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ARBUSCULAR MYCORRHIZAL *Glomus versiforme* INDUCED BIOPROTECTION OF APPLE TREE AGAINST SCAR SKIN DISEASE

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Abstract. Apple scar skin viroid (ASSVd) is a serious pathogen of apple fruits that results in severe loss in apple production. Up to nowadays, many ASSVd management options are applied to resist the disease, but the diserable resulits are not achieved. Therefore, this study was conducted in 2010–2012 at experimental field of Penglai City, Shandong Province, China (E 120°57'22'', N 370°38'33'') to investigate whether arbuscular mycorrhizal (AM) *Glomus versiforme* protects Red Fuji apple trees (*Malus* × *domestica* Borkh) against apple scar skin viroid. Red Fuji apple trees were inoculated with *Glomus versiforme* and then potential protection mechanism was explored and compared to non-inoculated plants. The results showed that inoculation with *Glomus versiforme* significantly increased root length colonization rate and clearly decreased the percentage of disease severity of apple scar skin disease. Compared to non-inoculated plants, *Glomus versiforme* obviously enhanced total nitrogen and phosphorus concentrations in leaves. Root colonization by *Glomus versiforme* induced an increase in defense-related enzyme activities in fruits, such as the enhanced activities of catalase, ascorbate peroxidase, chitinase and glucanase. Significant differences in acid content of fruit and fruit yield were observed as apple roots were colonized by *Glomus versiforme*. It is therefore concluded that *Glomus versiforme* can be regarded as a biocontrol agent to protect apple trees against the infection with ASSVd.

Key words: apple tree, pathogen, inoculation, protection mechanism

INTRODUCTION

Apple scar skin viroid (ASSVd), a member of the Pospiviroidae that replicates in the nucleus through an asymmetrical rolling circle mechanism, usually comprises 330 nucleotides and possesses no nuclease activity. ASSVd often induces serious diseases on pome fruit trees such as apple scar skin, dapple apple, pear rusty skin and pear dimple fruit in Europe, Asia and North America [Hashimoto and Koganezawa 1987, Hadidi et

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al. 1990, Zhu et al. 1995, Osaki et al. 1996, Koganezawa et al. 2003, Kyriakopoulou et al. 2003, Shamloul et al. 2004, Zhao and Niu 2008, Hadidi and Barba 2010].

Scar skin disease on apple concerns a development of greenish yellow or red yellow patches on the skin of apple. Sometimes these patches are small and have a somewhat circular shape and in other cases large and irregular shapes or elongated strips also can be seen both in the green and red parts of the skin. The symptoms occur first on the apples of one or some branches of an infected tree, but subsequently they appear also on the apples of other branches and finally they can be found over the entire crown. The disease can cause severe economic loss in apple production due to affected fruits that are only sorted in the lowest commercial grades and have little market value.

Regarding the transmission pathway of ASSVd, Wang and Lin [2002] reported that the ASSVd was efficiently spread by graft or contact with contaminated pruning tools and farm implements. Many ASSVd management options were proposed to control it including improving soil fertility levels, use of resistant cultivars, and especially use of chemical pesticides. Although damage caused by ASSVd could be retarded by carrying out these management measures, desirable results were not available. Only if the infected trees were thoroughly eradicated could the extension of scar skin disease be effectively inhibited, once apple trees were infected with ASSVd [Yang and Qiao 2010]. This reflected a dilemma in prevention of ASSVd. In the light of environmental and healthy concerns about extended use of chemicals together with recalcitrant pathogens resistant to chemicals, AM fungi may provide a more sustainable and environmentally acceptable alternative to these current practices [Raupach and Kloepper 1998, Harrier and Watson 2004].

AM fungi are the major components of the rhizosphere of most plants and play an important role in decreasing plant disease incidence [Akthar and Siddiqui 2008]. In several cases direct biocontrol potential has been demonstrated, especially for plant disease caused by *Phytophtora*, *Rhizoctonia* and *Fusarium* pathogens [Dalpé and Monreal 2004]. Different hypotheses have proposed to explain the bioprotection by AM fungi. These include improvement of plant nutrition and root biomass in mycorrhizal plants, which could contribute to an increased tolerance and compensate for root damage caused by pathogen, changes in root morphology, and induction of defense responses such as induced antioxidant and hydrolytic enzymes against pathogen by oxidation reduction reaction and fungi cell wall degradation [Baltruschat and Schönbeck 1975, Azcon-Aguilar and Barea 1996, Bolwell 2004, Khan et al. 2010]. These hypotheses seem to be plausible, but they are derived almost all from interactions between AM fungi association and fungal pathogen in soil. Different from fungus pathogen, ASSVd has no cellular structure and its replication depends only on host [Guo 2006]. Although AM fungi have been frequently reported to have protective effect against soil-borne pathogens, do they yet have protective effect on apple fruits against ASSVd? If they have, what mechanisms take effect in resisting the viroid. As a consequence, the aims of this study reported here were to investigate AM fungi as a biocontrol agent against apple scar skin disease under field conditions and further evaluate the mechanisms involved in the interactions between apple trees colonized by *Glomus versiforme* and infected by ASSVd.

MATERIALS AND METHODS

Field site. The experimental place was selected at a 18-year-old Red Fuji apple orchard, Penglai City, Shandong Province, China (E 120°57'22'', N 370°38'33''), where 150 apple trees (*Malus hupehensis* Rehd. root stock) were planted with spacing in each row of 3 m and row spacing of 4 m. The mean trunk diameter, height, root colonization rate of apple tree and disease severity of scar skin disease were 19 cm, 3.5 m, 7.7 and 53.6%, respectively. There were 15 lines of trees in the orchard and the trees in the lines of the 7^{th} to 9^{th} were dug up and subsequently replaced by other crops to avoid the mutual influence between treatment and control. The soil used in this study was brawn soil and its chemical properties were as followings: organic matter content 48.93 g kg^{-1}, available nitrogen 36.48 mg kg^{-1}, available phosphorus 9.05 mg kg^{-1}, available potassium 82.76 mg kg^{-1}, pH 6.9 (1: 2.5, soil: water suspension).

Experimental design. The experiment was established on March 28, 2010, with six replicates. *Glomus versiforme* was provided by Qingdao Agricultural University, Shandong Province, China. It was derived from pot culture prepared with *Trifolium repens* L. grown in 1:9 sterilized soil-sand and contained colonized pieces of root, soil, spores and colony-forming units per gram of 2.38×10^8. Along both sides of the rows, soil at distance of 80 cm from trunk was gently removed down to root appearance, with the width of 20 cm and length equivalent to crown diameter (about 4% of total root system), and subsequently *Glomus versiforme* inoculum was uniformly sprinkled around the root to assure a rapid colonization. Control counterparts were received volumetric sterilized soil-sand free of spores. In the procedure of grafting and stem pruning, man-made contaminations were all completely avoided. Watering and fertilization were fulfilled as necessary.

Data collection and determination method. Root length colonization and mineral nutrients in apple leaves were determined every two months, giving a total of three times in each experimental year (i.e. May, July and September). Defense-related enzyme activities, disease severity of apple scar skin disease, fruit quality and yield were tested when fruits were mature. Root length colonization by AM fungi was calculated using a gridline intersect method after staining the roots with trypan blue [Koske and Gemma 1989]. Fruit protein extraction was according to the method of Blilou et al. [2000] and protein concentration was determined as described by Lowry et al. [1951]. Catalase and ascorbate peroxidase activities in fruit were measured as described by Aebi [1984] and Amako et al. [1994] respectively. β-1,3-glucanase and chitinase activities in fruit were measured respectively by colorimetric assay of Kauffmann et al. [1987] and Reissig et al. [1955]. Total N, P and K concentrations in leaves were analyzed using the method of Lu [1999]. The yield of each treatment was recorded in kilograms. Fruit quality was analyzed for total sugar content with a photometer and for titrable acid content by titration [Liu and Yang 1996]. Disease severity of apple scar skin disease was estimated visually by assessing lesions on the fruits using a rating scale of 0–5 described according to Filion et al. [2003].

$$\text{Disease severity} = \frac{\sum(ab)\times 100}{AK}$$

where:

a = no. of diseased plants having the same degree of infection,
b = degree of infection,
A = total no. of examined plants,
K = highest degree of infection.

Statistical analysis. All experiments were repeated as indicated. Values presented are means. The effects of the treatments were tested by one-way analysis of variance (ANOVA). Means were compared between the treatments using the LSD (least significant difference) test at the 0.05 probability level.

RESULTS

Level of mycorrhizal colonization. Data in Table 1 show that mycorrhizal trees inoculated with *Glomus versiforme* possessed over 30% of roots being colonized in the whole experiment, while root colonization rate of non-inoculated trees always remained about 7.7%. The largest proportion of colonized root system of inoculated plants occurred in June, 2011 and was more 8 times higher than that of control plants. The result indicated that the level of root length colonization was significantly dependent on AM fungi.

Table 1. Effect of inoculation and non-inoculation with *Glomus versiforme* on root length colonization of apple plants in the years 2010 to 2012

Treatment	Root length colonization (%)								
	2010			2011			2012		
	May	Jun.	Sep.	May	Jun.	Sep.	May	Jun.	Sep.
Non inoculation	7.5b	7.8b	7.6b	7.6b	7.6b	7.7b	7.5b	7.7b	7.7b
Inoculation	39.3a	56.9a	48.1a	45.4a	67.6a	52.7a	42.2a	60.5a	44.4a

Explanation: Values of each column followed by the same letter are not significantly different at the 0.05 level. Each value represents the mean of 6 replicates

Estimation of mineral nutritional status in leaves. Compared to non-inoculated plants, the significant increases in both total nitrogen and phosphorus contents of leaves were detected in plants colonized by AM fungi. However, no differences in total potassium content of leaves were found, regardless of plants inoculated with *Glomus versiforme* or not (fig. 1). Although nitrogen and phosphorus concentration of mycorrhizal leaves both increased in comparison to control plants, the extent to which phosphorus concentration enhanced was greater than that of nitrogen concentration, indicating the effect conferred by *Glomus versiforme* was more obvious on phosphorus than nitrogen.

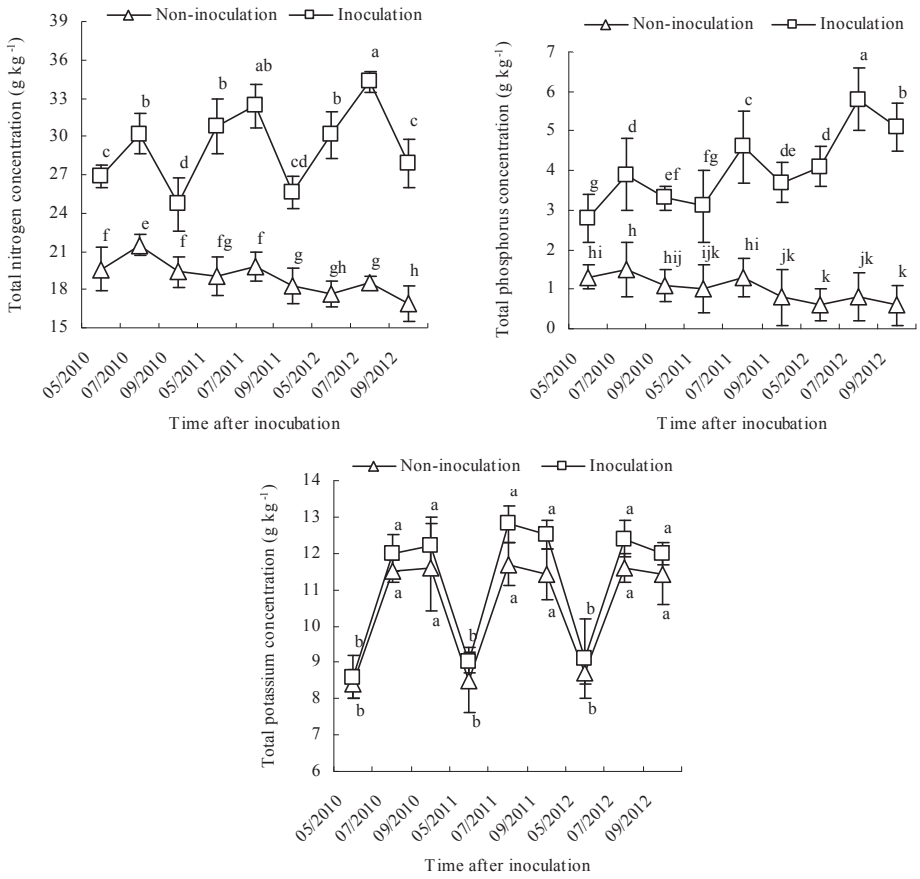

Fig. 1. Effect of mycorrhizal colonization on mineral nutrient concentration of apple leaves inoculated and non-inoculated with *Glomus versiforme*. Vertical bars represent means ± standard deviations. Values sharing the same letter are not significantly different at the 0.05 level. Each value represents the mean of 6 replicates

Estimation of enzymes activities in fruits. Catalase and ascorbate peroxidase activities in control fruit tended to decrease throughout the experiment (fig. 2). As compared to non-inoculated plants, the increases of both enzymatic activities were observed in *Glomus versiforme* inoculated plants. The peak values of enhanced activity occurred in catalase in 2010 and in ascorbate peroxidase in 2011 respectively. After reaching a maximum, the two enzymatic activities in inoculated plants subsequently decreased but still were higher than those found in control plants at the end of the experiment. Similar results were also obtained for chitinase and glucanase activities in non-inoculated and inoculated apple fruits (fig. 3). The maximum activities of both hydrolytic enzymes in inoculated and non-inoculated plants appeared in the year 2010 and within years they tended to decline.

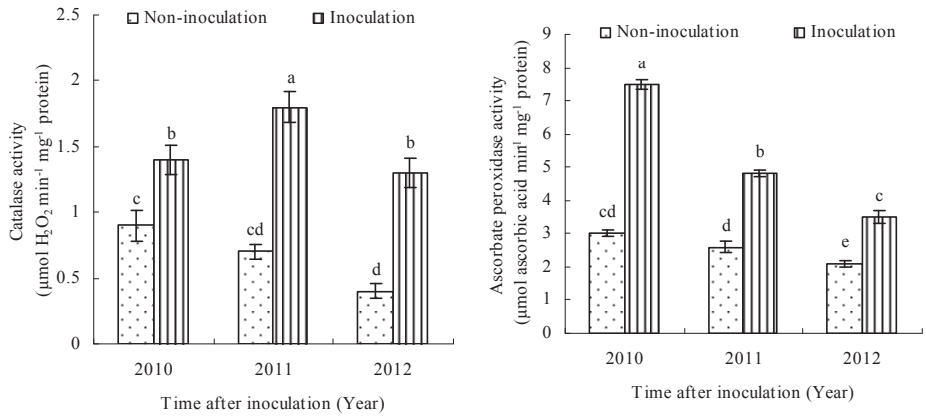

Fig. 2. Catalase and ascorbate peroxidase activities in fruit extracts from apple plants inoculated and non-inoculated with *Glomus versiforme*. Vertical bars represent means ± standard deviations. Values sharing the same letter are not significantly different at the 0.05 level. Each value represents the mean of 6 replicates

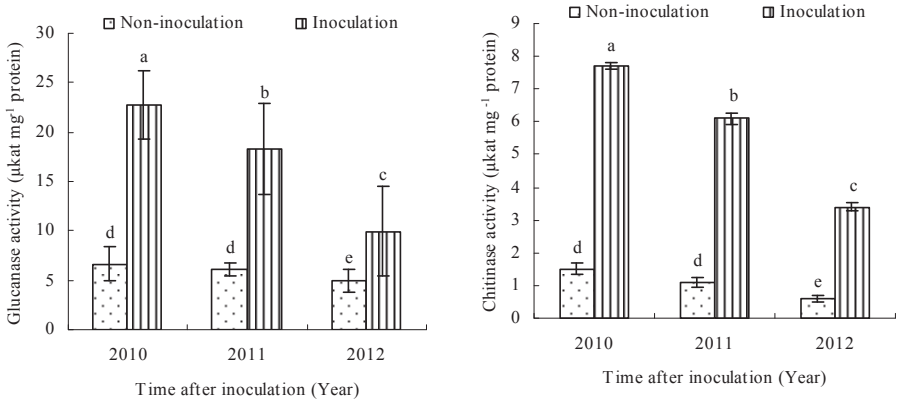

Fig. 3. Time-course of β-1,3-glucanase and chitinase activities in fruit extracts from apple plants inoculated and non-inoculated with *Glomus versiforme*. Vertical bars represent means ± standard deviations. Values sharing the same letter are not significantly different at the 0.05 level. Each value represents the mean of 6 replicates

Disease assessment. Data presented in Fig. 4 indicate that mycorrhizal colonization of apple plants obviously reduced disease severity of scar skin disease compared with control treatment. Disease severity in apple fruits inoculated with *Glomus versiforme* decreased from 27.4% in the year 2010 to 1.6% in the year 2012, however it increased from 53.6% in the year 2010 to 85.7% in the year 2012 in non-inoculated counterparts, especially in the year of 2012 disease severity in inoculated plants accounted for only 1.9% of control plants.

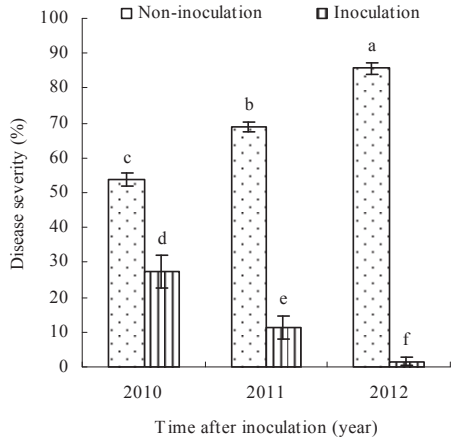

Fig. 4. Disease severity of scar skin disease in apple fruits inoculated and non-inoculated with *Glomus versiforme*. Each bar represents a mean ± standard deviations. Values sharing the same letter are not significantly different at the 0.05 level. Each value represents the mean of 6 replicates

Table 2. Effect of inoculation and non-inoculation with Glomus versiforme on sugar percentage, titrable acid content and yield of fruits in apple plants in the years 2010 to 2012

Treatment	Sugar percentage (%)			Acid content (mg L^{-1})			Fruit yield (kg $plant^{-1}$)		
	2010	2011	2012	2010	2011	2012	2010	2011	2012
Non inoculation	13.45a	13.11a	13.87a	1.3a	1.4a	1.3a	171b	169b	175b
Inoculation	13.66a	13.43a	13.71a	0.8b	0.7b	0.7b	185a	196a	192a

Explanation: Values of each column followed by the same letter are not significantly different at the 0.05 level. Each value represents the mean of 6 replicates

Effect on fruit quality and yield. Despite the fact that apple trees were inoculated with *Glomus versiforme*, there were no distinct differences in sugar content of fruit between inoculated and non-inoculated plants throughout the experiment. In *Glomus versiforme* inoculated plants a marked decrease of acid content and a significant increase of fruit yield were recorded when compared to non-inoculated plants. The peak

values of them occurred in the years of 2010 and 2011, respectively. The data in Table 2 indicate that the improvement in fruit quality and yield were attributed to root colonization by *Glomus versiforme*.

DISCUSSION

The obtained results revealed that mycorrhizal colonization significantly reduced the percentage of disease severity in plants with apple scar skin disease, indicating *Glomus versiforme* may protect apple fruits against the pathogenic ASSVd. In the present study we are interested in host responses of infected apple plants to interactions with *Glomus versiforme*.

Among the potential mechanisms involved in the resistance of mycorrhizal systems, the induction of plant defenses is the most controversial [Wehner et al. 2009]. A number of biochemical and physiological changes has been associated with mycorrhizal colonization [Al-Askar and Rashad 2010]. The findings in this study demonstrated that mycorrhizal colonization led to a significant increase in the activities of defense-related enzymes catalase, ascorbate peroxidase, β-1, 3-glucanase and chitinase, suggesting that these defense enzymes with high activity possibly involved in resistance to apple scar skin disease. However, the protection mechanisms of these antioxidant and hydrolytic enzymes against pathogens in AM are not clearly defined. It is likely that the first reaction of root cells to AM fungi invasion is priming that triggers plant tissues for a more effective activation of defense, with the induction of specific defense enzymes [Blilou et al. 2000, Conrath et al. 2006]. It is also possible that distinct signaling mechanisms operate between AM fungi and plant upon pathogens attack in AM. A short distance signaling may lead to the localized induction of specific defense-related genes and a long distance signaling may be involved in the general systemic suppression of these genes at enzyme activity levels [Lambais and Mehdy 1995]. The strong induction and accumulation of these defense enzymes may be the basis of high activity in inoculated fruits. Peroxidase and catalase are known to be involved in the defense mechanisms of plants in response to pathogens either by their direct participation in cell wall reinforcement, or by their antioxidant role in the oxidative stress generated during plant pathogen interaction [Mehdy 1994]. Cell wall thickening plays an important role as a physical barrier to stop ASSVd attack and antioxidation can also convert H_2O_2 to H_2O that conquers the damage in oxidative stress caused by ASSVd infection. For hydrolytic enzymes, β-1, 3-glucanase associated with chitinase may be involved in the degradation of arbuscular fungi cell walls, as proposed for plant – pathogen associations [Lambais and Mehdy 1995]. Alternatively, they may act in the partial degradation of plant cell walls infected with ASSVd, decreasing viroid parasitic sites.

The testing results indicated that *Glomus versiforme* colonization of apple plants markedly increased mineral nutrient concentrations in apple leaves, especially nitrogen and phosphorus nutrition. This finding is in agreement with that of Smith and Read [2008], who found that AM fungi improved the nutrient status of their host plants. It is therefore deduced that the increase in nutrients of apple plants through their AM fungi

may be another defense mechanism induced by mycorrhizal colonization against ASSVd infection.

Data obtained from the field trial demonstrated that a high degree of root colonization with *Glomus versiforme* showed high localized bioprotective effect against ASSVd, however low levels of root colonization that probably caused by other species of fungi existing in original soil showed no bioprotective effect. It is thus concluded that the local and general bioprotective effects of mycorrhization depend not only on the degree of AM root colonization but also on AM fungi species [Harrier and Watson 2004, Khaosaad et al. 2007]. Increase in mineral nutrient status, induction and accumulation of defense-related enzymes and decrease in apple scar skin disease severity may represent compensatory processes. These processes may possibly be mechanisms conferred by mycorrhizal colonization by which apple plants could compensate for the losses of yield and fruit quality caused by the infection of ASSVd as proved by data in Table 2.

CONCLUSION

1. Defense responses induced by *Glomus versiforme* colonization significantly protected apple plants from scar skin disease. The outcome is in fact complex interactions between apple plant, ASSVd and *Glomus versiforme*.

2. Different physical and physiological mechanisms have been shown to play a role in plant protection conferred by *Glomus versiforme*, namely, improved plant nutrition, enhanced induction and accumulation of defense-related enzymes and damage compensation.

3. At present, there is very limited knowledge and experience regarding the biocontrol of apple scar skin disease by AM fungi worldwide, further research efforts should be directed to understand what are the basic mechanisms controlling ASSVd infection with AM fungi.

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BIOOCHRONA DRZEW JABŁONI WYWOŁANA MIKORYZĄ ABUSKULARNĄ *Glomus versiforme* PRZED BLIZNOWATOŚCIĄ

Streszczenie. Wiroid bliznowatości skórki jabłek (ASSVd) jest poważnym patogenem jabłek powodującym znaczne straty w ich produkcji. Obecnie stosuje się wiele opcji kontrolowania ASSVd w celu uzyskania odporności na chorobę, ale nie osiągnięto jeszcze pożądanych rezultatów. Niniejsze badanie przeprowadzono w latach 2010–2012 na polu doświadczalnym miasta Penglai, prowincja Shandong w Chinach (E 120°57'22", N 370°38'33") w celu zbadania, czy mikoryza abuskularna (AM) *Glomus versiforme* chroni jabłonie Red Fuji (*Malus* × *domestica* Borkh) przed wiroidem bliznowatości skórki jabłek. Jabłonie Red Fuji zaszczepiono *Glomus versiforme* a następnie badano mechanizm potencjalnej ochrony oraz porównano go z roślinami nieszczepionymi. Wykazano, że inokulacja *Glomus versiforme* istotnie zwiększyła szybkość kolonizacji korzeni oraz wyraźnie zmniejszyła procent ostrej choroby bliznowatości skórki jabłek. W porównaniu

z roślinami nieszczepionymi, *Glomus versiforme* wyraźnie wzmagał stężenia całkowitego azotu i fosforu w liściach. Kolonizacja korzeni przez *Glomus versiforme* powodowała wzrost aktywności enzymatycznej związanej z mechanizmem obronnym, np. wzmożoną aktywność katalazy, peroksydazy askorbinianowej, chitynazy oraz glukanazy. Zaobserwowano istotne różnice w zawartości kwasów oraz plonie owoców w miarę kolonizowania korzeni jabłoni przez *Glomus versiforme*. Można więc wyciągnąć wniosek, że *Glomus versiforme* może być uważany za czynnik bio-kontroli chroniący jabłonie przed zakażeniem ASSVd.

Słowa kluczowe: jabłoń, patogen, inokulacja, mechanizm ochronny

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