Induced Systemic Resistance to Fusarium Root Rot and Changes in Antioxidative Ability by Arbuscular Mycorrhizal Fungus and Non-pathogenic \textit{Fusarium oxysporum} in Asparagus Plants

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Abstract

Induced systemic resistance (ISR) to Fusarium root rot and changes in antioxidative ability in asparagus (\textit{Asparagus officinalis} L.) plants inoculated with arbuscular mycorrhizal fungus (AMF) and non-pathogenic \textit{Fusarium oxysporum} (NPFO) by split root system were investigated. Single-inoculated plants of AMF and NPFO had higher dry weight of shoots and roots than control. Four weeks after \textit{Fusarium oxysporum} f. sp. \textit{asparagi} S. I. Cohen (Foa) inoculation, incidence and severity of symptoms became lower in all the AMF, NPFO and AMF+NPFO plants; the effects appeared in both the inoculated and non-inoculated roots. Before and after Foa inoculation, most of the AMF, NPFO-inoculated plants showed higher values than control in superoxide dismutase activity, 1,1-diphenyl-2-picyrhydrazyl radical scavenging activity, total polyphenol and ascorbic acid contents than control. From these findings, ISR to Fusarium root rot occurred through AMF and NPFO inoculation in asparagus plants, and the ISR was closely associated with the increase in antioxidative factors.

Key Words: antioxidative ability, biocontrol, \textit{Fusarium oxysporum}, \textit{Glomus intraradices}, induced systemic resistance.

Introduction

Asparagus decline is a serious and increasing threat in asparagus-producing regions over the world (Hamel et al., 2005; Wong and Jeffries, 2006; Knaflewski et al., 2008). Asparagus decline is supposed to be caused by the contribution of both biotic (Wong and Jeffries, 2006; Knaflewski et al., 2008) and abiotic factors (Miller et al., 1991; Lake et al., 1993). Abiotic factors are mainly related to the release of allelopathic compounds (Blok and Bollen et al., 1993), but also nutrient imbalance, deterioration of soil physiochemical conditions, cultural factors, and excessive harvesting pressure (González and del Pozo, 2008; Hamel et al., 2005; Yergeau, et al., 2006). As biotic factors, the most common phenomenon is Fusarium crown and root rot caused mainly by \textit{Fusarium oxysporum} f. sp. \textit{asparagi} S. I. Cohen and \textit{Fusarium proliferatum} (Matsushima) Nirenberg (Reid et al., 2002; Wong and Jeffries, 2006; Knaflewski et al., 2008; Elmer, 2015). Nahiyan et al. (2011) demonstrated that \textit{Fusarium oxysporum} f. sp. \textit{asparagi} and \textit{Fusarium proliferatum} are dominant in asparagus decline fields in Japan by PCR-SSCP (single-stranded conformational polymorphism) analysis. However, it is difficult to control this disease successfully with cultural and chemical methods due to the perennial nature of the crop, the pathogen is soil-borne, and highly resistant cultivars have not been developed (Pontaroli et al., 2000; Elmer, 2015).

Arbuscular mycorrhizal fungi (AMF) are wide-spectrum biocontrol agents (Yergeau et al., 2006) and promote host plant growth though many possible mechanisms, one enhancing phosphorus uptake (Marschner and Dell, 1994). Disease suppression using AMF has been reported in several crops (Pozo et al., 2002; Matsubara et al., 2004; Richter et al., 2011). Only a few reported induced systemic resistance (ISR) in mycorrhizal plants, such as citrus infected with \textit{Phytophthora parasitica} (Davis and Menge, 1980), barley with \textit{Gaumannomyces graminis} (Khaosaad et al., 2007) and tomato with \textit{Phytophthora parasitica} (Pozo et al., 2002). In addition, many points remain unclear about the mechanisms of disease tolerance in mycorrhizal plants, especially ISR aspects. The pathogen stress condition, the production of higher concentration of reactive oxygen species (ROS) such as \textit{H}_{2}\textit{O}_{2}, superoxide anion (\textit{O}_{2}^{-}) and hydroxyl radical (\textit{OH}^{-}) have been shown to create cytotoxic...
conditions in plant cells as oxidative stress (Sahoo et al., 2007). To overcome this negative consequence of ROS, plants have evolved various protective mechanisms either to reduce or completely eliminate ROS through antioxidative ability of producing antioxidative enzymes and substances (Mohgaddam et al., 2006). In mycorrhizal plants, Garmendia et al. (2006) reported that tolerance and an increase in superoxide dismutase (SOD) activity occurred in Verticillium dahliae-inoculated pepper. Richter et al. (2011) described that disease tolerance and increase in ascorbic acid content appeared in mycorrhizal St. John’s wort plants inoculated with Colletotrichum gloeosporioides. However, it is still unclear the relationship between ISR and antioxidative aspects in mycorrhiza-Fusarium disease interaction.

Isolates of non-pathogenic Fusarium oxysporum (NPFO) have provided effective protection against Fusarium oxysporum infected seedlings of numerous crops (Sneh, 1998; Larkin and Fravel, 1999). Disease protection by NPFO has been attributed by the competition between NPFO and other pathogens for nutrients and infection sites, or mycoparasitism (Sneh, 1998; Larkin and Fravel, 1999; Bao and Lazarovits, 2001). In asparagus, NPFO isolates have also been used for controlling Fusarium disease (Blok et al., 1997; He et al., 2002; Reid et al., 2002; Elmer, 2004). However, disease suppression mechanisms by NPFO have been still unclear, and it has not been reported on the relationship between ISR and antioxidative aspects in NPFO plants. In addition, it is also unclear synergistic effect of the combination use of AMF and NPFO on ISR and antioxidative ability in asparagus plants.

In this study, influence of AMF and NPFO inoculation of asparagus affected by Fusarium root rot was studied. We measured the changes in antioxidative ability and the ISR response in asparagus plants using a split root system to study the systemic activity of AMF and NPFO.

**Materials and Methods**

Plant culture and experimental treatments: A pot experiment was conducted in the greenhouse. Seeds of asparagus (Asparagus officinalis L., ‘Welcome’) were sown to commercial soil (autoclaved at 1.2 kg cm\(^{-2}\) and 121 °C for 1 h) in a plastic container (19.0 × 33.5 × 15.5 cm), and administered with mixed fertilizer (N : P : K= 13 : 11 : 13, 0.5 g per plant). Eight weeks after sowing, asparagus plants were treated with the split root system method. The plant root system was halved and each was placed in different compartment units with bedding soil. Half of the split root was inoculated with AMF (Glomus intraradices N.C. Schenck and G.S. Sm.) supplied by Idemitsuosukan Co. Ltd. (Tokyo, Japan), according to Matsubara et al. (2004). The other half of split root was not inoculated with AMF. In the control treatment, plants were inoculated with autoclaved AMF inoculum at one side of the split root system, and not inoculated at the other side.

NPFO (non-pathogenic Fusarium oxysporum, NF1) isolated from asparagus roots were grown on potato dextrose agar media. The conidia were harvested in potato sucrose liquid media and incubated at 25°C in the dark for 14 days. The conidial suspension was sieved and the concentration was adjusted to 10^6 conidia ml\(^{-1}\). Eighteen weeks after AMF inoculation, in single inoculation plot of NPFO, half of the split root was inoculated with NPFO, another half was not inoculated. In addition, in the combination plot, NPFO was inoculated to half of the split root, which was previously inoculated with AMF; the other half was not inoculated. Hence, this experiment was consisted of 4 treatments; control, AMF, NPFO, AMF+NPFO. Each treatment has 40 plants, so, total of 160 plants per experiment were used and repeated twice.

Fusarium oxysporum f. sp. asparagi (Foa; MAFF305556) were grown on potato dextrose agar media. The conidia were harvested in potato sucrose liquid media and incubated at 25 °C in the dark for 7 days. The conidial suspension was sieved and the concentration was adjusted to 10^6 conidia ml\(^{-1}\). Twenty weeks after AMF inoculation, each plant (both sides of split root system) was inoculated by pouring 50 ml of the conidial suspension onto the soil. Four weeks after Foa inoculation, the symptoms were categorized into 5 degrees: percentage of storage roots with root lesions in a plant: 1, less than 20%; 2, 20-40%; 3, 40-60%; 4, 60-80%; 5, 80-100%. The disease index was calculated by the following formula:

\[
\text{Disease index} = \frac{\sum (\text{number of plants} \times \text{number of degree in symptom})}{\text{Total number of plant} \times 5} \times 100
\]

**Evaluation of AMF colonization level:** Twenty weeks after AMF inoculation and 4 weeks after Foa inoculation, roots of asparagus were preserved with 70% ethanol and stained according to Phillips and Hayman (1970). The rate of AMF colonization in 1-cm segments of lateral roots (abbreviated RFCSL) was calculated. Hence, RFCSL expresses the percentage of 1-cm AMF-colonized segments to the total 1-cm segments of all lateral roots; the number of total segments was approx. 30 per plant. Average colonization level was calculated from the values of five plants in each time.
Analysis of antioxidative ability: Twenty weeks after AMF inoculation (just before Foa inoculation) and 4 weeks after Foa inoculation, were sampled and partitioned into shoots and roots and frozen in liquid nitrogen. Analysis of antioxidative enzyme activity and antioxidative substances were carried out as follows.

As for SOD activity, aliquots (0.1 g) of shoots and roots were homogenized in 4 ml of 50 mM phosphate-borate buffer (pH 7.8). The filtrate was centrifuged (EF-1300, Tomy Co., Ltd., Tokyo, Japan) at 13,000 rpm for 10 min. The supernatant was used for crude enzyme extract. The activity was determined using the Nitro blue tetrazolium (NBT) reduction method (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction measured at 560 nm using a spectrophotometer (U-1900, Hitachi, Tokyo, Japan).

DPPH radical scavenging activity was measured according to the method of Burits and Bucar (2000). One gram sample was ground in 40 ml of 90% methanol. The 10 μl of the extract was introduced into test tubes, and 2.7 ml of DPPH solution was added. The tubes were mixed and allowed to stand for 30 min in the dark. Absorbance was read against a blank at 520 nm using a spectrophotometer (U-1900, Hitachi, Tokyo, Japan). DPPH radical scavenging activity was calculated as the percent of inhibition relative to the control.

Ascorbic acid contents were determined by the 2,4-dinitrophenyl hydrazine method (Roe et al., 1948). Briefly, one gram sample was ground in 20 ml of 5% methaphosphate. The 2 ml of the extract was placed in a test tube, and 1 ml of 2,6-dichloroindophenol sodium (0.03%) was added. Then, 1 ml of 2% 2,4-dinitrophenyl hydrazine was mixed and incubated for 3 hrs. Absorbance was measured at 520 nm using a spectrophotometer (U-1900, Hitachi, Tokyo, Japan). Ascorbic acid content was determined using an equation obtained from a standard L-ascorbic acid graph.

Polyphenol contents were determined with Folin-Denis method (Folin and Denis 1915). One gram sample was ground in 10 ml of 90% methanol. The 400 μl of the extract was placed in a test tube, then 3 ml distilled water and 200 μl Folin-denis reagents. After 3 mins, 0.4 ml of Na₂CO₃ (10%) was added, the mixture was allowed to stand for 30 mins in the dark. Absorbance was measured at 700 nm using a spectrophotometer (U-1900, Hitachi, Tokyo, Japan). Polyphenol content was determined as quercetin equivalent using an equation obtained from a standard quercetin graph.

Statistical analysis: The data were compared by Tukey’s test at P<0.05. All analyses were performed using XLSTAT pro statistical analysis software (Addinsoft, New York).

Results

Twenty weeks after AMF inoculation, single-inoculated plants of AMF and NPFO had higher dry weight of shoots and roots than control; no growth promotion occurred in dual-inoculated plants (Fig. 1). AMF colonization occurred successfully and RFCSL reached more than 60% in all the plots, and no difference occurred before and after Foa inoculation including dual inoculation of AMF and NPFO (data not shown).

As for antioxidative ability after 20 weeks of AMF inoculation, SOD activity was higher in both shoots and roots of AMF, NPFO and AMF+NPFO plants compared to control; the increase occurred in both the inoculated and non-inoculated roots (Fig. 2). DPPH radical scavenging activity increased in shoots of single-inoculated plants in AMF and NPFO and all the roots compared to control. Ascorbic acid contents were higher in both shoots and roots in all the AMF, NPFO and AMF+NPFO plants compared to control, especially in AMF; the increase occurred in both the inoculated and non-inoculated roots. Polyphenol contents increased in most of the shoots and roots in AMF, NPFO and AMF+NPFO plants except non-inoculated roots in NPFO.

Four weeks after Foa inoculation, incidence of Fusarium root rot in roots reached 100% in control plants, but was lower in AMF plants. In addition, the severity of symptom in...
roots became lower in all the AMF, NPFO and AMF+NPFO plants than control, especially in AMF. The disease indices of Fusarium root rot reached more than 60 in control plants, whereas it was the lowest as less than 30 in AMF; the effect appeared in both the inoculated and non-inculated roots (Fig. 3). Four weeks after Foa inoculation, most of the antioxidative items increased in roots of the AMF, NPFO and AMF+NPFO plants compared to control; in shoots, difficult to analysis because of the withering (Fig. 4). In this case, SOD activity and DPPH radical scavenging activity increased in all the AMF, NPFO and AMF+NPFO plants. Ascorbic acid contents were higher in all the AMF, NPFO and AMF+NPFO plants, especially in AMF+NPFO. In polyphenol contents, most of the AMF, NPFO and AMF+NPFO plants showed increase except non-inoculated roots in NPFO; all of the increase occurred in both the inoculated and non-inoculated roots.

**Discussion**

In this study, dry weight of shoots and roots increased in AMF and NPFO plants compared to control, suggesting that growth-promoting effect through AMF and NPFO appeared in asparagus plants. However, no synergistic effect in growth promotion appeared in dual-inoculated plants, though no difference occurred in AMF colonization level between AMF and AMF+NPFO. The reason is still unclear.

Fig. 2 Superoxide dismutase (SOD) activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, ascorbic acid and polyphenol contents in shoots and roots before *Fusarium oxysporum* f. sp. *asparagi* inoculation. C, control; AMF, *Glomus intraradices*; NPFO, non-pathogenic *Fusarium oxysporum*; -, non-inoculated with AMF or NPFO; +, inoculated with AMF or NPFO; C+, inoculated with autoclaved AMF. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant difference according to Tukey’s test (P<0.05).
As for tolerance to Fusarium root rot in this experiment, the incidence and severity of symptom were alleviated by AMF and NPFO, and the effects appeared in both the inoculated and non-inoculated root parts; no synergistic effect in disease suppression occurred in AMF+NPFO. These results indicated that induced systemic resistance (ISR) to Fusarium root rot occurred in asparagus plants inoculated with AMF and NPFO.

SOD plays a primary role in defensive reactions and detoxifies superoxide (O$_2^-$) among the antioxidative enzymes; thus, SOD activity is considered the most important key enzyme in antioxidative abilities in plants (Fridovich, 1986). Garmendia et al. (2006) reported that alleviation of disease symptom and an increase in SOD activity occurred

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**Fig. 3** Incidence and disease indices of Fusarium root rot in asparagus roots 4 weeks after *Fusarium oxysporum* f. sp. *asparagi* inoculation. C, control; AMF, *Glomus intraradices*; NPFO, non-pathogenic *Fusarium oxysporum*. - , non-inoculated with AMF or NPFO; +, inoculated with AMF or NPFO; C+, inoculated with autoclaved AMF. Ratio of diseased roots in a root system; , 0-20; , 20-40; , 40-60; , 60-80; , 80-100 (%).

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**Fig. 4** Superoxide dismutase (SOD) activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, ascorbic acid and polyphenol contents in roots after *Fusarium oxysporum* f. sp. *asparagi* inoculation. C, control; AMF, *Glomus intraradices*; NPFO, non-pathogenic *Fusarium oxysporum*; - , non-inoculated with AMF or NPFO; +, inoculated with AMF or NPFO; C+, inoculated with autoclaved AMF. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant difference according to Tukey’s test (P<0.05).
in mycorrhizal pepper plants inoculated with *Verticillium dahliae*. In the present study, tolerance to *Fusarium* root rot appeared in mycorrhizal asparagus plants, and SOD activity increased in both sides of the split root parts in AMF, NPFO and AMF+NPFO plants before and after Foa inoculation. From these findings, changes in antioxidative enzyme activity would be associated with the ISR to *Fusarium* root rot in mycorrhizal and NPFO asparagus plants. However, in this experiment, antioxidant analysis was carried out only twice, and it took place after long period of Foa inoculation, so that it is difficult to clarify detailed relationship between reduction of disease symptoms and antioxidative ability. Further investigation would be needed on this point.

Antioxidative substances, such as polyphenol contents have lower electron reduction potential than the electron reduction potential of oxygen radicals; as a result polyphenol contents directly scavenge reactive oxygen intermediates without promoting further oxidative reactions (Ainsworth, 2007). Vanitha et al. (2009) mentioned that total phenol content increased in bacterial wilt in tomato plant. On the other hand, Sutton (1973) demonstrated that AMF colonization itself had no correlation with the mycorrhizal colonization levels in pepper. In the present experiment, we could not clarify AMF fungal species differences in ISR, and the relationship between AMF colonization level and ISR.

In addition, antioxidative ability was closely associated with the ISR in mycorrhizal and NPFO asparagus against *Fusarium* disease. These findings are advanced knowledges for understanding the ISR mechanism of AMF and NPFO. On the other hand, the combination use of AMF and NPFO also showed ISR, though disease suppression effect was lower than single inoculation of AMF, the reason is still unclear.

Norman et al. (1996) reported that the incidence of the symptom caused by *Phytophthora fragariae* in strawberry plants was reduced by the inoculation of AMF, though the effect differed with AM fungal species. Ozgonen and Erkilic (2007) reported that growth promotion and tolerance to *Phytophthora capsici* had no correlation with the mycorrhizal colonization levels in pepper. In the present experiment, we could not clarify AMF fungal species differences in ISR, and the relationship between AMF colonization level and ISR. On the other hand, Sutton (1973) demonstrated that AMF colonization consisted of three phases: (1) a lag phase during which spore germination, germ tube growth, and initial penetration occur; (2) a rapid growth phase, coinciding with the development of external mycelium, and spread of the fungus within the roots; and (3) a stable phase during which the proportion of infected roots to non-infected ones remain nearly constant. In this study, AMF colonization level was checked only twice, so that it is difficult to estimate when AMF reached maximum colonization level during the experimental period, and how the colonization level affects the ISR and antioxidative changes.

Some reports described that AMF colonization itself induced a temporal increase in antioxidative ability such as SOD, guaiacol peroxidase (G-POD), catalase (CAT), ascorbate peroxidase (APX), and flavonoid content, suggesting that colonization might be temporal stress for host plants (Wu et al., 2006; Zhu et al., 2010). On the other hand, Fuchs et al. (1997) described that tomato plants grown in autoclaved soil inoculated with NPFO (Fo47) showed increased activity of β-1,3-glucanase and β-1,4-glucosidase in stems compared to control plants. From this fact, NPFO could induce defense mechanisms in particular plant tissues or cells. In this study, several antioxidative factors increased in AMF- and NPFO-inoculated plants before Foa inoculation, so that the increase might be partially associated with the host defense response. However, further study would be needed to clarify the reason for antioxidative increase in AMF and NPFO inoculation itself, and the antioxidative increase in non-inoculated root parts in this study. In addition, AMF and NPFO plants showed the increase in most of the antioxidative ability in shoots, where no colonization occurred. From
these facts, we will estimate the ISR to shoot disease such as stem blight in asparagus plants inoculated with AMF and NPFO, and the relationship between antioxidative ability and the ISR. Our proposal in this study seeks to develop a sustainable practice to manage the disease and improve plant health, thus contributing to an improvement in asparagus decline.

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References


アーバスキュラー菌根菌及び非病原性フサリウム菌によるアスパラガス立枯病に対する全身誘導抵抗性及び抗酸化機能変動

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摘 要

アーバスキュラー菌根菌及び非病原性フサリウム菌（NPFO）によるアスパラガス立枯病に対する全身誘導抵抗性及び抗酸化機能変動をsplit root system法により調査した。菌根菌及びNPFOの単独・複合処理区では地上部・地下部乾物重は対照区より増大し、植物体成長促進効果がみられた。立枯病菌（Fusarium oxysporum f. sp. asparagi）接種4週間後、発病率・発病程度は菌根菌及びNPFO処理区で対照区より軽減された。この場合、接種根及び非接種根の両方で耐病性がみられたことから誘導抵抗性が確認された。また、抗酸化機能（SOD活性、DPPHラジカル捕捉能、総ポリフェノール・アスコルビン酸含量）は、菌根菌及びNPFOの接種根及び非接種根で地上部・地下部とも対照区より立枯病菌接種前後で増大する場合が多かった。これらの結果から、アーバスキュラー菌根菌及び非病原性フサリウム菌によるアスパラガス立枯病の全身誘導抵抗性が確認され、生物的防除に利用できる可能性が示唆されるとともに、抗酸化機能が全身誘導抵抗性に関連することが示された。

キーワード：抗酸化機能、Glomus intraradices、生物的防除、全身誘導抵抗性、non-pathogenic Fusarium oxysporum

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