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Suppression of Fusarium Crown Rot and Increase in Several Free Amino Acids in Mycorrhizal Asparagus

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ABSTRACT

Disease suppression of *Fusarium* crown rot and the changes in free amino acid contents in mycorrhizal asparagus (*Asparagus officinalis* L., cv. "Welcome") plants were investigated. Sixteen weeks after arbuscular mycorrhizal fungus (AMF; *Glomus intraradices*) inoculation, mycorrhizal plants showed higher dry weight of shoots than non-mycorrhizal plants, and AMF colonization level in a root system reached up to 73.3%. Ten weeks after *Fusarium proliferatum* (Fp; N1-31, SUF1207) inoculation, control plants showed 100% incidence and high severity in the 2 Fp isolates. However, AMF plants showed lower severity than non-AMF plants in the 2 Fp isolates. Ten weeks after Fp (N1-31) inoculation, the increase in 7 constituents of amino acids (glutamine, arginine, aspartic acid, alanine, citrulline, GABA, glycine) in shoots, and 9 in roots (asparagine, arginine, threonine, serine, glutamine, citrulline, valine, GABA, histidine) occurred in AMF plants. From these findings, plant growth promotion and suppression of *Fusarium* crown rot occurred in mycorrhizal asparagus plants, and the disease tolerance was supposed to be associated with the symbiosis-specific increase in free amino acids.

KEYWORDS

Asparagus Decline; *Fusarium proliferatum*; GABA; Growth Promotion; Symbiosis

1. Introduction

Asparagus decline is a serious and increasing threat in asparagus producing regions over the world [1-4]. It is supposed to be caused by the contribution of both biotic (disease) factors [1,2] and abiotic (allelopathy etc.) factors [5-7]. As biotic factors, the most common phenomenon is *Fusarium* crown and root rot, caused by *Fusarium proliferatum* (Fp), *Fusarium oxysporum* f. sp. *asparagi* (Foa), and *Fusarium redolens* etc. [1,2,8]. In Japan, Nahiyan *et al.* [9] demonstrated that Fp and Foa are dominant *Fusarium* species in asparagus decline fields by PCR-SSCP analysis. However, the diseases are still difficult to control because no resistant cultivar or disinfesting method has been developed. On the other hand, biological control of *Fusarium* disease was tried by inoculation with non-pathogenic isolates of the *Fusarium* species [10,11]. However, the method is not enough to control and has no growth promoting effect.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil inhabitants, and form a symbiotic relationship with roots of most of the terrestrial plants. AMF promotes host plant growth by enhancing phosphorus uptake through symbiosis [12], and hence an alternative to high inputs of fertilizers and pesticides in sustainable crop production systems. Previously, the author reported that tolerance to *Fusarium* root rot is caused by Foa in mycorrhizal asparagus (cv. Mary Washington 500 W) plants [13]. However, tolerance to Fusarium crown rot caused by Fp and the mechanisms on disease tolerance in mycorrhizal asparagus plants are still unclear.

As for the changes in amino acid constituents related to disease tolerance in mycorrhizal plants, Baltruschat and Schonbeck [14] demonstrated that the propagation of *Thielaviopsis basicola* was inhibited by the increase of

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arginine and citrulline in mycorrhizal tobacco plants. In addition, some reports mentioned that the free amino acid level in plants changes through AMF colonization. Sood [15], Fattah and Mohamedin [16] reported that increases in the contents of free amino acids occurred in mycorrhizal tomato and sorghum plants, respectively. On the other hand, Rolin *et al.* [17] reported that AMF colonization decreased total amino acid levels in mycorrhizal leek plants. However, it has been unclear how the contents of free amino acid change through symbiosis with AMF in asparagus plants and how the changes are associated with disease tolerance.

In this study, suppression of *Fusarium* crown rot and the changes in free amino acid contents in mycorrhizal asparagus plants were investigated in order to clarify the mechanisms of disease tolerance.

2. Materials and Methods

2.1. Inoculation of AMF

Seeds of asparagus (*Asparagus officinalis* L., cv. Welcome) were sown in commercial soil (autoclaved at 1.2 $kg \cdot cm^{-2}$ and 121˚C for 1hour) in plastic container (43 × 27 × 17 cm). During the time of seed sowing, plant holes were made, each hole contains 3g/plant commercial AMF (*Glomus intraradices*) inoculum supplied by Idemitsukosan Co. Ltd., Tokyo, Japan. Then, seeds were sown onto the inoculum, finally covered with soil and administered by mixed fertilizer (N: P: K = 13:11:13, 0.5 g per plant). Forty plants per plot with three replications were irrigated as regularly and grown in a greenhouse of Gifu University, Japan in 2011.

2.2. Inoculation of *Fusarium proliferatum*

Two isolates of *Fusarium proliferatum* (Fp:N1-31, SUF1207) 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 concentrations adjusted to 10^6 conidia per ml. Sixteen weeks after AMF inoculation, each plant was inoculated by 50 ml of the conidial suspension onto the roots.

2.3. Estimation of Symptoms of Fusarium Crown Rot

Ten weeks after inoculation of Fp, the symptoms of *Fusarium* crown rot were rated to 6 degrees as follows: 0, no symptom; frequency of diseased storage roots in a root system: 1, less than 20%; 2, 20% - 40%; 3, 40% - 60%; 4, 60% - 80%; 5, 80% - 100%.

2.4. Evaluation of AMF Colonization Level

Sixteen weeks after AMF inoculation, roots of asparagus were preserved with 70% ethanol and stained according to Phillips and Hayman [18]. 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 was calculated from the values of five plants.

2.5. Determination of Free Amino Acids in Plants

Sixteen weeks after AMF inoculation, plants were sampled and partitioned into shoots and storage roots from 10 plants, and all samplers were frozen in liquid nitrogen. The samples for free amino acid analysis were collected from 10 plants as follows: shoots (approx. 1 cm long from the base), storage roots (approx. 1 cm from the crown). Free amino acids in each 200 mg-weighed samples were extracted at 0˚C in 2 mL 0.2 N perchloric acid solution mixed with 1 mL 0.25 μM D, L-norleucine as an internal standard. Extracts were centrifuged at 14,000 rpm at 4˚C, and pH was adjusted to 4.0 with $KHCO_3$. Then, the extracts (20 μL in each time) were filtrated by a GL-chromatodisc (GL science Co., Ltd., Tokyo, Japan). Free amino acid concentrations (41 constituents) were measured using an automatic amino acid analyzer (JLC-500, JEOL Co., Ltd., Tokyo, Japan) using ninhydrin.

2.6. Statistical Analysis

Mean values were separated by *t*-test for dry weight and free amino acid contents at $P \leq 0.05$. All analyses were performed using statistical analysis software (SSRI, Tokyo, Japan).

3. Results

Sixteen weeks after AMF inoculation, AMF plants had greater dry weight of shoots and roots than non-AMF control plants (**Figure 1**). AMF colonization was occurred successfully and reached up to 73.3%, 16 weeks after AMF inoculation (data not shown). Ten weeks after Fp inoculation, control plants showed 100% incidence and high severity in the 2 Fp (N1-31, SUF1207) isolates (**Figure 2**). However, AMF plants showed lower severity than control plants in the 2 Fp isolates, especially in N1-31.

Sixteen weeks after AMF inoculation, 22 amino acids in shoots and 18 amino acids in roots were detected in both AMF and control plants (**Figures 3** and **4**). Contents of several free amino acids increased in both shoots and roots in AMF plants compared to control, and the number of increased free amino acids were greater in roots than shoots. AMF plants had increase in 8 constituents of free amino acids (glutamine, arginine, aspartic acid, alanine,

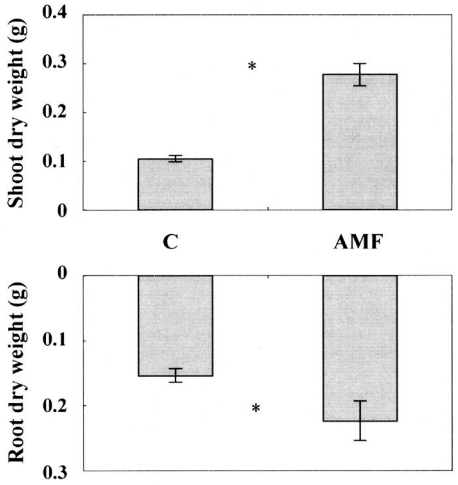

Figure 1. **Dry weight of asparagus plants 16 weeks after AMF inoculation. C, control; AMF, *Glomus intraradices*-inoculated. Bars represent standard errors (n = 10). *significantly different between control and AMF plants (*t*-test, $P \leq 0.05$); NS, not significant.**

citrulline, GABA, glycine, 2-aminoethanol) in shoots, and 9 (asparagine, arginine, threonine, serine, glutamine, citrulline, valine, GABA, histidine) in roots. Great increase occurred in glutamine, arginine, alanine, citrulline, GABA in shoots, while in roots, asparagine, serine,

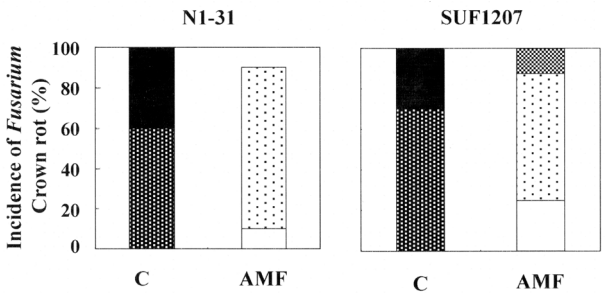

Figure 2. **Disease incidence of *Fusarium* crown rot in mycorrhizal asparagus plants 10 weeks after *Fusarium proliferatum* (N1-31, SUF1207) inoculation. C, AMF, See Figure 1. Ratio of diseased storage roots; □, 0 - 20; ▨, 20 - 40; ▩, 40 - 60; ▩, 60 - 80; ■, 80 - 100 (%).**

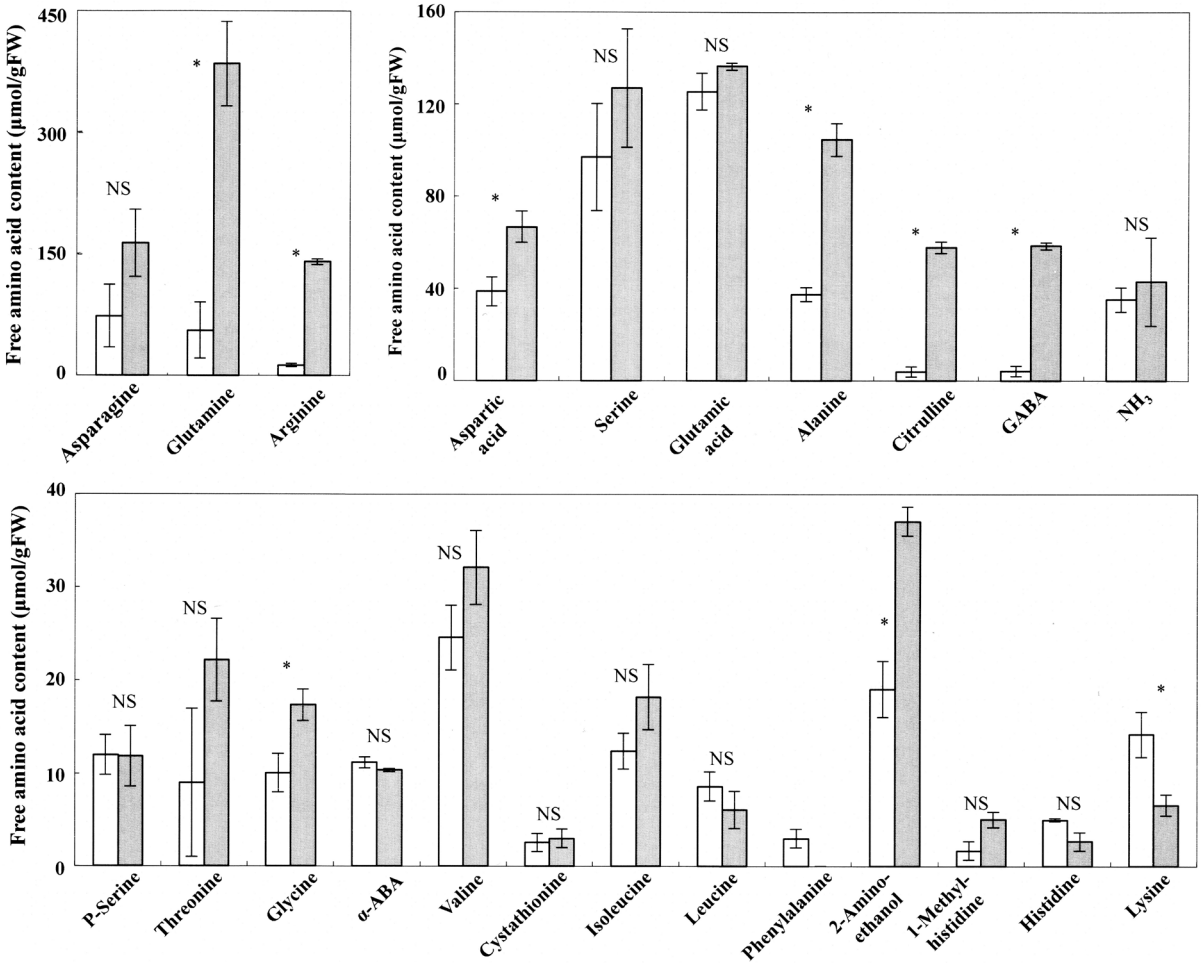

Figure 3. **Free amino acid contents in shoots of mycorrhizal asparagus plants 10 weeks after *Fusarium proliferatum* (N1-31) inoculation. □, C; ▩, AMF. C, AMF, see Figure 1. Bars represent SE (n = 10). *Significantly different between C and AMF plants (*t*-test, $P \leq 0.05$); NS, not significant.**

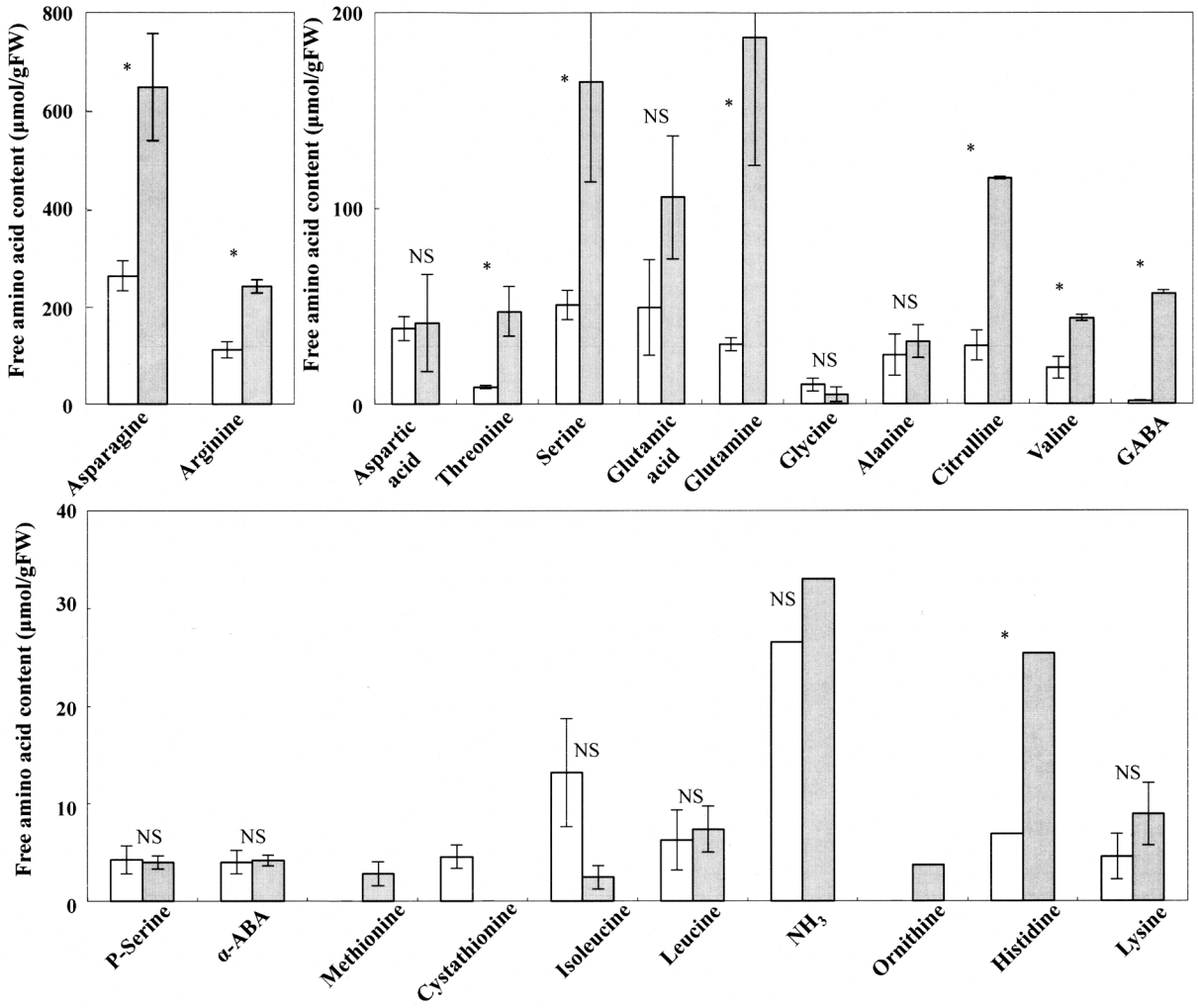

Figure 4. Free amino acid contents in roots of mycorrhizal asparagus plants 10 weeks after *Fusarium proliferatum* (N1-31) inoculation. □, C; ■, AMF. C, AMF, see Figure 1. Bars represent SE (n = 10). *significantly different between C and AMF plants (*t*-test, $P \leq 0.05$); NS, not significant.

glutamine, citrulline, GABA and histidine. In this case, arginine, glutamine, citrulline and GABA increased in both shoots and roots in AMF plants.

4. Discussion

In this study, increase in several free amino acids occurred in mycorrhizal asparagus plants, though the effect varied between the plant portions. Sood [15] reported the increases in glutamic acid, glycine, alanine and leucine in mycorrhizal tomato seedlings, and Fattah and Mohamedin [16] mentioned glutamic acid and serine increases in mycorrhial sorghum plants. On the other hand, Baltruschat and Schonbeck [14] demonstrated that in tobacco plants, an increase in both arginine and citrulline occurred in mycorrhizal plants. The results in this study have similar points as those reported for tomato, sorghum and tobacco. In addition, the increase in GABA concentration in mycorrhizal asparagus plants was confirmed. Recently, GABA acts an important function in plant stress responses [19], in addition, the blood pressure-lowing effect of GABA in humans was reported [20]. From these findings, GABA increase in mycorrhizal asparagus plants of this study is interesting in the aspects of both disease tolerance and quality in harvested products.

Increase of free amino acid contents in mycorrhizal plants has been reported [14-16], with concentrations varying for several host-fungus combinations. Previous and this reports, thus, only one AMF species, so that it remains unclear whether fungal difference in amino acid changes occurs in the same host. On the other hand, Fattah and Mohamedin [16] mentioned that the degree of the increase in amino acids was correlated with the level of mycorrhizal colonization in the sorghum-*Glomus intraradices* combination. Sutton [21] demonstrated 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 remains nearly constant. In our study, no significant difference in colonization level between before and after (data not shown) Fp inoculation. However, in this experiment, amino acids were investigated only after Fp inoculation. Hence, it was difficult to estimate the fluctuation in colonization level and the relationship between free amino acid contents and colonization level.

In this study, dry weight of shoots increased in AMF plants compared to control plants. From this finding, growth promoting effect through symbiosis appeared in mycorrhizal asparagus plants. As for tolerance to *Fusarium* root rot, Matsubara *et al.* [13] reported that AMF (*Glomus intraradices*) increased *Fusarium* root rot tolerance in asparagus (cv. Mary Washington 500 W) plants. Our results showed the tolerance to *Fusarium* crown rot in mycorrhizal asparagus (cv. Welcome) plants same as those findings. In the present study, AMF promoted the growth of asparagus plants, and the severity of symptoms in Fp was alleviated by pre-colonization with AMF. Baltruschat and Schonbeck [14] demonstrated that in tobacco plants, an increase in both arginine and citrulline occurred in mycorrhizal plants, which inhibited the propagation of *Thielaviopsis basicola.* Starratt and Lazarovits [22] reported low levels of the herbicide trifluralin induced resistance to *Fusarium* wilt and elevated levels of free amino acids in melon seedlings. In this study, the increase in several free amino acids through mycorrhizal symbiosis in asparagus plants was confirmed, and arginine and citrulline increased in both shoots and roots in AMF plants. From these findings, suppression of *Fusarium* crown rot in this study is closely associated with increase in free amino acids. On the other hand, Dehne and Schonbeck [23] reported that the lignification in the endodermis and the stele enhanced by AMF colonization suppressed *Fusarium* wilt in tomato plants. Matsubara *et al.* [13] reported that pectic substances in asparagus roots increased by AMF colonization, and they supposed that the resulting rigidity of root tissue suppressed *Fusarium* infection. Thus, some physiological and histological factors may be associated with disease tolerance in mycorrhizal plants.

On the other hand, Pozo *et al.* [24] reported that in tomato plants with a split root system, tolerance to *Phytophthora parasitica* appeared in both non-AMF inoculated roots and inoculated roots in AMF plants, so that induced systemic disease tolerance was recognized. In this study, several free amino acids increased in shoots, where no colonization occurred. From these facts, we will estimate the induced systemic disease tolerance in mycorrhizal asparagus plants with split root system, and further work is required to determine whether the changes in free amino acid contents have a direct or indirect relationship to the suppression of induced disease tolerance.

5. Conclusion

Our results suggest that AMF could induce suppression of *Fusarium* crown rot in asparagus plants, and several free amino acids increased through the symbiosis, lead to the disease suppression as a physiological factor. Thus, control of *Fusarium* diseases using AMF as a biocontrol agent 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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Abbreviations

AMF: arbuscular mycorrhizal fungus;
Fp: *Fusarium proliferatum*;
Foa: *Fusarium oxysporum* f. sp. *asparagi*;
RFCSL: The rate of AMF colonization in 1-cm segments of lateral roots;
GABA: Gamma-amino butyric acid.