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Agriculture (Poľnohospodárstvo), 63, 2017 (1): 3−13

Original paper DOI: 10.1515/agri-2017-0001

IMPACT OF ORGANIC FERTILISATION AND SUBSEQUENT GRASSLAND ABANDONMENT ON FLORISTIC COMPOSITION

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ŠTYBNAROVÁ, M. – DUFEK, A. – DELAGARDE, R.: Impact of organic fertilisation and subsequent grassland abandonment on floristic composition. Agriculture (Poľnohospodárstvo), vol. *63*, 2017, no. 1, p. 3–13.

The aim of this study was to evaluate changes in floristic composition of permanent grasslands after the cessation of their regular utilisation and organic fertilisation. A long-term small plot trial was established in 2004 in locality Rapotín. During 2004–2012, the experiment was fertilised with compost and slurry, both with the range of stocking rates 0.9, 1.4 and 2.0 livestock units (LU)/ha (corresponding to 54, 84, and 120 kg N/ha). The plots were cut 2–4 times per year depending on given dose of fertiliser. During 2013–2016, the regular management was ceased and the grasslands were completely abandoned. Before the grassland abandonment, the highest total number of species (24 species) was found in the treatments regularly fertilised with compost. The dominance of grasses was influenced by the grassland management, with decreasing intensity of utilisation, the dominance of grasses increased. Four years after the grassland abandonment, the species diversity in almost all treatments decreased and the dominance of grasses increased in all treatments, up to the value 67–80%. Based on the data about the soil chemical parameters from two investigated years, our results suggested not only the effect of grassland management and its subsequent abandonment, but also a residual effect of the both organic fertilisers. These findings indicated the importance of the maintenance of regular grassland management for sustainable conservation of grassland communities.

Key words: floristic composition, organic fertilisation, utilisation, meadows, abandonment

Pastures and meadows are the permanent source of the forage for farm animals. Their floristic composition is based on the naturally occurring grasses and legumes and the farmer can improve or deteriorate them by his interventions. In a long term, the grassland composition can be influenced by the fertilisation and by the intensity of utilisation, i.e. number and dates of harvests.

For the support of the growth, the development and the proper quality of the fodder crops is essential for their adequate nutrition. In most of the enterprises that are engaged in plant and animal production, it is usual to fertilise grasslands with mineral fertilisers. Therefore, many authors investigated the influence of mineral fertilisation on the floristic composition of grasslands (e.g. Mrkvička & Veselá 2002; Hejcman *et al*. 2007; Smits *et al*. 2008; Britaňák *et al*. 2009; Hrevušová *et al.* 2009; Rotar *et al.* 2016). The systematic utilisation of farm manures in grasslands is not common because of their preferred application in the intensive crop management on arable land. Existing methodical recommendations for the utilisation of organic fertilisers often do not take into consideration many important criterions, such as the estimation of the type and the dose of fertiliser in relation to the type of grassland, the altitude, or the date of application. That is the reason why in the Czech Republic it is used to apply the long-time experience from the abroad, mainly from Austria (Hrabě & Buchgraber 2004).

The organic fertilisers are the irreplaceable base for the rational agriculture. The high-quality farm

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manures support the soil fertility and have other positive effects (Samuil *et al.* 2009). By their correct systematic application, the important nutrients are returned back to the soil and the additional fertilisation with mineral fertilisers is not generally necessary in grasslands. The difference is that the nutrients in inorganic fertilisers can be directly taken up by plants in contrast to nutrients in organic fertilisers, which have to be released by microbial metabolism to make most of them available to plants (Böhme *et al*. 2005).

The influence of organic fertilisers (cattle slurry, in particular) has been studied by, for example, Schellberg & Lock (2009), Liu *et al.* (2010), Lalor *et al.* (2011, 2012), Duffková *et al*. (2015), Angeringer *et al*. (2016). Duffková & Libichová (2013) assessed the effect of different application rates of cattle slurry on the plant species composition of three-cut grassland. These authors mentioned that application of cattle slurry up to 120 kg N/ha/year can be considered as suitable compromise between maintenance of species-rich grasslands and requirements of farmers for sufficient forage production. Estavillo *et al*. (1997) referred that the difference between cattle slurry and N fertiliser was that the slurry behaved as a slow-release fertiliser, its supply of mineral N being greater in the periods of time when fertiliser was applied a long time ago. As Khalid *et al*. (2013) documented, compost also works quite differently from synthetic fertilisers, as amending soil with compost provides a slow-release source of nutrients. Significant quantities of nutrients (particularly N, P, K and micronutrients) became bioavailable with time as compost decomposed in the soil.

There is an advantage of organic fertilisers, which lies in the fact that their application can positively influence the soil organic carbon content (Gonet & Debska 2006). Microorganisms, for example, bacteria, fungi, actinomycetes and microalgae, play a key role in organic matter decomposition, nutrient cycling and other chemical transformations in soil (Murphy *et al*. 2007). The results of Šimon & Czakó (2014) indicated that additions of organic matter from various sources differ in the effects on soil organic matter and biological activity. According these authors, long-term application of cattle slurry + straw was rather similar to mineral fertilisation. Hlisnikovský *et al*. (2016) came to the conclusion that the decomposition and subsequent stabilisation of fresh organic matter in time, the microbial interactions and mineralisation of soil organic matter (Gude *et al*. 2012) and changes of contents of organic carbon were probable reasons for the subsequent decrease of easily available carbon fractions and increase of available metals in their experiment. It follows that there should be a long-term residual effect of organic fertiliser application (Diacono & Montemurro 2010), which was, however, only rarely investigated in grasslands.

Another question, which has been frequently asked by scientists, is addressed to changes of grassland floristic composition after the total grassland abandonment (Bohner *et al.* 2006; Öckinger *et al*. 2006; Bohner & Starlinger 2011; Ronch *et al.* 2013; Plesa *et al.* 2014; Păcurar *et al*. 2015). The abandonment of semi-natural grasslands become a major threat and raises a series of questions and situations, which have to be solved in the whole of Europe (Osterburg *et al.* 2010). This topic is still timely, because the grassland abandonment is happening currently mainly in the less-favoured areas near the borders of the Czech Republic.

Our study is based on the long-term investigation of the small-plot trial with permanent grassland and it broadens the current knowledge about these topics. The aim of this paper was to investigate the impact of the long-term grassland management (different cutting intensity and different types and doses of organic fertilisers) and the subsequent grassland abandonment from the viewpoint of the species diversity and floristic composition.

MATERIAL AND METHODS

Study site

The monitoring of the influence of intensity of utilisation and type and level of grassland fertilisation with organic fertilisers on the dynamics in a phytocoenosis was initiated in Rapotín in 2004. The experimental site was situated in the Czech Republic (50°00´32´´N and 17°00´83´´E) at 390 m above sea level on the east slope position (declination between 5.1 and 6.2°) in a moderately warm region without temperature extremes (Quitt 1971). Average annual

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temperature is 7.2°C and annual precipitation 693 mm. Further meteorological data are given in Table 1. The soil was sandy-loam, *Haplic Cambisol* with horizons Am–Bv-Bv/Cc–Cc (classification system according to IUSS Working Group WRB 2006). Table 4 shows chemical soil properties determined in spring 2012 and 2016 according to the methods of Zbíral *et al*. (2002). The vegetation of the experimental area was classified as *Cynosurion* with some elements of *Arrhenatherion* (Moravec *et al.* 1995). Before the experiment set-up, the grassland was utilised as the pasture for cattle.

Treatments

A long-term small-plot experiment (plot size: 12.5 m^2) in completely randomised blocks with four replicates was investigated on permanent grassland. Two types of organic fertilisers were applied during 2004–2012: (S) cattle slurry, and (C) compost. Organic fertilisers were used in annual doses of nitrogen: 54 kg/ha, 84 kg/ha and 120 kg/ha, which approximately corresponded to 0.9 LU/ha (LU = livestock unit), 1.4 LU/ha and 2.0 LU/ha. Average concentrations of elements in organic fertilisers are mentioned in Table 2. The first half of the doses of the cattle slurry (diluted with water in a ratio 1:3) as well as the compost was applied early in spring and the second one after the first cut. The fertilisers were analysed for the content of nutrients before their application, which was conducted annually during 2004–2012. The plots were cut two to four times per year depending on the given dose of fertiliser. Treatments of the fertilisation and the cutting regime are given in Table 3.

During 2013–2016, the regular management ceased and the grasslands were completely abandoned for this time.

Experimental measurements

The floristic composition was determined in spring (during 2004–2012 and in 2016) by means of the method of reduced projective dominance according Veselá *et al*. (2002). The projective dominance (*D*) of all vascular plant species was visually

T a b l e 1

Long-term annual average [1961–1990] in temperatures and precipitations in the locality of Rapotín

	Normal
Average temperature during the vegetation season [°C]	9.1
Average annual temperature [°C]	7.2
Average precipitation during the vegetation season [mm]	481
Average annual precipitation [mm]	693

T a b l e 2

Soil chemical properties determined in spring 2012 and 2016

Treatment	pH (0.01 $CaCl_2$)		C_{ox} [%]		N_{tot} [%]		Ratio C/N		P [mg/kg]		K [mg/kg]		Ca [mg/kg]		Mg [mg/kg]	
	2012	2016	2012	2016	2012	2016	2012	2016	2012	2016	2012	2016	2012	2016	2012	2016
S-0.9	4.83	5.10	1.59	0.88	0.19	0.20	8.44	4.32	59	46	186	150	2078	2118	204	269
S-1.4	4.70	5.18	1.44	0.85	0.17	0.20	8.52	4.21	55	61	155	124	1954	2125	185	291
S-2.0	4.78	5.17	1.41	0.93	0.17	0.19	8.13	4.85	65	57	162	107	1855	2107	195	296
C-0.9	5.13	5.43	1.58	1.20	0.20	0.24	8.10	5.06	85	75	196	138	2399	1760	228	279
C-1.4	5.06	5.12	1.61	1.13	0.19	0.22	8.39	5.13	71	64	151	114	2384	2823	213	321
C-2.0	5.21	5.52	1.74	1.17	0.19	0.22	9.13	5.22	92	93	180	127	2493	2742	247	323

Treatment – see Table 3; C_{ox} – oxidizable organic carbon; N_{tot} – total nitrogen in soil

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estimated directly in the percentages in each plot and each year of the study. The reduced projective dominance method was used, consequently, the sum of abundances for all plant species was 100%. Plant species were determined based on the descriptions of the vascular plants in the national flora (Kubát *et al*. 2002).

Species diversity was measured as the total number of species (richness) recorded in particular treatments, and it was further expressed in form of the Simpson´s diversity index (Begon *et al*. 1997) modified according Klimeš (2000):

$$DI = \frac{1}{\sum_{i=1}^{s} p_i^2}$$

where: p_i is the projective dominance of *i*-species and *S* is total number of species (richness).

Data analysis

Redundancy analysis (RDA) was used to investigate the relationships between floristic composition and applied management. Centering and standardisation by species was used in all analyses. The statistical significance of the first and all other constrained canonical axes was determined by the Monte Carlo permutation test within each block (499 permutations). All ordination analyses were performed in the CANOCO program (ter Braak & Šmilauer 2002). An ordination diagram was created in CanoDraw for Windows 4.14 for graphically visualising the results.

RESULTS

Species diversity

The amount of plants species presented in 2012 (before grasslands abandonment) and 2016 (after four years of grasslands abandonment) are shown in Table 4. From these results, it is evident that the highest total number of species (24 and 23 species) was found in the treatments regularly fertilised for 9 years with compost (by all types of intensities of utilisation) in 2012. It was due to the presence of rare plant species (dominance < 1%). On the contrary, treatments fertilised 9 years with slurry were characterised by the lower total number of species (15–19 species) in 2012. There is also obvious the

Table 3

Description of treatments with different grassland management (before abandonment)

Treatment	Fertilisation	Annual dose of nitrogen	Application	1st cut	2nd cut	3rd cut	4th cut
S-0.9	cattle slurry (diluted with water 1:3)	54 kg/ha	50% of dose – in spring, 50% of dose – after the 1st cut	June 15	Sept. 30	–	–
S-1.4	cattle slurry (diluted with water 1:3)	84 kg/ha	50% of dose – in spring, 50% of dose – after the 1st cut	May 30	July 30	Sept. 30	–
S-2.0	cattle slurry (diluted with water 1:3)	120 kg/ha	50% of dose – in spring, 50% of dose – after the 1st cut	May 15	June 30	Aug. 15	Sept. 15
C-0.9	compost	54 kg/ha	50% of dose – in spring, 50% of dose – after the 1st cut	June 15	Sept. 30	–	–
C-1.4	compost	84 kg/ha	50% of dose – in spring, 50% of dose – after the 1st cut	May 30	July 30	Sept. 30	–
C-2.0	compost	120 kg/ha	50% of dose – in spring, 50% of dose – after the 1st cut	May 15	June 30	Aug. 15	Sept. 15

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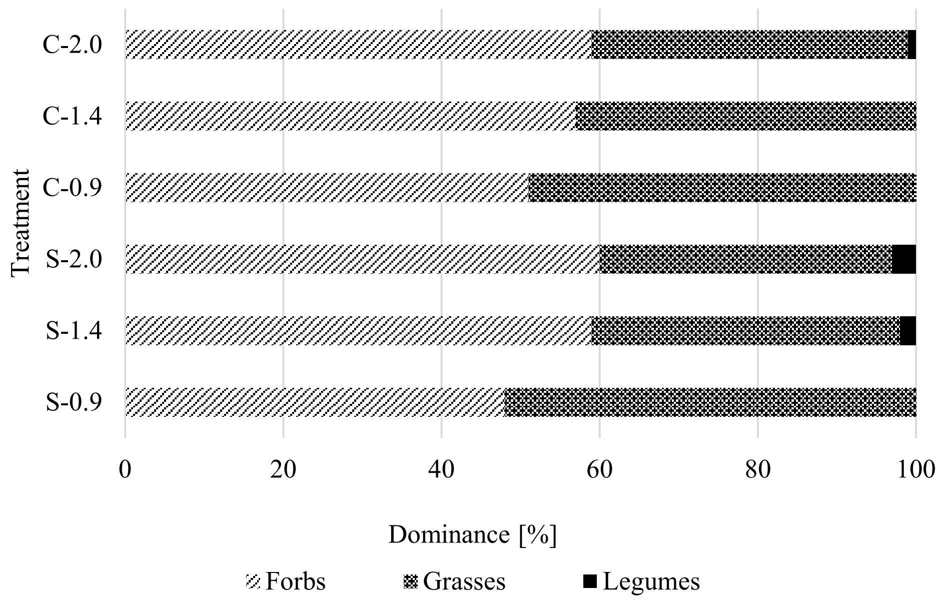
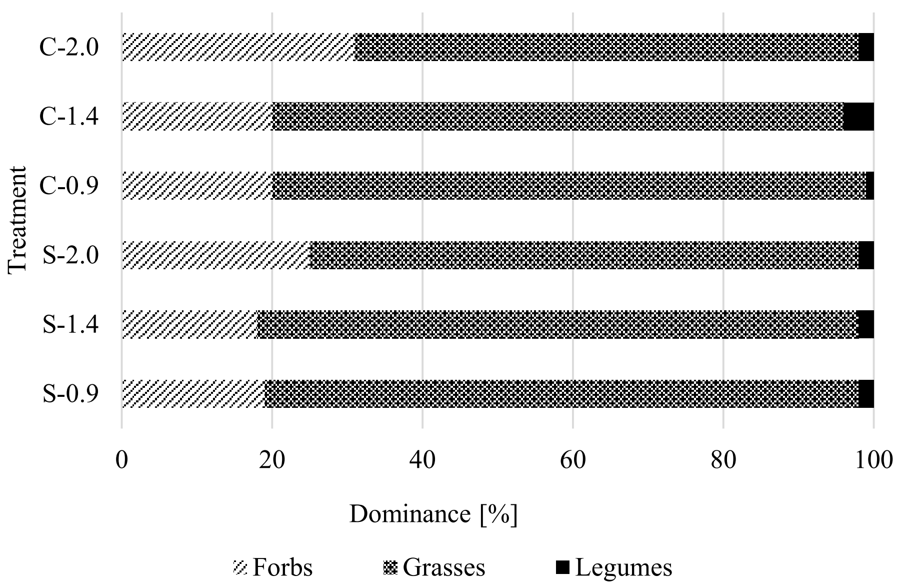
influence of the intensity of utilisation in the treatments fertilised with slurry in 2012 – with decreasing intensity of utilisation (by the simultaneous decreasing doses of the fertiliser) the total number of species decreased. The values of the Simpson´s diversity index (Table 4) indicated that the grassland fertilised with slurry were distinguished by the higher species diversity in terms of the equitability of the plant community (DI = 7.56–8.54) than the grassland fertilised with compost (DI = 5.59–6.22).

Four years after the grasslands abandonment, the total number of species in all treatments, but one



Treatment – see Table 3

Figure 1. The dominance of functional groups in grasslands in 2012



Treatment – see Table 3

Figure 2. The dominance of functional groups in grasslands in 2016

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(S-0.9), decreased. The most important changes in terms of the number of species were found in the treatments previously fertilised with compost. In case of the treatment fertilised with compost by the simultaneous extensive grassland utilisation (two cuts per year), the total number of species decreased from 23 species up to the minimal level of 13 species, whereas mainly the species with dominance < 1% disappeared from the grasslands. The Simpson´s diversity index decreased in all treatments, mainly in treatments formerly fertilised with slurry, which indicated the unbalanced communities in terms of their floristic composition. Overall, the differences in the species diversity among treatments decreased during four years of abandonment, which is apparent both on the values of the Simpson´s diversity index and on the values of the species richness.

Evaluation of the dominance of plant species and functional groups

Table 5 shows more detailed data about the dominance (in %) of particular species and functional groups (grasses, legumes and forbs) in 2012 and 2016. There were found differences between treatments in the dominances (% *D*) of functional groups in 2012 (Figure 1) and 2016 (Figure 2). In 2012, the dominance of grasses was influenced by the management, with decreasing intensity of utilisation (by simultaneous decreasing doses of fertilisers), the dominance of grasses increased. The dominance of legumes was the highest (*D* = 3%) by the most intensive grassland utilisation (four cuts per year) by simultaneous highest dose of slurry (2.0 LU/ha). The extensive grassland utilisation (by the fertilisation with both slurry and compost) had the negative effect on the dominance of legumes in the swards.

Four years after the regular management cessation, the differences between the two treatments decreased and the grasslands became to show the similar floristic composition in terms of the dominance of particular functional groups. From the Figure 2, it is obvious that the dominance of grasses increased in all treatments, up to the value 67–80%. It was mainly due to increasing the dominance of *Dactylis glomerata* L. and *Bromus hordeaceus* L., both plant species are the tall grasses with the high competitive ability. Occurrence of legumes was stabilised at the level of 2%.

The mentioned results about the floristic composition were statistically evaluated in more detail by means of the redundancy analysis (RDA). The ordination diagram RDA (Figure 3) shows the explanatory variables (management and year) and the dependent variables (dominance of a plant species in %). RDA explained 35.4% of the total variability, whereas the first two canonical axes get a share of 26.8%. The first canonical axis (which closely fits with the centroids of the variable ‘year’) explained 19.2% of the total variability. The second axis (which closely fits the variable ‘management’) explained 7.6% of the total variability. This ordina-

T a b l e 4

The values of species diversity in particular treatments in 2012 and 2016

Treatment	Simpson´s diversity index (DI)		Number of species with D > 1%		Number of species with D < 1%		Total number of species (S)	
	2012	2016	2012	2016	2012	2016	2012	2016
S-0.9	7.56	4.67	13	13	2	3	15	16
S-1.4	8.54	4.98	15	12	3	3	18	15
S-2.0	8.36	5.17	17	12	2	2	19	14
C-0.9	5.68	5.12	12	10	11	3	23	13
C-1.4	5.59	5.43	11	12	13	2	24	14
C-2.0	6.22	6.07	12	12	12	3	24	15

Treatment – see Table 3

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tion was found statistically significant (F = 3.288, p = 0.002).

Moreover, from the Figure 3, it is apparent that in 2012 (last year of the different managements), there was characteristic the higher occurrence of some forb species (e.g. *Taraxacum* sect. *Ruderalia, Cerastium holosteoides* Fr., *Veronica arvensis* L.) in the swards. On the contrary, in 2016 (four years after the abandonment), some tall grasses (e.g. *Bromus hordeaceus* L.), which were probably suppressed by the previous management, newly occurred. There is also information on which species positively responded to the long-term regular fertilisation with slurry (e.g. *Dactylis glomerata* L., *Stellaria graminea* L., *Rumex obtusifolius* L.), or to the fertilisation with compost (e.g. *Achillea millefolium* L., *Poa pratensis* L., *Geranium pyrenaicum* Burm. F.).

Table 2 shows differences in the content of soil nutrients between years and treatments, whereas mainly the ratio C/N changed during years. These changes can be attributed to microorganisms, e. g. bacteria, fungi, actinomycetes and microalgae, which play a key role in organic matter decomposition, nutrient cycling and other chemical transformations in soil.

DISCUSSION

Influence of the grassland management on the vegetation is not always definite and the results between authors can vary. The differences could be caused by different site conditions and research

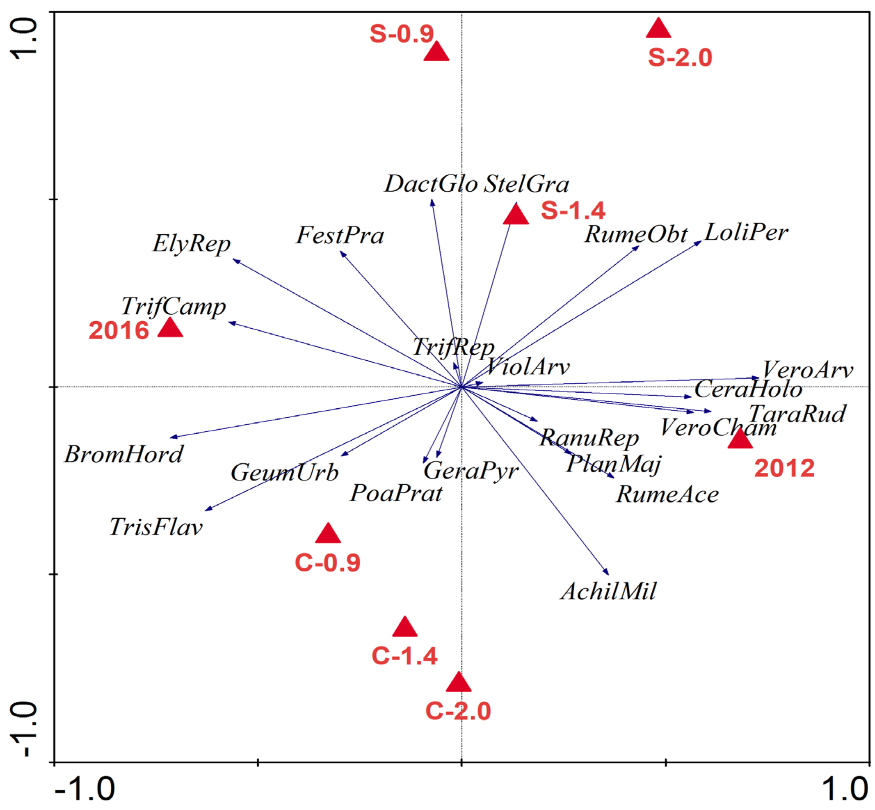

Figure 3. Ordination diagram showing differences between treatments in the dominance of particular species in 2012 and 2016

Note:
FORBS: *AchilMil = Achillea millefolium; CeraHolo = Cerastium holosteoides; GeraPyr = Geranium pyrenaicum; GeumUrb = Geum urbanum; PlanMaj = Plantago major; RanuRep = Ranunculus repens; RumeAce = Rumex acetosa; RumeObt = Rumex obtusifolius; StelGra = Stellaria graminea; TaraRud = Taraxacum* sect. *Ruderalia; VeroArv = Veronica arvensis; VeroCham = Veronica chamaedrys; ViolArv = Viola arvensis;* GRASSES: *BromHord = Bromus hordeaceus; DactGlo = Dactylis glomerata; ElyRep = Elymus repens; FestPra = Festuca pratensis; LoliPer = Lolium perenne; PoaPrat = Poa pratensis; TrisFlav = Trisetum flavescens;* LEGUMES: *TrifCamp = Trifolium campestre; TrifRep = Trifolium repens*

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methods. Our findings are in line with Ziliotto *et al.* (2002), who also mentioned that by the decreasing frequency of cuts there dominated tall grasses with the high competitive ability such as *Festuca arundinacea* Schreb. or *Dactylis glomerata* L. in the swards. Angeringer *et al.* (2016) recorded after three years of different management regime significant changes in coverage of the most frequent meadow species due to cutting regime, but few responses to the type of organic fertiliser. In spite of that, many other authors reported that the supply of nitrogen in fertilisers (either mineral or organic), as the part of the grassland intensification management, reduced the structural and floristic diversity of the sward

T a b l e 5

Floristic composition (the dominances of particular species in percentages) of the grasslands in 2012 and 2016

Latin name	S-0.9		S-1.4		S-2.0		C-0.9		C-1.4		C-2.0	
	2012	2016	2012	2016	2012	2016	2012	2016	2012	2016	2012	2016
Achillea millefolium L.	11	6	5	3	12	6	23	9	25	5	21	12
Capsella bursa-pastoris (L.) Medik.	1	1	3	–	3	–	–	–	–	–	–	–
Cerastium holosteoides Fr.	4	1	7	2	6	2	1	1	7	2	5	2
Crepis biennis L.	–	–	–	–	–	–	1	–	–	–	–	–
Geranium pyrenaicum Burm. F.	–	–	1	–	1	–	–	–	–	1	1	1
Geum urbanum L.	–	–	–	–	–	–	–	–	–	–	–	1
Plantago lanceolata L.	3	–	–	–	1	1	2	1	1	1	2	–
Ranunculus repens L.	–	–	1	1	–	1	1	–	–	–	2	–
Rumex acetosa L.	–	–	1	–	–	–	–	–	1	–	1	–
Rumex obtusifolius L.	1	–	–	–	2	–	–	–	–	–	–	–
Stellaria graminea L.	3	4	1	3	3	5	1	1	–	–	2	–
Taraxacum sect. *Ruderalia*	14	5	23	6	21	7	14	7	13	10	15	10
Urtica dioica L.	–	–	–	–	1	–	–	–	–	–	–	4
Veronica arvensis L.	3	–	3	–	2	–	–	–	1	–	3	–
Veronica chamaedrys L.	5	2	9	3	5	3	8	1	9	1	4	1
Veronica serpyllifolia L.	3	–	5	–	3	–	–	–	–	–	3	–
Forbs in total	48	19	59	18	60	25	51	20	57	20	59	31
Alopecurus pratensis L.	1	–	–	–	–	–	–	–	–	–	–	–
Arrhenatherum elatius L.	–	–	–	–	1	–	–	–	–	–	–	–
Bromus hordeaceus L.	–	9	–	8	–	9	–	19	–	25	–	24
Dactylis glomerata L.	28	41	16	37	12	38	21	18	24	8	17	6
Echinochloa crus-galli (L.) P. Beauv.	–	–	1	–	–	–	–	–	–	–	–	–
Elymus repens (L.) Gould	5	5	4	4	3	5	4	7	1	5	1	4
Festuca pratensis Huds.	2	10	2	11	4	5	3	3	1	1	2	4
Lolium perenne L.	1	–	2	–	5	–	–	–	–	–	–	–
Phleum pratense L.	–	–	–	–	–	–	1	–	–	–	–	–
Poa pratensis L.	15	12	14	20	11	15	14	13	15	19	20	14
Trisetum flavescens (L.) P. Beauv.	–	2	–	–	1	1	6	19	2	18	–	15
Grasses in total	52	79	39	80	37	73	49	79	43	76	40	67
Trifolium campestre Schreb.	–	1	–	1	–	–	–	–	–	2	–	–
Trifolium repens L.	–	1	2	1	3	2	–	1	–	2	1	2
Legumes in total	0	2	2	2	3	2	0	1	0	4	1	2

Treatment – see Table 3

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(Mrkvička & Veselá 2002; Rotar *et al*. 2006; Silva *et al.* 2008; Britaňák *et al.* 2009; Păcurar *et al.* 2011). Besides nitrogen, soil phosphorus content, in particular, is an important variable leading to a significant negative effect on species richness as documented by Hejcman *et al.* (2007, 2010), Helsen *et al.* (2014) or Riesch *et al*. (2016). These findings emphasised the importance of a reduction of nutrients for sustainable conservation of grassland communities.

The total grassland abandonment had a negative influence on the species diversity, which was demonstrated not only in our study, but this finding was also documented in studies of, for example, Isselstein *et al*. (2005) or Prévosto *et al.* (2011). Öckinger *et al*. (2006) referred that, when semi-natural pastures are abandoned, specialised grassland species are lost as a consequence of succession. There is no generally valid theory, which would describe changes in plant communities after the grassland abandonment. It is, in fact, the long-term process characteristic for each locality (Kahmen & Poschlod 2004; Pavlů *et al.* 2013). Nevertheless, many studies are in agreement that tall grasses with the high competitive ability and also the trees invade into the abandoned grasslands, thus, less light reaches the lower vegetation layers. The light is the basic requirement for the growth and the survival of many short plant species, so they are suppressed and the species diversity declines in the abandoned grasslands (Willems 1983). The site-climatic conditions, accumulation of the senescent material and of the nutrients play some role, as well. In such conditions, it is higher competitive ability of grasses than of forbs, which was also manifested in our experiment.

Results of Bohner & Starlinger (2011) show that the long-term effects of abandonment on grassland vegetation depend largely on local site conditions, and nutrient availability in the soil. Thus, in our study, the changes in floristic composition could be explained both by the effect of grassland abandonment and by the residual effect of application of organic fertilisers as data about the chemical soil properties indicated. Diacono & Montemurro (2010) mentioned that repeated long-term applications of organic amendments not only generally increase the size of the soil organic N pool, but also cause remarkable changes in soil characteristics, which influence N dynamics and can lead to a residual effect. Habteselassie *et al*. (2006) found an 89% increase in total soil N content after 5 years when dairy-waste compost at 200 kg/ha N was applied. The increase in total soil N content (although only in a small amount) was found also in our study four years after the last application of fertilisers, which was the most apparent in the treatments fertilised with compost. This finding is in line with the results of Šimon and Czakó (2014), who referred that additions of organic matter from various sources can differ in the effects on soil organic matter and biological activity. Generally, the composts are slowly decomposed in the soil, the continuous release of nutrients can sustain the microbial biomass population for longer periods of time (Murphy *et al*. 2007).

CONCLUSIONS

Four years after the grassland abandonment (in 2016), the differences between treatments decreased and the grasslands became to show the similar floristic composition in terms of the species diversity, as well as the dominance of particular functional groups. The total number of species as well as the Simpson's diversity index in almost all treatments decreased. The most important changes in species richness were found in the treatments previously fertilised with compost. The dominance of grasses increased in all treatments, up to the value 67–80%. Primarily the dominance of *Dactylis glomerata* L. and *Bromus hordeaceus* L. increased. Occurrence of legumes stabilised about the level 2%.

Our results suggested not only the effect of the grassland abandonment but also the residual effect of both organic fertilisers. Our findings indicated the importance of the maintenance of regular grassland management for sustainable conservation of grassland communities. As for the fertilisation, it is always important to consider in advance if a locality or grassland is suitable for the fertilisation, as the residual effect (mainly of organic fertilisers) can manifest itself for a long time.

Acknowledgements. This work was supported by the institutional support for the long-term conceptual development of the research organisation, Ministry

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of Agriculture Decision No. RO1216 from 26 February 2016 and by the project INGO No. LG15025.

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Received: August 31, 2016

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Agriculture (Poľnohospodárstvo), 63, 2017 (1): 14−22
DOI: 10.1515/agri-2017-0002 Original paper

CHITINASE ACTIVITIES IN WHEAT AND ITS RELATIVE SPECIES

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MORAVČÍKOVÁ, J. – UJVARIOVÁ, N. – ŽUR, I. – GÁLOVÁ, Z. – GREGOROVÁ, Z. – ZIMOVÁ, M. – BOSZORÁDOVÁ, E. – MATUŠÍKOVÁ, I.: Chitinase activities in wheat and its relative species. Agriculture (Poľnohospodárstvo), vol. *63*, 2017, no. 1, pp. 14–22.

Defense components such as chitinases (EC 3.2.1.14) are crucial for plants to cope diseases. Despite of that the pattern and activities of these enzymes in agronomically important Triticale is unexplored. This work is aimed to study chitinase activities in the leaves of plants of early developmental stages in two diploids (*Aegilops tauschii* Coss., *Triticum monococcum* L.), four tetraploids (*Ae. cylindrical* Host, *Ae. triuncialis* L., *T. araraticum* Jakubyz, *T. dicoccum* Schrank) and two hexaploids (*T. aestivum* L., *T. spelta* L.). The leaves were subjected to quantitative and qualitative activity assays using synthetic 4-methylumbelliferyl-β-D-*N*,*N*′,*N*′′-triacetylchitotrioside and glycolchitin as substrates, respectively. Our results showed that the activities of chitinases with specificity towards short oligomers were variable and genotype dependent. The enzyme activities in the tetra- and hexaploid genotypes were significantly higher than in diplod counterparts. In the gel detection assays were revealed up to four fractions (~20, 30, 42 and 95 kDa) of proteins with the chitinase activity towards long chain polymers. The isoform of ~30 kDa was identified in all analyzed genotypes. Among the seven acidic and three basic chitinase fractions identified, three acidic (ChiA, ChiB, ChiC) and two (ChiH, ChiI) fractions were present in all genotypes. None of the isoforms can be assigned as specific with respect to ploidy.

Key words: defense proteins, hydrolases, glycolchitin, chitinases, ploidy, PR protein, wheat

Chitinases are widely distributed enzymes in plants. Plant chitinases (EC 3.2.1.14) hydrolyze β-1,4-N-acetyl-D-glucosamine (GlcNAc) linkages of chitin. Their true substrate in plants is unknown since they lack chitin, but chitinases are suggested to cleave arabinogalactan proteins (AGPs) and N-acetylglucosamine-containing glycoproteins in the plant cell walls (van Hengel *et al.* 2001; van

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Hengel *et al.* 2002). Afterwards, the released substances can act as an elicitor of plant defence response (Fessel & Zuccaro 2016). Chitinases that are induced in response to pathogen attack are also referred as "Pathogenesis related proteins" (PR3, PR4, PR8 and PR11) (Kasprzewska 2003). They were shown to inhibit fungal growth *in vitro* (Sela-Buurlage *et al.* 1993), while their over-expression in transgenic plants enhanced fungal resistance (Moravcikova *et al.* 2004).

According to the CAZy database chitinases form two glycoside hydrolase families GH18 and GH19 which show different structures and catalytic mechanisms. The family GH19 chitinases are mainly found in plants. Based on their amino acid composition, plant chitinases are mainly grouped into five classes (Neuhaus 1999). The classes I, II and IV belong to the family GH19 while III and V to the family GH18. These enzymes can be expressed in plants constitutively (Colligne *et al.* 1993) or they can be induced upon pathogen infection (Žur *et al.* 2013), low temperature (Yeh *et al.* 2000), ethylene (Zhong *et al.* 2002), drought (Gregova *et al.* 2015) or heavy metals (Meszaros *et al.* 2014). Besides, it was suggested that they might take part in programmed cell death (Kim *et al.* 2015). Chitinases could also play a role in developmental processes such us pollination, senescence, seed germination and somatic embryogenesis (Kasprzewska 2003; Grover 2012).

Bread wheat is one of the most important grain crops worldwide. Its hexaploid genome is a result of the evolutionary hybridization, domestication and/or selection steps. However, genetic improvement for human purposes caused genetic erosion and increased susceptibility to environmental and biotic stresses. Not surprisingly that some wild ancestors and inter-crossable wheat relatives are more resistant to some disease than bread wheat (Peng *et al.* 2011).

In this work we studied the activities of chitinases in two wheat (*T. aestivum* L., *T. spelta* L.) and crop relative species (*Aegilops tauschii* Coss., *Ae. cylindrical* Host, *Ae. triuncialis* L., *T. monococcum* L., *T. araraticum* Jakubyz, *T. dicoccum* Schrank). Qualitative and quantitative chitinase activity assays were used to reveal in detail the profile and activities of individual isoforms in wheat and wild relative species at early developmental stage.

MATERIAL AND METHODS

Plant material

Seeds of eight wheat genotypes (Table 1) were obtained from the Gene Bank of the Slovak Republic (National Agricultural and Food Centre, Slovak Republic). The seeds were germinated on the watered sterile filter paper in dark at room temperature for 3 days. Then, germinated seeds were transferred to the pots with the commercial substrate BORA and cultivated at 22°C and 16 h/8 h light/dark photoperiod under 50 μE/m^2/s light intensity for 3 weeks. The leaves of wheat plants (10 plants/genotype) at two leaf stage (Zadoks stage 12) were collected and used for analyses.

Protein extraction

Crude protein extracts were isolated from the leaves of plants using an extraction buffer that contained 0.1 mol/dm^3 sodium acetate (pH 5.0) and 0.02% (v/v) β–mercaptoethanol according to the protocol described previously (Žur *et al.* 2013). Protein concentration was determined according to Bradford (1976).

Gel electrophoresis and chitinase activity staining

Protein extracts (30 μg) were separated on 12.5% (w/v) SDS-containing polyacrylamide slab gels (Laemmli 1970) with 0.01% (w/v) glycol chitin as an enzyme substrate. Glycol chitin was obtained by acetylation of glycol chitosan (Sigma G-7753) as described by Trudel and Asselin (1989). The gels were run at 8°C at a constant voltage of 120 V for 2 h. After electrophoresis, proteins were re-naturated by shaking the gel in 50 mmol/dm^3 sodium acetate buffer (pH 5.0), 1% (v/v) Triton X-100 for 1 hour.

Separation of proteins under native conditions (for acidic/neutral or basic/neutral proteins) was performed according to Konotop *et al.* (2012) using 11% (w/v) acrylamide gels with 0.01% (w/v) glycol chitin.

The chitinase activity was detected by staining with 0.01% (w/v) Fluorescent Brightener 28 (Pan *et al.* 1991).

Chitinase quantitative assays

The chitinase activity was assayed fluorimetrically using the synthetic substrate 4-methylumbelliferyl-β-D-N,N′,N′′-triacetylchitotrioside [4-MU-

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$(GlcNAc)_3$](Fluka 69615) as was described previously (Libantova *et al.* 2009). The fluorescence was measured using excitation/emission filters 360 nm/450 nm (Synergy H1,Bioetek). The chitinase activity was expressed in picomoles of methylumbelliferone (4-MU) generated per hour per milligram of soluble proteins.

The shown data represent the means of three replications. Statistical significance of the experimental results was evaluated by ANOVA/MANOVA and Duncan's tests, with help of STATISTICA® version 7.1.

RESULTS AND DISCUSSION

Chitinases from different plant species have been studied mainly for their inducibility upon biotic stresses. Their role in plant growth and development has also been proven. However, there are still limited information about chitinases in wheat and its relative species. So far, the Uniprot and NCBI databases contain up to 18 characterized sequences concerning chitinases in the hexaploid *T. aestivum* L. (Table 2). In literature, wheat chitinases were studied mainly for their antifungal potential (Liao *et al.* 1994; Caruso *et al.* 1999; Li *et al.* 2001; Kong *et al.* 2005), drought stress (Gregorova *et al.* 2015), heavy metal accumulation (Lyubka *et al.* 2008) or different concentration of nitrogen as a nutritional supply (Maglovski *et al.* 2017).

In this work we studied the activities of chitinases in wheat and relative species (Table 1). The plants were grown under controlled conditions up to two leaf stage. Afterwards, the leaves were collected and subjected to the chitinase activity assays.

The enzyme activities were evaluated quantitatively based on the ability of plant chitinases to release $(GlcNAc)_3$ from the tetramer 4MU-$(GlcNAc)_3$, a fluorogenic substrate for short oligomer-specific endochitinases. Our results showed that chitinases with hydrolytic activity towards oligomers were active in all analysed genotypes. Data are summarized in Figure 1a. The effect of genotype was found to be significant (at $p \leq 0.001$). The highest chitinase activity was detected for *T. diccocum* Schrank (59.05 nmol MU/h/mg) and the lowest ones for *T. monococcum* L. (5.89 nmol MU/h/mg) and *Ae. tauschii* Coss. (7.96

T a b l e 1

Wheat and crop relatives species used in the experiments and their resistance to important wheat diseases

Genotype	Disease resistance*					
	Gene bank**	Ploidy/ Genome	Powdery mildew Leaf/spike	Wheat rust leaf	Take-all of wheat	Blotch of wheat
Aegilops cylindrical Host	ARME N06-02	Tetraploid CCDD	9/9	1	9	9
Aegilops tauschii Coss.	ARME N06-40	Diploid DD	9/9	1	9	9
Aegilops triuncialis L.	ARME N06-06	Tetraploid UUCC	9/9	9	9	9
Triticum aestivum L.	Astella	Hexaploid $BBAA^uDD$	5/5	7	8	8
Triticum araraticum Jakubyz	AZESVK 2009-47	Tetraploid $GGAA^u$	7/6	5	8	8
Triticum dicoccum Schrank	AZESVK 2009-78	Tetraploid $BBAA^u$	8/7	6	6	6
Triticum monococcum L.	AZESVK 2 009-84	Diploid AA^m	9/9	8	9	9
Triticum spelta L.	Brun 5/9	Hexaploid $BBAA^uDD$	4/6	5	9	9

*Disease severity rating based on the data obtained from the Gene Bank of the Slovak Republic; 9 – very high resistance, 1 – very low resistance

**Accession number in the Gene bank of the Slovak Republic

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nmol MU/h/mg). The activities in the tetraploid and hexaploid species were significantly higher than in the diploid species (at $p \leq 0.01$) (Figure 1b). It might coincide with successive polyploidization of wheat genome since such genomic can reprogram gene expression patterns (Chen *et al.* 2007).

As is shown in Table 1 the analysed wheat relatives were evaluated as more resistant to the selected fungal diseases. However, no correlation between chitinase activities and disease severity rating was observed. Chitinases are multifunctional enzymes that take part not only in plant pathogenesis but

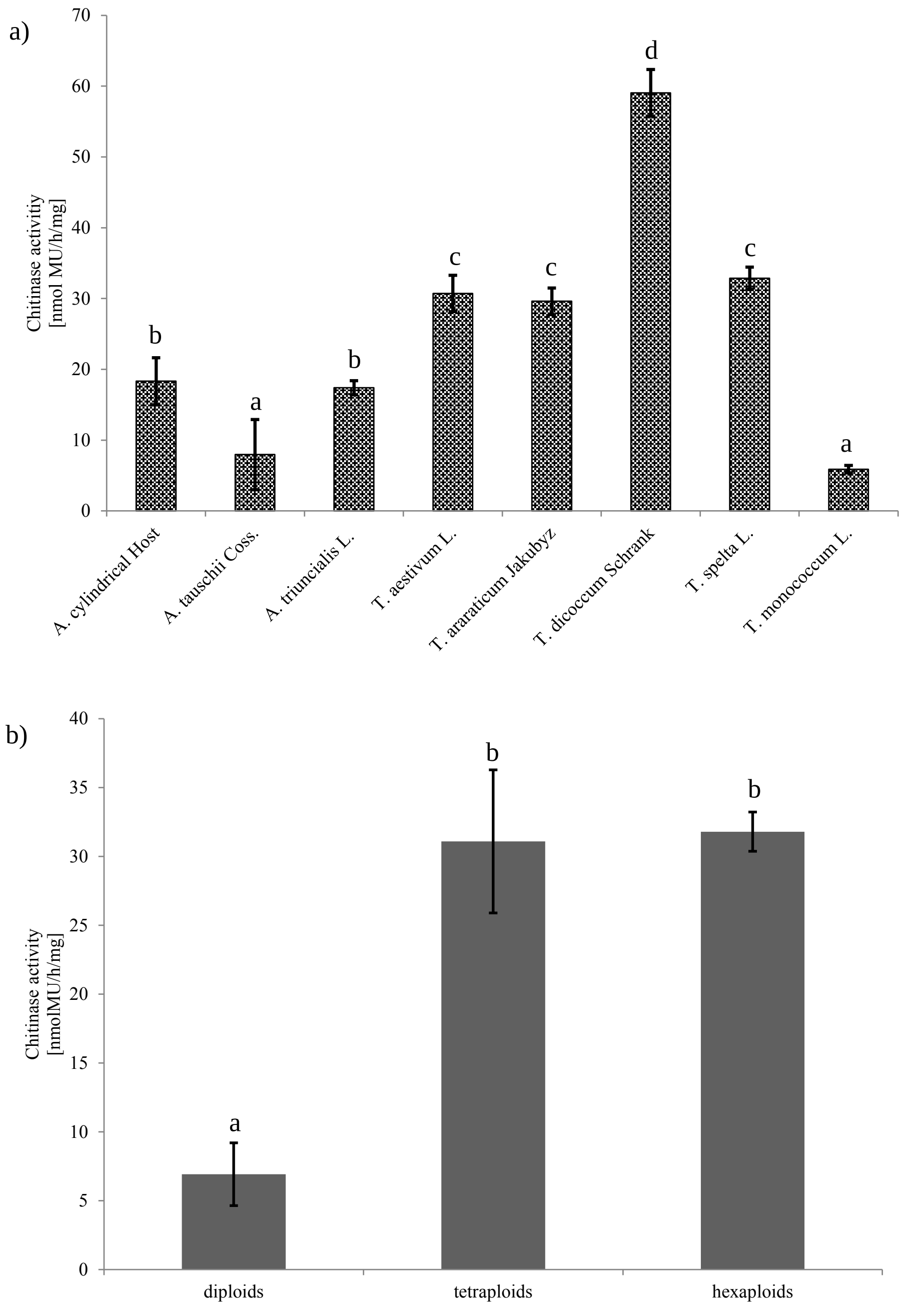

Figure 1. Chitinase activities in the studied wheat and its (wild) relatives (a) and in dependence on ploidy level (b). The enzyme activities were assayed fluorimetrically using [4-MU-$(GlcNAc)_3$] as a substrate. The activity was expressed in nmol of methylumberylferone (MU) released per hour per mg of soluble proteins. Bars represent means ± standard deviations of three replications. Distinct letters denote statistically significant differences with Duncan's test at $p \leq 0.001$ (a) and at $p \leq 0.01$ (b).

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T a b l e 2

Chitinases in *T. aestivum* L. and their characterization based on the data in the NCBI and UNIPROT databases (September 2016)

UNIPROT/ NCBI	Name	AA	MW [kDa]	DNA [bp]	Description/function	Literature
Q8W427/ AB029936	Chitinase 3, Chi3 cht1	319	33.5	960	Biotic stress *Puccinia striiformis West. f. sp. tritici*	–
Q8W428/ AB029935	Chitinase 2, Chi2 Chitinase 2	323	34.2	1,163	–	–
A0A0H4TIG5/ KR049249	Chitinase Chitinase, cht3	317	33.4	954	Biotic stress *P. striiformis West. f. sp. tritici*	–
Q6T484/ AY437443	Class I, chitinase Class I, chitinase	319	33.5	1,121	Botic stress *Fusarium graminearum*	Kong *et al*. (2005)
Q8W429/ AB029934	Chitinase 1, Chi 1 Chitinase 1	256	27.1	979	–	–
Q4Z8L7/ AY973230	Class II, chitinase Class II, chitinase	266	28.3	811	Seed development	–
Q4Z8L8/ AY973229	class II, chitinase class II,chitinase	266	28.2	892	Seed development	–
A0A0H4TM98/ KR049250	Chitinase cht4, chitinase	320	33.6	963	Biotic stress *P. striiformis West. f. sp. tritici*	–
A0A077RF77/ KR049248	Chitinasecht2, chitinase	320	33.6	963	Biotic stress *P. striiformis West. f. sp. tritic*i	–
Q9XEN3/ AF112963	Chitinase II, Cht2 Chitinase II, Cht2	230	24.7	956	Biotic stress *F. graminearum*	Li *et al*. (2001)
Q41539/ X76041	Endochitinase CHI Endochitinase CHI	320	33.6	1,985	Biotic stress *P. graminis*	Liao *et al*. (1994)
A0A023W638/ KJ507390	Endochitinase Endochitinase	320	33.6	963	–	–
A0A023W4F1/ KJ507387	Endochitinase Endochitinase	318	33.5	957	–	–
A0A023W636/ KJ507385	Endochitinase Endochitinase	319	33.5	960	–	–
A0A023W5U7/ KJ507389	Endochitinase Endochitinase	320	33.6	963	–	–
A0A023W4N4/ KJ507388	Endochitinase Endochitinase	318	35.3	957	–	–
A0A023W594/ KJ507386	Endochitinase Endochitinase	317	33.5	954	–	–

AA – amino acids; MW – molecular weight

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also they regulate processes of plant growth and development. It has been reported that upon (a)biotic stresses the activities of some constitutively synthesized chitinases can be increased (Žur *et al.* 2013).

The activities measured comprise several individual isoforms with different activities and functions. Therefore the substrate glycol chitin was used to detect chitinases with hydrolytic activities towards long polymers after their separation in gels. Our analyses identified up to four enzyme fractions (~20, 30, 42 and 95 kDa) with the chitinolytic activities (Figure 2b, Table 3). Only the ~30 kDa fraction was identified in all analysed genotypes. For comparison, the purified wheat chitinases in the sequence databases are of molecular mass in a range between 27 kDa to 36 kDa (Table 2). The molecular mass of plant chitinases commonly ranges from 25 kDa to 35 kDa, but plant isoforms of 20 kDa or ≥40 kDa were also described (Chang *et al.* 2014). Most plant chitinases of 30–46 kDa are referred as PR proteins (Ferreira *et al.* 2007) thus the identified chitinase isoforms of ~30 kDa and ~42 kDa might be significantly induced upon (a)biotic stresses.

Plant chitinases have been found to exist in acidic and basic forms according to their isoelectric points (Kasprzewska 2003). Based on the classification by Stintzi *et al.* (1993) the acidic isoforms are considered as extracellular while basic as vacuolar. We identified up to seven acidic (ChiA-ChiG) (Figure 2c) and three basic (Figure 2d) fractions with hydrolytic activities towards the long polymer glycolchitin (Table 3). The three acidic (ChiA, ChiB, ChiC) and two (ChiH, ChiI) isoforms were detected in all genotypes. In most of analysed genotypes, combination of four acidic fractions was found. However, none of the acidic isoforms can be associated with ploidy level.

Our results (Figure 1, Figure 2) showed that chitinases with specificities towards short oligomers and long chitin polymers are accumulated in the leaves of all analysed genotypes. The genotype had significant effect on short oligomer-specific chitinase ac-

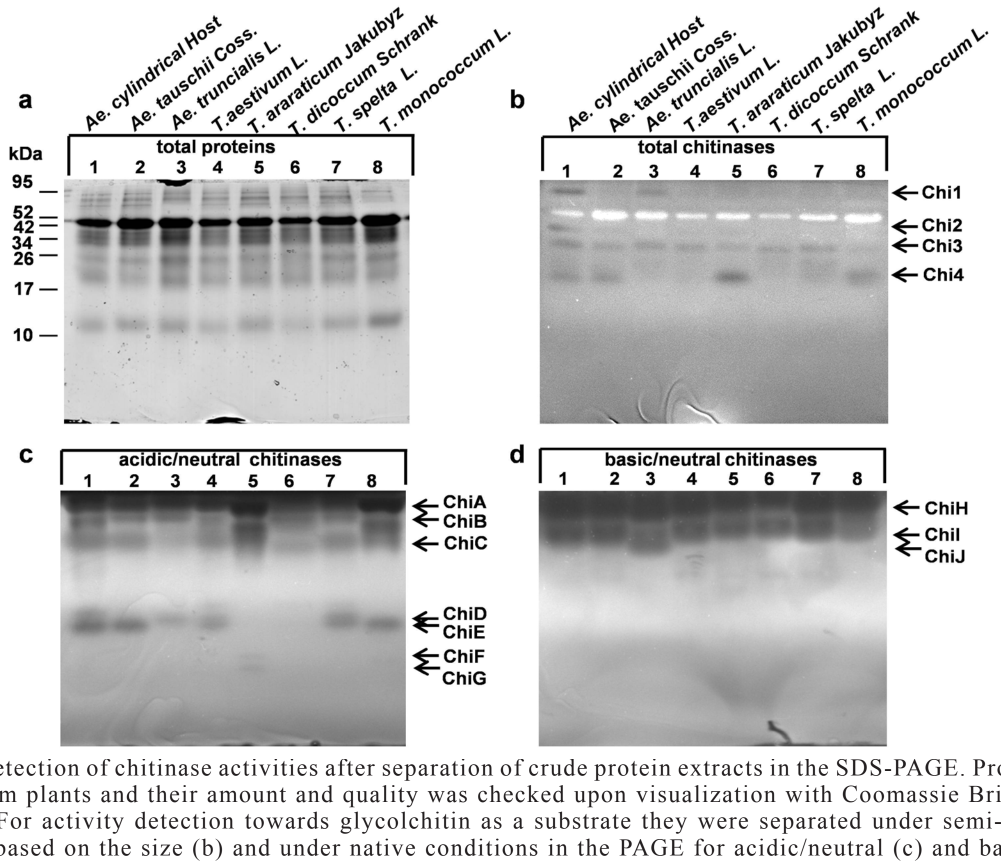

Figure 2. Detection of chitinase activities after separation of crude protein extracts in the SDS-PAGE. Proteins were isolated from plants and their amount and quality was checked upon visualization with Coomassie Brilliant Blue R 250 (a). For activity detection towards glycolchitin as a substrate they were separated under semi-denaturing conditions based on the size (b) and under native conditions in the PAGE for acidic/neutral (c) and basic/neutral proteins (d). Numbers on the left refer to the molecular mass marker. Arrows indicate the detected isoforms.

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tivities. These activities were significantly higher in tetra- and hexaploid than in diploid genotypes. The enzymes with hydrolytic activities towards oligomers and chitin polymers possess a potential for production of biologically active substances with antibacterial, antifungal, antitumor or immunity-enhancing effects (Li *et al.* 2016).

CONCLUSIONS

The activities of chitinases in two hexaploid wheat (*T. aestivum* L., *T. spelta* L.) and six their crop relative species (*Ae. tauschii* Coss., *Ae. cylindrical* Host, *Ae. triuncialis* L., *T. monococcum* L., *T. araraticum* Jakubyz, *T. dicoccum* Schrank) were studied. Our results showed that chitinases are active in the leaves of all analysed genotypes, while the gained values were variable and dependent on genotype. The activities in the tetra- and hexaploid genotypes appeared significantly higher than in their diplod counterparts. The gel activity assays revealed up to four fractions of proteins with chitinase activity of which the isoform Chi3 of ~30 kDa was identified in all analysed genotypes. More detailed analyses detected up to seven acidic and three basic

T a b l e 3

Overview of the chitinase activities in wheat and its relative species

	Chitinase isoforms						
	Total*				Acidic/neutral**		
	Chi1 ~95 [kDa]	Chi2 ~42 [kDa]	Chi ~30 [kDa]	Chi4 ~20 [kDa]	ChiA	ChiB	ChiC
Ae. tauschii Coss.	–	–	+	+	+	+	+
T. monococcum L.	–	–	+	+	+	+	+
T. araraticum Jakubyz	–	–	+	+	+	+	+
T. dicocum Schrank	–	–	+	–	+	+	+
Ae. cylindrical Host	+	+	+	+	+	+	+
Ae. truncialis L.	+	–	+	–	+	+	+
T. aestivum L.	–	–	+	–	+	+	+
T. spelta L.	–	–	+	+/	+	+	+

Table 3 continued

	Chitinase isoforms						
	Total*				Basic/neutral***		
	ChiD	ChitE	ChiF	ChiG	ChiH	ChiI	ChiJ
Ae. tauschii Coss.	–	+	–	–	+	+	–
T. monococcum L.	–	+	–	–	+	+	–
T. araraticum Jakubyz	–	–	+	+	+	+	–
T. dicocum Schrank	–	–	–	–	+	+	–
Ae. cylindrical Host	+	+	–	–	+	+	–
Ae. truncialis L.	+	–	–	–	+	+	+
T. aestivum L.	+	–	–	–	+	+	–
T. spelta L.	+	–	–	–	+	+	–

*Size of the isoforms (Chi1-Chi4) detected in the gel after re-naturation of separated proteins in the SDS-PAGE (Figure 2b)
**Fractions detected in the gel after separation of proteins in the PAGE under conditions for acidic/neutral proteins (Figure 2c)
***Fractions detected in the gel after separation of the proteins in the PAGE under conditions for basic/neutral proteins (Figure 2d)

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enzyme fractions of which some were specific for individual genotypes. However, a larger set has to be studied to assign them with respect to ploidy level. The presence of additional isoforms in the wild relatives of wheat brings a promise for identifying novel sources of defence compounds potentially interesting for breeding purposes. Besides, wheat chitinases can be studied for potential use in biotechnological programs focused on production of bioactive chitoologosacharides.

Acknowledgements. This work was supported by the bilateral project APVV SK-PL-2015-044, by the project APVV-15-0051, UGA VIII/32/2017 and by the Operational programme Research and Development for the project: 'Implementation of the research of plant genetic resources and its maintaining in the sustainable management of Slovak republic' (ITMS: 26220220097), co-financed from the resources of the European Union Fund for Regional Development.

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Received: February 11, 2017

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Agriculture (Poľnohospodárstvo), 63, 2017 (1): 23−32
Original paper DOI: 10.1515/agri-2017-0003

HERBIVOROUS INSECTS DIVERSITY AT *MISCANTHUS* × *GIGANTEUS* IN UKRAINE

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STEFANOVSKA, T. – PIDLISNYUK, V. – LEWIS, E. – GORBATENKO, A.: Herbivorous insects diversity at *Miscanthus* × *giganteus* in Ukraine. Agriculture (Poľnohospodárstvo), vol. *63*, 2017, no. 1, p. 23–32.

Miscanthus × *giganteus* is considered as a perspective energy crop for biomass production in Ukraine where its commercial production has been observed. The herbivorous pest may pose a risk of yield reduction when an energy crop is growing on monoculture. The herbivorous diversity, species composition and potential damage associated with growing *M.* × *giganteus* were studied on seven experimental sites at three locations in Ukraine. The different life stages of herbivorous insects from seven orders representing thirteen families were found on *M.* × *giganteus* during the herbivorous survey and most of the insects had a pest status. Research indicated that crop was an alternate host for key cereal pest the Hessian fly *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). A comparative analysis of the biodiversity of herbivorous insects across research locations was done using statistical analysis. It was found that site location played a significant role in the level of biodiversity and an increase in the insect's herbivores diversity was associated with the type of researched lands. The massive scale commercial use of *M.* × *giganteus* should take into account a responsible consideration of the benefits and risks associated with that crop in order to protect agroecosystems.

Key words: survey of herbivorous insects, pest, Hessian fly, statistical method

M. × *giganteus* is a sterile hybrid of two species: *M. sinensis* and *M. sacchariflorus*, and is native to Southern Asia (Hodkinson *et al*. 2002). Plant belongs to C-4 photosynthetic pathway type and has a capacity for effective and substantial biomass production (Zub & Brancourt 2010; Agostini *et al*. 2015). The biological feature of that crop is its ability to grow at the contaminated and abandoned sites, which makes it appropriate for phytotechnologies (Otepka *et al*. 2011; Pidlisnyuk *et al*. 2014; Nsanganwimana *et al*. 2016). The *M.* × *giganteus* potential for biofuel production has been extensively researched in Europe (Lewandowski *et al*. 2003; Gauder *et al*.2012), including Slovakia (Porvaz *et al*. 2012; Jurekova *et al*. 2013; Gubisova *et al*. 2016) and Czech Republic (Vaprova & Knapek 2010; Strasil 2016). *M.* × *giganteus* perennial rhizomatous grass's feature makes its establishment and reproduction very costly. Recently, significant progress was achieved in the development of *M.* × *giganteus* hybrid seed; the planting of this hybrid seed leads to a more economically feasible crop production than using the rhizomes (Clifton-Brown *et al*. 2017). Nowadays,

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the crop is used commercially for heating and generating electricity in several EU countries. In the US, the crop has been proposed for commercial production in Midwest and Northern States, particularly in locations where the precipitation is not a limiting factor. The number of growing sites has been expended in the US rapidly; however, the commercial production has not been well established yet (Heaton *et al.* 2008).

Currently, no pests of economic importance are found in *M.* × *giganteus* in Europe or the US. The European experience with *M.* × *giganteus* planting showed that the crop has low level of risk from pest damage. However, taking in consideration a hybrid nature of the crop, any pest associated issues that do arise may cause a serious problem (Thomson & Hoffmann 2010). Only few pests have been reported that directly damage the crop (Semere & Slater 2007). The survey of invertebrates of *M.* × *giganteus* in the United Kingdom indicated that there were ‘‘no major pests found’’ (Hugget *et al*. 1999). Results of the two-year research in Germany showed the damage of crop by two-spotted spider mite *Tetranychus urticae.* Koch Gottwald and Adam (1998), Semere and Slater (2007) recorded that aphids were the dominated Homoptera group found on field trials of Miscanthus. This finding may raise the issue of how aphids indirectly affect the crop by transmitting viruses. Several researches indicated that the crop, that may contribute to the distribution of viruses, were transmitted by aphids. It was observed that corn leaf aphid *Rhopalosiphum maidis* (Fitch) may colonize Miscanthus and lay eggs on the established plants. The laboratory studies showed that aphid feeding on *M.* × *giganteus* transmitted viruses: Barley Yellow Dwarf virus (Christian *et al*. 1994; Hugg *et al.* 1999) and the sorghum mosaic virus (Grisham *et al.* 2012).

The results of recent studies in the Northern France, where the cultivation of *M.* × *gigantheus* started in the early 2000s, gave a new argument regarding the concern on increasing the risk of speeding phytoviruses by aphids and Miscanthus acting as reservoir for aphids’ pests from neighbouring food crops. Coulette *et al*. (2013) showed that *M. sacchariflorus* (parental species for M. × *giganteus*) did not appear as an appropriate host for the three aphid species *Aphis fabae* Scop, *Myzus persicae* (Sulzer) and *Rhopalosiphum padi* L. Ameline *et al.* (2015) reported that the host suitability for the four major aphis depends on the degree of specialization to Poaceae and appeared as moderate for specialist *Rhopalosiphum padi* L., low for polyphagous *Aphis fabae* (Scop) and *Myzus persicae* (Sulzer) and as very low for Brassicae specialist *Brevicoryne brassicae* L. In controversy*,* this study illustrated that the cultivation of Miscanthus in large scale might not always aggravate the problem of creating reservoir aphids from adjusting food crops; it could be assumed that *M.* × *giganteus* acted as a barrier crop helping to reduce the risk of transmission and spread of phytoviruses.

The recording of direct increasing damage by several insects in the US may be evidence to suggest that *M.* × *giganteus* has pests; its effect on biomass is still unknown and its severity will depend on the scale and the time of plant’s growth. The fact that all insects reported to feed on *M.* × *giganteus* in the US are pests of corn, sugarcane or sorghum, raises concerns that the production of that crop in the large scale will increase pest numbers in existing food crops. It can be well illustrated by the relationship between *M.* × *giganteus* and corn (Bradshaw *et al*. 2010). *M.* × *giganteus* appeared as a host to the yellow sugarcane aphid and may cause damage in young stands in the field condition. The potential of *Sipha flava* (Forbes) to damage *M.* × *giganteus* in case of a large-scale cultivation was confirmed in the laboratory research (Pallipparambil *et al*. 2014), when the crop served as a host plant for corn rootworm determined as dangerous maize pest (Spencer & Raghu 2009). It was indicated (Gloyna *et al*. 2011) that the larvae of Western corn rootworm, of *Diabrotica virgifera virgifera* (Le Conte) originating from a Central and South Eastern European population, could be developed at *M.* × *giganteus*. Armyworm and stem borer, which were host of corn and sorghum species, were able to feed on the crop as well.

Prasifka *et al.* (2009) showed that *M.* × *giganteus* along with switch grass was a host of fall armyworm *Spodoptera frugiperda* (J. E. Smith) determined as a pest of corn was observed in the

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infesting plots. Laboratory test showed that *S. frugiperda* larva preferred corn leaves over Miscanthus ones. The pest was able to complete the development on Miscanthus and switchgrass at the green house conditions; however, it did not survive well during the field experiment. As it was reported by Prasifka *et al*. (2012), the stem-boring caterpillars: *Elasmopalpus lignosellus* (Zeller) (Pyralidae), *Diatraea saccharalis* (F.) (Pyralidae), and Mexican Rice Borer *Eoreuma loftini* (Dyar) (Crambidae) might cause *M.* × *giganteus* biomass reduction, and a long-term investment in breeding for host plant resistance might be requested.

Since 2004 *M.* × *giganteus* was under evaluation for commercial production in eight regions of Ukraine (Kvak 2013), an emerging commercial production has been currently observed and the area under cultivation is about 1,500 ha with expectation of significant extending in the nearest future (Pidlisnyuk & Stefanovska 2016). This trend is due to the increasing biofuel demands, energy security concern and political desire to increase the share of bioenergy in the country's energy balance (Geletukha *et al*. 2015). The fact is illustrated in Figure 1.

Growing interest and commercialization of *M.* × *giganteus* production in Ukraine will lead to land use changes and possible cultivation of the crop in monoculture. The land use change is a significant contributor of biodiversity changing (Whittaker *et al*. 2001). Some previous studies indicated a positive effect of *M.* × *giganteus*, growing to biodiversity service (Semere 2007; Gauder *et al*. 2012; Dauber *et al*. 2015), when the crop hosted several arthropods, particular predatory ground beetles and parasitoids – natural enemies of important agricultural pests. Stanley and Stout (2013) indicated that *M.* × *giganteus* supported higher abundance and diversity of pollinators and hymenopteran wasps comprised to traditional food crops. Another study concerned about the possible negative impact of *M.* × *giganteus* growing to the state of biodiversity (Stefanovska *et al*. 2011).

There are several factors that determine species diversity, particular spatial arrangement of habitat elements and the spatial-temporal heterogeneity of the landscape (Schluter & Ricklefs 1994; Lewinsohn *et al*. 2005; Rocca & Greko 2011). In comparison to Western Europe and the US, very limited data exists on that topic while growing *M.* × *giganteus* in Eastern Europe, including Ukraine. The purpose of this study was to do a survey of herbivorous insects in field conditions at different regions of Ukraine and to analyse the abundance, richness and biodiversity of common insect herbivores while growing *M.* × *giganteus* in seven sites at three different locations: Vinnytsia, Zhytomyr and Kyiv regions.

MATERIAL AND METHODS

The selected locations represented the areas which have high potential for biofuelcrops' production (Geletukha *et al.* 2015). A total of seven established plots of *M.* × *giganteus* varying in time of cultivation were chosen for herbivorous sampling. Plot sizes varied from 25 m^2 to 100 m^2. Some characteristics of the plots are presented at Table 1. For all the plots, *M.* × *giganteus* was planted manually in the soil depth of 0.05–0.1 m with spacing of 0.70 m × 0.70 m, which was equal to 20 pieces of plant per m^2; planting was done in the period of end of April to middle of May, depending of the annual weather conditions that determined the features of the vegetation season. The way of planting Miscanthus was same for each year of planting. The insect sampling was conducted at 2010 and 2011. Visual observation and sampling was conducted at five subplots randomly located within the planting at a minimum of 10 m from the plot edge to reduce edge effects. Above ground insect samples were collected every four weeks during the growing season using both active and passive sampling methods (Binns *et al*. 2000). Active sampling, through sweep net and stem was performed prior to the passive sampling methods. Stem counts were geared towards sedentary aphids. Sweep net sampling was a direct sampling method that would collect insects on the foliage of the plant. Twenty sweeps were taken down off the alternate crop rows and the specimens were collected and preserved in 90% ethanol solution until identification. In passive samples, Pitfall traps (Prasifka *et al*. 2007) were used for the collection of ground dwelling insects and sticky cards were used for the collection of

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small arboreal insects. Pitfall traps and yellow sticky cards were set up in a grid pattern with 1 m spacing. The traps were replaced weekly from the beginning of June till the end of July. All the traps were transferred to the laboratory for further insects' identification. For the sampling of Elateridae and Scarabaeidae species, a method of spring soil excavation was implemented. At each plot, the holes 0.50 × 0.59 m at depth 0.50 m were dug. The excavated soil samples were sieved and extensively screened for larvae at all stages of wireworms, grubs, mole crickets and cutworms, which were counted further.

The insect presented in the traps was identified till higher taxon and the selected herbivorous trophic groups were identified till species or at least till genera.

The data obtained from 2010 and 2011 years of insect sampling data are presented together in the same diagrams.

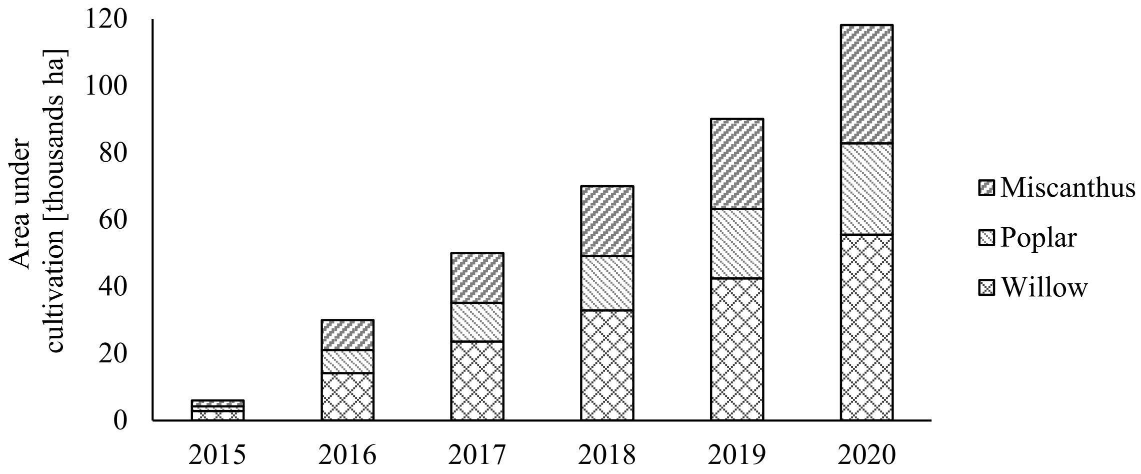

Figure 1. Prediction of energy crops area cultivation (Geletukha *et al*. 2015)

T a b l e 1

Characteristics of the research sites

Site location	Site and plot number	Location (GPS)	Year of the study	Year of the growing	Type of the soil
Zhytomyr	site1, plot 1	50.252385 28.70011	2010 2011	3 4	Podzolic gleyed
	site 1, plot 2	50.252385 28.70011	2010 2011	6 7	
Vinnytsia	site 1, plot 1	48.997958 27.462125	2010 2011	1 2	Flooded grey podzolic soil
	site 1, plot 2	48.997958 27.462125	2010 2011	3 4	
Kyiv	site 1, plot 1	50.42945 30.494808	2010 2011	1 2	Dark grey soil
	site 1, plot 2	50.42945 30.494808	2010 2011	3 4	
	site 2, plot 1	50.415087 30.55705	2010 2011	1 2	

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In order to assess the insect biodiversity, the Shannon index (Shannon 1948) was measured.

$$H = -\Sigma p_i \log_2 (p_i) \quad (1)$$

where:
$P_i = n_i/N$
(n_i) – sharing of the specie; (N) – total number of individuals

In order to determine the diversity of herbivorous taxa, we referred to the different research sites and to find the maximum entropy for each taxa of herbivores, the Hartley approach was used (2), in which N – number of taxa, as for the case:

$$H_{max} = \log_2 N \quad (2)$$

The Pielow's evenness index (3) was calculated by dividing the Shannon index ratio (1) by its maximum value (2), in order to align data from one site with other sites:

$$H' = \frac{H}{H_{max}} \quad (3)$$

Parametric statistical method, student's t-test and ANOVA were used to compare herbivore diversity across the sites in 2010–2011.

RESULTS AND DISCUSSION

Results of the survey of herbivorous insects provided at different sites where *M.* × *giganteus* grown are shown at Figure 2. The analysis of outcomes shows the different life stages of insects from seven orders and families observed during the 2010–2011 growing seasons across the sites. Specifically, among the recorded herbivorous generalists and highly specialists for Poaceae family pests were found for all the sites. The soil dwelling polyphagous insect (*Gryllotalpa gryllotalpa* L.*, Melolontha melolontha* L. and *Agrotis segetum* S.) were recorded at three sites located in Vinnytsia and Kyiv in the first year of growing *M.* × *giganteus*; no significant plant's injury by insects was observed with one exception of the Hessian fly. Its larvae and pupae were observed inside the stems of the plant in one plot located in Zhytomyr region. It was evident that Aphididae (Homoptera) and Thripidae (Thysanoptera) families were dominated at three locations. The *Rhopalosiphum padii* L. was the most widely represented species for all the researched sites and the Herbivorous trips were found in three sites. That fact is in correlation with results reported by Hurej and Twardowski (2009), who observed this group of sub sucking insects at *M.* × *giganteus* plots in Poland during the first years of cultivation.

Overall, 50 species of herbivorous trips were registered at the agricultural landscapes in Ukraine. In this study, the high population trips' density was observed in three locations (Figure 3). That trend can be due to the weather conditions in the research years, which were very favourable for the trips' development because of warm and dry summer. Leaf hopper, *Psammotettix striatus* L. were additionally recorded in the site located at Vinnytsia region, which was more southern in geographical location in comparison with other sites.

Shannon and Pielow's evenness indexes were used for the comparison of biodiversity of herbivorous insects across research locations. Results of the calculation of average data in 2010–2011 are presented at Figure 4, Table 2 and Table 3. It was hypothesized that the factor of location influenced the biodiversity of herbivorous insects. Following the performing the share of influence of considering and not considering that factors location was determined. The results showed that the impact of the factor 'location 'was 51%. Since *F* Theoretical > *F* Critical (Table 2), the null hypothesis might be rejected, that is, the site location played a significant role in the level of biodiversity. Results also showed that the increase in the diversity of insect herbivores was associated with the type of observed lands. Thus, Vinnytsia site located in more agricultural setting (with many crops types) illustrated a significantly higher level of species diversity in comparison with other locations in Zhytomyr and Kyiv regions, which showed few types of crops.

Analysis of previous research results indicated that *M.* × *giganteus* did not have many herbivorous pests, which in turn confirmed the statement that *M.* × *giganteus* cultivated fields could be

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served as a refuge for insect herbivores. Hence, the more the fields with various agricultural crops surround Miscanthus field, the more likely it is that the pests from those fields will move to the Miscanthus field. It can be concluded that the introduction of *M.* × *giganteus* in crop rotation may help to improve the environmental sustainability of the agricultural landscapes through the conservation of natural species and territory, and the plant's growth may work as ecological corridors. Profound research is needed for studying the interaction of *M.* × *giganteus* with other trophic groups of insects, particularly entomophagous and soil micro and mezofauma.

The importance of documenting trips, aphids and leafhoppers population data is three-fold. First, the pests may have a direct impact on crop production and profitability. Second, many of these insects are also associated with current crops, such as wheat, and may build up large populations

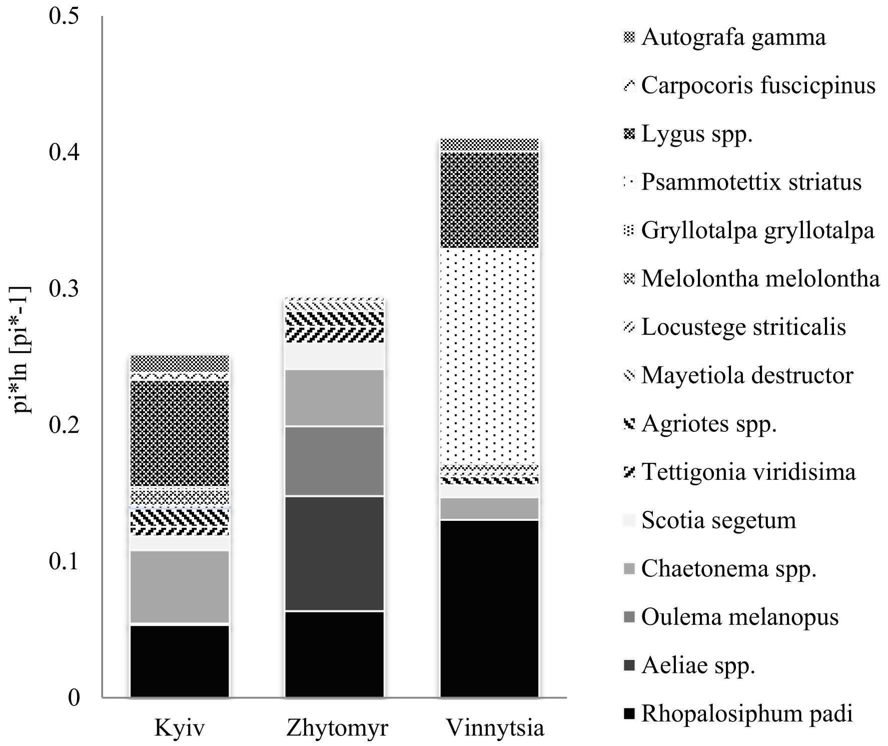

Figure 2. Variation of insect species and genera on *Miscanthus* × *giganteus* at three research locations in 2010–2011 growing seasons

T a b l e 2

Shannon index in sites

Site	Zhytomyr	Vinnytsia	Kyiv, site 1	Kyiv, site 2
1	0.46	0.59	0.42	0.46
2	0.46	0.67	0.44	0.58
3	0.76	0.78	0.47	0.32
4	0.33	0.93	0.52	n/d
M	0.50	0.74	0.46	0.46
±m	0.09	0.07	0.02	0.08

M – mean; SE – Standard error

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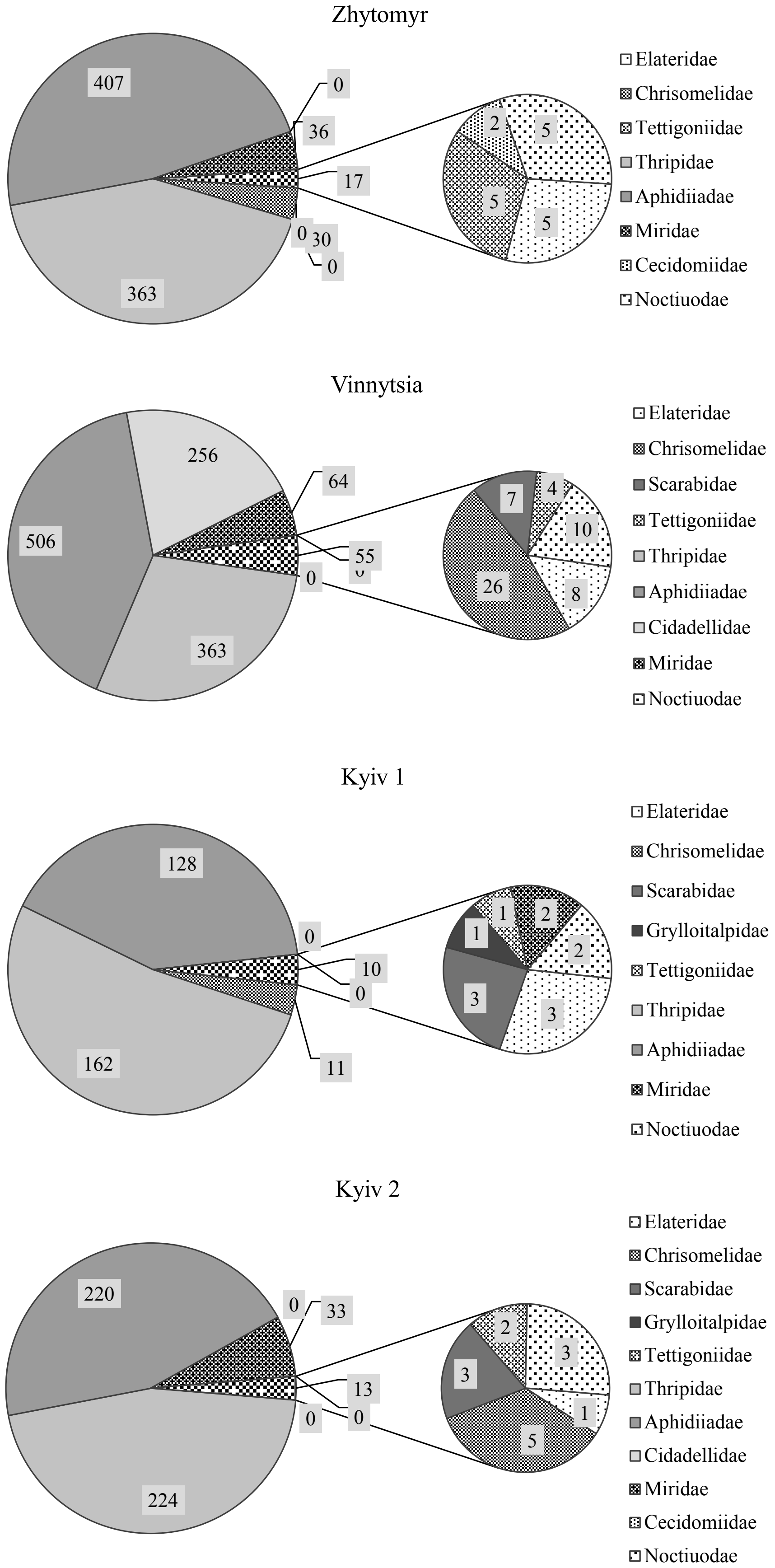

Figure 3. Number of herbivorous individuals in different stages at three locations in 2010–2011 growing seasons

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that could then migrate into production fields. Third, two of the most commonly found insects: the aphid *Rhopalosiphum padi* L. and the leafhopper *Psammotettix striatus* L. are both vectors of wheat diseases; thus, *M.* × *giganteus* could potentially serve as harbourage for vectors and disease.

The survey found that *M.* × *giganteus* was an alternate host for a key cereal pest the Hessian fly *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). Since Hessian fly, along with other species from the families Chrolopidae and Antomyidae, is a destructive pest for several cereal crops, there is a potential risk that the insect may reduce the yield of Miscanthus and damage adjacent food crops. Because intercropping is a common practice in Ukraine wheat, barley and oats may be potentially affected by this insect as well. It highlights that the current studies of Hessian fly colonization of Miscanthus plantings should be prolonged for the evaluation of potential risks to other crops and wild species sharing the agricultural landscape.

The fact that *M.* × *giganteus* appeared as a good host for the Western root corn beetle brings a new challenge for the commercial growing of that plant, which may increase the risk of further distribution of *Diabrotica virgifera virgifera* (Le Conte) throughout the country (Andreyanova & Sikura 2010).

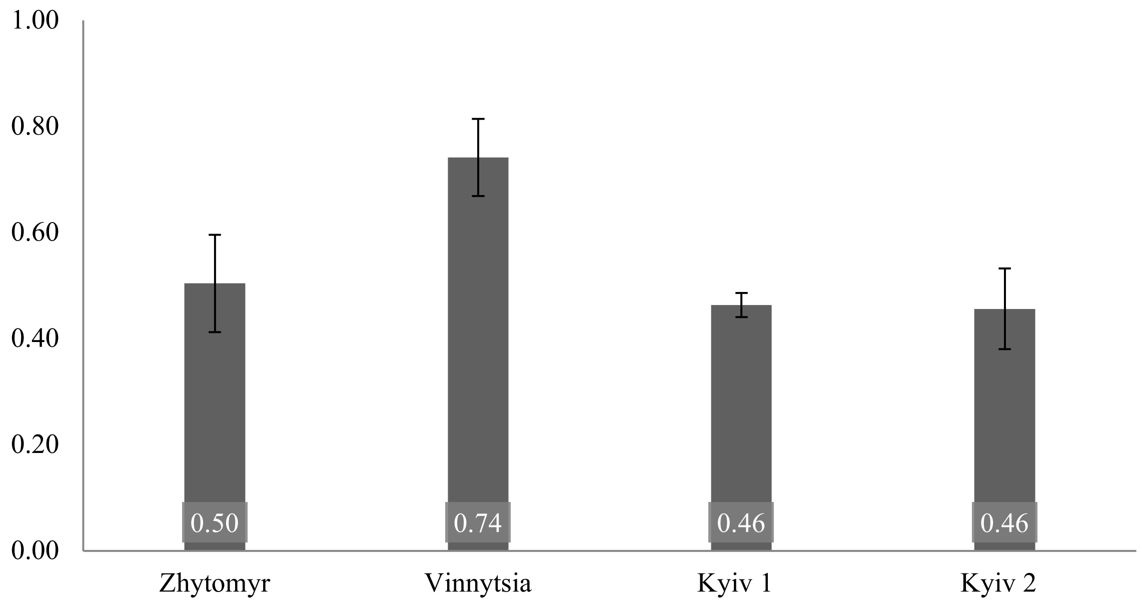

Figure 4. Pielow's evenness index (average value) for three location in 2010–2011 growing seasons

T a b l e 3

Variations between groups as for ANOVA

Variation	SS	df	MS	*Fc*	*P*-value	*Fd*
Between groups	0.211	3	0.07	3.78	0.05	3.59
Inside groups	0.205	11	0.02			
All together	0.416	15				

Fc – *F* calculated; *Fd* – *F* distribution

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CONCLUSIONS

The study indicates that intensive involvement of perennial grass *M.* × *giganteus* into the agricultural landscape in Ukraine, dominated by cereal crops from the same family Poaceae (wheat, corn, rye), stimulates numerous indirect interactions between that crop and small grain cereals and can pose a risk to agricultural landscapes as all. The conversion of marginal and abandoned lands, where the growing of *M.* × *giganteus* is profitable into monoculture production areas can bring new environmental challenge. Hence, the massive scale commercial use of *M.* × *giganteus* should take into account a responsible consideration of the benefits and risks associated with that crop, in order to protect the agricultural ecosystems that supply food, feed and increasingly, the fuel.

Acknowledgements. The research was supported by CRDF UKB1-2959-KV-08 and NATO SPS MYP#G4687.

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Received: February 15, 2017

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Agriculture (Poľnohospodárstvo), 63, 2017 (1): 33−49

Original paper DOI: 10.1515/agri-2017-0004

ECONOMIC EVALUATION OF NANO AND ORGANIC FERTILISERS AS AN ALTERNATIVE SOURCE TO CHEMICAL FERTILISERS ON *CARUM CARVI* L. PLANT YIELD AND COMPONENTS

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MAHMOUD, A.W.M. – EL-ATTAR, A.B. – MAHMOUD, A.A.: Economic evaluation of nano and organic fertilisers as an alternative source to chemical fertilisers on *Carum carvi* L. plant yield and components. Agriculture (Poľnohospodárstvo), vol. *63*, 2017, no. 1, p. 33–49.

To show the benefits of organic agriculture, safe and sustainable production, the present research was performed in an open field (new reclaimed area of desert) of Wadi El-Notron, Beheira Governorate, Egypt, for two successive years (2013 and 2014) aimed at a better understanding and to investigate the role of alternative source of chemical fertilisers represented by humic substances, natural nano-zeolite-loaded nitrogen and biofertilisers (HNB) on yield, morphological, leaf and seed anatomy, chemical compositions reflected in macro and micro nutrients, indigenous hormones, plant pigments, total carbohydrates, ascorbic acid, thiamine, total phenolics, total flavonoids, total fatty acids, oil yield and constituents of caraway (*Carum carvi* L.) plants. Our results revealed that plants receiving a combination treatment (HNB) recorded significant increases over control in both growing seasons. Moreover, economic evaluation reflects the profound influence of combination treatment (HNB) that realized the maximum gross income and minimum production cost. These findings emphasize the magnitude of the role of natural soil additions and organic fertilisers in mitigating environmental pollution while providing safe production and also minimizing total costs of chemical fertilisers.

Key words: biofertilisers, caraway, economic evaluation, humic, hormones, zeolite, oil yield

In the last few years, utilization of fertilisers has risen exponentially throughout the world, causing serious environmental problems. Overload application of fertilisers may accumulate heavy metals in soil and plant system; hence they possibly enter the food chain. Nitrogen and phosphorus as chemical fertilisers applied to farming land can provide valuable plant nutrients. On the other hand, if not handled in the right way, N and P can have harmful environmental effects. Meanwhile, the total nitrogen demand for agriculture all over the world is estimated to be 112.9 million tons for 2015. In the meantime, total loss in nitrogen fertiliser application is about 60–75% (FAO 2013). Therefore, a greater part of nitrogen fertilisers are not absorbed, and they consequently pollute both underground and surface water. Intemperance N in the form of nitrate may infiltrate the groundwater, causing nitrate contamination, which causes blue baby syndrome disease (Fewtrell 2004). Additionally, an overdose of N and P fertilisers leads to eutrophication, subsequently creating anoxic areas called dead zones (Savci 2012). Generally, there has been an increasing awareness about the undesirable impact of artificial or petrochemical fertilisers on the environment, over and above the potentially dangerous effects of

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chemical residues due to heavy metal accumulation in plant tissues on human health and animal consumers.

An example an aromatic plant is caraway (*Carum carvi* L.), which belongs to Apiaceae family, indigenous to northern Africa, Europe, and western Asia. The caraway fruit is a schizocarp, which at harvest is separated into two equally shared parts called "seeds" (Oosterhaven *et al.* 1995). Caraway plant is an important source of monoterpene, as it has carvone and limonene as its major monoterpene components. The seeds are used as a spice in food due to its pleasant flavour, and traditionally used as a remedy for dyspepsia and intestinal colic and antispasmodic (Lawless 1992) problems. Caraway essential oil has antioxidant (Yu *et al.* 2005; Wojdylo *et al.* 2007), antibacterial (Alzoreky & Nakahara 2003; Shan *et al.* 2007), fungicidal (Soliman & Badeaa 2002) and insecticidal (Lopez *et al*. 2008) properties. It has a lethal effect on mites (El-Zemity *et al*. 2006) and also has larvicidal (Pitasawat *et al.* 2007) and molluscicidal activities (Kumar & Singh 2006). Focusing on organic farming, we found that carvone is applied as a natural inhibitor of vegetative growth, mostly with stored onions and potatoes (Hartmans *et al*. 1995; Oosterhaven *et al.* 1995). Also, essential oil plays a vital role in pharmaceutical applications as medicine for humans due to its diuretic (Lahlou *et al*. 2007), anti-hyperglycaemic (Ene *et al.* 2008), anti-hypercholesterol (Lemhadri *et al.* 2006) plus anti-cancerous (Naderi-Kalali *et al*. 2005; Kamaleeswari *et al.* 2006) properties.

Nanoscience involves the development of fundamental understanding applied to nanoscale phenomena such as physical properties of materials and objects such as clusters of atoms, molecules and biological structures. Nanoscience is sometimes further broken down into sub-disciplines such as nanochemistry, nanophysics and nanobiology. Nanoparticles, natural or synthetic, are materials that vary in size between 1 and 100 nm (Kelkar *et al.* 2014). Nanotechnology opens up a large scope for novel applications in the fields of biotechnology, agriculture, fertiliser industries since nanoparticles have unique physico-chemical properties because of their high surface area, high reactivity, tunable pore size and particle morphology (Siddiqui *et al.* 2015).

The present research aims at a better understanding and investigation of the role of alternative sources of chemical fertilisers using natural zeolite in the

T a b l e 1

Some physical and chemical properties of experimental site

Physical properties		Chemical properties	
Particle size distribution [%]		Electrical conductivity (EC) [dS/m]	1.68
Coarse sand 2000–200 μ	80.20	pH (1:2.5) soil : water suspension	7.68
Fine sand 200–20 μ	12.50	Soluble cations [meq/l]:	
Silt 20–2 μ	4.25	Ca^{2+}	5.20
Clay < 2 μ	3.05	Mg^{2+}	4.18
Bulk density [g/cm^3]	1.52	K^+	2.40
Total porosity [%]	52.8	Na^+	5.20
Pore size distribution as % of total porosity		Soluble anions [meq/l]:	
Macro (drainable) pores (> 28.8 μ)	82.98	CO_3^{2-}	0.00
Micro pores (< 28.8 μ)	17.02	HCO^{3-}	1.70
Water Holding Capacity (WHC)*	20.33	Cl^-	3.60
Field capacity (FC)*	8.55	SO_4^{2-}	11.50
Wilting percentage (WP)*	4.10	Total $CaCO_3$ [%]	0.20
Available moisture (FC-WP)*	4.45	Organic matter [%]	0.20
Hydraulic conductivity [cm/h]	6.25		

* % on weight basis

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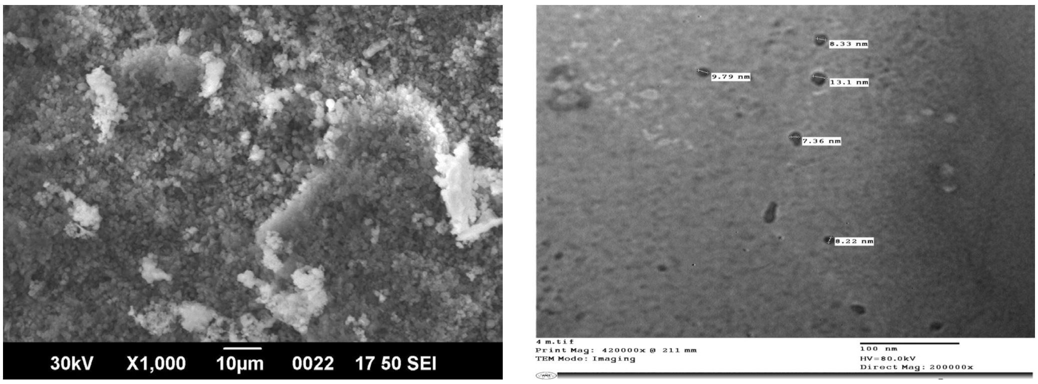
form of nano particles loaded by nitrogen, bio-fertilisers and humic substances on yield, morphological, leaf and seed anatomy, chemical compositions and oil yield and constituents of caraway plants in order to reduce environmental pollution and provide safety as well as minimize economic costs that arise from the use of chemical fertilisers.

MATERIAL AND METHODS

This research was carried out at the new reclaimed area of desert located in Wadi El-Notron, Beheira Governorate (Longitude 28°54' E, Latitude 28°20 N and Altitude 130 m) in Egypt, as an open field work, during two continual seasons (2013/14 and 2014/15), at Soil, Water and Environment Research Institute, Agriculture Research Center (A.R.C). Mechanical and chemical analyses of the reclaimed soil were performed according to Richards (1954) and Jackson (1973) and the results are shown in Table 1.

Plant material, transplant and harvest dates

The fruits of *Carum carvi* L. were obtained from experimental farm of Faculty of Pharmacy, Cairo University, and planted in the experimental open field on 13 October 2013 in the first season and 15



(a) (b)

Figure 1. (a, b) Structure of nano-zeolite using scanning electron microscope (SEM)

T a b l e 2

Chemical composition of zeolite before loaded by N

Chemical composition [%]	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	FeO	MnO	MgO	CaO	Na_2O	K_2O	SrO	P_2O_3	Loss on ignition
	45.50	2.81	13.30	5.40	8.31	0.51	6.30	9.52	2.83	0.87	0.22	0.67	3.76
Trace elements [ppm]	Ba	Co	Cr	Se	Cu	Zn	Zr	Nb	Ni	Rb	Y	–	–
	10	1.2	35	0.8	19	64	257	13	55	15	22	–	–

T a b l e 3

Chemical composition of nano zeolite after loaded by N

Chemical composition [%]	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	FeO	MnO	MgO	CaO	Na_2O	K_2O	SrO	P_2O_3	N
	45.50	2.81	13.30	5.40	8.31	0.51	6.30	9.52	2.83	0.87	0.22	0.67	2.70
Trace elements [ppm]	Ba	Co	Cr	Se	Cu	Zn	Zr	Nb	Ni	Rb	Y	–	–
	10	1.2	35	0.8	19	64	257	13	55	15	22	–	–

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October 2014 in the second season, with a distance of 60 cm among rows, with a 40 cm spacing between plants in plots of 6 × 7 m^2 under drip irrigation. The plants were harvested from 20 to 24 May 2014 for the first season and from 18 to 21 May 2015 for the second one.

Land preparation

Before planting, the soil was first mechanically ploughed and planked twice till the soil surface settled.

Fertilisers added

Chemical fertilisers in doses recommended by the Ministry of Agriculture and Land Reclamation were added at the rate of 100 kg/fed. (one Fadden equal 4,200 m^2). Ammonium sulphate (20.5% N) was divided into two doses; the first was added after 2 weeks of planting, while the second was four weeks later. Both calcium superphosphate (15.5% P) at the rate of 200 kg/fed. and potassium sulphate (48% K) at the rate of 25 kg/fed. were added one day before planting. Natural zeolite (Table 2) in the form of granules was obtained from Indonesia and converted into nano size according to Hassan and Mahmoud (2015), then loaded by nitrogen (Table 3) by soaking in ammonium sulphate 1 M solution for 8 days (Li *et al.* 2013) at 25°C. Total N content was analysed using the Kjeldahl digestion method (Helrich 1990). The structure was characterized using scanning electron microscope (SEM) model JSM.6390LA (JEOL) analytical (Figure 1) at Holding Company for Drinking Water and Waste Water, Greater Cairo Company for Drinking Water, Central Laboratory. Transmission electronic microscopy (TEM) was performed in TEM lab (FA-CURP), Faculty of Agriculture, Cairo University Research Park. Nano-zeolite-loaded nitrogen was added to the soil at the transplanting date and then as foliar at 20, 40 and 60 days after transplanting in both seasons.

Biofertilisers

Two bacterial cultures as shown by light microscope (Figure 2) containing 1 × 110^8CFU/ml from *Bacillus megaterium* and *Azotobacter chroococcum* were prepared individually in the biofertiliser unit, Soils Water and Environmental Research Institute, Department of Microbiology (ARC), Giza, Egypt. Then, they were mixed well together in liquid at equal portions (1:1 v). Roots of seedlings were dipped into the mixture of biofertilisers for 20 min immediately before planting. This mixture of biofertilisers was added to plant rhizosphere through view holes already made in the soil surface for inoculation (4 holes, 20 cm depth) at 30, 45 and 60 days after transplanting in both seasons (3 l mixture of biofertilisers/100 l water/fed.).

Humic acids

Humic substances obtained from Soil and Water and Environment Research Institute, Agriculture Research Center (ARC) were applied as a solution of 1 l/fed. at the transplanting date, and then 30 and

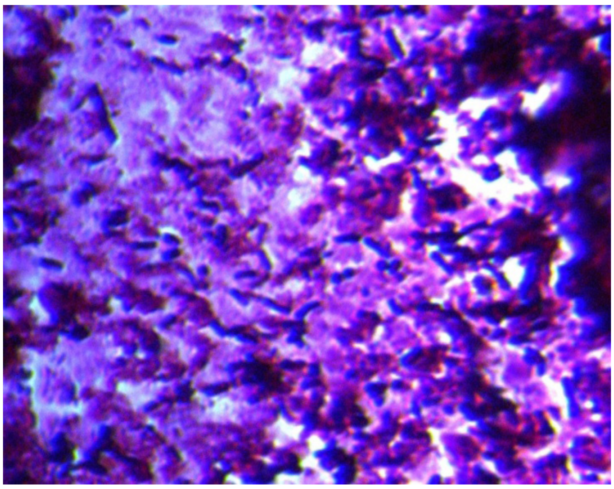

Figure 2. *Bacillus megaterium* and *Azotobacter chroococcum* mixture as shown by light microscope

Table 4

Chemical composition of humic substances

Chemical composition	Value
Humic acids	42%
Fulvic acid	48%
Total Nitrogen (N)	0.5 %
Phosphorus (P)	0.06%
Potassium (K)	0.8%
Calcium (Ca)	1.0%
Magnesium (Mg)	0.1%
Sulfur (S)	1.3%
Iron (Fe)	0.21%
Boron (B)	0.01%
Solubility in water	83%
Colour of aqueous solution	Medium brown
Electrical conductivity (EC) [mS/cm]	3.140
pH	6.92

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60 days respectively from planting as a soil drench per season. All agricultural practices were followed as recommended during both seasons. Chemical composition of humic substances are presented in Table 4.

Leaf and seed anatomy

The specimens were taken from the leaves at the end of vegetative growth and seeds at harvest and then fixed in formalin-acetic acid alcohol (FAA) using 70% ethanol. The specimens were gradually dehydrated in a *tert*-butyl alcohol (TBA) series (Johansen 1940) and embedded in paraffin wax (m.p. 56°C). Sections were cut on a rotary microtome at a thickness of 8–10 µm (Model RM2245, Leica Microsystems). Paraffin was removed with xylol and slides were stained with safranin FCF methanol and fast green and then mounted in Canada balsam (Johansen 1940). The selected sections were examined and photographed using a light microscope (Model BX51; Olympus Optical).

Treatments

- NPK fertilisers (recommended doses) as control
- Humic substances;
- Nano-zeolite-loaded nitrogen;
- Biofertilisers;
- Humic substances + Nano-zeolite + Biofertilisers (HNB) as shown in Figure 3.

Data recorded

Growth parameters

Plant height [cm], number of branches/plant, number of umbles/plant, plant fresh weight [g], plant dry weight [g], weight of umbles/plant [g], fruit weight/plant [g], fruit yield/ha [t].

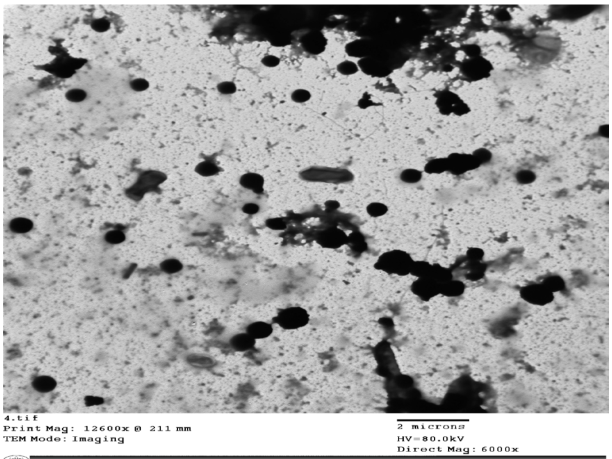

Figure 3. TEM of mixture Humic substances + Nano-zeolite + Biofertilisers (HNB treatment)

Chemical Analysis

The chemical constituents such as N, P, K, Ca, Mg, Fe and Zn were measured in the dry material. The wet digestion of 0.2 g plant material with sulphuric and perchloric acids was carried out on herbs by adding concentrated sulphuric acid (5 ml) to the samples and the mixture was heated for 10 min. Then 0.5 ml perchloric acid was added and heating continued till a clear solution was obtained. The digested solution was quantitatively transferred to a 100 ml volumetric flask using deionized water as reported by Piper (1950).

The total nitrogen content of the dried leaves was determined by using the modified micro-Kjeldahl method as described by Helrich (1990).

Phosphorus was determined calorimetrically by using the chlorostannous molybdophosphoric blue colour method in sulphuric acid according to Jackson (1973).

Potassium concentrations were determined by using the flame photometer apparatus (CORNING M 410, Germany).

Vitamin C as ascorbic acid [mg] was determined in seeds and estimated per 100 ml fresh weight, according to Helrich (1990) method.

Concentrations of Ca, Mg, Fe and Zn in plant samples were determined using atomic absorption spectrophotometer with air-acetylene and fuel (Pye Unicam, model SP-1900, US).

Plant pigments

Total chlorophyll and carotenoid content were measured by spectrophotometer and calculated according to the equation described by Moran (1982).

Total carbohydrates in plant herbs were determined by phosphomolybdic acid method according to Helrich (1990).

Total phenolic contents of the extracts were determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method (Singleton & Rossi 1965).

Total flavonoid was determined using the method of Meda *et al.* (2005).

Total fatty acids in seeds were converted into their methyl esters using 3% sodium methylate in methanol according to the method described by Cecchi *et al.* (1985).

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HPLC analysis of thiamine

Assays of thiamine in caraway seeds were carried out using a method described by Rapala-Kozik *et al*. (2008).

Endogenous phytohormones

The analysis was performed according to Fales *et al.* (1973) for the determination of gibberellic acid (GA), abscisic acid (ABA) and indole-acetic acid (IAA). The quantification of the endogenous phytohormones was carried out with Ati-Unicum gas–liquid chromatography, 610 Series, equipped with flame ionization detector according to the method described by Vogel (1975).

Essential oil isolation

Air-dried seeds (50 g) were subjected to hydrodistillation for 90 min (time fixed after a kinetic survey during 30, 60, 90 and 120 min). The optimal yield was obtained at 90 min.

Gas chromatography-mass spectrometry

The GC–MS analyses were performed on a gas chromatograph HP 6890 (II) interfaced with a HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with electron impact ionization (70 eV). An apolar HP-5MS capillary column (60 m × 0.25 mm, 0.25 m film thickness) was used.

Data analysis

The experimental design was randomized complete block design with six replicates. Data were subjected to statistical analysis using ANOVA at 5% significance level. The difference between treatments then analysed using DMRT (Duncan Multiple Range Test) at 5%.

Economical evaluation

The yield components were calculated and economic analysis was performed using the following equations proposed by Sarwar *et al.* (2007); FAO (2000) and Mubashir *et al.* (2010).

Gross income = yield × price

Profitable return (PR) = gross income – total production cost

PR% over control = PR – control treatments

Benefit cost ratio (BCR) = PR over control/total production cost

Investment factor (IF) = gross income/total production cost

(IF) must equal or more 3

RESULTS AND DISCUSSION

Growth characters

The data representing caraway morphology (Tables 5 and 6) revealed that a combination of humic substances, nano-zeolite-loaded nitrogen and biofertiliser (HNB) treatment had pivotal effect on plants. They significantly increased all growth parameters of caraway plants in comparison with either control (plants receiving recommended dose of NPK fertilisers) or all=other treatments in both growing seasons. These increases were (67% and 68%) respectively in fruit yield/ha compared to that in control treatment. The results of the combined humic substance and nano-zeolite-loaded nitrogen treatments as well individual treatments were significantly higher than the results of control plants. It was clear also that the application of biofertilisers alone significantly limited the growth of caraway and its biomass production. As a result, the reduction from biofertilisers treatment was 46% and 62% compared to control in the first and second season, respectively.

Our data provide a plausible mechanism for how the combination of humic substances, nano-zeolite-loaded nitrogen and biofertilisers (HNB) together boost all growth parameters as compared to control. The beneficial effects of these treatments on caraway plant are due to nutrient availability and improvement of soil's physical, chemical and biological properties as evident by higher water retention, decreased soil pH, higher CEC, increased soil organic matter in addition to availability of elements to be absorbed by plant roots. Given the significant role of nanoscale (nano-zeolite-loaded nitrogen), the authors confirmed that nanoparticles interact with plants, causing many morphological and physiological changes, depending on their properties and chemical composition, size and surface covering (Khodakovskaya *et al.* 2012). Other suggested positive effects on plant growth and development and their impact on plants depend on the composition, concentration, size and physical and chemical

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properties as well as plant species (Ma *et al.* 2010). More evidence of positive effects was reported on kale (*Brassica alboglabra* Bailey). Li *et al.* (2013) indicated that the application of ammonium- and potassium-loaded zeolite resulted in an increase in the total harvest weight over control plants. Ranjbar *et al.* (2004) and Bernardi *et al.* (2008) reported on similar treatments on tobacco and oat, respectively and found that zeolite application increased leaf area, plant height and stem diameter relative to the control without zeolite. Additionally, Hassan *et al.* (2006) observed that rosemary plants receiving compost in combination with mixture of bio-fertilisers recorded considerable increases in growth characteristics represented in fresh herb, dry weight and number of branches when compared to inorganic fertiliser treatment.

Effect on elements content

The highest content of both macro and micro elements resulted from the application of mixed humic substances, nano-zeolite-loaded nitrogen and biofertilisers (HNB). This increase was statistically significant with N, P, Ca, Fe and Zn compared to control plants (Table 7) since it recorded 79% for nitrogen, 72% and 74% for phosphorus, 83% and 76% for calcium, 52% and 54% for iron and 81% and 79% for zinc, respectively, for both first and second seasons. Also, it was noticed that the same previous treatment had recorded an increase of potassium over control in both growing seasons although this increase was not significant in the first season (91%), but it was statistically significant (89%) in the second one. The same result was found with magnesium as a result of HNB treatment

T a b l e 5

Effect of different treatments on plant height, number of branches and number of umbles of caraway plant during two seasons 2013 and 2014

Treatment	Plant height [cm]		No. of branches/plant		No. of umbles/plant	
	F	S	F	S	F	S
NPK	91.7^{b}	94.0^{b}	8.56^{b}	8.95^{b}	31.48^{b}	35.23^{b}
Humic substances (H)	80.3^{c}	83.6^{c}	8.07^{b}	8.84^{b}	28.72^{b}	30.21^{b}
Nano Zeolite (N)	82.5^{c}	83.2^{c}	7.89^{b}	8.80^{b}	30.22^{b}	34.55^{b}
Biofertilisers (B)	66.8^{d}	69.3^{d}	6.44^{c}	7.36^{b}	22.40^{c}	25.36^{c}
HNB	111.6^{a}	115.7^{a}	10.89^{a}	11.12^{a}	40.65^{a}	49.28^{a}

Means with the same letters in a column are not significantly different by DMRT 5%
F – first season; S – second season

T a b l e 6

Effect of different treatments on plant fresh weight, plant dry weight, weight of umbles, fruit weight and fruit yield of caraway plant during two seasons 2013 and 2014

Treatment	Plant fresh weight [g]		Plant dry weight [g]		Weight of umbles/plant [g]		Fruit weight/ plant [g]		Fruit yield [t/ha]	
	F	S	F	S	F	S	F	S	F	S
NPK	219.6^{b}	225.5^{b}	51.42^{b}	55.61^{b}	96.24^{b}	100.3^{b}	60.54^{b}	65.47^{b}	2.61^{b}	2.51^{b}
Humic substances (H)	200.3^{c}	213.3^{c}	44.81^{c}	48.89^{b}	89.17^{c}	93.51^{c}	54.87^{c}	61.55^{b}	2.16^{b}	2.41^{b}
Nano Zeolite (N)	215.5^{b}	221.0^{b}	48.56^{b}	50.77^{b}	91.50^{c}	98.10^{b}	57.21^{c}	63.12^{b}	2.25^{b}	2.48^{b}
Biofertilisers (B)	185.7^{d}	190.3^{d}	41.33^{c}	45.27^{c}	72.46^{d}	79.54^{d}	38.91^{d}	45.71^{c}	1.20^{c}	1.56^{c}
HNB	286.6^{a}	300.2^{a}	68.58^{a}	70.97^{a}	112.83^{a}	120.60^{a}	86.31^{a}	90.64^{a}	3.40^{a}	3.67^{a}

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where its increase over control, although not significant, was recorded at 90% in the first season while in the second season, it gave significant increases (88%). Many researches confirmed the important role of zeolite, which has specific ion-exchange, adsorption, catalytic properties, and promotes keeping nutrients in the plough horizon, which makes it possible to reduce their losses in soil from washing out and to obtain beneficial results as an after-effect (Ryakhovskaya & Gainatulina 2009), besides producing long-term soil improvements as well as slow release of the fertiliser in the form of nano nitrogen. Zeolites can also act as water moderators, in which they will adsorb up to 55% of their weight in water and slowly release it upon plant demand (Monnier & Dupont 1983). This has a great effect in desert reclamation processes particularly in arid and semi-arid areas.

Moreover, zeolite containing macro and micronutrients, loaded with nano nitrogen and its channels, provide large surface areas on which chemical reactions can take place by making nutrients more effective by enabling ammonium nitrate, potassium, magnesium and calcium as well as trace elements for slow release as needed (Kallo *et al*. 1986). Along with humic substances and biofertilisers acting as excellent sources of nutrients, they also improve physical and chemical properties of soil, decrease soil pH, which leads to solubilization of nutrients and increases its availability, minimizes the loss of nutrients by leaching and stimulates increase of population and activities of micro-organisms in the soil (Bernardi *et al.* 2008).

Effect on plant pigments, total carbohydrates, ascorbic acid and thiamine

The combination of humic substances, nano-zeolite-loaded nitrogen and biofertilisers (HNB) increased total chlorophyll and carotenoids (Table 8). Whereas total chlorophyll significantly increased by 72% in both seasons over control, carotenoids content also increased significantly (85%) in the first season compared with control. This increase continued in the second season, but was not significant. The elevated amount in total chlorophyll and carotenoids content may be due to the beneficial effects of humic acids and positive role of zeolite in the presence of biofertilisers, which give excellent combination for more available nutrients and overcoming most macro and micro nutrient deficiency, particularly of nitrogen, iron and zinc, in sandy soil, which is efficient in the photosynthesis process. They also build high capacity of the plants for building metabolites, which in turn contributes much to the increase of nutrient uptake. Our results are in agreement with those obtained by Al-Qadsia (2004) on *Ocimum basilicum* and Ranjbar *et al*. (2004) on tobacco.

As far as total carbohydrates are concerned, data in Table 8 illustrated that the increase in total carbohydrates as a consequence of HNB application was significant (84%) in the first season compared to control plants, while the increase over control treatment in the second season was insignificant. On the other hand, during both seasons, application of biofertilisers alone led to significantly lower carbohydrate content (80% and 84%, respectively)

T a b l e 7

Effect of different treatments on nitrogen, phosphor, potassium, calcium, magnesium, iron and zinc in the herb of caraway plant during two seasons 2013 and 2014

Treatment	N [%]		P [%]		K [%]		Ca [%]		Mg [%]		Fe [ppm]		Zn [ppm]	
	F	S	F	S	F	S	F	S	F	S	F	S	F	S
NPK	2.53^{b}	2.71^{b}	0.26^{b}	0.28^{b}	3.40^{a}	3.70^{b}	1.53^{c}	1.55^{c}	0.35^{a}	0.37^{b}	170^{c}	183^{c}	49.3^{c}	53.5^{b}
Humic substances (H)	1.81^{c}	2.08^{c}	0.30^{b}	0.33^{a}	3.20^{a}	3.50^{b}	1.58^{c}	1.80^{b}	0.37^{a}	0.36^{b}	191^{b}	198^{b}	52.7^{b}	55.6^{b}
Nano Zeolite (N)	2.31^{b}	2.55^{b}	0.35^{a}	0.36^{a}	3.50^{a}	4.05^{a}	1.71^{b}	1.61^{c}	0.33^{a}	0.35^{b}	200^{b}	215^{b}	56.8^{b}	60.0^{a}
Biofertilisers (B)	1.86^{c}	2.16^{c}	0.21^{c}	0.24^{b}	2.70^{b}	3.03^{b}	1.21^{d}	1.37^{d}	0.29^{b}	0.32^{c}	155^{d}	169^{d}	42.5^{d}	48.7^{c}
HNB	3.21^{a}	3.44^{a}	0.36^{a}	0.38^{a}	3.73^{a}	4.18^{a}	1.83^{a}	2.03^{a}	0.39^{a}	0.42^{a}	324^{a}	341^{a}	60.2^{a}	67.4^{a}

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in comparison with control (NPK). The increase in total carbohydrates may be due to the increase of photosynthesis as a result of increase in photosynthetic pigment content in leaves. HNB appears to be a source of a number of essential elements that may play an important role in plant metabolism, notably the most significant function would appear to involve carbohydrate metabolism and photosynthesis (Tisdale & Nelson 1975). Similar results were found by Jin *et al*. (2008) on acer plant. Interestingly, the same trend was obtained with ascorbic acid where in the first season, HNB treatment significantly increased the amount of ascorbic acid (80.5%) over control. Also in the second season, there was an increase recorded for aforesaid treatment compared to control in spite of that increase being insignificant. Matching results were obtained by Soliman and Mahmoud (2013) on *Adansonia digitata* L.

During both seasons, there was significant increase in thiamine, which recorded increase of 92.5% and 91%, respectively, over control as a result of HNB application (Table 8). Thiamine content recorded insignificant results from other treatments represented in humic substances, nano-zeolite and biofertilisers individually in comparison with the results of control treatment in both seasons. The increase in ascorbic acid and thiamine may be due to advantageous effects of the combination of humic substances, nano-zeolite and biofertilisers together resulting in more release of nutrients in an available form for plant uptake, which resulted in higher efficiency of the photosynthesis process, increasing

T a b l e 8

Effect of different treatments on total chlorophyll content [mg/g fresh weight], total carbohydrates, carotenoids content, ascorbic acid and thiamine of Caraway plant during two seasons 2013 and 2014

Treatment	Total chlorophyll [mg/g]		Total carbohydrates [%]		Carotenoids content [mg/g]		Ascorbic acid/100 g seed		Thiamine /100 g seed	
	F	S	F	S	F	S	F	S	F	S
NPK	2.29[b]	2.32[b]	27.41[b]	30.72[a]	0.52[b]	0.59[a]	17.22[b]	17.86[a]	0.371[b]	0.384[b]
Humic substances (H)	1.97[c]	2.28[b]	25.31[b]	28.44[a]	0.43[c]	0.49[b]	17.37[b]	19.13[a]	0.381[b]	0.388[b]
Nano Zeolite (N)	2.3[b]	2.54[b]	27.39[b]	31.62[a]	0.55[b]	0.58[a]	18.09[b]	18.67[a]	0.387[b]	0.391[b]
Biofertilisers (B)	1.73[c]	2.01[c]	21.89[c]	25.82[b]	0.40[c]	0.46[b]	14.98[c]	15.12[b]	0.359[b]	0.366[b]
HNB	3.17[a]	3.19[a]	32.49[a]	35.11[a]	0.61[a]	0.65[a]	21.38[a]	21.42[a]	0.401[a]	0.422[a]

Means with the same letters in a column are not significantly different by DMRT 5%
F – first season; S – second season

T a b l e 9

Effect of different treatments on total phenols, total flavonoids and total fatty acids of caraway plant during two seasons 2013 and 2014

Treatment	Total phenols [μg CE/g]		Total flavonoids [μg CE/g]		Total fatty acids [mg/g DW seed]	
	F	S	F	S	F	S
NPK	8.69[b]	8.65[b]	42.11[a]	45.27[a]	55.42[c]	60.33[b]
Humic substances (H)	8.72[b]	8.77[b]	40.32[a]	41.53[b]	52.28[c]	56.88[c]
Nano Zeolite (N)	8.78[b]	8.81[b]	41.21[a]	44.36[a]	60.12[b]	58.43[c]
Biofertilisers (B)	9.15[a]	9.33[a]	37.32[b]	38.89[b]	39.46[d]	35.22[d]
HNB	8.55[b]	8.70[b]	45.48[a]	47.51[a]	68.95[a]	69.71[a]

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amino acid and vitamin synthesis in plant tissue. These results are in agreement with those obtained from Leclerc *et al.* (1991) on carrot and celery root and Soliman and Mahmoud (2013) on *Adansonia digitata* L.

Effect on total phenolics, total flavonoids and total fatty acids

Previous researches indicated that fertilisation type had an influence on the phyto-nutritional quality of crops. Inorganic fertilisers are believed to reduce the antioxidant levels while organic fertilisers were proven to enhance the antioxidant content in plants (Dumas *et al.* 2003). Our data shown in Table 9 revealed that biofertiliser treatment significantly increased total phenolics in both seasons (94% and 92.7%) compared either with control and all other treatments including mixed HNB treatment. Meanwhile there were insignificant differences recorded during both seasons with humic substances, nano zeolite and mixed HNB treatments compared to control plants. On the other hand, total flavonoids were found to be significantly having lowest content (88.6% and 86%, respectively) in the first and second seasons with biofertiliser treatment compared with control. Mixed HNB treatment gave the highest content of flavonoids against control and all other treatments in both seasons, although that increase was insignificant. There was insignificant difference in total flavonoids resulting from humic substances, nano zeolite and mixed HNB treatments in comparison with control, particularly in the first season. The results obtained with regard to total phenolics and total flavonoids are in parallel with those obtained by Omar *et al.* (2012) on cassava tubers and Asami *et al*. (2003) on dried marion berry, strawberry and corn.

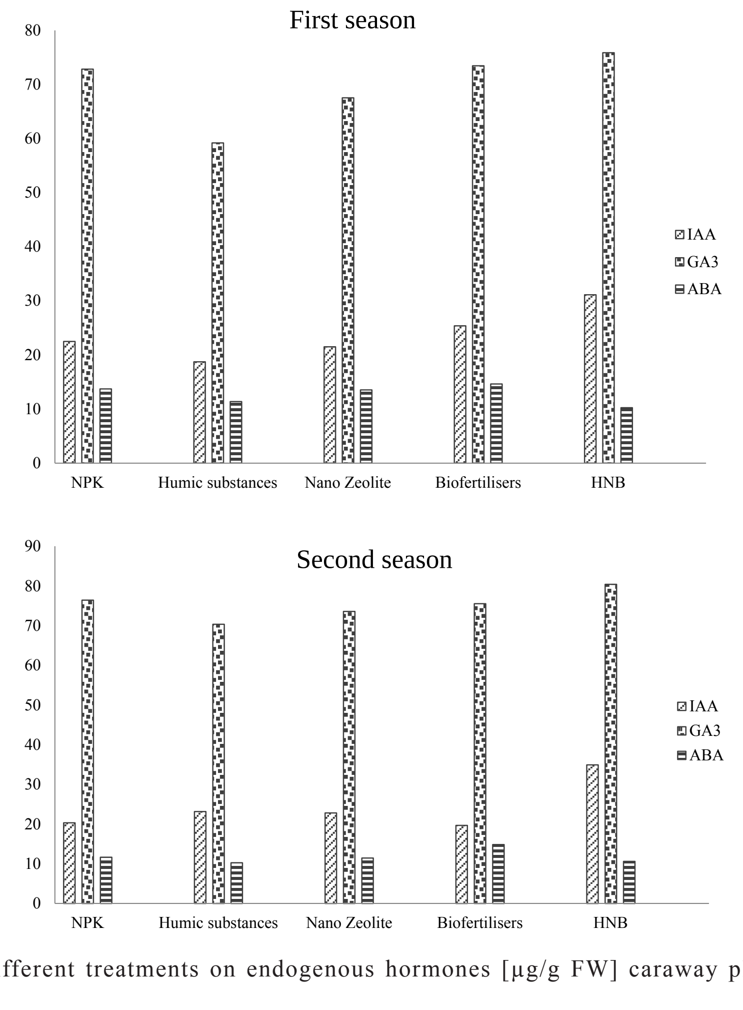

Figure 4. Effect of different treatments on endogenous hormones [μg/g FW] caraway plant during two seasons 2013 and 2014

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In this connection, data in Table 9 show that, in both first and second seasons, total fatty acids significantly increased (80% and 87%, respectively) as a result of HNB treatment compared with control. Also it was noteworthy that mixed HNB treatment significantly had the upper hand in total fatty acid content compared to all other treatments during both seasons. On the contrary, the lowest total fatty acids resulted from the application of biofertilisers alone either in first or second season in comparison to control and all other treatments. Our previous data are in conformity with the results obtained by Sinkovič and Žnidarčič (2016) on Radicchio plant. The different influences of humic substances, nano zeolite and biofertilisers mixed (HNB) on total phenolics, total flavonoids and total fatty acids and physiological activities could be due to the power of releasing and chelating available nutrients for plant uptake along

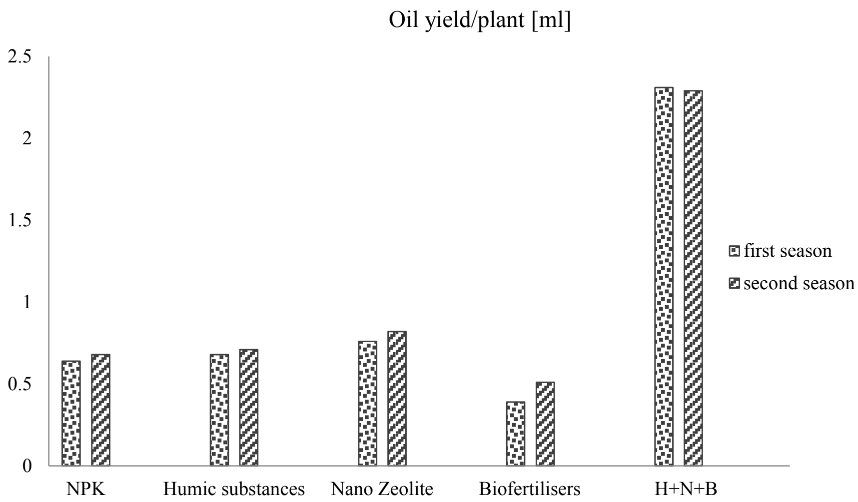

Figure 5. Effect of different treatments on essential oil in the herb of caraway plant as oil yield per plant during two seasons 2013 and 2014

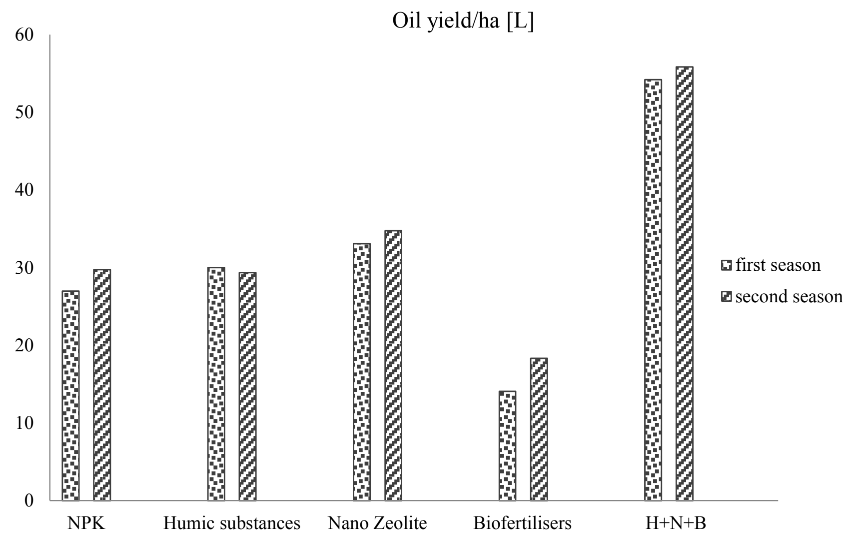

Figure 6. Effect of different treatments on essential oil in the herb of caraway plant as oil yield per hectare during two seasons 2013 and 2014

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with their impacts on physiochemical and biological properties, which also enable efficient use of biofertilisers and organic fertilisers. Generally seed composition constituents were reported to be genetically controlled (Simpson & Wilcox 1983). However, seed composition has also shown to be affected by environment (Dardanelli *et al.* 2006), genotype, maturity, diseases, planting date (Bellaloui *et al*. 2011), temperature (Dardanelli *et al*. 2006), drought (Bellaloui *et al.* 2012), nutrients in soil and seed (Kravchenko & Bullock 2002).

Effect on phytohormones

The results of hormonal analysis as evident from levels of indole-3-acetic acid (IAA), gibberellic acid (GA_3) and abscisic acid (ABA) in caraway herb are affected by different treatments, as displayed in Figure 4. The increase of growth parameter was associated with elevated levels of growth promoters (IAA, GA_3) and low level of ABA. Hence it was found that mixed (HNB) treatment significantly gave the highest content of IAA, particularly in comparison to control (72% and 58%, respectively) in both seasons. As for GA_3, it was clear that mixed (HNB) treatment resulted in an increase over control in GA_3 content, although this increase was insignificant but, in the second season (HNB), the treatment resulted in significant increases compared with all other treatments and mainly (95%) over control. Concerning ABA content, in the first season, both humic substances and mixed HNB treatments significantly resulted in the lowest ABA content (83% and 75%, respectively) compared with control plants. In contrast, in the second season, it was unequivocal that the highest ABA content significantly resulted from biofertiliser treatment over control (78%) and in general over all other treatments. The above-mentioned results are in consonance with those obtained by Soliman and Mahmoud (2013) on *Adansonia digitata* L.

The stimulated effects of mixed treatment (HNB) may be attributed to the production of phytohormones from biofertilisers as mentioned by Mehnaz *et al*. (2001) who reported that IAA produced by rhizobacteria as plant growth promoting Rhizobacteria PGPR is believed to increase root growth and root length, resulting in better nutrient uptake from soil. Also Marek and Skorupska (2001) provided evidence that four different forms of GA are produced by many *Bacillus* sp., which effectively reverses a chemical-induced inhibition of stem growth, working together in the presence of organic

T a b l e 10

Effect of different treatments on percentage essential oil constitutes of caraway plant during two seasons 2013 and 2014

Treatment	NPK		Humic substances (H)		Nano Zeolite (N)		Biofertilisers (B)		HNB	
Components [%]	F	S	F	S	F	S	F	S	F	S
α-pinene	0.244	0.211	0.321	0.305	0.421	0.436	0.295	0.358	0.441	0.506
Camphene	0.364	0.297	0.391	0.331	0.305	0.479	0.405	0.412	0.351	0.401
Carvone	80.38	78.11	75.08	73.56	82.38	80.09	70.48	75.95	77.62	84.35
β-Pinene	0.38	0.33	0.310	0.36	0.28	0.35	0.26	0.24	0.34	0.32
Limonene	7.97	8.52	10.29	10.06	9.56	9.24	15.41	11.58	8.91	6.73
Linalool	0.01	0.01	0.020	0.04	0.03	0.02	0.04	0.05	0.03	0.04
β-Selinene	0.50	0.51	0.590	0.65	0.54	0.53	0.6	0.58	0.56	0.55
α-Selinene	0.38	0.36	0.250	0.23	0.24	0.21	0.27	0.23	0.26	0.25
α-Terpineol	0.01	0.01	0.020	0.02	0.03	0.01	0.03	0.02	0.00	0.00
Unknown	0.31	0.29	0.410	0.39	0.52	0.49	0.25	0.23	0.58	0.61
Unknown	0.03	0.05	0.060	0.08	0.07	0.06	0.04	0.05	0.09	0.08
Unknown	0.00	0.00	0.000	0.02	0.01	0.01	0.00	0.00	0.02	0.03

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matter present in humic substances, which is considered a source of hormone-like substances.

Effect on essential oil productivity

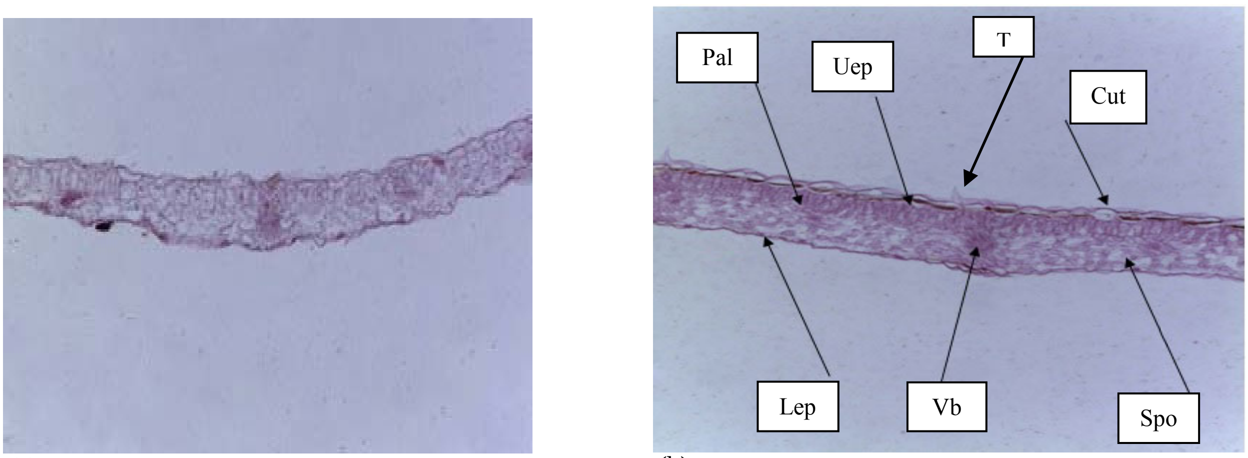
The data of essential oil yield as yellow colour and aromatic spicy odour are shown in Figures 5 and 6. The combination of humic substances, nano-zeolite-loaded nitrogen and biofertilisers (HNB) treatment significantly resulted in rising essential oil yield either per plant or per hectare in comparison with control since they recorded 28% and 50%, respectively for the first season and 30% and 53%, respectively for the second one. On the other hand, the lowest significant result of essential oil yield was recorded with biofertiliser treatment alone compared with all other treatments and particularly control plants (61% and 52%) and (75% and 58%) for both seasons respectively.

The increase in essential oil productivity with mixed treatment (HNB) over all other treatments including control treatment could be explained on the basis of available elements, vitamins, hormone-like substances, amino acids and sugars, which have a significant effect on the physiological or biochemical processes within the plant (the case of luxury of metabolism) and consequently boost essential oil yield. These results coincide with those obtained by Mahmoud (2012) on yarrow plant *(Achillea millefolium*) who stated that application of compost mixed with zeolite, humic acids and biofertilisers increased oil yield.

Effect on essential oil constitutes

The data for essential oil components are presented in Table 10. It clearly=indicates that mixed treatment (HNB), particularly in the second season, resulted in the highest percentage of carvone (84.35%) and α-pinene (0.506 %) over all treatments including control plants. While the lowest percentage of carvone (70.48%) was obtained from biofertilisers, but unexpected results were obtained from biofertiliser treatment alone since it gave the highest percentage of limonene (15.41%) compared to either control or all other treatments.

With components, we found that the highest percentage of camphene (0.479%) resulted in the second season as a result of nano-zeolite treatment, while humic substances alone gave the highest percentage of β-selinene (0.65%) in the second season. But the highest percentage of α-selinene (0.38% and 0. 36%, respectively) was obtained from control plants in both seasons. The aforesaid results are in concurrence with those of Sedláková *et al.* (2003) who mentioned that carvone and limonene account for the main proportion, that is, about 95% of caraway essential oil components. However, Jalali-Heravi *et al.* (2007) reported that the major constituents in caraway seeds were terpinene (24.40%), 2-methyl-3-phenylpropanal (13.20%) and 2,4(10)-thujadien (14.02%).



(a) (b)

Figure 7. Leaves: (Pal) palisade; (Uep) upper epidermis; (T) trichomes, (Cut) cuticle; (Lep) lower epidermis; (Vb) vascular bundle; (Spo) spongy tissue

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Effect of different treatments on anatomical structure (leaves and seeds) of caraway plant

Effect on leaves

Generally caraway leaves are dorsiventral where the palisade tissue is located on the adaxial side of the blade and the spongy tissue on the abaxial one. Compact arrangement of epidermal cells and the presence of cuticle layer, stomata and trichomes are the main features of epidermis.

As revealed in Figure 7a and 7b, the epidermis cells of the control (Figure 7a) were similar in shape and size, while the epidermal cells of the HNB-treated leaves (Figure 7b) became larger in size and reached a maximum size. Additionally, it was remarkable that the thickness of mesophyll tissue, which specializes in photosynthesis and contains chloroplasts in palisade, and size of spongy parenchyma tissue, air spaces and trichomes after HNB treatment became larger compared to control. Moreover, vascular bundles of the principal veins, which are accompanied from above and below by parenchyma tissue in which a secretory canal lies above and below the vein were larger and greater in HNB treatment leaves compared to control leaves. All these findings were clear based on the chlorophyll concentration, which was higher in HNB treatment leaves compared to control.

This confirmed the profitable effect of mixed treatment of HNB on the anatomical structure of caraway leaves. In this sense, these data were similar to those obtained by El-Feky *et al.* (2013) on basil who found that foliar application of nano Fe_3O_4 improved anatomical structure of leaves.

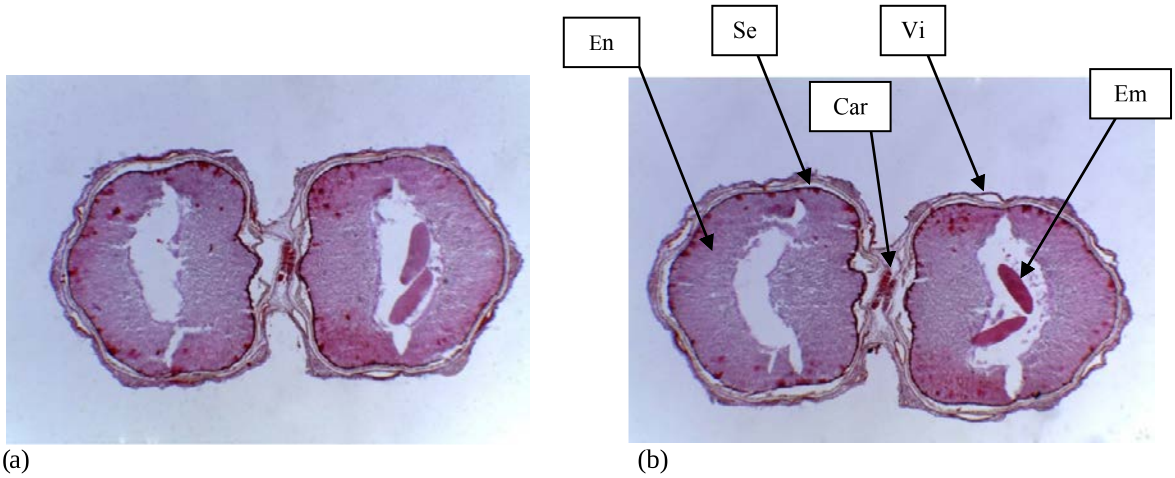

Figure 8. Seed: (En) endosperm; (Se) seed coat; (Car) carpophores; (Vi) vittae, (Em) embryo

T a b l e 11

Economical evaluation of different treatments

Treatments	Total yield [L/ha]	Total production cost [US dollar/ha]	Gross income [US dollar/ha]	Profitable return (PR) [US dollar/ha]	PR over control [US dollar/ha]	PR% increase [US dollar/ha]	BCR	IF
NPK as control	29.75	315	17,850	17,535	–	–	–	56.6
Humic substances	29.36	241	17,616	17,375	160	1.0	0.66	73.0
Nano-zeolite	34.77	240	20,862	20,622	3,087	17.6	12.8	87.0
Biofertilisers	18.34	238	11,004	10,766	6,769	38.6	28.4	46.2
HNB	55.85	304	33,510	33,206	15,671	89.3	51.5	110.0

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Effect on seeds

Caraway fruit is a schizocarp (cremocarp) consisting of two mericarps. Each mericarp has two (sometimes three) large vittae on the commissural surface and about 20 to 30 small vittae on the dorsal surface. Between the vittae, the pericarp is ridged externally and a vascular strand is contained in each of these ridges.

The seed contains a small dicotyledonous embryo at the apical end, embedded in an abundant oily endosperm (Parry 1945). It was found that vittae size and numbers in fruits were higher in HNB treatment (Figure 8b) in comparison with control (Figure 8a). These findings confirm the beneficial role of the mix of humic substances, nano-zeolite-loaded nitrogen and biofertilisers on anatomical structure and oil yield as mentioned before. The obtained results are in agreement with Nassar *et al*. (2001) on anise.

Economical evaluation

Data in Table 11 undoubtedly explained that chemical fertilisers resulted in a maximum production cost of US$315/hectare. On the other hand, the minimum production cost of biofertiliser treatment was US$238/hectare. Meanwhile, a combination of humic substances, nano-zeolite-loaded nitrogen and biofertilisers (HNB) gave the maximum gross income of $33,510/hectare, profitable return (PR) of $33,206/hectare and benefit–cost ratio (BCR) 51.5. On the subject of investment factor (IF), the highest IF value (110) was recorded in the combination treatment (HNB). Generally, data analysis revealed that the cost of commercial production of caraway plant (*Carum carvi* L.) varied among treatments. In the interim, all treatments realized reasonable profitability since their IF is more than 3 (FAO 2000).

CONCLUSIONS

On the basis of previously mentioned data, which lead to the conclusion that application of humic substances, natural nano-zeolite-loaded nitrogen and biofertilisers mixture (HNB) gave good results on either plant under investigation caraway (*Carum carvi* L.) and environment that presents with higher growth characteristics and chemical composition in comparison with results derived from chemical fertilisers NPK considering both quality and quantity. Meanwhile it was clear that economic evaluation showed a beneficial profitable return, which increases the income of farmers and producers. This influential mixture (HNB), which is inexpensive, could replace the chemical fertilisers used for plant growth enhancement. In a nutshell, application of organic fertilisers, natural soil amendments and near to the ground quantity of chemical fertilisers (nano form) could bring good economic benefits and multiple advantages for consumers, producers, farmers and our ecosystem.

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Received: February 10, 2017

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