrease in K^+ was noted in plants of cultivar MILLAT 11 and the least in NAYAB 11 growing under normal conditions. Potassium uptake increased with the application of K_2SO_4 fertilizer on wheat plants under salt stress, but the minimum increase was exhibited by wheat cultivar MILLAT 11 (*Fig. 1f*). Similarly, maximum reduction in K (37.5%) was observed in plants growing at 150 mM NaCl, however, an increase in K uptake was noted at 200 mM K_2SO_4 under NaCl salinity stress (*Fig. 1f*). Under salinity stress, plants of both wheat cultivars maintained lower shoot K^+ contents. Wheat cultivars responded positively to K_2SO_4 fertilizer. Adverse effects of saline medium were also detected on P uptake (*Fig.2a*). On addition of K_2SO_4 plants exhibited an increase in the uptake of P under NaCl salinity stress in both NAYAB 11 and MILLAT 11. Wheat Cultivar MILLAT-11 showed the least uptake of P as compared to NAYAB-11. Maximum reduction (54.5%) in P uptake was observed at 150 mM of salt stress, however, the least reduction (18.2%) was observed at 200 mM K_2SO_4 under saline conditions (*Fig. 2a*).

Table 4. *Mean square values from analysis of variance (ANOVA) of data for phosphorus (P), potassium (K^+), sodium (Na^+) and nitrogen (N) contents at different levels of K_2SO_4 in wheat cultivars under NaCl stress*

Source of variation	df	Plant P contents	Plant K^+ contents	Plant Na^+ contents	Plant N contents
Treament	5	0.7363912 ns	169.75993***	169.75993***	24.917318***
Cultivars	1	1.9904507*	0.0879219 ns	0.0879219 ns	25.662667**
Treament x Cultivar	5	2.602809***	36.436648***	36.436648***	37.879022***
Error	24	0.3628611	4.0945417	4.0945417	3.1693667
Total	35				

*** , **, * , ns = Significant at 0.05, 01, .001 %, non-significant respectively

Application of K_2SO_4 has improved the Ca^{2+} and Mg^{2+} contents under saline medium, however, maximum was in the plants growing in normal environment in both cultivars (*Fig 2b*). The contents increased with increasing level of K_2SO_4 fertilizer. The maximum Ca^{2+} content was observed at 200 mM K_2SO_4 in NIAB-11followed by MILLAT-11 under NaCl stress as compared to other treatments of K_2SO_4 (2b). Interactive effect of cultivars and treatment showed non significant behavior for Ca^{2+} content (*Table 5*). An increase in nutient uptake like K^+, Ca^{2+}, Mg^{2+}, N and P was observed by addition of K_2SO_4 fertilizer in plants of both the cultivars grown under saline conditions. The increase was further prominent with an increase in K_2SO_4 levels. The increase was further prominent with an increase in fertilizer application. The application of salinity decreased significantly the total proteins and increased the total free aminoacids in both wheat cultivars. However with the utilization of fertilizer there was less decrease in total proteins (*Fig. 2 e)* and total free aminoacids and with the increasing concentrations of potash fertilizer (*Fig. 2 f*).

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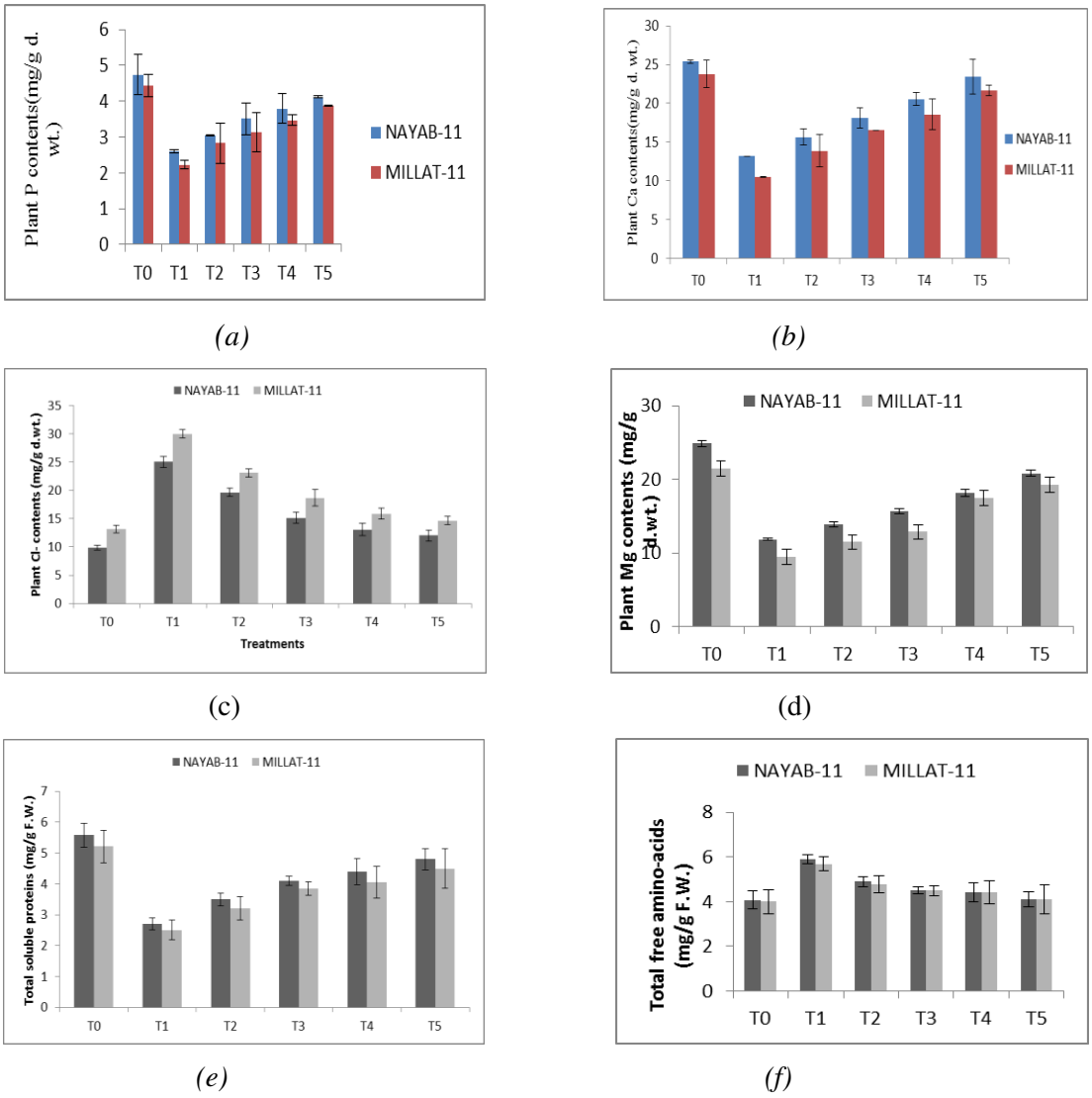

Figure 2. *Effect of different levels of K_2SO_4 on phosphorus (a) calcium (b), chloride (c)and magnesium (d), total soluble proteins and (total free aminoacids) of plants of two wheat cultivars at 150 mM of NaCl stress*

Table 5. *Mean square values from analysis of variance (ANOVA) of data for chloride (Cl^-) and magnesium(Mg^{2+}) contents at different levels of K_2SO_4 on two wheat cultivars under 150 mM of NaCl stress*

Source of variation	df	Plant Cl^- contents	Plant Mg^{2+} contents	Total soluble proteins (mg/g F.W)	Total free aminoacids (mg/g F.W)
Treament	5	109.55973***	27.031512***	4.16732***	4.76432**
Cultivars	1	0.0679228 ns	41.212351**	5.43261***	5.32451**
Treament x Cultivar	5	30.336628***	86.539162***	2.9245**	1.45621*
Error	24	3.0744515	3.1274646	1.09821	0.12431
Total	35				

*** , **, * , ns = Significant at 0.05, 01, .001 %, non significant respectively

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Discussion

Environmental stresses are the main problem to plant growth and development and salinity is one of the major stresses causing severe losses in crop productivity all over the world (Hakeem et al., 2013; Zafar et al., 2015). However, there are other environmental factors like; drought, water logging, heavy metal toxicity and low or high temperatures, which are also involved in decreasing the growth and productivity of crops (Bray et al., 2000; Hakeem et al., 2013). In the present study fresh and dry biomass decreased by salinity stress (*Fig. 1b and c*) as indicated by many investigators (Ashraf et al., 2011; Ali et al., 2012; Kausar et al., 2012; Kausar and Gull, 2014b). The reason for this could be due to the lowering of stomatal conductance, fixation of carbon dioxide, and disturbance in biochemical reactions/activities (Ashraf et al., 2011; Ashraf et al., 2013, 2015). The application of K_2SO_4 alleviated the adverse effect of salinity stress on the plant height and was effective in enhancing the growth of wheat in stress medium (*Fig. 1a*). Similar results have been reported by Tzortzakis (2010) and Ashraf et al. (2013), who showed that application of potash fertilizer decreases the toxic effects of salinity on growth, as well as plant biochemical and physiological processes.

It is a well known fact that use of K_2SO_4 in saline soils is effective in having high fresh biomass production in many crops (Hussain et al., 2013; Saffa et al., 2013; Ashraf et al., 2015), because of the activation of some enzymes necessary for plant growth. Addition of K_2SO_4 in growth medium is an excellent way for working of enzymes by mainting pH required for proper growth of plant cell (Hussain et al., 2013, Saffa et al., 2013; Ashraf et al., 2015). A reduction in the biomass and uptake of essential nutrients under salt stress may be due to the presence of excessive Na^+ and Cl^- concentrations in the growth medium (Hussain et al., 2013; Saffa et al., 2013; Ashraf et al., 2015). Similarly a reduction in dry weight of the plant due to salt stress can be alleviated by an application of potash fertilizer in order to mitigate the toxic effects of salts (Kausar et al., 2012; Kausar et al., 2014; Ashraf et al., 2013). These findings are in agreement with those of other workers (Tzortzakis, 2010; Kausar et al., 2012, Ashraf et al., 2013). In saline conditions the uptake, absorption and accumulation of toxic ions (Na^+ or Cl^-) are enhanced which reduce the uptake and translocation of essential nutrients in plants resulting in a decrease in leaf water potential, rate of photosynthesis, growth and overall plant productivity (Ali et al., 2012; Hussain et al., 2013, Saffa et al., 2013).

Soils affected by salinity are in need of more nutrient uptake predominantly P, Mg^{2+} and K^+, which play key role in physiological and biochemical processes (Kausar and Gull, 2014a). Development of plant depends particularly on the accessibility of appropriate amounts of potassium fertilizer (Kausar and Gull, 2014b), which improves plant growth and productivity (Hussain et al., 2013, Saffa et al., 2013) especially in the plants facing salt stress. These findings too are in accordance with those of Ashraf et al. (2003, 2015), and Kausar et al. (2014). They showed that N and K^+ contents decrease in stressed environment and with the application of nutrients like potassium, the uptake of essential nutrients i.e., Ca^{2+} , K^+ and Mg^{2+} can increase followed by proper regulation of all metabolic activities, and resulting in an improvement of growth and productivity. Tzortzakis (2010) and Ashraf et al. (2015) also state that K^+ is a vital nutrient and growth of plants depends upon its availability. Both wheat cultivars showed positive response to K_2SO_4 and absorption of potassium nutrients (*Fig. 2 a, b and d*), as these enhance growth leading towards an improvement in fresh and dry biomass production. These results are in agreement with those of Ashraf and Sarwar (2002); Ashraf et al.

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(2003, 2013); Golldack et al. (2003); Hussain et al. (2013); Saffa et al. (2013) and Kausar and Gull (2014b).

In the present study, potassium sulphate was used to alleviate the negative effects of salt stress and an improvement in plant height, fresh and dry weights was recorded in both wheat cultivars. Positive influence of potassium on plant growth and productivity under saline environments has been reported by Rashid et al. (2001) and Hussain et al. (2013) as well. Indirect evidences are also available indicating that potassium and sulphate have central role in the construction of some important proteins (Khan et al., 2008; Khan et al., 2010), which are necessary to impart stress tolerance in plants (Lee et al., 2009). Our findings too have confirmed that K_2SO_4 increases both growth and productivity of wheat plants by increasing the absorption of essential nutrients in saline and non saline medium. On the bases of earlier and present findings K_2SO_4 application in saline soils can be recommended to alleviate the adverse effects of salinity and to have economical crop yield.

REFERENCES

[1] Akhtar, J., Ahmad, R., Ashraf, M. Y., Tanveer, A., Waraich, E. A., Oraby, H. (2013): Influence of exogenous application of salicylic acid on salt stressed mung bean (Vignaradiata): growth and nitrogen metabolism. – Pakistan Journal of Botany 45: 119-125.

[2] Alam, S. M., Naqvi, M. H. (2003): Pakistan and agriculture 2003. - Pakistan and Gulf Economist.

[3] Ali, A., Basra, S. M. A., Hussain, S., Iqbal, J. (2012): Increased Growth and Changes in Wheat Mineral Composition through Calcium Silicate Fertilization under Normal and Saline Field Conditions. – Chilean J Agric Res 72 (1).

[4] Ali, C.K., Javed, M., Javaid, M.A. (1999): Growth promotion of wheat by potassium application in saline soils. – J. Ind. Soc. Soil Sci. 47: 510-513.

[5] Ashraf, M., Kausar, A., Ashraf, M.Y. (2003): Alleviation of salt stress on growth of pearl millet (*Pennisetum glaucumm* L. (R. Br.) by pre-sowing treatment of seeds with chilling, heating, distilled water, NaCl and PEG. – J. Agron. 23(3): 253-259.

[6] Ashraf, M.; Ozturk, M.; Athar, H.R. (Eds.) (2009): Salinity and Water Stress: Improving Crop Efficiency. (Tasks for Vegetation Science 44). - Springer Science+Business Media,NY, 244 pp.

[7] Ashraf, M., Afzal, M., Ahmad, R., Ali, S. (2011): Growth and yield components of wheat genotypes as influenced by potassium and farm yard manure on a saline sodic soil. - Soil and Environment 30: 115-121.

[8] Ashraf, M., Ahmad, M. S. A., Ozturk, M., Aksoy, A. (2012): Crop improvement through different means. – In: Ashraf et al. (Eds) Crop Production for Agricultural Improvement, Springer, pp. 1-15.

[9] Ashraf, M., Muhammad, S.S., Arif, M.S., Riaz, M., Ali, S., Abid, M. (2015): Effects of potassium sulfate on adaptability of sugarcane cultivars to salt stress under Hydroponic Conditions. – Journal of Plant Nutriton 38(13): 2126-2138.

[10] Ashraf, M. Y., Sarwar, G. (2002): Salt tolerance potential in some members of Brassiaceae. Physiological studies on water relations and mineral contents. In prospects for saline agriculture. –Academic Publishers Netherlands 237-245.

[11] Ashraf, M.Y., Rafique, N., Ashraf, M., Azhar, N., Marchand, M. (2013): Effect of supplemental potassium (K^+) on growth, physiological and biochemical attributes of wheat grown under saline conditions. –Journal of Plant Nutrition 36: 443-458.

[12] Ashraf M. Y., Iqbal N., Ashraf M., Akhter J. (2014): Modulation of physiological and biochemical metabolites in salt stressed rice by foliar application of zinc. – Journal of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_137147

Plant Nutrition 37: 447-457.
[13] Bray, E.A., Bailey-Serres, J., Weretilnyk, E. (2000): Responses to abiotic stresss. - In: Buchanan, B. Gruissem, W., Jones, R. (Eds) Biochemistry and molecular biology of plants. Plant Physiology 1158-1203.
[14] Bremner, J. M. (1965): Total nitrogen and inorganic forms of nitrogen. In: Methods of Soil Analysis. Ed. C. A. Black. – American Soc. Agron., Madison, Wisconsin 2: 1149-1237.
[15] Ebrahimi, R. F., Rahdari, P., Vahed, M. S., Shahinrokhsar, P. (2012): Rice response to different methods of potassium fertilization in salinity stress condition. –International Journal of Agricultural and Crop Science 4: 798-802.
[16] Golldack, D. Q., Michalowski, F., Kamasani, U. R., Bohnert, H. J. (2003): Salinity stress tolerant and sensitive rice regulate AKT1- type potassium channel transcript differently. – PIant Mol Biology 51: 71-81.
[17] Hakeem, K. R., Parvaiz, A., Ozturk, M. (Eds.) (2013): Crop Improvement-New Approaches and Modern Techniques. - XXVII, Springer Science+Business Media, NY, 493.
[18] Hamilton, P. B, Van Slyke, D. D. (1953): Aminoacid deterimination with ninhydrin. – J. Biol. Chem.150: 231-233.
[19] Han, L., Gao, Y., Li, D. (2014): Ion Uptake in Tall Fescue as Affected by Carbonate, Chloride, and Sulfate Salinity. - PLOS ONE 9(3): e91908. doi:10.1371/journal.pone.0091908.
[20] Hussain , Z., Khattak, R.A., Irshad, M., Eneji, A.E. (2013): Ameliorative effect of potassium sulphate on the growth and chemical composition of wheat (Triticum aestivum L.) in salt-effected soils. – J. Soil Sci. Plant Nutr. 13 (2).
[21] Hasanuzzaman, M., Nahar, K., Fujita, M., Ahmad, P., Chandna, R., Prasad, M. N. V., Ozturk, M. (2013): Enhancing Plant Productivity under Salt Stress- Relevance of Poly-omics. – In: Salt stress in Plants: Signalling, Omics and Adaptations (eds. Ahmad et al.), , SpringerVerlag,NY, USA, 113-156.
[22] Irshad, M., Honna, T., Eneji, A. E., Yamamoto, S. (2002): Wheat response to nitrogen source under saline conditions. – Journal of Plant Nutrition 25(12): 2603-2612.
[23] Jackson, M. L. (1962): Soil chemical analysis. – Prentice Hall of Englewood cliff, New Jersey, USA.
[24] Kausar, A, Ashraf, M.Y, Ali, I, Niaz, M., Abbass, Q. (2012): Evaluation of sorghum varieties/lines for salt tolerance using physiological indecies as screening tool. – Pak. J Bot. 44(1): 47-52.
[25] Kausar, A., Gull, M. (2014a): Effect of potassium sulphate on the growth and uptake of nutrients in wheat (Triticum aestivum L.) under salt stressed conditions. – Journal of Agricultural Sciences 6(8): 1-12.
[26] Kausar, A., Gull, M. (2014b): Nutrients uptake and growth analysis of four sorghum (Sorghum bicolor L.) genotypes exposed to salt stress. – Pensee Journal 76(4): 102-110.
[27] Kausar A., Khurshid A., Ashraf M.Y., Ghafoor R., Gull M. (2015). Photosynthesis, transpiration, stomatal conductance potential and water uptake in selected rice cultivars (*Oryza sativa*.) under various saline conditions. – Sylwan 159(3): 258-265.
[28] Khan, A. M., Islam, E., Shirazi, M. U., Mumtaz, S., Mujtaba, S. M. (2010): physiological response of various wheat genotypes to salinity. – Pakistan Journal of Botany 42: 3497-3505.
[29] Khan, M. H., Panda, S. K. (2008): Alterations in root lipid peroxidation and antioxidant responses in two rice cultivars under salinity stress. – Acta Physiology Plant 30: 89 -91.
[30] Kosova, K., Prasil, I.T., Vitamas, P. (2013): Protein Contribution to Plant Salinity Response and Tolerance Acquisition. – Int. J. Mol. Sci. 14:6757-6789.
[31] Lee, B.V., Dikiy, A., Kim, H.Y.,Gladyshev, V.N. (2009): Functions and evolution of selenoprotein methionine sulfoxide reductases. – Biochimica et Biophysica Acta (BBA) - General Subjects 1790(11): 1471-1477.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_137147

[32] Lowry, O. H., N. J., Rosebrough, A. L., Randall, F. R. J. (1951): Protein measurement with folin phenol reagent. – J. Biol.Chem. 193: 265-275.
[33] Marjan F., Ashraf M.Y., Rehman, R. (2012): Bio-chemical respose by elevated salinity stress in two barley lines. – Int. J. Biol. Biotech. 9(4): 353-358.
[34] Mesbah, E. A. E. (2009): Effects of irrigation regimes and foliar spraying of potassium on yield, yield components and water use efficiency of wheat in sandy soils. – World Journals of Agricultural sciences 5: 662-669.
[35] Rashid, H. H., Ranjha, A. M., Mehdi, S., Saifullah, m. (2001): Relative efficiency of muriate and sulphate of potash for wheat. – International Journal of Agricultural Biology 3: 403-405.
[36] Safaa R. El-Lethy, Magdi T. Abdelhamid and Fatma R. (2013): Effect of Potassium Application on Wheat (Triticum aestivum L.) Cultivars Grown Under Salinity Stress. – World Applied Sciences Journal 26 (7): 840 -850.
[37] Steel, R. G. D., Torrie, J. H., Dickey, D. A. (1997): Principles and procedures of statistics, A biometrical approach. - McGraw Hill Co., New York, pp. 178-182.
[38] Tzortzakis, N.G. (2010). Potassium and calcium enrichment alleviate salinity-induced stress in hydroponically grown endives. – Hort. Sci. (Prague) 37: 155-162.
[39] Wolf, B. (1982): A comprehensive system of leaf analysis and its use for diagnosing crop nutrient status. – Communications in soil science and plant Analysis 13: 1035-1059.
[40] Zafar S., Ashraf M.Y., Niaz M., Kausar A., Hussain J. (2015). Evaluation of wheat genotypes for salinity tolerance using physiological indices as screening tool. – Pakistan Journal of Botany 47: 397- 405.

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IMPACT OF FLOWERING STAGE ON NUTRITIVE VALUE, PHYSICAL QUALITY AND DIGESTIBILITY OF SILAGES MADE FROM CEREAL FODDERS

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Abstract. The objective of current study was to investigate the effects of flowering phase (maturity) i.e. early bloom (20% flowering), mid bloom (50% flowering) and full bloom (100% flowering) on chemical and physical characteristics of silages, made from *Zea mays* L., *Sorghum bicolor* (L.) Moench and *Avena sativa* L. cereal fodders grown in subtropical conditions. The three fodders were harvested at three different maturity stages, chopped and ensiled in laboratory silos for 30 days fermentation period. The analysis of variance revealed that the increase in maturity from early to full bloom significantly ($P < 0.05$) increased dry matter (DM %) in maize (18.82±0.02 to 25.80±0.05), sorghum (20.65±0.01 to 29.47±0.01) and oat (17.68±0.01 to 26.54±0.01) silages, and decreased crude protein in all. The NDF and ADF increased with increase in maturity for sorghum and oats silage, while it decreased for maize silage. Lactic acid increased linearly ($P < 0.05$) with increasing maturity while pH, ME and in vitro dry matter digestibility (*IVDMD*) decreased. Stage of maturity also had significant effect on physical characteristics (*color, smell and structure*) of silage in all fodder crops. The highest flieg score was recorded in full bloom stage of maturity (100% flowering) due to low pH and higher DM recovery in all cereal silages. It was concluded from the current study that quality silages can successfully be made from cereal fodders grown in subtropical conditions.
Keywords: *cereals maturity, bloom stages, lactic acid, physical quality, flieg score*

Introduction

The shortage of good quality fodder, round the year, is the major constraint for livestock production in Pakistan (Khan et al., 2011). The seasonal fodder production poses a great challenge for livestock farmers to feed their animals when fodder supply is limited especially during summer (May - July) and winter (November – January) months (Rasool et al., 1996). The conservation of fodder in the form of silage is a viable solution to ensure its supply during those lean periods (Khan et al., 2011).

The quality of silage is dependent on the availability of fermentable substrates (McDonald et al., 1981) energy density, and water content in plants (Bal et al., 1997). As the plant matures, the water-soluble carbohydrates decrease, thereby

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decreasing the fermentation activity of bacteria (Jhonson et al., 2003). Too early or too late harvesting stage not only impairs the energy density of whole plant but also affects the optimum moisture level required for good silage preservation (Bal et al., 1997). Therefore, optimum stage of maturity is important to harvest maximum nutrients for livestock feeding.

Optimal harvesting stage to increase yield and silage quality of maize, in literature, varied from tasseling stage (Fu et al., 2011), one-third milk line (Johnson et al., 2002), late dough stage (Vecchiettini et al., 2003) to two-thirds milk line stage (Fariani et al., 1994). When sorghum was harvested at late-milk, late-dough and hard-grain stages of maturity, higher nutritive values were noticed at late milk stage silages (Sonon and Bolsen, 1996). Also, reported that advancing development (maturity) of corn from 30.0 to 42.0% (i.e. black layer stage) dry matter (DM) at ensiling did not affect DM intake, milk production and composition in dairy animals (Khan et al., 2012). Despite the difference in optimal harvesting stage for silages, it is also worth mentioning that there are even very few studies investing the effects of maturity stage on silage quality in subtropical environment as in Pakistan. Under such scenario, the studies are needed to tackles the issue of silage production for maximum feed supply to the livestock.

The objective of current study was to examine the effect of varying maturity stages of three different fodders (*Zea mays* L., *Sorghum bicolor* (L.) Moench and *Avena sativa* L.) on fermentation characteristics and nutritive value of silages under local environmental conditions.

Materials and Methods

Sowing and harvesting of cereal fodder crops

The three fodder crops i.e. *Zea mays* L. (maize), *Sorghum bicolor* (L.) Moench (sorghum) and *Avena sativa* L. (oats) were used for silage making. The maize, sorghum and oats were planted during the month of June, July and November, respectively on agriculture field of Dairy Animals Training and Research Center, University of Veterinary and Animal Sciences, Ravi Campus Pattoki, Pakistan (31°1'0" North, 73°50'60" East with an altitude of 186 meters (610 ft.). Each fodder crop was harvested at three different stages of maturity. The maturity stages were based on the flowering in the field and categorized as; 1) 20 % flowering (20% of plants in the field had shown flowers), 2) 50% flowering, 3) 100 % flowering. The detail of planting and harvesting at different maturity stages has been presented in *Table 1*. At each harvesting time, the respective fodder was randomly cut during a clear day from four different parts of the field and chopped by mechanical chopper (Fimax, V-Belt Driven, MC10X and Turkey) with a chop size of about 2 cm.

Table 1. *Date of sowing and harvest stages of cereal fodders*

Fodder type	Date of sowing	Date of harvest		
		Early bloom (20% flowering)	Mid bloom (50% flowering)	Full bloom (100% flowering)
Maize	15 July	25 September	1st October	6th October

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Sorghum	15 June	15th August	25 August	4th September
Oats	15 November	25 February	7th March	12th March

Ensiling of chopped fodder in bag silos

The chopped forage taken from different parts of the field was mixed to make a representative sample for silage making. The mixed sample was packed in laboratory silos (transparent thick polyethylene bags) with capacity of 40 kg having dimensions 80×40 cm. The chopped materials were packed in the bag silo step by step. To fill the bag, about 3-6 kg of chopped forage was placed in the bag each time and pressed manually for compaction to remove air. The procedure was repeated till the bag was full and then sealed immediately. All the bags were labeled and stored under shed at room temperature for fermentation.

Physical quality evaluation of silages

After 30 days of fermentation period, the bags were opened and samples were taken for physical and chemical analysis. For physical analysis, the quality of silages was determined by color, smell, and structure along with total flieg score described by Kilic (1986). For color evaluation the scale 1- 4 was used on the basis of change in green color from dark brown , dark green to pale yellow, for smell the scale 1-7 was used on the basis of repugnant putrid smell to acidic sweet pleasant smell, for structure the scale 1-4 was used on the basis of softness of leaves and stem as well as its ability to remain intact after squeezing the silage tightly in hand and then opening from breaking into small pieces to break into two or three pieces. The same person scored the silages for smell, color and structure to avoid any bias. Flieg score was calculated using a formula (Flieg Score = 220 + (2 x Dry Matter% - 15) - 40 x pH) reported by Kilics (1986). The flieg score with value 81-100, 61-80, 41-60, 21-40 and 0-20 represented the silage quality a very good, good, medium, low and poor respectively. The overall silage quality was classified into categories as poor, medium, good and very good on the basis of cumulative score obtained from color, smell and structure flieg score.

Chemical quality and pH analyses of silages

Chemical analyses were done to determine pH, lactic acid, dry matter content (DM), crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF). Approximately 25g sample was taken from each bag immediately after opening. The sample silage was adulterated with 100 ml of distilled water (Hart and Horn., 1987). After hydration for 10 min using blender, the diluted material was then filtered through cheese cloth and then pH was determined by using a digital pH meter. The liquid obtained was further filtrated through Whatman 54 filter paper, centrifuged and kept at 20˚C for lactic acid determination by high pressure liquid chromatography (Muck and Dickerson., 1988). Also, approximately 250g sample (in triplicate) was taken from each bag dried in a hot-air oven (Memmert, Beschickung-Loading Model 100-800, Germany) at 60°C for 72 hours (for DM%) then ground through hammer mill (Wiley laboratory Mill, Standard Model No. 2, Arthur H. Thomas Company, USA) making particle size of about 0.5

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to1mm and stored in pre labeled bottles for further laboratory analyses. Nitrogen (N) contents of samples were determined by procedure AOAC (1990) using Kjeldahl apparatus (ID 984.13) and then multiplying the N concentration by a factor 6.25 to calculate CP content. The NDF and ADF contents were determined according to Van Soest et al. (1991). The gross energy of the silage samples was determined through the IKA C-2000 Bomb Calorimeter, while metabolizable energy (ME) was calculated as 63% of the gross energy (Mandal et al., 2003).

In-vitro dry matter digestibility of silages

The *in vitro* dry matter digestibility (*IVDMD*) trials were conducted at the University of Sydney, Camden. The dried samples were taken from Pakistan to Camden by air cargo. For IVDMD study, rumen liquor (inoculant) was collected from rumen of cannulated lactating Holstein cows managed on pasture and cereal-based concentrate (9kg DM/cow/day), at Corstorphine farm, University of Sydney. The collected rumen liquor was filtered through various layers of cheese cloth and mixed with buffered minerals solution in 1:2 ratio and placed at 39˚C under O_2 free environment. Dry matter digestibility (DMD) was determined *in vitro* by batch incubation of samples in rumen liquor (Wang et al., 1999). All the dried samples from respective cereal silages were incubated in duplicate using ANKOM filter bags technology (New York, USA). The open side of the bag (having 0.5g ground sample) was sealed with heat sealer impulse and then put into a 50ml dark bottle. All the bottles were injected with 25ml of buffer solution under anaerobic condition then sealed with rubber lid. Bottles including blank (inoculums only) were incubated in rotary incubator for 48hrs at 39˚C and rotated 90 times per minute. After 48 h of incubation the bags having digested sample were removed from the flasks, washed under running tap water then dried in oven at 60˚C for 48 hours. The IVDMD percent (%) was calculated from the difference of the dry weight of sample and residues remained in the bag after 48 h of digestion divided by weight of sample×100 (Wang et al., 1999).

Statistical analyses

The data were analyzed by analyses of variance, using General Linear Model's procedures of SAS (SAS 9.1.3). Differences of means among main effects were compared by Fisher's least significant difference test (Steel et al., 1997).

Results

Effect of maturity on chemical composition of cereal silages

Chemical composition of three silages (maize, sorghum and oats) harvested at three different stages of maturity i.e. early bloom (20% flowering), mid bloom (50% flowering) and full bloom (100% flowering) has been presented in *Table 2*. DM content % increased with increasing maturity from early to full bloom ($P < 0.05$) in maize (18.82±0.02 to 25.80±0.05), sorghum (20.65±0.01 to 29.47±0.01) and oats (17.68±0.01 to 26.54±0.01) silages respectively, but in contrast CP and ME contents were significantly ($P<0.05$) decreased in all cereals silages with increasing maturity (*Table 2)*. However, with advance maturity increasing pattern

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of NDF and ADF were also observed in sorghum and oats silage with progressed age form early to full bloom stage, but in contrary tended to be decreased NDF and ADF in maize silage with advancing maturity.

Table 2. *Effect of maturity stages on chemical composition of cereal silages*

Maturity stages				
Silages	Parameters	Early bloom	Mid bloom	Full bloom
Maize	DM%	18.82±0.02^{c}	22.38±0.03^{b}	25.80±0.05^{a}
	CP%	9.84±0.02^{a}	8.60±0.01^{b}	7.89±0.02^{c}
	NDF%	66.26±0.01^{b}	66.83±0.01^{a}	62.3±0.01^{c}
	ADF%	33.25±0.03^{b}	34.76±0.02^{a}	31.6±0.03^{c}
	ME(Mcal/kg)	2.92±0.003^{a}	2.87±0.001^{b}	2.85±0.02^{b}
Sorghum	DM%	20.65±0.01^{c}	26.56±0.03^{b}	29.47±0.01^{a}
	CP%	7.45±0.31^{a}	7.00±0.33^{ab}	6.19±0.32^{b}
	NDF%	62.42±0.04^{b}	56.50±0.08^{c}	64.68±0.14^{a}
	ADF%	33.68±0.01^{b}	29.38±0.01^{c}	35.25±0.05^{a}
	ME (Mcal/kg)	2.83±0.009^{a}	2.79±0.005^{b}	2.78±0.009^{b}
Oats	DM%	17.68±0.01^{c}	23.61±0.03^{b}	26.54±0.01^{a}
	CP%	8.86±0.02^{a}	7.40±0.02^{b}	6.82±0.01^{c}
	NDF%	52.70±0.36^{c}	63.31±0.11^{b}	65.33±0.03^{a}
	ADF%	33.42±0.03^{b}	33.41±0.03^{b}	34.26±0.02^{a}
	ME (Mcal/kg	2.80±.003^{a}	2.79±0.005^{a}	2.77±0.002^{b}

Means within each row followed by different superscripts are significantly different ($p<0.05$).

Fermentation characteristics and IVDMD of silages

The results showed that with the increase in maturity stage of silages, fermentation characteristics significantly got better (*Table 3*). The pH with advancing maturity linearly decreased ($P < 0.05$) in maize 4.29±0.005, 4.24±0.005 and 3.94±0.008, sorghum 3.95±0.01, 3.83±0.008 and 3.62±0.017 and oats silage 4.04±0.008, 3.95±0.02 and 3.71±0.008 from early to full bloom stage respectively (*Table 3*). Whereas, lactic acid (LA) concentration significantly ($P<0.05$) increased with advance maturity. However, IVDMD decreased ($P < 0.05$) with increasing maturity for sorghum and oats but not or maize (*Table 3*).

Table 3. *pH, LA, and IVDMD of cereal silages at different stage of maturity*

Maturity stages					
Silages	Parameters	Early bloom	Mid bloom	Full bloom	P-V
Maize	pH	4.29±0.005^{a}	4.24±0.005^{b}	3.94±0.008^{c}	<.0001
	LA%	4.03±0.04^{c}	4.46±0.08^{b}	7.06±0.05^{a}	<.0001
	IVDMD%	68.80±0.17	67.53±0.26	67.00±0.76	0.087

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Sorghum	pH	3.95±0.01[a]	3.83±0.008[b]	3.62±0.017[c]	<.0001
	LA%	4.67±0.09[c]	5.76±0.09[b]	6.18±0.13[a]	0.0002
	IVDMD%	65.30±0.90[a]	63.03±0.78[a]	60.26±0.49[b]	0.009
Oats	pH	4.04±0.008[a]	3.95±0.02[b]	3.71±0.008[c]	<.0001
	LA%	3.22±0.067[c]	3.74±0.19[b]	4.91±0.07[a]	0.0002
	IVDMD%	60.60±0.49[a]	57.46±0.76[b]	55.33±0.41[c]	0.002

Means within each row followed by different superscripts are significantly different (p<0.05).

Physical quality of silages

Effect of maturity on physical characteristics including color, smell and structure and flieg score has been shown in *Table 4*. Maturity had non-significant (P>0.05) effect on color of the silages in all cereals. However, the score for smell and structure positively increased with the increase in maturity for maize, sorghum and oat silages from early bloom to full bloom (P<0.05). Numerically higher flieg score was observed in full bloom followed by mid bloom and early bloom stage of maturity for all silages respectively. The cumulative effect of all physical traits indicated that silages had highest quality at 100 % maturity stage of flowering (full bloom) in all cereal silages.

Table 4. *Effect of maturity stages on physical quality of silages*

Maturity stages					
Silages	Parameters	Early bloom	Mid bloom	Full bloom	P-V
Maize	Color	3.66±0.08	3.50±0.05	3.70±0.05	0.172
	Smell	3.76±0.14[b]	4.77±0.14[a]	5.27±0.40[a]	0.018
	Structure	2.63±0.08[c]	3.43±0.12[b]	3.90±0.05[a]	0.000
	Sensory score	10.05	11.7	12.87	
	Flieg score	71.04	80.16	99.00	
	Quality class	Good	Very good	Very good	
Sorghum	Color	3.36±0.08	3.66±0.08	3.36±0.08	0.083
	Smell	4.31±0.09[b]	4.40±0.09[ab]	4.91±0.21[a]	0.056
	Structure	2.80±0.11[c]	3.37±0.17[b]	3.88±0.04[a]	0.002
	Sensory score	10.47	11.43	12.15	
	Flieg score	78.76	94.22	109.68	
	Quality class	Good	Very good	Very good	
Oats	Color	4.53±0.14	4.10±0.20	4.70±0.32	0.26
	Smell	3.33±0.08[b]	3.40±0.05[b]	3.90±0.05[a]	0.002
	Structure	2.80±0.15[c]	3.36±0.08[b]	3.83±0.08[a]	0.002
	Sensory score	10.66	10.86	12.43	
	Flieg score	88.3	104.92	119.14	
	Quality class	Good	Very good	Very good	

Means within each row followed by different superscripts are significantly different (P<0.05)

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Discussion

Chemical composition and nutritive value

The finding of increased DM and decreased CP and ME with increasing maturity in our study was consistent with previous studies (Khorasani et al., 1997; Khan et al., 2011). Corn silage DM also increased quadratically and CP% tended to decrease as maturity increased (Row, 2015). The current results are in agreement to Khan et al. (2011) who reported that NDF and ADF contents in maize silage decreased with increasing maturity while it increased in other cereal silages like sorghum and millets. But, in contrast to Row (2015) who presented that NDF contents increased quadratically in corn silage as maturity increased. The increased DM and lower CP and ME with increasing age could be attributed to increased lignification and decreased content of leafy part of plant. The decreased NDF and ADF in maize silage with increasing maturity could be due to the deposition of starch into grains. Similarly, Khan et al. (2012) who found that difference in maturity at harvest during grain filling had a major effect on the carbohydrate structure (starch:NDF ratio) and fatty acid (FA) content of corn silages.

Fermentation characteristics

The linear decrease in pH values of maize, sorghum and oats silages from early to full bloom stage of maturity was in agreement with the findings of Sarwatt et al. (1989) who described that pH values of maize silages decreased with advancing growth of fodder. Similarly, Khan et al. (2011) also reported that ensiled fodder (maize, sorghum and millet) at initial stage of growth did not decrease pH quickly. The decrease in pH is mainly due to the accumulation of lactic acid as a result of fermentation. Lactic acid contents in the our study were supported by the findings of Khan et al. (2011) who ensiled maize, sorghum and millet fodder at pre-heading, heading and milk stage of maturity and concluded that lactic acid contents increased with the advancement of age of fodder. Similarly, Bal et al. (1997) and Harrison et al. (1998) reported that maize silage harvested at milk stage of maturity have highest concentration of lactic acid. The pre-requisite for the development of the lactic acid bacteria during the early stages of ensilage are the contents derived from the plant juices (water soluble carbohydrates) released by plasmolysis as a result of plan cell wall breakdown (McDonald, 1981). Khan et al. (2011) reported that ensiled fodder (maize, sorghum and millet) at initial stage of growth had low level of available water soluble carbohydrates (WSC). Bergen et al. (1991) reported that WSC was greater in silages made from barley, wheat and oats fodders harvested at milk stages. The range of pH values in current study ranging between 3.62 and 3.94 at full stage of maturity (100% flowering) in all cereals silages were consistent with the reports of McCullough (1978) who observed that pH value less than 4.2 was indicative of good quality preserved silage.

In-vitro dry matter digestibility

The decreased IVDMD in sorghum and oats silages, but unchanged in maize silage with advancing maturity was supported by Edmisten et al. (1998) who reported that IVDMD of small grain cereals (barley, oat, rye, and wheat) silages harvested at six growth stages, decreased from vegetative to the milk stage and then remained similar or declined marginally to the hard dough stage. The decline in IVDMD from growing to

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the boot stage was probably due to the consistent small increase in lignification of stems. Russell et al. (1992) also investigated the effect of growth on IVDMD harvested at 0, 14 and 28 days intervals after physical maturity and suggested that later harvest did not affect IVDMD of the maize forages. Though, digestibility of NDF decreased linearly as maturity advanced (Row, 2015).

Effect of maturity proceeding to physical quality of cereals silages

The improvement in physical traits of silages with increasing age was in agreement to previous studies (Khan et al., 2011; Khan et al., 2012). Higher values for color, smell, structure and flieg score in full bloom followed by mid bloom and lower in early bloom stage of maturity could be due to low pH and higher DM as well as higher lactic acid concentration across all crops silages in advanced maturity stage. Türemiş et al. (1997) reported that low pH and acetic acid contents resulted in high physical quality scores.

Conclusion

It was concluded from the current study that quality silages can successfully be made from cereal fodders grown in subtropical conditions.

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REFERENCES

[1] Association of Official Analytical Chemists, AOAC. (1990): Official Methods of Analysis. 15th Ed. - Association of Official Analytical Chemists, Arlington, Virginia, USA.

[2] Bal, M.A., Coors, J.G., Shaver, R.D. (1997): Impact of the maturity of corn for use as silage in the diets of dairy cows on intake, digestion, and milk production. - J. Dairy. Sci. 80: 2497–2503

[3] Bergen, W.G., Byrem, T.M., Grant, A.L. (1991): Ensiling characteristics of whole-crop small grains harvested at milk and dough stages. - J Anim Sci. 69: 1766-1774.

[4] Edmisten, K.L., Green, J.T., Mueller., J.P., Burns, J.C. (1998): Winter annual small grain forage potential. ii. Quantification of nutritive characteristics of four small grain species at six growth stages. Commun. - Soil Sci. Plant Anal., 29 (7 and 8): 881-899.

[5] Fu, F., Guo, C., Tang, Q., Liu, J., Li, W. (2011): Growth dynamics and optimal harvesting stage of two forage maize varieties. - Agric. Sci. China 10: 220-227.

[6] Fariani, A., Warly, L., Matsui, T.A., Fujihara, T., Harumoto, T. (1994): Rumen degradability of Italian Ryegrass (Lolium multiflorum, L) harvested at three different growth stages in sheep. - Asian-Aust. J. Anim. Sci. 7 (1): 41-48.

[7] Harrison, J.H., Johson, L., Riley, L.R., Xu, S., Loney, K., Hunt, C.W., Sapienza, D. (1998): Effect of harvest maturity of whole plant corn silage on milk production and component yield and passage of corn grain and starch into feces. - J. Dairy Sci. 79(1):149.

[8] Hart, S.P., Horn, F.P. (1987): Ensiling characteristics and digestibility of combinations of turnips and wheat straw. - J. Anim. Sci. 14: 1790-1800.

[9] Khan, N.A., Tewoldebrhan, T.A., Zom, R.L.G., Cone, J.W., Hendriks, W.H. (2012): Effect of corn silage harvest maturity and concentrate type on milk fatty acid composition of dairy cows. - J. Dairy Sci. 95: 1472–1483.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_149157

[10] Khan, S.H., Azim, A., Sarwar, M., Khan, A.G. (2011): Effect of maturity on comparative nutritive value and Fermentation characteristic of maize, Sorghum and millet silages. - Pak. J. Bot. 43(6): 2967-2970.

[11] Khorasani, G.R., Jedel, P.E., Helm, J.H., Kennelly, J.J. (1997): Influence of stage of maturity on yield components and chemical composition of cereal grain silages. - J. Anim. Sci. 77: 259-267.

[12] Kilic, A. (1986): Silo feed. Instruction, Education and Application Proposals. - Bilgehan Press, Izmir: 327.

[13] Mandal, A.B., Paual, S.S., Pathak, N.N. (2003): Nutrient requirements and feeding of buffaloes and cattle. - Published by Int. Book Distributing Co, Charbagh, and Lucknow, India. 23.

[14] Row, C.A. (2015): Corn Plant Maturity Effect on Yield and Nutritional Quality; Corn Silage Inoculation on Performance of Cattle Fed Silage with or without Live Yeast Added. M.Sc. thesis (Published). Dept. of Animal Sci., University of Nebraska – Lincoln. 41.

[15] Rasool, S., Ahmad, G., Abdullah, M. (1996): Fodder conservation, crop residues and by products in livestock production system. - Proceeding of national conference on the improvement production and utilization of fodder crops in Pakistan. March 21-27, Pakistan Agriculture Research Coubcil / FAO, Islamabad, Pakistan. pp. 176-186.

[16] Russell, J.R., Irlbeck, N.A., Hallauer, A.R., Buxton, D.R. (1992): Nutritive value and ensiling characteristics of maize herbage as influenced by agronomic factors. Anim. - Feed Sci. Technol. 38: 11–24.

[17] Steel, G.D., Torrie, J.H., Dickey, D.A. (1997): Principles and procedures of statistics. 3rd ed. - Mc Graw-Hill, New York.

[18] Sonon, R.N., Bolsen, K.K. (1996): Effects of cultivar and stage of maturity on agronomic characteristics, chemical composition and nutritive value of forage sorghum silages. - Adv. Agric. Res. 5(3):1-17.

[19] Sarwatt, S.V., Mussa, M.A., Kategile, J.A. (1989): The nutritive value of ensiled forages cut at three stages of growth. - Anim. Feed Sci. Technol. 22: 237-245.

[20] Johnson, L.M., Harrison, J.H., Davidson, D., Mahanna, W.C., Shinners, K., Linder D. (2002): Corn silage management: Effects of maturity, inoculation and mechanical processing on pack density and aerobic stability. - J. Dairy Sci. 85(2): 434-444.

[21] Muck, R.E., Dickerson, J.T. (1988): Storage temperature effects on proteolysis in alfalfa silage. - Trans. ASAE. 31:1005-1009.

[22] McDonald, P. (1981): The biochemistry of silage. - John Wiley and Sons, Ltd. Chichester, United Kingdom.

[23] McCullough, M.E. (1978): Silage-some general consideration. - In: McCullough, M.E. (Ed.) Fermentation of Silage-A Review. National Feed Ingredients Assoc, West Des Moines, IA; 1-26

[24] Turemis, A., Kizilsimşek, M., Kizil, S., Inal, I., Saglamtimur, T. (1997): Farklı katkı maddelerinin çukurova koşullarında yetiştirilen bazı yazlık yem bitkisi ve karışımlarından yapılan silajlar üzerine etkisi. - Türkiye I.Silaj Kong. Bildiri Kitabı. Bursa, s. pp. 166-175.

[25] Vecchiettini, M., Cinti, F., Sandrini, E. (2003): Effect of harvest stage on maize silage production. - In: Kirilov, A., Todorov, N., Katerov, I. (Eds.) Optimal forage production for animal. Production and the environment. - Proc. 12th Symp. Eur. Grassl. Fed.Pleven, Bulgaria. - 303-306.

[26] Van-Soest, P.J., Robertson, H.B., Lewis, B.A. (1991): Method of dietary fiber and non-starch polysaccharides determination in relation to animal material. - J. Dairy. Sci. 74: 3583-3591.

[27] Wang, Y., McAllister, T.A., Xu, Z.J., Gruber, M.Y., Skadhauge, B., Jende-Strid, B., Cheng, K.J. (1999): Effects of proanthocyanidins, dehulling and removal of pericarp on digestion of barley grain by ruminal micro-organisms. - J. Sci. Food Agric. 79: 929−938.

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ANALYSIS OF ANTIMICROBIAL POTENTIAL OF SOME FICUS TAXA FROM DISTRICT BHIMBER AZAD JAMMU AND KASHMIR, PAKISTAN

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Abstract. Some important species of the genus Ficus (*Ficus racemosa* L.*; Ficus auriculata* Lour.*; Ficus palmata* Forssk. and *Ficus religiosa* L.) from district Bhimber Azad Kashmir were analyzed for examining their antimicrobial potential against different clinical human pathogens *viz* Bacteria like *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and Fungi like *Aspergillus flavus, Fusarium solani, and Candida albicans.* Plant leaves were extracted in Petroleum ether (PE), Chloroform, Methanol and Water in sequential order and antimicrobial activity was tested by using Agar Well Diffusion method and Micro dilution method. The significant activity was shown by plant extracts of four species of genus Ficus against all disastrous pathogens. As methanolic extract of *Ficus* species showed maximum zone of inhibition (ZI)) 19.3mm with minimum inhibitory concentration (MIC) 42.7 (µg/ml) against *S. aerus* and ZI (21.9mm) with MIC (52.9 µg/ml*)* against *A. flavus*. Moderate activity was found in Chloroform and Petroleum ether extracts for *Ficus* species with ZI (47.3 mm) against *S. aerus* and ZI (57.6mm) against *A. flavus*. The least ZI (10.4mm) and MIC (43.4 µg/ml) against *P. aeruginosa* and (10.6mm) and MIC (48.4 µg/ml) against *C. albicans* were shown by aqueous extract against all experimental human pathogens. Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were also evaluated by a serial micro-dilution method. It was found that the MBC and MFC is normally two folds of the MIC. The present study depicted that *P. aeruginosa* and *C. albicans* were maximum resistant against controlled antibiotics and crude plant extracts of *Ficus* species while *S. aerus* and *A. flavus* showed maximum infection against plant extract of *Ficus* species. *E. coli* and *F. solani* showed moderate resistance to leaf extract of *Ficus* species. The current study results also revealed that *F. racemosa* and *F. auriculata* have more antimicrobial effect than *F. palmata* and *F. religiosa.* The conclusions of present study may be helpful in developing possible source of new and effective herbal medicines to treat such infectious diseases which are caused by disastrous human pathogens. These research findings can be better source of novel drug discovery and drug development.
Keywords: *methanol, agar well diffusion method, antimicrobial activity, drug development, minimum inhibitory concentration*

Introduction

It has long history of many centuries that plant and plant parts have been used as folk and traditional medicines for the treatment of many disastrous diseases and minor

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ailments. In current era of science and technology there is tremendous development in field of medicine which leads to discovery of potential natural and synthetic drugs. From many centuries different types of plants are used as source of potential and powerful drugs and millions of people has benefited from such natural blessing of Almighty Allah. The importance of traditional medicines like Ayurveda, Siddha, Unani and Homeopathy has pre-history in sub-continent of Indo-Pak. According to survey conducted by WHO, 80% of world population depends on folk and traditional medicines instead of allopathic, primarily due to commercially synthesized and cost effective medicines like antibiotics and secondly the antibiotic resistant clinical pathogens due to misuse of antibiotics (WHO, 2001; Aibinu et al., 2003; Aibinu et al., 2004). The depriveness of such expensive and low efficacy drugs have increased mortality rate particularly Morbidity rate (Williams, 2000). Due to frequent and excess use of commercially synthetic drugs like expensive and ineffective antibiotics make pathogens more resistant. So, efficacy of such drugs becomes very low and also has adverse side effects on the body. So for, safe, effective, cheaper and no side effect treatment of common infectious diseases with alternative source of medicines, including crude plant extracts with potential antimicrobial and other ethnopharmacological abilities, should be discovered. There is necessary to determine alternative substances from sources with proved antimicrobial activity (Pretorius et al., 2003; Moreillion et al., 2005). Some important secondary metabolites of low molecular weights isolated from plant source by aqueous or organic solvents extraction method or steam distillation method. According to estimation, more than 110 such crude chemicals are commonly involved in drug synthesis throughout the world. As there are number of medicinal plants and plant parts are used as extensive source of novel medicines to treat numerous devastating diseases which are commonly caused by harmful human pathogens.

Among medicinal plants, Ficus genus belongs to family Moraceae are re-known medicinally important group which has over 800 species with shrubs, vines and woody trees habits present in most sub-tropical and tropical zone in almost all parts of world (Hameed, 2006). The Ficus genus is collectively named as Fig tree or common Fig. There are more than 500 species of Ficus genus in Asia and 29 native species are reported in Pakistan. The most common species in Pakistan are *F. carica* L., *F. benghalensis* L., *F. religiosa* L., *F. palmate* Forsok, *F. elestica* Roxb. ex Hornem, and *F. auriculata* Lour. etc. Phytochemical screening of Ficus depicted number of useful chemical constituents with more important are phenolic (Abdel- Hameed, 2009; Veberic et al., 2008; Basudan et al., 2005; Lee et al., 2002). As Ficus species are potential source of different promising pharmacological activities like anti-histamine, anti-cancerous (Lansky et al., 2008; Kitajima et al., 1999) and antimicrobial like jaundice, epilepsy (Noumi and Fozi, 2003; Betti, 2004), toothache, whooping cough, tonsillitis, bacillary dysentery, bronchitis, and influenza enteritis are reported to be treated by Ficus extracts. Antioxidant activities were also reported for Ficus extracts (Abdel-Hameed, 2009; C.aliskan and Polat, 2011). There was a dire need to make a comprehensive study of antimicrobial activity against different clinical human pathogens including Bacteria and Fungi.

The antimicrobial activity of different plants have been explored various techniques such as by calculating diameter of zone of inhibition (ZI), minimum inhibitory concentration (MIC) against pathogens. The investigation of such bio-active compounds is done through phytochemical screening and pharmacological especially antimicrobial activity by using different methods like agar well diffusion and micro-dilution methods

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for mycofloral analysis (Tanveer et al., 2014). These methods have been proved good for determining antimicrobial potential of some taxa of Ficus from Bhimber area of Azad Kashmir.

The selected field area of the current study was district Bhimber Azad Kashmir, the gate way of entrance of Kashmir State by great Mughal emperors, 50 Km from Mirpur (Divisional Headquarter) and almost 60 km from Gujrat (district of Punjab Province) consist of mostly hilly areas of Peer panjal and Shiwalik ranges and part of sub tropical ecological zone (Ishtiaq et al., 2013). District Bhimber consists of three sub-divisions / Tehsils viz Smahni, Bhimber and Barnala. The samples of Ficus species were collected from different localities of all three Sub-divisions of District Bhimber Azad Kashmir.

The need of present study is to determine significance of Ficus species in terms of antimicrobial activity by using crude leaf extract against disastrous human pathogens (Westh et al., 2004). No doubt, there were already different research projects on assessment of antimicrobial activity by different solvent extracts of medicinal plants against harmful microorganisms have been done but in current investigations, antimicrobial activity of organic and aqueous plant extracts of important Ficus species of selected area against clinical human pathogens (bacteria and fungi) and calculation of susceptibility of such microbes through MIC was done first time.

The major objective of current study was to determine antibacterial and antifungal activity of plant extract of some important species of genus Ficus from district Bhimber Azad Kashmir against different human pathogens. The secondary objective of this research was to measure the MBC and MFC for comparison of different extracts utilized and different species used and recommend the best solvent for extraction.

Materials and Methods

Collection of plant material

Samples of Ficus species were collected from different localities of all the three Tehsils i. e. Samahni, Bhimber and Barnala of district Bhimber AJK. Plant samples were identified by and authenticated by renowned taxonomist (*Dr M Ishtaiq*) of Botany Department MUST, AJK and herbarium specimens were placed in Departmental herbarium.

Culture and maintenance of microorganisms

Pure stock cultures of all experimental bacteria and fungi were obtained from the Biotechnology Department, main campus MUST, Mirpur Azad Kashmir. The pure bacterial cultures were maintained on nutrient agar medium (NA) and fungal culture on potato dextrose agar medium (PDA). Each bacterial and fungal culture was further maintained by sub-culturing regularly on the above mentioned medium and stored at 4 °C before use in experiments. Dilutions of the inoculums were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculums.

Preparation of the extract

The leaves of selected Ficus species were cleaned, chopped into small pieces and shade dried in open air at room temperature for 8-10 days. The dried leaves were then powdered using electric grinder and stored in air tight polythen bags for further

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antimicrobial investigations. Solvent like Petroleum Ether, Chloroform, Methanol and Water were used for leaf extraction of Ficus species.

Maceration optimization

Maceration is well-known extraction method. Plant material 10 gm is mixed in each solvent like petroleum ether, chloroform, methanol and aqueous (100 ml) and it is placed for few days (Handa et al*.,* 2008). Components are taken into pre weighed empty boxes after filtering with Whaatman no.1 filter paper. The filtrate should be air dried completely then the components were weighed to know the crude extract content. All extracts were stored in sterile glass bottles at room temperature until further use.

Microbiological screening

Antimicrobial activities of different extracts were evaluated by agar well diffusion method (Murray et al., 1995) modified by (Olurinola, 1996) and minimum inhibitory concentration (MIC) (Kelmanson et al., 2000). The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values were determined by serial micro dilution assay.

Agar well diffusion method

For agar well diffusion method (Murray et al., 1995, later modified by Olurinola, 1996) antimicrobial susceptibility was tested on solid (Agar-agar) media in Petri plates. For bacterial assay nutrient agar (NA) (40 gm/L) and for fungus PDA (39 gm/L) was used for developing surface colony growth. All the media prepared was then sterilized by autoclaving the media at 121°C for 20 min.

Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with 8 hour old - broth culture of respective bacteria and fungi. Wells (10 mm diameter and about 2-3 cm apart) were made in each of these plates using sterile corkborer. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different solvents viz. Petroleum Ether, Chloroform, Methanol, and Water. About 100 µl of different concentrations of plant solvent extracts were added through sterile syringe or micropipette into the wells and allowed to diffuse at room temperature for 2 hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens and 28°C for 48 hours fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded.

Micro dilution method

The minimum inhibitory concentration (MIC) is the lowest concentration able to inhibit any visible bacterial growth on the culture plates. Serial dilutions of products are made in bacterial and fungal growth media. The test organisms are then added to the dilutions of products, incubated and stored for growth. This procedure is a standard assay for antimicrobials (WHO, 2006).

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MIC is important in diagnostic laboratories to confirm resistance of microorganisms against antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC is used, clinically, not only to determine amount of antibiotic that patient will receive but also type of antibiotic used, which in turn lowers the opportunity for microbial resistance to a specific antimicrobial agents (Mitscher et al.,1972).

The minimum inhibitory concentrations (MIC), MBC and MFCs were performed by a serial dilution technique using 96-well micro titer plates. The different plant extracts viz., Petroleum Ether, Chloroform, methanol and Aqueous were taken (1 mg/ml) and serial dilution of the extract with Luria broth for bacterial culture and for fungus, potato dextrose broth medium with respective inoculums were used. The micro plates were incubated for 72 hours at 28 °C, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC (Gautam et al., 2007).

The MBCs were determined by serial sub-culturing of 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 72 hours. The lowest concentration with no visible growth was considered as MBC, indicating 99.5 % killing of original inoculum and compared with standards tetracycline for Bacteria control. The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of 2 µl into microtitre plates containing 100 µl of broth per well and further incubation 72 hours at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5 % killing of original inoculums. Penicillin was used as positive controls (1–3000 µg/ml) for fungi. All experiments were performed in duplicate and repeated three times (Mitscher et al., 1972).

Results

The antimicrobial activity of leaf extract of some important species of Ficus genus (viz. *F. racemosa, F. auriculata, F. palmata vaerigata* and *F. religiosa*) of study area was determined by Agar well diffusion method and micro dilution method in different polar and non-solvents (Petroleum Ether, Chloroform, Methanol and distilled waters) against different clinical human pathogens of Bacteria and Fungi. Antimicrobial activity of some selected Ficus species against different microbial pathogens were evaluated by zone of inhibition (ZI) and Activity Index (AI) as shown in *Tables 1a-4a*. Minimum inhibitory concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum fungicidal concentration (MFC) was also calculated (*Tables 1b-4b*).

The result of potential antimicrobial activity of different species of Ficus genus in terms of zone of inhibition against clinical human pathogens were compared with ZI value of standard antibiotics i.e. penicillin (1 mg/disc) and tetracycline (1 mg/disc). The methanolic extract of *F. racemosa* showed maximum activity against *S. aerus* with ZI (19.3mm) and AI (0.97) and moderate activity against *E. coli* with ZI (14.1mm) and AI (0.94) but *P. aeruginosa* proved as most resistant strain among all experimental bacteria with ZI (17.1mm) and AI (0.80). After the methanol, chloroform has moderate antibacterial activity with diameter of ZI (12.3mm) and AI (1.11) and Petroleum ether has ZI (11.1mm) with AI (1.05) against *E. coli.* The most resistant bacterial pathogen were *P. aeruginosa* and *S. aerus* have ZI (12.9mm) with AI (0.60) and ZI (102.7mm) with AI (0.59), respectively. The aqueous extract of *F. racemosa* showed maximum ZI (8.9mm) with AI (0.70) against *E. coli* and *S. aerus* proved as most resistant among bacterial strains with 8.1mm ZI and 0.42 AI (*Table 1a*)

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On the other end, methanol extract of *F. racemosa* considered as most valuable antifungal agent than all other solvent extracts with maximum ZI diameter (19.3mm) and AI (1.21) against *C. albicans* and least ZI (16.7mm)with AI(0.78) against *F. solani* considered as more resistant fungal stain. While *A. flavus* showed moderate resistance against methanol extract of *F. racemosa* has ZI (18.1mm) with AI (1.06). Chloroform and petroleum ether, next to methanol, proved as moderate antifungal agent with maximum control against *C. albicans* have ZI(16.5mm) with AI (1.03) and ZI (16.1 mm) with AI (1.02), respectively. The *F. solani* again proved as most resistant fungal strain have ZI (15.1mm) with AI (0.70) and ZI (14.9 mm) with AI (0.70) against chloroform and petroleum ether solvent extract, respectively. Aqueous extract showed the least effect against *C. albicans* with ZI (10.6 mm) and AI (0.68) and *F. solani* proved as most resistant with ZI (9.7 mm) and AI (0.45) (*Table 1a* and *Fig. 2*).

By developing a sub-culture on fresh NA medium for bacteria and on PDA for fungi by diluting used extract for one day to determine MBC and MFC. The least Minimum Inhibitory Concentration against Bacteria strains was observed as 40.3 µg/ml in methanol extract of *F. racemosa* against *P. aeruginosa* with 82.3 µg / ml in methanol extract as MBC value and among experimental fungal strains 40.8 µg / ml MIC in methanol extract of *F. racemosa* against *F. solani* and 96.3 µg / ml in methanol extract as MFC (*Table 1b*).

The methanol extract of *F. auriculata* showed maximum antimicrobial activity against bacteria and fungi. Maximum antibacterial activity was observed against *S. aerus* with ZI (18.3 mm) and AI (0.94) and minimum ZI (17.1 mm) and AI (0.90) against *P. aeruginosa.* In terms of antifungal activity *F. solani* again proved as most resistant fungal strain among all experimental fungi with ZI (16.9 mm) and AI (0.78) by aqueous extract of *F. auriculata* and maximum antifungal agent against *C. albicans* with ZI (19.8 mm) and AI (1.21). Similarly, aqueous solvent extract of *F. auriculata* showed least antimicrobial activity with maximum ZI (8.9 mm) and AI (0.70) against *E. coli* and maximum ZI (10.6 mm) with AI (0.68) against *C. albicans*. In the contrast, *P. aeruginosa* bacterium and *F. solani* fungus were proved most resistant strains than other against aqueous extract of *F. auriculata* with minimum ZI (10.4 mm) and AI (0.45) and ZI (10.2 mm) with AI (0.45), respectively (*Table 2a* and *Fig. 2*).

While the methanol extract of *F. auriculata* showed 37.9 µg/ml least MIC value against *E. coli* among all experimental bacterial pathogens with 79.4 µg/ml in methanol extract as MBC value and among fungal pathogens the least MIC was observed against *C. albicans* 48.6 µg/ml in methanol extract and 97.1 µg/ml in methanol extract as MFC (*Table 2b* and *Fig. 2*).

Similarly, the methanol extract of *F. palmata vaerigata* showed maximum antibacterial and antifungal activity than remaining other solvent extracts with maximum ZI (20.7mm) and AI (1.73) against *E. coli* and maximum antifungal ZI (18.1mm) with AI (1.21) against *C. albicans*, respectively. The least activity was shown by aqueous extract of *F. palmata vaerigata* as maximum antibacterial agent against *E. coli* with ZI (7.9 mm) and AI (0.70) and maximum antifungal agent against *C. albicans* with ZI (9.7 mm) and AI (0.68). P. aeruginosa proved more resistant bacterial strain with ZI (7.3 mm) and AI (0.33) and *F. solani* was more resistant fungus than other experimental fungi with ZI (10.4 mm) and AI (0.48) against aqueous extract of *F. palmata virigata* (*Table 3a*)

Similarly, least MIC value as 38.6 µg/ml in methanol extract of *F. palmata vaerigata* was observed against *E. coli* among bacterial strains with 79.1 µg/ml in methanol

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extract as MBC and among fungal pathogens the least MIC in methanol extract of *F. palmata* was observed against *F. solani* as 47.4 µg/ml with 97.3 µg/ml in methanol extract as MFC (*Table 3b*).

The maximum ZI (16.3mm) with AI (1.73) of methanolic extract of *F. religiosa* against *E. coli* and maximum antifungal activity with ZI (18.4mm) and AI (1.21) against *C. albicans* fungal pathogen. The minimum antibacterial value of aqueous extract of *F. religiosa* was observed ZI (18.2mm) with AI (0.82) against *S. aerus* and most resistant fungal pathogen was *F. solani* with ZI (9.3 mm) and AI (0.45) against aqueous extract of *F. religiosa.* (*Table 4a*).

In the same way, least MIC was observed as 40.5 µg / ml of methanol extract of *F. religiosa* against *E. coli* among bacterial strains and 83.6 µg / ml in methanol extract as MBC and 49.9 µg/ml in methanol extract as least MIC against *F. solani* and 97.6 µg/ml in methanol extract as MFC (*Table 4b*).

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Table 1a. *Antimicrobial activity (zone of inhibition in mm and activity index) of various extracts of Ficus racemosa against clinical pathogens along with controlled antibiotics*

Micro-Organism	**Bacteria**									**Fungi**								
Extract Solvent	*S. aereus*			*E. coli*			*P. aeruginosa*			*A. flavus*			*F. solani*			*C. albicans*		
	ZI mm	AI	St ZI Mm	ZI Mm	AI	St ZI Mm	ZI Mm	AI	St ZI Mm	ZI mm	AI	St ZI Mm	ZI mm	AI	St ZI Mm	ZI Mm	AI	St ZI mm
Petroleum ether	13.9± 0.35	0.70	19.6 ±0.3 5	11.1 ±0.4 0	1.05	14.5 ±0.2 0	12.7 ±0.4 0	0.59	21.5 ±0.3 0	16.5 ±0.4 0	0.97	17.0 6±0. 30	14.9 ±0.7 0	0.70	21.1 ±0.5 0	16.1 ±0.3 0	1.02	15.7 ±0.2 0
Chloroform	15.1± 0.25	0.72	19.5 ±0.4 0	12.3 ±0.6 0	1.11	14.4 ±0.4 0	12.9 ±0.2 0	0.60	21.3 ±0.3 0	16.8 ±0.7 0	0.98	17.0 7±0. 50	15.1 ±0.3 0	0.70	21.3 ±0.4 0	16.5 ±0.4 0	1.03	15.9 ±0.5 0
Methanol	19.3± 0.35	0.97	19.8 ±0.2 5	14.1 ±0.1 5	0.94	14.9 ±0.4 0	17.1 ±0.7 0	0.80	21.9 ±0.6 0	18.1 ±0.2 0	1.06	17.0 5±0. 70	16.7 ±0.6 0	0.78	21.2 ±0.4 0	19.3 ±0.5 1	1.21	15.6 ±0.4 0
Water	8.1± 0.25	0.42	19.1 ±0.3 0	8.9± 0.60	0.70	12.7 ±0.5 0	9.9± 0.20	0.45	21.7 ±0.6 0	11.2 ±0.2 0	0.65	17.0 9±0. 80	9.7± 0.40	0.45	21.5 ±0.5 0	10.6 ±0.7 1	0.68	15.4 ±0.3 0

IZ= Inhibition zone (in mm) includes the diameter of disc (6 mm); Standards: Tetracycline (1.0 mg/disc), Penicillin (1.0 mg/disc); AI- activity index = IZ of test sample / IZ of standard; Values are mean of triplicate readings (mean ±0.05 S.D

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Table 1b. *MIC (µg / ml), MBC and MFC performance of different extracts of Ficus racemosa against pathogenic organisms*

Microorganism	**Bacteria**						**Fungi**					
Microbial Strains	*S. aereus*		*E. coli*		*P. aeruginosa*		*A. flavus*		*F. solani*		*C. albicans*	
Extract Solvent	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC
Pet. Ether	47.2±0.50	94.1±0.30	45.5±0.10	94.8±400.	44.7±0.50	95.1±0.30	57.5±0.30	114.2±0.30	58.3±0.40	116.4±0.70	55.8±0.70	99.1±0.20
Chloroform	46.9±0.40	91.7±0.50	45.1±0.70	93.1±0.70	43.8±0.30	88.7±0.50	56.7±2.05	113.9±0.50	57.7±0.60	115.8±0.70	55.1±1.20	101.2±0.90
Methanol	42.1±0.80	85.6±0.30	41.5±0.30	78.2±0.70	40.3±0.40	82.3±0.50	52.2±0.50	103.1±1.60	48.7±0.50	96.3±0.60	49.1±0.70	98.3±0.80
Aqueous	49.6±3.46	96.8±0.20	47.1±0.30	95.3±0.40	46.3±0.30	97.1±0.20	56.1±0.60	113.1±0.65	58.7±0.40	115.2±0.50	48.4±0.60	98.8±0.70

Values are mean of triplicate readings (mean ±0.05 S.D).

Table 2a. *Antimicrobial activity (zone of inhibition in mm and activity index) of various extracts of Ficus auriculata against clinical pathogens along with controlled antibiotics*

Microorganism	**Bacteria**									**Fungi**								
Extract Solvent	*S. aereus*			*E. coli*			*P. aeruginosa*			*A. flavus*			*F. solani*			*C. albicans*		
	ZI mm	AI	St ZI mm	ZI Mm	AI	St ZI Mm	ZI Mm	AI	St ZI Mm	ZI mm	AI	St ZI Mm	ZI mm	AI	St ZI Mm	ZI Mm	AI	St ZI mm
Petroleum ether	14.3 ±0.5 0	0.72	19.6 ±0.7 0	13.3 ±0.8 0	0.85	14.1 ±0.6 0	12.9 ±0.3 0	0.60	21.5± 1.10	15.1±0 .90	0.97	17.06 ±1.15	14.7±1. 30	0.70	21.1±1. 0	16.6± 1.30	1.02	15.7± 1.40
Chloroform	14.9 ±0.5 0	0.76	19.5 ±0.6	12.9 ±1.1 0	0.83	14.2 ±0.7 0	13.1 ±0.6 0	0.61	21.3± 0.80	15.9±1 .90	0.98	17.07 ±1.46	15.0±0. 40	0.70	21.3±1. 40	17.6± 1.20	1.03	15.9± 1.70

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Methanol	18.3 ±0.5 0	0.94	19.3 ±0.4	13.9 ±0.7 0	0.92	14.9 ±0.7 0	18.1 ±0.8 0	0.90	21.9± 1.80	17.1±0 .80	1.06	17.05 ±1.82	16.9±1. 60	0.78	21.2±0. 90	19.8± 0.50	1.21	15.6± 1.30
Water	9.1± 1.30	0.47	19.1 ±1.0	8.9± 0.40	0.70	14.7 ±0.5 0	10.4 ±1.2 0	0.45	21.7± 1.10	11.8±0 .90	0.65	17.09 ±0.88	10.2±1. 10	0.45	21.5±0. 80	10.6± 1.10	0.68	15.4± 1.10

IZ= Inhibition zone (in mm) includes the diameter of disc (6 mm); Standards: Tetracycline (1.0 mg/disc), Penicillin (1.0 mg/disc); AI- activity index = IZ of test sample / IZ of standard; Values are mean of triplicate readings (mean ±0.05 S.D).

Table 2b. *MIC (µg / ml), MBC and MFC performance of different extracts of Ficus auriculata against pathogenic organisms*

Microorganism	**Bacteria**						**Fungi**					
Microbial Strains	*S. aereus*		*E. coli*		*P. aeruginosa*		*A. flavus*		*F. solani*		*C. albicans*	
Extract Solvent	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC
Pet. Ether	48.5±0. 40	88.1±0.5 0	46.4±0.7 0	94.8±0.3 0	45.1±0.4 0	97.9±0.90	58.1±1.8 9	115.8±1.5 0	58.8±0.2 0	117.2±0.6 0	54.9±1.5 0	99.7±.1.5 0
Chloro- Form	47.2±0. 30	86.3±0.4 0	45.7±0.6 0	95.7±0.4 0	43.3±0.8 0	97.1±0.70	55.5±1.4 0	113.6±1.2 0	57.9±1.3 0	114.8±1.4 0	54.3±1.2 0	98.9±1.4 0
Metha- Nol	42.4±0. 60	85.8±1.7 0	39.9±1.1 0	79.4±0.5 0	38.6±0.6 0	94.5±1.20	53.8±1.5 0	105.1±1.5 0	48.4±1.0 0	98.3±1.00	48.6±1.0 0	97.1±0.9 0
Aque- Ous	50.1±0. 50	97.8±0.3 0	45.1±0.7 0	96.2±1.5 0	49.0±0.5 0	100.3±1.0 0	56.1±0.8 0	116.2±1.3 0	59.3±0.9 0	116.1±0.7 0	55.4±2.0 0	99.2±1.0 5

Values are mean of triplicate readings (mean ±0.05 S.D)

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Table 3a. *Antimicrobial activity (zone of inhibition in mm and activity index) of various extracts of Ficus palmata against clinical pathogens along with controlled antibiotics*

Microorganism	**Bacteria**									**Fungi**								
	S. aereus			*E. coli*			*P. aeruginosa*			*A. flavus*			*F. solani*			*C. albicans*		
Extract Solvent	ZI mm	AI	St ZI mm	ZI Mm	AI	St ZI mm	ZI Mm	AI	St ZI mm	ZI mm	AI	St ZI Mm	ZI Mm	AI	St ZI Mm	ZI Mm	AI	St ZI mm
Petroleum ether	12.8 ±0.70	0.70	19.6 ±0.70	12.9 ±0.40	1.05	14.4 ±0.50	11.9 ±0.70	0.59	21.5 ±0.70	15.1 ±0.46	0.97	17.06 ±0.28	14.3± 0.60	0.70	21.5± 0.70	15.1 ±0.40	1.02	15.5 ±0.60
Chloroform	13.3 ±0.90	0.72	19.5 ±0.60	12.1 ±0.80	1.11	14.2 ±0.70	12.3 ±0.80	0.60	21.3 ±0.40	15.9 ±0.90	0.98	17.0± 0.19	15.9± 1.66	0.70	21.3± 0.40	15.8 ±0.60	1.03	15.9 ±1.70
Methanol	17.8 ±1.20	0.84	19.3 ±1.20	16.7 ±0.70	1.73	14.9 ±1.30	15.6 ±0.80	0.90	21.9 ±1.40	18.1 ±0.70	1.06	17.05 ±0.60	17.3± 0.50	0.78	21.9± 0.50	18.1 ±0.80	1.21	15.3 ±1.40
Water	8.6± 0.80	0.42	19.1 ±0.55	7.9± 0.60	0.70	12.7 ±0.60	7.3± 0.40	0.45	21.7 ±1.40	11.8 ±0.40	0.65	17.09 ±0.88	10.4± 1.20	0.45	21.5± 0.70	09.7 ±0.90	0.68	15.1 ±0.70

IZ= Inhibition zone (in mm) includes the diameter of disc (6 mm); Standards: Tetracycline (1.0 mg/disc), Penicillin (1.0 mg/disc); AI-activity index = IZ of test sample / IZ of standard; Values are mean of triplicate readings (mean ±0.05 S.D

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Table 3b. *MIC (µg / ml), MBC and MFC performance of different extracts of Ficus palmata against pathogenic organisms*

Microorganism	**Bacteria**						**Fungi**					
Microbial Strains	*S. aereus*		*E. coli*		*P. aeruginosa*		*A. flavus*		*F. solani*		*C. albicans*	
Extract Solvent	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC
Pet. Ether	46.4±0.60	86.7±1.4	45.7±0.90	94.7±1.50	42.9±1.20	97.6±1.51	57.3±0.50	115.1±0.40	57.1±0.70	116.9±1.60	55.2±0.50	101.1±0.30
Chloroform	45.8±1.60	85.9±1.2	44.9±1.1	95.2±0.50	41.5±0.70	95.6±0.80	55.7±0.80	114.4±0.70	56.3±0.50	116.1±0.40	54.5±0.60	99.4±0.80
Methanol	41.7±0.90	84.3±0.60	38.6±1.62	79.1±0.30	40.8±1.40	92.9±1.20	51.1±0.40	102.7±1.40	47.4±1.20	97.5±1.30	50.3±1.40	97.3±1.10
Aqueous	48.9±1.30	95.2±0.40	46.4±0.60	95.8±0.50	43.4±0.80	99.8±0.50	58.0±0.60	116.9±1.60	58.7±0.80	117.1±0.30	58.3±0.70	103.7±1.40

Values are mean of triplicate readings (mean ±0.05 S.D)

Table 4a. *Antimicrobial activity (zone of inhibition in mm and activity index) of various extracts of Ficus religiosa against clinical pathogens along with controlled antibiotics*

Microorganism	**Bacteria**									**Fungi**								
Extract Solvent	*S. aereus*			*E. coli*			*P. aeruginosa*			*A. flavus*			*F. solani*			*C. albicans*		
	ZI mm	AI	St ZI mm	ZI Mm	AI	St ZI mm	ZI Mm	AI	St ZI mm	ZI Mm	AI	St ZI Mm	ZI mm	AI	St ZI Mm	ZI Mm	AI	St ZI mm
Petroleum ether	14.8 ±0.9	0.70	19.6 ±1.4	12.9 ±1.7	1.05	14.4 ±0.6	12.7 ±0.70	0.59	21.5 ±1.20	16.4 ±0.60	0.97	17.06± 0.12	14.2±0.50	0.70	21.1±1.20	16.7 ±1.32	1.02	15.7 ±1.50
Chloroform	15.9 ±1.7	0.72	19.5 ±0.8	13.2 ±0.7	1.11	14.2 ±0.6	12.9 ±0.70	0.60	21.3 ±0.5	17.3 ±0.40	0.98	17.07± 0.13	14.9±1.70	0.70	21.3±0.60	17.5 ±0.70	1.03	15.9 ±1.20

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Methanol	17.2 ±0.5	0.84	19.3 ±0.6	16.3 ±0.6	1.73	14.9 ±1.8	15.3 ±1.0 0	0.90	21.2 ±1.1 0	18.7 ±1.4 0	1.06	17.05± 0.20	16.5±0 .70	0.78	21.2±0 .80	18.4 ±0.5 0	1.21	15.6 ±0.8 0
Water	9.7± 1.4	0.42	18.9 ±0.7	8.9± 0.4	0.70	14.7 ±0.5	8.2± 0.70	0.45	21.7 ±0.6 0	11.8 ±1.6 0	0.65	17.09± 0.46	9.3±0. 50	0.45	21.5±0 .70	10.9 ±0.4 0	0.68	15.4 ±1.2 0

IZ= Inhibition zone (in mm) includes the diameter of disc (6 mm); Standards: Tetracycline (1.0 mg/disc), Penicillin (1.0 mg/disc); AI- activity index = IZ of test sample / IZ of standard; Values are mean of triplicate readings (mean ± 0.05S.D

Table 4b. *MIC (µg / ml), MBC and MFC performance of different extracts of Ficus religiosa against pathogenic organisms*

Microorganism	**Bacteria**						**Fungi**					
Microbial Strains	*S. aereus*		*E. coli*		*P. aeruginosa*		*A. flavus*		*F. solani*		*C. albicans*	
Extract Solvent	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC
Pet. Ether	48.1±060.	87.5±1.10.	49.5±1.30	95.4±0.40	43.7±0.60	97.7±1.33	57.9±0.40	115.7±0.50	59.6±0.70	116.7±1.00	56.2±0.70	101.9±1.20
Chloroform	47.6±0.90	85.7±1.10	48.2±1.10	94.9±120	42.9±0.60	96.2±0.40	57.3±1.20	114.3±0.60	58.3±0.50	114.9±1.50	55.8±1.70	100.8±0.60
Methanol	42.7±0.80	85.1±0.40	40.5±0.70	83.6±0.70	41.8±0.1.30	94.3±0.50	52.9±1.40	104.6±0.80	49.9±1.80	97.6±0.70	50.3±80	99.3±0.40
Aqueous	49.8±0.70	96.9±1.20	45.1±0.40	95.9±160	44.8±1.10	99.8±1.50	58.8±1.70	113.9±1.20	60.3±1.10	117.1±0.40	59.4±1.10	102.3±0.60

Values are mean of triplicate readings (mean ±0.05 S.D

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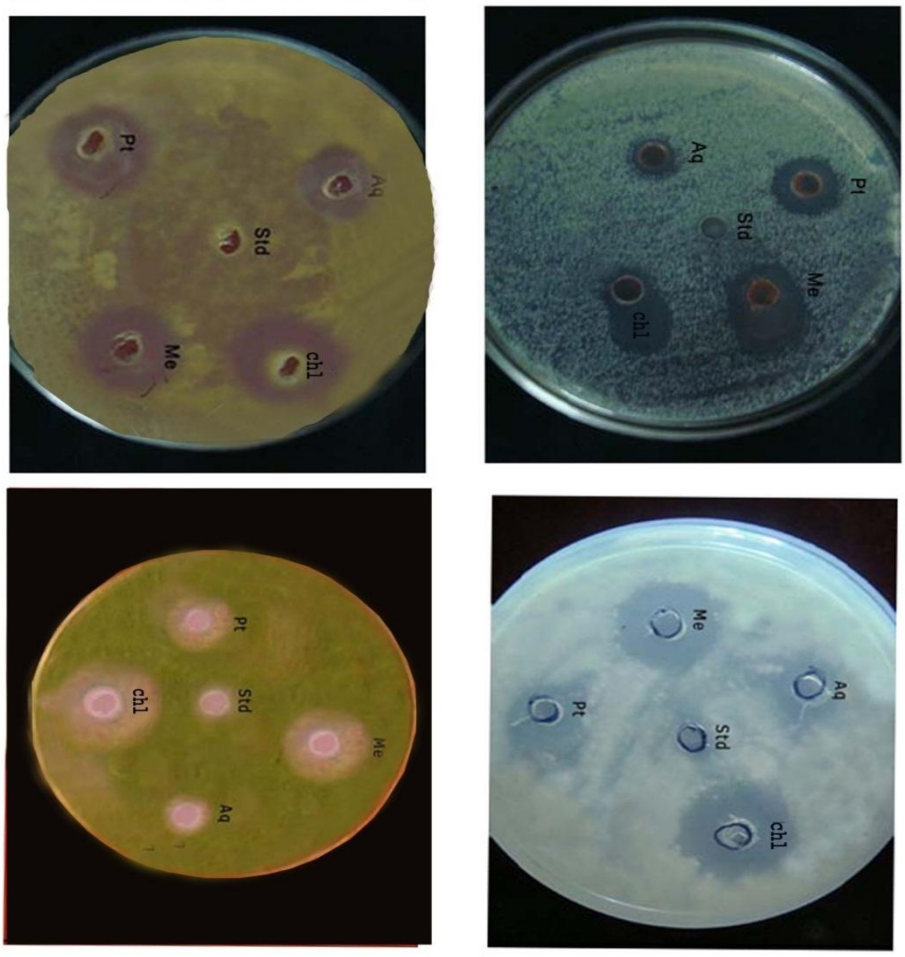

Figure 1. *Showing antimicrobial activity of : i. Escherichia coli, ii. Staphylococcus aerus, iii. Aspergillus flavus, iv. Fusarium solani*

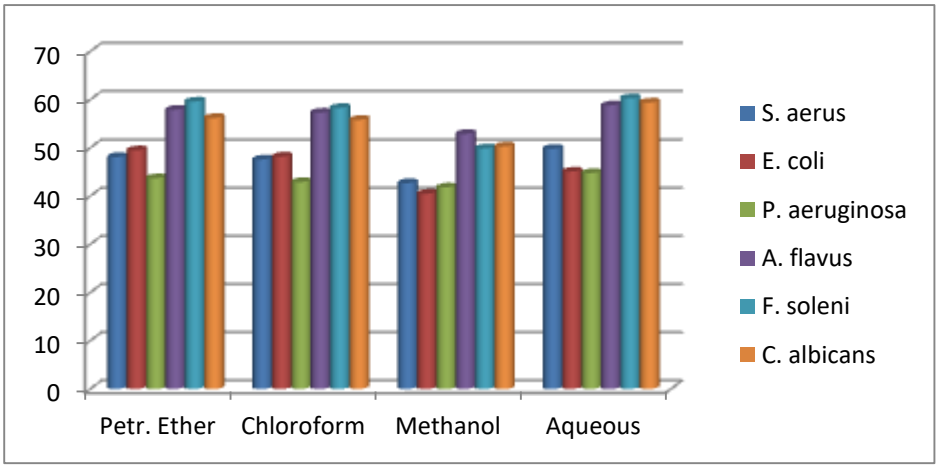

Figure 2. *Showing antimicrobial activity against selected fungi and bacteria with four different extracts*

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Discussion

The treatment of common ailments and diseases caused by clinical human pathogens has become unaffordable for a common man due to high cost and low efficacy of allopathic drugs. So there was severe need of finding alternative source of drugs and bioactive chemical compounds from plant source which have not merely served as alternative cheaper, less toxic and high effected antimicrobial agent but also more effective against highly resistant clinically tested pathogens. Therefore, comprehensive study is made on pharmaceutical activity of different clinical human pathogens. A number of plants have been investigated, as alternative source of medicinal drug especially antimicrobial agent (Kelmanson et al., 2000; Ahmad and Beg, 2001; Guleria et al., 2006; Zakaria et al., 2007).

The antimicrobial activity results depicted that all plant extracts have considerable activity against experimental human pathogens. Methanol extract has maximum antibacterial as well as antifungal activity against different experimental pathogens as the same results were also observed in different medicinal plants by different microbiologists (Ilango et al., 2009; Geethalakshmi et al., 2010; Rahman et al., 2011; Upadhyay et al., 2011).

The methanol extracts have most significant effect, against drug resistant microbial strains. The action of bioactive compounds of plant extracts is not yet known fully, but organic plant extract proved as more antimicrobial agent as compared to aqueous which showed presence of non-polar residual in extract showed strong abilities of bacteriostatic. These results were concised with Cowan (1999) that most antibiotic compounds in plant extracts are usually saturated organic compounds which easily soluble in organic solvent. Similar results also found in previous research of Preethi et al. (2010) and Seyydnejad et al. (2010). Similarly, methanol extract of *F. carica* was proved maximum potent antimicrobial agent than chloroform, petroleum ether which showed moderate activity and aqueous extract of *F. carica* was least potent agent. Many researchers reported that methanol extract was observed as most effective antimicrobial agent than chloroform and petroleum ether as mentioned in *Figure 1a and 1b* (Sekar et al., 2012).

The results of current research work indicated that *P. aeruginosa*, *E. coli* and *C. albicans* were most resistant pathogens against leaf extract of Ficus species as antimicrobial agent. The least effect of plant extracts of Ficus species as antimicrobial agent was observed by *S. aerus* and *F. soleni*. Chloroform and petroleum ether plant extracts proved as moderate antimicrobial agent than methanol and least effect was observed by aqueous extract of all selected species of Ficus. The same results were also reported by Murugesan et al., (2011) and several other studies that petroleum ether and aqueous extract also have considerable antimicrobial activity against many clinically isolated bacteria and fungi (Thatoi et al., 2008).

The methods employed in current research for assessment of antimicrobial activity were agar well diffusion and MIC of extracts of Ficus species against pathogenic microbial organisms were determined by Micro-dilution method. The same methods were also used by many researchers to find antimicrobial activity of different crude plant extracts against many pathogens (Arora et al., 2007; Gurudeeban et al., 2010; Pavithra et al., 2010). The MIC is an important feature of laboratories to diagnose resistance of tested pathogens and to check application of alternative antimicrobial agent other than antibiotics. The MIC of Ficus species extracts was found less than MBC and MFC values which were almost two fold than MIC, revealing that plant

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extracts of Ficus species were regarded as microbisidic at higher concentration and microbistatic with low concentration. Maji et al. (2010) were also analyzed similar findings in past. They explain that ficus species crude extracts showed high concentration as microbisidic and low concentration as microbistatic.

Conclusion

The conclusion of current research study is that analyzed selected species of Ficus possess bioactive chemical compounds which depict potential antimicrobial activity against different pathogens. The extract of leaf obtained from methanol; showed better results than other solvents. The maximum inhibitory activity was found against *E. coli* bacterium and *C. albicans* fungi. The *F. racemosa* and *F. auriculata* were found the best ficus with maximum antimicrobial potential. These herbal recipes can serve as great antimicrobial potential drug in pharmaceutical industry to avoid and protect many harmful infectious. All used solvent extract of Ficus species have significant inhibitory role against tested pathogens as compared to standard antibiotics. The results of the current study also favor the folklore importance along with positive directives for synthesis of new antimicrobial medicine from different these Ficus species.

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REFERENCES

[1] Abdel, H.E.S. (2009): Total phenolic contents and free radical scavenging activity of certain Egyptian Ficus species leaf samples. - Food Chem. 114: 1271–1277.
[2] Ahmad, I., Beg, A.Z. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multiple drug resistant human pathogens. - J. Ethanopharma 74: 113-123.
[3] Aibinu, I., Adenipekun, E., Odugbemi, T. (2004): Emergence of Quinolone Resistance amongst *Escherichia coli* strains isolated from clinical infections in some Lagos State Hospitals in Nigeria.- Nigerian Journal of Health and Biomedical Science 3(2): 73-78.
[4] Aibinu, I., Adenipekun, E., Odugbemi, T. (2004): Emergence of Quinolone Resistance amongst *Escherichia coli* strains isolated from clinical infections in some Lagos State Hospitals in Nigeria.-Nigerian Journal of Health and Biomedical Science 3(2): 73-78.
[5] Aibinu, I.E., Ohaegbulam, V.C., Adenipekun, E.A., Ogunsola, F.T., Odugbemi, T.O., Mee, B.J. (2003): Extended-Spectrum Beta-Lactamase Enzymes in Clinical Isolates of *Enterobacter* species from Lagos, Nigeria. - Journal of Clinical Microbiology 41(5): 2197-2200.
[6] Alikan, O.C., Polat A.A. (2011): Phytochemical and antioxidant properties of selected fig (*Ficus carica* L.) accessions from the eastern Mediterranean region of Turkey.- Sci. Hortic. 128: 473–478.
[7] Arora, D.S., Kaur G.J. (2007): Antibacterial activity of some Indian medicinal plants.- Journ. Nat. Med. 61: 313–317.
[8] Basudan, O.A., Ilyas, M. Parveen, M. Muhisen, H.M.H., Kumar, R. (2005): A new chroming from *Ficus lyrata*.- Asian Nat. Prod. Res. 7: 81–85.
[9] Betti, J.L. (2004): An ethnobotanical study of medicinal plants among DJA biosphere reserve, Cameroon. African Study Monogr. 25: 1–27.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_159176

[10] Gautam, R., Saklani, A., Jachak, S.M. (2007): Indian medicinal plants as a source of antimycobacterial agents. - J. Ethnopharmacol. 110: 200–234.
[11] Cowan, M. (1999): Plant products as antimicrobial agents. - Clinical Microbiology 12: 564-582.
[12] Geethalakshmi, R., Sarada, D.V.L., Marimuthu, P. (2010): Evaluation of antimicrobial and antioxidant potentials of *Trianthema decandra* L. - Asian J. of Biotech. 2(4): 225-231.
[13] Guleria, S., Kumar, A. (2006). Antifungal activity of some Himalayan medicinal plants using direct bioautography. - J. Cell Mol. Bio. 5: 95-98.
[14] Gurudeeban, S., Rajamanickam, E., Ramanathan, T., Satyavani, K. (2010): Antimicrobial activity Of *Citrullus colocynthis* in Gulf of Mannar.- Int. J. of Curr. Res., 2: 078-081.
[15] Hameed, N., Sabbir, A. Ali, A., Bajwa, R. (2006): *In vitro* micropropagation of disease free rose (*Rosa indica)* L.- Mycopath. 4: 35-38.
[16] Handa, S.S., Khanuja, S.P.S., Longo, G., Rakesh, D.D. (2008): Extraction Technologies for Medicinal and Aromatic Plants. - International centre for science and high technology, Trieste, pp. 21-25.
[17] Ishtiaq, M., Ahmed, F. Maqbool, M., Hussain, T. (2013): Ethnomedicinal inventory of flora of Maradori valley, district Forward Khahuta, Azad Kashmir, Pakistan. - American Journal of Research Communication 1-23.
[18] Ilango, K., Chitra, V., Kanimozhi, P., Balaji, G. (2009): Antidiabetic, Antioxidant and Antibacterial Activities of Leaf extracts of *Adhatoda zeylanica*. Medic (Acanthaceae). - J. Pharm. Sci. & Res. (2): 67-73.
[19] Kelmanson, J.E., Jager, A.K., Vaan, S.J. (2000): Zulu medicinal plants with antibacterial activity. -J. Ethanopharmacol. 69: 241-246.
[20] Kitajima, J., Kimizuka, K., Tanaka, Y. (1999): New dammarane-type acetylated triterpenoids and their related compounds of Ficus pumila fruit. - Chem. Pharm. Bull., 47: 1138–1140.
[21] Lansky, E.P., Paavilainen, H.M. Pawlus, A.D., Newman, R.A. (2008): Ficus spp. (fig): Ethnobotany and potential as anticancer and anti-inflammatory agents. - J. Ethnopharmacol. 119: 195–213.
[22] Lee, J.H., Stein, B.D. (2011): Antimicrobial Activity of a Combination of *Mume fructus*, *Schizandrae fructus* and *Coptidis rhizoma* on enterohemorrhagic *Escherichia coli* O26, O111, and O157 and its effect on Shiga toxin releases.- Foodborne Pathogen Dis. 8(5): 643-646.
[23] Maji, S., Dandapat, P., Ojha, D., Maity, C., Halder, S.K., Das, P.K., Mohapatra, T., Pathak, K., Pati, B.R., Samanta, A., Mondal, K.C. (2010): In vitro antimicrobial potentialities of different Solvent extracts of ethnomedicinal plants against clinically isolated human pathogens. - Journal of Phytology 2(4): 57–64.
[24] Mitscher L.A., Harone, J.B., Irvine, F.R. (1972): Antibiotics from Higher plants Introduction, rationale and Methadology. - J. Nat. products 135(2): 257- 258.
[25] Moreillion, P., Que, Y.A., Glauser, M.P. (2005): *Staphylococcus aureu*s (Including Staphyloccal Toxic shock). – In: Mandell, G.L, Bennett, J.E, Dolin, R. (Eds.) Principles and Practice of Infectious diseases. Published by Churchill Livingstone Pennyslyvania, 6th ed. 2: 2333-2339.
[26] Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, H.R. (1995): Manual of Clinical Microbiology, 6th Ed. -ASM Press, Washington DC, 15-18.
[27] Murugesan, S., Pannerselvam A., Chanemougame, T. (2011): A Phytochemical screening and Antimicrobial activity of the leaves of *Memecylon umbellatum* Burm. - F.J. of App. Pharma. Sci. 1 (1): 42-45.
[28] Noumi, E., Fozi, F.L. (2003): Ethnomedical botany of epilepsy treatment in fongo-tongo village, western Province. - Cameroon Pharm Bio. 41: 330–339.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_159176

[29] Nostro, A., Germano, M.P., Angelo, V.D., Marino, A., Cannatelli, M.A. (2000): Extraction methods and bio-autography for evaluation of medicinal plant antimicrobial activity. - Lett. Microbiol. 30(1): 379-384.
[30] Olurinola, P.F. (1996): A laboratory manual of pharmaceutical microbiology. - Idu, Abuja, Nigeria 69-105.
[31] Pavithra, P.S., Janani, V.S., Charumathi, K.H., Indumathy, R., Potala, S., Verma, R.S. (2010). Antibacterial activity of the plant used in Indian herbal medicine. - Int. J. of green pharma. 10: 22-28.
[32] Preethi, R., Devanathan, V.V., Loganathan, M. (2010): Antimicrobial and Antioxidant Efficacy of Some Medicinal Plants Against Food Borne Pathogens. - Adv. in Bio. Res. 4 (2): 122-125.
[33] Pretorius, J.C., Magama, S., Zietsman, P.C. (2003): Growth inhibition of plant pathogenic bacteria and fungi by extracts from selected South African plant species.- South African Journal of Botany 20: 188-192.
[34] Rahma, M.S., Salehin, M.F., Jamal, M.A., Pravin, H.M., Alam, A. (2011): Antibacterial activity of *Argemone mexicana* L. against water brone microbes.- Res. J. of Medicinal plant 5(5): 621-626.
[35] Seyydnejad, S.M., Niknejad, M., Darabpoor, I., Motamedi, H. (2010): Antibacterial Activity of Hydroalcoholic Extract of *Callistemon citrinus* and *Albizia lebbeck.*- American J. of App. Sci. 7(1): 13-16.
[36] Sekar, D., Kolanjinathan, K., Saranraj, P., Gajendiran. K. (2012): Screening of *Phyllanthus amarus*, *Acalypha indica* and *Datura metel* for its antimicrobial activity against selected pathogens.- Int. J. Pharmaceut. Biol. Arch. 3: 1231–1235.
[37] Tanveer, H., Ishtiaq, M., Azam, S., Jawad, W., Haq, I.U. (2014): Comparative Analysis of Air, Soil and Water Mycoflora of Samahni Area, Distract Bhimber Azad Kashmir Pakistan. - African J. of Microbiology Research 8(23): 2295-2306.
[38] Thatoi, H.N., Panda, S.K. Rath, S.K., Dutta, S.K. (2008): Antimicrobial Activity and Ethnomedicinal Uses of Some Medicinal Plants from Similipal Biosphere Reserve, Orissa.- Asian J. of Plant Sci. 7: 260-267.
[39] Upadhyay, R.K., Tripathi, R., Ahmad, S. (2011): Antimicrobial activity of two Indian medicinal plants *Tinospora cordifolia* (Family: Menispermaceae) and *Cassia fistula (*Family: Caesalpinaceae*)* against human pathogenic bacteria. - J. of Pharma. Res. 4(1): 167-170.
[40] Westh, H., Zinn, C.S., Rosdahl, V.T. (2004): An international multicenter study of antimicrobial consumption and resistance in *Staphylococcus aureus* isolates from 15 hospitals in14 countries. - Microb. Drug Resist. 10: 169-176.
[41] Williams, R. (2000): Antimicrobial resistance a global threat. - Essential Drug Monitor 1: 28-29.
[42] World Health Organization (WHO). (2001): Traditional medicine. Fact sheet number 134. Revised May, 2003. (Available on http/www.who.int/media centre fact sheet/fs/134).
[43] Zakaria, Z., Sreenivasan, S., Mohamad, M. (2007): Antimicrobial Activity of *Piper ribesoides* Root Extract against *Staphylococcus aureus*.- J. App. Biol. Sci. 1 (3): 87-90.

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GENOME–WIDE ANALYSIS OF ETHYLENE RESPONSIVE FACTOR IN MAIZE: AN IN SILICO APPROACH

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Abstract. Transcription factors are usually considered as key player for gene regulation. Among various transcription factor families, AP2/ERF superfamily is well known for regulating various stress responses in plants. The family encompasses AP2/ERF domain, which is involved in DNA binding comprises of about 60 to 70 amino acids. To date, there is no detailed report presenting structural and functional prediction of ERF genes in *Zea mays* (L.). The current study presents a comprehensive genome-wide analysis of 105 ERF genes in maize (ZmERF) using several computational techniques. We performed phylogenetic analysis, conserved motif analysis, chromosomal localization, gene structure analysis and multiple sequence alignment of ERF genes. The phylogenetic analysis led to classification of these ERF members into 10 major groups and various subgroups and inferred evolutionary relationship among the groups on the basis of various protein motifs as well as intron/exon structure. The mapping of ERF genes on 10 maize chromosomes revealed their existence on all chromosomes with most number (17 genes) carried by chromosome 1 and least number (8 genes) found on chromosome 3. Interestingly, a very limited intron frequency was resulted in gene structure analysis. Gene ontology analysis concludes that ZmERF are involved in responses to various stresses including both biotic and abiotic. The results of the present study provide important structural information to design functional analyses of the ERF genes in *Z. mays.*

Keywords: *in silico study, phylogeny, gene structure analysis, chromosomal mapping, protein structure, motif analysis, Z. mays*

Introduction

Abiotic stresses are serious threat to agricultural crops and reduce their yield up to 50 % (Boyer, 1982). These stresses are regulated at cellular, biochemical, physiological and molecular level by adapted plants. Various stress responsive genes associated with molecular signaling have been discovered which are regulated and expressed at molecular level (Seki et al., 2003; Zhang et al., 2004). Transcription factors are referred as DNA binding proteins, which bind at cis-regulatory element and initiate transcription process. On the basis of function difference and similarities, transcription factors are classified into many families AP2/ERF, WRKY, ARF, FAR1 etc (Pabo and Sauer, 1992).

AP2/ERF play a key role in gene expression, related to cell proliferation, hormone secretion, plant reproduction, and biotic as well as abiotic stress responses (Hattori et al., 2009; Hinz et al., 2010). In past decade, AP2/ERF family has become more attentional gene family. AP2/ERF superfamily originated as a result of horizontal

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transfer from bacteria/viruses to plants (Magnani, 2004; Shigyo et al., 2006). This is one of the most important transcription factors having AP2-DNA binding domain (Kim et al., 2011), which consists of 60 to 70 amino acid residues first discovered in *Arabidopsis thaliana* (Jofuku et al., 1994). Later on, these genes were cloned in tobacco plants (Takagi and Shinshi, 1995). At present, AP2/ERF gene have been identified in several species of plants including barley, soya bean, grape, poplar, wheat, foxtail millet, peach, sorghum, brassica, maize, moso bamboo (Zhang et al., 2008; Zhang et al., 2012; Song et al., 2013; Yan et al., 2013; Du et al., 2014; Lata et al., 2014; Wu et al., 2015). AP2/ERF superfamily is further divided into three families: AP2, ERF, and RAV (http://planttfdb.cbi.pku.edu.cn) on the basis of the domain observed in it. AP2 family have two AP2/ERF domain and possess very important function in regulation of developmental process like leaf formation, flower (Elliott et al., 1996), embryo, ovule (Boutilier et al., 2002; Song et al., 2013) and fruit development (Zhang et al., 2012). AP2 family is further classified into AP2 and ANT subfamilies (Shigyo et al., 2006). ERF family have single AP2/ERF domain and carry crucial function in ethylene signal transduction in response to environmental stresses (Takagi and Shinshi, 1995; Dubouzet et al., 2003), pathogen related stimuli and regulated pathogenesis-related gene expression (Zarei et al., 2011). Recently, overexpression of ERF gene was studied in rice (Zhang and Huang, 2010), tomato and tobacco under saline and drought environment (Guo et al., 2004; Zhang and Huang, 2010). In RAV, in addition to one AP2/ERF domain, there is another DNA binding B3-like domain, which is plant specific binding and also present in other transcription factors (Kagaya, et al., 1999). Moreover, RAV also show ethylene response as well as brassinosteroid hormonal response (Alonso, 2003; Hu et al., 2004).

Further studies on ERF family shows its further division into two subfamilies: the CBF/DREB (dehydration response element binding) and ERF (Ethylene responsive factors) (Sakuma et al., 2002). These two sub families are further arranged into many groups, 15 in Rice and 12 in *Arabidopsis* (Nakano et al., 2006), 10 in grape (Licausi et al., 2010) 10 in cucumber (Hu and Liu, 2011) and 107 in *Z. mays* (Huang et al., 2014). Even high conservation in domain sequence, each family shows different DNA binding site. ERF subfamilies bind to the GCC box (AGCCGCC) (Takagi et al., 1995), where G2, G5 and C7 are considered to be the important residues (Hao et al., 2002), DREB bind TACCGACAT where C4, G5, and C7 are basic residues for binding (Jiang et al., 1996; Sakuma et al., 2002). AP2 binds to GCAC(A/G)N(A/T)TCCC(A/G)ANG(C/T) (Gong et al., 2008; Wilson and Krizek, 2000). The ERF-associated amphiphilic repression (EAR) motif was also identified in many species of plant like *Arabidopsis*, *Z. mays* and sorghum (Yan et al., 2013). The three dimensional analysis of AP2/ERF protein domain shows it consist of 3 anti-parallel beta-sheet and one alpha-helix (Yamasaki et al., 2013) arginine and tryptophan in beta-sheet have basic function in formation of GCC box binding domain and serine/threonine also important residue for DNA binding domain (Shanker et al., 2012).

Though, AP2/ERF superfamily has been thoroughly studied, yet there is no detailed report in *Z. mays* which could provide information about the structure, characteristic and function of each gene keeping in view the contribution of ERF members in plant stress regulation. Current study is focused on genome-wide analysis of ERF subfamily in *Z. mays*, which is the most important cereal crops in the world after wheat and rice. The crop has a nutritional value vital in our daily life (Verheye, 2010). The study encompasses computational analysis of ZmERF *viz.* analysis of conserved domain,

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phylogenetic evolutionary studies, gene structure analysis, chromosomal distribution, gene ontology and BLAST hits distribution of ERF genes among different plant species. This prediction-based study might help to design wet-lab experiments against abiotic stress-resistance in *Z. mays*. The study also extends significant information related to ZmERF genes to make possible solution of constraints in growing the crop in unfavorable ecological conditions.

Materials and methods

Identification and bioinformatics analysis of ZmERF protein

AP2/ERF superfamily is already studied in *Z. mays* which identified 107 ERF subfamily members (Du et al., 2014). These 107 ERF genes accession were used for current study. Sequences and related data of these genes were retrieved from different databases including Plant Transcription Database (http://planttfdb.cbi.pku.edu.cn/) (Jin et al., 2014), Phytozome (http://phytozome.jgi.doe.gov/) and maize GDB (http://www.maizegdb.org/) (Andorf et al., 2015). As result we were able to collect data and sequences of 105 ERF genes, excluding two ERF genes; GRMZM2G061227 and GRMZM2G16097I which were not found from these databases. New Annotation was also given according to their chromosomal location e.g GRMZM1.8 and GRMZM 6.5 is given to GRMZM2G039112 and GRMZM2G085964, respectively, where digits before point represents chromosome number and after point location of gene on respective chromosome in ascending order. Bioinformatics analysis of ERF genes was performed which involve amino acid (a.a) length, molecular weight (kDa), isoelectric point (Ip) using ExPASy server (http://www.expasy.ch/tools).

Conserved domain analysis and phylogenetic analysis

To observe conserved residues in AP2 domain, multiple sequence alignment of these 105 ERF genes was executed using CLC work bench software package (Knudsen et al., 2011) with parameters: gap opening cost: 10.0, gap extension cost: 0.1, end gap cost: free. The aligned sequences of ERF genes were used to construct phylogenetic tree in CLC viewer 7.6 (Knudsen et al., 2011) with neighbor joining method, Distance measure: Jukes-Cantor and Bootstrap: 1000 Replicates.

Analysis of ZmERF proteins motifs and gene ontology annotation

Motif analysis was conducted using MEME online software (Bailey and Elkan, 1994; Bailey et al., 2006). Attributes used to accomplish data analysis include; number of different motifs: 20, minimum motif width: 15, maximum motif width: 54 amino acids, distribution of the motif occurrence: zero or one per sequences. The gene ontology of ERF protein sequence was performed using BLAST GO (Conesa and Götz, 2008; Gotz et al., 2011) with default parameters. GO analysis of ZmERF described the biological process, species distribution, molecular function and cellular localization (Gotz et al., 2011). Databases employed to search out the sequence homologies were: NCBI non-redundant protein (Nr), NCBI nucleotide sequence (Nt), Protein family, Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-PROT protein, Cluster of Orthologous Groups (COGs) and Gene Ontology (GO).

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Chromosomal distribution, gene structure and sub-cellular localization of ZmERF genes

Location of all these genes on chromosomes is determined as per data of Phytozome and NCBI for instance, chromosome number, chromosome length and start position of gene. Graphical map is made by designing scale on the longest chromosome basis (chromosome number 5), and gene position on chromosome is considered as the gene annotation: like GRMZM1.14, GRMZM1.4 and GRMZM6.5 for AC206031, AC206951 and GRMZM2G085964, respectively. The genomic data was retrieved from phytozome and NCBI for gene gene structure analysis. Longest gene was found after sequence analysis to design a scale. The graphical representation of exon and intron was constructed according to numeric value and position of exon taken from NCBI. Prediction of sub-cellular localization was carried by two online bioinformatics tools namely WoLF PROST and Plant-mPLoc (Horton et al., 2007; Chou and Shen, 2010).

Results

Bioinformatics analysis of ethylene responsive factors

Comparative analysis of ERF genes in different species are given in *Table 1*, showing *Z. mays*, sorghum and peach have almost similar number of ERF gene e.g 107, 105 and 104 respectively. Chines cabbage and carrot have higher number of ERF, while *Arabidopsis*, moso bamboo, and foxtail millet have fewer number than *Z. mays* (*Table 1*). The individual genes of *Z. mays* are listed in *Table 2*, with their predicted features, including annotation chromosome number, exon number, intron number, gene length (ORF), amino acid (a.a) sequence length, number of exons, and isoelectric point. The length of genes ranged from 392 bp (GRMZM1.1) to 6700 bp (GMZM1.6), amino acid sequence length 130 (GRMZM1.1) to 2000 (GMZM1.6) a.a, exon number 1 to 6 (GRMZM1.7) and Ip ranged from 4.23 (GRMZM2.5) to 13.03 (GRMZM6.5) (*Table 2*).

Table 1. *Comparison of AP2/ERF genes in different species of plants*

Plants	***Total AP2/ERF***	***Classification***					***Reference***
		AP2	***ERF***	***DREB***	***RAV***	***Soloist***	
Foxtail millet	171	28	90	48	5	*	(Lata et al. 2014)
Chines cabbage	291	49	139	109	14	1	(Song, Li, and Hou 2013)
Peach	131	21	104	*	5	1	(C. H. Zhang et al. 2012)
Sorghum	126	16	105	*	4	1	(Yan et al. 2013)
Z. mays	184	22	107	51	3	1	(Du et al. 2014)
Moso bamboo	116	28	80	*	7	1	(Wu et al. 2015)
Carrot	267	38	143	71	12	3	(Yao et al. 2015)
Arabidopsis	147	18	65	57	6	1	(Sakuma et al. 2002)
Vitis venefera	149	20	86	36	6	1	(Licausi et al. 2010)
Oryza sativa	164	26	79	52	7	-*	(Wu et al. 2015)
Musa acuminata	265	67	119	81	16	3	(Lakhwani et al. 2016)
Musa balbisiana	318	71	144	99	22	4	(Lakhwani et al. 2016)

*Data not found in cited paper

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Table 2. *Ethylene responsive factors and their predicted characteristic in Z. mays*

New annotation	Accession #	ORF * Length	a.a*	Ip*	Chr #	Exons #	Intron #
GRMZM1.1	AC198979	392	130	8.46	1	1	0
GRMZM1.2	GRMZM2G129674	1667	302	5.15	1	3	2
GRMZM1.3	GRMZM2G018984	2001	340	4.91	1	2	1
GRMZM1.4	AC206951	482	160	11.65	1	1	0
GRMZM1.5	AC200038	1152	339	7.18	1	2	1
GRMZM1.6	GRMZM2G703514	6700	2000	10.58	1	5	4
GRMZM1.7	GRMZM2G461905	3971	1185	10.31	1	6	5
GRMZM1.8	GRMZM2G039112	1836	414	4.37	1	1	0
GRMZM1.9	GRMZM2G016079	837	195	10.94	1	1	0
GRMZM1.10	GRMZM2G480434	608	202	4.83	1	1	0
GRMZM1.11	GRMZM2G033656	857	242	7.93	1	1	0
GRMZM1.12	GRMZM2G309731	510	160	10.88	1	1	0
GRMZM1.13	GRMZM2G369472	1137	269	6.37	1	1	0
GRMZM1.14	AC206031	839	170	10.82	1	1	0
GRMZM1.15	GRMZM2G010100	1256	418	4.77	1	1	0
GRMZM1.16	GRMZM2G009598	1320	238	11.43	1	1	0
GRMZM1.17	GRMZM2G381441	1183	285	9.95	1	1	0
GRMZM2.1	GRMZM2G067463	623	177	5.35	2	2	1
GRMZM2.2	GRMZM2G068967	756	222	10.23	2	1	0
GRMZM2.3	GRMZM2G475678	1070	356	4.58	2	1	0
GRMZM2.4	GRMZM2G055180	1336	279	5.18	2	1	0
GRMZM2.5	GRMZM2G079825	1365	271	4.23	2	1	0
GRMZM2.6	GRMZM2G087059	2385	445	9.94	2	2	1
GRMZM2.7	GRMZM2G148333	2313	423	4.57	2	2	1
GRMZM2.8	GRMZM2G138396	1074	263	6.28	2	1	0
GRMZM2.9	GRMZM2G011236	1219	311	8.12	2	1	0
GRMZM2.10	GRMZM2G125460	1138	284	9.05	2	1	0
GRMZM2.11	GRMZM2G458437	939	296	7.06	2	1	0
GRMZM3.1	GRMZM2G142179	1519	354	4.49	3	1	0
GRMZM3.2	GRMZM2G133168	4945	201	12.14	3	1	1
GRMZM3.3	GRMZM2G169382	1274	227	7.34	3	2	1
GRMZM3.4	GRMZM2G105266	1839	299	7.58	3	1	0
GRMZM3.5	GRMZM2G149756	3202	357	6.24	3	2	1
GRMZM3.6	GRMZM2G002119	849	186	10.73	3	1	0
GRMZM3.7	GRMZM2G310368	1047	237	9.02	3	1	0
GRMZM3.8	GRMZM2G474326	1148	229	9.12	3	1	0
GRMZM4.1	AC213666	491	163	7.64	4	1	0
GRMZM4.2	GRMZM2G089995	955	203	7.35	4	1	0
GRMZM4.3	GRMZM2G060206	625	124	9.09	4	1	0
GRMZM4.4	GRMZM2G165257	1295	294	8.30	4	1	0
GRMZM4.5	GRMZM2G018398	2689	361	4.44	4	2	2
GRMZM4.6	GRMZM2G173771	1052	230	8.50	4	1	0
GRMZM4.7	GRMZM2G060465	823	253	7.87	4	1	0
GRMZM4.8	GRMZM2G076896	2637	329	730	4	2	1
GRMZM4.9	GRMZM2G146028	1284	208	8.14	4	1	0
GRMZM4.10	GRMZM2G052720	1413	196	5.29	4	1	0
GRMZM5.1	GRMZM2G024871	522	137	7.53	5	1	0
GRMZM5.2	GRMZM2G322672	2499	393	9.48	5	4	3
GRMZM5.3	GRMZM2G079653	1109	211	7.88	5	1	0
GRMZM5.4	GRMZM2G085678	1672	213	10.03	5	2	1
GRMZM5.5	GRMZM2G073258	1003	290	6.40	5	1	0
GRMZM5.6	GRMZM2G016434	2720	408	8.86	5	2	1

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GRMZM5.7	GRMZM2G047999	941	301	4.40	5	1	0
GRMZM5.8	GRMZM2G466044	1529	294	5.81	5	1	0
GRMZM5.9	GRMZM2G057386	1525	329	6.10	5	1	0
GRMZM5.10	GRMZM2G103085	1219	236	8.94	5	1	0
GRMZM5.11	GRMZM2G110333	2746	363	4.69	5	2	2
GRMZM6.1	GRMZM2G135452	989	173	6.96	6	2	1
GRMZM6.2	GRMZM2G328197	969	316	4.50	6	1	0
GRMZM6.3	GRMZM2G175543	1698	197	6.35	6	1	0
GRMZM6.4	GRMZM2G106591	1406	230	10.47	6	2	1
GRMZM6.5	GRMZM2G085964	1225	223	13.03	6	2	1
GRMZM6.6	GRMZM2G100727	1348	297	8.57	6	1	0
GRMZM6.7	GRMZM2G020016	1815	210	10.92	6	1	0
GRMZM6.8	GRMZM2G020150	1857	220	8.20	6	1	0
GRMZM6.9	GRMZM2G317596	1008	290	6.94	6	1	0
GRMZM7.1	GRMZM2G379652	989	329	6.34	7	1	0
GRMZM7.2	GRMZM2G478965	1088	362	5.79	7	1	0
GRMZM7.3	GRMZM2G019443	1022	322	9.64	7	1	0
GRMZM7.4	GRMZM2G171569	1449	307	10.06	7	1	1
GRMZM7.5	AC233933	788	262	10.25	7	1	0
GRMZM7.6	GRMZM2G123119	998	257	6.51	7	1	0
GRMZM7.7	GRMZM2G060517	608	136	8.45	7	1	0
GRMZM7.8	GRMZM2G363052	3043	283	4.42	7	1	4
GRMZM7.9	GRMZM2G052667	3074	418	5.17	7	2	2
GRMZM7.10	GRMZM2G131281	1041	227	9.79	7	2	1
GRMZM7.11	GRMZM2G018138	737	218	10.42	7	1	0
GRMZM7.12	GRMZM2G384386	1425	308	7.18	7	1	0
GRMZM7.13	GRMZM2G307119	1496	315	7.11	7	1	0
GRMZM7.14	GRMZM2G025062	1211	235	6.29	7	2	1
GRMZM8.1	GRMZM2G081892	2010	336	4.75	8	1	1
GRMZM8.2	AC187157	1101	291	7.68	8	2	1
GRMZM8.3	GRMZM2G044077	1597	325	4.43	8	1	0
GRMZM8.4	GRMZM2G053503	1177	230	8.87	8	2	1
GRMZM8.5	GRMZM2G021790	854	281	7.59	8	1	0
GRMZM8.6	GRMZM2G120401	1295	294	8.30	8	1	0
GRMZM8.7	GRMZM2G457562	1657	235	6.97	8	1	0
GRMZM8.8	GRMZM2G066158	1235	205	10.69	8	1	0
GRMZM8.9	GRMZM2G174347	1192	233	8.46	8	1	0
GRMZM8.10	GRMZM2G151542	2021	303	4.41	8	1	0
GRMZM8.11	GRMZM2G132223	855	165	10.29	8	1	0
GRMZM8.12	GRMZM2G132185	1121	241	9.36	8	1	0
GRMZM9.1	GRMZM2G171179	2960	363	4.97	9	2	2
GRMZM9.2	GRMZM2G156006	762	169	6.26	9	2	1
GRMZM9.3	GRMZM2G429378	947	315	4.75	9	1	0
GRMZM10.1	GRMZM2G544539	2253	349	9.42	10	2	1
GRMZM10.2	GRMZM2G020054	1295	186	7.92	10	1	0
GRMZM10.3	GRMZM2G055070	593	186	7.20	10	1	0
GRMZM10.4	GRMZM2G425798	2212	422	10.10	10	3	2
GRMZM10.5	GRMZM2G023708	1029	272	4.35	10	1	0
GRMZM10.6	GRMZM2G175525	761	253	9.88	10	1	0
GRMZM10.7	GRMZM2G080516	1955	270	5.73	10	1	0
GRMZM10.8	GRMZM2G438202	1671	222	10.52	10	1	0
GRMZM10.9	GRMZM2G104260	904	172	6.67	10	2	1
GRMZM10.10	GRMZM2G164591	1115	178	10.30	10	1	0

**ORF=Origin of replication frame, A.A= Amino acid, Ip=Isoelectric Point , Chr=Chromosome*

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Multiple sequence alignment

Analysis of AP2/ERF conserved domain showed that most of the sequences have conserved amino acid residues. The motif AAEIR is almost conserved in all amino acid sequences of ZmERF. Consensus sequence established that the active site Y41 is highly conserved in all sequences. G4, A37 and A38 sites also harbor conserved residues in most of the ERF protein sequences. At the N-terminal, residues are more conserved as compared to C-terminal residues of the domain (*Fig. 1*).

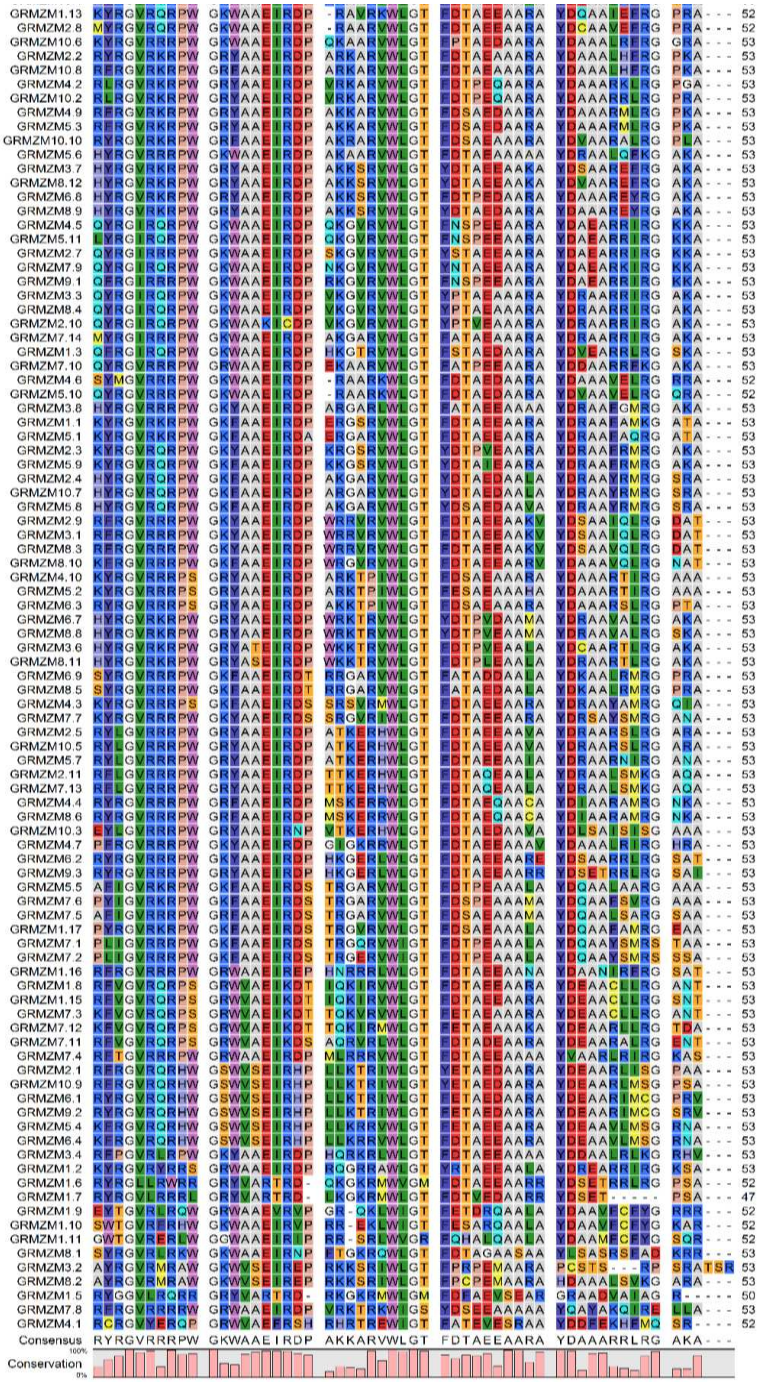

Figure 1. *Multiple sequence alignment of 105 ZmERF amino acid sequences, performed through CLC bio software package*

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Phylogenetic relationship between the ERF genes of Z. mays

Phylogenetic analysis revealed that 105 ERF genes are distributed into 38 sister groups and 29 genes are single gene. On the basis of phylogenetic analysis, ERF protein family is divided into 10 subgroups, namely I-X. Group I contain 4 ERF genes, II contain 7 genes, III 9 genes, IV has 12 genes, V has 9 genes, VI has 15 genes, VII have 9 genes, VIII have 15 genes, IX have 16 genes and X have 9 genes (*Fig. 2*). In order to better understand relative relationship of the same gene family among different species, an evolutionary analysis is performed between *Z. mays* (105 ERF genes) and Sorghum (53 ERF genes) (Yan et al., 2013), as both species belong to monocot and sorghum represented a model plant. Phylogenetic tree showed all *Z. mays* and sorghum ERF genes are distributed into 59 sister groups, containing 27 ZmERF-ZmERF, 28 ZmERF-SbERF and 4 SbERF-SbERF (*Fig. 3*).

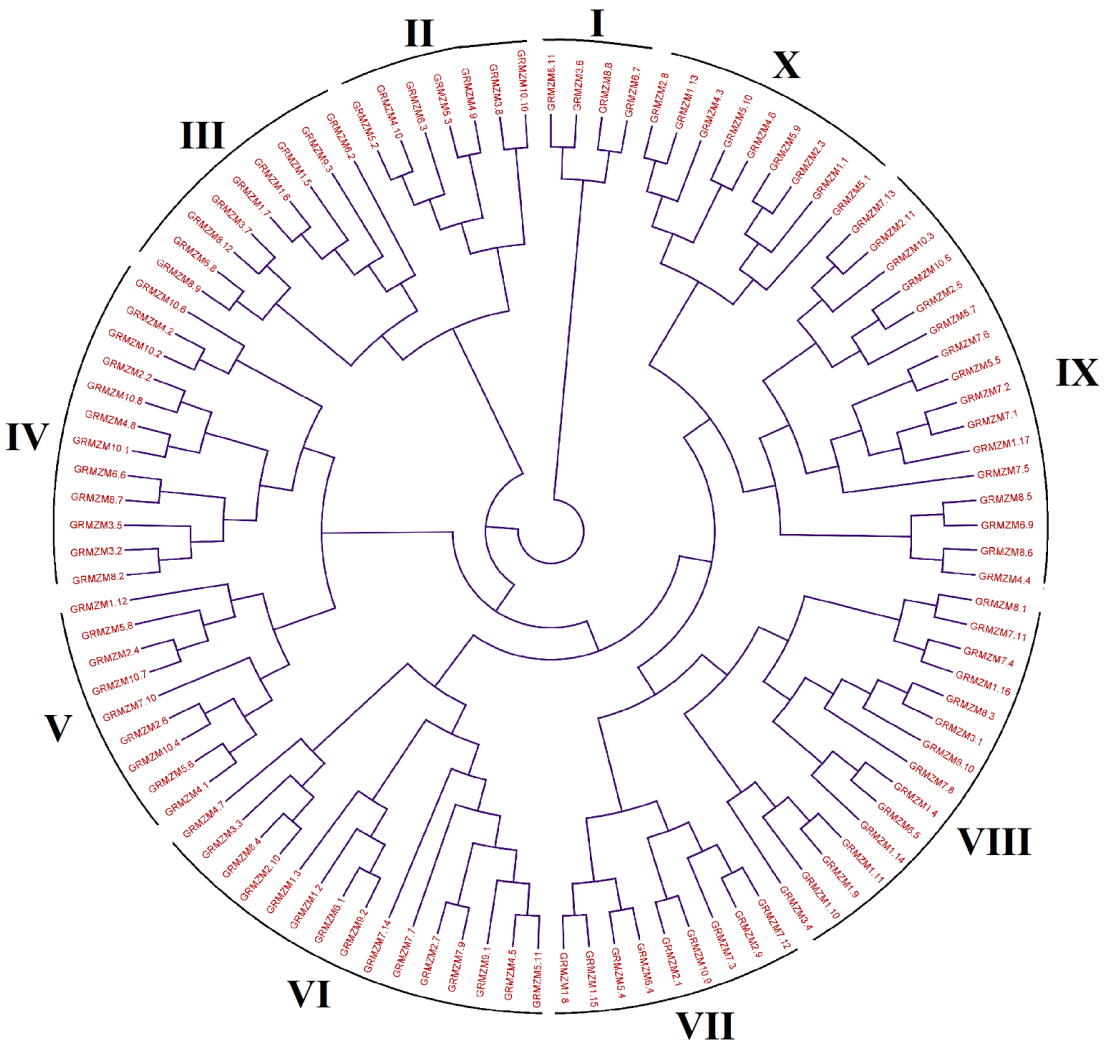

Figure 2. *Phylogenetic analysis of Z. mays ERF genes. An uprooted tree was generated using the CLC sequence viewer 7.0 program by the neighbor-joining method with bootstrap 1000 replicates*

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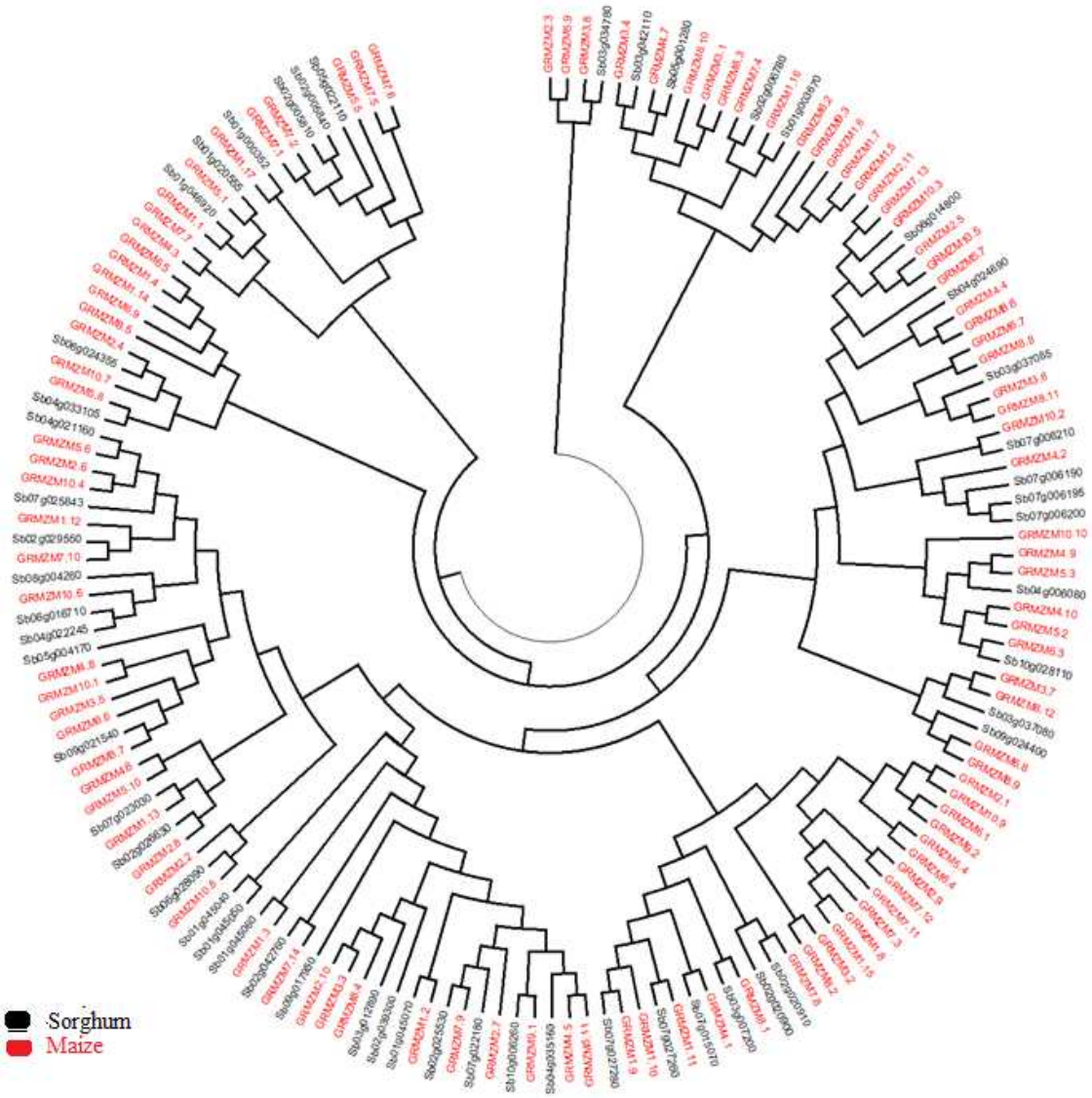

Figure 3. *An uprooted phylogenetic tree constructed by MEGA6 using neighber-joining method of Z. mays and sorghum ERF genes.*

Analysis of conserved motifs in ZmERF family

MEME motif discovery depicted that there were many other motifs then ERF motif. In *Figure 4*, motif 1, 2 and 3 were conserved in all 105 ERF protein sequence while other motifs were group specific. In 1st group Motif 7th was added at the N-terminal. Similarly in group II, motif 8th and 11th were observed in addition to the motifs in I group. 3rd group (III) contained motif 6th while there is deletion of motif 8th. In 4th group some gene had 7th motif in addition to 1st, 2nd, 3rd and 11th motifs. Group 5th had 10th motif with the motifs present in group I. IV group had motif 5th, motif 9th some proteins had motif 12th and 4th. In VII group, motif 14th is added with other motifs. In group VIII motif 6 is dominant with motifs 1, 2 and 3. In IX, motif 13th was present in addition to special motif, 4 and 11. In the last group(X) there were no any new motifs other than mentioned above (*Fig. 4*).

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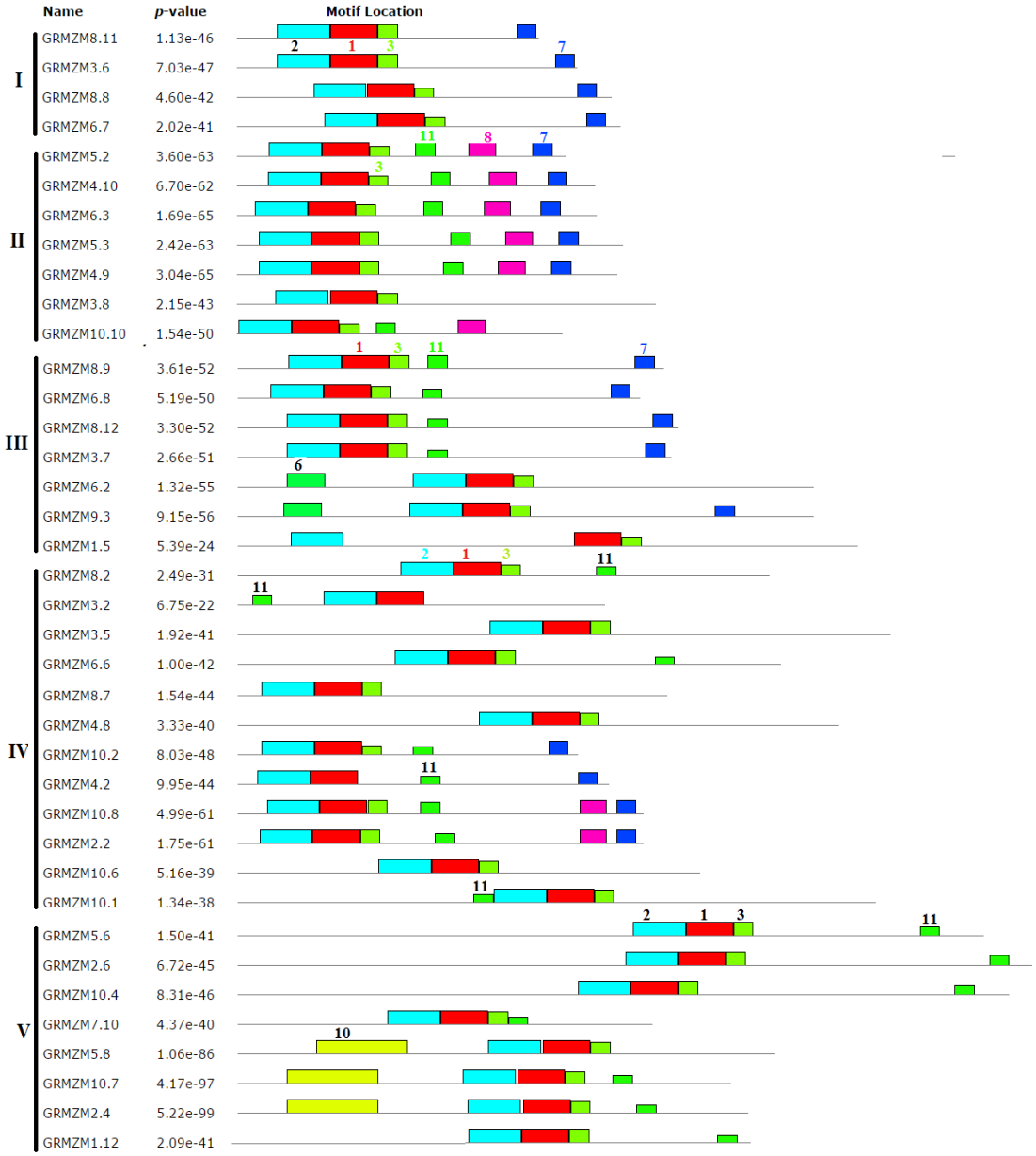
Some ZmERF protein sequences also showed LWSY(Motif 12) and EAR-like (Motif 7) motifs just like other plant species *viz. Arabidopsis*, rice and sorghum (Yan et al., 2013). In *Z. mays* LWSY motif is modified in LWSF pattern like in A-3 group of sorghum (Yan et al., 2013), and only three ERF genes have LWSF motif, GRMZM4.5, GRMZM5.11 and GRMZM9.1 belong to group VI. EAR-like motif with conserved residues DLNXP were present in 17 ERF genes covering group I, II and III. These motif along their gene name, start point and p-values are given in *Fig. 5*.



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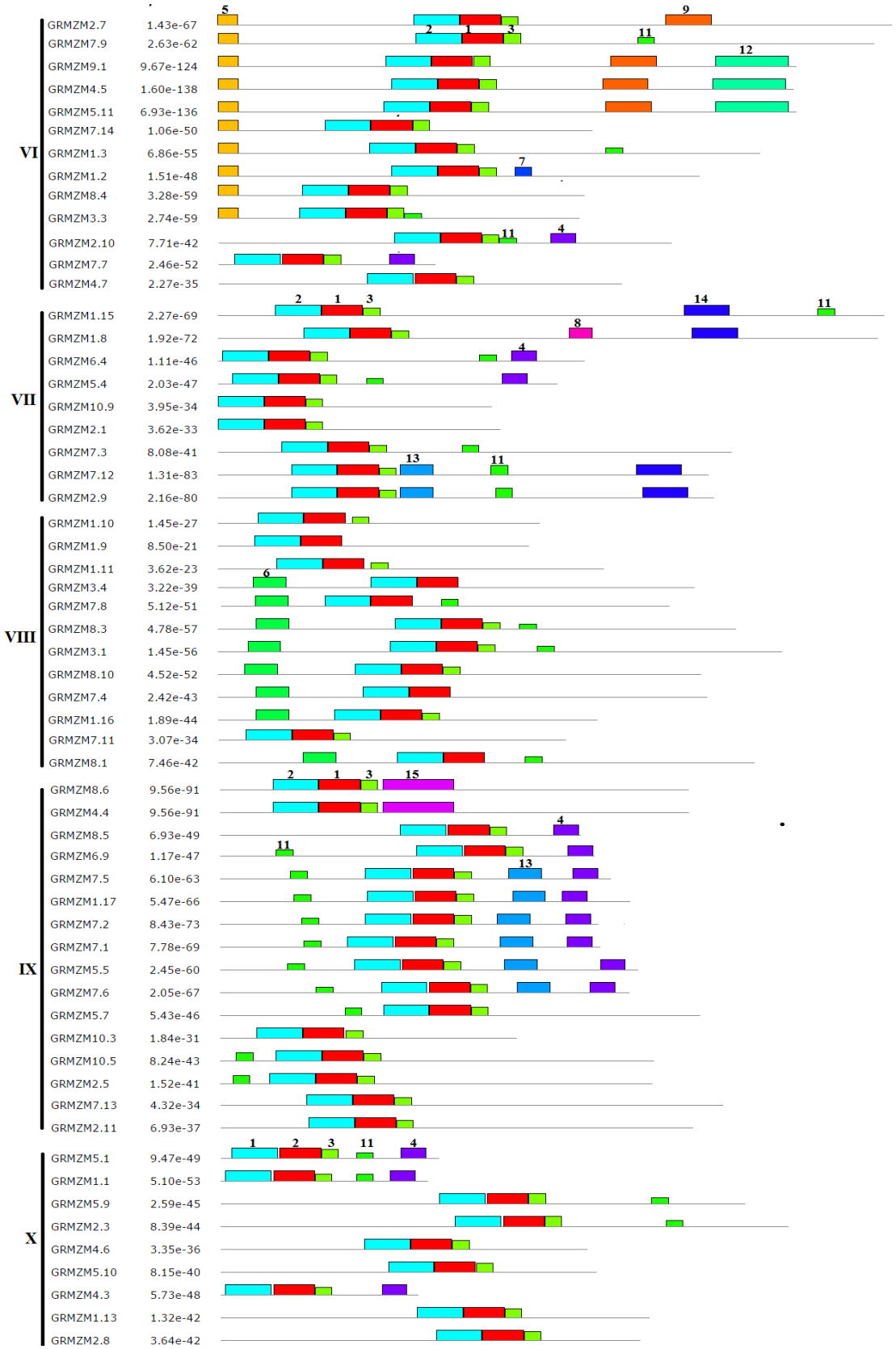

Figure 4. *Conserved Motif identified among group I-X of ERF family in Z. Mays, using MEME Motif discovery analysis. Gene name and combined E-velue are given at left side, each color represents specific motif and block length represent length of motif*

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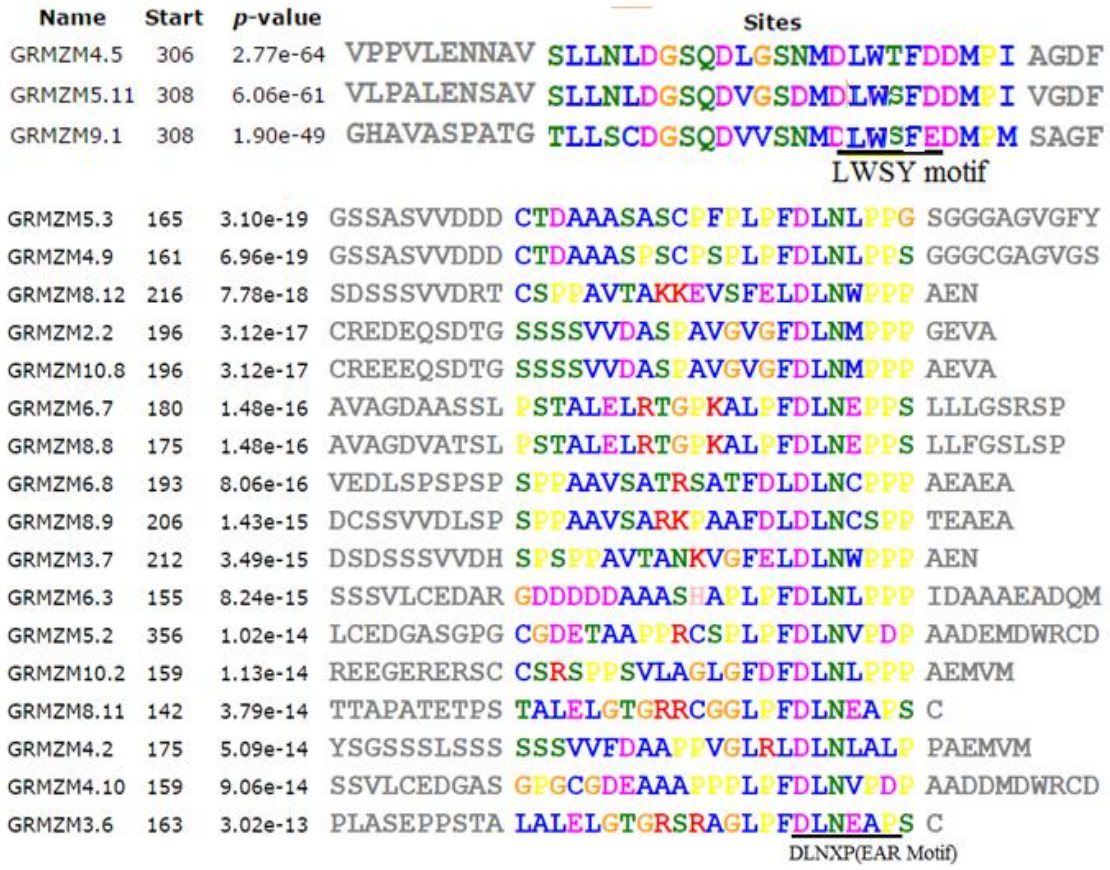

Figure 4. *Conserved LWSY motif and ERF-associated amphiphilic repression (EAR) motif sequences in the c-terminal region of ERF proteins. Consensus amino acid residues were identified using the MEME program and the conserved motifs are underlined respective motif 12 and Motif 7 respectively*

Mapping of ERF genes on Z. mays chromosomes

According to various sources data collection, chromosomal location of 105 genes was predicted graphically. Comparison showed chromosome 1 has highest number of gene (17 genes), followed by chromosome 7 and 8 (14 and 12 genes, respectively). Chromosome 2 and 5 had 11 genes, 4 and 10 carried 10 genes and 6, 3 and 9 harbor 9, 8, 3 genes, respectively. Result of chromosomal location showed most of the ZmERF genes were present in cluster form. Interestingly, almost all genes are either on top or bottom of the chromosome. Many genes in the same group are located on the one chromosome, for instance, GRMZM1.5 GRMZM1.6 GRMZM1.7 belongs to group III clustered in a ~34 kbp segment (chr1);.GRMZM7.1 and GRMZM7.2 belong to IX group, are clustered in ~16 kbp segment (chr7) (*Fig. 6*).

Gene structure analysis of ZmERF

In order to observe detailed evolutionary relationship among ZmERF genes, intron-exon structure was analyzed. Result of the current study showed that most of the genes have no intron and some of them have one or two introns. GRMZM1.7

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gene has highest number of exons (5), followed by GRMZM1.6, GRMZM1.2 and GRMZM10.4 (4, 3, 3 exons, respectively) (*Fig. 7*).

Subcellular localization of ZmERF

In subcelular localizatoin analysis, most of ZmERF proteins were confined in nuclues. According to Plant-mPLoc prediction, 94 ZmERF genes were localized only in nuclues, two genes GRMZM8.1 and GRMZM8.2 were present in cytoplasm and 8 ZmERF genes shared multiple locations (cytoplasm, nucleus, cell membrane and chloroplast). As shown in *Table 3*, Wolfpsort predicted 22 ZmERF genes located in Nucleus, 42 proteins shared dual locations (nucleus and chloroplast) and some proteins were found at multi-located (Cytoplasm, Chloroplast, Nucleus, Mitochondria).

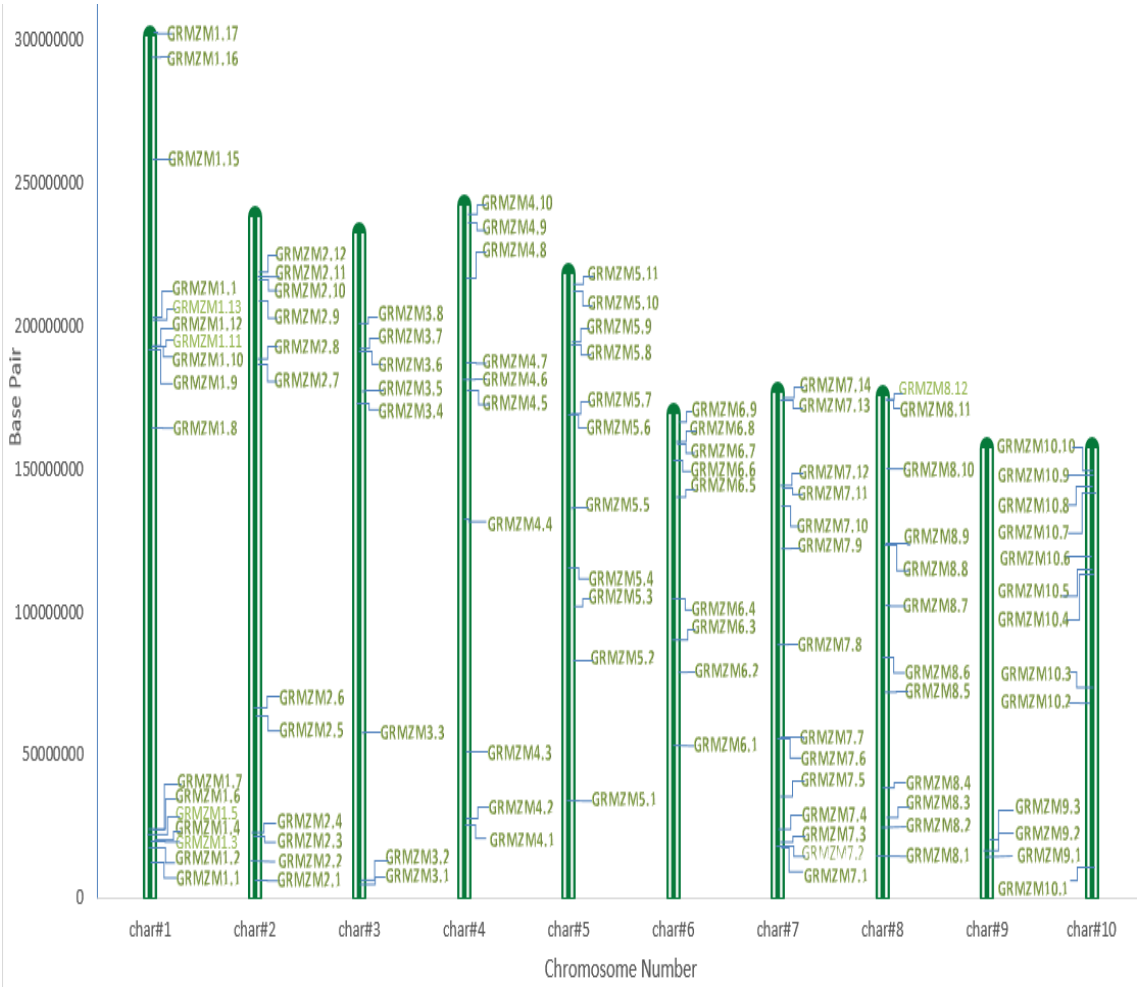

Figure 5. *Chromosomal mapping: The localization of 105 ZmERF genes on Z. mays chromosomes. The chromosomes number is shown at the bottom of each bar. ERF Genes are named according to their position and size on the chromosome and mentioned in the Table 2. The comparative position of ERF gene and size of chromosome are characterized using vertical scale.*

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Figure 6. *Gene structure analysis: intron/exon structure ERF genes from Z. Mays. Scale is drawn according to largest gene. Brown color is showing exon portion of gene while blue line represent intron. The size of intron and exon are drawn according to the scale at the top*

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***Table 3**. Predicted subcellular localization of 105 ZmERF, with their annotation, molecular weight and isoelectric point*

Annotation	Mw (kDa)	pI	wolfpsort	Plant-mPLoc[1]
GRMZM1.1	13.89	8.46	nucl: 13	Nucleus
GRMZM1.2	32.69	5.15	chlo: 5, cyto: 4	Nucleus
GRMZM1.3	36.89	4.91	nucl: 13	Nucleus
GRMZM1.4	15.51	11.65	chlo: 7	Chloroplast. Nucleus
GRMZM1.5	36.43	7.18	nucl: 5, mito: 5, cyto: 4	Cytoplasm. Nucleus
GRMZM1.6	216.02	10.58	nucl: 14	Cytoplasm. Nucleus
GRMZM1.7	127.97	10.31	nucl: 12, cyto: 1	Cytoplasm. Nucleus
GRMZM1.8	43.94	4.37	chlo: 6, nucl: 5, cyto_nucl: 4,	Nucleus
GRMZM1.9	20.96	10.94	chlo: 11, nucl: 3	Cytoplasm. Nucleus
GRMZM1.10	21.97	4.83	nucl: 7, cyto: 4, chlo: 2	Cytoplasm. Nucleus
GRMZM1.11	25.49	7.93	chlo: 6, mito: 4,	Cytoplasm
GRMZM1.12	16.75	10.88	nucl: 12, plas: 1	Nucleus
GRMZM1.13	29.03	6.37	nucl: 9, chlo: 4	Nucleus
GRMZM1.14	18.9	10.82	cyto: 9, chlo: 3	Nucleus
GRMZM1.15	44.44	4.77	cyto: 7, chlo: 4,	Nucleus
GRMZM1.16	25.63	11.43	nucl: 9, cyto: 4	Nucleus
GRMZM1.17	37.6	9.95	chlo: 9, nucl: 5	Nucleus
GRMZM2.1	25.92	5.35	chlo: 5, mito: 4	Nucleus
GRMZM2.2	29.77	10.23	chlo: 13	Nucleus
GRMZM2.3	44.62	4.58	cyto: 8, nucl: 5	Nucleus
GRMZM2.4	36.72	5.18	nucl: 12, chlo: 2	Nucleus
GRMZM2.5	35.1	4.23	chlo: 8, nucl: 6	Nucleus
GRMZM2.6	54.52	9.94	chlo: 7, nucl: 4	Nucleus
GRMZM2.7	51.36	4.57	chlo: 9, nucl: 3	Nucleus
GRMZM2.8	34.76	6.28	chlo: 7, cyto: 5,	Nucleus
GRMZM2.9	40.35	8.12	nucl: 14	Cytoplasm. Nucleus
GRMZM2.10	37.49	9.05	chlo: 10, nucl: 3	Nucleus
GRMZM2.11	39	7.06	nucl: 9, chlo: 4	Nucleus
GRMZM3.1	45.03	4.49	nucl: 12, cyto: 1	Nucleus
GRMZM3.2	29.03	12.14	nucl: 13	Nucleus
GRMZM3.3	24.49	7.34	nucl: 11, chlo: 2	Nucleus
GRMZM3.4	31.73	7.58	chlo: 9, nucl: 5	Cytoplasm. Nucleus
GRMZM3.5	36.75	6.24	nucl: 14	Nucleus
GRMZM3.6	19.65	10.73	chlo: 6, cyto: 3	Nucleus
GRMZM3.7	24.43	9.02	chlo: 13	Nucleus
GRMZM3.8	23.29	9.12	nucl: 10, chlo: 4	Nucleus
GRMZM4.1	18.51	7.64	nucl: 6, mito: 5, cyto: 2	Nucleus

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GRMZM4.2	20.76	7.35	chlo: 8, nucl: 4, mito: 2	Nucleus
GRMZM4.3	13.24	9.09	nucl: 8, mito: 5	Nucleus
GRMZM4.4	31.34	8.30	nucl: 12, cyto: 2	Nucleus
GRMZM4.5	39.47	4.44	nucl: 13	Nucleus
GRMZM4.6	23.97	8.50	nucl: 10, chlo: 3	Nucleus
GRMZM4.7	27.05	7.87	chlo: 7, cyto: 3	Nucleus
GRMZM4.8	34.31	730	nucl: 13	Nucleus
GRMZM4.9	21.69	8.14	nucl: 10, mito: 3	Nucleus
GRMZM4.10	19.75	5.29	nucl: 7, chlo: 4	Nucleus
GRMZM5.1	14.1	7.53	nucl: 14	Nucleus
GRMZM5.2	42.58	9.48	chlo: 7, nucl: 5	Nucleus
GRMZM5.3	22.16	7.88	nucl: 10, chlo: 3	Nucleus
GRMZM5.4	23.36	10.03	nucl: 13	Nucleus
GRMZM5.5	30.61	6.40	nucl: 10, cyto: 2	Nucleus
GRMZM5.6	43	8.86	nucl: 12, chlo: 2	Nucleus
GRMZM5.7	32.81	4.40	nucl: 13	Nucleus
GRMZM5.8	30.72	5.81	nucl: 12	Nucleus
GRMZM5.9	34.51	6.10	nucl: 13	Nucleus
GRMZM5.10	25.02	8.94	nucl: 7, chlo: 5	Nucleus
GRMZM5.11	39.63	4.69	nucl: 14	Nucleus
GRMZM6.1	18.81	6.96	mito: 10, chlo: 3	Nucleus
GRMZM6.2	33.16	4.50	nucl: 14	Nucleus
GRMZM6.3	20.61	6.35	nucl: 6, mito: 5	Nucleus
GRMZM6.4	23.83	10.47	chlo: 7, nucl: 4, mito: 3	Nucleus
GRMZM6.5	24.08	13.03	chlo: 6, nucl: 6, mito: 2	Nucleus
GRMZM6.6	31.26	8.57	nucl: 9.5, cyto_nucl: 6,	Nucleus
GRMZM6.7	21.73	10.92	cyto: 6, nucl: 4,	Nucleus
GRMZM6.8	23	8.20	chlo: 10, mito: 3	Nucleus
GRMZM6.9	31.03	6.94	nucl: 14	Nucleus
GRMZM7.1	35.08	6.34	nucl: 14	Nucleus
GRMZM7.2	38.19	5.79	nucl: 14	Nucleus
GRMZM7.3	34.37	9.64	nucl: 12, mito: 2	Nucleus
GRMZM7.4	33.2	10.06	nucl: 13	Nucleus
GRMZM7.5	27.06	10.25	nucl: 7, chlo: 5	Nucleus
GRMZM7.6	27.61	6.51	nucl: 13	Nucleus
GRMZM7.7	15.19	8.45	nucl: 13	Nucleus
GRMZM7.8	31.47	4.42	nucl: 7, chlo: 4	Nucleus
GRMZM7.9	44.16	5.17	chlo: 12, nucl: 1	Nucleus
GRMZM7.10	23.92	9.79	nucl: 11, mito: 2	Nucleus
GRMZM7.11	22.97	10.42	chlo: 11, mito: 2	Nucleus

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GRMZM7.12	33.03	7.18	nucl: 14	Nucleus
GRMZM7.13	33.34	7.11	nucl: 13	Nucleus
GRMZM7.14	25.74	6.29	nucl: 12, cyto: 2	Nucleus
GRMZM8.1	35.99	4.75	chlo: 10, nucl: 2	Cytoplasm
GRMZM8.2	29.78	7.68	chlo: 6, nucl: 3, mito: 3	Cytoplasm
GRMZM8.3	35.77	4.43	nucl: 7, cyto: 3, chlo: 2	Nucleus
GRMZM8.4	24.77	8.87	nucl: 12, chlo: 1	Nucleus
GRMZM8.5	30.14	7.59	nucl: 13	Nucleus
GRMZM8.6	31.34	8.30	nucl: 12, cyto: 2	Nucleus
GRMZM8.7	24.19	6.97	nucl: 10.5, nucl_plas: 6	Nucleus
GRMZM8.8	21.62	10.69	cyto: 6, nucl: 4	Nucleus
GRMZM8.9	23.88	8.46	chlo: 11, nucl: 2	Nucleus
GRMZM8.10	31.77	4.41	nucl: 12, chlo: 2	Nucleus
GRMZM8.11	17.84	10.29	nucl: 7, chlo: 6	Nucleus
GRMZM8.12	24.94	9.36	chlo: 14	Nucleus
GRMZM9.1	39.31	4.97	chlo: 9, nucl: 4	Nucleus
GRMZM9.2	18.03	6.26	chlo: 6, mito: 4, nucl: 3.5	Nucleus
GRMZM9.3	32.99	4.75	chlo: 13	Nucleus
GRMZM10.1	35.68	9.42	nucl: 9.5, plas: 5.5	Nucleus
GRMZM10.2	19.91	7.92	nucl: 13	Nucleus
GRMZM10.3	19.84	7.20	nucl: 10, mito: 4	Nucleus
GRMZM10.4	45.36	10.10	nucl: 14	Nucleus
GRMZM10.5	28.37	4.35	chlo: 9, nucl: 5	Nucleus
GRMZM10.6	26.06	9.88	nucl: 10, pero: 4	Nucleus
GRMZM10.7	29.01	5.73	nucl: 11.5, plas: 6.5	Nucleus
GRMZM10.8	22.83	10.52	chlo: 13	Nucleus
GRMZM10.9	18.38	6.67	nucl: 6.5, mito: 5, cyto_nucl: 4, chlo: 2	Nucleus
GRMZM10.10	18.94	10.30	nucl: 7, chlo: 5, mito: 2	Nucleus

nucl=nucleus, chlo = chloroplast, mito = mitochondria, cyto = cytoplasm

Gene ontology annotation

Gene ontology analysis resulted in the association of ZmERF proteins in diverse biological, cellular, and molecular activities (*Fig. 8*). The analysis of physiological processes connected to these ZmERF proteins revealed that most of the proteins were involved in stresses responses and regulations. Stress responses include water stress, salt stress, abiotic, hormone, lipid , cold, alcohol, while, in regulation processes, all ZmERF proteins were found to involve in primary metabolic process, nitrogen compound metabolism, macromolecule metabolism, cellular metabolism, biosynthetic process. In molecular process, all ZmERF proteins showed that all proteins possess nucleic acid binding activity including, catalytic activity (found in

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one gene only) and cellular transportation (found in 2 genes). Moreover, cellular compartment by Blast2GO also predicted the localization of ZmERF proteins in nucleus (99 ZmERF genes), followed by plastids (10 ZmERF genes), mitochondria (5 ZmERF genes), and other intracellular membrane bounded organelles. In addition species distribution determination through Blast2GO tool resulted that *Oryza sativa* has highest blast hits with ZmERF proteins (~272 Blast hits value), followed by *Setaria italica* (~205 hits), *Sorghum bicolor* (~128 hits) and lowest blast hits is shown by *Solanum lycopericum* (~19 blast hits) (*Fig. 9*).

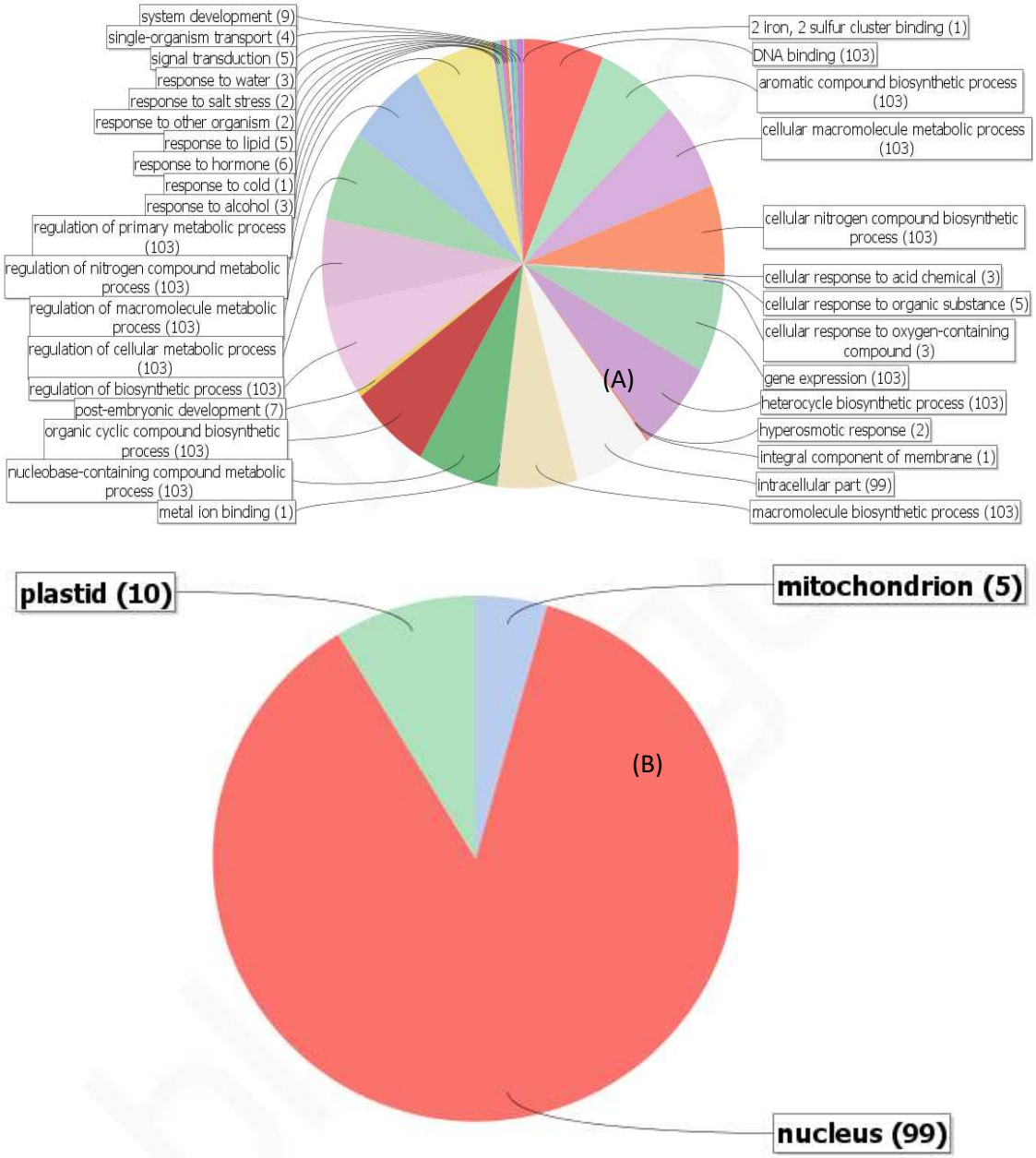

Figure 7. *Gene Ontology of ZmERF proteins .The result of Blast2GO showing (A) Biological Process and (B) Cellular Component , of 105 ZmERF proteins*

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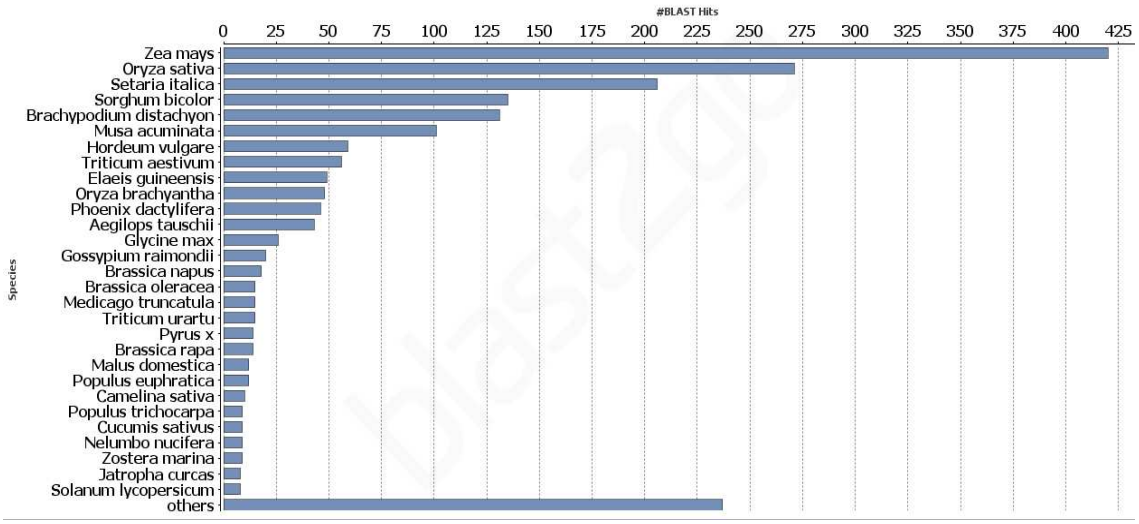

Figure 9. *Species distribution of top BLAST hits obtained using Z. Mays amino acid residues BLAST hits against NCBI-Nt and other default databases in Blast2GO tool , are in x-axis and species distribution are on y-axis*

Discussion

Transcription factors play an important role in modulating the adaptation response of plants to various internal or external signals (Sharma et al., 2010). They possibly change downstream gene expression in stress signal transduction pathways via activation and repression of genes after experience to stress. Plant genomes comprise a large number of transcription factors like AP2/ERF, WORKY, MYB, SPL ARF, FAR1 (Zheng et al., 2007; Park et al., 2010; Chen et al., 2012; Ambawat et al., 2013; Padmanabhan et al., 2013; Mathelier et al., 2014). It has been expected that the *Arabidopsis* genome codes for at least 1533 transcription factors, comprising of over 5.9% of its total predicted genes (Rao et al., 2014). According to (Du et al., 2014), 105 AP2/ERF genes were identified in ERF subfamily having one AP2-like domain.

Throughout the last decade, a huge amount of research has been directed that showed overexpression of ERF family genes increases the resistance of plants to environmental stresses (Shinozaki and Shinozaki, 2012). For example, overexpression of ERF genes in chillies (Shin et al., 2002), *Arabidopsis* (Berrocal-Lobo et al., 2002), and tomato (Gu et al., 2002). Therefor it is one of the most important challenges to identify the genes which have resistant against abiotic factor. The phylogeny, conserved motifs, gene structure and gene ontology annotation were extensively analyzed in several plant species including genome-wide analysis in model plant, *Arabidopsis*, rice (Nakano et al., 2006) and sorghum (Yan et al., 2013), while few ERF gene is *Z. mays* were also studied in waterlogging stress (Liu et al., 2008). With the completion of *Z. mays* genome sequencing projects, phylogenetic analysis of ZmERF genes would be helpful to investigate general function of ERF genes and the evolutionary process of the AP2/ERF domain in plants.

We found that *Z. mays* has 105 ERF genes which are greater than *Arabidopsis*, sorghum, peach and moso bamboo, while less than Chines cabbage and Carrot (*Table 1*). Within conserved domain, G4, G11, W27 and G29 are conserved in all 105 ERF genes while R6, R8, A15, E16, I17, R18, R25, L28, T30, A37, A38, Y41, D41 and G50

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are conserved in 97% of 105 ERF genes (*Figure 1*). A similar pattern of conserved residues was reported in sorghum ERF genes (Yan et al., 2013) and in cucumbers (Hu and Liu, 2011). These conserved residues have significant effect in the function of AP2-domain and also help for point mutation on individual protein. Comparative analysis of amino acid residues among different crops have shown that the most conserved motifs in the AP2/ERF superfamily were present in other species, including *Arabidopsis*, rice, maize and other plants like LWSF (Motif 12) and EAR (Motif 7) (Yan et al., 2013) are also observed in ZmERF proteins in which LWSF function was in gene repression transcriptional regulatory cascades (Ohta et al., 2001). Apart from commonly present motifs in *Arabidopsis* and rice, other motifs in *Z. mays* also play important role in biological processes. In contrast to the groupings (B1-B6) described by (Nakano et al., 2006), we have classified ERF genes in to ten groups (I-X) which will provide more detailed information, where IX had largest number followed by VI and VIII. A relatively great number of genes in these groups might be the significance of evolutionary adaptations to numerous environmental changes. The 105 ZmERF have found 39 sister pairs which showed strong nitration and duplication among ZmERF proteins. The comparative analysis of ZmERF with sorghum ERF showed 28 ZmERF-SbERF sister pair with close homology in ERF family among monocots species.

In chromosomal maps, distribution of ZmERF genes in all the 10 chromosomes was similar to the findings of AP2/ERF in Sorghum (Yan et al., 2013) and Chinese cabbage (Song et al., 2013). Interestingly, ERF gene comprising of same group and present on same chromosome forming cluster, was also observed in *Arabidopsis* (Sakuma et al., 2002) sorghum (Yan et al., 2013), brassica (Song et al., 2013) and cucumbers (Hu and Liu, 2011). The clusters of genes with same product function are useful for drawing recent evolutionary history (Anollés, 2001).

It has been found that intron/exon position configuration provides hint in evolutionary relationship (Hu and Liu, 2011). A very limited number of introns were found in ZmERF genes, while most of the genes were dominated by exons. In previous studies, it has been reported that most of the ERF genes in *Arabidopsis* and peach (Sakuma et al., 2002; Zhang et al., 2012) have no introns. Current schematics representation showed that the absence of intron in ERF genes is also a feature of *Z. mays* (*Table 2*; *Fig. 6*).

Out of 105, 103 ZmERF genes were annotated by Blast2GO. Annotation resulted in the putative participation of ZmERF in diverting the biological, cellular and chemical processes including abiotic stress responses like drought, cold, temperature, salinity etc. Our finding is similar to previous study of AP2/ERF genes in other species, like expression of *PeDREB2* in *Populus euphratica* carried enhancement of drought and cold (Chen et al., 2009; Kumar and Venkateswarlu, 2011) and expression of Sl-ERF.B.3 gene in tomato was only observed under stress condition (Klay et al., 2014). The molecular process of ZmERF showed that all 105 genes have sequence-specific DNA-binding activity which is already reported as a feature of AP2/ERF gene (Yamasaki et al., 2013) and also studied in cucumbers (Hu and Liu, 2011). Cellular localization showed 99 genes are located in nucleus, 10 in plastid and 5 in mitochondria. This means that some ZmERF have multiple locations in the cell. This characteristic of AP2/ERF genes was reported in foxtail millet (Lata et al., 2014).

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Conclusion

To our knowledge, this is the first study in *Z. mays* regarding genome-wide analysis of ERF genes. The study elucidated the ERF gene family role in regulations and defense responses and stress signaling pathways. Characterization and analysis of these functional TF genes may contribute to improve molecular basis of *Z. mays* for better gene pool development and stress adaptation. Our study may also attribute functional gene resources for genetic future engineering approaches for making the *Z. mays* plants tolerant again abiotic stresses. However, further research is needed to explore the biological roles of these TF genes.

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REFERENCES

[1] Alonso, J.M. (2003): Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana.* - Science 301: 653–57.
[2] Ambawat, S., Sharma, P., Neelam, R., Yadav, S., Yadav, R.C. (2013): MYB Transcription factor genes as regulators for plant responses: An overview. - Physiology and Molecular Biology of Plants 19: 307–21.
[3] Andorf, M. et al. (2015): Maize GDB update: New tools, data and interface for the maize model organism database. - Nucleic Acids Research 5: 1007-1009.
[4] Bailey, T.L., Elkan, C. (1994): Fitting a mixture model by expectation maximization to discover motifs in biopolymers. proceedings / international conference on intelligent systems for molecular biology. - ISMB International Conference on Intelligent Systems for Molecular Biology 2: 28–36.
[5] Bailey, T.L., Williams, N., Misleh, C., Li., W.W. (2006): MEME: Discovering and analyzing DNA and protein sequence motifs. - Nucleic Acids Research 34: 69–73.
[6] Boutilier, K., Offringa, R., Sharma, V. K., Kieft, H., Ouellet, T., Zhang, L. (2002): Ectopic expression of baby boom triggers a conversion from vegetative to embryonic growth. - The Plant Cell 14: 1737–49.
[7] Boyer, J.S. (1982): Plant productivity and environment. - Science 218: 443–48.
[8] Anollés, G. (2001): Novel strategies to study the role of mutation and nucleic acid structure in evolution. - Plant Cell, Tissue and Organ Culture 67: 115–32.
[9] Chen, G., Jinhuan, L., Xinli, X., Weilun, Y. (2009): Expression profiling and functional characterization of a DREB2-type gene from populus euphratica. - Biochemical and Biophysical Research Communications 378: 483–87.
[10] Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., Yu, D. (2012): The role of WRKY transcription factors in plant abiotic stresses. - Gene Regulatory Mechanisms 1819: 120–28.
[11] Chou, C. K., Shen, H.B. (2010): Plant-mPLoc: A top-down strategy to augment the power for predicting plant protein subcellular localization. - Plos One 5: e11335.
[12] Conesa, A., Stefan, G. (2008): Blast2GO: A comprehensive suite for functional analysis in plant genomics. - International Journal of Plant Genomics: 1–12.
[13] Du, H., Huang, M., Zhang, Z., Cheng, S. (2014): Genome-wide analysis of the AP2/ERF gene family in maize waterlogging stress response. - Euphytica 198: 115–26.
[14] Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, M. (2003): OsDREB genes in rice, *Oryza Sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. - Plant Journal 33: 751–63.
[15] Elliott, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q. (1996): AINTEGUMENTA, an APETALA2-like gene of *Arabidopsis* with pleiotropic roles in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_177200

ovule development and floral organ growth. - The Plant cell 8: 155–68.
[16] Gong, W., He, K., Covington, M., Kumar, S.P., Snyder, M., Harmer, N. (2008): The development of protein microarrays and their applications in dna-protein and protein-protein interaction analyses of *Arabidopsis* transcription factors. - Molecular plant-microbe Intercation 1: 27–41.
[17] Gotz, S., Arnold, R., Sebastián-León, P., Martín-Rodríguez, S., Tischler, P. (2011): B2G-FAR, a species-centered GO annotation repository. - Bioinformatics 27: 919–24.
[18] Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X. (2002): Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in *Arabidopsis.* - The Plant cell 14: 817–31.
[19] Guo, Z.J., Chen, X.J., Wu, X.L., Ling, J.Q., Xu, P. (2004): Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. - Plant Molecular Biology 55: 607–18.
[20] Hao, D. , Yamasaki, K., Sarai, A., Ohme-Takagi, M. (2002): Determinants in the sequence specific binding of two plant transcription factors, CBF1 and NtERF2, to the DRE and GCC Motifs. - Biochemistry 41: 4202–4208.
[21] Hattori, Y., Nagai, K., Furukawa, S., Song, X.J., Kawano, R., Sakakibara, P. (2009): The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water - Nature 460: 1026–1030.
[22] Hinz, M., Wilson, I.W., Yang, J., Buerstenbinder, K., Llewellyn, D., Dennis, S. (2010): *Arabidopsis* RAP2.2: An ethylene response transcription factor that is important for hypoxia survival. - Plant Physiology 153: 757–72.
[23] Horton, H.P., Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C. (2007): WoLF PSORT: protein localization predictor. - Nucleic Acids Research 35: 585–87.
[24] Hu, L., Liu, S. (2011): Genome-wide identification and phylogenetic analysis of the ERF gene family in cucumbers. - Genetics and Molecular Biology 34: 624–33.
[25] Hu, X.Y., Wang, Y.H., Liu, X.F., Li, Y.J. (2004): *Arabidopsis* RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development. - Cell Research 14: 8–15.
[26] Jiang, C., Iu, B., Singh, J. (1996): Requirement of a CCGAC cis-acting element for cold induction of the BN115 gene from winter *brassica napus.* - Plant molecular biology 30: 679–84.
[27] Jin, J., Zhang, H., Kong, L., Gao, G., Luo, J. (2014): PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors. - Nucleic Acids Research 42: 1182–87.
[28] Jofuku, K.D., Boer, B.G., Montagu, M.V., Okamuro, J.K. (1994): Control of *Arabidopsis* flower and seed development by the homeotic gene APETALA2. - The Plant cell 6: 1211–25.
[29] Kagaya, Y. Yasuaki, K., Hattori, K. (1999): RAV1, a novel dna-binding protein, binds to bipartite recognition sequence through two distinct dna-binding domains uniquely found in higher plants. - Nucleic Acids Research 27: 470–78.
[30] Kim, I.J.S., Mizoi, J., Yoshida, T., Fujita, Y., Nakajima, J., Ohori, T. (2011): An ABRE promoter sequence is involved in osmotic stress-responsive expression of the dreb2a gene, which encodes a transcription factor regulating drought-inducible genes in *Arabidopsis.* - Plant and Cell Physiology 52: 2136–46.
[31] Klay, I., Pirrello, J., Riahi, L., Bernadac, A., Cherif, A., Bouzayen, M. (2014): Ethylene response factor Sl-ERF.B.3 is responsive to abiotic stresses and mediates salt and cold stress response regulation in tomato. - The Scientific World Journal 167681.
[32] Knudsen, B., Knudsen, T., Flensborg, M., Sandmann, H., Heltzen, M., Andersen, A., Mønsted, S. (2011): CLC Sequence Viewer. A/S Cb 6.
[33] Kumar, A., Venkateswarlu, B. (2011): Abiotic stress response in plants-physiological , biochemical and genetic perspectives. - InTech, Rijeka Croatia: 1-358
[34] Lakhwani, D., Pandey, A., Dhar, Y.V., Bag, S.K., Trivedi, P.K., Asif, M. (2016):

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_177200

genome-wide analysis of the AP2/ERF family in musa species reveals divergence and neofunctionalisation during evolution. - Scientific Reports 6: 18878.

[35] Lata, C., Mishra, A.K., Muthamilarasan, M., Bonthala, V.S., Khan, Y., Prasad, M. (2014): Genome-wide investigation and expression profiling of ap2/erf transcription factor superfamily in foxtail millet (*Setaria italica* L.). - PLoS ONE 9: e113092.

[36] Francesco, L., Francesco, M.G., Sara, Z., Fabio, O., Mario, P., Pierdomenico, P. (2010): Genomic and transcriptomic analysis of the AP2/ERF superfamily in vitis vinifera. - BMC genomics 11: 719.

[37] Xihui, L., Junji, F., Dan, G., Wenxin, L., Tingsong, L., Yunling, P., Jianhua, W., Gouying, W. (2008): Genome-wide analysis of gene expression profiles during the kernel development of maize (*Zea mays* L.). - Genomics 91: 378–87.

[38] Magnani, E., Sjölander, K., Hake, S. (2004): From endonucleases to transcription factors: evolution of the AP2 DNA binding domain in plants. -The Plant cell 16: 2265–77.

[39] Mathelier, A., Zhao, X., Zhang, A.W., Parcy, F., Worsley-Hunt, R., Arenillas, D. J., et al. (2014): JASPAR: An extensively expanded and updated open-access database of transcription factor binding profiles. - Nucleic Acids Research 42: 142–47.

[40] Mizoi, J., Shinozaki K., Yamaguchi-Shinozaki, K. (2012): AP2/ERF family transcription factors in plant abiotic stress responses. - Biochimica et Biophysica Acta-Gene Regulatory Mechanisms 1819: 86–96.

[41] Nakano, T., Suzuki K., Fujimura T., Shinshi H. (2006): Genome-wide analysis of the erf gene family. - Plant Physiology 140: 411–32.

[42] Nole-Wilson, S., Krizek, B.A. (2000): DNA binding properties of the *Arabidopsis* floral development protein aintegumenta. - Nucleic Acids Research 28: 4076–82.

[43] Ohme-Takagi, M., Shinshi H. (1995): Ethylene-Inducible DNA binding proteins that interact with an ethylene-responsive element. - The Plant cell 7: 173–82.

[44] Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., Ohme-Takagi, M. (2001): Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. - Plant Cell 13: 1959–68.

[45] Pabo, C.O., Sauer R.T. (1992): Transcription factors: structural families and principles of dna recognition. - Annual Review of Biochemistry 61: 1053–95.

[46] Meenu, S.P., Shisong, M., Tessa, M.B.S., Kirk, C., Peter, H., Savitharamma, P.D.K. (2013): Novel positive regulatory role for the SPL6 transcription factor in the N TIR-NB-LRR receptor-mediated plant innate immunity. - PLoS Pathogens 9: e1003235.

[47] Park, W., Scheffler, B.E., Bauer, P.J., Campbell, B.T. (2010): Identification of the family of aquaporin genes and their expression in upland cotton (*Gossypium hirsutum* L.). - BMC Plant Biology 10: 142.

[48] Rao, G., Sui, J., Zeng, Y., He, C., Duan, A., Zhang, J. (2014): De novo transcriptome and small RNA analysis of two chinese willow cultivars reveals stress response genes in *Salix matsudana*. ed. Matty Knight. - PLoS ONE 9: e109122.

[49] Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., Yamaguchi. (2002): DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. - Biochemical and Biophysical Research Communications 290: 998–1009.

[50] Seki, M., Kamei, A.,Yamaguchi-Shinozaki, K., Shinozaki, K. (2003): Molecular responses to drought, salinity and frost: common and different paths for plant protection. - Current Opinion in Biotechnology 14: 194–99.

[51] Shanker, A.K., Maddaala, A., Kumar, M.A., Yadav, S.K., Maheswari, M. (2012): In silico targeted genome mining and comparative modelling reveals a putative protein similar to an *Arabidopsis* drought tolerance DNA binding transcription factor in chromosome 6 of *Sorghum bicolor* genome. - Interdisciplinary Sciences: Computational Life Sciences 4: 133–41.

[52] Sharma, M.K., Kumar, R., Solanke, A.U., Sharma, R., Tyagi, A.K., Sharma, A.K. (2010): Identification, phylogeny, and transcript profiling of ERF family genes during

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_177200

development and abiotic stress treatments in tomato. - Molecular Genetics and Genomics 284: 455–75.

[53] Shigyo, M., Hasebe, M., Ito, M. (2006): Molecular evolution of the AP2 subfamily. - Gene 366: 256–65.

[54] Song, X., Li Y., Hou X. (2013): Genome-wide analysis of the AP2/ERF transcription factor superfamily in chinese cabbage (*Brassica aapa* Ssp. Pekinensis). - BMC Genomics 14: 998–1009. http://www.biomedcentral.com/1471-2164/11/719.

[55] Verheye, W. (2010): Growth and production of maize: traditional low-input cultivation. - Encyclopedia of Life Support Systems 1.

[56] Wu, H., Lv, H., Li, L., Liu, J., Mu, S., Li, X., Gao, J. (2015): Genome-wide analysis of the AP2/ERF transcription factors family and the expression patterns of DREB genes in moso bamboo (Phyllostachys Edulis). - Plos One 10: e0126657.

[57] Kazugo, M., Liu, Q., Miura, S., Shinozaki, Y.K., Shinozaki, K. (1999): Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. - Nature Biotechnology 17: 287–91.

[58] Yamasaki, K., Kigawa T., Seki, M., Shinozak, K., Yokoyama, S. (2013: DNA-binding domains of plant-specific transcription factors: structure, function, and evolution. - Trends in Plant Science 18: 267–76.

[59] Yan, Yan, H.W., Hong, L., Zhou, Y.Q., Jiang, H.Y., Zhu, S.W., Fan, J . (2013): A genome-wide analysis of the erf gene family in sorghum. - Genetics and Molecular Research 12: 2038–55.

[60] Yao, M., Xu, Z.S., Huang, Y., Tian, C., Wang, F., Xiong, A.S. (2015): Genome - wide analysis of AP2 / ERF transcription factors in carrot (*Daucus carota* L.) reveals evolution and expression profiles under abiotic stress. - Molecular Genetics and Genomics 290: 2049-6.

[61] Zarei, A., Körbes, A.P., Younessi, P., Montiel, G., Champion, A. (2011): Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the PDF1.2 promoter in *Arabidopsis*. - Plant Molecular Biology 75: 321–31.

[62] Zhang, Kori, N.K., Zhang, C.H., Shangguan, L.F., Ma, R. J., Sun, X., Tao, R. (2012): Genome-wide analysis of the AP2/ERF superfamily in peach (*Prunus persica*). - Genetics and molecular research 11: 4789–4809.

[63] Zhang, G., Chen, M., Chen, X., Xu, Z., Guan, S., Li, L. C. Ma, Y. (2008): Phylogeny, gene structures, and expression patterns of the ERF gene family in soybean (*Glycine max* L.).- Journal of Experimental Botany 59: 4095–4107.

[64] Zhang, J.Z., Creelman, R., Zhu, J. (2004): From laboratory to field. using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. - Plant Physiology 135(2): 615–21.

[65] Zhang, Z., Huang, R. (2010): Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor TERF2/LeERF2 is modulated by ethylene biosynthesis. -Plant Molecular Biology 73: 241–49.

[66] Zheng, Z., Mosher, L.S., Fan, B., Klessig, D.F. Chen, Z. (2007): Functional analysis of *Arabidopsis* WRKY25 transcription factor in plant defense against *Pseudomonas syringae*. -BMC Plant Biology 7(1): 2.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1405_177200