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ARTICLE

The disappearance of three archaeophyte species in Hungary can be explained by their marked sensitivity to fertilizers

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ABSTRACT The archaeophytes are the component of segetal plant communities, and ensure biodiversity in arable field margins. Unfortunately, the number of these species decreased in the last decades because of the changing structure of agricultural production and increasing chemical application. In this study, the fertilizer sensitivity of three archaeophyte species was characterized using germination test, outdoor observation and proline content measurement. *Papaver rhoeas* had the most favourable germination parameters with promptness indices (1.5-14.0) and germination rates (0.39-0.81) decreasing with the concentration of fertilizer. On the other hand, mean germination time (7.32-10.03 days) decreased with elevated fertilizer concentration. *Consolida regalis* was characterized by the weakest development in laboratory. Slow early development was detected in case of *Cyanus segetum* (promptness index: 0.25-1.75; mean germination time: 12-13 days). *Co. regalis* responded to fertilization with higher blooming intensity. The proline accumulation indicated pronounced salt sensitivity of *Cy. segetum* (0.49-0.54 mg/100 mg), which could be one reason of the disappearance of this species from fields under cultivation. Our results suggest that at least *Co. regalis* and *Cy. segetum* are highly sensitive to mineral fertilizers and hence natural protection techniques for example arable weed margins must be widely used to block the disappearance of those species.

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Introduction

Archaeophytes are those species, which originally cannot be found in the flora of European area, but naturalized between Neolithic and the end of middle ages due to human influence. Survival of segetal species depends on the nature transformer activity of human beings. These are usually annual, ephemeral, pioneer species with high adaptation ability to arable field cereals (Preston et al. 2004; Williamson et al. 2008).

Many rare, endangered or extinct species can be found in the list of European archaeophytes since the development of agriculture poses a serious risk on their life conditions (Richner 2014). Fertilization has direct and indirect effects on arable field plants including archaeophytes. One of the most important challenges is the changing quantity and method of fertilization (Albrecht 1995; Šarić et al. 2011). Its direct effect is based on different nutrient uptake mechanisms: the bred grain cultivars are able to uptake quickly the mineral fertilizers, while segetal weed species prefer organic nutrients (Pyšek and Lepš 1991; Kleijn and van der Voort 1997). In addition, as an indirect effect, the increasing use of fertilizers results in the loss of plant diversity due to increased competition ability of crops (Pyšek and Lepš 1991; Meyer et al. 2013). Therefore, poor light competitors (e.g., *Co. regalis*) are disappearing and the stronger nitrophilous neophytes are spreading (Svensson and Wigren 1986; Albrecht 1995). Competitive species will have higher size due to limited light resource but their total biomass weight decrease (Kleijn and van der Voort 1997). The frequency of small and subordinate species decreases, and the number of species reduce over time (Schmitz et al. 2014). It depends on species whether direct or indirect effects will predominate (Pyšek and Lepš 1991).

The perennials of arable field border community uptake the remaining fertilizer that cannot be utilized by crops. This effect leads to stronger competition against annual archaeophytes, and structure changes (Stoate et al. 2001). Decrease of archaeophytes was observed in Germany between 1953 and 2000 (Baessler and Klotz 2006). The applied potassium

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and nitrogen fertilizers caused significant reduction of anthropochorous species while the number of invasive weeds increased. The number of *Co. regalis* and *Papaver rhoeas* plants showed significant reduction between 1957 and 1979. The habitat-specific species (such as *Co. regalis*) were forced and the weed diversity decreased due to fertilizer application (Baessler and Klotz 2006).

The loss of archaeophytes was also observed in Denmark when comparing the 1967-1970 and 1987-1989 periods (Andreasen et al. 1996). The frequency of segetal species including *Cy. segetum*, *Co. regalis* and *P. rhoeas* also decreased in France from 1970s to 2000s (Fried et al. 2009).

Wassmuth et al. (2008) stated that *Cy. segetum* had higher biomass production in fertilized soil. This is a compensation mechanism caused by low number of individuals. However, this low number may cause population extinction (Bischoff and Mahn 2000). In the studies conducted by Mohammaddoust et al. (2008) the soil covering of *Cy. segetum* increased because of phosphorus application, while the nitrogen application had positive effect on dry biomass production. The germination rate of seeds decreased due to nitrogen application in contrast to phosphorus which improved germination. Optimal phosphorus level has positive effects on the appearance of species and improves soil fertility. On the other hand, potassium had no positive effects (Andreasen and Skovgaard 2009), its high level caused frequency reduction (Andreasen et al. 1991).

The aim of our investigation was to determine fertilizer sensitivity of three archaeophyte species (*Cy. segetum*, *Co. regalis,* and *P. rhoeas*) using laboratory examination and outdoor observations. The chosen species are easy to apply as indicator plants of archaeophytes because they are common, easy to determine at the early developmental stage, flexible but sensitive for environmental changes (Büchs 2003; Bellanger et al. 2012). The original (semi-natural) habitat of archaeophytes is the cereal fields and the border of arable crops. For the successful *in situ* protection of these species is necessary to identify the reasons of their disappearance. The problem of over-fertilization has already been known, but this observation often lacks statistical support (Wörz and Thiv 2015). Therefore, we wanted to identify and statistically confirm the destructive mechanisms of fertilizer application and give recommendation to reduce the chemical damage of segetal vegetation.

Materials and Methods

Ex situ germination test

Four replications of 25 seeds from each of the three species (*Cyanus segetum* Hill, *Consolida regalis* Gray, *Papaver rhoeas* L.) were used for germination test. *Cyanus* and *Consolida* seeds were placed in Petri dishes between two filter papers, while *P. rhoeas* seeds were placed on top of the filter papers. Applied fertilizer (N:P:K 6:12:24 + 8S - DC 42, TIMAC AGRO Düngemittelproduktions und Handels GmbH; mixed, spherical granulated form) were powdered and dissolved in distilled water. Treatments were carried out by adding 10 ml of 0.5; 1; 2 and 3 g/l the fertilizer solution. Control seeds were watered only with 10 ml distilled water. Germination tests were conducted under controlled conditions (10 hours dark period in 10 °C and 14 hours light period in 20 °C, 27.3 µmol/m^{2}s luminous photosynthetic photon flux). During the 14-20 days long examination period, germinated seeds containing two millimetres long radicles were removed. This was taken as a criterion of germination.

The following parameters were calculated:

Promptness index (PI): $PI = nd_2 \times (1.00) + nd_4 \times (0.75) + nd_6 \times (0.50) + nd_8 \times (0.25)$, where nd_2 nd_4 nd_6 nd_8 showed the number of seeds germinated on the 2nd 4th 6th and 8th day, respectively (Zafar et al. 2015),

Germination stress tolerance index (GSTI) expressed in %: GSTI = (PI of stressed seeds /PI of control seeds) × 100 (George 1967), where stressed seeds indicate fertilizer treated seeds,

Mean germination time (MGT) expressed in days: $MGT = (\sum ni \times ti)/\sum n$, where ni showed the number of seeds germinated on ti time, ti showed the number of days from start, n showed the number of germinated seeds at the end of the test,

Germination speed (GS): GS = 1/MGT × 100 (Hartmann et al. 1997), and

Germination rate (GR) = the number of germinated seeds until the end of the test / total number of planted seeds.

Outdoor experiments

The experiment took place in the Experimental and Research Farm (Faculty of Horticultural Science, Budapest, Hungary) in Soroksár (47° 24' 1" N latitude, 19° 8' 37" E longitude and 115 m altitude). The soil was sandy and poor in humus, strongly infected by perennial weeds. The soil was rotated with cultivator on 23th September 2014. A total of 20 g/m^2 fertilizer (N:P:K 6:12:24 + 8S) was dispersed and rotated into the soil in 50% of the whole area. Plants were examined in separate parcels containing only the studied species; the size of parcels was 1.5×1.5 m. Two hundred seeds of all three species were sown three times (on 8th, 14th and 21st of October). Seeds were covered with 2 cm soil and irrigated with 10 l of water. Size, phenological development and decoration value were determined weekly after germination from 21st of October to 14th of July (except the winter period from 24th of November to 14th of April).

Size parameters (horizontal sizes - width and length,

Table 1. Changes in the germination factors of two archaeophyte species exposed to different fertilizer (N:P:K 6:12:24 + 8S) treatments.

Treatment	*PI*	*GSTI* (%)	*MGT* (day)	GS	GR
C. segetum					
control	1.75^{a} ± 0.52	--	12.06^{a} ± 21.82	8.29	0.52^{a} ± 2.71
0.5 g/l	0.75^{a} ± 0.38	42.86	12.45^{a} ± 33.42	8.03	0.44^{ab} ± 1.63
1 g/l	1.50^{a} ± 0.48	85.71	12.26^{a} ± 8.71	8.16	0.39^{ab} ± 4.79
2 g/l	0.50^{a} ± 0.25	28.57	12.74^{a} ± 16.18	7.85	0.27^{bc} ± 1.26
3 g/l	0.25^{a} ± 0.13	14.29	13.00^{b} ± 8.05	7.69	0.09^{c} ± 1.50
P. rhoeas					
control	14.00^{a} ± 5.20	--	7.32^{a} ± 62.64	13.66	0.81^{a} ± 2.50
0.5 g/l	10.00^{a} ± 2.89	71.43	8.30^{a} ± 62.37	12.05	0.72^{a} ± 1.71
1 g/l	3.00^{a} ± 1.19	21.43	8.90^{a} ± 67.45	11.23	0.61^{ab} ± 4.44
2 g/l	2.25^{a} ± 1.13	16.07	9.61^{a} ± 43.90	10.41	0.51^{b} ± 1.71
3 g/l	1.50^{a} ± 0.75	10.71	10.03^{a} ± 45.27	9.97	0.39^{b} ± 1.71

GR: germination rate; GS: germination speed; GSTI: germination stress tolerance index; MGT: germination time; PI: promptness index. For each species, different letters within the same column indicate significantly different values at $p \leq 0.05$ according to the Tukey test. GSTI and GS data are derived from PI and MGT, respectively, and hence statistically not analysed.

vertical size - height) were measured by tape measure. Ranking was used to determine the phenological statement and ornamental value. The values represented the following categories:

5. Very decorative, full blooming, healthy wildflower.

4. Moderately decorative, begin or finish blooming, healthy wildflower.

3. Slightly decorative, decorated only by vegetative parts. Healthy plant.

2. No decoration value because of phenological stage, or some kind of stress (*e.g.*, sunshine, wind, insects or pathogen).

1. Plants could not be found in the area.

Proline content determination

The proline content was measured according to Ábrahám et al. (2010). Leaves were collected from plants analysed in the outdoor experiment on 24th November 2014 and on 8th June 2015. Samples were stored at -20 °C until use. The samples (approx. 100 mg fresh weight) were ground with 3% sulfosalicylic acid (5 µl/mg fresh weight), and were centrifuged (15. 689 g, 5 min, 25 °C) using a 5418 R (Eppendorf, Hamburg, Germany). A total of 100 µl from the supernatant was mixed with the reaction mixture (100 µl 3% sulfosalicylic acid, 200 µl glacial acetic acid and 200 µl acidic ninhydrin). After 60 minutes of incubation at 96 °C, the reaction was terminated on ice. Then, 1 ml toluene was added to the reaction mixture and vortexed for 20 seconds. After the separation (5 minutes) the chromophore was removed into a fresh tube. Finally, the absorbance was measured at 520 nm using a GeneSys VIS-10 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The proline content was calculated on fresh weight basis using a standard concentration curve.

Statistical evaluation

Data presented for each species represent the mean values determined from 4 independent measurements. After tested for normal distribution and equality of variances, 1-way analysis of variance (ANOVA) was carried out and significant differences were calculated according to Tukey test, with $P \leq 0.05$ being considered significant in all analyses. Statistical analyses were carried out using the SPSS 20 software (IBM, New York, US).

Results and Discussion

In vitro germination test

Changes in the germination promptness index indicate that *P. rhoeas* is characterized by rapid development (Saeb et al. 2013), while the germination speed of *Cy. segetum* is slower than crop cultivars, but similar to the value of other *Centaurea* species (Turkoglu et al. 2009). This developmental response of archaeophytes to fertilizer effects might be in association with the disappearing of such plants from arable lands in Europe (Albrecht 1995; Svensson and Wigren 1986). The slowest development was shown in case of *Co. regalis*. This species has not germinated during the test period of 14-20 days. The stress tolerance index of examined species was low and decreased rapidly with increasing fertilizer concentra-

Table 2. Pairwise comparison of flower number depending on fertilizer application for three archaeophyte species.

Species	Treatment	*N*	Average flower number in parcel
P. rhoeas	control	33	0.705361[a]
P. rhoeas	fertilized	33	0.759147[a]
Cy. segetum	control	33	1.734274[a]
Cy. segetum	fertilized	33	2.183164[ab]
Co. regalis	control	33	3.529449[b]
Co. regalis	fertilized	33	5.162685[c]

N: number of examined plants. Different letters indicate significantly different values at $p \leq 0.05$ according to the Tukey test.

Table 3. Pairwise comparison of proline content depending on fertilizer treatment by three archaeophyte species – samples collected in November (mg/100 mg fresh weight).

Species	Treatment	*N*	Average proline content
P. rhoeas	control	5	0.47403020[a]
P. rhoeas	fertilized	5	0.47569120[a]
Co. regalis	control	5	0.47450840[a]
Co. regalis	fertilized	5	0.47756320[a]
Cy. segetum	control	5	0.48867940[a]
Cy. segetum	fertilized	5	0.54261496[b]

N: number of examined samples. Different letters indicate significantly different values at $p \leq 0.05$ according to the Tukey test.

tion (Table 1). GSTI values were 10-15% at 3 g/l fertilizer concentration that are considerably lower values than those of wheat cultivars (70-90%). For example, the salt tolerance index of three barley cultivars was 67.07-91.24% when 5 g/l NaCl was added into the medium (Goumi et al. 2014). The same index of sunflower cultivars ranged between 80% and 90% due to 5 g PEG added in 100 ml distilled water, but one cultivar had just 50% (Ahmad et al. 2009).

Comparing mean germination time (MGT), more pronounced sensitivity to 3 g/l fertilizer concentration was detected for *Cy. segetum*, as for the other two species ($SL < 0.05$). The germination rate of *P. rhoeas* was decreased by 3 g/l fertilizer solution to 39%, while *Cy. segetum* showed only 9% germination rate due to 3 g/l fertilizer treatment ($SL < 0.05$).

Outdoor experiments

Differences were not shown in phenological development and plant size between treated and control parcels. Examining the covering shares a significant difference occurred for *Cy. segetum* ($SL < 0.001$): 39.27% in treated parcels, while 27.79% in control parcels. Significantly higher number of flowers was shown by *Co. regalis* (Tukey test $SL < 0.05$) when comparing fertilized and control parcels (Table 2).

The top of *P. rhoeas* blooming time (ornamental value 5) occurred in the third decade of June. Main blooming time of *Co. regalis* continued from the end of May until first decade of June. The number of blooming plants was 60-100% of all germinated seedlings in this period. Minor second decoration period could be observed in the last decade of June in treated parcels. *Cy. segetum* had the highest ornamental value (5) also from the end of May until the beginning of June. The blooming period of *Cy. segetum* was longer than those of *Co. regalis* and *P. rhoeas* blooming time. The number of flowering *Cy. segetum* individuals decreased continuously while the number of flower heads of the flowering individuals increased until the end of June. The decrease of individuals (Baessler and Klotz 2006; Fried et al. 2009) was not observed for *Consolida* and *Papaver* because of the short experimental period. However, positive effects of the fertilizer could be realized in the increased number of *Cyanus* plants (Wassmuth et al. 2008). Many studies confirmed that both herbaceous crops and trees become progressively more tolerant as the plants grow older with a salt-sensitive early-vegetative growth (emergence) stage and a less sensitive stage during flowering (Läuchli and Grattan 2007; Niinemets 2010). Regardless of the more abundant flowering of archeophytes, their number will be lowered by fertilizer induced stress effects in the most sensitive seedling stage, resulting in a definite loss of established plants. The treated *Cyanus* and *Consolida* plants were slightly and non-significantly higher than control that is associated with the bigger biomass weight due to fertilizer application (Bischoff and Mahn 2000).

Proline content determination

A number of environmental stresses (*e.g.*, high salinity) cause proline accumulation (Ahmad et al. 1981; Kubala et al. 2015). This physiological response is hypothesized to be a consequence of the osmoprotective and osmolyte role of proline (Fougére et al. 1991). It is able to reduce the damage of oxidative stress and protect protein structures (Samuel et al. 2000). The determination of proline content may provide useful information on the actual physiological status and stress tolerance of plants (Ábrahám et al. 2010).

A significant difference was shown in samples of *Cy. segetum* (Table 3) in November. Proline content of fertilized stand was higher in comparison with the control ($SL < 0.01$). This difference was not observed in June (0.4537 mg/100 mg in treated parcels, 0.4545 mg/100 mg in control parcels). Such a difference was not shown by the other two species, but the values were similar. The effect of salt stress was decreased in comparison with two collecting times (paired samples test: $t = 4.504$, $SL < 0.001$). The proline content of the three examined species was higher than bred grain cultivars (Pyšek and Lepš 1991; Kleijn and van der Voort 1997). Ten times lower

fresh weight proline concentration (0.5 mg/g) was measured in tomato after daily complex nutrient application (Claussen 2005). Only treatments applied eleven times caused a similar value (3 mg/g) to our results (4.7-5.4 mg/g).

Conclusions

The used parameters and analyses were useful to explain the disappearance of three archaeophyte species in Hungary due to their marked sensitivity to fertilizers.

Co. regalis had the weakest germination factors in laboratory tests. Germination rate of *P. rhoeas* and *Cy. segetum* was significantly decreased and rapid declining in germination stress tolerance index was observed due to fertilizer application in case of *P. rhoeas*. Furthermore, the slow early development may give an explanation for disappearing of archaeophytes (especially *Co. regalis* and partly *Cy. segetum*). Main blooming time continued from the end of May to the first decade of June; however, a significant second decoration period occurred at the end of June in fertilizer applied parcels. Nevertheless, the soil covering of *Co. regalis* was the least (8-10%). The applied fertilizer did not have any significant effect neither on blooming intensity nor on blooming period. Analysing proline concentration, higher salt sensitivity was determined for *Cy. segetum* seedlings, especially at the early stage of development. This sensitivity lowers the surviving and spreading opportunities of this species among crops. This stress factor can be prolonged under non-irrigated conditions, which is confirmed by the small variations in proline contents of samples collected from November to June.

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