

## Effect of mycorrhizal inoculation of leek *Allium porrum* L. on mineral nitrogen leaching

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### Abstract

KUČOVÁ L., ZÁHORA J., POKLUDA R. (2016): **Effect of mycorrhizal inoculation of leek *Allium porrum* L. on mineral nitrogen leaching.** Hort. Sci. (Prague), 43: 195–202.

This study evaluated the influence of the arbuscular mycorrhizal fungi (AMF) inoculation of leek (*Allium porrum* L.) on the leaching of ammonia and nitrate nitrogen from the experimental pots filled with either sterile or non-sterile soil mix, consisting equally of the arable soil and horticultural substrate. Leek plants were inoculated by *Funneliformis mosseae*, *Claroideoglomus claroideum*, *Rhizophagus intraradices* and by their combinations. Based on the obtained data, it can be concluded that: (a) the influence of AMF on nitrate leaching does not follow some simple rules, (b) the amounts of percolating nitrates can be affected also by sterilization and by the combination of AMF inocula, (c) AMF can, in general, reduce the nitrate leaching from soil even though mycorrhizal colonization of roots did not achieve extremely high rates. These conclusions may be useful in the horticultural practice and ecological sustainability of the soil quality.

**Keywords:** arbuscular mycorrhizal fungi; nitrate leaching; soil; root colonization

Nitrogen (N) is a key nutrient, which can limit productivity of any ecosystem. Soil N availability has been investigated extensively, and the study of the soil N cycle has become major component of the ecosystem ecology (VERESOGLOU et al. 2012). The sustainability of agricultural ecosystems depends on techniques that reduce  $\text{NO}_3^-$  losses and provides benefits for soil and for producers (GABRIEL et al. 2012). These could be techniques like the use of selected cover crops, crop rotation on fields and optimizing N fertilizer applications (JÉGO et al. 2012) or techniques which substitute

mineral N fertilizers with N input via diazotrophs. Recent studies demonstrated that N availability in mycorrhizosphere depends not only on the activities of microbial populations, but also on their microbial grazers (i.e. protozoa, nematodes) (IRSHAD et al. 2013). Consequently, the availability of N in the soil and the movement of unutilized mineral N downstairs are controlled synergistically by many living soil organisms (KUZYAKOV, XU 2013). In this way the presence of nitrate N in leachates or in percolated soil solutions can be interpreted as the insufficiency of the whole soil-microbe-plant system

doi: 10.17221/182/2015-HORTSCI

to effectively use or immobilize such an attractive mineral N compound (JOHNSON, REUSS 1986).

Arbuscular mycorrhizal fungi (AMF) are natively present in the soil and create a mutualistic symbiosis with a huge amount of plants from which both partners profit. The main benefit for plant is an increase of plant nutrient uptake, especially P and N, and a better water intake by plant roots (SMITH, READ 1997, 2008). AMF can also positively stimulate plant growth, increase photosynthesis and stimulate disease and pest resistance as well as osmotic diffusion under drought stress conditions (OZTEKIN et al. 2013). In return, up to 20% of plant-fixed carbon is transferred to the mycorrhizal fungus depending both on the physiological status of its host plant and on the plant – AMF relationship (PARNISKE 2008; SMITH, READ 2008). Despite the fact that mycorrhizal symbiosis can improve a lot of factors, a number of key questions still must be answered (BENDER et al. 2015; VAN DER HEIJDEN et al. 2015). Based on the research on the role of AMF in plant N nutrition it has been suggested that AMF can also enhance plant N acquisition (MATSUMURA et al. 2013). Nevertheless, our molecular knowledge regarding the metabolic and transport pathways involved and their regulatory mechanisms are still rudimentary (TIAN et al. 2010). Leaching of  $N_{\min}$  represents one of the key threats to environmental sustainability in agriculture. The most dangerous forms are nitrates ( $\text{NO}_3^-$ -N) for their negative charge and for their mobility in the soil environment. It is a well-known fact that mineral N from the arable land can quickly contaminate surface water and also underground sources of drinking water (NEUMANN et al. 2012). While many studies have focused on the nutrient uptake, relatively few have considered that various AMF could also contribute to the different reduction of N losses. Therefore, the objective of this

experiment was to confirm the decrease of nitrate leaching effect by AMF.

## MATERIAL AND METHODS

**Experimental design.** To determine losses of the mineral N from soil via leaching, the simple field pot experiment was established. The experimental soil was prepared as a mixture of arable soil with horticultural substrate (vol/vol ratio 1:1; basic characteristics of both are summarized in Table 1). After appropriate pre-incubation time – 2 weeks under constant soil temperature (25°C), the soil samples were inoculated by different types of AMF species. Soil sterilization (at 80°C for 24 h in a laboratory dryer) was done primarily to inactivate naturally occurring mycorrhizal fungi for non-mycorrhizal control treatments and secondly to allow colonization of the sterile soil environment (treatment A) by the selected inocula of AMF spores. Non-sterile soil mix was left untreated (treatment B). Pot experiment was established in the experimental facility of the Faculty of Horticulture in Lednice, Mendel University Brno. Leek (*Allium porrum* L.), cv. Terminal, was chosen as a well-suited model plant. Leek plants were pre-cultivated in the multi-trays (cell size 20 × 20 mm) in a greenhouse for 2 months (from April to June). The 1.0 g of AMF inoculum was added to each leek seed at sowing to the each cell. Leek plants for the combined treatment were inoculated by a mixture of 0.5 g + 0.5 g of selected AMF inocula. After two months of pre-cultivation the plants were transferred to the containers (volume of 2 dm<sup>3</sup>). Leek plants grew under the same conditions till the harvest time (beginning of October). Twelve weeks after planting all plants were destructively harvested.

Table 1. Basic characteristics of the experimental soil

Monitored parameter	Unit	Value	Monitored parameter	Unit	Value
N-NH <sub>4</sub>	mg/kg	3.64	humus	%	1.75
N-NO <sub>2</sub>	mg/kg	0.03	Mg	mg/kg	440
N-NO <sub>3</sub>	mg/kg	1.20	K	mg/kg	280
N <sub>min</sub>	mg/kg	4.87	Ca	mg/kg	2.930
P	mg/kg	113	pH		6.44
C <sub>ox</sub>	%	1.02			

Table 2. Experimental setup of the treatments – abbreviations

Type of treatment	Sterile substrate (A)	Non-sterile substrate (B)
<i>R. intraradices</i>	A-RI	B-RI
<i>C. claroideum</i>	A-CC	B-CC
<i>F. mosseae</i>	A-FM	B-FM
<i>R. intraradices</i> + <i>C. claroideum</i>	A-RI+CC	B-RI+CC
<i>C. claroideum</i> + <i>F. mosseae</i>	A-CC+FM	B-CC+FM
<i>R. intraradices</i> + <i>F. mosseae</i>	A-RI+FM	B-RI+FM
Control	A-C	B-C

**Fungal material.** This experiment used three strains of the AMF inocula and their three combinations and also the non-inoculated control treatment. Symbiom Ltd., Czech Republic supplied all the AMF inocula. The exact strains of AMF used in the experiment were: *Funneliformis mosseae* BEG 95 (T.H. Nicolson & Gerd.) C. Walker & A. Schüssler 2010, *Claroideoglomus claroideum* BEG 210 (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüssler, *Rhizophagus intraradices* BEG 140 (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüssler (SCHÜSSLER 2010). All the AMF inocula were mixed with dry zeolite to ensure better application. Table 2 shows the experimental setup of the treatments. Treatments compared 10 leek plants from containercultivation. Overall, there were 14 different treatments used in the whole experiment, so the pot experiment contained the total of 140 plants.

**Evaluation of leaching of mineral nitrogen.** Mineral nitrogen ( $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$ ) leaching from the soil was captured by special discs with mixed ion exchange resin (IER), which were located under each experimental container (Figs 1 and 2).

The discs were made from plastic Petri dishes (PD). The outer part of PD was 90 mm in diameter and 10 mm high. There was a small hole created on a bottom side of inner dishes, where there was subsequently glued a nylon mesh (grid size of 0.1 mm) to ensure controlled percolation through IER. Mixed IER (Cation [Purolite C100E] and Anion [Purolite A520E] Exchange Resin at volume ratio 1:1) were then placed onto the inner PD dishes. For the quantification of  $\text{N}_{\text{min}}$  trapped by the resin, the IERs were allowed to dry at room temperature. The trapped  $\text{N}_{\text{min}}$  was then extracted from resin using the 100 ml of 1.7 M NaCl. The released  $\text{N}_{\text{min}}$  was determined by distillation and titration method. The results obtained by extraction from the IERs were expressed as  $\text{mg N}_{\text{min}}/\text{dm}^3$  of soil.

**Measurement of the root colonization.** After the harvest of leek plants, the roots were separated from the soil and thoroughly washed using tap water. Then, the treated roots were stained by a modified Trypan blue method (PHILLIPS, HAYMAN 1970). After staining, the colonization was determined by a modified gridline intersect method

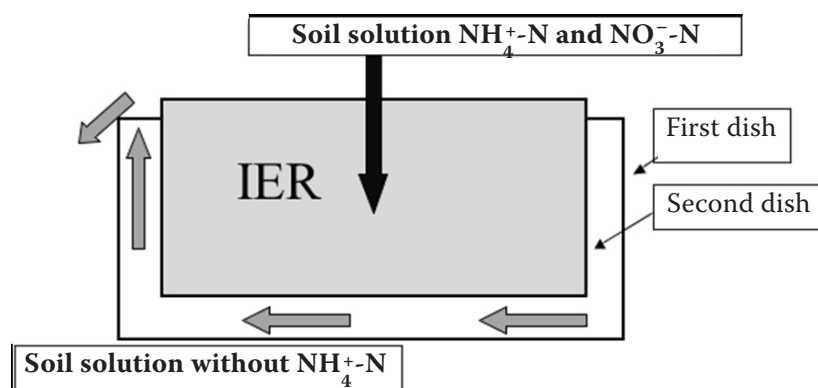


Fig. 1. Schematic illustration showing the percolation of soil solution and the trapping of mineral nitrogen  
IER – ion exchange resin

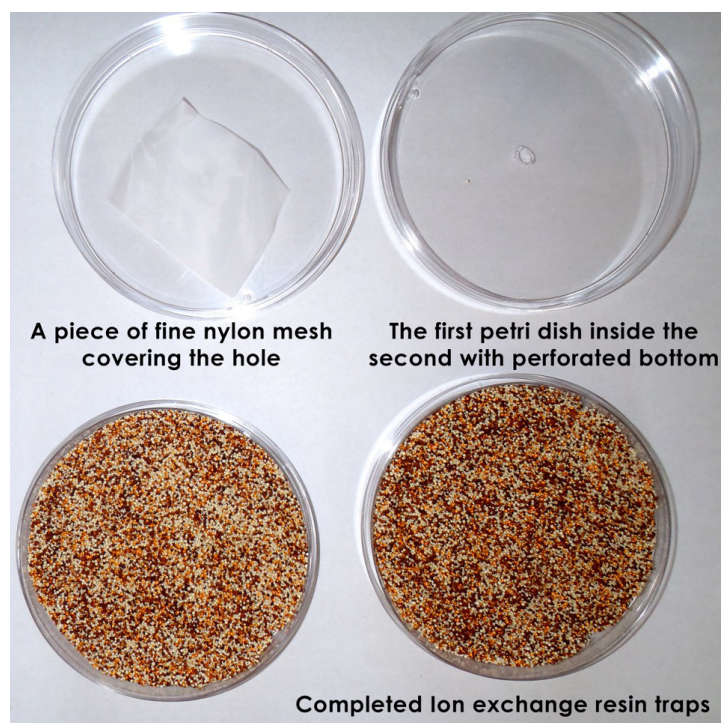


Fig. 2. Preparation of the plastic Petri dishes with a mixed ion exchange resin

(GIOVANNETTI, MOSSE 1980). Root colonization was expressed as a percentage of infection of AMF in root cortex.

**Statistical analyses.** Differences in the amount of leached mineral nitrogen and root colonization by AMF were evaluated by one-way analysis of variance (ANOVA) in combination with the Tukey's HSD test. All analyses were performed by Statistica 12, Inc., (Statsoft.com).

## RESULTS AND DISCUSSION

### Leaching of mineral nitrogen

Even though the relationship between AMF and nitrogen leaching has not been thoroughly studied (VERESOGLOU et al. 2012), the leaching of  $N_{\min}$  is a mechanism of a great ecological relevance (SCHIMEL, BENNETT 2004).

The applied method of trapping mineral N from percolating soil solution into the structure of ion exchange resin offers the following advantages: it (a) enables simulation of the real horticultural cultivation during the whole experiment and (b) minimizes reverse microbial immobilization from the once-trapped  $-N_{\min}$ , because ions trapped into IER are present there in the form which is hardly available for the microorganisms (BINKLEY, MAT-

Table 3. Ammonia nitrogen leaching from experimental pots as related to various mycorrhizal inoculation and sterilization treatments of soil mix

Substrate	Treatment	Average ( $\text{mg NH}_4^+\text{-N/dm}^3$ ) $\pm$ SD
A	RI	$0.767^a \pm 0.937$
B	RI	$0.653^a \pm 0.924$
A	CC	$0.391^a \pm 0.151$
B	CC	$0.475^a \pm 0.287$
A	FM	$0.824^a \pm 0.905$
B	FM	$0.434^a \pm 0.311$
A	RI + CC	$0.601^a \pm 0.161$
B	RI + CC	$0.570^a \pm 0.424$
A	CC + FM	$0.429^a \pm 0.122$
B	CC + FM	$0.546^a \pm 0.497$
A	RI + FM	$0.384^a \pm 0.191$
B	RI + FM	$0.460^a \pm 0.133$
A	C	$0.524^a \pm 0.252$
B	C	$0.744^a \pm 0.221$

substrate: A sterilized, B non sterilized; mean values  $\pm$  means standard deviation,  $n = 5$ , ANOVA, values within a column followed by different letters differ significantly ( $P < 0.05$ ) based on the Tukey's HSD test; treatments – see Table 2 for abbreviations



Table 4. Root colonization by AMF (%)

Substrate	Treatment	Average (Root colonization by AMF (%)) $\pm$ SD
A	RI	40.8 <sup>b</sup> $\pm$ 16.7
B	RI	45.3 <sup>b</sup> $\pm$ 18.6
A	CC	35.8 <sup>ab</sup> $\pm$ 20.1
B	CC	35.5 <sup>ab</sup> $\pm$ 10.7
A	FM	45.5 <sup>b</sup> $\pm$ 20.4
B	FM	66.7 <sup>c</sup> $\pm$ 10.6
A	RI + CC	49.0 <sup>b</sup> $\pm$ 10.3
B	RI + CC	40.3 <sup>b</sup> $\pm$ 11.9
A	CC + FM	55.2 <sup>b</sup> $\pm$ 12.7
B	CC + FM	29.7 <sup>ab</sup> $\pm$ 4.8
A	RI + FM	46.8 <sup>b</sup> $\pm$ 22.7
B	RI + FM	44.5 <sup>b</sup> $\pm$ 8.4
A	C	10.1 <sup>a</sup> $\pm$ 4.5
B	C	36.5 <sup>ab</sup> $\pm$ 8.8

substrate: A sterilized, B non sterilized; treatments – see Table 2 for abbreviations; mean values  $\pm$  means standard deviation,  $n = 5$ , ANOVA, values within a column followed by different letters differ significantly ( $P < 0.05$ ) based on the Tukey's HSD test; AMF – arbuscular mycorrhizal fungi

SON 1983). Microbial immobilization could in fact represent up to one third or even one half of the amount of soil mineral N in the course of one day (BURGER, JACKSON 2003) and this has to be otherwise prevented by immediately collecting leachates from the pots and storing them in a freezer before they are analysed for mineral nitrogen in the laboratory.

The concentration of  $\text{NH}_4^+$ -N in the leachate was very low and did not differ significantly between any of the experimental treatments (Table 3). Leaching losses of  $\text{NH}_4^+$ -N fluctuated in a quite narrow range of 0.384 mg  $\text{NH}_4^+$ -N/dm<sup>3</sup>, for the treatment of sterile soil mix with leek plants inoculated simultaneously by *F. mosseae* and by *R. intraradices*, up to 0.824 mg  $\text{NH}_4^+$ -N/dm<sup>3</sup> at the treatment of sterile soil mix with leeks inoculated by *F. mosseae*. It should be taken into account that our control treatment does not represent a “pure” non-mycorrhizal treatments – the control leek plants were inoculated unintentionally by unknown propagules during the pre-cultivation procedure from soil substrate (Table 4: root colonization by AMF

of the sterilized treatment A-C reached 10.1% and non-sterilized B-C 36.5%). Root colonization in the sterile soil mix treatment A-C (10.1%) may be due to contamination of the substrate during the cultivation of plants; note, however, that for the latter case, the root colonization is very low. There were at least two main reasons for only little ammonium N amount in the leachates from our experimental pots; first, because of relatively low mobility of ammonia cations in soils generally, and second, because of low amount of  $\text{NH}_4^+$ -N in the soil mix at the beginning of the experiment (3.64 mg/kg; Table 1).

Although only a few studies investigated the effects of AM colonization on N under nursery conditions, FATIMA et al (2006) showed decreased leaching of  $\text{NH}_4^+$ -N from mycorrhizal plants of *Encelia californica* (California sunflower) (CORDIKI et al. 2011). Similarly, low amounts of  $\text{NH}_4^+$ -N in leachates were measured in a microcosm-based study with a mycorrhizal defective tomato mutant (0.035 mg  $\text{NH}_4^+$ -N/dm<sup>3</sup>) and its mycorrhizal wild-type progenitor (0.029 mg  $\text{NH}_4^+$ -N/dm<sup>3</sup>) by ASGHARI, CAVAGNARO (2012). However, there is only a limited possibility to compare the results, because the columns used in their work were flushed with water only once, which is ten weeks after planting tomato plants at the end of the experiment.

Leaching of N in the form of nitrates is an important pathway of N-loss from soil or soil microsite in the sense of SCHIMEL, BENNETT (2004). According to VERESOGLOU et al. (2012), individual organisms coexisting in a given system of plant, AMF and rhizosphere microorganisms, can reduce N-leaching simply by reducing the amount of leachate as a result of the enhanced evapotranspiration, faster soil-drying, and by improving the water holding capacity of soil in the mycorrhizosphere. Moreover, the efficient uptake of inorganic N recorded for AM plants should result in a decrease in the levels of inorganic N in the soil solution as well as in a decline in N-losses through leaching in the AM mycorrhizosphere.

The content of  $\text{NO}_3^-$ -N in our soil mix at the beginning of the experiment was even lower than that of  $\text{NH}_4^+$ -N (1.20 versus 3.64 mg/kg, Table 1). Nevertheless, this value is not crucial for the nitrate leaching, because the latter process is determined by both above-mentioned factors as well as by the interactions between microorganisms and plant roots in the rhizosphere (KUZYA-

doi: 10.17221/182/2015-HORTSCI

Table 5. Nitrate nitrogen leaching from experimental pots across various mycorrhizal inoculation and sterilization treatments of soil mix

Substrate	Treatment	Average (mg NO <sub>3</sub> <sup>-</sup> -N/dm <sup>3</sup> ) ± SD
A	RI	0.142 <sup>a-d</sup> ± 0.0920
B	RI	0.112 <sup>abc</sup> ± 0.0990
A	CC	0.050 <sup>a</sup> ± 0.0133
B	CC	0.060 <sup>a</sup> ± 0.0090
A	FM	0.315 <sup>a-d</sup> ± 0.0850
B	FM	0.282 <sup>a-d</sup> ± 0.1500
A	RI-CC	0.437 <sup>e</sup> ± 0.2500
B	RI-CC	0.384 <sup>bcd</sup> ± 0.1260
A	CC-FM	0.083 <sup>ab</sup> ± 0.0380
B	CC-FM	0.092 <sup>ab</sup> ± 0.0440
A	RI-FM	0.261 <sup>a-d</sup> ± 0.0390
B	RI-FM	0.335 <sup>bcd</sup> ± 0.1140
A	C	0.337 <sup>bcd</sup> ± 0.1070
B	C	0.397 <sup>d</sup> ± 0.2330

treatment: A – sterile, B – non sterile; treatments: mean values ± means standard deviation,  $n = 5$ , ANOVA, values within a column followed by different letters differ significantly ( $P < 0.05$ ) based on the Tukey's HSD test; treatments – see Table 2 for abbreviations

kov, Xu 2013). Leaching losses of NO<sub>3</sub><sup>-</sup>-N from our experimental pots fluctuated in a range practically from zero (0.05 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>) for the treatment of non-sterile soil mix with leek plants inoculated by *C. claroideum*, up to 0.437 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>, for the treatment of sterile soil mix with leek inoculated simultaneously by *R. intraradices* and by *C. claroideum*. In fact, these values of the leached nitrate-N reached only half of the values of leached NH<sub>4</sub><sup>+</sup>-N. The values close to zero at the treatment B-CC (0.060 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>), A-CC (0.050 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>) and A-CC+FM (0.083 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>) represent the main significant differences between them and the treatments of A-RI+CC (0.437 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>), B-RI+CC (0.384 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>) and B-C (0.397 mg NO<sub>3</sub><sup>-</sup>-N/dm<sup>3</sup>) (Table 5). However, if we compare our low values of nitrate leachates with the similar results obtained by e.g. (Plošek et al. 2014) of the order of units of mg NO<sub>3</sub><sup>-</sup>-N, there is no rea-

son to overestimate the results from our statistical analyses. Nevertheless, we can conclude, that our results of the NO<sub>3</sub><sup>-</sup>-N leaching from the AM plants with one inoculum were in all six treatments lower than that obtained in control treatments. Interestingly, the simultaneous inoculation with two inocula of *R. intraradices* and *C. claroideum* in both soil mixes were followed by higher leaching of NO<sub>3</sub><sup>-</sup>-N when compared to control treatments in sterile and non-sterile conditions.

### Root colonization

The root colonization by AMF could be very different and variable, such as in the experiment with *Nicotiana tabacum* where the mean root colonization was 11%, varying from 2% to 28% (CosME, WURST 2013). Our results showed few statistical differences between the treatments B-FF (66.7%) and A-C (10.1%) (Table 5).

Leek plants growing in the control treatment with sterile soil mix had the lowest root colonization compared to the other tested treatments (A-C 10.1%). The experiment expects that some percentage of the root colonization by AMF will occur in the sterile soil mix, due to contamination from the cultivation conditions. The highest root colonization was observed at the treatment B-FM (66.7%), (Table 5). It is apparent that the influence of sterilization of soil mix was not very important. Similar conclusions for the higher differences between the root colonization are brought by the experiment with 4 species of the AMF and their interactions with *T. harzianum* (MARTÍNEZ-MEDINA et al. 2011). Each of the AMF used in this experiment, had different percentage of root colonization (i.e. *G. constrictum* 35–45%, *R. intraradices* 8–38%). These data show that root colonization was related to numerous interactions between the AMF genotype, soil structure, and also to the interactions between the host plant (Tajini et al. 2012). In our work, in spite of the expected low colonization in the sterile substrate, the recorded data showed higher values of the root colonization also for some sterile treatments. It may be an example of the fact that the AMF added to the sterile substrate have been developed better and rapidly than the same AMF in non-sterile substrate treatment. The AMF in the non-sterile substrate could have been limited by naturally occurring AMF.

## CONCLUSION

This paper has shown that AMF inoculation can reduce nitrate losses from the soil, and that the rate of this reduction depends on the different type of inoculum and different inocula combinations. The nitrate losses from the soil were generally low, lower than 0.5 mg  $\text{NO}_3^-$ -N/dm<sup>3</sup> throughout the duration of the experiment. Despite the low values of leached nitrates it was found that the non-sterile treatment with *C. cla-roideum* reduced  $\text{NO}_3^-$ -N by 84.9% when compared with the non-sterile control treatment. From an agricultural perspective, this experiment may be valuable not only for the fact that it was focused on the potential of arbuscular mycorrhiza to reduce the nitrate leaching from soil, but also because it gave support for the usage of the AMF to improve soil conditions in agronomic industry and also better utilization of plant nutrients from the soil.

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doi: 10.17221/182/2015-HORTSCI

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Received for publication August 20, 2015

Accepted after corrections March 24, 2016

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