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Effect of two neem-derived pesticides on Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Coleoptera: Chrysomelidae) under laboratory conditions

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Abstract: Mortality and antifeedant activity of two different neem-derived pesticides were investigated on larvae of Colorado potato beetle (*Leptinotarsa decemlineata* Say). In no-choice tests, mortality of larvae increased with increase in time period, meanwhile the feeding damage decreased with the increase of neem leaf extract concentration in contrast to NeemAzal T/S (1% azadirachtin) in which neither there was any significant difference in mortality nor on feeding damage. In the choice test, none of the treatments were lethal to the larvae tested. The larvae fed on the leaves irrespective of the treatment.
Keywords: azadirachtin, neem leaf extract, biological control, Colorado potato beetle, potato
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Introduction

Colorado potato beetle (CPB) (*Leptinotarsa decemlineata* Say, Coleoptera: Chyrsomelidae) is an important pest of potato causing significant economic losses world-wide. CPB destroys all the green vegetative parts of potato, sometimes resulting in 100% yield loss and is also a vector of bacterial potato ring rot disease (*Clavibacter michiganensis* subsp. *Sepedonicus* Smith 1910 Davis et al. 1984) (Alkan et al. 2015). CPB is a multivoltine insect and uncontrolled populations can destroy the whole yield during the growing season (Alkan et al. 2017). CPB feeds mostly on solanaceous crops as they contain high concentrations of toxic glycoalkoloids in their foliage which the beetle detoxifies and excrete them with the diet (Wimer et al. 2015). Management of CPB using chemical insecticides is a common control measure that is applied since many decades. (Alkan et al. 2017). As a result of regular chemical control, CPB is currently resistant to most classes of synthetic insecticides (Kutas and Nádasy 2005). This ability of detoxifying the active compounds can explain their ability to develop resistance to different insecticides (Wimer et al. 2015).

Combination of chemical insecticides is a simple approach to prevent the development of resistance (Trisyono and Whalon 1999), but the damage to the environment and the beneficial organisms dwelling in such environments is still inevitable. The growing challenges and concerns about the negative impacts on the environment and resistance to various insecticides lead researchers to look for alternative solutions to these. An alternative control method is biological control using entomopathogenic microbes such as *Bacillus thuringiensis* var. *tenebrionis* Berliner, 1915 (*Btt*). It is considered as a promising agent against CPB but frequent usage of *Btt* could result in resistance to it (Trisyono and Whalon 1999). Apart from microbes, several plant extracts have been screened for their toxic and/or antifeedant effects on CPB. Plant derived pesticides and insect feeding inhibitors for crop protection are gaining attention (Kutas and Nádasy 2005) but are still not exploited to their maximum potential. There could be several advantages of these plant-derived pesticides such as they are of natural origin, harmless to humans and non-target organisms and as such environmentally friendly. Combined application of *Btt* and plant derived insec-

ticides can prevent the development of resistance to either of them. They represent a sustainable control method permitted in organic farming (Skuhrovec et al. 2017).

Azadirachtin, one of the most active insecticidal compounds found in Neem (*Azadirachta indica* A. Juss.) has been studied previously for its effects on CPB. It is a tetranortriterpenoid and is known to possess strong antifeedant properties (Isman et al. 1990). Zabel et al. (2002) demonstrated the effect of neem extracts on CPB third instar larvae under laboratory and field conditions. They found a satisfying antifeedant activity of neem on CPB larvae under laboratory conditions and foliage protection under field conditions and suggested neem as a part of integrated pest management (IPM) programs in small orchards, private gardens and tree rows. Schmutterer (1985) found that there is a strong insecticidal effect of neem seed kernel extract on CPB larvae. In addition, there was a significant reduction in the feeding damage in the treated plots. In another study conducted by Moreau et al. (2006), the effect of companion planting along with different botanical extracts was evaluated. They found that 2% of neem extract sprayed on the potatoes on the field resulted in lower CPB densities, lower leaf damage and higher yields as compared to control plots as compared to other treatments Novodor, companion planting, garlic and capsaicin extracts. When Hiiesaar et al. (2000) applied different water dilutions of NeemAzal-T/S (1% azadirachtin) on CPB eggs, they found that the embryonic development of the eggs was almost complete but only 47% eggs hatched, while the rest perished inside the eggshell. Additionally, they found a direct mortal effect on 2-day-old larvae of first instar, whereas fourth instar larvae showed varied effects along with potent antifeedant properties.

Our aim of this study is to validate the effects of water extract of dried neem leaves, which has been used for centuries in the trop-

ical and sub-tropical countries by the growers and farmers because of its easy availability and cheap costing; as compared to commercially available neem product (containing only 1% azadirachtin as the active ingredient) which is much more expensive, on CPB larvae under laboratory conditions in Hungary.

Materials and Methods

Preparation of neem leaf extracts (NLE)
The method was followed as per Doshi et al. (2018) and Petrikovszki et al. (2019) with modified working concentrations. Working concentrations of 1, 5, 10, 15 and 20% of NLE was prepared from a stock concentration of 20% using distilled water.

Preparation of azadirachtin (AZA)
A modified methodology of Doshi et al. (2018) and Petrikovszki et al. (2019) was used. The working concentrations used were 0.001, 0.003, 0.005, 0.01, 0.1% prepared from a stock concentration of 0.1% azadirachtin which was prepared by dissolving 10 mL of NeemAzal T/S (1% azadirachtin) in 100 mL distilled water.

Preparation of Bacillus thuringiensis var. tenebrionis (Btt)
Btt was prepared as a positive control. A 2% solution of commercially available Btt was made from Novodor (3.0% *Bacillus thuringiensis* var. *tenebrionis*) by mixing 2 mL of Novodor in 100 mL distilled water.

Collection of CPB larvae
Freshly hatched, first and second instar larvae from the untreated leaves of potato cv. 'Balatoni Rózsa' were collected in the experimental field of Szent István University, Gödöllő campus. Fresh non-infected potato leaves of the same potato variety were collected for different treatments and serve as a food source.
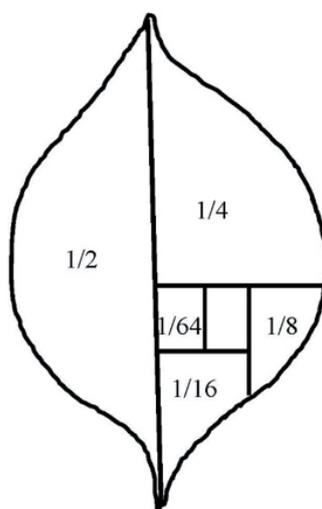
Figure 1. Diagrammatic representation of a potato leaf and used for assessing the feeding damage caused by Colorado potato beetle larvae.

a. No-choice test

The fresh undamaged potato leaves were dipped in the respective treatment solution for 10 seconds and kept outside for 1 min for drying at room temperature before placing them on moist filter paper in 9 cm glass Petri dishes. A total of 5 individuals, which included freshly collected mixed population of newly hatched and 1st instar larvae were placed on the top of the leaves using a fine brush. A negative control was performed by dipping the leaves in distilled water and positive control was by using 2% of Novodor. Each treatment was replicated 3 times. The Petri dishes were closed with the lid and kept at a temperature of 25±2°C, relative humidity of 60±5%, light intensity of 16L:8D conditions. Larval mortality and feeding damage (represented diagrammatically in Fig. 1) on the leaves was observed and recorded for a time period of 24, 48, 72, 96 hours. One-way ANOVA post-hoc Tukey's test was performed on the data using RStudio v 3.4.0 (2017) to compare the different treatments against each other and graphs were made in the excel.

b. Choice test

The setup for choice test was the same as the no-choice test except that it was performed in 15 cm diameter glass Petri dish with 2 fresh undamaged potato leaves, one treated with different concentrations of neem products and the other with distilled water and placed on the opposite side of Petri dishes on moist filter paper. Five individuals consisting random mixture of first, second and third instar larvae were placed in the centre of the Petri dish and the dish was closed with a glass lid. A negative control was performed by dipping both the leaves in distilled water and a positive control was performed by dipping one leaf in 2% Novodor (*Bacillus thuringiensis* var. *tenebrionis*) (*Btt*) solution and the other in distilled water. The conditions were the same as that in no-choice test. Larval mortality and feeding damage (Fig. 1) on the leaves was observed and recorded for a time period of 24, 48, 72, 96 hours. One-way ANOVA post-hoc Tukey's test was performed on the data using RStudio v 3.4.0 (2017) to compare the different treatments against each other and graphs and graphs were made in the excel.

Table 1. Effect of different concentrations (%) of two different neem-derived pesticides on mortality of CPB larvae at different time interval under no-choice condition. Different letters represent significant difference at 95% confidence level. Data are mean of 3 replicates.

Treatment	Conc (in %)	24h mortality (mean ± SE)	48h mortality (mean ± SE)	72h mortality (mean ± SE)	96h mortality (mean ± SE)
Control 0	0	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Neem Azal T/S (AZA)	0.001	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	0.003	0.0 ± 0.0 a	0.0 ± 0.0 a	6.66 ± 6.66 a	6.66 ± 6.66 a
	0.005	0.0 ± 0.0 a	7.00 ± 6.66 ab	6.66 ± 6.66 a	13.33 ± 13.33 a
	0.01	0.0 ± 0.0 a	7.00 ± 6.66 ab	13.33 ± 6.66 a	33.33 ± 6.66 a
	0.1	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
neem leaf extract (NLE)	1	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	5	0.0 ± 0.0 a	7.00 ± 6.66 ab	6.66 ± 6.66 a	6.66 ± 6.66 a
	10	0.0 ± 0.0 a	20.00 ± 20.00 ab	33.33 ± 13.33 a	40.00 ± 11.54 a
	15	0.0 ± 0.0 a	53.00 ± 24.03 b	66.66 ± 17.63 b	80.00 ± 11.54 bc
	20	0.0 ± 0.0 a	13.00 ± 13.33 ab	66.66 ± 13.33 b	93.00 ± 6.66 c
Btt	2	0.0 ± 0.0 a	0.0 ± 0.0 a	6.66 ± 6.66 a	26.66 ± 13.33 a
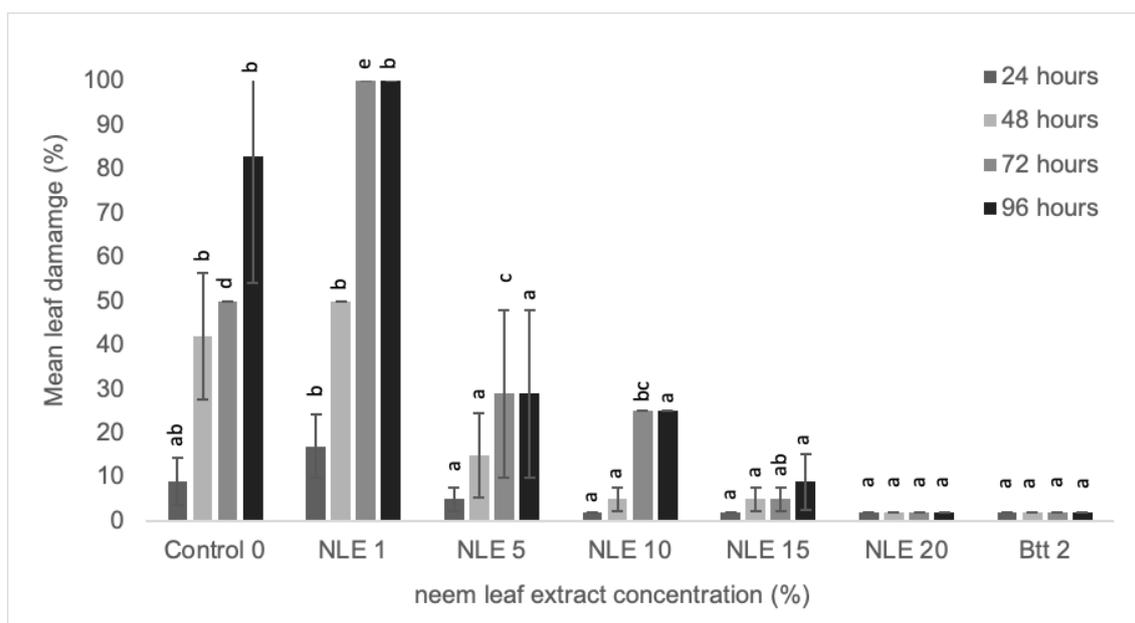
Figure 2. Effect of different neem leaf extract (NLE) concentrations (%) on mean leaf damage (%) caused by CPB larvae at different time interval under no-choice condition. Different letters indicate significant difference at 95% confidence level ($p<0.05$). Data are mean of 3 replicates.

Results

a. No-choice test

Two different neem-derived pesticide products were used for this experiment with different concentrations to check their efficacy against CPB larvae (Table 1). In case of AZA, there is no significant difference in the mortality after 96 hours post-treatment even at the highest concentration of 0.1%. The
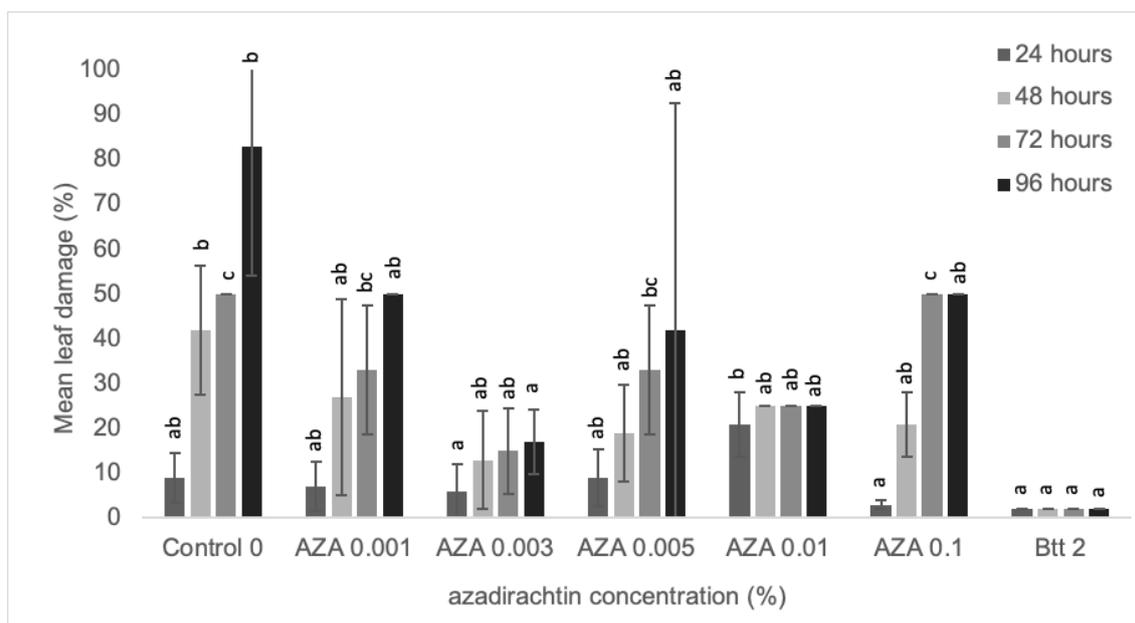
Figure 3. Effect of different azadirachtin concentrations (AZA) (%) on the mean leaf damage (%) at different time interval caused by CPB larvae under no choice condition. Different letters indicate significant difference at 95% confidence level (p<0.05). Data are mean of 3 replicates.

NLE was much more lethal as compared to AZA for CPB larvae. There was a significant difference (p<0.05) in mortality of CPB larvae with the increase in concentration as the time progressed. NLE 15% and 20% showed the highest mortality of 80 and 93% at 72h and 96h respectively and were significantly different from the rest of the treatments. *Btt* did not show any significant difference in the mortality of the larvae at the given working concentration.

CPB feeds mainly on potato leaves which is why the different concentrations of neem leaf extract and azadirachtin were tested on the feeding of CPB and leaf damage (%) was assessed (Fig 2, 3 respectively) at different time interval under no-choice condition. After 24 hours post-treatment, there was no significant difference between the feeding damage caused by the CPB larvae throughout the different NLE concentrations (Fig 2) compared to negative control. After 48 hours post-treatment, significant reduction in feed-

ing damage was observed in the case of NLE 5-20% and *Btt* whereas NLE 1% did not show any difference as compared to Control 0. At 72h post-treatment, all NLE concentrations showed significant difference in feeding damage compared to negative control and for 96h post-treatment, NLE 5-20% and *Btt* showed significant difference compared to negative control, which coincides with the high mortality as seen in Table 1 after 72 and 96h post-treatment respectively.

In the case of azadirachtin (Fig 3), the feeding damage was not consistent. At 24h post-treatment, no significant feeding damage was observed. At 48h post-treatment, only *Btt* showed significant reduction in feeding damage while in the case of 72h AZA 0.003 and 0.01% and *Btt* significantly reduced the feeding damage. In the case of 96hpost-treatment, only AZA 0.003% an *Btt* showed significant reduction in feeding damage.

b. Choice test

In this test, the effect of different neem de-

Table 2. Effect of different concentrations (%) of two different neem-derived pesticides on mortality of CPB larvae at different time intervals under choice condition. Different letters represent significant difference at 95% confidence level. Data are mean of 3 replicates.

Treatment	Conc (in %)	24h mortality (mean ± SE)	48h mortality (mean ± SE)	72h mortality (mean ± SE)	96h mortality (mean ± SE)
Control 0	0	0.0 ± 0.0 a	13.33 ± 6.66 a	13.33 ± 6.66 a	13.33 ± 6.66 a
Neem Azal T/S (AZA)	0.001	0.0 ± 0.0 a	0.0 ± 0.0 a	20.00 ± 11.547 a	20.00 ± 11.547 a
	0.003	0.0 ± 0.0 a	0.0 ± 0.0 a	13.33 ± 13.33 a	13.33 ± 13.33 a
	0.005	6.66 ± 6.66 a	6.66 ± 6.66 a	13.33 ± 13.33 a	26.66 ± 17.64 a
	0.01	13.33 ± 6.66 a	13.33 ± 6.66 a	40.00 ± 0.00 a	40.00 ± 0.00 a
	0.1	6.66 ± 6.66 a	20.00 ± 11.547 a	26.66 ± 6.66 a	33.33 ± 6.66 a
Neem leaf extract (NLE)	1	6.66 ± 6.66 a	13.33 ± 13.33 a	20.00 ± 11.547 a	33.33 ± 6.66 a
	5	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	10	0.0 ± 0.0 a	6.66 ± 6.66 a	13.33 ± 6.66 a	13.33 ± 6.66 a
	15	0.0 ± 0.0 a	6.66 ± 6.66 a	13.33 ± 13.33 a	13.33 ± 13.33 a
	20	0.0 ± 0.0 a	6.66 ± 6.66 a	6.66 ± 6.66 a	13.33 ± 13.33 a
Btt	2	0.0 ± 0.0 a	6.66 ± 6.66 a	13.33 ± 6.66 a	26.66 ± 6.66 a
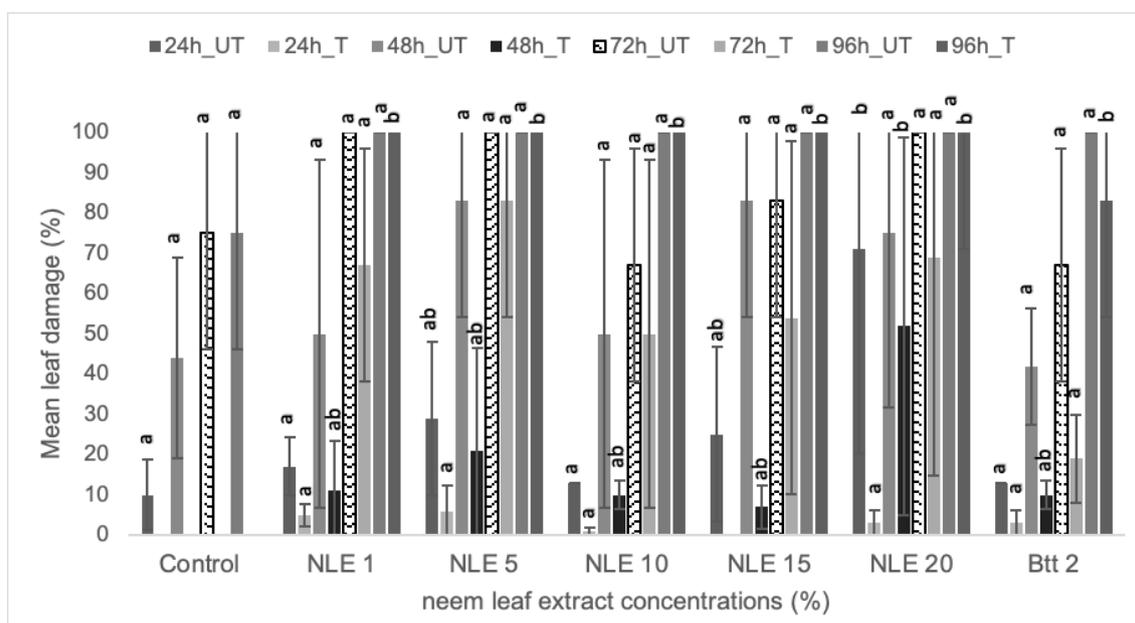
Figure 4. Effect of different neem leaf extract (NLE) concentrations (%) on the mean leaf damage (%) at different time intervals caused by CPB larvae under choice condition. Different letters represent significant difference at 95% confidence level (p<0.05). Data are mean of 3 replicates.

rived pesticide products on the mortality of CPB larvae and the feeding damage can be investigated better (Table 2). There is no significant difference between different treatments for the entire time period throughout the experiment. NLE 5% showed no mortal-
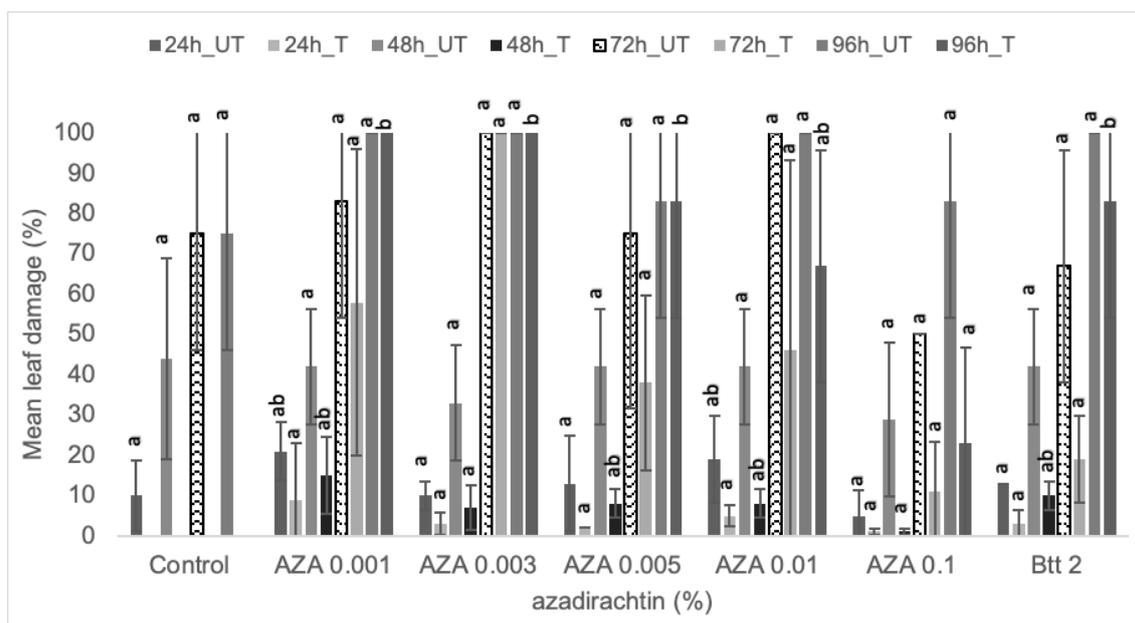
Figure 5. Effect of different azadirachtin (AZA) concentrations (%) on the mean leaf damage (%) at different time intervals caused by CPB larvae under choice condition. Different letters represent significant difference at 95% confidence level (p<0.05). Data are mean of 3 replicates.

ity even after 96 h post-treatment. The maximum mortality (%) was seen for AZA 0.01% after 96 hr post-treatment followed by AZA 0.1% yet the difference was not significant.

In the case of neem leaf extract, leaves treated with NLE 20% showed a significant difference in the leaf damage after 48h. In addition, it is also evident that all treatments had a significant reduction in the mean leaf damage at 96 h when compared to their respective untreated leaves (Fig. 4). Similarly, in the case of azadirachtin, all treatments had a significant reduction in the mean leaf damage at 96 h when compared to their respective untreated leaves (Fig. 5).

Discussion

It is evident that neem leaf extract is toxic to the newly hatched and first instar larvae. Intoxication of CPB larvae when treated with different but higher neem leaf extract concentrations showed delayed but high mortality as seen from the no choice test as com-

pared to azadirachtin. Delayed larval mortality in the case of neem leaf extract might be due to the antifeedant activity of different compounds found in NLE and larvae as seen from the results. Another possible reason could be that the various compounds present in the NLE are slow in their action (Trisyono and Whalon 1999) or the accumulation of lower concentrations of neem compounds in the gut system and then acting on the hormonal system as suggested by Zehnder and Warthen (1988) and Trisyono and Whalon (1999).

On the contrary, weak mortality results were obtained in the case of azadirachtin in the no choice test for both the products in choice test. This might be because of the mixed population of the larvae and there is a possibility that the second and third instar larvae have more evolved gut system to digest neem and excrete out the toxic compounds Wimer et al (2015) and sparing the untreated leaf for the first instar larvae with weaker gut system.

Another possibility can be the uneven distribution of different compounds on the leaf extract. Perhaps there was not enough of concentration of different compounds found in neem leaves on the leaf surface which in turn was not enough for larval mortality. Another reason can be the slow toxic effect of the different neem compounds.

With respect to antifeedant properties, a strong antifeedant activity was observed in the case of neem leaf extract in the no choice experiment which might be due to different compounds present in the leaf extracts acting either alone or in combinations. Similar results were obtained by Alford et al. (1987) when they tested antifeedant activity of Limonin against Colorado potato beetle larvae. Also, Zabel et al, (2002) found that neem extract had a strong antifeedant activity against Colorado potato beetle larvae under laboratory conditions which is like our results from the no choice test but contradicts the results from choice test. In the case of azadirachtin the antifeedant activity was weak in our experiment-, Our results contradict the work done by Hiiseer et al. (2000) where the azadirachtin from the same commercial product showed only 12% consumption is Howver, our findings are consonant with the results reported by Klocke and Barnby (1989). and with the work done by Hiiseer et al. (2009) where they could not find any significant effect on feeding activity. Kutas and Nadasy (2005) experienced similar results of low antifeedant activity in the case of azadirachtin (NeemAzal T/S) and they argued that this can be possible due to the low concentration of azadirachtin used for the experiment while the recommended dose is 0.3-0.5%.

Conclusion

In our experiments, we found mixed results according to the antifeedant and lethal effects of commercial azadirachtin and neem leaf extract, respectively. We found that in these aspects traditional neem leaf extract was superior to the commercial product. The reason for it could be that it contains not only azadirachtin but many other biologically active different compounds which exhibit different plant protection properties. Field trials are necessary to validate our hypothesis. In addition, detailed analysis of different compounds present in the neem leaf extract should be done to estimate their concentration.

Acknowledgements

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Riparian conservation management needs habitat quality mapping

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Abstract: Riparian habitat quality has a significant influence on the water quality of rivers, primary resources for urban and agricultural use. River water quality deteriorates where normal ecological functioning is disrupted by harmful impacts from nearby land-use types. Important rivers are typically managed and protected by government-led conservation programs. These programs often lack a key tool for efficient conservation management, habitat quality mapping. The Berg River, an important water source in South Africa, was used as a case-study to assess how habitat quality mapping could broaden the current scope of river conservation programs. The river faces threats from nearby urban settlements, industrial areas, mining, encroachment, and agricultural practices. The aim of this study was to develop habitat quality and habitat degradation maps for a section of the Berg River to assess the value that mapping holds for conservation managers and spatial planners. InVEST modelling software and ArcGIS was used to produce these habitat quality maps based on land-use/land-cover and threat impact data. The resulting maps showed several specific locations of heavily threatened and degraded riparian habitat that had not specifically been included in current government conservation management or spatial planning. Habitat quality mapping is an important tool that conservation managers and spatial planners can use to successfully address habitat degradation and protection while facing resource limitations, such as lack of funding. Oversight of degraded riparian habitats will lead to further decreases in river water quality, adversely affecting human welfare and local economies.
Keywords: habitat quality, environmental monitoring, river conservation, InVEST

Introduction

Habitat quality of rivers and riparian zones are subject to degradation when flowing through man-managed landscapes (Tóth 2014). This degradation can cause exponential deterioration of river water quality from biological and physical edge effects that have negative impacts like facilitating entry of invasive species and competitors, pollution, and toxic chemicals (Perry et al. 2018).

Land-use and land management nearby rivers have diverse effects on the ecological functioning of rivers (Perry et al. 2018). Biodiversity loss and declining water quality of riparian habitats occur due to threats like land encroachment into rivers and pollution from adjacent land (Fierro et al. 2017).

Vörösmarty et al. (2010) detailed threats to water catchments and summarized that 80% of the global population faces high levels of threats to water security. Developing countries, such as South Africa, are particularly

vulnerable to water threats as relatively little precautionary investment is made in ecological (green and blue) infrastructure and biodiversity conservation (Angelstam et al. 2017).

The Berg River in the Western Cape, South Africa (Figure 1), is used as a case-study in this assessment. The river is an important water source for urban and agricultural use in the Western Cape province and its riparian habitat and water quality face many threats. The Western Cape recently faced a looming water shortage crisis that has impacted widely on water availability and quality which in turn adversely affected agricultural production, harming the stability of local economies and human welfare (Botai et al. 2017).

Agricultural encroachment, agricultural runoff, polluted stormwater runoff from urban settlements, invasive alien species, and poorly treated wastewater effluent are some of the major threats that cause poor water quality in the Berg River (Tererai et al. 2013;
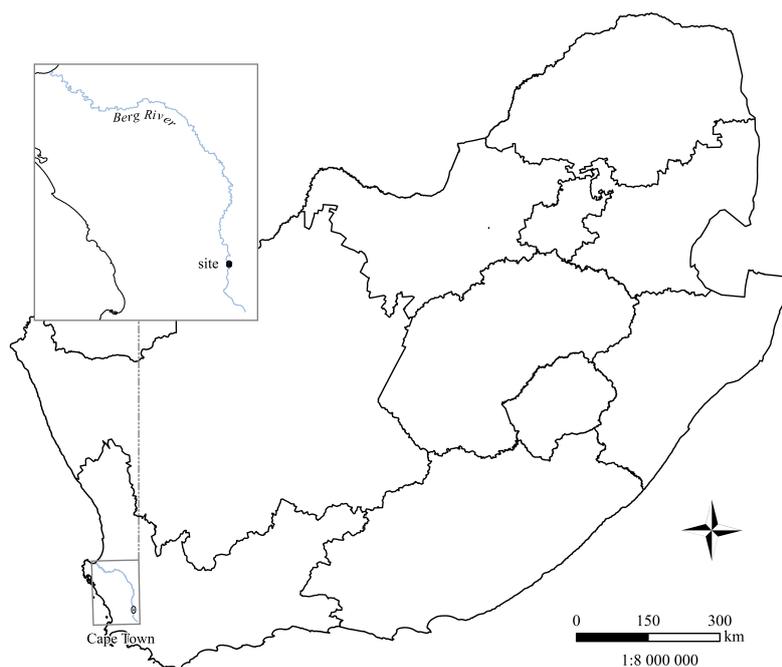
Figure 1. The Republic of South Africa and the location of the study site along the Berg River in the Western Cape province.

Locke 2016). Further habitat degradation is caused by activities associated with certain land-use such as urban settlements, industrial areas, mines, access roads, river encroachment, and agricultural practices (DEADP 2012; McLean et al. 2017).

The Berg River is a highly modified river and subject to increasing environmental degradation from habitat fragmentation, edge effects, and degradation in neighbouring habitats (DWS 2016; Locke 2016). Kamish (2008) discovered that clearing of natural vegetation along the Berg River led to a decrease in overall species richness, as well as increased concentrations of dissolved salts in the river due to a rising of the water table. Other land-use types are linked to an increase in chemical pollutants (ammonium, phosphates, and inorganic nitrates) and E. coli in Berg River water (De Villiers 2007; DEADP 2012; Struyf et al. 2012).

As water is a scarce and valuable resource, the Western Cape provincial government de-

veloped programs to improve the efficient usage and quality of water by alleviating environmental pressures on important water catchments like the Berg River. Government-led programs such as the Berg River Improvement Plan (BRIP) and the Western Cape Biodiversity Spatial Plan (WCBSP) guide land management and the development of river conservation management (DEADP 2012; Pool-Stanvliet et al. 2017). The BRIP aims to support water quality monitoring, upgrade wastewater treatment works, support riparian zone ecological rehabilitation, instil 'best-practices' for land-use types, and improve ecosystem resilience (DEADP 2012). The WCBSP represents ecological infrastructure and priority biodiversity areas that need to be protected over the long-term to fulfil core biodiversity management mandates (Pool-Stanvliet et al. 2017).

These programs strive to support integrative ecological and biodiversity conservation management approaches to rivers and

riparian habitat to regenerate proper biodiversity functioning. Yet, GIS mapping tools designed for this purpose are not utilised by government officials that work as conservation managers in the Berg River catchment. GIS mapping tools have been developed to help conservation managers implement restoration actions and detect location-specific ecological stress (Zlinszky et al. 2015). Such tools are essential in improving water quality as maps assist in discovering direct and indirect sources of impact on riparian zones, such as habitat quality (Geist 2011; Mika and Farkas 2017).

Land-use and land-cover (LULC) data provide area-covering information on variables that impact river habitat quality. Currently, data on land-use and land-cover for South Africa is publicly available as GIS data (CapeNature 2014). Software that works with GIS data formats have been developed that analyse LULC data and produce habitat quality maps. The Habitat Quality InVEST (Integrated Valuation of Ecosystem Services and Trade-offs) Model analyses data on land-use and threats to biodiversity to produce habitat quality and degradation maps. This model is a rapid assessment technique of habitat quality to inform natural resource managers of an area's conservation needs (Sharp et al. 2018).

The aim of this study is to develop habitat quality and habitat degradation maps for a section of the Berg River to assess the value that mapping holds for conservation managers and spatial planners. For this purpose, ArcGIS and the InVEST Habitat Quality Model is used to produce habitat quality maps to determine their utility in current river conservation programs. The output maps are compared to the current WCBSP and BRIP to determine whether degraded riparian areas are considered in the current river conservation program.

Materials and Methods

Study Area

The 7 km^2 study area, 100 meters above sea level, covers a 3 km stretch of the Berg River in the Western Cape Province, South Africa (Figure 2). The Western Cape (WC) is one of nine provinces of the Republic of South Africa located at the south-western tip of the African continent. It has a dry Mediterranean climate with warm, dry summers and cold, wet winters (mean annual rainfall: 515 mm) (Tyson and Preston-Whyte 2000). The Berg River is the second largest river in the Western Cape province. It is approximately 285 km long with a catchment area of 8 980 km², flowing north from Franschhoek to Velddrif, and opens into the Atlantic Ocean. It is considered to be one of the most important water sources for the agricultural industry and as drinking water for the City of Cape Town (just outside the catchment, c. 70 km to the southwest), with a population of 4,52 million (DWS 2016). About 65% of the Berg River catchment area is under agricultural activities.

Various soil types are found along the Berg River, from sandy sediments in the lower catchments to distinct clay accumulations in the middle catchment (Clark and Ratcliffe 2007). These rich clayey soils have attracted agricultural development that has caused extensive transformation of riparian habitat along the river (Kamish 2008). Conversion of natural vegetation to other land-uses along the Berg River has impacted vulnerable species, endemic to the Cape Floristic Region, leading to a high concentration of threatened species (RHP 2004). SANBI (2006) listed 457 native plant species as threatened within this catchment and 270 of these are listed as either endangered or critically endangered.

The study area falls within the Cape Winelands Biosphere Reserve, forming part of the Swartland Alluvium Fynbos ecosys-
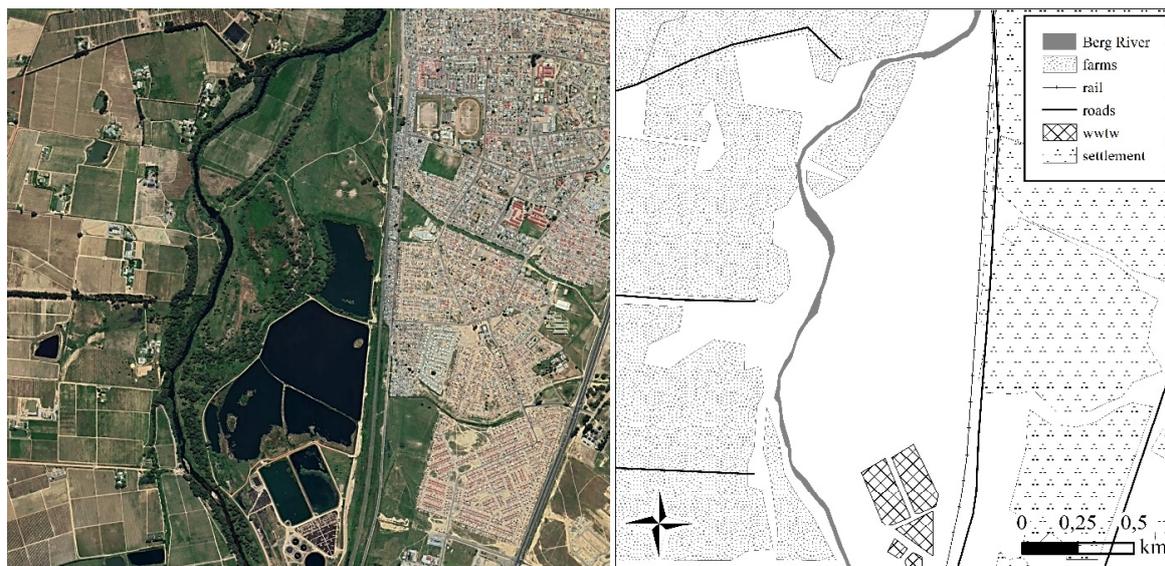
Figure 2. The study site along the Berg River for habitat quality mapping, satellite image (left) and threats map layer (right) (CapeNature 2017; Google Earth Pro 2019).

tem and has Category 1 and 2 terrestrial critical biodiversity areas, as identified by the WCBSP. These are "areas in a natural condition that are required to meet biodiversity targets, for species, ecosystems or ecological processes and infrastructure" (Driver et al. 2012). Vegetation types such as fynbos, renosterbos, and strandveld are characteristic of this region (Mucina and Rutherford 2006).

This study site was chosen due to the presence of sensitive habitats like wetlands and multiple land-use types which may act as sources of environmental degradation, i.e. commercial agriculture (farms), urban settlements, wastewater treatment works (wwtw), roads, and rails. These land-use types threaten habitat quality and biodiversity by directly degrading habitat area, displacing and eradicating species, and endangering population viability (Locke 2016; Fierro et al. 2017).

Process

LULC GIS data was collected from Cape-Nature, a governmental organisation responsible for maintaining wilderness areas and public nature reserves in the Western Cape Province. This dataset represents the 2014 LULC for the Western Cape and has been updated in 2016 (CapeNature 2014).

The LULC GIS data was converted and transformed with ArcGIS (ESRI version 10.4.1) to create separate layers of the study area as input for the InVEST Habitat Quality Model (naturalcapitalproject.stanford.edu/invest/), as detailed in the InVEST 3.5.0 User Manual (Sharp et al. 2018). The model was used to analyse habitat degradation and quality, with reference to threats and habitat sensitivity to threats. Habitats included grasslands, wetlands, bush, shrubland, and thicket. Threats included commercial agricultural fields, a waste water treatment plant, urban settlements, roads, and rails.

The models' outputs were raster GIS map data which represent the current (1) relative level of habitat degradation, and (2) relative level of habitat quality of the mapped area. These two maps were overlaid and examined to identify critical conservation areas within the study area (indicated as red circles on

Figure 3). The identified critical conservation areas were compared to the current Western Cape Biodiversity Spatial Plan and Berg River Improvement Plan and the associated GIS data layer, the WCBSP Bergrivier [Vector] (CapeNature 2017; Pool-Stanvliet et al. 2017).

Results

The InVEST Habitat Quality Model output maps, the relative level of habitat degradation and of habitat quality, show several specific locations of riparian habitat in need of critical conservation action (Figure 3). In the habitat degradation map, habitat was classified into levels of no concern (nc), least concern (lc), low degradation (ld), medium degradation (md), and high degradation (hd). Habitat classified as not habitat (nt), unsuitable habitat (uh), low quality (lq), medium quality (mq), and high quality (hq) showed the specific distribution of the conservation priority of riparian areas.

Compared to the Western Cape Biodiversity Spatial Plan and Berg River Improvement Plan, specific conservation priority areas like those identified here have not specifically been included in current conservation management and planning. The WCBSP outlines general policy guidelines for landuse and delineates critical biodiversity areas, but excludes location-specific information and does not use habitat quality as an indicator. The Berg River Improvement Plan only summarises management goals, critical success factors, and strategy implementation and omits spatially specific conservation information and planning.

Figure 3 shows red circles which indicate critical conservation priority areas identified by this study that have not specifically been included in local government conservation planning. This result demonstrates the practical use of the InVEST Habitat Quality Model to locate conservation priority areas for on-the-ground conservation action.

Discussion

Maps of the relative level of habitat degradation and quality of the research site indicated conservation priority areas and high threat impact areas, based on LULC data and threats to habitat. Several conservation priority areas important in mitigating environmental degradation were identified in the study site. It was found that current government river conservation plans do not include location-specific details informed by habitat mapping.

Habitat mapping can therefore be added to the WCBSP and the BRIP as spatial data for conservation managers and spatial analysts to work with, with a regional level map on habitat quality of the Western Cape. Habitat Quality modelling provides an opportunity to categorize conservation areas as ordinal conservation sites, i.e. high conservation needs (high priority), intermediate conservation needs (medium priority), and low conservation needs (low priority). This practical application offers a powerful tool to government officials working as conservation managers to identify the most important areas in which to dedicate scarce resources like funding, time, and labour.

The results of this study are similar to results obtained in the studies of Lin et. al. (2016), Terrado et al. (2016), and Li et al. (2019). All three studies found priority conservation sites in riparian zones that were previously overlooked. Terrado et al. (2016) found the InVEST Habitat Quality Model to be highly accurate by validating it with in-field observations and bio-physical sampling. Li et al. (2019) used the model to analyse changes in bird species presence over a decade which was attributed to human-related land-use and activities. Ecological security patterns were calculated by using the model along with structural connectivity designs to produce an efficient ecological network in Lin et al. (2016).
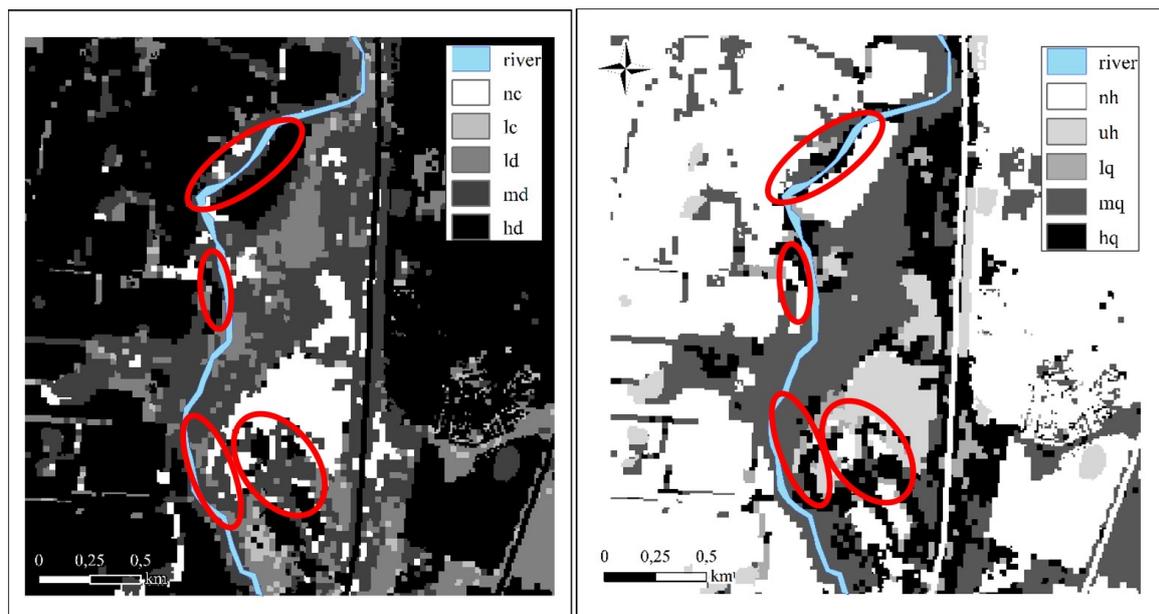
Figure 3. InVEST Habitat Quality Model outputs; Habitat Degradation map (left) and Habitat Quality map (right) with critical conservation priority areas (red circles). Legend; no concern (nc), least concern (lc), low degradation (ld), medium degradation (md), high degradation (hd), not habitat (nt), unsuitable habitat (uh), low quality (lq), medium quality (mq), high quality (hq).

A location-specific river conservation plan for the Berg River has to be developed through habitat mapping. Identifying conservation priority areas is important for improved resource management, conservation, water quality, and habitat protection. Long-term climate change impacts on the Western Cape will decrease water availability and suitable arable land which may result in further land encroachment into the Berg River (Midgley et al. 2005; Weber et al. 2018).

Mapping habitat quality and conservation priority areas provide a visual aid to conservation managers and spatial planners to identify the most important areas for conservation and to monitor environmental pressures and threats (Buhl-Mortensen et al. 2015). This technique can be used to inform which locations require more resources for environmental protection (Zlinszky et al. 2015). Field work can be optimized by detecting changes and allowing pre-selection of sites of interest.

Further research can be done by mapping the impact of long-term land-use change on the Berg River riparian zones. He et al. (2017) have developed a framework for using the InVEST Habitat Quality Model together with cellular automata simulation, this facilitates predictive scenario analysis based on LULC and habitat threats. This method provides an opportunity to test and analyse the impacts of policy interventions and ecological network development on river conservation. Used in conjunction with ecosystem trade-off analysis, managers would better understand which impacts are caused by specific stakeholders (Kovács et al. 2015).

Further riparian habitat quality studies in the Berg River should involve nature-based solutions' transdisciplinary research by following the framework set out by Nesshöver et al. (2017). This integrative, systemic approach allows for efficient natural resource manage-

ment which would lead to a comprehensive, holistic solution to improving river water quality. Given the high probability of land-use change along the river in the future, such research would be invaluable for conservation managers and spatial planners.

The use of InVEST models and mapping software has many advantages; it is free to use, presents a useful visual reference for conservation management, and it allows for sensitivity of habitats to be specified (useful when considering wetlands, grasslands, and forests). The model enables specification of the level of impact of threats to biodiversity and habitat sensitivity to threats. Maps then reflect the diverse impacts of threats such as agricultural runoff, soil erosion, and urban settlements. Limitations of using this mapping technique include poor data presentation on rare or cryptic species and of abiotic environmental conditions, with a cer-

tain level of subjective valuation of biodiversity. These maps are a single snapshot of the spatio-temporal distribution of landscape elements and should always be used in conjunction with in-field validation, research results, and qualitative data collection. Repeated habitat quality mapping over time forms part of a comprehensive ecological monitoring program led by conservation managers and it is a valuable tool for riparian zone and water conservation.

Acknowledgements

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Impact of nutrient supply on the relative development of yield components of winter wheat

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Abstract: In the long-term fertilization experiment, at Fülöpszállás, on calcic meadow chernozem soil we carried out experiments in seven growing seasons (2003/2004, 2004/2005, 2005/2006, 2006/2007, 2007/2008, 2008/2009, 2009/2010) with two winter wheat variety (GK Kalász, GK Petur,) in 4 replications, on 20 square meter random layout plots. The yield components were evaluated by kind of Sváb cumulative yield analysis. It can be determined that one-sided N, PK and NPK 2:1:1 rate applications had significant effect not only on yield of winter wheat, but also on yield components determining yield. Compare with the use of different nutrient rates it can be determined that in Fülöpszállás production site of high humus content, good P_2O_5 and K_2O providing ability; in the case of one-sided N application only slightly, but under PK application higher increase in yield component could be realized compared to plants of unfertilized control plots. The NPK 2:1:1 rate application has spectacularly represented the cumulative effect of nutrients, as the appropriate rate of nutrients caused not only the aggregation of the effects of certain nutrient rates, but intensifying influences resulted in redoubling of its results. The higher rates of PK and certain 2:1:1 rate NPK treatments increase the values of yield components only to a lower extent compared to control treatment.
Keywords: winter wheat, nitrogen, phosphorus, potassium, yield components
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Introduction

Nutrition supplementation is almost as old as crop production, although for thousands of years farmers have been more instinctively based on traditions, beliefs, habits, and observations rather than following scientifically sound principles. Processing data from several countries, Bocz (1963) clearly found that there was a strong correlation between wheat yield and fertilizer use. Harmati (1987) and Jolánkai (1981) demonstrated the relationship between yield and fertilizer use through domestic examples.

Based on the results of the National Fertilization Continuous Experiments, Debreczeni and Dvoracsek (1994) and Debreczeniné and Ragasits (1996) found that the required quantity of nutrients differs from one production site to another. The optimum nutrient level is influenced not only by conditions of production site but also by the cultivation purpose and economic factors. Thus, the farmers are able to supply nutrients for winter wheat with knowledge of soil analysis, ecological conditions and economic parameters.

According to Harmati (1987) nutrient supply of wheat is mainly (about 85%) by fertilization. In the previous years, winter wheat was rarely fertilized with manure in Hungary (Késmárki and Petróczki 2003), but numerous publications proved the important role of stable manure in the supply of microelements of winter wheat (Kádár and Lásztity 1979, Németh et al. 1987, Kismányoky and Kiss 1998). In contrast, mainly for economic reasons, only nitrogen, phosphorus and potassium are used in fertilization practices (Ragasits 1998).

Lönhardné et al. (1995) concluded that nutrient supply has a great effect on the quantitative parameters of the ears (length of ears, weight of ears, number of grains per ear). In contrast Harmati (1987) the largest part of extra yield caused by fertilizers-which can be appeared in productive tillering (increase number of ear) not in the quantitive parame-

ters of the ears.

According to Lesznyákné (2001), the thousand grain weight is a genetically highly determined yield component that can only be slightly influenced by agrotechnical factors. While Harmati and Gyuris (2002) investigated the effect of P on yield components, it was found that increasing P fertilizers positively changed yield components, thus increasing the number of ears, the number of grains per ears and the thousands grains weight.

According to Jakab, et al. (2017) the fertilization had different effect on the examined generative factors. The thousand seed weight did not change significantly, but the change of length of spike and number of spiklets under the influence of fertilization was significant. Fertilization had a great effect on the length of spike, weight of spike and grain number of spike (Jakab, et al. 2016). Kristó, et al. (2008) have found that the PK and the NPK treatments significantly increased the number of shoots, the number of ears, the number of spikelets.

Materials and Methods

In the long-term fertilization experiment on calcic meadow chernozem soil in Fülöpszállás, evolved by Istvan Harmati in 1982, we made 16 different type of fertilization treatment, which can be use as a different fertilization strategy. We have selected 10 typical treatment from the 16 (Table 1). Fertilization experiments have been carried out in 7 growing seasons (2003/2004, 2004/2005, 2005/2006, 2006/2007, 2007/2008, 2008/2009, 2009/2010) with 2 winter wheat variety (GK Kalász, GK Petur) with 500 seed/m^2 density, in 4 replications, on 20 m^2 random layout plots.

The yield components were evaluated with Sváb-type cumulative yield production analysis (Sváb 1961, 1962). In our yield component investigations plants and shoots (de-

riving from unit area of 0.25 running meter) have been removed from the internal rows of the plots. The samples have been marked with the help of a measuring rod. The cumulative yield production analysis gives opportunity for graphic representation of plant development, where horizontal axle (x) represents yield components (end products of different development stages) per unit area in developmental order, and vertical axle (y) indicates the percent value of yield components referring to a basis for comparison. In the course of cumulative yield production analysis the followings are considered as yield components: A=number of seeds per unit area, B=number of shoots per unit area, C=number of ears per unit area, D=number of spikelets per unit area, E=number of grains per unit area, F=grain weight per unit area.

Results

As a result of the investigation in Fülöpszállás, data from yield components were made by variance analysis of are in Table 2. All of the treatments have a significant effect on the number of shoots, grains, and grain weight of winter wheat per unit area in 0.1%. It was significant 1% on the number of spikelets, 5% on the number of ears.

All of the investigated yield components and the fertilizer (A) were 0,1% level of significance. While the winter wheat species (B) was 5% level of significance in the case of the number of ears per sample. From the other parameters we couldn't get statistically reliable results. Growning seasons (C) has a significant effect on the number of shoots, spikelets, and grain weight per unit area in 5%, but there was no influence on the number of ears and grains.

From the interactions fertilizer × winter wheat species (A × B) no significant difference could be proved concerning to the yield components. While the fertilizer ×

Table 1. Data of nutrient applications in the experiment.

№ of the application	Name of application	N	P_2O_5	K_2O
		kg ha^{-1} active agent		
1	control	0	0	0
2	*N1*	30	0	0
3	*N2*	60	0	0
4	*N2*	90	0	0
5	*PK1*	0	30	30
6	*PK2*	0	60	60
7	*PK3*	0	90	90
8	*NPK1*	60	30	30
9	*NPK2*	120	60	60
10	*NPK3*	180	90	90

Table 2. Results of variance analysis of yield components (MS).

	df	number of shoots	number of ears	number of spikelets	number of grains	grain weight
Repeat	3					
Total treatment	559	211266.30***	145157.60*	29245401.29**	106041703.5***	188175.05***
Fertilizer (A)	9	443.64***	363.25***	220919.21***	1179419.18***	2934.15***
Variety (B)	1	232.72*ns*	92058*	127912.2*ns*	46537.55*ns*	16.35*ns*
Growing season(C)	6	852.84*	418.38*ns*	167867.90*	198952.26*ns*	821.59*
Intercepts:						
A × B	9	9.80*ns*	9,85*ns*	2569.28*ns*	4221.37*ns*	11.39*ns*
A × C	54	20.95*	1245*ns*	4355.68*	1282.85*	30.49*
B × C	6	124.35***	115.18***	30328.22***	5622.59***	167.00***
A × B × C	54	12.38***	8.67***	2397.28***	767798***	16.87*ns*
Error	420	3.07	2.24	813.84	2118.67	15.98

*The mean difference is significant at the P=5% level.
**The mean difference is significant at the P=1% level.
***The mean difference is significant at the P=0.1% level.
ns: The mean difference is non- significant.

growing seasons (A × C) interaction have a significant effect on the number of shoots, spikelets, grains and grain weight per unit area in 5%. Number of ears we couldn't get statistically reliable results. In the winter wheat species × growing seasons (C) interaction have significant effect on all of the yield components in 0.1% Fertilizer × winter wheat species × growing seasons (A × B × C) interactions have a significant effect on the number of shoots, ears, spikelets, and grains per unit area in 0.1%. Grain weight we couldn't get statistically reliable results.

We can see the effect of different level fertilizer treatments of the relative process of development of winter wheat in

Figure 1, where the level 100% is the yield components of the control, unfertilized treatment for a long time ago. On the development graph yield components were signed capital letters of the ABC. A= number of seeds/sample, B=number of shoots/sample, C=number of ears/sample, D=number of spikelets/sample, E=number of grains/sample, F=grain weight/sample. In the investigation of the effect of different nutrient rates, the $N_{30}P_0K_0$ (N1) application had almost no effect to the winter wheat tillering tendency, furthermore $N_{60}P_0K_0$ (N2) and $N_{90}P_0K_0$ (N3) applications increased 6% and 5% of the number of shoots per unit area. Based on the graph of the different level of N applications influenced the number of spikelets per unit area positively, compared with the control unfertilized treatment in the case of the 30 kg ha^{-1} N application (N1) increased the number of spkelets per unit area in 4%, 60 kg ha^{-1} N application (N2) increased in 3%, and 90 kg ha^{-1} N application (N3) increased in 10%. By linking C and D end products of different development stages with line facing up, which means the different level of N applications had obviously positive effect on the number of spikelets. The effect of one-sided, different level nitrogen applications increased the number of grain yield by 11%, or 23%, compared with the control unfertilized plots. By linking E and F end products of different development stages with line facing down, in the case of 30 kg ha^{-1} (N1) and 90 kg ha^{-1} (N3) applications, which means these treatments had an effect on thousand-seed weight.

The different levels of phosphorous and potassium applications without nitrogen (PK1, PK2, PK3) show very similar development graph. Compared with the control, unfertilized plots these applications increased the number of shoots per unit area in 11-22%, the number of ears per unit area in 11-23%, the number of spikelets per unit area in 23-33%, and the number of grains per unit area in 15-27%. The end products of different development stages, that is grain weight per unit area increased compared with the control, unfertilized plots, in case of the $N_0P_{30}K_{30}$ (PK1) application in 28%, $N_0P_{60}K_{60}$ (PK2) application in 47%, $N_0P_{90}K_{90}$ (PK3) application in 43%. It means the greatest PK application (PK3) without nitrogen increased much less extent in yield than $N_0P_{60}K_{60}$ (PK2) application. The PK treatments influenced tillering tendency, spikelets development, and thousand-seed weight favorably, in contrast had negative effect on the number of grains.

By investigating the winter wheat average growing seasons, and species NPK 2:1:1 rate applications (NPK1, NPK2, NPK3), compared with the control, unfertilized plots, their tendency of the development lines were similar. In case of $N_{60}P_{30}K_{30}$ application (NPK1) the number of shoots/sample were 30%, the number of ears were 33%, number of spikelets were 55%, number of grains were 72%, and the grain weight were 105% more than the control, unfertilized plots. At the application $N_{120}P_{60}K_{60}$ (NPK2) the number of shoots/sample were 39%, the number of ears were 37%, number of spikelets were 73%, number of grains were 102%, and the grain weight were 155% more than the control, unfertilized plots. At the application $N_{180}P_{90}K_{90}$ (NPK3) the number of shoots/sample were 51%, the number of ears were 56%, number of spikelets were 99%, number of grains were 147%, and the grain weight were 184% more than the control, unfertilized plots.

On the Figure 2 we represented the development graph of the two determined variety: yield components of GK Kalász variety gives level 100%, compared with GK Petur's development line. By investigated the average years and nutrient treatments of GK Petur it seems had less tillering tendency than GK Kalász. Moreover, shoots in
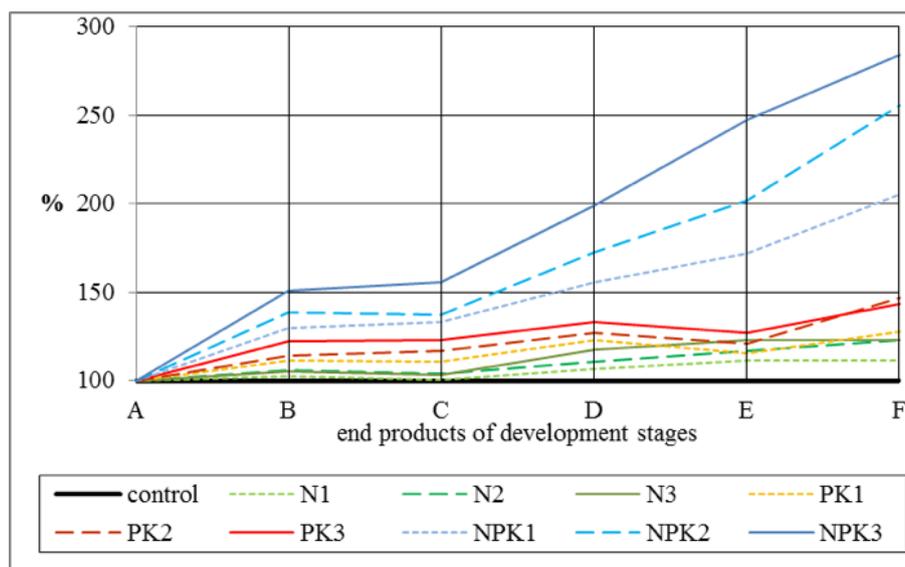
Figure 1. Relative development of winter wheat on different fertilizer treatments in average of 7 year and 2 varieties.
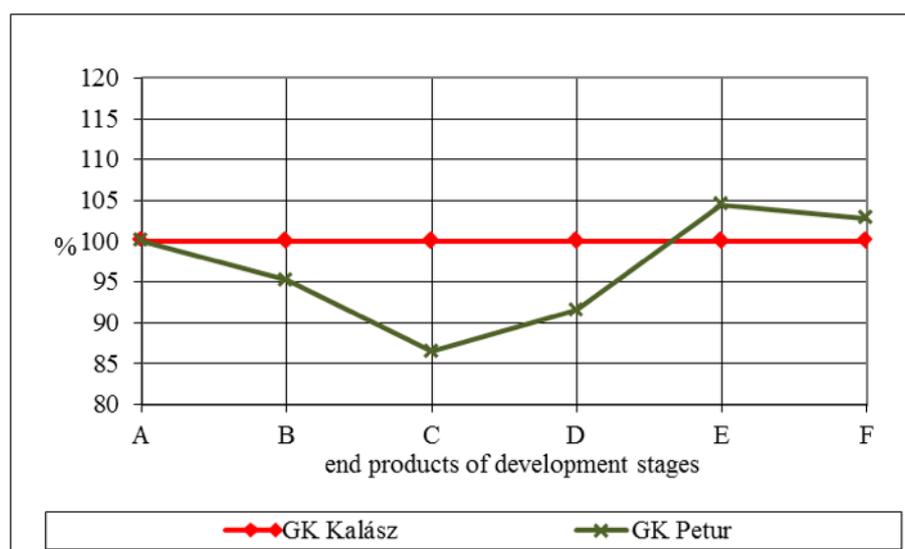
Figure 2. Relative development of winter wheat on experimented two varieties.

GK Petur were unproductive, which means there is no spikelets. We can see it by B and C end products of different development stages line tendency. Development line tendency of C-D and D-E show that GK Petur has more spikelets and number of grains, than GK Kalász. It means the number of grains per unit area is also much more than GK Kalász. Although in the investigation of the average years and nutrient treatments of the thousand-seed weight of GK Kalász was much bigger (0.7g), than GK Petur, grain yield per unit area is less in 3%.

On the Figure 3 we can see the relative development of winter wheat on different growing seasons. The 100% level means the determined yield components during the 7 growing season. In the year of 2005/2006,
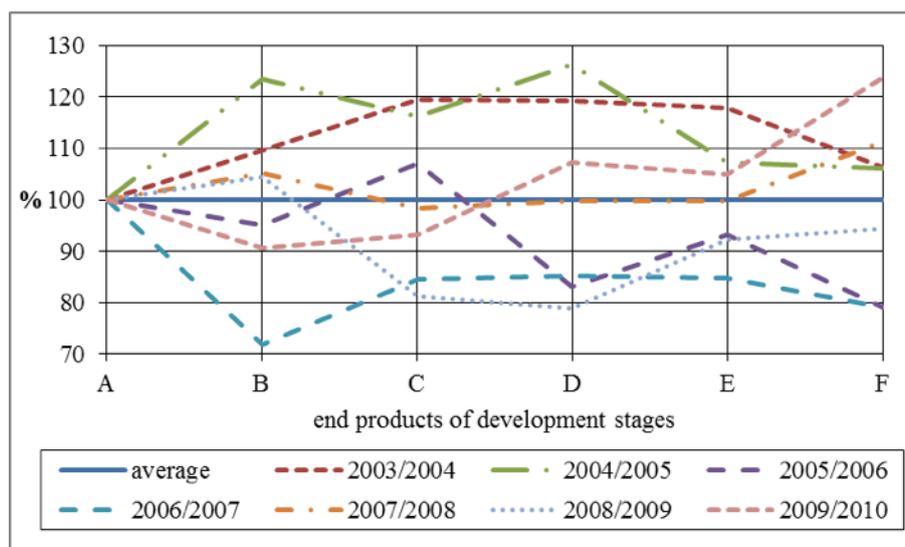
Figure 3. Relative development of winter wheat on different growing seasons.

2006/20007 and 2009/2010 tillering tendency of winter wheat was below the average because of the low amount of precipitation in the autumn and winter. In contrast in the year of 2004/2005 it was rainy moderately warm in autumn, which was quite favourable for initial development and tillering tendency of winter wheat. Because of the rainy, mild weather of March and April was positive for the number of ears and spikelets in the years of 2003/2004, 2004/2005 and 2009/2010. In 2007/2008 and 2009/2010 the weather in May and June were favourable for graining, this was the reason why thousand-seed weight of winter wheat was much above than the determined average years.

Discussion

Nutrient supply for winter wheat is very important in the current level of management, usually most part of it means the use of fertilizer. Nowadays there are a lot of new winter wheat variety in commercial growing, it plays much significant role to recognize nutrients supply treatments to get economical, sustainable and environmentally friendly wheat cultivation. In our nutrient rate investigations the effect of different nutrient application strategies on the most important yield components influencing yield of winter wheat varieties have been studied.

It can be determined that one-sided, different level nitrogen applications increased yield components (number of shoots, ears, spikelets, grains, and grain weight per unit area) in slightly rate. On the basis of our results it can be determined the same like Lásztity (1987) that N applications had a great effect on grain weight, number of grains, in contrast one-sided N applications decreased thousand-seed weight of winter wheat.

All of the determined species and growing seasons the different levels of phosphorous and potassium had positive effect on the yield components of winter wheat all the time, compared with the end products of different development stages of the control, unfertilized plot. In our experiments is the same with Liakas et al. (2001), as PK rations increased as the number of shoots increased. Only P and K application plots had higher tillering tendency, increased number of spikelets and seed size, according to Rag-

asits (1983) too. In contrast PK application without nitrogen was negative for the fertilization of flowers, because the number of grains per one spikelets was lower than the control, unfertilized plots. NPK application in 2:1:1 rate show nutrients cumulative effect spectacularly. The end products of different development stages, yield per unit area also increased, just like the number of shoots, ears, spikelets, and grains. From the derived yield components the number of spikelets per ears, number of grains per spikelets and thousand-seed weight were also increased.

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Transcriptome analysis of an ochratoxin-A biodegrading bacteria

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Abstract: Fighting against and decreasing the effect of mycotoxins is an emerging problem. Among post-harvest methods are physical, chemical, and biological ones. This study is focusing on the biological tools for minimalizing the harmful effect of the ochratoxin-A (OTA) occurring on crops and fodders. The bacteria *Cupriavidus basilensis* ÖR16 strain has very good ability to detoxify ochratoxin-A to phenylalanine and ochratoxin-alfa. In previous studies the degradation rate of the ÖR16 bacteria was over 98%. The whole genome sequencing was also performed by our group in 2012. During this research, the enzymes, and genes responsible for the OTA degradation were characterized via transcriptome analyses. 15 genes were identified, which could play role in the degradation of OTA. Testing and investigating these nominated genes and enzymes could lead for a prepared fodder additive, which can help in the elimination of the negative effects of OTA in the future.
Keywords: OTA, genes, biodegradation

Introduction

Ochratoxin-A (OTA) is one of the most important mycotoxins, which is produced as toxic metabolites by genera belonging to *Aspergillus* and *Penicillium* species, mainly *Aspergillus niger*, *A. ochraceus*, *A. carbonarius*, and *Penicillium verrucosum* (De Bellis et al., 2015). The chemical structure of OTA is (N-[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl-L-phenylalanine) includes a b-phenylalanine-dihydro isocoumarine derivative, which is very constant at intense temperature and resistant to hydrolysis. The currently used managing methods of raw materials in feed and food production does not reduce the OTA, therefore the toxin persists in the final food and feed products (Ferenczi et al., 2014). OTA is considered to be one of the important contaminants which targets cereal grains and crop products, peanuts, coffee beans, red wine and pork products (Bragulat et al., 2008). The occurrence of OTA can be detected in all regions due to inappropriate storage of human foods and animal derived products and weather conditions. The International Agency for Research on Cancer (IARC) has been classified OTA as group 2B - possible carcinogenic to humans (IARC, 1993). OTA mainly target the kidney in humans as well as to its ability to induce porcine nephropathy (Duarte et al., 2012).

There are several strategies focusing on the reduction or elimination of OTA concentration in human food and animal feeds. The physical adsorbents procedure, besides, has various disadvantages despite that its widely used method, such as nonspecific bindings of some important nutrients (vitamins, minerals, and therapeutic agents), high cost and limited efficiency. According to the literature, the biological methods are the most effective and promising methodology for controlling the contamination of OTA in food and animal feeds, thus diminish the risk on animals and human health. More than ten species of bacteria, including our selected bacteria, have the ability to degrade OTA: *Acinetobacter calcoaceticus* (De Bellis et al., 2015), *Acinetobacter sp.* (Liuzzi et al., 2017), *Alcaligenes faecalis* (Zhang et al., 2017), *Bacillus licheniformis* (Petchkongkaew et al., 2008), *Bacillus amyloliquefaciens* (Chang et al.,

2015), *Brevibacterium spp.* (Rodríguez et al., 2011), *Lactobacillus acidophilus* (Fuchs et al., 2008), *Pediococcus parvulus* (Abrunhosa et al. 2014), *Lactobacillus spp.* (Luz et al., 2017).

In this study the *Cupriavidus basilensis* ÖR16 strain OTA degradation was investigated in the level of RNA expression. The genus *Cupriavidus* was identified in 2004 (Coenye et al., 2003). Members of this genus are Gram-negative, chemoorganotrophic and facultative chemolithotrophic bacteria that can be found in diverse habitats such as soil, root nodules and aquatic environment. The genus *Cupriavidus* belongs to the family Burkholderiaceae and the class β-proteobacteria. The genus consists of nineteen type strains. Remarkable heavy metal tolerance of environmental isolates has been confirmed (Goris et al., 2001). According to the literature in the case of 7 strains different xenobiotic biodegradation was observed. For example chlorinated aromatic chemicals; halo benzoate and nitrophenols were degraded by *Cupraividus necator* CCUG 52238T (Makkar and Casida, 1987) and some xenobiotic genes and enzymes such as benzoate1,2-dioxygenase and chlorocatechol-degradative for this strain were reported (Ogawa and Miyashita, 1999). *Cupriavidus basilensis* RK1 DSM 11853T strain was originally isolated as a 2,6-dichlorophenol degrading strain (Steinle et al., 1998). Other isolates of the species are also capable for degradation of various xenobiotics such as furfural, 5-hydroxymethyl furfural, bisphenol-A, chlorophenols and atrazine (Stamper et al. 2003, Koopman et al., 2010).

In the case of *Cupriavidus basilensis* ÖR16 one study in mice demonstrated the effectiveness of the strain's detoxification ability (Ferenczi et al., 2014). There was efficient degradation for the OTA by the 5th day of the experiment by the strain. The by-product group which arrived from OTA degradation process of the *C. basilensis* ÖR16 was not toxic on the mice kidney cells. In 2012 the total genome project was preceded of the *C. basilensis* ÖR16 strain for making a foundation of future studies (Cserháti et al., 2012).

Materials and Methods

The experiment reagents
Ochratoxin-A mycotoxin (OTA) (Sigma-Aldrich Co., USA). Luria-Bertani medium (LB) (100%: 10 g tryptone, 5 g yeast extract, 9 g sodium-chloride). Minimal buffer medium (3.1 g of K_2HPO_4, 1.7 g of $NaH_2PO_4 \cdot 2H_2O$, 4.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 20 mg of EDTA, 4 mg of $ZnSO_4 \cdot 7H_2O$, 2 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.4 mg of $CuSO_4 \cdot 5H_2O$, 0.8 mg of $CoCl_2 \cdot 6H_2O$, 2 mg of $MnCI_2 \cdot 2H_2O$).

Bacterial strain and culture conditions in the biodegradation experiment
The strain *Cupriavidus basilensis* ÖR16, was isolated from a Hungarian pristine soil sample. It was deposited in the National Collection of Agricultural and Industrial Microorganisms (NCAIM BO2487). It was grown on LB agar plates and incubated at 28 °C for 72 h. Single colonies were inoculated into 50 ml liquid LB medium and incubated at 170 rpm at 28 °C for 72 h. After resuspension, the optical density (OD600) of the culture was measured at 600 nm (OD 600) (IMPLEN SpectroPhotometer, GENESIS 10S, Thermo Fischer Scientific) and adjusted to 0.6 (OD600 = 0.6) to prepare bacterial inoculum, from this 10 ml was added to 45 ml minimal buffer, which contained 7 mg/l OTA in final concentration.

OTA degradation experiment for getting the RNA to transcriptome analysis
The *C. basilensis* ÖR16 strain was cultured in LB media for growing and getting the exact cell number. The OTA degradation was

carried out in a minimal buffer, *C. basilensis* ÖR16 was grown on LB agar plates and incubated at 28 °C for 72 h. Single colonies were inoculated into 50 ml liquid LB medium and incubated at 170 rpm at 28 °C for 72 h. Cultures then centrifuged and cleaned from LB media via minimal buffer.

10 ml of the ÖR16 was added to 45 ml minimal buffer and 45 ml 2% fructose (200 ml Demineralized Water, 4.0 g D-Fructose), only with fructose as carbon source, to activate just those genes, which are responsible or act in the presents of OTA or OTA degradation and incubated for 11 hours (till reaching the log phase of ÖR16).

For control *E. coli* TOP10 was used in LB and in minimal buffer media, incubated in the same circumstance as ÖR16. After 11 hours incubation, OD was measured to reach 0.4-0.8 (to be suitable with the requirement of the RNA isolation kit). 7 ppm of OTA was added to the target groups (ÖR16 + OTA). Samples were set in duplicates.

Remaining OTA concentrations in the supernatant and pellet were analysed by High Performance Liquid Chromatography (Szent Istvan University, Advanced Chemistry Department) and by Neogen Accuscan Gold ELISA equipment (Szent Istvan University, Environmental Protection and Safety Department).

RNA extraction, RNA quality test

In order to obtain good quality RNA, 100 ml of the matrix (45 ml of 2% fructose + 45 ml minimal buffer + 10 ml of culture of ÖR16 + 7 mg/l of OTA) was used for the biodegradation experiment for the transcriptome analysis. Samples were centrifuged at 4600 rpm at 4 °C for 30 minutes after reaching the log phase (11 h). Total RNA was extracted from the pellets using the Trizol Plus RNA Purification Kit (Thermo Fisher Scientific Co., USA) at SZIU, Gödöllő, according to the manufacturer's instructions. The quality and the quantity or RIN (RNA integrity number)

of the RNA sample were analysed by Agilent 2200 Technologies and using TapeStation software (Seqomics Ltd, Hungary) (Table 1.).

Transcriptome analysis

Whole transcriptome sequencing was performed using TrueSeq RNA Library Preparation Kit v2 (Illumina Co., USA) according to the manufacturer's instructions. Briefly, RNA quality and quantity measurements were performed using RNA ScreenTape and Reagents on TapeStation (all from Agilent Co., USA) and Qubit (Thermo Fisher Scientific Co., USA); only high quality (RIN 7 and 8) total RNA samples were processed. Next, 1 μg of RNA was treated with DNaseI (Thermo Fisher Scientific Co., USA), the ribosomal RNA depleted using RiboZero Magnetic Kit for Gram-negative bacteria (Epicentre Co., USA) and the leftover was ethanol precipitated. The success of rRNA removal was determined by measurement on TapeStation using high-sense RNA ScreenTape and Reagents (Agilent Co., USA).

RNA was purified and fragmented; first strand cDNA synthesis was performed using SuperScript II (Thermo Fisher Scientific Co., USA) followed by second strand cDNA synthesis, end repair, 3′-end adenylation, adapter ligation, and PCR amplification. All the purification steps were performed using AmPureXP Beads (Beckman Coulter Co., USA). Final libraries were quality checked using D1000 ScreenTape and Reagents on TapeStation (Agilent Co., USA). The concentration of each library was determined using the KAPA Library Quantification Kit for Illumina (KAPA Biosystems Co., USA). RNA quality control, RNA preparation and sequencing were performed by Seqomics, Ltd, Hungary on an Illumina NextSeq instrument using the NextSeq 500/550 High Output Kit v2 (300 cycles; Illumina Co., USA) generating ~10 million clusters for each sample.
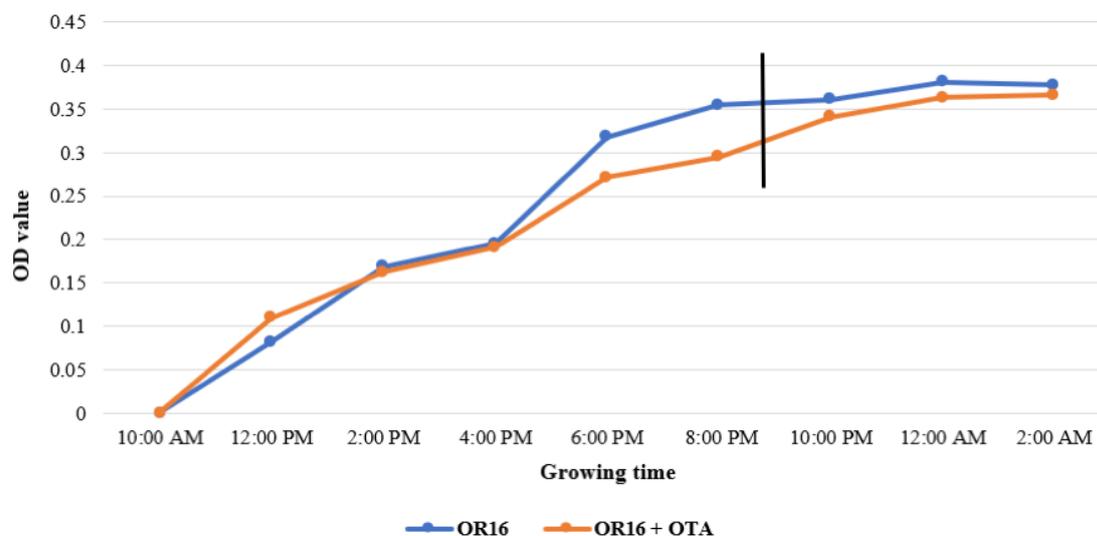
Figure 1. The log phase age of *C. basilensis* ÖR16 in the minimal buffer, black line means the end of the log phase.

Bioinformatics analysis of RNA-sequencing data

After sequencing, paired-end Illumina reads were quality trimmed in CLC Genomics Workbench Tool (v.11.0, Qiagen Bioinformatics Co., Denmark) applying an error probability threshold of 0.01. No ambiguous nucleotide was allowed in trimmed reads. For filtering, reads were mapped on CLC with a length fraction of 0.9 and a sequence identity threshold of 0.95. RNA-Seq analysis package from CLC was then used to map filtered reads on a custom-masked *C. basilensis* ÖR16 genome version. Only those reads were considered that displayed an alignment longer than 80% of the read length while showing at least 95% sequence identity against the reference genome. Next "Total gene read" RNA-Seq count data was imported from CLC into R 3.3.2 for data normalization and differential gene expression analysis. Function "calcNormFactors" from package "edgeR" v.3.12.1 was applied to perform data normalization based on the "trimmed mean of M-values" (TMM) method. Genes displaying at least one -fold gene expression change with an FDR (false discovery rate) value below 0.05 were considered as significant (Seqomics Ltd, Hungary).

Results

Log phase identification of Cupriavidus basilensis ÖR16 strain

Estimating the log phase of *Cupriavidus basilensis* ÖR16 was important, to find the correct time for extracting the best quality RNA from the inoculum. During the pre-experiments, the RNA extraction was according the peak of the OTA degradation process on the 3rd day, but at that time the RNA was already broken, not useful for transcriptome analysis. The 11th hour was the proper time for making the RNA extraction, getting good quality of RNA, which can be used for the analysis (Figure 1).

Results of the RNA isolation

The biodegradation in minimal buffer was stopped at the 11th hour, according the log phase peak for getting good quality RNA. There were two parallel settings from each
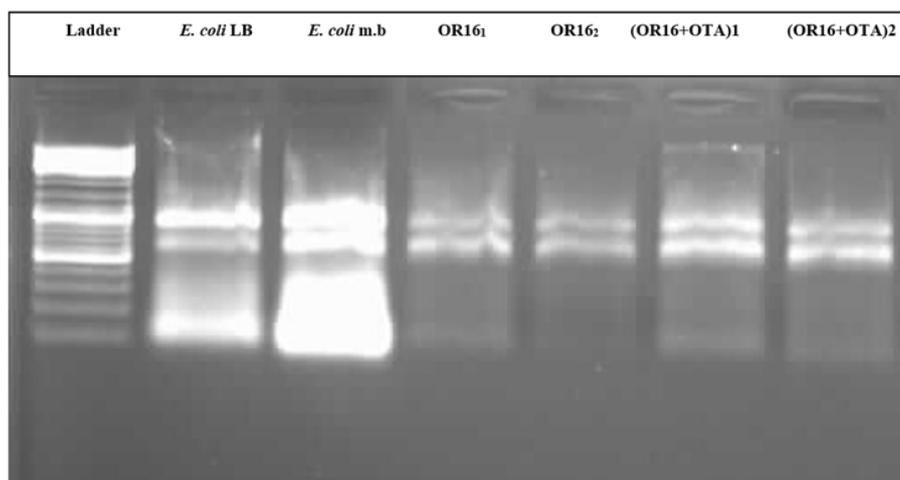
Figure 2. RNA bands of *Cupriavidus basilensis* ÖR16 with and without OTA from the OTA degradation experiment conducted in minimal buffer for transcriptome analysis, sampled after 11 hour of incubation.

Table 1. RNA quality results of the different setting from the OTA degradation matrix in minimal buffer from the Agilent 2200 Technologies (Seqomics Ltd, Mórahalom, Hungary), RIN =RNA integrity number.

Sample description	23S/16S (Area)	Conc. [ng/μl]	RIN
Electronic Ladder	-	84.9	-
OR 16_1	0.8	99.2	8.2
OR 16_2	0.7	57.8	8.2
OTA OR 16_1	1.3	92.3	7.0
OTA OR 16_2	0.5	70.1	7.6

set: OTA+ÖR16 strain; ÖR16 strain; *E.coli* LB (in LB media) and *E. coli* MB (in minimal buffer media). RNA was extracted and the RNA integrity was confirmed in 1% agarose gel electrophoresis (Figure 2). RNA quality was tested by Agilent 2200 Technologies and using TapeStation software (Seqomics Ltd, Mórahalom, Hungary) (Table 1).

Results of the transcriptome analysis

During bioinformatics analysis it turned out that 3500 genes were up regulated in the ÖR16 strain in the presents of OTA. A decision system had to be developed for the identification of the potential genes and enzymes, which could play a role in the OTA degradation. The decision system concluded the following circumstances:

- At least 2-fold expression
- Playing role in any aromatic ring opening
- Should be a protease
- Could be connected to the hypothetical degradation pathways in literature
- There is any literature about the role in degradation of xenobiotics or OTA

The ideal case was when all the circumstances were standing. Of course there were exceptions like the low fold expression genes. Out of the 3500 gene, only 15 genes

could be enrolled into the criteria of the decision system (Table 2).

Discussion

In the literature there are two hypothetical microbiological degradation pathways involved in the OTA degradation as illustrated in Figure 3. The first pathway (a) is the hydrolysis occurred in the amide bond, which links the L-β-phenylalanine molecule to ochratoxin-alpha (OTα) moiety both of them are non-toxic (Abrunhosa et al., 2010). The second pathway (b), the lactone ring hydrolysis can be considered a more hypothetical process in the OTA degradation and detoxification (Bruinink et al., 1998; Abrunhosa et al., 2010).

According to the literature, there are several enzymes which might be involved in the biodegradation of OTA. There are two types of carboxypeptidases among microbes, which may be involved in the OTA-biodegradation (Chang et al., 2015; Liuzzi et al., 2017). The first one is carboxypeptidase-A (CPA), where the "A" refers to aromatic compound, carboxypeptidases that have a stronger preference for those amino acids containing aromatic or branched hydrocarbon chains. The CPY is the second enzyme, where the "Y" refers to yeast origin. A CPY was isolated from *Saccharomyces cerevisiae*, it could degrade 52% of OTA and converted it to OTA-α after five days of incubation with pH 5.6 at 37 °C (Abrunhosa et al., 2010).

There are different enzymes besides the carboxypeptidases, which can degrade OTA. *Aspergilus niger* strains have a few enzymatic tool for OTA degradation: a lipase enzyme can hydrolyse OTA through the amide bond (Stander et al., 2000) and Protease-A have been reported to degrade around 87.3% of 1μg OTA respectively with pH 7.5 in 25 hour-incubation period (Abrunhosa et al., 2010). At last, amidase 2, which is encoded by open reading frame (ORF) of *Aspergillus niger* has the hydrolytic activity to degrade 83% of 50 µg/ mL of OTA (Loi et al., 2017).

Among the 15 identified genes, there are interesting members, which can degrade different chemicals according to the literature. For example, *dienelactone hydrolase* can degrade protoanemonin, which is a toxic metabolite, which may be formed during the degradation of some chloroaromatic compounds, such as polychlorinated biphenyls (Brückman et al. 1998). The *Aromatic Ring hydroxylase* can convert closed-ring structures to non-aromatic cis-diols (Neidle et al., 1991) The *Predicted metal-dependent hydrolase* are acting on carbon – nitrogen bonds and *2-keto-4-pentenoate hydratase* participates in 9 metabolic pathways: phenylalanine metabolism, benzoate degradation via hydroxylation, biphenyl degradation, toluene and xylene degradation, 1,4-dichlorobenzene degradation, fluorene degradation, carbazole degradation, ethylbenzene degradation and styrene degradation (Zhen et al., 2006). In 2017 Luizzi and colleagues cloned and investigated CPA genes of the *Acinetobacter* sp. *neg1* strain responsible for OTA biodegradation. In our transcriptome result the ÖR16 CPAs showed weak fold expression, not matching by the criteria of the decision system. Even so the future investigation of these CPAs is still important.

According to the results of the transcriptome analyses there is a chance to identify the enzymes via cloning and expression. Testing the expressed proteins in OTA degradation system the OTA degraders can be identified. The following genes should be investigated in the future for their OTA-biodegradation and detoxification potential: OR16_12645 coded membrane CPA (penicillin-binding protein), OR16_24100 coded membrane proteins related to metalloendopeptidases, OR16_31869 coded membrane CPA (penicillin-binding proteins PbpC), OR16_31894 coded phenylalanine-4-hydroxylase and OR16_16257 coded aro-
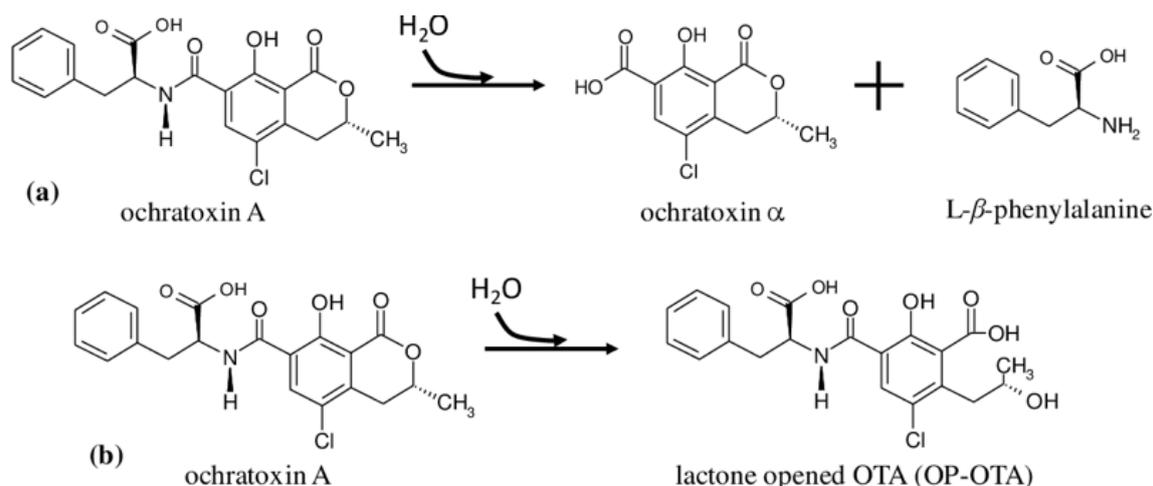
Figure 3. The biodegradation pathways of OTA adapted from Abrunhosa et al., 2010).

matic ring hydroxylase.

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Table 2. Fifteen nominated genes out of 3500 according the transcriptome analyses of the ÖR16 strain in present of OTA in minimal buffer.

Nominated genes	Chromosome	Gene name	Region	Fold expression	Size in bp	Contig number
Aromatic Ring hydroxylase	AHJE01000038	OR16_16257	Complement (106897..108471)	8.1	1575	38
Shikimate 5-dehydrogenase	AHJE01000021	OR16_09609	Complement (8185..8595)	7.6	411	212
Predicted metal-dependent hydrolase	AHJE01000108	OR16_35215	Complement (47971..48636)	6.5	666	108
Dienelactone hydrolase and related enzymes	AHJE01000063	OR16_25377	Complement (58185..59420)	6	1236	63
Amidases related to nicotinamidases	AHJE01000045	OR16_19156	Complement (94947..95645)	4.8	699	45
Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	AHJE01000019	OR16_08912	Complement (70900..71211)	4.3	312	19
Membrane proteins related to metalloendopeptidases	AHJE01000060	OR16_24100	Complement (30086..30808)	3.8	727	60
2-keto-4-pentenoate hydratase	AHJE01000003	OR16_01040	Complement (14846..17032)	3.2	2187	3
Metal-dependent amidase/aminoacylase/carboxypeptidase	AHJE01000017	OR16_07981	Complement (138451..139662)	2.5	1212	17
Phenylalanine-4-hydroxylase	AHJE01000094	OR16_31894	Complement (17995..18927)	2.3	927	94
Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	AHJE01000003	OR16_01015	Complement (11210..11980)	2.1	771	3
Membrane carboxypeptidase/penicillin-binding protein PbpC	AHJE01000094	OR16_31869	Complement (8236..10443)	1.9	2208	94
Membrane carboxypeptidase (penicillin-binding protein)	AHJE01000029	OR16_12645	Complement (43999..46188)	1.8	2190	29
D-alanyl-D-alanine carboxypeptidase	AHJE01000058	OR16_23878	Complement (72768..73976)	1.3	1209	58
D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4)	AHJE01000027	OR16_12223	Complement (91672..93207)	1.1	1536	27

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Effects of fertilisation on development and nutrient uptake of black locust saplings grown in pots

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Abstract: Currently, black locust is the most important tree species in Hungary with significant economic value. Intensification of its cultivation and the improvement of the timber quality should include the use of highly productive clones and reasonable fertilization. Nutrition management should be based on reliable data from exact experiments. In our trial, nutrition intake of Turbo Obelisk OBE01 clone saplings was examined during a four-month period. Osmocote Pro (18:9:10 + 2Mg) was used as fertilizer at a dose of 2.5 and 5 kg m^{-3} mixed to a peat-based substrate. At the end of the growing period, saplings reached a height of 260-280 cm and a stem diameter of 16-18 mm. Nutrient intake order was found to be the following: Ca (3.3-4.2 g) > N (3.1-3.6 g) > K (2.1-2.9 g) > Mg (0.35-0.5 g) = P (0.3-0.5 g). Based on our results, a lower N:P and N:K rate fertilizer is recommended, especially if a non-peat based substrate and longer growing period is planned with a higher rate of nitrogen fixation. Considering nitrogen resource, a dose of 5 kg m^{-3} was proved to be less effective than a concentration of 2.5 kg m^{-3}. However, the higher concentration of phosphorous, potassium and magnesium were well-utilized by the plants.
Keywords: nutrient concentration, nutrient distribution, dry matter, Turbo Obelisk clone
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Introduction

Black locust (*Robinia pseudoacacia* L.) originated from North America, is the second most important cultivated deciduous tree species in the World after the eucalyptus (Bartha et al. 2006). It has global economic importance and widely cultivated in temperate North America, Europe, and Asia. It was introduced to Hungary between 1710 and 1720 and spread widely in the 19th century (Rédei et al. 2008). In 2018, the surface of 454 000 hectares of black locust forest meant a quarter of Hungarian forest surface (KSH). Considered as an important and recognized tree species, black locust was declared as Hungaricum in 2014 (http1).

The main aim of black locust plantation is to exploit its fast and intensive growing. Although it is widely planted on medium quality or on poor soils where it is only able to produce firewood quality, the species can provide high-quality timber on suitable sites with nutrient-rich, well-aerated soil. Under favourable conditions, height growth of trees can have a peak in five years while it can reach the final diameter after 10 years, producing 8-12 tonnes of dry timber for a hectare. These characteristics make black locust one of the most important tree species of timber plantations in Hungary. At the same time, technology applied for less intensive species cannot be adopted for black locust (Rédei et al. 2008; Rédei 2015).

Due to the huge surface of Robinia forests and plantations, a great amount of black locust timber is available in the market but still at low quality, useless for furniture or construction industry. Improvement of timber quality can be reached by the use of selected varieties and intensive cultivation technology (Keresztesi 1988; Rédei 2015). Besides poplar and willow, black locust is also used in energy forests to provide the

highest aboveground biomass in the shortest time (Orlovic and Klasnja 2004). Wood and his colleagues (1977) called the attention to the problem that intensive forest management practices and short rotation cycles can severely deplete the nutrient pool of the soils. Short rotation forestry, therefore, requires reasonable nutrient management but Hungarian literature on the topic is scarce, especially under nursery conditions.

Black locust fixes 75-150 kg/ha atmospheric nitrogen in a year (Boring et al. 1981), being one of the most effective tree species (Olesniewicz and Thomas 1999). Consequently, black locust can enhance the growing of poplars (*Populus* sp.) or oriental arborvitae (*Platycladus orientalis*) when planted in mixed forests (Shen et al. 1998; Chen et al. 2018). However, careful nutrient management should be carried out until the associated nitrogen-fixing Rhizobium bacteria develop on the roots.

Pope and Andersen found (1982) that phosphorous and potassium fertilization significantly improved dry biomass in the first and second years of planting, especially on poor sites. Based on the results of Keresztesi (1988), optimal available phosphorous and potassium content in the black locust nursery beds should reach 150-200 mg kg^{-1} and 100-150 mg kg^{-1}, respectively. The findings of Tsiontsis and his colleagues (2001) revealed that available calcium and magnesium determines the improvement of Robinia. In Hungary, 2,5 kg m^{-3} Osmocote (18 : 9 : 10 + 2Mg) is generally applied in nurseries while the manufacturer, although for ornamental purposes, recommends 5 kg m^{-3}.

Nutrient demand of Robinia is not considered high: Wen and his colleagues (1998) calculated that 11.7 kg nitrogen, 0.7 kg phosphorous, 3.66 kg potassium, 15.1 kg calcium, 2.3 kg magnesium and 0.3 kg sulphur is necessary for 1 ton of dry biomass. Pope and Andersen (1982) revealed that nutrient content and distribution between leaf and stem of Robinia is stable. Site quality, spacing and fertilization had no effect on the distribution of dry matter, nitrogen and phosphorous between the foliage and the stem. Seed provenance had no considerable effect on nutrient status either when comparing seeds from Hungary and Iran (Moshki et al. 2012). Even site quality proved to have a minor effect on the order of nutrients quantities in thirty-year-old plantations, resulting in a sequence of Ca > N > K > Mg > P (Moshki and Lamersdorf 2011).

'Turbo' Robinia is a variety bred for intensive early-stage growing with faster timber mass development than common black locust by 30-50%. The variety is both suitable for energy plantation and forest establishment. 'Turbo Obelisk' is an asexually propagated clone group selected from 'Turbo' variety for its very fast early-stage growth. It can produce higher timber mass by 100% compared to traditional varieties, being able to reach maturity with a straight stem at the age of 15 on good sites (Pataki et al. 2016; Silvanus Forestry homepage).

Our study aimed to investigate the effect of two fertilizer doses (2.5 and 5 kg m^{-3}) on growth, dry matter and nutrient uptake and distribution of 'Turbo Obelisk' clones during a four-month pot trial.

Materials and Methods

The experiment was carried out in the research unit of Szent István University in Gödöllő-Szárítópuszta (N 47°58' E 19°37'). Micro-propagated saplings of Turbo Obelisk OBE01 provisional variety was planted on the 15th May 2019 to seedling trays of 66 cells with a cell size of 3.5 × 3.5 × 4.5 cm filled with perlite. Saplings were placed in an unheated, plastic sheet covered greenhouse and were fertigated on demand. After 22 days of growing, properly rooted saplings were planted to polypropylene pots of 12cm

diameter filled with a potting mixture of 90 v/v% peat and 10% v/v% perlite. Peat mix of Klassman TS3 Medium Basic contained 140 mg/L nitrogen, 41 mg L^{-1} phosphorous, 149 mg L^{-1} potassium and 100 mg L^{-1} magnesium based on manufacturer's information. Three treatments were carried out: control did not receive additional fertilizer, the standard treatment was supplied by 2.5 kg m^{-3} of Osmocote Pro (18 : 9 : 10 + 2Mg) 8-9 months, while elevated fertilizer treatment included the adding of 5 kg m^{-3} of the above fertilizer. 28 saplings per treatment were grown until 28th June 2018 when height and stem diameter were measured. Then 16 saplings with average values were planted into 12-litre polypropylene plant bags filling them with 10 litres of the potting mix of the same composition used before. Plant bags were placed outdoor on plastic pallets covered by geotextile to separate from the soil surface. Treatment units of 4 bags were randomly placed. Bamboo sticks and a high wire trellis system were used. Shading net was used for the first two weeks. Further fertilization was not made during the growing period. Daily irrigation was made by drip irrigation sticks. During the growing period from 28th June to 2nd October, each plant bag received a total of 282 L water from irrigation and a total of 135 mm rainfall. The average air temperature was measured at 20.7 °C.

Development of saplings was monitored by measuring height and stem diameter of each plant four times during the growing period (28th June, 28th July, 27th August and 27th September). Plant height from potting mix surface to the apex was measured by a metric gauge and was recorded in centimetres. Stem diameter was described by a digital vernier calliper measuring at the height of 5 cm from potting mix surface with the accuracy of 0.1 mm.

During the growing period, lateral shoots were removed three times (23rd July, 11th August and 12th September). Cut lateral shoots were grouped in the same block of four pots and were dried at 65 °C. Dry weight was measured with a scale at an accuracy of 0.01 g. Based on the results measured on the 27th September, from every four-pot unit two plants with middle values were selected and cut on the 2nd of October. Then leaves and stem were separated, milled and were dried. Dry weight of both stem and leaves was measured. Lateral shoots were mixed with previous cuttings to give one sample/treatment. Nitrogen, phosphorous, potassium, magnesium and calcium content of lateral shoot and stem samples were measured in an accredited laboratory based on MSZ-08-1783 standard. Nutrient uptake of aboveground biomass was calculated based on nutrient content and dry matter weight. Nutrient uptake values of fertilized plants were reduced by nutrient values of control plants, resulting in the amount of nutrients taken up from the fertilizer. This value was compared to the total nutrient quantity of the fertilizer added to each plant bag.

Statistical analysis was made with Microsoft Excel Analysis Tool Pack. In the case of height and stem diameter, all the plants were measured therefore 16 data from each treatment was available. In the case of dry weight and nutrient uptake, 4 data were calculated from the 4-unit blocks for each treatment. After the normality test and the verification of homoscedasticity of the data, one-way ANOVA was used to analyse the data. Fischer test was used as post-hoc test at 95% likelihood.

Results and Discussion

Plant size

No significant differences in size were measured among control and fertilized plants of the small-pot experiment (Figure 1), which means that nutrient included in the peat mix was sufficient for the development of the

plants in the first month. Therefore, the second trial period was started with saplings of the same size in case of all the three treatments.

After one month (28th July) the size of the control plants was significantly smaller than those of the two fertilized treatments. This trend remained true for the subsequent two measuring dates. A higher concentration of fertilization resulted in slightly higher and thicker plants compared to standard dosage (2.5 kg m^{-3} Osmocote) on the 28th July, but the difference did not prove to be significant either in the case of height or stem diameter (Figure 1.). At the last two measuring dates (27th August and 27th September) height data was still not significantly different between the two fertilized treatments. However, the stem diameter of elevated fertilizer dose showed a significantly higher number than standard dosage at both dates. Therefore, stem diameter proved to be a more efficient parameter of plant development than height. It can be concluded that elevated fertilizer dose resulted in bigger, more developed plants than standard dose. At the same time, differences between control and standard dose were more pronounced than those between standard and elevated dose of fertilizer. Hence, the 'first' dose of 2.5 kg fertilizer eventuated a higher rate of production growth than the 'second' dose of 2.5 kg fertilizer.

Heights of 260-280 cm and stem diameter of 16-18 mm of fertilized plants mean an outstanding result during the growing period of 4.5 months, compared to international literature. Pope and Andersen (1982) measured a height of 57-139 cm and a stem diameter of 7.1-17.3 mm for a one-year breeding of bare-root black locust seedlings, strongly depending on site quality. Moshki and his colleagues (2012) planted out 3-week-old saplings to plastic pots. After breeding them for four months, height was measured 150 cm and stem diameter was 5 mm. This value

changed to 200 cm and 6 mm, respectively, at the end of the 6-month-experiment. The exceptional values of the current study can be explained by the genetic potential of Turbo Obelisk OBE01 clone and the effect of irrigation.

Dry matter production
Similarly to the tendencies occurred in plant sizes, dry matter weight showed significant differences among treatments. Control plants had significantly the lowest aboveground biomass and elevated fertilizer treatment showed significantly the highest values (Table 1). The standard dose of fertilizer doubled the biomass compared to control one, while elevated fertilizer dose improved a further 30% on it. When analysing stem, leaf and lateral shoot biomass values separately, similar tendencies can be observed. Treatments had no significant effect on the rate of the stem of the whole biomass. Notwithstanding the tendency that enhanced fertilizer level improved the rate of stem biomass, although still not at a significant level (Table 1).

The above dry biomass data confirm again the exceptional growth capacity of Turbo Obelisk clone. Eigel and his colleagues (1980) measured a dry weight of 164 g as an average of seven different plantations. Plants were in situ sown, they were fertilized during the two-year trial period and plant density was 28,700 plant ha^{-1}. Pope and Andersen (1982) measured a dry weight between 50 and 600 g after a three-year trial. Weight was dependent on fertilization and site quality. Leaf rate of 46-49% in our experiment considered high but fits the age of the plants while it will decrease with the time. Eigel and his colleagues (1980) measured 38% leaf-rate at a five-year-old stand whilst it was 11% at the age of 13 years.

Nutrient content and nutrient uptake
The tendency of different nutrients was variable in the stem. Nitrogen content was sig-
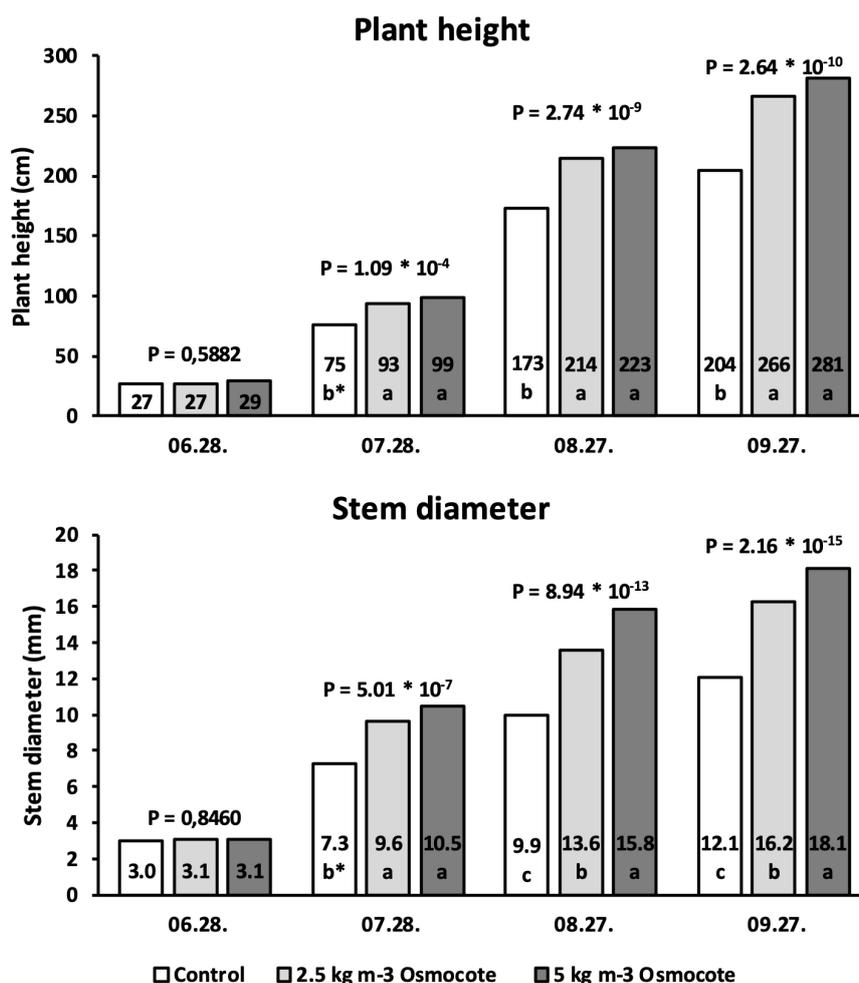
Figure 1. Effect of different fertilization doses (kg m^{-3}) on the height and stem diameter of pot-grown Black locust saplings. (*Average values of the same date and letter mean no significant differences based on Fischer's least significant difference test.)

nificantly the lowest in the stem in the elevated fertilizer treatment while it was non-significant between the standard treatment and the control, although control values were the highest (Table 2). The reason for this phenomenon could be the low dry biomass of control plants (Table 1). The high nitrogen content of the stem of control plants was observed even though plants definitely showed nitrogen deficiency. The absolute nitrogen values of 6-9 mg kg^{-1}, which is considerably higher than the values measured by Moshki and Lamersdorf (2011) on elder plants (2-3 mg kg^{-1}) is one of the signs of juvenile stage.

Phosphorous content of the plants was significantly different from each other at all the treatments and it increased with the elevation of the doses (Table 2). The measured values can be considered low compared to the results (2-3 mg kg^{-1}) of a previous study on pot-grown Robinia plants (Moshki et al. 2012). In the case of calcium, magnesium and potassium, no significant effect ($P > 0.05$) of the treatments was detected. The reason for these results can be

Table 1. Effect of fertilizers on aboveground biomass of pot-grown black locust saplings

	Aboveground parts (g plant^{-1})	Stem (g plant^{-1})	Leaves and lateral shoots (g plant^{-1})	Ratio of stem weight (%)
Control	78.8 c*	39.9 c	38.9 c	50.6%
2.5 kg m^{-3} Osmocote	178.1 b	93.5 b	84.6 b	52.7%
5 kg m^{-3} Osmocote	229.7 a	125.3 a	104.4 a	54.5%
P-value	2.59×10^{-7}	4.10×10^{-7}	5.37×10^{-6}	0.1930
LSD 5%	21.8	12.9	13.6	-

(*Average values of the same parameter and letter mean no significant differences based on Fischer's least significant difference test.)

that the potting mix contained a relatively high amount of magnesium, while irrigation water, given equally to all the plants, contained a considerable amount of calcium. Among the above mentioned mineral elements, fertilization had the greatest impact on potassium, resulting in considerable, but not significant differences among treatments. Nitrogen, potassium and calcium content of the stem varied between 8 and 9 mg kg^{-1}. These results are in harmony with the findings of Moshki and his colleagues (2012) except calcium which was measured almost twice more in our experiment.

Considering the nutrient uptake of the stem, the same tendency occurred for every examined nutrient except the nitrogen. With the elevation of fertilizer doses, nutrient uptake increased significantly (Table 2). However, similarly to the dry matter values, the difference between control and standard dose was greater than that of between standard and elevated dose. Nitrogen uptake of the stem did not differ between standard and elevated level of fertilizer treatments. Thus, the significantly bigger stem dry matter compensated the significantly lower nutrient concentration. Probably, higher carbohydrate accumulation can explain the lower rate of nitrogen-based compounds and therefore lower nitrogen concentration for the elevated level treatment.

Nitrogen concentration of leaves showed different tendencies than that of the stem. Despite the smaller dry weight, control had lower values (Table 2), so data reflected the well visible nitrogen deficiency. The values of the two fertilized treatments were nearly the same, so leaf-nitrogen was not affected by the adding of extra 2.5 kg m^{-3} fertilizer to the plants. Potassium showed the same tendency, although elevated dose treatment resulted in a slightly higher concentration of potassium than standard-dose treatment. The tendency of phosphorous and magnesium values was equal: with the elevation of fertilizer doses, nutrient content of the leaves increased significantly. The values of calcium showed a totally different trend. The highest value was detected in the control treatment while it was equally lower in the two fertilized treatments. It is important to emphasize that all the treatments received an equal quantity of calcium through the irrigation water. It is worth to note that the concentration of calcium in the leaves was higher than those of potassium and, even the level of nitrogen. All the concentration of the measured nutrients, except potassium, showed equality to values published by Moshki and Lamersdorf (2011) and Moshki and his colleagues (2012). However, potassium levels

Table 2. Effect of fertilization doses on the nutrient content and uptake of aboveground biomass of pot-grown black locust saplings

	Nitrogen	Phosphorus	Potassium	Magenesium	Calcium
Nutrient concentration in the stem (mg g^{-1} DW)					
Control	9.45 a*	0.43 c	7.40	0.90	8.75
2.5 kg m^{-3} Osmocote	8.15 a	1.08 b	8.53	0.90	7.88
5 kg m^{-3} Osmocote	6.60 b	1.28 a	8.73	0.95	8.68
P-value	0.0138	3.40×10^{-6}	0.0653	0.9087	0.3628
LSD 5%	1.71	0.17			
Nutrient uptake of the stem (g plant^{-1})					
Control	0.374 b	0.017 c	0.295 c	0.036 c	0.349 c
2.5 kg m^{-3} Osmocote	0.765 a	0.100 b	0.796 b	0.085 b	0.736 b
5 kg m^{-3} Osmocote	0.817 a	0.160 a	1.093 a	0.119 a	1.093 a
P-value	0.0002	1.55×10^{-7}	0.0059	0.0003	0.0166
LSD 5%	0.155	0.019	0.122	0.029	0.206
Nutrient concentration in the leaves and lateral hoots (mg g^{-1} DW)					
Control	18.05 b	1.15 c	11.88 b	2.38 c	35.50 a
2.5 kg m^{-3} Osmocote	27.80 a	2.65 b	15.98 a	3.28 b	29.70 b
5 kg m^{-3} Osmocote	26.88 a	3.35 a	17.15 a	3.70 a	29.75 b
P-value	0.0067	1.78×10^{-7}	0.0002	1.81×10^{-5}	0.0075
LSD 5%	5.96	0.31	1.71	0.32	3.59
Nutrient uptake of the leaves and lateral hoots (g plant^{-1})					
Control	0.703 b	0.045 c	0.464 c	0.092 c	1.379 b
2.5 kg m^{-3} Osmocote	2.353 a	0.223 b	1.343 b	0.276 b	2.538 a
5 kg m^{-3} Osmocote	2.789 a	0.349 a	1.785 a	0.386 a	3.099 a
P-value	8.05×10^{-5}	1.29×10^{-8}	1.11×10^{-7}	1.09×10^{-6}	$3.03 \times 10{-4}$
LSD 5%	0.622	0.031	0.174	0.050	0.589
Total nutrient uptake of above-ground parts (g plant^{-1})					
Control	1.077 b	0.062 c	0.758 c	0.128 c	1.727 c
2.5 kg m^{-3} Osmocote	3.118 a	0.323 b	2.139 b	0.361 b	3.274 b
5 kg m^{-3} Osmocote	3.606 a	0.509 a	2.878 a	0.505 a	4.191 a
P-value	2.51×10^{-5}	5.48×10^{-9}	8.32×10^{-9}	2.77×10^{-6}	5.09×10^{-5}
LSD 5%	0.656	0.041	0.207	0.071	0.664
Total amounts of nutrient provided by the fertilizer (g container^{-1})					
2.5 kg m^{-3} Osmocote	4.05	0.88	1.87	0.27	-
5 kg m^{-3} Osmocote	8.10	1.76	3.74	0.54	-
Utilisation of nutrients provided by the fertilizer (%)					
2.5 kg m^{-3} Osmocote	50%	30%	74%	86%	-
5 kg m^{-3} Osmocote	31%	25%	57%	70%	-

(*Average values of the same parameter and letter mean no significant differences based on Fischer's least significant difference test.)

in our experiment were only half of those reported by their trial made of young, pot-grown plantlets.

Nutrient uptake tendencies of the leaves were almost identical to the results of stem analysis except that in the case of calcium no significant difference was detected between the two fertilizer treatments. The same trend was

described in the case of nitrogen content, no significant difference was measured between the two fertilizer dozes. Significant differences were observed in the case of phosphorous, potassium and magnesium as higher fertilizer dose produced higher nutrient uptake.

Consequently, the total nutrient uptake consisting of the stem and leaf nutrient uptake followed the earlier described tendencies (Table 2). Only nitrogen uptake remained nonsignificant between the two fertilizer dose treatments. The nutrient uptake of the five elements was obviously significantly the lowest in the case of control plants. Compared to the standard treatment, calcium uptake of control plants was less than half of standard-dose treatment, in the case of phosphorous, this number was around 20%. Results of the elevated fertilizer treatment revealed that during the four-month-trial, potential nutrient uptake of the plants was nearly 4 g per plant in the case of nitrogen, more than 4 g per plant in the case of calcium and more than 3 g per plant in the case of potassium. Phosphorous and magnesium uptake ended around 0.5 g per plant.

These results can serve a base for a fertilization system which allows exploiting the maximal growing potential of the sapling. Our results support the high growing capacity of Turbo Obelisk clone as Eigel and his colleagues (1980) reported similar nutrient uptake (N 3.5, P 0.2, K 1.7, Ca 1.1, Mg 0.3 g per plant) in the case of two-year-old, in-situ sown, fertilized Robinias. Interestingly, the nutrient uptake order by quantity was proved to be identical what Moshki and Lamersdorf (2011) found in plantations.

The nutrient uptake tendencies underpin the previous statement that the 'second' dose of 2.5 kg m^{-3} fertilizer was not as effective, as the first one. The highest decrease in efficiency was observed in the case of the nitrogen (Table 2), which means that the dose of 8 g per container seemed to be a luxury quantity, based on our results. An extra dose of phosphorous and potassium proved to be more beneficial, 1.75 g of phosphorous and 3.75 g of potassium per container did not seem as excessive quantities. The highest rate of nutrient consumption was described in the case of magnesium. The reason for this result could be that – similarly to calcium uptake – plants partly covered their nutrient need not only from the fertilizer but also from the irrigation water.

Conclusions

Based on our results, Turbo Obelisk clone could produce very fast growing and high nutrient uptake under optimal fertilization conditions. Nutrient uptake order by quantity was Ca > N > K > Mg = P. Based on our results, a lower rate of N : P and N : K should be used than the fertilizer applied in the experiment (18 : 9 : 10). Between the two doses of fertilizer (standard: 2.5 kg m^{-3} and elevated: 5 kg m^{-3}) elevated amount proved to be less effective than the standard one, especially in the case of the nitrogen. Moreover, nitrogen fixation was probably still at low efficiency due to peat-based potting mix and a relatively short, four months growing period.

Acknowledgements

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Portrait of Columella, in Jean de Tournes, Insignium aliquot virorum icones.
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Lucius Junius Moderatus Columella

(AD 4 – 70) is the most important writer on agriculture of the Roman empire. His De Re Rustica in twelve volumes has been completely preserved and forms an important source on agriculture. This book was translated to many languages and used as a basic work in agricultural education until the end of the 19th Century.