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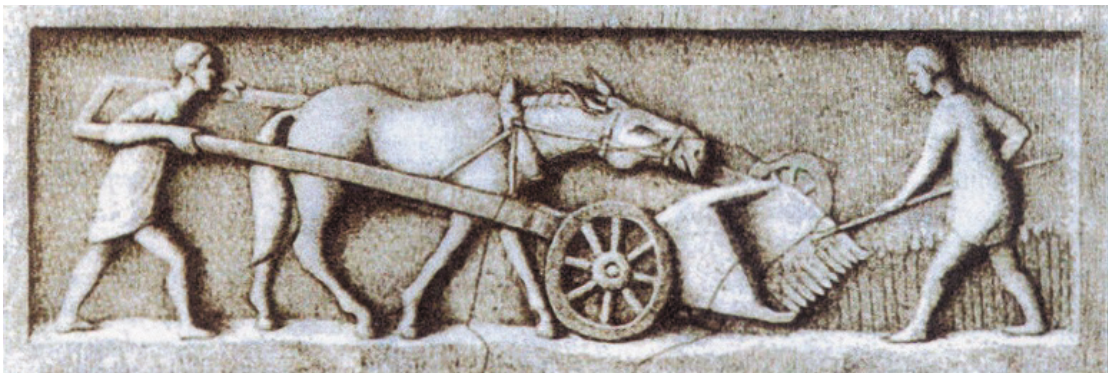

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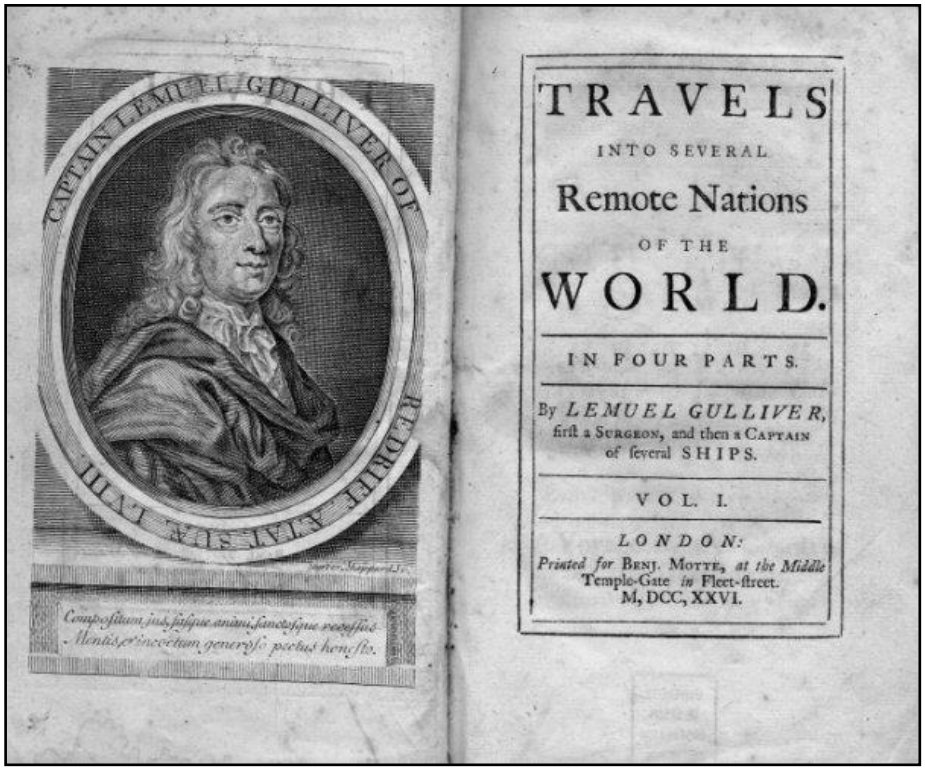

The first edition of Swift's Gulliver's Travels published by Benjamin Motte in 1726

Scientific publication has a twofold mission. Once it is the outcome of any scientific research disseminating the results achieved. On the other hand it may provide a basis for scientific qualification. If the yield of the scientific activity is manifested in the means and the way of publication, this yield has to be measured, evaluated and marketed. The higher is the rank of the journal it is published in, and the broader is the sphere of dissemination, the higher will be the reputation of the scientist. Since the beginning of the history of the human race grading or qualification has been a most essential institution of any society. In favour of a more successful ranking, criteria have always been improved. Also, as a result of this perpetual improvement of the qualification methodology, systems became more and more sophisticated.

Jonathan Swift the dean of St. Patrick's in Dublin wrote his evergreen social pamphlet titled "Gulliver's travels into several nations of the World". In a systematic assessment he criticized most fields of failures and deviations within the human society. He was presenting a detailed description of the qualification process done by the emperor of Lilliput, an imaginary country which is one of the scenes of the novel.

"There is likewise another diversion, which is only shown before the emperor and empress, and first minister, upon particular occasions. The emperor lays on the table three fine silken threads of six inches long; one is blue, the other red, and the third green. These threads are proposed as prizes for those persons whom the emperor has a mind to distinguish by a peculiar

mark of his favour. The ceremony is performed in his majesty's great chamber of state, where the candidates are to undergo a trial of dexterity very different from the former, and such as I have not observed the least resemblance of in any other country of the new or old world. The emperor holds a stick in his hands, both ends parallel to the horizon, while the candidates advancing, one by one, sometimes leap over the stick, sometimes creep under it, backward and forward, several times, according as the stick is advanced or depressed. Sometimes the emperor holds one end of the stick, and his first minister the other; sometimes the minister has it entirely to himself. Whoever performs his part with most agility, and holds out the longest in leaping and creeping, is rewarded with the blue-coloured silk; the red is given to the next, and the green to the third, which they all wear girt twice round about the middle; and you see few great persons about this court who are not adorned with one of these girdles."

What is the message of this short description? Maybe two postulates can be derived from that. One is the humorous introduction to the qualification system that depends on the ever changing requirements of the process. The other is a really tragic conclusion. It suggests that the qualification process serves the compliance to the criteria dictated by the person in charge rather than providing a chance to evaluate the person's contribution to the society.

This is the point where we may return to the field of scientific publication. This journal, Columella, provides a forum for scientific publications in the field of agricultural and environmental sciences. Columella is a newborn periodical having yet no considerable records in the world of scientific journals. However it is the journal of one of the most ambitious agricultural faculties of Hungary with a mission to disseminate novel research results in favour of creating a better world.

Thanks to the authors of the present issue, and also thanks to the readers who may read, use and broadcast the scientific information compiled. Hope to welcome them as future authors as well.

Katalin Posta editor-in-chief

Márton Jolánkai guest editor

NON-DESTRUCTIVE AND DESTRUCTIVE MEASUREMENTS' CHLOROPHYLL CONTENT IN SUNFLOWER AND MAIZE PLANTS UPTAKEN DIFFERENT CHEMICAL FORMS OF SELENIUM

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Abstract: Selenium (Se) is an example of an essential element becoming more and more insufficient in food crops as a result of intensive plant production in many countries. Se is an essential biological trace element. Accordingly, controlling the Se uptake and metabolism in plants will be important to reaching to adequate methods for bio fortification. Furthermore, chlorophyll content (chl) is one of the most important physiological parameters which is related to plant photosynthesis and is usually used to predict plant potential. In this regard, during and end of the experiment in hydroponic culture, chlorophyll content of sunflower and maize plants' leaves treated different concentrations of Se in two forms of sodium selenite (Se^{IV}) and sodium selenate (Se^{VI}) was measured in two methods of non-destructive and destructive ones to clarify the relationship between Se and chl. Both measurements were done on old and young leaves and results showed that Relative Chlorophyll Content (RCC) and Chl a and b were not impaired at the end of experiment from Se exposure up to 3 mg L^{-1} of both Se^{IV} and Se^{VI} in two plants. Although high doses of sodium selenite caused toxicity in sunflower treatments.

Keywords: Sodium selenite/Sodium selenate, Relative chlorophyll content, Chlorophyll a and b content, Sunflower, Maize

1. Introduction

Selenium is one of the elements playing a most important role in human and animal health and is essential to all other organisms including bacteria and algae.

Most plants contain rather low foliar Se, around 25 μg kg^{-1} and rarely exceed 100 μg kg^{-1}. However, some plants exhibit a great capability to accumulate Se and they may concentrate Se to extremely high levels over 1000 mg kg^{-1} that may be toxic to humans and animals. Although Se is not an essential element for plants, with some exceptions, it is being added to soil to ensure that both food and feed products contain adequate amounts for the dietary needs. It should be emphasized that the margin of safety of Se concentrations is rather narrow (Kabata-Pendias 2011).

The chemical properties of Se are relatively similar to those of sulphur. Its speciation is highly dependent on the pH and Eh (Elrashidi et al., 1987; Masscheleyn et al., 1990) inducing a complex behaviour and a large variety of selenium compounds in the environment. Se has four stable redox states: selenide (Se (-II)), elemental selenium (Se (0)), selenite (Se (IV)) and selenate (Se (VI)) (Fernández-Martínez and Charlet, 2009; Seby et al., 1998).

As an essential trace mineral, Se is indispensable for cells to function properly. Two inorganic species, selenite (Se^{IV}) and selenate (Se^{VI}) are important in the bio geological and biochemical cycle of Se, but they exhibit different biochemical properties and their energy consumption during uptake and metabolism are different (Shen et al., 1997; Weiller et al., 2004).

In addition, chlorophyll is a frequent organic chemical component because it is naturally present in plants, giving their specific colouration (Withnallas et al., 2003) as a photosynthetic pigment and an essential

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component of the plant photosystem. Leaf chlorophyll content affects photosynthetic ability and thus is one of the most important physiological traits affecting plants (Czyczyło-Mysza et al., 2013; Teng et al., 2004; Wang et al., 2008) so that content of photosynthetic pigments is highly correlated with the nutrition condition (Gitelson et al., 2003) and as an indicator for growth and survival of plants (Foyer et al., 1982; Peng and Gitelson, 2012). Despite of a substantial literature on Se uptake by plants and crops such as wheat, little consideration has been given to sunflower and maize plants (Longchamp, 2011).

In this study we selected sunflower (*Helianthus annuus* L.) and maize (*Zea mays* L.) because they are widely grown crops providing with important sources of Se for human diet. To achieve our goals we selected the non-destructive and destructive chlorophyll content measurements that could be valuable and effective ways for estimating the effect of Se in sunflower and maize plants.

2. Materials and methods

2.1. Test plants and growing conditions

Sunflower (*Helianthus annuus* L. cv. Arena PR) as a dicotyledon and maize (*Zea mays* L. cv. Norma SC) as a monocotyledon plant were chosen for our research. Disinfected sunflower and maize seeds were geotropically germinated between moist filter papers at 22°C. Sunflower seedlings with 1.5-2.0 cm hypocotyl and maize seedlings with 2.5-3.0 cm coleoptile were placed into aerated nutrient solution pots. Sunflower and maize plants were grown in a climate room under strictly regulated environmental conditions. Relative humidity was maintained between 65-75%, the light/dark cycle was 16/8 hrs. with a respective 25/20°C temperature periodicity, and light intensity was kept at a 300 μmol $m^{-2}s^{-1}$ during daytime.

2.2. Nutrient supply and selenium treatments

The nutrient solution used for plant growth had the following compositions: 2.0 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.1 mM KCl, 10 μM H_3BO_3 for sunflower and 0.1 μM H_3BO_3 for maize, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$ and 0.2 μM $CuSO_4$. In addition, iron was supplied in the form of 10^{-4} M Fe-EDTA (Cakmak and Marschner, 1990).

Selenium was supplemented to the nutrient solution as either selenite in the form of Na_2SeO_3 or selenate in the form of Na_2SeO_4 in five different concentrations, as follows: 0 (control), 0.1, 0.3, 0.9 and 3 mg L^{-1}. Nutrient solution was changed every 3 days and evaporated water was replenished regularly. The experiment ended 3 weeks for sunflower and 2 weeks for maize after planting when the third leaf of the control treatment had completely grown and seedlings had approximately 30-20 cm and 40-30 cm long shoots and roots, for sunflower and maize, respectively. Experiments were carried out in triplicates (three pots) that every pots had four seedlings.

Sodium selenite, sodium selenate and N,N-Dimethylformamide (N,N-DMF) were obtained from Sigma-Aldrich Ltd. (Poole, UK).

2.3. Measurement of chlorophyll content

RCC average of five different parts in leaves from two seedlings in each pot, were measured in three times (when every leaf of sunflower and maize plants grew completely and at the same time, RCC of older leaves were measured, too) by portable, non-destructive chlorophyll meters (Minolta SPAD-502, Japan).

Chlorophyll a and b contents were calculated in destructive measurement. Two first and second mature, intact and erect leaves from two seedlings in each pot, sampled for extraction and determination of the chlorophyll a and b. 50 mg of each leaf were collected and with 5ml N,N-Dimethylformamide (N,N-DMF) blended. This solution cooled at 4°C for 72 hours and finally, the extraction content of the pigment was determined using UV–vis

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Table 1. Fresh and dry weight (g) of sunflower shoot and roots affected by applied different Se forms

treatments	**Weight of shoots (g)**				**Weight of roots (g)**			
Applied Se (mg L^{-1})	**Selenite (Se IV)**		**Selenate (Se VI)**		**Selenite (Se IV)**		**Selenate (Se VI)**	
	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight
0.0	12.19^{ab}	0.84^{a}	12.19^{ab}	0.84^{a}	4.76^{abc}	0.15^{a}	4.76^{c}	0.158^{c}
0.1	10.61^{abc}	0.73^{a}	12.47^{ab}	0.92^{a}	5.48^{abc}	0.17^{a}	9.29^{bc}	0.276^{bc}
0.3	9.36^{bc}	0.65^{a}	14.25^{ab}	1.01^{a}	6.29^{ab}	0.21^{a}	8.57^{bc}	0.263^{abc}
0.9	4.96^{d}	0.39^{b}	11.85^{abc}	0.90^{a}	3.20^{cd}	0.13^{a}	6.91^{abc}	0.239^{abc}
3.0	1.30^{e}	0.13^{c}	0.41^{bc}	0.10^{b}	1.59^{cd}	0.09^{a}	1.06^{d}	0.066^{c}

Significant differences in the mean value of each treatment group are indicated by different lower case letter based on LSD test ($p < 0.05$, n = 3 ± s.e.)

Table 2. Fresh and dry weight (g) of maize shoot and roots affected by applied different Se forms

treatments	**Weight of shoots (g)**				**Weight of roots (g)**			
Applied Se (mg L^{-1})	**Selenite (Se IV)**		**Selenate (Se VI)**		**Selenite (Se IV)**		**Selenate (Se VI)**	
	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight
0.0	3.48^{ab}	0.26^{a}	3.48^{a}	0.26^{a}	1.64^{a}	0.09^{ab}	1.64^{a}	0.10^{a}
0.1	2.77^{b}	0.21^{b}	4.11^{b}	0.30^{a}	1.4^{a}	0.08^{b}	1.87^{a}	0.11^{a}
0.3	2.96^{ab}	0.23^{ab}	3.26^{a}	0.25^{a}	1.72^{a}	0.10^{ab}	1.62^{a}	0.10^{a}
0.9	2.66^{b}	0.23^{ab}	2.99^{a}	0.23^{a}	1.78^{a}	0.11^{a}	1.55^{a}	0.10^{a}
3.0	0.54^{c}	0.06^{c}	3.29^{a}	0.27^{a}	0.49^{b}	0.04^{c}	1.45^{a}	0.09^{a}

Significant differences in the mean value of each treatment group are indicated by different lower case letter based on LSD test ($p < 0.05$, n = 3 ± s.e.)

spectrophotometry (Metertech SP-830 PLUS, Taiwan) at two characteristic wavelengths, 647 and 664 nm, which are the maximum absorption wavelengths for chlorophylls b and a, respectively (Moran and Porath 1981). According to the formula that was proposed by Wellburn (1994), the following was processed mathematically for quantifying chlorophyll a and b content:

- Chlorophyll a ($mg.g^{-1}$) = (11.65 a664-2.69 a647)
- Chlorophyll b ($mg.g^{-1}$) = (20.81 a647-4.53 a664).

2.4. Plant weight measurement

At the end of the experiment, shoots were separated from roots and weighted immediately. Plant parts were dried at 70°C until constant weight was achieved, then cooled to room temperature and weighed by an analytical scale (OHAUS, Swiss).

2.5. Statistical analyses

All data were statistically analyzed using *SPSS 19.0* software (2010), and the mean values of each treatment group were subjected to multiple comparisons analysis. The bars indicate the standard error of the mean. Significant differences in the mean value of each treatment group are indicated by different lowercase letters based on the *LSD test* ($p<0.05$, n = 3).

3. Results and discussion

3.1. Effect of different applied Se forms on fresh and dry weight of sunflower and maize

The fresh and dry weight of sunflower and maize organs decreased with increased concentrations of both Se^{IV} and Se^{VI} (**Table 1. and 2.**). It was found that the Se tolerance in the selenite treatments can make lower biomass than selenate at different concentrations. But fresh and dry biomass of both decreased when their concentrations in the growth medium reached 3 mg L^{-1} in two plants. Although sunflower plant was more sensitive than maize for these biomass reductions.

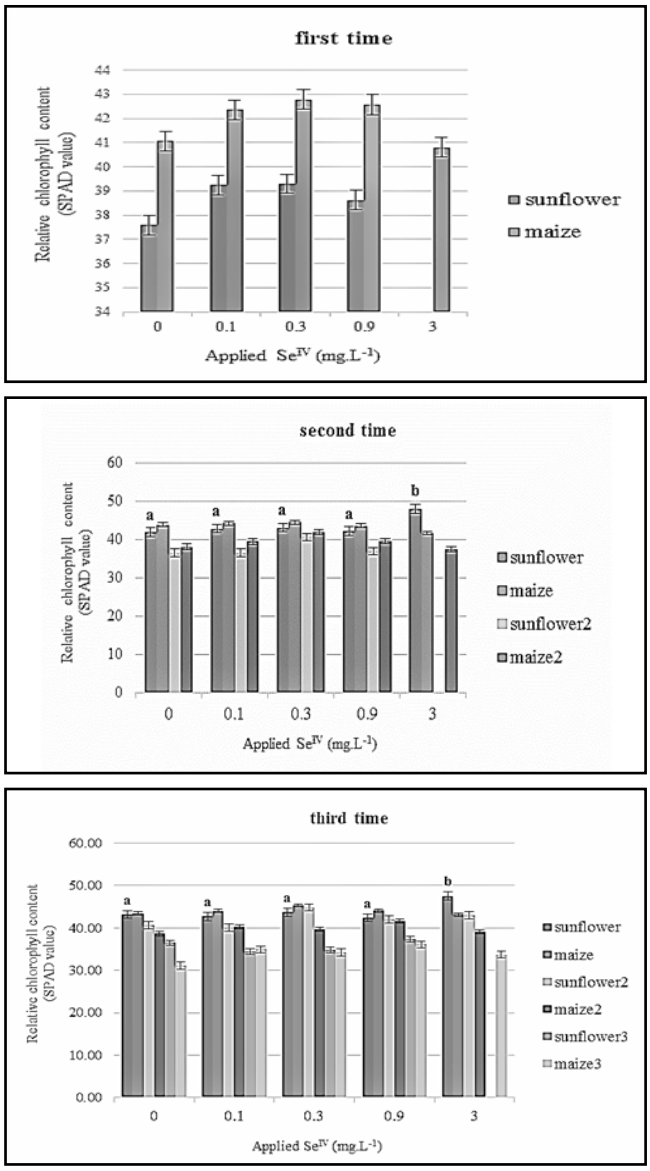
3.2. Effect of different applied Se forms on physiological parameters

3.2.1. Relative Chlorophyll Content (RCC)

Figure 1. shows the relative chlorophyll contents according to SPAD value in sunflower and maize leaves at different concentrations of Se^{IV} in three times of measurement. Since high doses of 3 mg kg^{-1} Se^{IV} caused toxicity in sunflower, the youngest leaf did not grow well enough in every time of measurement and then, RCC measurement was impossible for it. Also, SPAD value of first leaf (the oldest leaf) at the second and third time of measurement significantly increased at this concertation. On the other hand, RCC did not changed significantly with increasing the application of Se^{IV} in maize plants even at the highest concentration of 3 mg L^{-1} for three times of measurement.

Figure 2. displays relative chlorophyll contents according to SPAD value in sunflower and maize leaves at different concentrations of Se^{VI} in three times of measurement. RCC of sunflower treatments changed significantly in the first time of measurement but this state was not same in the other times. Moreover, high doses of 3 mg kg^{-1} Se^{IV} caused toxicity in sunflower and the youngest leaf did not grow in third time of measurement. Then, RCC

Figure 1. Se^{IV} uptake effects on RCC of sunflower and maize leaves in three time of measurement. Significant differences in the mean value of each treatment group are indicated by different lowercase letter based on the LSD test ($p < 0.05$, n = 3±s.e.)



measurement was impossible for it.

Furthermore, maize plants' RCC did not changed significantly with increasing the application of Se^{IV} even at the highest concentration of 3 mg L^{-1} in all three times of measurement.

3.2.2 Chlorophyll a and b content

The main kinds of chlorophyll in plants are chlorophyll a and b (Chl *a* and *b*). They differ only slightly in the composition of a side

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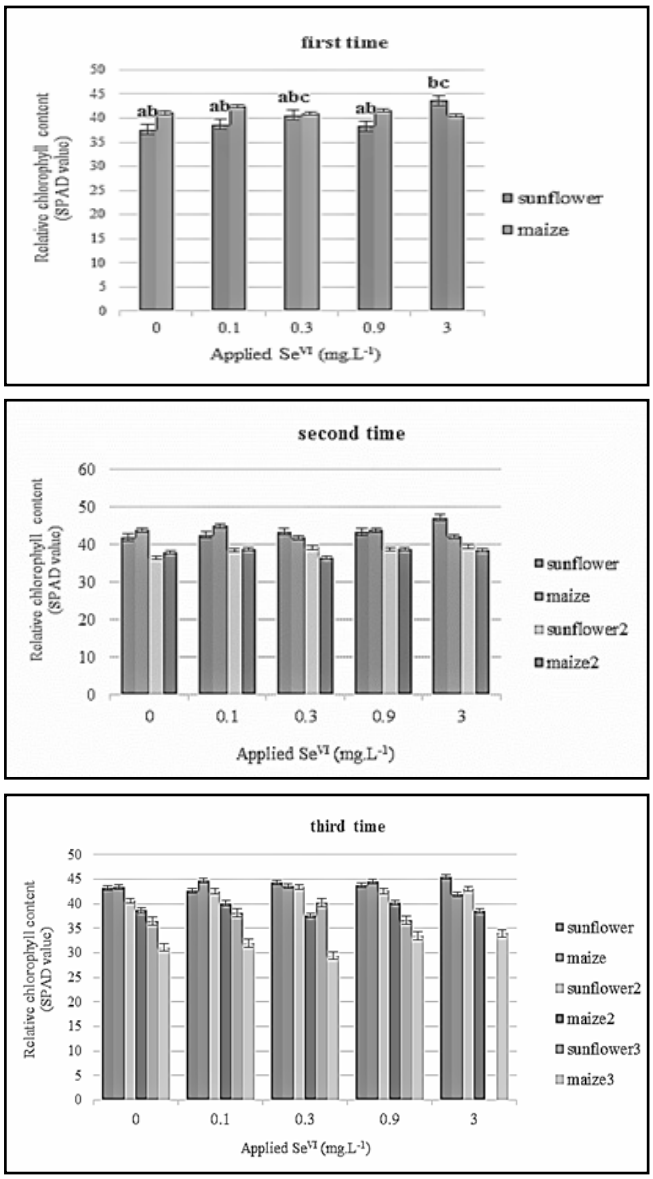
Figure 2. Se^{VI} uptake effects on RCC of sunflower and maize leaves in three time of measurement. Significant differences in the mean value of each treatment group are indicated by different lowercase letter based on the LSD ($p < 0.05$, n = 3±s.e.)



chain, where CH_3 and CHO in both Chl *a* and *b*, respectively. Both Chl *a* and *b* are genuine components of the photosynthetic membranes. These two chlorophylls are very effective photoreceptors because they contain a network of alternating single and double bonds, and the orbitals can delocalise stabilizing the structure. Such delocalized polyenes have very strong absorption bands in the visible regions of the spectrum, allowing the plant to absorb the energy from sunlight (Streitweiser and Heathcock 1981).

Effect of different applied concentrations of selenite on Chl a and b contents in first and second leaves of sunflower and maize can be observed in **Figure (3)**. No significant difference in these chlorophyll contents was recorded by increasing the application of this Se form. Whereas, **Figure (4)** displays the response of Chl a and b contents in first and second leaves of sunflower and maize at different selenate concentrations. The previous trend for selenite also recorded for selenate, where no significant difference in these chlorophyll contents was seen by increasing the application of selenate form.

4. Conclusion

The function of Se in plants has been investigated in many studies and there is still little evidence that Se is essential for all plants. However, there are some indications that this element may be required for Se-

Figure 3. Se^{IV} uptake effects on chlorophyll a and b contents of first and second leaf of sunflower and maize based on the LSD test ($p < 0.05$, n = 3±s.e.)

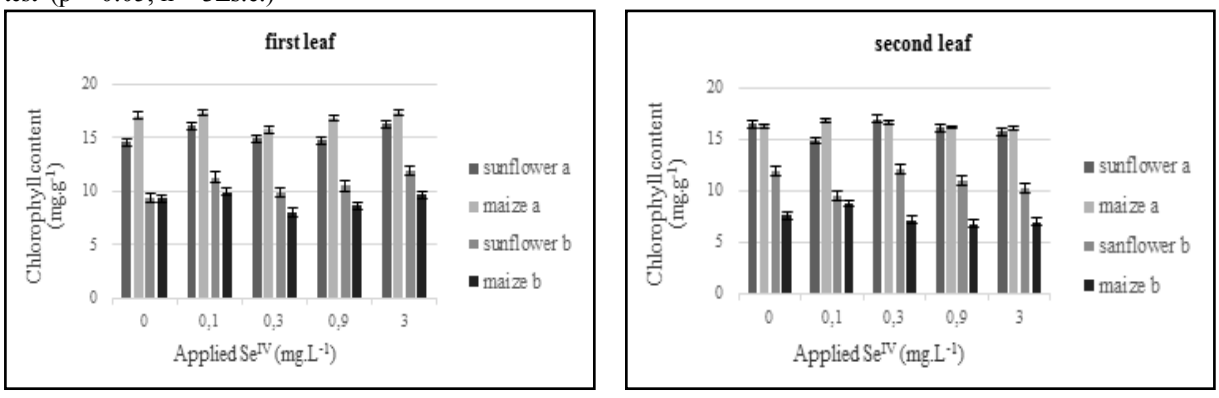
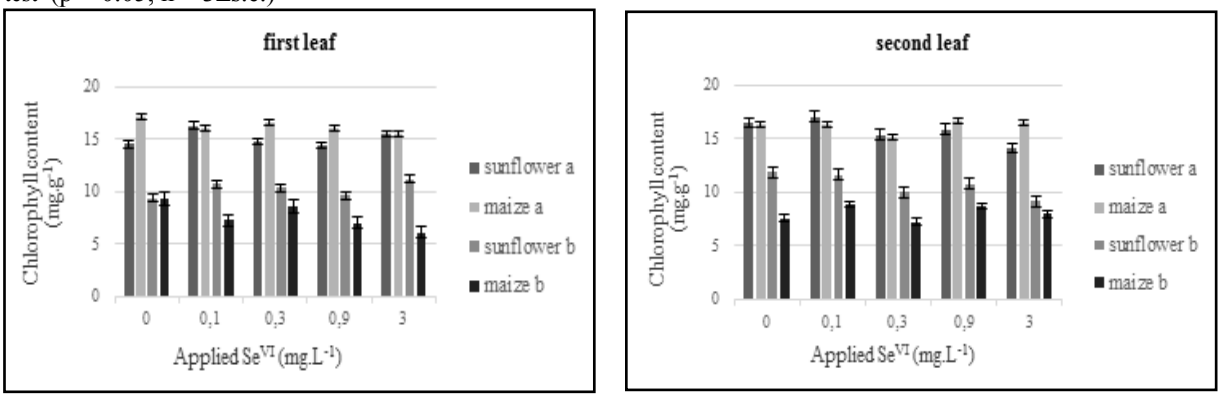


Figure 4. Se^{VI} uptake effects on chlorophyll a and b contents of first and second leaf of sunflower and maize based on the LSD test ($p < 0.05$, n = 3±s.e.)



accumulating plants and at proper Se addition, the growth rate of plants may be enhanced (Hartikainen, 2005). In addition, chlorophylls are the most common green pigments found in plants that play a key role in photosynthesis (Schoefs, 2002) and its content in agricultural crop leaves is of great importance for nutritional state diagnosis, yield prediction, studying the mechanisms of plant and environment interaction. The presented results allow us to conclude the effects of different Se species uptake by sunflower and maize plants on chlorophyll contents that were achieved by both non-destructive and destructive measurements. RCC content in sunflower samples that had been treated with Se^{IV}, due to increasing the concentration to 3 mg kg^{-1} and high dose Se toxicity, had significant difference in the oldest leaf at the second and third time of measurement. Whereas, this state was not seen in Se^{VI} treated sunflower and maize samples.

Moreover, chlorophyll a and b in destructive method of chlorophyll content measurement, did not change significantly in both first and second leaves of sunflower and maize samples which had been treated with both Se^{IV} and Se^{VI}.

Finally, collected data shows both forms of Se^{IV} and Se^{VI} uptake by sunflower and maize, do not change chlorophyll content of these plants leaves, significantly.

Acknowledgment

The authors declare that they have no conflict of interest.

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IMPACT OF NITROGEN TOPDRESSING ON THE PERFORMANCE OF WHEAT YIELD AND GRAIN PROTEIN

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Abstract: Yield samples of winter wheat *Triticum aestivum* L. varieties taken from the Nagygombos experimental site of the Szent István University in two different crop years have been evaluated. Impact of N topdressing on the performance of yield and protein was studied. In case of five high quality wheat varieties yield and protein values were examined. The results suggest that ascending doses of N topdressing, and split applications had a beneficial effect on the yield figures, the amount of protein content as well as the total protein yield of the wheat varieties examined.

Keywords: yield, grain protein, total protein yield, winter wheat

Introduction

Wheat is the most widely spread basic staple for mankind. Wheat is also one of the most important cereals in Hungary with a high economic value. Utility, market and alimentation value of this crop is highly affected by agri-environmental conditions and within that crop year effects, as well as agronomic impacts (Győri 2006; Várallyay 2008). The aim of wheat production is twofold; to provide quantity and quality. Milling and baking quality of wheat is mainly determined by the genetic basis however it can be influenced by management techniques (Grimwade et al 1996; Pollhamerné, 1981; Pepó 2010; Vida et al. 1996).

The protein content of the wheat crop may have important impacts on the nutritional quality for humans and livestock and on the functional properties in food processing. The amount of wheat yield, as well as the grain quality is highly influenced by the nitrogen supply of the crop. Nowadays yield levels of wheat production exceed five to ten times that of the natural yielding ability of this grain crop. Unless mankind would risk the deterioration of nutrient supply of arable land, fertilization has to be implemented in favour of maintaining nutrient balance of the field and to avoid exploitation (Hegedűs et al. 2002).

Also, the means and the way of fertilization in general and N supply in particular may induce changes in wheat quality. The protein content of the grain is responsible for both bread-making quality and the value of animal feed. (Lásztity1999; Shewry and Halford 2001, Kismányoky and Ragasits 2003; Győri 2008). Long term trials have proved that ascending doses of N applications resulted in dry matter and quality improvement of wheat varieties (Berecz and Ragasits 1990; Ragasits et al 2000).

The present study is dealing with the impact of nitrogen supply on the performance of yield and protein content of wheat grain in the case of different varieties.

Materials and methods

In a long term field trial a wide range of high milling and baking quality winter wheat *Triticum aestivum* L. varieties were examined under identical agronomic conditions. The small plot trials have been run at the Nagygombos experimental field of the Szent István University, Crop Production Institute, Hungary since 1998. Soil type of the experimental field is chernozem (calciustoll). Annual precipitation of the experimental site belongs to the 550-600 mm belt of the Northern edges of the Hungarian Great Plain.

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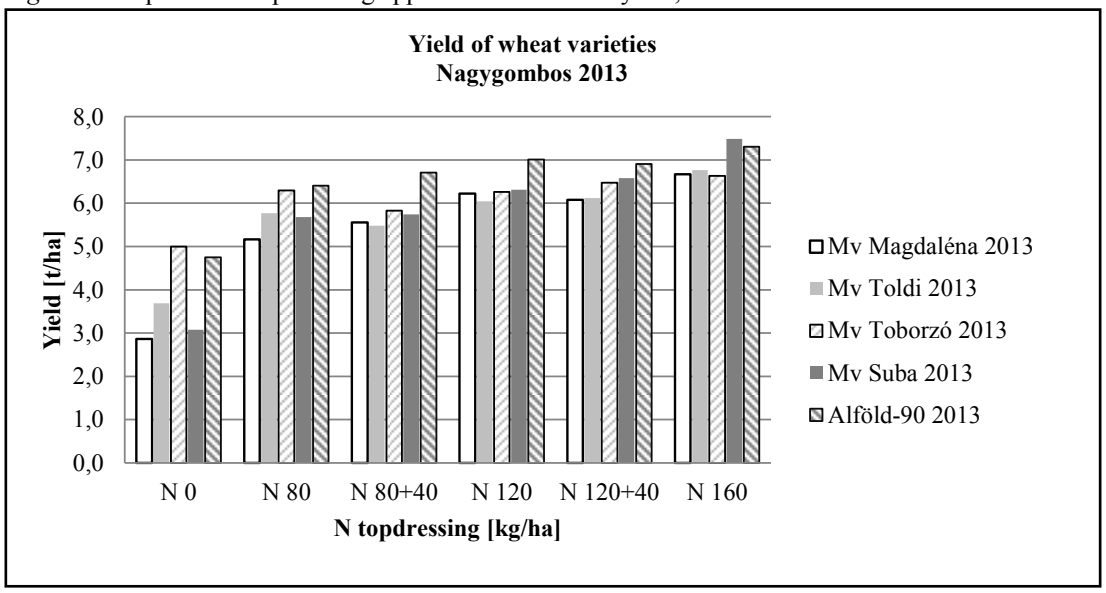
Experiments were conducted in split-plot design with nine replications. The size of each plot was 10 m^2. Plots were sown and harvested by plot machines (standard Wintersteiger cereal specific experimental plot machinery series). Various identical agronomic treatments were applied to plots. Plant nutrition applications were done in single and combined treatments. N topdressing variants were applied by single and repeated topdressings representing 4 levels: 0, 80, 120 and 160 kg/ha N in single applications, whereas 80+40 kg/ha and 120+40 kg/N in two applications (at the time of tillering and heading). All plots were sown with identical series of wheat varieties for studying their performance in relation with agronomic impacts. The recent study presents the performance and evaluations of five winter wheat cultivars (Alföld 90, Mv Magdaléna, Mv Suba, Mv Toborzó and Mv Toldi) of two consecutive crop years; 2013 and 2014. laboratories according to Hungarian standards (MSZ 1998). The protein figures and the total protein yield figures were correlated with the treatments applied. Statistical analyses were done by Microsoft Office 2003 programmes.

Results and discussion

The results obtained suggest, that ascending doses of N supply resulted in yield increase regarding all varieties and both crop years. The amount of grain yields are shown by figures 1 and 2. There were differences between the yield levels of the two crop years. In 2013 yield figures were detected within the range of 2.9 to 7.4 t/ha. There were varietal differences as well. Untreated control has shown significant differences between varieties. Highest yields were obtained on the plots of Alföld 90 and Mv Toborzó. The highest yields were found in the case of 160 kg/ha topdressing. Mv Suba proved to be the highest yielding variety.

Figure 1. Impact of N topdressing applications on wheat yield, 2013



Wheat yield were measured by each plot harvested. Protein content was determined from grain samples, as well as other quality characteristics at the Research Laboratory of the SIU Crop Production Institute, RET Regional Knowledge Centre and the NÉBIH National Food Chain Safety Office

In the 2014 crop year wheat yields were recorded within a narrower range of 4.8 to 7.3 t/ha. There were also varietal differences. Untreated control has shown minor differences between varieties only. The highest yields were found in the range of 80 to 120+40 kg/ha topdressing applications. In this crop year

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Figure 2. Impact of N topdressing application on wheat yield, 2014

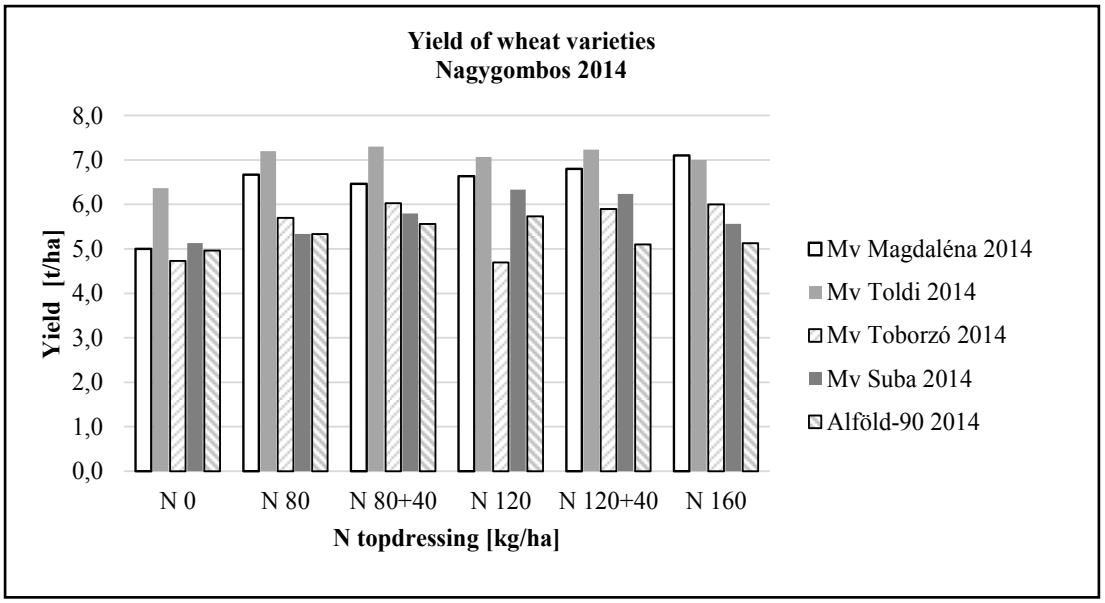
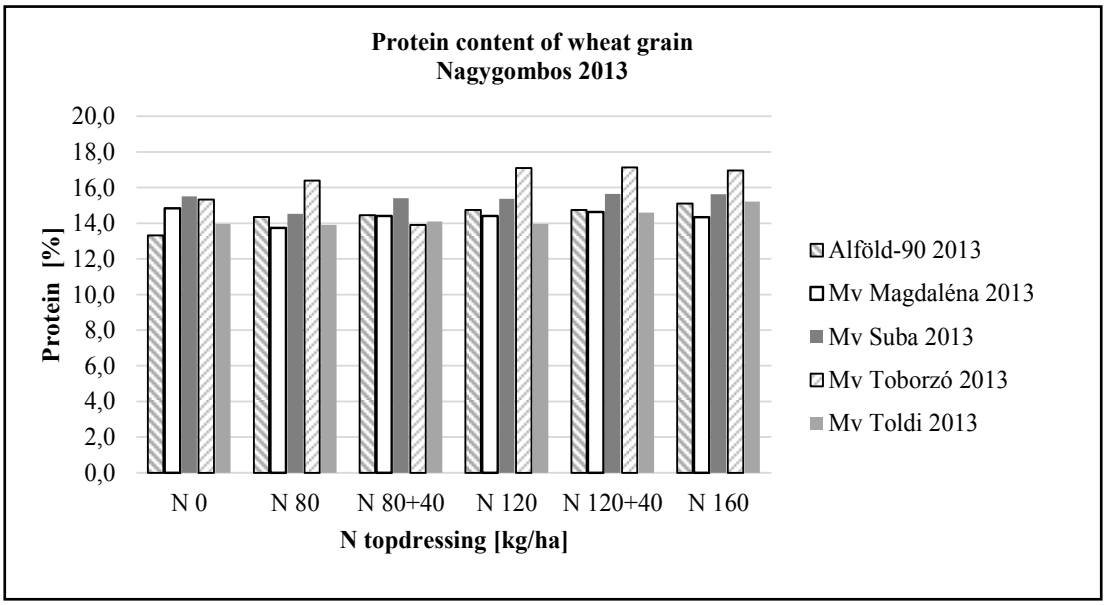


Figure 3. Impact of N topdressing applications on wheat grain protein content, 2013



160 kg/ha doses induced yield decrease in all varieties. Mv Toldi proved to be the highest yielding variety in this year.

Analyzing wheat grain samples protein content proved to be highly affected by N applications as well as varieties examined. There were also significant differences between the protein levels of the two crop years. Figures 3 and 4 comprise data of protein performance in 2013 and 2014.

2013 proved to be a high protein crop year with small, but significant differences between applications. Protein figures ranged from 13.2 to 17.2 %. The smallest figure was obtained in the untreated control, while the highest was observed in the treatments 120 to 160 kg/ha. Mv Toborzó proved to be the best variety in this crop year along most of the treatments.

2014 can be considered an average crop year concerning protein figures. Protein figures ranged between 10.1 to 14.0 %. The smallest figure was also recorded in the untreated control, while the highest was obtained in the treatments 120 to 160 kg/ha. Mv

Figure 4. Impact of N topdressing applications on wheat grain protein content, 2014

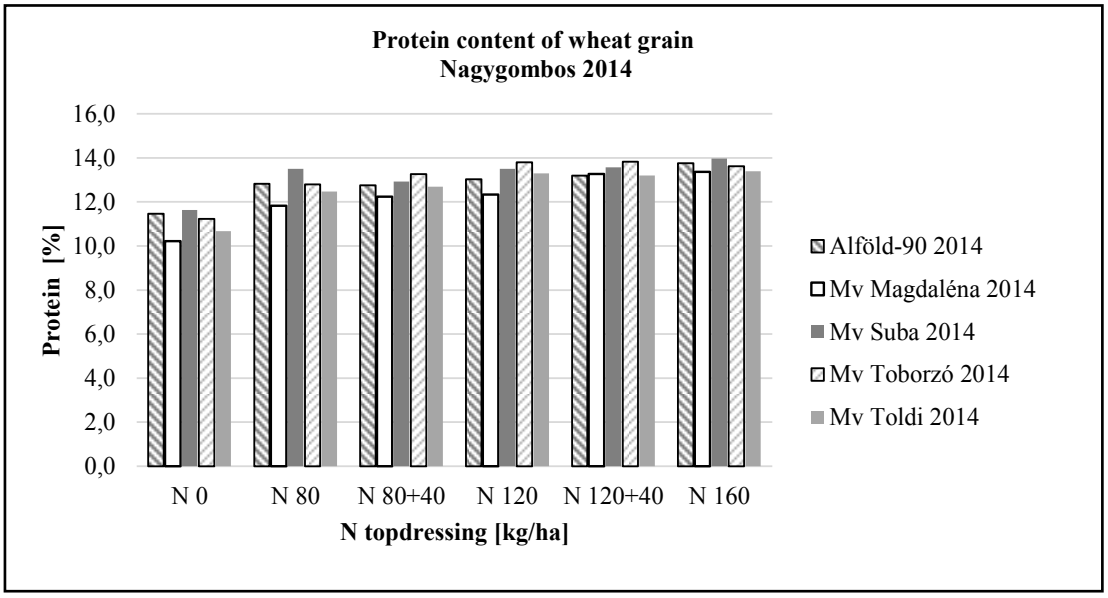
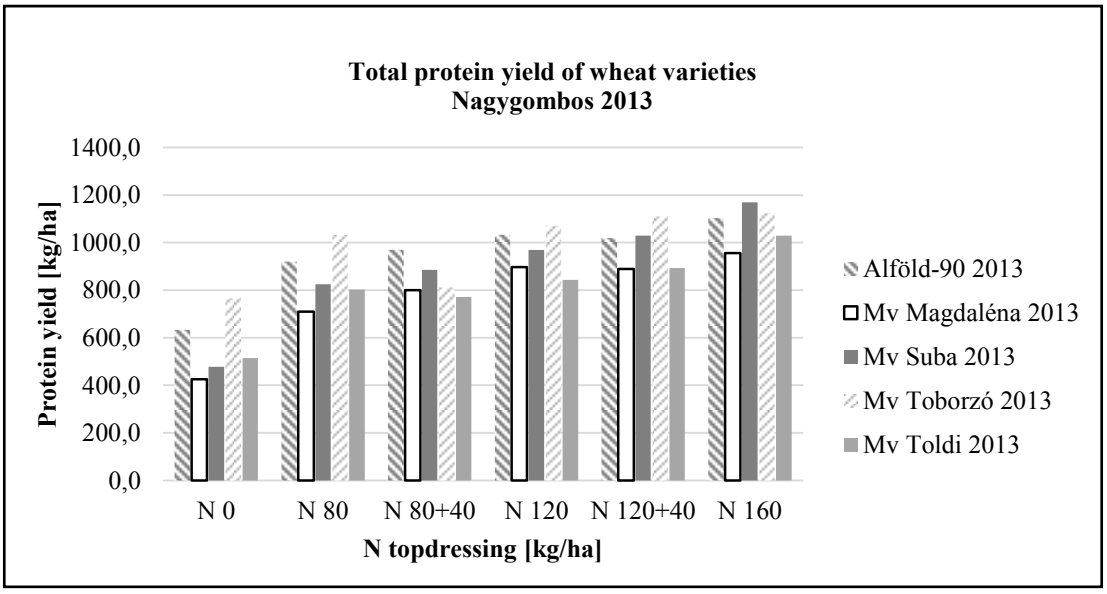


Figure 5. Impact of N topdressing applications on total protein yield, 2013



Toborzó and Mv Suba proved to be the best varieties in this year in most of the treatments.

Figure 5 and 6 give information on the total protein yield of the varieties examined. Yield and quality evaluations of grain crops very seldom make attempts to estimate protein yields, however the amount total protein content of grains exceed the level of that of so called proteinous field crops, like pulses.

Total protein yields were higher in 2013. The amount has been varied by treatments and varieties within the range of 410 to 1190 kg/ha. In 2014 the range was almost similar but narrower. Protein yields ranged between 515 and 970 kg/ha. Differences between N applications were bigger than that of varieties in both crop years.

Table 1 and 2 presents correlation figures between treatments and varieties concerning yield figures, protein values and protein yield amounts. With a few exception the treatments and the parameters examined have been highly correlated with each other. In 2013 there were

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Figure 6. Impact of N topdressing applications on total protein yield, 2014

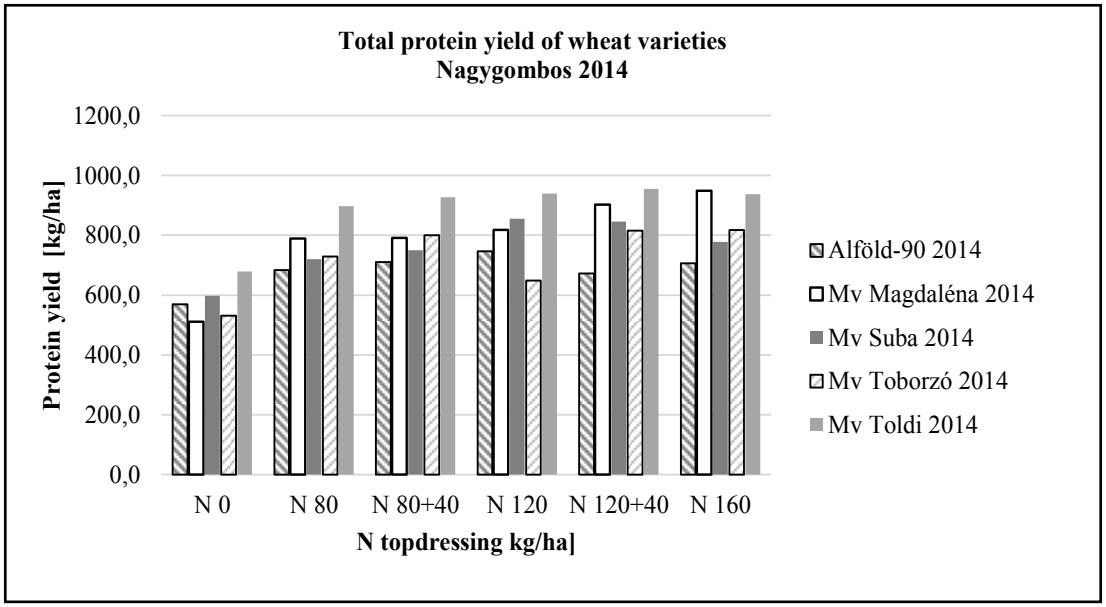

Table 1. Correlation between N topdressing applications and performance of wheat varieties in the 2013 crop year

Wheat varieties	Impact of N doses on		
	Yield, t/ha	Protein content, %	Protein yield kg/ha
r (Alföld-90)	0.9837	0.9962	0.9909
r (Mv Magdaléna)	0.9883	0.4003	0.9901
r (Mv Suba)	0.9936	0.1305	0.9990
r (Mv Toborzó)	0.9452	0.9509	0.9661
r (Mv Toldi)	0.9802	0.6782	0.9863

Table 2. Correlation between N topdressing applications and performance of wheat varieties in the 2014 crop year

Wheat varieties	Impact of N doses on		
	Yield, t/ha	Protein content, %	Protein yield kg/ha
r (Alföld-90)	0.4578	0.9877	0.8826
r (Mv Magdaléna)	0.9463	0.9954	0.9828
r (Mv Suba)	0.5977	0.9463	0.8592
r (Mv Toborzó)	0.5716	0.9536	0.8645
r (Mv Toldi)	0.7506	0.9729	0.9308

two varieties (Mv Magdalena and Mv Suba), where weak correlations were found only. In 2014 all versions correlations could be detected, however the level of correlations was not so strong in the case of yield performance of some varieties.

The results of the experiment highlight that both quantity and quality manifestation was influenced by N supply. The recent study was based on the evaluations of five cultivars in two crop years.

Conclusions

Ascending levels of N topdressing. and

increased number of fertilizer broadcasting applications proved to have a positive effect on the crop yield and on the amount of protein content of winter wheat varieties. The results suggest that ascending doses of N topdressing. and split applications had a beneficial effect on the yield figures. the amount of protein content as well as the total protein yield of the wheat varieties examined. There were differences between both the yield and protein figures of the two crop years.

In the study definite differences were found between the performance of the wheat varieties. Mv Suba and Mv Toborzó were found to be the highest protein yielding varieties.

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THE EFFECT OF CROP SPECIES AND N FERTILIZATION ON SOIL ORGANIC MATTER

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Abstract: Soil organic matter (SOM) is one of the most influential properties regarding soil fertility. Various crop species and varieties, as well as plant nutrition application may have an impact on the amount of soil organic matter. In a small plot field experiments the most characteristic agronomic impacts (biological bases, production sites, plant nutrition and crop year effects) influencing the efficiency of carbon sequestration of two major crop plants: - wheat Triticum aestivum and maize Zea mays have been studied. The aim of the research was to observe, identify and quantify agronomic impacts and their interactions that may have an influence on organic matter formation and so on carbon sequestration. Crop variety and plant nutrition proved to be the most important factors influencing organic matter production. Interactions have been found between crop plant genotypes and N levels applied.

Keywords: carbon sequestration, maize, winter wheat, plant nutrition, soil organic matter

Introduction

Climate change is one of the major issues of mankind. There is a continuous rise in temperature escorted by the increasing frequencies of weather anomalies. In case of: Hungary two facts can be observed; the ascending levels of temperature rise, with a magnitude of 1 ^{o}C and the annual precipitation decrease with increasing territorial and temporal variabilities Human activities are significantly altering the natural carbon cycle (Lal 2004). Long-term rise in atmospheric CO_2 highlights crop production regarding both adaptation and mitigation (Jolánkai et al 2005). The negative effects of climate change can be limited by changes in crops and crop varieties, improved water-management and irrigation systems, adapted plant nutrition, protection and tillage practices, and better watershed management and land-use planning (Berzsenyi and Lap 2005; Márton 2005; Sárvári 2005, Pepó 2010). The global potential of carbon sequestration through crop production, land use and soil management practices may offset one-fourth to one-third of the annual increase in atmospheric CO_2, a most endangering GHG (Lawlor 2005). Soil organic matter (SOM) is a result of carbon sequestration based on the photosynthetic activities of plants. Any organic matter manufactured by plants is originated from atmospheric CO_2. Plants (crop plants and natural vegetation) capture C and produce vegetative material, a biomass that comprises yield and by products. The prior one is regularly taken away from the crop site however the latter remains there providing a resource for SOM formation. Recent land use technologies aim the removal of plant residues in favour of using them as biofuels and so endangering soil remediation. The pool of organic C exists in dynamic equilibrium between gains and losses; soil may therefore serve as either a sink or source of C, through sequestration or greenhouse gas emissions respectively, depending on exogenous factors (Lal 2004). As biomass material undergoes decomposition, some microbial resistant compounds are formed. These include modified lignins, oils, fats and waxes. Also, some new compounds are synthesized, like polysaccharides and polyuronids. These materials form the basis for humus (Brady 1984).

When plant residues are returned to the soil, various organic compounds undergo decomposition. Decomposition is a biological process that includes the physical breakdown and biochemical transformation of complex organic molecules of dead material into simpler

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organic and inorganic molecules (Juma, 1999). The continual addition of decaying plant residues to the soil surface contributes to the biological activity and the carbon cycling process in the soil. Breakdown of soil organic matter and root growth and decay also contribute to these processes. Carbon cycling is the continuous transformation of organic and inorganic carbon compounds by plants and micro- and macro-organisms between the soil, plants and the atmosphere. Decomposition of organic matter is largely a biological process that occurs naturally and determined by three factors: soil organisms, the physical environment and the quality of the organic matter (Brussaard, 2012). Soil organic carbon (SOM) contains approximately 58 % C, therefore a factor of 1.72 can be used to convert organic carbon (OC) to SOM. There is more inorganic C in calcareous soils in general (Edwards et al. 1999).

Materials and methods

The Szent István University Crop Production Institute has recently started a new research programme on exploring the most characteristic agronomic impacts (biological bases, production sites, plant nutrition and crop year effects) influencing the efficiency of carbon sequestration of two major crop plants; wheat *Triticum aestivum* L. and maize *Zea mays* L.: The aim of the research is to observe, identify and quantify agronomic impacts and their interactions that may have an influence on organic matter formation and so on carbon sequestration. The trials were set up at the Nagygombos experimental site with a parallel version sown at Szárítópuszta in a three years consecutive series between 2007-2010. Six Martonvásár high starch maize hybrids were used in the trials representing different genotypes (Mv

Table 1. Carbon sequestration of maize (*Zea mays* L.) hybrids, Nagygombos

	Hybrid	Grain yield kg/m^2	Plant dry matter kg/m^2	AG biomass C content estimate, kg
0 N	Mv-251	0.48	0.34	0.33
	Maraton	0.86	0.61	0.59
	Norma	0.38	0.37	0.30
	Gazda	0.69	0.48	0.23
	Mv-454	0.70	0.46	0.45
	Mv-500	0.56	0.42	0.39
80 N	Mv-251	0.79	0.71	0.60
	Maraton	1.06	0.90	0.78
	Norma	0.79	0.67	0.55
	Gazda	0.76	0.77	0.61
	Mv-454	0.78	0.58	0.54
	Mv-500	1.36	1.04	0.96
120N	Mv-251	0.83	0.75	0.63
	Maraton	0.79	0.63	0.57
	Norma	0.80	0.78	0.63
	Gazda	1.14	1.17	0.92
	Mv-454	1.17	1.13	0.92
	Mv-500	1.43	0.62	0.82

C value $LSD_{0.05}$ – Hybrid:0.112; Nitrogen: 0.048

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250, Maraton, Norma, Gazda, Mv 454 and Mv 500) and a broad range of maturity groups (FAO 200-500). Also five wheat varieties (Mv Magdaléna, Alföld 90, Mv Suba, Mv Csárdás, Mv Toborzó) were exposed to ascending levels of nitrogen applications. Experimental double row 10 m^2 plots were designed in randomized blocks for maize, and full 10 m^2 plots in split-plot arrangement for wheat crop were sown both with four replications. The nitrogen applications were as follows: N 0, N 80 kg/ha, and N 120 kg/ha respectively. Basic plant nutrition and plant protection treatments were identical and appropriate regarding the agronomic requirements of the experimental field and providing ceteris paribus conditions to the trial.

Phenological, herbological, phytosanitary observations and yield characteristics have been evaluated. Yield samples were analysed at the Research Laboratory of the SIU Crop Production Institute, RET Regional Knowledge Centre and the NÉBIH National Food Chain Safety Office laboratories according to Hungarian standards for quality features (protein, carbohydrate, starch, cellulose, fat, ash etc). Carbon sequestration values were estimated on the basis of grain yield and total above ground biomass dry matter production. Statistical analyses were done by Microsoft Office 2003 programmes. The paper presents three years average data of the experiment

Results and discussion

The results obtained suggest that crop variety and plant nutrition proved to be the most important factors influencing organic matter production. Interactions have been found between crop plant genotypes and N levels applied.

Data of the maize trial are presented by Table 1. Grain yield, plant dry matter and carbon content values are presented by each hybrid in N application variants. Both hybrids and nitrogen applications proved to be significant concerning C values. Plant nutrition proved to be a strong factor in comparison with hybrid effects. However maize hybrids have shown differences as well. In general the

Table 2. Carbon sequestration of winter wheat (*Triticum aestivum* L.) varieties, Nagygombos

	Hybrid	Grain yield kg/m^2	Plant dry matter kg/m^2	AG biomass C content estimate, kg
0 N	Mv Magdaléna	0.54	0.59	0.45
	Alföld 90	0.68	0.75	0.55
	Mv Suba	0.76	0.83	0.62
	Mv Csárdás	0.69	0.76	0.58
	Mv Toborzó	0.70	0.75	0.58
80 N	Mv Magdaléna	0.74	0.85	0.64
	Alföld 90	0.71	0.77	0.59
	Mv Suba	0.80	0.89	0.68
	Mv Csárdás	0.81	0.88	0.65
	Mv Toborzó	0.84	0.91	0.69
120 N	Mv Magdaléna	0.78	0.85	0.65
	Alföld 90	0.84	0.84	0.67
	Mv Suba	0.75	0.82	0.63
	Mv Csárdás	0.79	0.75	0.62
	Mv Toborzó	0.80	0.86	0.66

C value $LSD_{0.05}$ – Variety: 0.092; Nitrogen: 0.067

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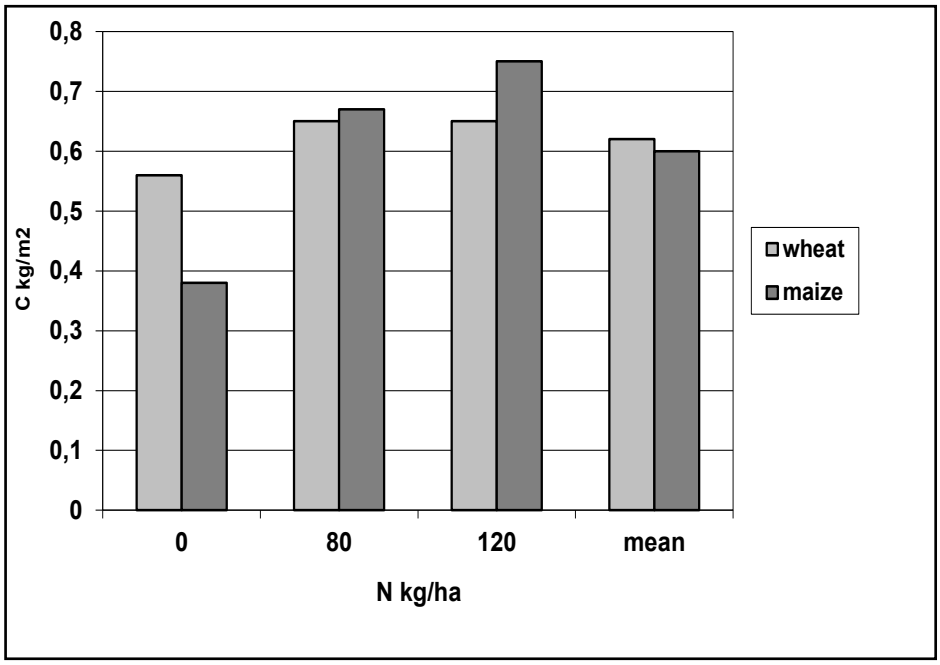
Figure 1. Nitrogen impact on carbon sequestration of grain crops, Nagygombos



results suggest, that late maturity hybrids had higher carbon sequestration abilities, but this difference was influenced by the nutritional levels, too.

Experimental results of winter wheat trials are summarised in Table 2. Identical to the other experiment, grain yield, plant dry matter and carbon content values are presented by each wheat variety in respective N applications. Compared to the maize trial data, wheat varieties were found to have less consequent specificity regarding C content, however N applications had really strong interactions with varieties. The difference between untreated control and N applications proved to be significant in all varieties, however 80 versus 120 kg/ha treatments had less impact on that.

Figure 1. presents a graphical evidence of carbon sequestration performance differences of the two crops examined.

Figure 2. Carbon content of above ground biomass, straw/stem residues, and the possibly derived soil organic matter, kg/m^2

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The photosynthetic activities of plants determine soil organic matter (SOM). The organic matter manufactured by plants is originated from atmospheric CO_2. All plants, like maize and wheat in this study capture C and produce vegetative material, a biomass that comprises yield and by products. Figure 2 provides information on the magnitude of carbon sequestration in relation with the crop species studied. Wheat and maize, regardless to the yield differences of the crops produced almost identical amount of C within the above ground biomass. Wheat straw had a higher and maize stalks had less C content, and as a result of that expected SOM has shown differences suggesting a better performance of wheat by products.

Conclusions

Wheat in general was more stable regarding C values, however maize crop was proved to be more efficient concerning ascending N doses. All together in mean values there was no measurable difference between the two crop species. Crop variety and plant nutrition proved to be the most important factors influencing organic matter production. Interactions have been found between crop plant genotypes and N levels applied. Atmospheric C budget can be balanced by photosynthetic dry matter production of natural vegetation and agricultural crops. The latter can be influenced by agronomic applications. Soil organic matter is based on the sequestration of C derived from plant residues. Wheat represents a better source for organic carbon in comparison with maize crop.

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COMPARISON OF THE DRAWDOWN OF SUBSIDIES FROM THE EUROPEAN AGRICULTURAL FUND FOR RURAL DEVELOPMENT IN THE ROMANIAN AND HUNGARIAN COUNTIES BETWEEN 2007 AND 2013

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Abstract: Hungary and Romania rely on rural development support programs in order to alleviate the economic, social and environmental problems of rural areas, which would not be possible using only internal resources. It is worthwhile to analyse the results of these programs and their benefits for the recipient countries. The purpose of the study is to examine the 2007-2013 rural development programs in these two neighbouring countries, together with the application patterns relevant in this programming period. In the secondary research statistical methods are used to analyse the amount of support specific for the given regions and assess whether application activity is in line with the economic and social situation of these regions. In summary, Romania and Hungary have not exploited EU rural development resources fully. In the case of Romania, it made possible for LEADER action groups to learn and acquire experience and they have had significantly less latitude than member states with more experience with the program. The program resulted in the deepening of rural development cooperation between Romania and Hungary and the implementation of a large number of joint projects between 2007-2013.

Keywords: common support for rural development, Hungary, Romania, county distribution, factor and cluster analysis, 2007-2013's program period

Introduction

The convergence of the regional differences has been since the beginning one of the principles of the European Community (Rechnitzer and Smahó, 2011), but regional policy in its modern sense is the result of a long development process. Roberts and Springer (2001) stress that the feasibility of EU policies depends on the will of the Member States to make the policies achievable. The combination of the wishes and priorities of Member States can shape the design of policies and although the EU seeks consensus this may become more complicated with the accession of the new Member States which increased considerably economic and cultural differences, and created new problems to be solved. At the 1999 Berlin Forum the EU recognized that *in addition to regional policy it is also a necessity to think in terms of rural areas* and as part of the "Agenda 2000" – containing proposals from the Commission for the 15 Member States concerning the developments to be carried out in the period after 2000 – the Common Agricultural Policy was reformed, with rural development as the second pillar. Glatz (2008, p. 33.) writes: "It has been realised that the countryside must be reinforced by business supporting, job-creating actions because of the prosperity of rural areas can only be achieved if there is work in the countryside".

There is no uniformly accepted definition to describe rural areas, although this is one of the important tasks of rural development. The exact definition of rural area is very difficult, since it is a very complex socio-economic and cultural concept. In most cases, the word 'rural' is coupled with negative values, as an opposite of the more developed urban areas or as the location of agricultural farming. Many of the approaches do not target the unambiguous and exclusive definition, but rather a kind of classification. (Tóth and Máté, 2013) The European Union does not give a description of what should be considered as rural area, each Member State must define this concept on the basis of their individual, specific characteristics, giving content to the definition. "Clarifying the concept of rural areas is essential so that the areas whose

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development require methods, tools and measures that differ from the average can be outlined. The definition of rural areas may take place according to different criteria, depending on what point of view of rurality – geographical, social, economic or cultural – is considered" (Sarudi, 2003, p. 211.). According to the European Charter of Rural Communities (1995): rural areas are land, internal or coastal areas that typically include small towns and villages and the area is used for the following purposes: agriculture, forestry, aquaculture, fishing, economic and cultural activities of residents, non-urban recreation, conservation, and housing. In the decade prior to AGENDA 2000 rural areas corresponded to areas where population density at NUTS 5 level did not exceed 100 persons/km^2. The OECD criterion in the same period was 150 persons/km^2. Agenda 2000 contains a simplified concept for rural areas which are areas where population density is below 100 persons/km^2 and is declining, and where the proportion of people employed in agriculture is twice the EU average (Csete and Láng, 2009).

In Hungary, a rural region is a region which does not have town status or has the town status but with a population inferior to 10,000 inhabitants. The concept of rural areas is relatively new in Romania where the issue of rural development gained importance in the second half of the 1990s especially with the launching of the EU's SAPARD Programme for Agriculture and Rural Development. In Romania the status of the settlements are determined by law, so there are rural areas with a population of over 10,000 and there are towns with lower population (Kerekes et al., 2010). Villages with own local governments are considered rural settlements and several rural settlements form rural areas (Vincze, 2012).

In Romania and Hungary EU rural development programs contribute to a great extent to the development of rural areas and the increase of living standards, as national sources would not be sufficient to cover the costs of these developments. The aim of the study has been to analyse the efficiency of the use of the rural development grants financed by the European Agricultural Fund for Rural Development (EAFRD) for the 2007 to 2013 program period in the case of the two countries. It was also examined whether there were significant regional differences in terms of retrieval of resources, at the county level. On the basis of the literature studied and other secondary information the following hypotheses were formulated tested by statistical methods:

H1: There is a significant relationship between the GDP of a given county and the amount of the approved grant requests

H2: The Romanian and the Hungarian counties may be ordered in clusters according to the spatial distribution of grants financed by the European Agricultural Fund for Rural Development

H3: Romanian and Hungarian rural development show different trends in relation to the 2007-2013 grant specificities.

Material and Methods

The research was based on the compiled database containing the cumulative data of the

Table 1. Variables used in the factor- and cluster analysis

Romania	Hungary
Approved grant per one agricultural farm (EUR/farm)	
Approved grant per one farm employee (EUR/person)	
Approved grant per one inhabitant (EUR/person)	
Approved grant per one rural inhabitant (EUR/person)	-
Approved grant per one unemployed (EUR/person)	
Approved grant per one hectare of cultivated agricultural land (EUR/ha)	

Source: own secondary research (2013)

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EAFRD financed Romanian and Hungarian approved grant applications. The database included data from 41 Romanian and 19 Hungarian counties excluding Bucharest and Budapest, as major urban cities. Data concerning Romania were extracted from the website of the Paying Agency for Rural Development and Fisheries (APDRP), while data concerning Hungary were extracted from the website of the Agricultural and Rural Development Agency (ARDA). The data cover the period between 2007-08/29/2012 for Romania and 2007-14/10/2013 for Hungary. Data for Romania were available in Euro while data for Hungary were given in Forint. For the sake of comparison the latter were converted into Euro at the exchange rates applied by the European Investment Bank.

Besides to descriptive statistics, factor and cluster analysis were also used with the help of the SPSS software package. In order to ensure comparability between the countries and classification into groups on the basis of the granted rural development support, relative indicator values were calculated that were used in the factor and cluster analysis (Table 1). The indicators were derived from the databases of Eurostat, the KSH (Hungarian Central Statistical Office) and INSS (Romanian Statistical Office). The differences in euro were so considerable that it was not possible to use conjointly variables of the Romanian and Hungarian side (a separate cluster analysis was used for that). Nevertheless, trends in the application practices of the two countries can be observed.

For the factor and cluster analysis the Kaiser-Meyer-Olkin test was used first and as the obtained values for both countries were higher than 0.5 the factor analysis could be performed. The component matrix obtained during the factor analysis was rotated through the Varimax method and the cluster analysis was also performed by means of the Centroid weight centered method. The Sajtos and Mitev (2007) research and data analysis SPSS guidebook was very useful for the analysis.

With a view of a better understanding and in order to complete the results of the research, *professional interviews with five Romanian and five Hungarian farmers and entrepreneurs were carried out*. According to Malhotra (2001), this method helps researchers to have a better overview and become more familiar with certain problem areas. The interviews were focused on the applications for EU support and also on the possible experiences and opinions. The interviews were conducted between 19-23 August 2013.

Results and Discussion

Table 2 contains the relevant data and main variables of the two countries which are the subject of the study. The population of Romania is the double of that of Hungary, which is understandable by comparing the territory of the two countries. Despite the higher number of its population Romania is economically less developed since on purchasing power parity basis its GDP exceeded the Hungarian GDP by only EUR 85.674 million. According

Table 2. Main indicators of Romania and Hungary

Indicators	Romania	Hungary
Population (2012, people)	21 355 849	9 931 925
Population (2010, people)	21 462 186	10 014 324
GDP PPP (2010, million EUR)	244 507	158 833
Number of unemployment (2010, people)	626 960	474 757
Number of employees in agriculture (2010, people)	1 639 000	439 955
GVA created in agriculture (2010, EUR/capita)	5 200	8 100
SO created in agriculture (2010, EUR)	10 420 314 210	5 241 037 240

Source: own edition on the basis of AMÖ, KSH, INSSE, EUROSTAT

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to Gross Value Added per capita in agriculture and Standard Output in agriculture (2010 data) the performance of Hungary is better compared to that of Romania.

48% of the Romanian population lives in rural areas and 67% plays an active role in agricultural activities. 30% of the rural population works in either totally or partially self-sufficient farms on 1.17-3.3 hectares. 97% of the farms are small scale farms.

In Romania the average size of farms is 3.5 hectares, the average size of individual farms is 2.3 hectares, and the average size of commercial enterprises is 270.4 hectares. The share of agriculture in the GDP is the highest among European countries (6% in 2010). Romania is the second largest agricultural producer (after Poland) among the Central and Eastern European countries and the sixth among the EU27 countries (Tánczos, 2012).

Rural development in Romania and Hungary between 2007 and 2013

Between 2007 and 2013 Romania could spend EUR10 billion while Hungary could spend EUR 5.3 billion on rural development including member state contributions (Table 3). In the period examined 61 855 grant applications for rural development were allocated to Romania representing 47% spending from the funds. Alba County has to be mentioned in relation to the number of successful grant applications with the outstanding result of 4 561 effective applications. It was followed by Bistrița-Năsăud with 3 897 successful applications and Mehedinți with 2 803 applications. The three counties are among the ten counties that have won the largest amounts.

București and Ilfov Counties received the least amount of grants (10 and 188). There were much more winner applications in Hungary, but with fewer amounts than Romania. In the analysed period, 70% of spending from the rural development funds was represented by the 201 244 successful applications in Hungary. Regarding the number of grants, Bács-Kiskun county delivered remarkable results: 31 905 applications had won, followed by Szabolcs-Szatmár-Bereg (25 732) and Hajdú-Bihar (23 928) counties. Right after Pest county they could take advantage of the rural development funds to the greatest extent. With 2 855 winning applications there is Komárom-Esztergom county at the end of the list.

As far as the scope of measures is concerned, the modernization of agricultural assets, as well as the increase in the added value of agricultural and forest products were crucial both in Hungary and Romania. In the former agricultural environmental protection payments, in the latter the modernization of villages were the major priorities. The smallest amount of subsidies went to the axis of LEADER (EUR 424 million in Romania

Table 3. The budget of the Romanian and Hungarian rural development (2007-2013)

Name	Romania	Hungary
Total grants, EUR (EAFRD+Member States contributions)	10 billion	5,3 billion
Number of successful applications	61 855	201 244
Contracted amounts of support, EUR	4 727 401 911	3 724 200 800
UTILISATION OF RESOURCES	**47%**	**70%**
Axis 1 (Competitiveness), EUR	2 545 400 451	2 256 681 782
Axis 2 (Environment protection), EUR	37 085 311	663 792 528
Axis 3 (Quality of life), EUR	2 097 806 221	669 555 268
Axis 4 (LEADER), EUR	47 109 928	134 171 222
Romanian data: 2007-2012.08.29., Hungarian data: 2007-2013.10.14.		

Source: own edition on the basis of APDRP.ro, MVH.hu (2007-2013)

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and EUR 273 million in Hungary). 17% of LEADER funds was utilized in Hungary, while a scarce 2% was used in Romania during the investigation period. This can be explained by the 2.5 year delay (2009) in starting the first cycle of LEADER programme by Romania. There were no ministerial regulations on the work plan when task forces worked out their regional strategies: the documentation, the implementation of procedures and (as a result) the release of applications were considerably delayed. According to the head of the South Satu Mare Action Group (as one of the interviewees), in Romania, the LEADER is a way to decentralization by bringing decision-making down to local level. The Romanian Ministry provides a strong influence, because the Rural Development, Fisheries and Paying Agency only supplements the operation of LEADER. Romania will not lag behind in the 2014-2020 period and more funds will be available for bottom-up initiatives.

After the 2007 EU accession of Romania, the Romanian-Hungarian trade links and rural development cooperation intensified significantly. Between 2007 and 2013, EUR 248 million was provided to develop cross-border co-operation within the framework of Hungary-Romania Cross-border Co-operation Programme, partially financed from ERDF, as well from national funds. The programme – that will continue in the 2014-2020 period – aims to bring people, communities and actors closer in border regions, in order to promote the joint development of co-operating regions. The Carpathian Region Business Network and the Rural Development Cooperation with Hungarians Living Beyond the Borders national strategic programmes are also worth mentioning. The latter specifies the major goals and areas of Romanian-Hungarian co-operation during the 2014-2020 term, emphasizing the smart, sustainable and inclusive growth. The European Regional Associations, the LEADER programme, the co-operation and networking, the national strategic framework programs and professional cross-border co-operations are the proposed frameworks for implementation. (Vidékfejlesztési Minisztérium, 2012) It is obvious that the links between tourism and regional development are very complex including; the regionalisation type of the given country; the typologies of the different regions (outlying and remote, intermediate or economically integrated); and their economic development level and tourism potential. (Bujdosó et al. 2015/a) Although the microregions among the two countries can be considered as heterogeneous in terms of tourism and can be characterised by significant spatial disparities, the tourist potential of the Hungarian-Romanian border region is very important. (Bujdosó et al. 2015/b)

Table 4. The correlation between the effectiveness of the proposals counties and GDP (Pearson Correlation)

Name	Romania	Hungary
	Cumulative amount of aid approved (EUR)	
GDP purchasing power parity (million EUR)	0.391*	0.602**
** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).		

Source: result of own research (2013)

Contact Investigation

The first hypothesis, which presumes that the relationship between the GDP of a given county and the amount of approved grant requests is significant, was verified by Pearson correlation for both countries. In Hungary the positive correlation is much stronger than in Romania. Hence, it is statistically proved that the economically more developed counties received larger amounts of subsidies than the less developed ones (Table 4).

This is because the farmers and entrepreneurs in less developed counties often do not have enough deductibles for the applications (as it was also confirmed by the interviews). On either side of the border, almost each respondents had negative opinion about borrowing. The procedure of the requests for funds was diagnosed as bureaucratic and cumbersome, even by the successful candidates.

The factor analysis of the support indicators of the Romanian and Hungarian counties

As a result of factor analysis approx. the same two factor variables can be identified for both countries (Table 5-6). Because of its too big impact (and distortion of the results) the approved grant per one hectare of cultivated agricultural land (EUR/Ha) indicator was excluded from the analysis in the case of Romania.

The name of the first factor is 'factor of grants for agricultural activity', the second's name is 'factor of grants for inhabitants', which means that these two theoretical factors were in the factor analysis instead of the actual indicators, thereby eliminating the excessive weight of the multitude of variables that are strongly correlated with each other.

Clusters of the Romanian and Hungarian counties

By the two factor variables the counties of both states can be classified into five-five clusters. So, the second hypothesis (H2) which assumed that the Romanian and Hungarian counties can be arranged into clusters on the basis of the spatial distribution of grants financed by the European Agricultural Fund for Rural Development is herewith justified. The Romanian and Hungarian clusters and the corresponding counties are shown on Table 7.

The counties of the first cluster received the least subsidies for rural development related to the agricultural activity factor. On regional level, except for the administratively non-existing Székely Land, North East, South West and Ialomita, every county in the

Table 5. The correlation of the Romanian factors with the variables

Factor Components	Factors	
	1	2
First factor (from three variables)		
Approved grant per one farm employee (EUR/person)	0.906	0.358
Approved grant per one agricultural farm (EUR/farm)	0.877	0.452
Approved grant per one rural inhabitant (EUR/person)	0.811	0.505
Second factor (from two variables)		
Approved grant per one unemployed (EUR/person)	0.391	0.875
Approved grant per one inhabitant (EUR/person)	0.440	0.857

Source: result of own research (2013)

Table 6. The correlation of the Hungarian factors with the variables

Factor Components	Factors	
	1	2
First factor (from three variables)		
Approved grant per one inhabitant (EUR/person)	0.953	0.171
Approved grant per one unemployed (EUR/person)	0.889	-0.041
Approved grant per one farm employee (EUR/person)	0.696	0.668
Second factor (from two variables)		
Approved grant per one agricultural farm (EUR/farm)	-0.084	0.909
Approved grant per one hectare of cultivated agricultural land (EUR/ha)	0.635	0.688

Source: result of own research (2013)

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Table 7. Clusters of the Romanian and Hungarian counties

	Romania	Hungary
Cluster 1	Argeș, Bacău, Botoșani, Buzău, Călărași, Covasna, Dâmbovița, Dolj, Galați, Giurgiu, Gorj, Harghita, Iași, Ilfov, Maramureș, Mehedinți, Mureș, Neamț, Olt, Prahova, Suceava, Teleorman, Vâlcea, Vaslui, Vrancea	Békés, Csongrád, Fejér, Heves, Jász-Nagykun-Szolnok, Somogy
Cluster 2	Alba, Arad, Bihor, Brăila, Caraș-Severin, Cluj, Ialomița, Timiș	Bács-Kiskun, Borsod-Abaúj-Zemplén, Hajdú-Bihar, Pest, Szabolcs-Szatmár-Bereg
Cluster 3	Bistrița-Năsăud, Sălaj, Satu Mare, Tulcea;	Baranya, Győr-Moson-Sopron, Veszprém
Cluster 4	Conștanța, Huneadoara, Sibiu	Komárom-Esztergom, Nógrád, Vas
Cluster 5	Brașov	Tolna

Source: result of own calculation (2013)

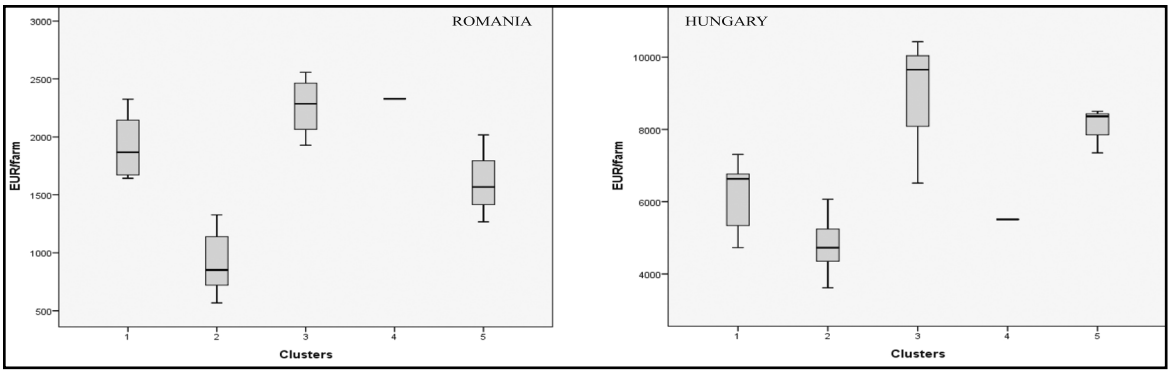
statistical regions of South Romania were in an unfavourable situation considering the effectiveness of receiving funds. Brasov County was in the best position, and formed a separate cluster by itself. The other clusters performed at about the same level.

The counties of the Hungarian second cluster received the least amount of rural development subsidies, compared to the agricultural factor. In this regard the third cluster was in an unfavourable position as well. The value of the indicator is reasonably established in the first group's counties. Tolna county forms a separate cluster and regarding the received grants for agricultural activity it belongs to the top level, similarly to the fourth cluster. In contrast with Romania, the regions show an entirely diverse image. As it can be seen in Figure 1, there are counties in each region whose performance was worse while other counties received subsidies more successfully. Based on the factor and cluster analysis the third hypothesis (H3) which states that the Romanian and Hungarian rural development displays different trends in terms of the 2007-2013 application features, can be partially accepted.

The characteristics of clusters by indicators

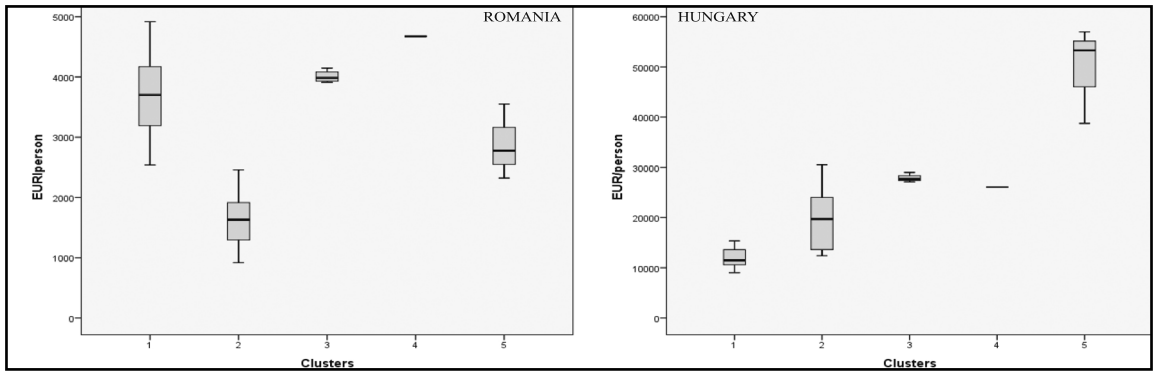
Further the characteristics and relative position of clusters are illustrated in boxdiagrams, where the boxes contain the standard deviation of half of the sample. The upper and lower sole of the box displays the minimum and maximum of a particular cluster. Based on the approved grant per one agricultural farm, with a EUR 2286 median, the Romanian Brasov county is followed by Bistrița-Năsăud, Sălaj, Satu Mare and Tulcea from the third cluster. The value of this indicator was much larger in Hungary. Baranya, Győr-Moson-Sopron

Figure 1. Approved grant per one agricultural farm (EUR/farm, clusters)



Source: result of own calculation (2013)

Figure 2. Approved grant per one farm employee (EUR/person, clusters)



Source: result of own calculation (2013)

and Veszprém, the counties of the third cluster performed best with a EUR 9651 median. The results can be seen on Figure 1.

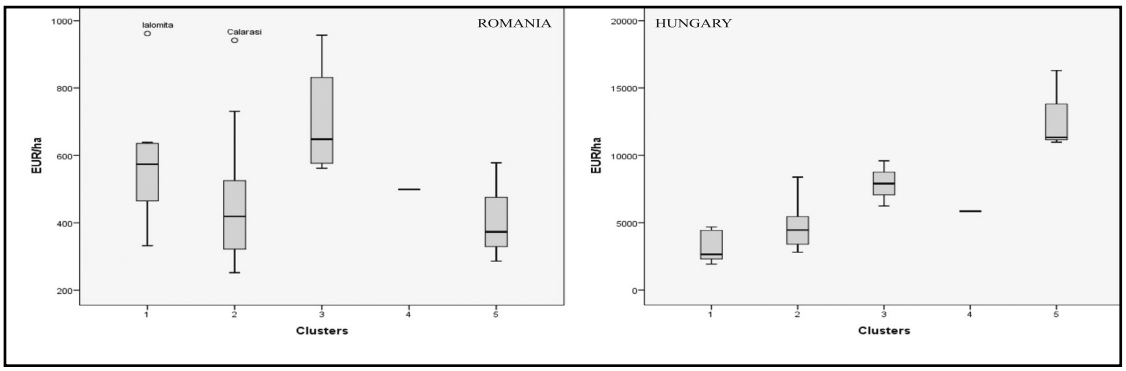
Even in case of the groups in least favourable situations, the approved grant per one farm employee is higher in Hungary than in Romania. The value of this indicator is the biggest in the third cluster's counties after Brasov in Romania, similarly to the previous indicator. In Hungary, with a EUR 53 292 median the highest value of this indicator belongs to the fifth cluster (Komárom-Esztergom, Nógrád, Vas). Mathematically, the number of agricultural employees has a growing potential in these counties (Figure 2).

As stated by Schlett (2013), job seekers and agricultural entrepreneurs could be primarily targeted by the expansion of agricultural employment. In case of Romania, the approved grant per one hectare of cultivated agricultural land caused the smallest differences between clusters. Based on the median, the counties of the third cluster are in the most favourable situation. In terms of this indicator, Ialomita county from the first cluster and Călăraşi county from the second cluster are in an outstanding position. The Hungarian index values outbid the values of Romania, and the best position is held by the counties of the fifth cluster with a EUR 11 337 median, as it can be seen in Figure 3.

Conclusion

The allocation of rural development fund took place in consideration of the characteristics of the rural areas and without favouritism but there are certain critical points. The economically more developed counties were more effective in their applications, especially in Hungary. Grant application activities in the economically underdeveloped counties could be encouraged in the 2014-2020 period by counselling within the framework of national

Figure 3. Approved grant per one hectare of cultivated agricultural land (EUR/ha, clusters)



Source: result of own calculation (2013)

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programs and real program assistance based on actual intellectual capital. Rural development in Hungary between 2007 and 2013 was more diverse and more efficient than in Romania. There were more measures, a larger number and smaller projects won support and 70% of the grants were utilised compared to the 47% in Romania. The situation of the Hungarian clusters according to the indicators are much better than in Romania. The value of subsidies for the unemployed are significant both in Romania and in Hungary therefore support should primarily be given to job-creating investments. According to Schlett (2013) the primary target group for the expansion of employment in agriculture could be job seekers and agricultural entrepreneurs. On the basis of the examined variables five-five clusters can be considered different types of counties. In both countries a greater emphasis should be placed on the LEADER program as bottom-up initiations are more likely to provide a solution to the old and new problems of the regions. It would be appropriate to create a standard and publicly available database on the utilisation of rural development funds. The research results may provide useful information for decision-makers involved in rural development and we believe that the results are indicative as to which areas and factors should be focussed on during the implementation of the 2014-2020 rural development program.

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REGULATION OF THE JUB1 STRESS-RELATED TRANSCRIPTION FACTOR IN *ARABIDOPSIS THALIANA* IN RESPONSE TO BIOTROPHIC FUNGUS *OIDIUM NEOLYCOPERSICI*

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Abstract: Biotic stresses influence fitness of the plants and may decrease their productivity. Especially biotrophic fungi govern complex regulation of host metabolism to fulfill their nutritional needs, while the plant tries to avoid this fungal activity. The defense system of the plant may involve transcription of stress-dependent genes, activation of signaling pathways or production of antimicrobial compounds. The responsible signal molecule in biotrophic pathogen-host interaction was found to be mostly the salicylic acid (SA). We studied here, whether the salicylic acid (SA) is required for the induction of a stress-related transcription factor JUB1 by the biotrophic fungus of tomato *Oidium neolycopersici* or not. To prove the regulation of JUB1, we isolated the upstream region of the gene and transcriptionally fused to *gus* reporter gene. This promoter::reporter fusion was then transferred into wild type and two salicylic acid insensitive mutants of *Arabidopsis thaliana* (*nim1-1* and *nahG*). The transgenic plants were infected by powdery mildew (PM) *O. neolycopersici*. We found an obvious increase of reporter gene expression in all three lines mostly at the area, where the pathogen was contacted to the plants. Based on the result, the stress-dependent JUB1 transcription factor is probably not influenced by the SA mutations of *nim1-1* and *nahG*. However, the induction appeared along with hydrogen-peroxide development, which suggests that this gene regulation in response to powdery mildew is probably regulated via H_2O_2 and not by SA signaling.

Keywords: biotic stress, H_2O_2 signaling, *Oidium neolycopersici*, salicylic acid, transcription factor

Introduction

Pathogens may cause extreme decrease in crop yield, which may have serious economic consequences. The *Oidium neolycopersici* powdery mildew (PM) is one of the major diseases of tomato (*Solanum lycopersicum*). Additionally it has a broad host range (over 60 species) in 13 plant families, mostly in *Solanaceae* and *Curcubitaceae*. High humidity promotes pathogen proliferation, therefore the *O. neolycopersici* causes disease mostly in greenhouse grown tomatoes. In spite of the PM does not infect the fruit, a marked decrease is observed in fruit size and quality on the infected plants (Jones et al., 2001), which results in significant shortfall for growers. Although plant-pathogen interactions have been under intense scientific research, many details of the interaction are still unclear. Studies searching the genetic and molecular backgrounds of these interactions may provide solution for enhancement of resistance in cultivated plants.

The biotrophic pathogens subsist on living plants only, since the host plant metabolism satisfies their nutritional needs. Therefore, these organisms are able to modulate plant metabolic pathways to support their growth and reproduction. Especially in the case of fungi, this manipulation places at the direct contact site between fungus and the plant. Fungi, during their infection process, develop a specific feeding structure – named haustorium – inside the plant cell lumen without puncturing the cytoplasm membrane. This particular organ is through which the nutrient exchange and signal transduction take place. The metabolic system of host plant may be modified by fungal effector molecules, which trigger increased expression of certain genes. However, the plants try to avoid the parasitic activity of the attacker since this may decrease their fitness and survival rate. Therefore, after recognition of infection a signal transduction process is activated, mediating by the plant defense responses against the pathogen (Blumwald et al., 1998).

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In biotrophic-host interaction usually the salicylic acid (SA) is the specific signal, which induces the components of immunity (Glazebrook, 2005). This defense mechanism may involve the activation of resistance *(R)* genes, which recognize effector molecules along with induction of immune response, or may include the production of antimicrobial compounds such as the phytoalexins. Earlier studies demonstrated that the transcription factors regulate the expression of defense genes in immune response, such as the WRKYs, MYBs and NACs does (Ambawat et al., 2013; Nuruzzaman et al., 2013; Pandey and Somssich, 2009). The NAC (NAM/ATAF/CUC) transcription factors compose a large gene family and are specific to plants (Riechmann et al., 2000). The members of this family are found to regulate both abiotic and biotic stress responses, especially the JUNGBRUNNEN 1 encoding *JUB1/ANAC042* gene, which reacted to necrotrophic fungus *Alternaria brassicicola* infection. The *jub1* knock-out mutants represented high susceptibility to this fungus and failed to accumulate camalexin, which is a specific *Arabidopsis* phytoalexin (Saga et al., 2012). Camalexin has protective function against fungal and bacterial pathogens, and *JUB1* was found to participate in biosynthesis of this molecule (Saga et al., 2012). Additionally, this gene was found to negatively regulate senescence, while overexpressorion delayed bolt development, and repressed intracellular H_2O_2 levels along with increase in abiotic stress tolerance. During abiotic stress the JUB1 was found to interact with 5'- RRYGCCGT-3' consensus core sequence of *DREB2A* promoter. DREB2A (DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2) acts as regulator in drought and high-temperature stress (Lim et al., 2006; Liu et al., 1998). Shahnejat-Bushehri et al. (2012) have found that the JUB1 associates with thermomemory genes, such as Heat Shock Proteins (HSP) and Heat Shock Factors (HSF). The heat-stress tolerance is probably regulated via activation of these genes, since *JUB1* overexpression resulted in up-regulation of *HSPs* and *HSFs* (Wu et al., 2012). Although the *JUB1* was found to be slightly influenced by the mutation of isochorismate synthase (*ics1*) gene in response to *Golovinomyces orontii* (Chandran et al., 2009), however, the SA induction–deficient (*sid2-2*) did not alter the induction of *JUB1* in response to Flg22 elicitor compared to the wild type. Our aim was to study the regulation of this *Arabidopsis NAC* gene in response to *O. neolycopersici*, whether its PM-mediated induction is dependent on SA or not.

Materials and methods

Cloning process using Gateway® Technology

For analyzing the regulation of the *JUB1* gene in response to powdery mildew infection, the upstream region of the gene was isolated using PCR with the following primers:

(P15'-TTACAGCGAGGGAGATAATGA-3', P2 5'-TCGATCTCTTTAGAACACCAATCA-3').

The primers were designed based on the sequence of *JUB1* gene (AT2G43000) of The Arabidopsis Information Resource (TAIR) database. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used for the PCR reaction. This enzyme provides blunt end for the amplified fragment, which is required for the process of Gateway Cloning Technology (Invitrogen). The PCR product was loaded on the agarose (1% TAE) gel, excised and isolated back using the Wizard® SV Gel and PCR Clean-Up System of Promega. The fragment was cloned into the pENTR D-Topo vector (Invitrogen), and then subcloned into pGWB633 binary vector (Nakamura et al., 2010) mediated by the LR clonase enzyme of Invitrogen. The recombinant positive binary vector contained the promoter of *JUB1* (*pJUB1*), which was transcriptionally fused to the *gus* reporter gene. The cloning success was confirmed by PCR and sequencing. Additionally, the pGWB633 vector contains the *bar* glufosinate-ammonium

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resistance gene in the T-DNA region as a selection marker of positive transformants.

For control experiments a no-promoter (*pØ::GUS*) fusion was also created in pGWB633, which was also confirmed by PCR and sequencing.

Plant preparation

The GV3101::pMP90 *Agrobacterium tumefaciens* strain was transformed with the recombinant pGWB633 binary vectors containing the *pJUB1::GUS* and *pØ::GUS* fusion. The surviving colonies were checked by PCR, and the positive bacteria were used for transformation of *Arabidopsis thaliana* lines. The seeds of the lines were obtained from Arabidopsis Biological Resource Center (ABRC): three lines were selected for the experiment; wild type (WT), *nim1-1* mutant and *nahG* transgenic line. All the lines originated from Wassilewskija ecotype. The *nim1-1* is defective in SA-mediated signal transduction, while the *nahG* eliminates all the innate SA by constitutively expressing the salicylate-hydroxylase gene (Delaney et al., 1995; Gaffney et al., 1993). If the *pJUB1::GUS* fusion is regulated in response to PM in these lines, the SA is neither the responsible signal for regulation of *gus*, nor of *JUB1*. The *Arabidopsis* seeds were sowed on water-saturated soil and kept on 4ºC for two days. After germination the plants were cultivated in growth chamber for four weeks with the following conditions: 22-24ºC, cool white light, long day illumination, 60% RH. The first bolts were cut back to induce multiple bolt development. The six-week old *Arabidopsis* lines were transformed with the GV3101::pMP90-pGWB633 (*pJUB1::GUS*) and with the GV3101::pMP90-pGWB633 (*pØ::GUS*) after the description of Clough and Bent (1998). After transformation the plants, T0s were transferred back to the growth chamber and cultivated for further weeks till the siliques were ripen. The seeds of T0 plants were harvested and sowed into soil as described above. Two weeks after germination the T1 seedlings were sprayed with 10 mg/l glufosinate-ammonium containing herbicide (Finale). The spray was repeated three times, when the positive transformants were clearly distinguishable from non-transgenic plants. The survived plants were selected and transplanted. The plants were grown to T3 generation to select transformants containing one copy of the transgene in homozygote form. Three transgenic lines for each genetic background (WT, *nim1-1*, *nahG*) were selected for further experiments. The selected T3 plants were used for testing the basal expression of the *gus* and its induction in response to PM infection.

Four-week old transgenic plants were mock-inoculated and inoculated with *O. neolycopersici* following the description of Huibers et al. (2013). The plants were grown in growth chamber (22-24ºC, cool white light, long day illumination, 60% RH) for further two weeks. Fourteen days after inoculation (dai) the PM colonies were visible on the leaves and these leaves were harvested to stain them histochemically.

Histochemical staining of transgenic plants

Histochemical staining was performed after the descriptions of Jefferson et al. (1987), with the modification in the GUS-Buffer: 100 mM phosphate buffer (pH 7.0), 10 mM EDTA, 1% Triton X-100, 0.3% H_2O_2, 0,5 mg/ml X-Gluc/5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt. The leaves were incubated in the GUS-buffer overnight at 37ºC, and on the next day the chlorophyll was removed using 70% ethanol wash. The fungal filaments were stained with cotton blue solution. The leaves were observed using stereo and light microscopes.

DAB-staining of hydrogen-peroxide

Wild type non-transgenic plants were inoculated with *O. neolycopersici* as described above. The accumulated H_2O_2 in response to PM was stained using the method

of Thordal-Christensen et al. (1997). After 8 hour incubation the leaves were dipped into 70% ethanol to remove chlorophyll. The fungal filaments were stained with cotton blue solution. The leaves were observed using stereo and light microscopes.

Results

Histochemical staining of non-infected transgenic plants with all three types of genetic background (WT-*pJUB1::GUS*, *nim1-1-pJUB1::GUS* and *nahG-pJUB1::GUS*) expressed the β-glucuronidase on a basal level, which expression is not tissue specific. The expression of the reporter gene was detected in the root caps (the meristematic zone was free of *gus* expression (Figure 1F, G)), in axillary buds, in the junction of root and hypocotyl, in style tissue of the carpel and at the margins of leaves (Figure 1). The *gus* expression was lacking from the no-promoter (*pØ::GUS*) control plants (Figure 1H).

After the infection of these transgenic plants we observed the colonies covered leaves at 14 dai. The *nim1-1* and *nahG* lines were infected in a higher rate compared to the wild type, since the SA deficiency increase susceptibility to PM (Delaney et al., 1995; Gaffney et al., 1993). The histochemical staining demonstrated that *gus* expression was induced significantly in the infected plants compared to the mock-infected ones. This phenomenon was detected in all three types of transgenic plants, especially in the plants with *nim1-1* and *nahG* genetic background. During microscopic observation of the infected leaves we detected the induction mostly at that area, where the pathogen was in direct contact with the host (Figure 2). The uninfected area displayed basal expression of the *gus*. The no-promoter (*pØ::GUS*) control plants did not display *gus* expression after the infection.

Earlier studies demonstrated the hydrogen-peroxide accumulates in response to biotrophic pathogen invasion (Wang et al., 2009). Therefore, we tested the location of the increased H_2O_2 in wild type non-transgenic plants in response to *O. neolycopersici*. We found that the hydrogen-peroxide accumulated at the site of infection. Microscopic observation demonstrated that the increased H_2O_2 was detected in the epidermal pavement cells and only around the haustorium, in the extrahaustorial matrix (EHM) (Figure 3). Since the *JUB1* promoter was induced in SA signal defective plants, it is probably not regulated by SA. Inversely, the hydrogen-peroxide may be an inducer in response to pathogenic infection, since this molecule was detected at the site of infection.

Figure 1. Expression of pJUB1::GUS *in transgenic* Arabidopsis thaliana *plants without powdery mildew (PM) fungus infection. A) 4-week old seedling leaf margin, B) Axillary bud, C) Junction of hypocotyl and root, D) Stigma and style of the carpel with pollen grains, E) 5-day old seedling cotyledon, F) Root tip, G) Root cap, H) No-promoter* (pØ::GUS) *control plant. Scale bars indicate 100 µm.*

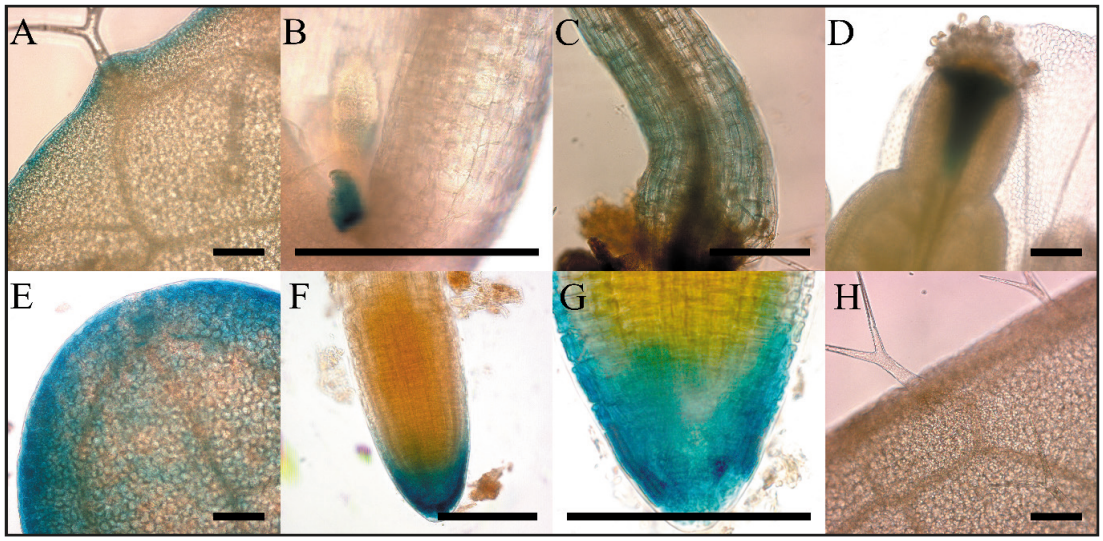

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Figure 2. Stereomicroscopic observation of PM-induced gus *expression in* pJUB1::GUS *transgenic* Arabidopsis thaliana *plants compared to the mock-infected plants. Genetic background of transgenic plants: WT – wild type;* nim1-1 *– non-induced immunity;* nahG *– contains the gene which encodes salicylate hydroxylase. Scale bars indicate 1 mm.*

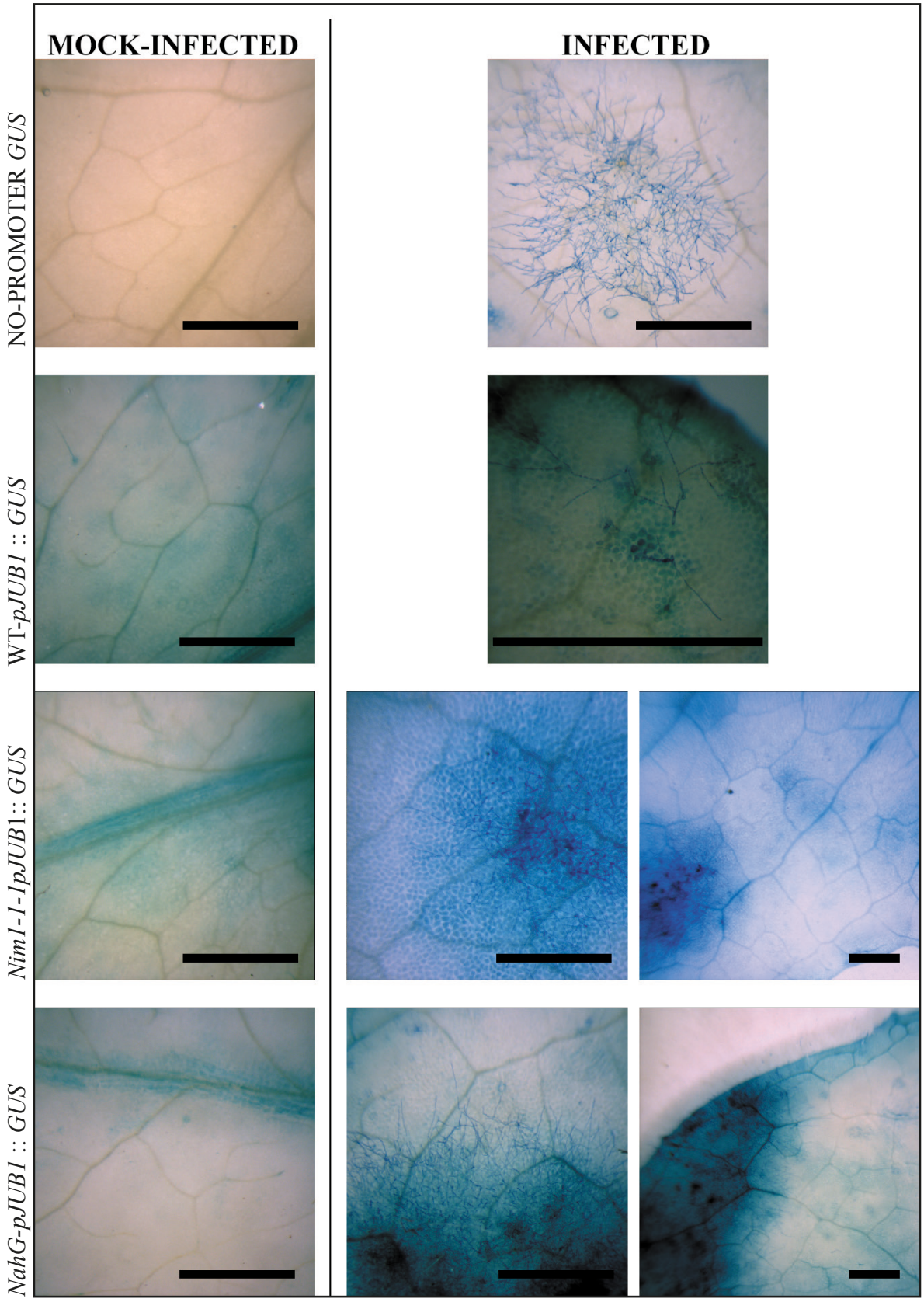
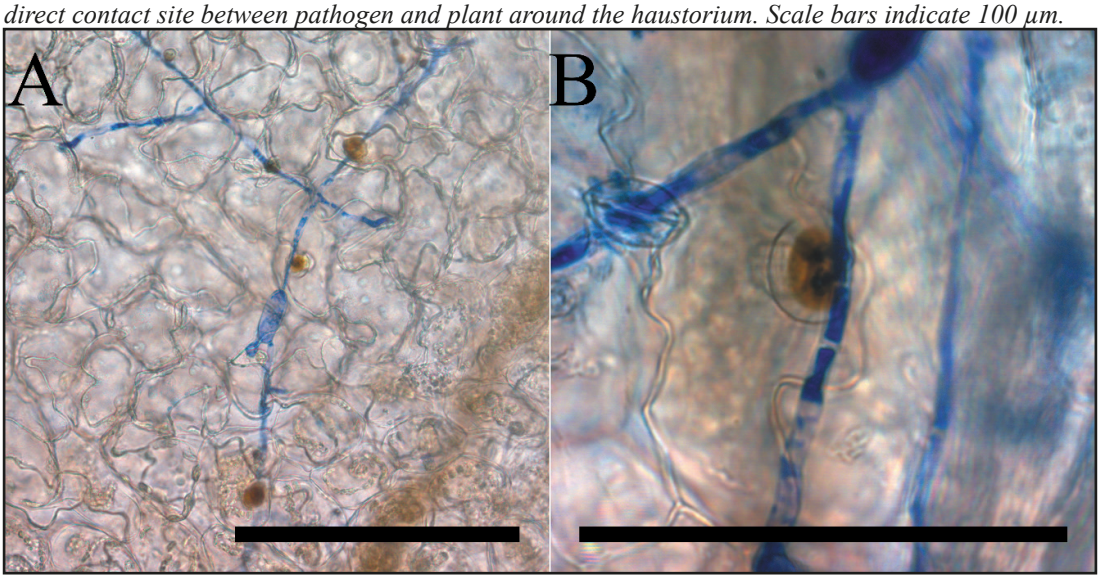


Figure 3. Accumulated hydrogen-peroxide in response to Oidium neolycopersici *infection in* Arabidopsis thaliana*. A) H_2O_2 oxidized DAB (brown stain) only at the area where the pathogen induced the hydrogen-peroxide accumulation. B) The increased level of H_2O_2 was detected specifically at the direct contact site between pathogen and plant around the haustorium. Scale bars indicate 100 µm.*



Discussion

The goal of this study was to observe the regulation of an *Arabidopsis* stress-related transcription factor in response to a biotrophic fungus *O. neolycopersici*. In preliminary experiments we found that the *pJUB1* induces basal expression in various tissues such as in the root cap, in axillary buds, in the junction of root and hypocotyl, in style tissue of the carpel and at the margins of cotyledons and young leaves. This basal expression was detected in all three types of transgenic plants (WT-*pJUB1::GUS*, *nim1-1-pJUB1::GUS* and *nahG-pJUB1::GUS*), meaning that SA is probably not required for this basal regulation.

On the effect of the infection with *O. neolycopersici*, a significant induction of *gus* expression was observed in the infected leaves compared to the PM-free ones, which suggests that this gene probably plays a role in the defense reaction against the pathogen. The induction was observed in all three types of transgenic plants, meaning that neither the SA, nor the nim1-1-mediated SA signal transduction is required for induction by the biotrophic *O. neolycopersici*. Although, the *G. orontii* is also a biotrophic fungus, it was found that the SA biosynthesis mutant *ics1* slightly modulated the induction of *JUB1* in response to *G. orontii* (Chandran et al., 2009). However, the *JUB1* reacted to PM in this mutant, but in a bit lower rate (7-fold) compared to the induction in the wild type (8-fold) (Chandran et al., 2009). Saga et al. (2012) demonstrated that *JUB1* expression was significantly lower in *ein2-1* (ethylene-insensitive) mutant in response to Flg22 in the primary root apex compared to the wild type. This suggests that *JUB1* may be induced by the elicitor via ethylene signaling. Additionally the *JUB1* responded intensively to *Sclerotinia sclerotiorum* to regulate camalexin against the pathogen, but the coi1-2 (coronatine-insensitive) mutation did not influence the up-regulation (Stotz et al., 2011).

Microscopic observation of the infected leaves showed that the induced expression was mostly around the PM colonies, in the haustorium containing plant cells. Recent study has demonstrated that plant cells around the haustoria express a different gene set compared to the uninfected area of the leaf. Interestingly, among these specifically induced genes the most are not previously associated with defense responses. Many of them manage

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endoreduplication process in mesophyll cells, which is activated by the fungus. The multiplication of chromosomes results in overstrain of overall host metabolism, which benefit the fungus survival and reproduction. Interestingly among the specifically induced genes the *pathogenesis-related 1* (*PR-1*) was identified, which participate in SA-mediated defense response (Chandran et al., 2010). However, ortholog of *JUB1* in grape was found to co-express with the *Vitis PR-1* in response to *Erysiphe necator* (Fung et al., 2008).

Earlier results showed that biotrophic fungus infection induce hydrogen-peroxide accumulation at the site of infection (Wang et al., 2009). Observation of infected wild-type non transgenic plants lead to recognize, the increased H_2O_2 is constricted around the developed haustorium, in the extrahaustorial matrix. Based on the phenomenon the H_2O_2 may be the inducer signal of *JUB1* expression, since this molecule also has signaling function during biotic stress (Slesak et al., 2007). The *JUB1* was found to be H_2O_2 inducible along with back-regulation of innate H_2O_2 content (Shahnejat-Bushehri et al., 2012; Wu et al., 2012), which suggests that *JUB1* probably responds to *O. neolycopersici* via H_2O_2 homeostasis. Wu et al. (2012) also represented that *JUB1* functions as a regulator of plant longevity. The overexpression of *JUB1* resulted in delayed senescence and increased cytokinin level, which suggests that this gene is up-regulated at the site of infection to retard aging of the leaf. Additionally, a recent study demonstrated that the highest cytokinin level in the primary root apex was detected in the root cap (Antoniadi et al., 2015). Adding our results to this, the JUB1 may participate in cytokinin biosynthesis. Therefore, during infection, the up-regulation of *JUB1* by the biotrophic fungi may corporate in the well-known 'green island' symptom (Thomas and Ougham, 2014), putatively regulating susceptibility and not defense. Although *JUB1* gene was found to play a role in biosynthesis of camalexin, and it acts as defense gene against necrotrophs (Saga et al., 2012), its activation may benefit the pathogen in the host-biotroph interaction.

Conclusion

The goal of this study was to observe the regulation of the stress-related *JUB1* gene during biotic stress. We have found that *JUB1* is inducible by biotrophic fungus *O. neolycopersici*, and the up-regulation can be detected at the area of the infection site. The expression of *JUB1* is probably dependent on hydrogen-peroxide and not SA homeostasis during the infection. *JUB1* possibly play a role in cytokinin biosynthesis, therefore incorporates in the 'green-island' effect.

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EVALUATION OF DIFFERENT VIETNAMESE SOILS AS POTENTIAL SOURCE OF ARBUSCULAR MYCORRHIZAL FUNGAL INOCULUM IN *CAPSICUM FRUTESCENS*

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Abstract: Consumption of chili peppers (*Capsicum frutescens* L.) represents an important aspect of the daily diet for Vietnamese population because of its high content for antioxidant compounds. To increase the economic benefits related to chili peppers cultivation and reduce negative impacts of the high input agriculture on the environment, biological alternatives to chemical fertilizers are strongly demanded. Arbuscular mycorrhizal fungi (AMF) are well-known soil microorganisms of great interest for their potential application in agriculture as 'bio-enhancers' of plant performance. However selection of suitable AMF strains is time-costing and the outcome of field inoculation can be affected by the weak ability to compete among the native AMF population. In the present study we proposed a "bulk" approach to identify soil hosting AMF strains suitable for the development of inocula for *C. frutescens*. Three different soils were tested as source of AMF inoculum in bi-compartmented pot cultures. All the inoculated treatments performed significantly better, in terms of plant growth, compared with the non-inoculated control plants. Pots inoculated with soil from tropical forest showed the best growth performances. Molecular characterization of the AMF root assemblages highlighted differences in the composition among treatments, with the "tropical forest soil" treatment characterized by the highest number of AMF taxa colonizing the roots.

Keywords: arbuscular mycorrhizal fungi; chili pepper; *Capsicum frutescens* L.; Vietnam; inoculation; bi-compartmented pot culture

Introduction

Chili peppers belong to the plant genus *Capsicum* (family Solanaceae) and are among the most largely consumed spices throughout the world. In Vietnam, chili peppers (*Capsicum frutescens* L.) play an important role in the daily diet of the population because of the high content of vitamins, minerals such as Ca, P, K, Fe, and the antioxidant capsaicin responsible for the pungent taste (Malik et al., 2011). Vitamins such as C, E, pro- vitamin A and B are present in high concentrations in various chili pepper types (Howard et al., 2000; Bae et al., 2012). The intake of these antioxidant compounds in food is an important health-protecting factor when they are taken daily in adequate amounts (Sies, 1991).

Because of their importance on the market chili peppers provide high economic returns to farmers and thereby contribute to the Gross Domestic Product (GDP) of Vietnam, with 4% of the agricultural GDP (Office of Ministry of Agriculture and Rural Development). To increase yield of chili peppers most farmers use chemical fertilizers, but their high price significantly reduces their profit. Furthermore high input agriculture practices have a considerable negative impact on the environment and represent a matter of concern for the health of consumers (Van Bruggen, 1995; Atkinson et al., 2002).

An increasing demand for biological alternatives has promoted the interest on application studies of the subset of soil microorganisms known to improve plant growth and health (Willis et al., 2013). Some of them play an important role in the rhizosphere, influencing the nutrient acquisition by the plant. Among the soil biota responsible for key ecological services, arbuscular mycorrhizal fungi (AMF) occupy a

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special ecological niche because they represent the most ancient and widespread symbiosis involving 70–90% of land plant species (Parniske, 2008). The majority of agricultural crops have the potential to host AMF as root symbionts (FAO, 2012), with benefits from improved mineral nutrient uptake in exchange of photosynthetates. In addition to enhanced plant productivity AMF can provide increased resistance to soil pathogens, to abiotic stress factors like drought, salinity and heavy metal toxicity and contribute in improving soil structure (Jeffries et al., 2003).

Over the last decades, application of AMF in agriculture has increased greatly and a significant effort has been dedicated to develop suitable formulations for fungal propagules (Gianinazzi and Vosatka, 2004). However the application of AMF inocula on a field scale can be problematic for the unpredictable outcome of the establishment among the native AMF community in concurrence with unfavourable edaphic factors (Berruti et al., 2014). The use of an inoculum based on locally sourced AMF might be a suitable choice because of a better adaptation to the environmental conditions (Lambert et al., 1980), avoiding at the same time the ecological risks of the introduction of foreign species (Schwartz et al., 2006). Furthermore even though experiments with cultured isolates suggested AM fungi had very low host plant specificity (Klironomos, 2000), recent findings highlighted preferential host-symbiont associations as a factor to consider for a successful outcome of inoculation (Magurno et al., 2015). In the present study we propose a "bulk" approach to identify soil hosting AMF strains suitable for the development of inocula for *C. frutescens*. The use of AMF natural consortia from soils of different origin, instead of single AMF isolates, was chosen in order to:

- avoid the time-costing step of isolation of single AMF strains
- associate the beneficial effects on the plant growth to AMF strain/strains competitive in the root colonization

Materials and methods

Pot design

Three types of soils were chosen as a source of AMF inoculum, following a land-use gradient. Soil from tropical forest (FS) was sampled at Nam cat Tien (11°23'15.0"N; 107°28'05.0"E) in Dong Nai province, Vietnam. The soil was described as sandy and clay-rich. The dominant resident plant was *Tetrameles nudiflora*, a large deciduous tree species widespread in Southeast Asia. Agricultural (AS)and grassland (GS) soils, both sandy, were sampled at the district of Ho Chi Minh City (10°53'17.2"N; 106°40'03.1"E), Vietnam, from a field cultivated with cassava (*Manihot esculenta*) and from an adjoining meadow. All samplings were performed on March 2013, by collecting five soil cores per soil type at a depth of 30 cm. Cores from the same soil type were grinded and mixed together homogeneously.

In order to evaluate the contribution of the AMF consortia to the plant growth the tests were carried out in plastic pots (18cm x 18cm x14cm) divided by a nylon mesh filter (40µm pore size) in two compartments (Figure 1). Peat, mixed with sand, was used for its ability to retain moisture and as source of mineral nutrients at low concentration. The mixture of sand-peat was autoclaved at 121˚C for two hours and its pH was adjusted to 6.5. The upper part was filled with sand and peat with a ratio 5:1, while the bottom part was filled with sand and peat with a ratio 2:1. The bottom compartment was inoculated with 45 g of soil (1.5% w/w) close to the interface with the upper compartment. Because of the size of its pores the nylon mesh filter was representing a physical barrier for the roots to spread into the bottom compartment where peat was present at higher concentration while AMF hyphae could have access to both compartments (Smith and Read, 2008).

Five pots were prepared for each soil type and five pots without soil inoculation were added to the trials as control (CON). Totally 20 pots were used in the experiment.

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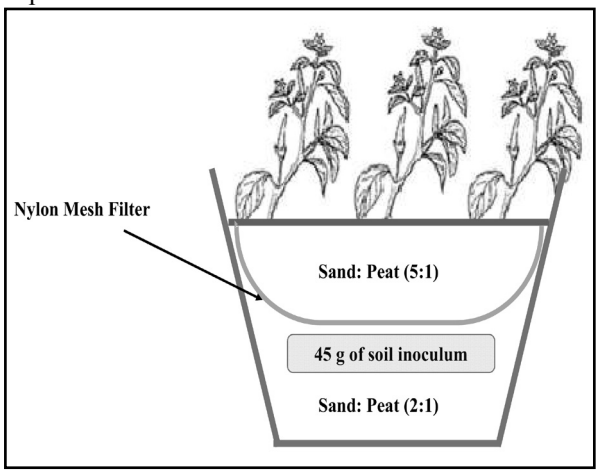
Figure 1. Schematic representation of the bi-compartmented pot culture system adopted in the experiments



A nylon mesh filter (pore size 40 µm) was used to separate two compartments with different amounts of peat as source of nutrients. The soil, source of AMF inoculum, was mixed with the substrate in the top part of the bottom compartment. Pots were planted with 3 seedlings/pot of *Capsicum frutescens* L. in a way that roots could spread only in the upper compartment with low peat content. The access to the peat present in higher concentration in the bottom compartment was achieved by the establishment of symbiosis with AMF hyphae (diameter 2-10 µm) free to move across the mesh membrane.

Plant growth conditions

Seeds of *Capsicum frutescens* L. were collected at The Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City (Vietnam). Seeds were surface sterilized with alcohol 80% and pre-germinated in Petri dishes under moist conditions. When two cotyledons appeared, after about three days, seedlings were transplanted into pots (three seedlings per pot). After transplanting seedlings were watered with Long Ashton nutrient solution for 7 days and subsequently with distilled water. Pots were kept in a climatic chamber EKOCHL 1500 (18/24°C, 60% RH, 16h light) for 14 weeks.

Spore counts and assessment of root colonization

Spore abundance was measured in the soils used for inoculation (FS, AS and GS) and in the substrate of the pots after plant harvesting. AMF spores were isolated in 3 replications from 35 g of air-dried soil/pot-substrate by wet sieving through 200 and 30 µm sieves, followed by sucrose gradient centrifugation (Ianson and Allen, 1986). After centrifugation, spores were transferred into Petri dishes and counted under stereomicroscope at 100X magnification. Spore abundance was expressed as the number of AMF spores per gram of soil.

To evaluate the level of root colonization, fifteen root fragments (1 cm long) from each plant were collected, washed with tap water and stained with trypan blue (Trouvelot et al., 1986). Root pieces were observed under stereomicroscope at 100X magnification and the root colonization was determined according to Trouvelot et al. (1986), using MYCOCALC software.

Agronomic variables

During the growth period, shoot length was measured at an interval of two weeks. After 14 weeks, plants were harvested paying attention to not damage the root system. Because of the extremely low nutrient conditions it was not possible to reach the flowering stage. The following variables were recorded: a) shoot length, b) root length, c) shoot dry weight, d) root dry weight.

Root DNA extraction, PCR and cloning

Molecular analyses were performed on one plant per treatment (FS, AS, GS, CON) chosen according to the best growth performances. DNA was extracted from three root fragments (1 cm long) for plant following the protocol described by Khan et al. (2007).

The AMF species composition inside the roots was analyzed by a PCR approach targeting a portion of the ribosomal Short Sub Unit (SSU). PCR was performed using the primers AML1 (5'-ATC AAC TTT CGA TGG TAG

GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3'), specific for Glomeromycota (Lee et al., 2008).

Amplifications were carried out using the Phusion High-Fidelity DNA Polymerase (Thermo Scientific) with the following thermal profile: initial denaturation at 98°C for 1 min, followed by 35 cycles at 98°C for 10 sec (denaturation), 64°C for 15 sec (annealing), 72°C for 24 sec (extension), followed by a final extension at 72°C for 5 min.

PCR products were analyzed by gel electrophoresis. Bands at the expected size of 800 bp were cut out and DNA was extracted with the Illustra GFXTM PCR DNA and Gel band purification kit (GE Healthcare Life Sciences) according to the manufacturer's instructions.

Purified DNA fragments were cloned into CloneJET™ PCR Cloning Kit (Thermo Scientific) and transformed into *Escherichia coli* DH5α according to the manufacturer's instructions.Transformants were checked by PCR for the presence and size of the insert.

Restriction fragment length polymorphism analysis

Positive clones were analyzed for restriction fragment length polymorphism (RFLP) by digestion with Hinf*I* (Promega) and electrophoretic run on 2.5% TBE agarose gel. Representative clones were selected for each restriction profile found. Plasmids were extracted with the Wizard® Plus SV Minipreps DNA Purification System Kit (Promega) and sent for sequencing to Biomi Ltd (Agricultural Biotechnological Center, Gödöllő).

Phylogenetic analysis

Sequence similarities were determined using the blastn sequence similarity search tool provided by GenBank. Only sequences belonging to Glomeromycota were selected for the subsequent analyses and the others were discarded. Sequence editing was conducted manually using MEGA 4.0 (Tamura et al., 2007) and Chromas Lite 2.01. Sequences were aligned by MUSCLE with reference sequences identified with blastn and sequences representing the major taxonomic groups of Glomeromycota. Phylogenetic tree inference, using neighbour-joining method, was computed with MEGA 4.0 software assessing Kimura-2p model as distance method and 1000 replicates of non-parametric bootstrapping.

Statistical analysis

The data about the agronomic variables measured, spore abundance and root colonization were analyzed by SPSS software version 20 (IBM). The homogeny of variance of data were verified using Levene's test. When the p-value of the Levence's test was found to be less than 0.05, data were analyzed with Kruskal-Wallis test for non-parametric test.

In addition, Bonferroni test was used for post-hoc testing for multiple comparisons of the data measured. When the p-value of the Levence's test was found to be higher than 0.05, data were analyzed with ANOVA one way test and Tukey test was used as post-hoc test for multiple comparisons of the data measured.

Results

Spore counts and assessment of root colonization

Before substrate inoculation spore abundance was measured in the three different soils chosen as source of AMF inoculum. The amount of spores (average of three independent replications) observed per gram of soil was 4.34±1 in the tropical forest soil (FS), 6.26± 1 in the agricultural soil (AS) and 8.57±2 in the grassland soil (GS).

After plant harvesting the sand-peat substrate was collected and the spore abundance measured. In the control pots no spores were detected. Similar spore abundance was found among the different treatments (1.2 spores/g). Bonferroni test confirmed no significant differences among the three sets of pots

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inoculated with different soils.

After staining no root colonization was found in the roots from the control plants. Fungal mycelium and structures were observed in roots from all inoculated pots. Among the treatments FS showed the highest percentage of root colonization (81.78%) followed by GS (63%) and AS (56%). According to Bonferroni test FS treatment was significant different from AS and GS (Table 1).

Table 1. Percentage of AMF colonization in chili pepper roots. The percentages represent the average of the values measured for all the plants in every treatment. Standard deviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Treatment	Root colonization (%)
CON	0^{c}±0
FS	81.78^{a}±13.91
AS	56^{b}±11.75
GS	63.4^{b}±14.43

Table 1. Percentage of AMF colonization in chili pepper roots. The percentages represent the average of the values measured for all the plants in every treatment. Standarddeviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Plant growth

Shoot length. The plant growth after two weeks started to show a perceptible change among the treatments. At the moment of the plant harvesting, after 14 weeks, the average shoot length of FS plants was the highest (14.32 cm) followed by GS (11.57 cm), AS (11.01 cm) and CON (6.18 cm) plants respectively (Table 2). Bonferroni test showed that the CON plants were significantly different ($p< 0.05$) from the inoculated plants belonging to the FS, AS and GS treatments. According to the test the FS plants were significantly higher than AS and GS plants. No significant differences were observed between AS and GS treatments (Table 2).

Table 2. Growth of chili pepper plants measured in the assay after 14 weeks. Values are given as average of measures collected among all the plants for each treatment. Standard deviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Table 2. Agronomic variable of chili pepper plants measured in the assay after 14 weeks. Values are given as average of measures collected among all the plants for each treatment. Standard deviation is provided with the values. Shared uppercase letters indicate no statistical difference (p>0.05) between the treatments, as determined by Bonferroni test. CON: control plants; FS: plants inoculated with forest soil; AS: plants inoculated with agricultural soil; GS: plants inoculated with grassland soil.

Treatment	Shoot length (cm)	Root length (cm)	Dry shoot weight (mg)	Dry root weight (mg)
CON	5.18^{c}±1.9	9.88^{a}±0.09	20^{c}±7	10^{c}±3.5
FS	14.32^{a}±1.15	13.43^{a}±0.02	74^{a}±13	26^{a}±3.3
AS	11.01^{b}±3.09	12.70^{a}±0.02	47^{b}±14	20^{b}±2.2
GS	11.57^{b}±2.69	12.44^{a}±0.02	45^{b}±12	18^{b}±1.9

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Root length. The assessment of root length after 14 weeks showed a positive effect of the substrate inoculation on the root growth. The average root length of FS plants was the highest (13.43 cm) followed by AS (12.70 cm), GS (12.44 cm) and CON (9.88 cm) plants respectively (Table 2).Bonferroni test showed no significant difference among the different types of inoculation ($p>0.05$).

Dry shoot and root biomass. The dry shoot and root weight averages measured after 14 weeks confirmed the trend seen on the shoot length measurements. The weight of inoculated plants were significantly higher compared to the control plants. FS plants had significantly higher shoot and root dry biomass (74 and 26 mg respectively) than the plants belonging to the AS and GS treatments (Table 2). No significant differences were observed between AS and GS treatments.

Molecular analysis

DNA was extracted successfully from all the root samples (4).

Amplification from FS, AS and GS DNA gave an expected product of approximately 800bp while no amplicons were detected for CON DNA. After cloning a colony screening was performed to obtain 20 clones per treatment, positive for the PCR insert. The PCR products digested with Hinf*I* resulted in a total of six restriction profiles (RP1-6). The restriction profile 5 (RP5) was the most abundant, with 52 clones of 60 clones analyzed. All six restriction patterns were represented among the clones analyzed for the FS treatment. In the treatment AS only two restriction patterns were found as well as in the treatment GS. Fourteen clones, representative of the six restriction profiles, were sent for sequencing. The sequences were deposited at the National Center for Biotechnology Information (NCBI) GenBank with accession numbers KU195704-KU195714.

Phylogenetic analyses

After editing, sequences were analyzed by blastn. Three sequences, representing two restriction profiles (RP3 and RP4), were discarded because they were related either to plant or Basidiomycota DNA.

All four AMF restriction patterns were represented by the FS clones. Among the GS and AS clones only two (RP2 and RP5) and one (RP5) restriction patterns, respectively, were found. Eleven AMF sequences were used to build a phylogenetic tree (Figure 2). Sequences related to the restriction patterns RP5 and RP6 clustered inside the Claroideoglomeraceae family with the exception of one clone RP5 clustering with RP2 inside the *Gigaspora* genus. RP1 clustered with the reference sequence from *Glomus macrocarpum* in the family of Glomeraceae.

Discussion

The pot culture system adopted in the present study represented a challenge for the plants to survive. With the exception of the first week, when Long Ashton nutrient solution was provided, the only source of nutrients was provided by peat mixed to sand with different ratio in the two compartments (sand-peat 5:1 and sand-peat 2:1 respectively). Nevertheless, all the control and inoculated plants survived for the complete duration of the experiments. As expected, because of the extreme deficiency of nutrients, it was not possible to reach the reproductive stage of the plants, even after 14 weeks of growth.

The nylon mesh filter was used with the aim to give access to the high-peat-content compartment only to those plants able to establish a symbiotic relationship with AM fungi whose hyphae (diameter = 2-10 μm) could cross the membrane.

In the inoculation treatments AMF spores of new formation were found in both compartments of the pots and the plant roots showed a certain degree of mycorrhization, indicating that the experiment was successful in the establishment of AMF symbiosis. Even

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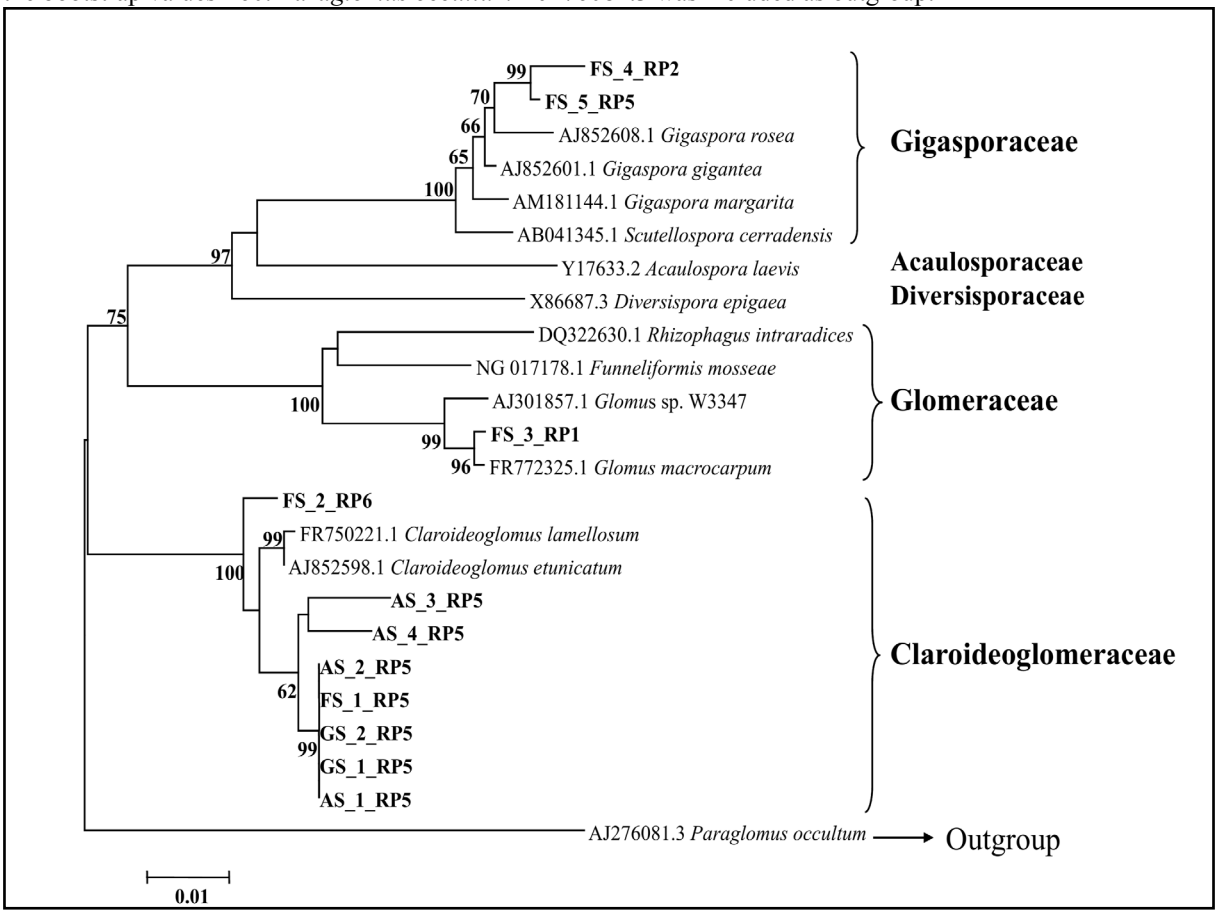
Figure 2. Neighbour-joining phylogenetic tree displaying the relationship between the 11 AMF sequences recovered from chili pepper roots and 13 reference sequences from GenBank, representing some of the main Glomeromycota families. Sequences obtained in this study are in bold and labelled with the corresponding restriction pattern and soil of provenience (FS: forest soil, AS: agricultural soil, GS: grassland soil). Numbers next to the nodes indicate the bootstrap values >60. *Paraglomus occultum* AJ276081.3 was included as outgroup.



if the soil source of AMF inoculum was placed in the bottom compartment the distance from the developing roots was not preventing the establishment of symbiosis. In a previous study extensive mycelial growth was observed in the absence of the host plant with germinating hyphal length up to 5 cm 15 days after spore germination (Logi et al., 1998).

During the 14 weeks, a significant difference in plant growth between the inoculation treatments and the control was observed.

This result indicated that the plants in the FS, AS and GS treatments could receive a better nutrition through the AMF mycelium spread in the compartment rich in peat content, where the plant roots could not have had access.

Plants from the FS treatment showed overall a better development compared to the plants from other treatments, directly correlated with the highest percentage of root colonization (≈ 82%). Generally a strong mycorrhization is associated with a reduced root system because the plant, supplied with nutrients by the AMF mycelium, does not need to invest resources in the roots development (Bonfante and Perotto, 1995). In the present study this was not observed probably due to the deficit of nutrients in the pot system.

Considering the spore abundance measured in the different soils before inoculation, the best growth performances of FS plants appeared not to be correlated merely with the number of spores present in the inoculum. Therefore the identity of AMF strains colonizing the roots could have played an important role in the outcome of the symbiosis.

Specific primer AML1 and AML2 were used to verify the presence and identify of the AMF taxa in the plant roots. All the treatments inoculated gave a positive PCR product and, as expected, no amplification was detected in the control plant roots. The phylogenetic analysis showed a good correlation between the restriction patterns observed and the phylotypes identified. Sequences were distributed in three families even if most of them, associated with two restriction patterns, clustered in the family of Claroideoglomeraceae.

In the FS treatment (inoculated with tropical forest soil) the highest number of taxa (4) was found, followed by GS (2) and AS (1) treatments. These results are in agreement with the data reported by Öpik et al. (2006), where the number of AM fungal taxa per host species differed between habitat types: the highest richness belonged to tropical forests (18.2 AMF taxa per plant species), followed by grasslands (8.3), temperate forests (5.6) and habitats under anthropogenic influence (arable fields and polluted sites, 5.2). In our assay molecular analyses were limited to a low number of clones (20 per treatment) thus our data could just resemble the richness gradient, not the taxa numbers, mentioned above.

Considering the distribution of clones among the phylotypes detected, it was not possible to recognize exclusive dominant taxa associated with the best plant growth performances observed in FS plants. In fact the same phylotype belonging to the Claroideoglomeraceae family was strongly dominant in all the treatments. However, the molecular target used for AMF identification does not allow a deep resolution at species/strain level. It was demonstrated that some functional traits could be different among strictly related species and among strains belonging to the same species (Takács et al., 2006). On this point of view, it could be hypothesized that the highest root colonization, as well as the growth response observed in the FS plants could be explained by species or strains different from those present in the AS and GS plants, even if belonging to the same AMF genus.

Furthermore, one of the two FS sequences clustering in the Gigasporaceae was associated to the dominant restriction profile RP5, mainly related to the Claroideoglomeraceae family. As a consequence an underestimation of the abundance of FS clones related to the Gigasporaceae family could have occurred. Members of the *Gigaspora* genus colonize soil more extensively than plant roots, thus their contribution to plant nutrition should not be correlated with the percentage of root colonization (Hart and Reader, 2002).

Conclusion

We described here an effective methodology to test and select useful AMF strains under controlled conditions. Contrary to the classical procedures of developing formulations for AMF propagules, we proposed an approach involving uncharacterized AMF assemblages with the aim to evaluate beneficial effects for the plant growth and ability of the AMF strains to compete in the root colonization in the same trial. Therefore this experimental system, when applied on a large scale with the purpose of commercial inocula development, could be suitable to test a high number of AMF strains at the same time, avoiding the time-costing step of strain isolation and propagation. The system we proposed could be applied also for other tests beyond those related to nutritional benefits of the symbiosis.

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HUMIC SUBSTANCES APPLICATIONS IMPACT QUALITY AND YIELD OF COMMERCIALLY-PRODUCED POMEGRANATE SAPLINGS IN NANGARHAR, AFGHANISTAN

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Abstract: The efficacy and reliability of humic substances for increasing crop yields have not been widely established in the scientific literature. The aim of this research was to measure the effect of humic and fulvic acid application on the number of harvested pomegranate saplings, which meet the required standards and to compare it with saplings produced without humic substances. The application of humic substances increased by 34% the number of harvested pomegranate saplings meeting the requirements of the established standard. It improved quality characteristics of the saplings as well, such as increased weight and volume of the root system and increased diameter and height of the plant.

Keywords: Humic acid, nutrient uptake, yield components, saplings quality standards

Introduction

Pomegranate (*Punica granatum*) production is a significant contributor to the Afghan agricultural economy and a significant source of income for farmers and rural communities of many provinces. However, varietal and nursery technology degradation have been caused by years of war. For example, in Nangarhar Province, commercial fruit nurseries have been in operation just since 2006. That is why a survey study for that year indicated that only 45% of the saplings were acceptable for transplant (Sandor 2007). The large majority of unacceptable saplings were too small to transplant. Climatic and soil conditions in this Eastern region are challenging, characterized by low average annual precipitation, high temperature during the growing season and limited soil development. The use of biostimulants such as humic substances may significantly improve plant growth and yields.

Diercks (1983) concluded that the way to increase soil nutrient capacity is to introduce organic matter bound nutrients into the soil or to mix free nutrient based fertilizers with organic carbon compounds. He emphasized that in order to maintain the long-term balance of the soil and surrounding ecosystems, agriculturalists should pay greater attention to the practical application of organic manure and humus substances; a practice that has been historically neglected. Because of its molecular structure, it provides numerous benefits to crop production. Humic acid is the end product of organic matter decay and is primarily found in manure, peat, lignite coal, and leonardite. Humic acid is characterized by forming chelates with metallic micronutrients, iron, copper, zinc and manganese (Kussow 2002). The cation exchange capacity (CEC) of humic acid is in the range of 500 to 600 milliequivalents per 100 grams. This is about five times greater than the CEC of good quality peat moss and twice higher as the CEC of soil humus (Bigman 1996, Stevenson et al. 1982). Albayrak and Camas (2005) indicated that humic acid significantly affects most yield components. Root and leaf yields and their yield components increase along with increase rates of humic acid. Further studies by Kotob (2009) show that humic acid applications

mitigate the harmful effects of salinity and enhance seedling emergence and plant growth.

Katkat et al. (2009) published their results of a humic acid experiment with wheat indicating that humic acid applications increase dry matter and N, P, K, Ca, Mg, Na, Fe, Cu, Zn and Mn uptake of plants in non-limed pots at 0.1% rate of humic acid. Higher rates, such as 0.2% was found much more effective on increasing dry matter and nitrogen uptake at high lime conditions. The foliar application of humic acid significantly affected Mg, Fe and Mn uptake with the highest dry matter accumulation and nutrient uptake obtained at the rate of 1g kg-1 of humic acid treatment. Khattab et al. (2012) indicated that the use of humic acid and amino 3 acids enhanced vegetative growth and fruiting in pomegranate production. In contrast, Mackowiak et. al. (2001) were unable to show any increase in wheat yield caused by humic acid applications, but successfully cured nutrient deficiency symptoms in the crop. Timothy and Bottoms (2010) reported similar results regarding phosphorus deficiency in lettuce and tomato. Cimrin and Yilmaz (2005) established that additional phosphorus application into the soil increased the uptake of nitrogen in the plant but they could not observe this in the humic acid treatment.

The main purpose of this study was to measure yield and quality changes in commercial pomegranate sapling production as affected by applications of humic substances. Also studied

Table 1. Comparison between reference values and average soil test results

Characteristic	Measured Value	Reference Range	Description	Conditions
$CaCO_3$ (%)	10.56	5,0-19,9	Moderately Alkaline	
K_A	35	30-38	Sandy-Loam	
pH_{H2O}	8.14	7.2-7.9	Moderately Alkaline	
Salt (%)	0.02	<0.05	Low	
P_2O_5 (mg/l)	47.03	26.0-50.0	Moderate	If $CaCO_3$ >1% and K_A value is >30
K_2O (mg/l)	129.5	101-160	Moderate	If K_A value is >30
NO_3-N (ppm)	3.5	<10	Low	-

Table 2. Methods for the pomegranate saplings production in the experiments

Characteristics	Description
Land Area	2.0 ha
Crop	Pomegranate saplings
Date of planting	March 2007 (Year 1) and 2008 (Year 2)
Date of harvesting	December 2007 (Year 1) and December 2008 (Year 2)
Planting distance	70 x 10 cm
Physical layout	Ridges
Starter fertilizer	150 kg DAP/ha and 75 kg urea/ha
Top dressing	May-June 2007 – 50 kg DAP/ha and 25 kg urea/ha by side dressing method
Water source	Canal (River)
Irrigation method	Furrow
Irrigation efficiency	40.08%
Irrigation interval	From 4 to 7 days
Weeds control	During growing season weeds were controlled by mechanical methods (hand labor)

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were changes of the soil physical and chemical properties and soil nutrient availability when compared to the baseline soil test.

Materials and Methods

The humic and fulvic acid used in this experiment was produced by Farmfert Formulators INC from South Africa and registered under the code number PCT WO 2006/092720AI. The four farms of the experiments were located in Behsod District, Nangarhar Province in the Eastern Region of Afghanistan. The water table can be found between 4 to 6 m. The initial soil characteristics of the four farms showed little differences. The soil pH is slightly-moderately alkaline with low salt accumulation. The soil was developed under forest and has a tendency to become sodic. The cation exchange capacity value indicates the dominance of 2:1 type secondary clay minerals, mainly Montmorrilonit and Smectit. The value of exchangeable sodium is very low. The phosphorus and potassium content in the soil is moderately good (Table 1).

The experiments were conducted in commercial pomegranate nurseries managed according to traditional production techniques described in Table 2.

For propagation we used simple non-rooted cuttings containing 5-6 buds each. The selected branches for cuttings were purchased from a commercial nursery located in Kandahar Province, Afghanistan. The cuttings were a year old, approximately 25-30 cm in length, populated with buds approximately 5 cm apart, and the resulting cuttings had a green cambium ring inside indicating that they were alive and healthy.

The four experiments consisted of two randomized treatments with four repetitions each within the four pomegranate nurseries (0.4 ha each) managed under traditional production methods. Additionally to the 4 treated plots per experiment, each one of them included one control (untreated) plot. In 2007, the four future experimental areas were standardized to a uniform production method (Table 2) and baseline data was collected. We used these data as a background information for the four experiments conducted in 2008. Standardization of the experimental areas was important to reduce the impact of soil neglect caused by more than 20 years of internal war. In 2008, the experiments followed the standard production method established in 2007 and treated plots received two applications of humic acid applied in shallow furrows with 15 cm distance from the plants. The application depth was 10 cm. The humic acid solution contained humic acid powder (50% concentration) mixed with water using 1:8 ratio during the preparation. The total application rate of humic acid was 100 kg/ha. The first soil application (50 kg/ha) occurred 60 d after planting and the second at d 90 at a rate of 50 kg/ha. After 120 d of planting we applied fulvic acid (10 l/ha) on the forming canopy of the saplings using a backpack sprayer. The saplings were harvested after 210 day.

During the field trial we conducted weekly measurements of sapling stem diameter and height of 20 saplings from treated and untreated plots. The sampling selection method is known as a point-intercept transect (laying down the center of a quadrat over each point of a line transect and then counting every plant inside the square) using transect tools, which are straight lines typically established through the use of a cord, wire or measuring tape, in this case a metal "Z" frame with a one meter length. The data was measured from the plants between the two ends of the frame. Harvested saplings that meet acceptable quality standards for commercial use were defined as those meeting a minimum height of 0.8 m and minimum diameter of 8-9 mm (Cselòtei at al. 1985). The diameter of the 10 saplings was measured at three levels: root-stem transition region, stem midsection, and upper-third section. In addition, 10 plants per plot were measured for root weight and quantity after sapling harvest. Roots were separated from soil using a 6 mm sieve.

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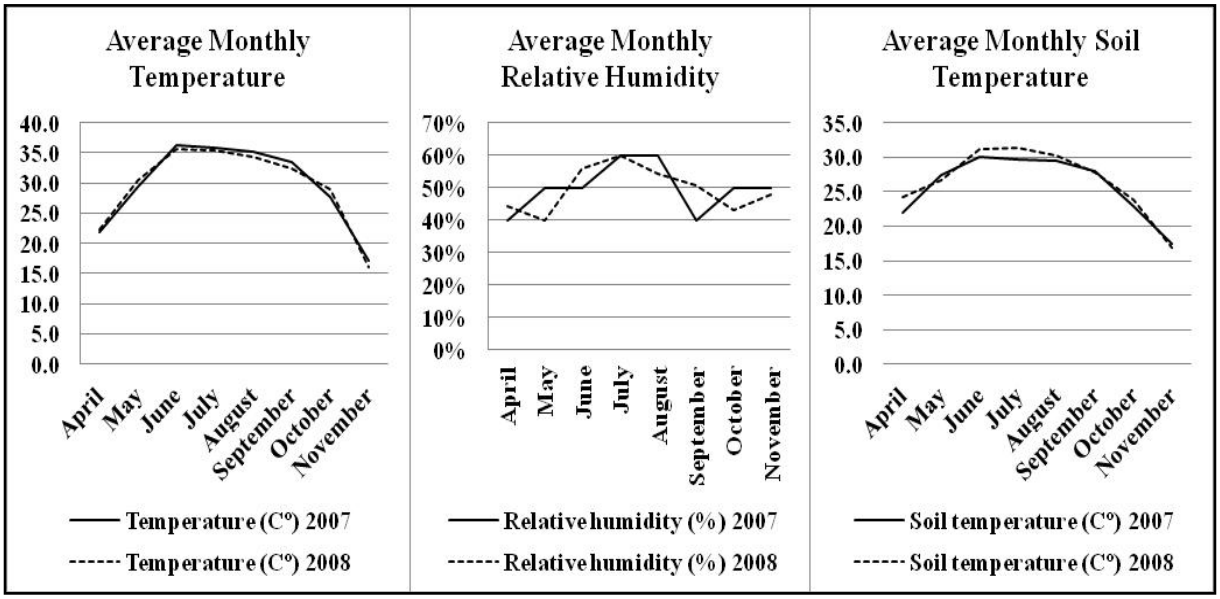
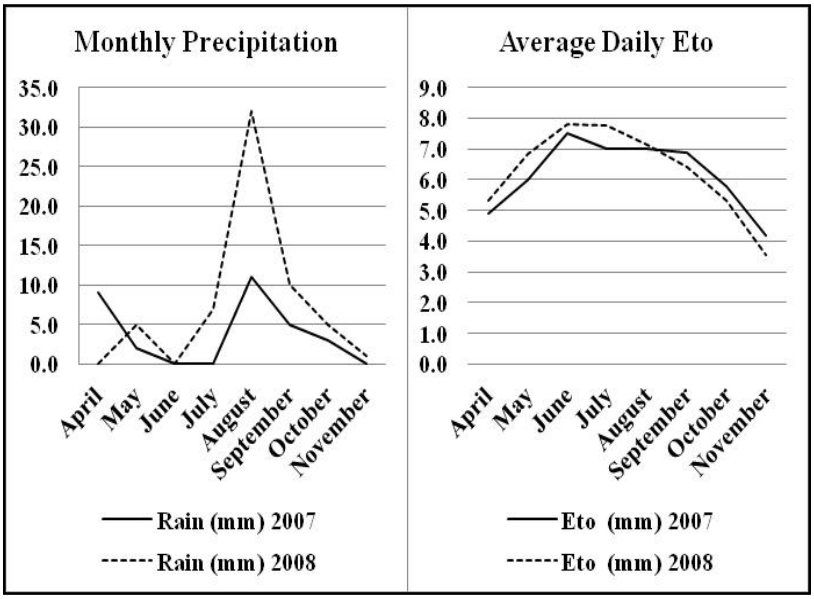
Table 3. Methods used for testing the soil samples from the area of the experiment

HACH Soil Testing Methods
Calcium sulphate extraction and cadmium reduction method for $N\text{-}NO_3$
Mehlich 2 extraction and ascorbic acid method for PO_4^-
Mehlich 2 extraction and tetraphenylborate method for potassium
Aqueous extract and electrode method for pH
Aqueous extract and electrode method for EC and salinity (Twin Cond B-173)

Soils were sampled twice: before and two months after the humic substances applications. Tests were performed to determine the main changes in the soil as a result of the treatment. The tests were conducted using HACH kits (Table 3). The soil samples were taken from 0-15 cm, 15-30 cm, and 30-45 cm depth. We registered local climatic data from the experimental site (Figure 1) using a Min Max Thermometer of 25 cm, an Outdoor Metal Barometer of 16 cm, a Class A Evaporation Pan, Rain Gauge Standard, and Soil Probe Thermometer.

Figure 1. Climatic conditions in the area of the experiment (Samarkhel, Afghanistan)





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The climatic conditions in 2007 and 2008 were similar which allowed for comparison of experimental data from both years. The analysis included the comparison between the baseline data from 2007 and untreated saplings data from 2008. Resulting data were processed using the Student t-test to examine characteristics of the pomegranate saplings. Additionally, the results of the four experiments were tested with one and two-factor analysis of variance (ANOVA) and Fisher test. The differences between the means of the samples were analyzed with the standard error of the mean which is a good estimate for standard deviation of a large number of samples drawn from the population. In order to compare the effects of the humic substances treatment, the differences between the samples means were compared with the value of the $SD_{5\%}$ and a 95% confidence interval.

Figure 2. Average monthly values between the saplings height of treated (Test) and untreated (Control) plants

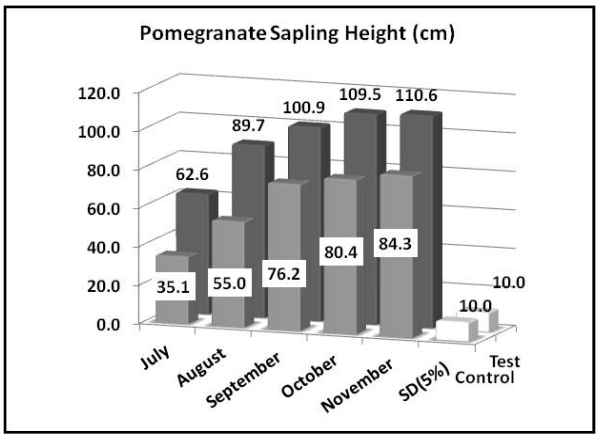
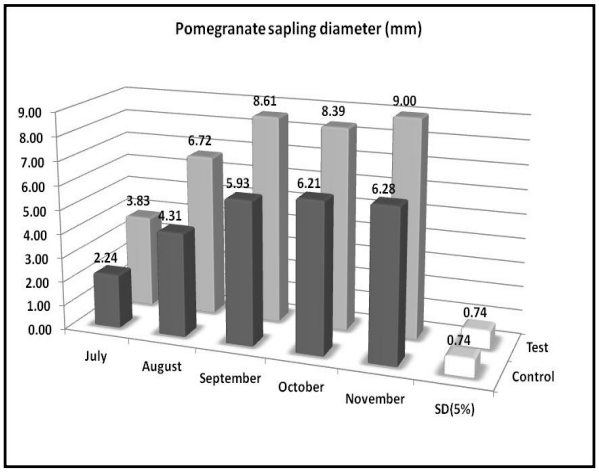


Figure 3. Average monthly diameter values of treated and untreated pomegranate saplings



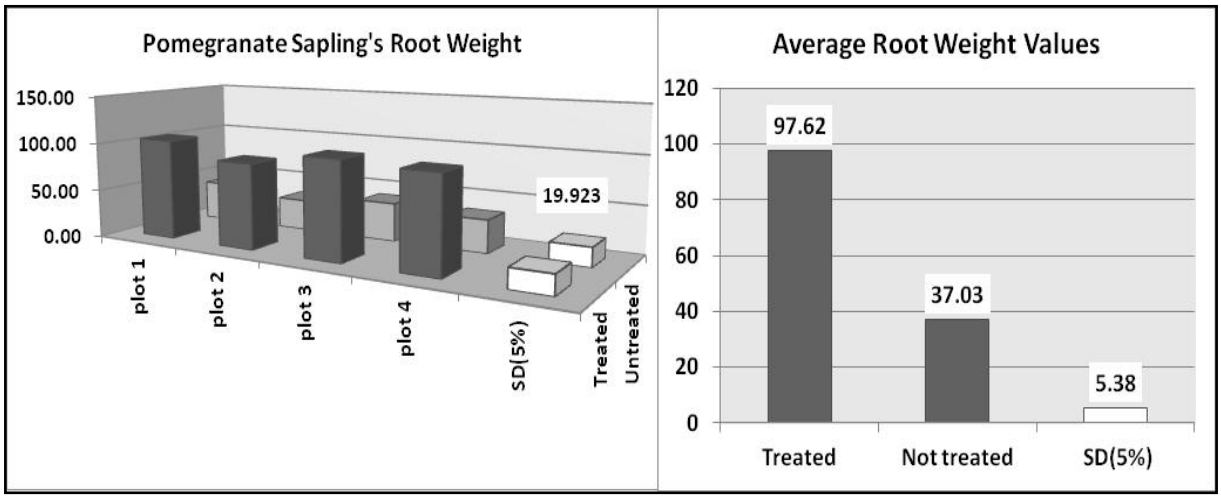
Results and Discussion

At the end of the production cycle, plant height and stem's diameter from the treated plants were 30 - 35% greater than those from the untreated plants. The analysis and comparison of the average monthly values between the saplings from treated and untreated plants showed similar tendency for growth and significant differences between the two groups (Figure 2).

The treated samples always resulted in significantly higher values for both diameter and height when compared to the untreated samples (Figure 3).

Besides the analysis of sapling's height and diameter, several other tests were used for the qualification of the harvested pomegranate saplings. For example, the analysis of sapling's

Figure 4. Weight comparison between treated and untreated pomegranate saplings



average root weight values showed similar differences between the treated and untreated plants. While the pomegranate saplings from the treated soil developed a roots system with an average weight of 98 g, the root system of the control plants averaged of 37 g. The difference was considerably larger than SD(5%)=5.38 value of the one-way ANOVA analysis (Figure 4).

The data from the untreated plants supports the positive effects of humic substances application. However, what would be the effect on the variance generated by variability of the root weight? Would the measured values show a more scattered data base or the variability of the data would be negligible? During the Fisher test, the comparison of the highest variance value of the treated samples (34.0 for the samples of the 4th plot) with the lowest variance value of the untreated samples (9.0 for the samples of the 2nd plot) resulted in significant difference (p=0.026). Considering the fact that the average root weight from the treated plants are considerably higher than those from the untreated plants, in order to determine the effect of humic acid on the variance of the root weight, it seemed to be more trustable result the comparison between the calculated coefficients of variance of the untreated and treated samples. The Fisher test for the comparison between the CV values (respectively $CV_{(TEST)}$=35.0% and $CV_{(CONTROL)}$=26.0%) did not show significant difference (p=0.194), which means that humic substances does not negatively affect the variance in the values of root weight. The comparative analysis of the differences in the root weight between treated and untreated plants showed higher value than the calculated value of the $SD_{(5\%)}$ ($SD_{5\%}$=19,923 for plots and $SD_{5\%}$=5.38 for average values). Based on the received "F" value from the analysis of variance it can be proved statistically that the observed differences in the root weight between treated and untreated samples are significant and the weight of the treated samples are always higher than the root weight of the control plants (Figure 4).

Root volume plays an important role in the sapling survival. The analysis of volume of the root system indicated that the average number of roots over 2 mm diameter was 63 for the treated saplings in comparison to 18 for the untreated saplings. The largest difference was found in the number of roots with a diameter range of 4-7 mm. The number of roots of the pomegranate saplings from the humic substances treated soil was 4 times higher than those found in the untreated sapling population. Number for the roots over 8 mm and in the range of 2-4 mm was 4 times and 3 times higher, respectively, than the number of roots in the untreated population. The occurrence of the different root diameters was tested with two-factor analysis of variance. In both samples (test and control) the occurrence of thin roots (2-4mm diameter) was the most frequent. In all three diameter's categories (root-stem transition region, stem mid-section, upper-third section) the difference between treated and untreated samples was highly significant. The difference increased drastically with the decrease of root's diameter (Figure 5).

The collected data were compared using two-factor analysis of variance. The difference between treated and untreated samples was highly significant at all three points. The largest difference was measured in the diameters of the root-stem transition region. In the cases of stem mid-section and upper-third section the observed differences were also significant (Figure 6).

The research team expected similar results from the baseline (2007) and the untreated samples (2008) because the climatic conditions were similar and the applied technology was the same in both years. Usually, in the area where the experiment was conducted climatic conditions vary very little from one year to other (Figure 1). The collected data during

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Figure 5. The occurrence of different root diameters in control and test samples (>8, 4-7, 2-4mm) and the calculated difference between the categories

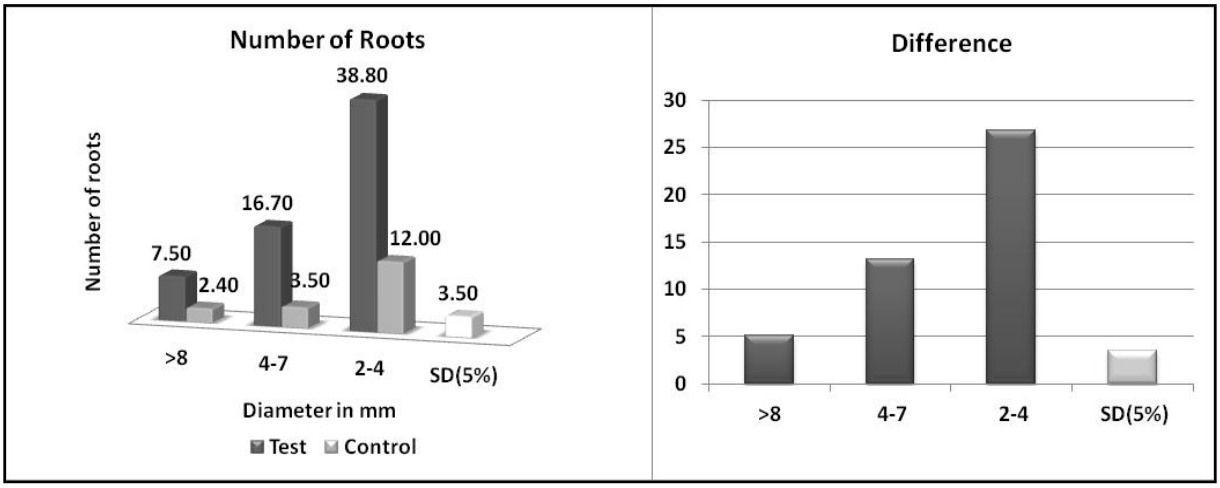
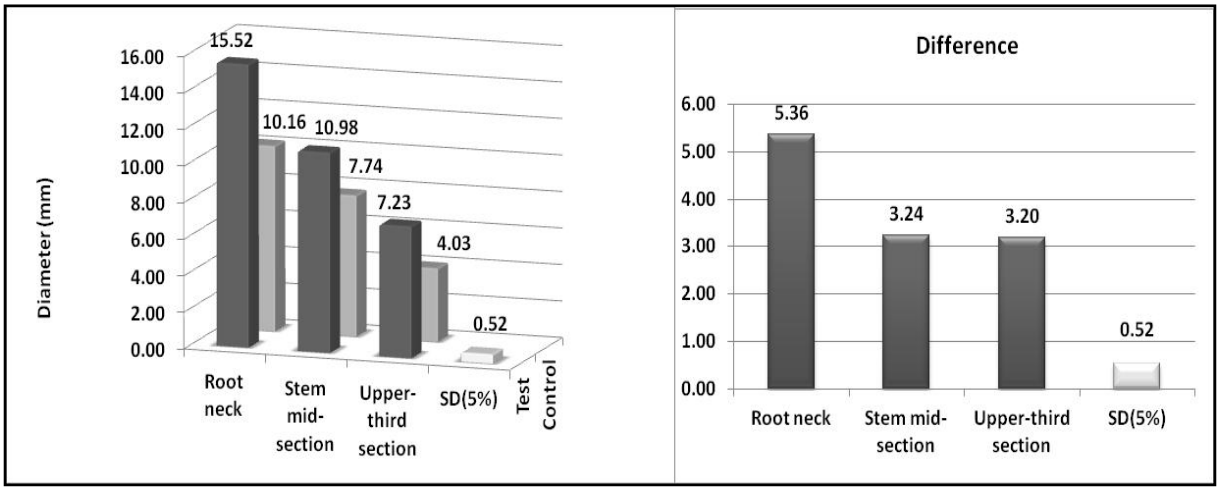


Figure 6. Sapling diameters at different points (Root-stem transition region, Stem mid-section, Upper-third section) and the calculated difference between the categories



the experiment indicated that the differences between treated plants and untreated plants (based on the quality standards established for commercial sapling production) were considerably high. The treated soil produced significantly higher number of saplings that met the standards when compared to those untreated. In addition to meeting a minimum height of 0.8 m and minimum diameter of 8-9 mm, harvested saplings meeting the quality standard were free of pests, diseases, and physical damage. No differences were observed between the 2007 and 2008 untreated samples. The use of humic substances reduced the number of substandard saplings from 57% to 22% (Figure 7).

The soil test results revealed significant differences in all three sampling depths. However, the major changes were observed in the 0-15 cm sampling depth. These major changes after 2 months were the increase in nitrogen, phosphorus and exchangeable

Figure 7. Comparison of pomegranate sapling's samples according to the quality standards

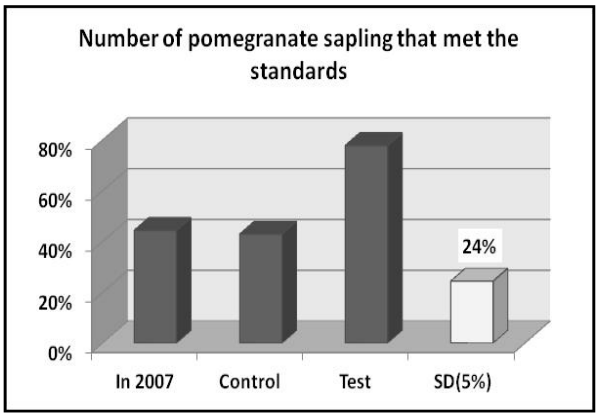


Table 4. Soil properties before and two months after humic substances treatment

Soil depth (cm)	Before humic substances application			After 2 months of humic substances application		
	Nitrate-nitrogen (mg/L)	Phosphate-phosphorus (mg/L)	Exchangeable potassium (mg/L)	Nitrate-nitrogen (mg/L)	Phosphate-phosphorus (mg/L)	Exchangeable potassium (mg/L)
0-15	3.50	49.97	181.50	11.30	85.53	211.17
15-30	3.00	47.03	129.50	7.12	69.44	227.67
30-45	2.08	20.02	118.50	5.12	59.81	156.25

Soil depth (cm)	Before humic substances application			After 2 months of humic substances application		
	$pH_{(H2O)}$	EC (mS/cm)	Salinity (%)	$pH_{(H2O)}$	EC (mS/cm)	Salinity (%)
0-15	8.14	0.41	0.01	7.75	0.78	0.01
15-30	8.07	0.45	0.02	7.83	0.36	0.02
30-45	7.90	0.44	0.02	7.93	0.29	0.01

potassium content in the soil, the increase of electrical conductivity and the decreased pH value. The tests did not reveal significant difference in soil salinity (Table 4).

This nursery experiment contrast the work of Feibert et al. (2000) which questions the effect of humic substances for yield and quality characteristics of crops. However, observations by Albayrak and Camas (2005) support the results of this experiment indicating strong positive effect of the humic substances on plant growth in various aspects (diameter, height, and root growth). The significant increase in volume and number of the root system, especially in the number of roots with a diameter less than 2.0 mm indicates higher capacity for nutrient and water uptake. This is essential for the sapling to survive hard climatic conditions and for healthy development. Also, the large differences measured in the sapling's diameter and height between treated and untreated plants, the positive effect of humic substances was confirmed by the experiment.

Conclusions

The humic substances application significantly increased the number of pomegranate saplings, which met the standard requirements for nursery production. This fact showed the efficacy and reliability of the application of humic substances in order to increase crop yields and quality. The trial in Afghanistan was conducted under harsh environmental conditions (very arid area with extremely high temperatures and poorly developed alkaline soil showing nutrient deficiencies and lack of organic matter). Under these conditions, which are also typical for other areas in the region, the application of humic substances in nursery production can have a significant impact on the feasibility and sustainability of the nursery and horticultural business in Afghanistan.

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PROFESSOR GYÖRGY VÁRALLYAY – 80 YEARS JUBILEE

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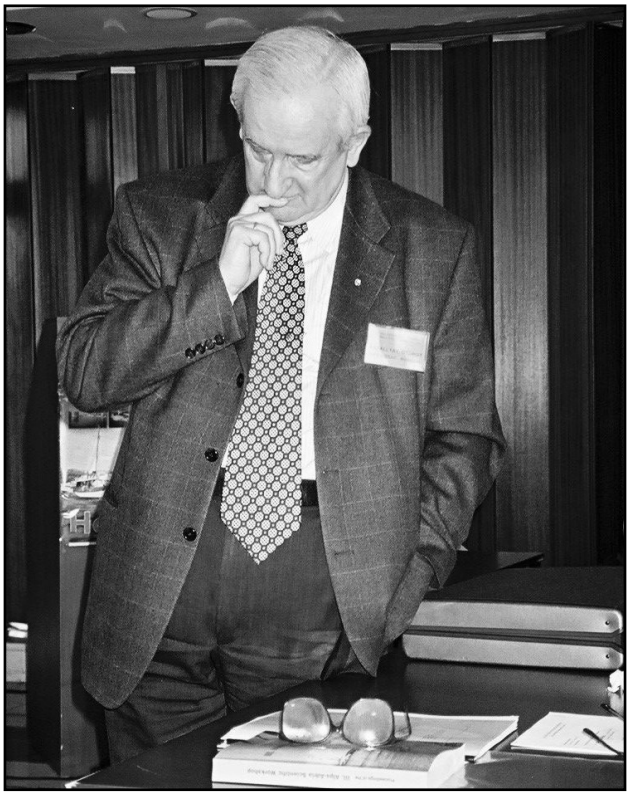
Soil science may be considered as one of the most profound scholarly fields of mankind. A prominent scientist of soil science, Professor György Várallyay has reached a remarkable jubilee; he was born 80 years ago. His life, his intellectual behaviour has been performing an everlasting record in the field of pedology.



He was born in an academic family on the 17th of July 1935 in Debrecen. His father himself was a professor engaged in soil science at the famous Mosonmagyaróvár College. We may assume that György Várallyay has been initiated to follow the family traditions from his childhood.

He completed his agricultural studies at the Gödöllő Agricultural University, wherefrom he graduated in 1957. From the very beginning he was involved in soil research. Between 1957 and 1960 he was employed by the National Institute of Agricultural Quality Testing (OMMI) where he was a junior research fellow experiencing in soil mapping, melioration and extension.

In 1960 he was appointed by the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences (RISSAC), his second and last workplace. He has been involved in various research programmes aiming the exploration, remediation and utilization of saline soils which represent a huge tract within arable lands in Hungary. This research have yielded his first scientific degrees; a PhD in 1964 and a CSc in 1968. During these years he started his active participation in the international scientific community. He became a member of the International Soil Science Society, and also, he joined an expert mission in Jemen.

1969 can be considered as the beginning of a new phase in his scientific research. After completing a scholarship in the Netherlands, he introduced novel methodology in the field of soil physics and water management in Hungary. His scientific research results contributed to almost all methodology standards in these fields.

From 1976 he was the head of the Soil Science Department of RISSAC. He was a key member of the nationwide research programme "The determination of the agro-ecological potential of Hungary" lead by István Láng. During this work he managed to design a series of 1:100.000 scale soil maps of Hungary. Between 1981 and 1997 he was the director of the institute. According to his high skills in management and coordination, he became one of the most successful leaders within the scientific network of the Hungarian Academy of Sciences.

The scientific activities, his contribution to the national and international scientific organisations is enormous. He has been a member, secretary and later president of the Soil Science Committee of the Hungarian Academy of Sciences. He has also been an active participant of high level governmental bodies in the field of scientific qualification and environmental decision making. He has been a member of the highest scientific committees of all the four Hungarian agricultural university faculties.

He defended his DSc thesis in 1988. He was elected to be the member of the Hungarian Academy of Sciences (1993 CM; 1998 FM). He was awarded to be an external member of the Slovak Academy of Sciences. He has been a founder of the Alps Adria Scientific Cooperation, an organisation integrating scientists of various countries of the geographic region.

Source: cms.talaj.hu 2015

His exceptional scientific output can be labelled with more than 800 scientific publications and almost 2000 citations referring to those. He has been member of a wide range of scientific journals (Acta Agronomica Hungarica, Agrokémia és Talajtan, Archives of Agronomy and Soil Science, Columella, Geoderma, Hidrológiai Közlöny, International Agrophysics, Land Degradation and Rehabiliation, Soil Technology).

He was appreciated by many national and international scientific awards. Some of the most important ones: Magyar Köztársasági Érdemrend Középkeresztje 1997, Széchenyi Prize (2004), and last but not least the highest award in the field of crop production - the Surányi insignum (2015).

Professor György Várallyay is 80 years old by now, just in the year of soil. However he is active as he has always been during his life. A caring society of colleagues, students, theoretical and practical experts, scientists and farmers surround him, and he is permanently at service of all of us. Simply we may state that he is a man of spiritual power, with a mission to enrich society.

Source of the graphics

Front cover:
Gallo-Roman harvesting machine, called Vallus. Source: U. Troitzsch - W. Weber (1987): Die Technik : Von den Anfangen bis zur Gegenwart

Rear cover:
Portrait of Columella, in Jean de Tournes, Insignium aliquot virorum icones. Lugduni: Apud Ioan. Tornaesium 1559. Centre d'Études Supérieures de la Renaissance - Tours

POSTA Katalin, editor-in-chief

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Lucius Junius Moderatus Columella

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