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Tolerance to Fusarium Wilt and Changes in Antioxidative Ability and Free Amino Acid Content in Mycorrhizal Strawberry Plants

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ABSTRACT

The influence of arbuscular mycorrhizal fungi (AMF) colonization on tolerance to fusarium wilt and the changes in antioxidative ability and free amino acid content in strawberry (*Fragaria×ananassa* Duch. cv. 'Nohime') plants was investigated. Strawberry runner plants were treated with split root system and inoculated with *Glomus mosseae*. Mycorrhizal plants showed higher dry weight of shoots and roots than did non-mycorrhizal plants among most of the plots. Five weeks after *Fusarium oxysporum* f. sp. *fragariae* (Fof) inoculation, severity of disease symptoms was eased in shoots and roots of mycorrhizal plants. Plant mycorrhization did not modified the antioxidative ability before Fof inoculation. However, 5 weeks after Fof inoculation, mycorrhizal plants showed higher SOD activity, DPPH radical scavenging activity, polyphenol content, ascorbic acid content in some plant portions. Free amino acid content GABA, aspartic acid, threonine, serine, glycine, citrulline and arginine increased in mycorrhizal plants with different entity depending on plant portions. In addition, some among the amino acids increased in mycorrhizal plants showed suppressed by 20 to 70% the propagation of Fof cultured by Czapec-Dox media *in vitro* depending their concentration. From these findings, plant growth enhancement and tolerance to fusarium wilt including induced tolerance occurred in mycorrhizal strawberry plants. In this case, the disease tolerance might be associated with the increase in antioxidative ability and symbiosis-specific amino acids.

Keywords: *Fragaria*×*ananassa* Duch., GABA, *Glomus mosseae*, polyphenol, SOD, symbiosis

INTRODUCTION

In strawberry cultivation, Fusarium wilt and anthracnose have been the serious diseases in the major strawberry producing regions in Japan, and the diseases caused heavy losses during nursery and fruit production period (Tezuka and Makino 1991; Okayama 1993; Mori and Kitamura 2003). Recently, capillary watering as a cultural control method of these diseases has been introduced to strawberry cultivation, but the diseases are still difficult to control (Okayama 1993; Akita 2001). As for biological control of Fusarium disease, an attempt has been made to use non-pathogenic isolates of *Fusarium oxysporum* in strawberry (Tezuka and Makino 1991). However, the non-pathogenic isolates have no growth-promoting effect and the method is still not enough to control.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil inhabitants, and form a symbiotic relationship with the roots of most terrestrial plants. AMF promote host plant growth by enhancing phosphorus uptake through symbiosis (Marschner and Dell 1994) and hence an alternative to high inputs of fertilizers and pesticides in sustainable crop production systems. As for strawberry, growth enhancement through AMF inoculation was reported in several combinations of fungal species and strawberry cultivars (Robertson *et al*. 1988; Chavez and Ferrera 1990; Williams *et al*. 1992; Niemi and Vestberg 1992; Varma and Schuepp 1994). In addition, the inoculation of strawberry plants with AMF resulted in a reduced disease when challenged with *Phytophthora fragariae*; however, the effect differed with host cultivar and AMF species (Baath and Hayman 1984; Mark and Cassells 1996; Norman *et al*. 1996). Previously, the author found the tolerance to Fusarium wilt in strawberry (*Fragaria*×*ananassa* Duch. cv. 'Nohime') plants inoculated with five species of AMF (*Gigaspora margarita*, *Glomus fasciculatum*, *Gl. mosseae*, *Gl.* sp. R10 and *Gl. aggregatum*) in greenhouse experiment, and the effect mostly appeared in *Gl. mosseae*-inoculated plants (Matsubara *et al*. 2004). The mechanisms of disease tolerance in mycorrhizal plants are unclear. However, for mycorrhizal plants, disease tolerance and increase in superoxide dismutase (SOD) activity show correlation in tomato (Pozo *et al*. 2002) and pepper (Garmendia *et al*. 2006), drought tolerance and increase in SOD took place in lettuce (Lozano *et al*. 1996) and shrub (Roldan *et al*. 2008), tolerance to SO_2 and SOD increase occurred in *Avena nuda* (Huang *et al*. 2008). In addition, Li *et al*. (2008) demonstrated that tolerance to high temperature stress and increase in antioxidative enzymes occurred in mycorrhizal strawberry plants. However, the relationship between antioxidative abilities and disease tolerance in mycorrhizal strawberry plants still remains unclear.

As for the changes in constituents related to disease tolerance in mycorrhizal plants, Baltruschat and Schonbeck (1975) demonstrated that in tobacco plants, an increase in both arginine and citrulline occurred in mycorrhizal plants, which inhibited the propagation of *Thielaviopsis basicola*. In addition some reports mentioned that the free amino acid level in plants changes through AMF colonization. Sood (2003) and Fattah and Mohamedin (2000) reported that increases in the contents of free amino acids occurred in mycorrhizal tomato and sorghum plants, respectively. Rolin *et al*. (2001) reported in leek plants that total amino acid levels in roots decreased in mycorrhizal plants. However, it has been unclear how free amino acid content changes through symbiosis with AMF in strawberry plants and how the changes are associated with disease tolerance.

In this study, the influence of AMF colonization on tolerance to Fusarium wilt and the changes in antioxidative

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ability and free amino acid content in mycorrhizal strawberry plants was investigated in order to clarify the mechanisms of disease tolerance.

MATERIALS AND METHODS

Inoculation of AMF

Strawberry (*Fragaria×ananassa* Duch. cv. 'Nohime', a commercial cultivar in Japan) runner plants were treated with split root system method. The plant root system was separated into halves and each was placed in a different pot (10.5 cm in diameter): one with disinfested bedding soil (autoclaved at 1.2 kg cm^{-2} and 121°C for 1 h), and one with the same soil inoculated with *Glomus mosseae* (supplied by Idemitsukosan Co. Ltd.), according to Matsubara *et al.* (2004). Two weeks after AMF inoculation, the plants were administered by mixed fertilizer (N: P: K = 13: 11: 13, 1 g per pot). Twenty plants with split root system were raised per treatment with two replications, were irrigated by the capillary watering method (Matsubara *et al.* 2004) and grown in a greenhouse (28 ± 3°C under natural light and day length).

Inoculation with *Fusarium oxysporum* f. sp. *fragariae*

Fusarium oxysporum f. sp. *fragariae* (Fof: strain 2S, supplied by National Agricultural Research Center for Kyusyu Okinawa Region in Japan) was grown on potato-dextrose agar media. The conidia were harvested in potato-sucrose liquid media (Nissui pharmaceutical Co., Ltd.) and incubated at 25°C in the dark for 7 days. The conidial suspension of Fof was sieved (45 μm), and the concentration was adjusted to 10^6 conidia mL^{-1}. Eight weeks after AMF inoculation, all the plants were inoculated by each 10 mL of the Fof conidial suspension onto all the split roots. Plants were grown in a growth chamber by capillary watering at 28 ± 3°C, 60 ± 5% relative humidity under natural light and day length.

Estimation of symptoms of Fusarium wilt

Five weeks after Fof inoculation, the severity of fusarium wilt was categorized into 5 degrees on the base of the percentage of diseased petioles in a plant: <20%, 20-40%, 40-60%, 60-80%, 80-100%.

Evaluation of AMF colonization level

Roots of AMF plants were harvested 8 weeks after AMF inoculation and 5 weeks after Fof inoculation. The root samples were stained according to Phillips and Hayman (1970) and the rate of AMF colonization in 1-cm segments of lateral roots (RFCSL) was calculated. Hence, RFCSL expresses the percentage of 1-cm AMF-colonized segments to the total 1-cm segments of all the lateral roots; the number of total segments was approx. 50 $plant^{-1}$. The average colonization was calculated from the values of 3 plants.

Determination of plant dry weight

Plants were sampled 8 weeks after AMF inoculation and 5 weeks after Fof inoculation. Plant samples were separated into shoots and roots and dry matter was weighed after drying at 100 °C for 2 days.

Determination of antioxidative abilities

Eight weeks after AMF inoculation (just before Fof inoculation) and 5 weeks after Fof inoculation, plants were sampled and partitioned into petioles, crowns and main roots with no colonization and frozen in liquid nitrogen. Analyses of antioxidative abilities were carried out as the methods as follows.

1. SOD activity

One gram of sample was ground in 4 ml of 0.1 mol phosphate-borate buffer (pH7.8) with 1 mmol ethylenediaminetetra acetic acid, 3 mmol dichlorodiphenyltrichloroethane and 4% (w/v) polyvinylpyrrolidone. The filtrate was centrifuged (EF-1300, Tomy Co., Ltd., Tokyo, Japan) at 13,000 rpm for 20 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). All the chemicals used in this study were analytical grade and obtained from Nacalai Tesque, Co., Inc., Kyoto, Japan.

2) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was measured according to the method of Brurits 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 4ml distilled water and 1 ml of 250 μl 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 517 nm using a spectrophotometer (U-1900, HITACHI). DPPH radical scavenging activity was calculated as the percent of inhibition relative to the control.

3) Polyphenol content

Polyphenol content was determined with Folin-denis method (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 (10% $Na_2WO_4 \cdot 2H_2O$, 2% $H_3(PMo_{12}O_{40}) \cdot nH_2O$, 5% phosphoric acid). After 3 min, 0.4 ml of Na_2CO_3 (10%) was added, the mixture was allowed to stand for 30 min in the dark. Absorbance was measured at 700 nm using a spectrophotometer (U-1900, HITACHI). Polyphenol content was determined as quercetin equivalent using an equation obtained from a standard quercetin graph.

4) Ascorbic acid content

The assay was performed 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, and1ml of 2,6-dichloroindophenol sodium (0.03%) was added. Then, 1 ml of 2% 2,4-dinitrophenylhydrazine was mixed and incubated for 3 hrs. Absorbance was measured at 520 nm using a spectrophotometer (U-1900, HITACHI). Ascorbic acid content was determined using an equation obtained from a standard L-ascorbic acid graph.

Determination of free amino acids in plants

Eight weeks after AMF inoculation, plants were sampled and partitioned into petioles, crowns and main roots (in AMF plants, sampled only AMF-inoculated roots), and all samplers were freeze-dried using a freeze dryer (FDU-1200, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The samples for free amino acid analysis were collected from 10 plants as follows: petioles (approx. 1 cm long from the base), main 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.

Fof culture with several amino acids *in vitro*

The conidia of Fof grown on PDA media was subcultured for 2 weeks on Czapec-Dox media (Ohata 1995) containing $NaNO_3$ 3 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, sucrose 30 g, agar 8 gL^{-1} (pH 5.8). Then, the conidia were further subcultured (10^6 conidia mL^{-1}) in liquid Czapec-Dox media with or

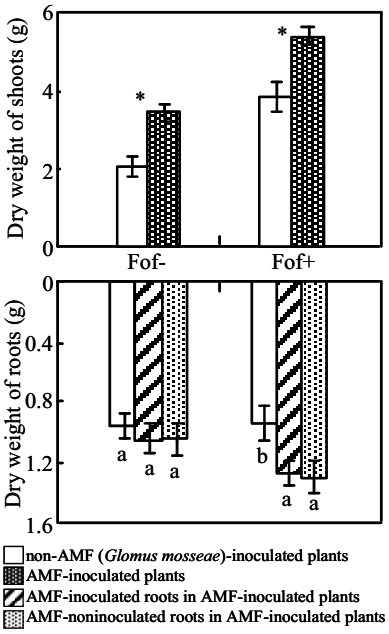

Fig. 1 Dry weight of strawberry plants. Fof-, before *Fusarium oxysporum* f. sp. *fragariae* (Fof) inoculation; Fof+, 5 weeks after Fof inoculation. Bars represent standard errors. *Data significantly different following *t*-test, *P*=0.05. Columns denoted by different letters indicate significant difference according to Tukey's multiple range test (*P*=0.05).

without addition of several filter-sterilized amino acids (gamma-aminobutyric acid (GABA), aspartic acid, threonine, serine, glycine, citrulline, leucine and arginine; 0.1, 1%, w/v) at 25°C in the dark for 7 days by shaked culture (100 rpm). Then, the density of conidia was investigated using hemocytometer and calculated the propagation index of amino acid-added plots to amino acid-non-added plots. The average was calculated from 5 replications.

Experimental design and statistical analyses

Most of the values were expressed as the mean of three measurements for each treatment. Mean values were separated by *t*-test at $P < 0.05$ and Tukey's multiple range test at $P < 0.05$ via analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Effect of AMF inoculation on plant growth and Fusarium wilt

AMF plants had higher dry weight of shoots and roots than did NAM ones except roots of before Fof inoculation (**Fig. 1**), suggesting that growth promotion effect appeared in mycorrhizal strawberry plants. AMF colonization was successful, reaching values about 40% and 48% just before and 5 weeks after Fof inoculation, respectively (**Fig. 2**). The severity of fusarium wilt symptoms decreased in shoots of AMF plants (**Fig. 3**). In addition, reduction in symptom severity in roots became lower in both AMF+ and AMF- than in NAM one. Thus, AMF- roots showed disease tolerance. From these findings, tolerance to Fusarium wilt and induced tolerance were recognized in mycorrhizal strawberry plants. Our results agreed with those of Pozo *et al*. (2002), which reported an induced tolerance to *Phytophthora parasitica* in tomato plants with split root system.

Influence of AMF inoculation on antioxidative ability

In this study, AMF did not cause variations in SOD and DPPH activities, polyphenols and ascorbic acid content in leaf petioles, crown and main roots of strawberry (**Fig. 4**). Fof caused a reduction of ascorbic acid and DPPH, but an

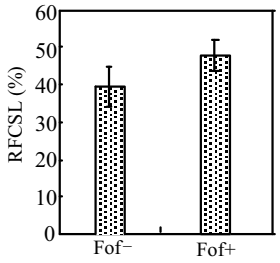

Fig. 2 AMF colonization level (RFCSL) in mycorrhizal strawberry plants. Fof-, Fof+, see **Fig. 1**. Bars represent standard errors. *Data significantly different following *t*-test, *P*=0.05.

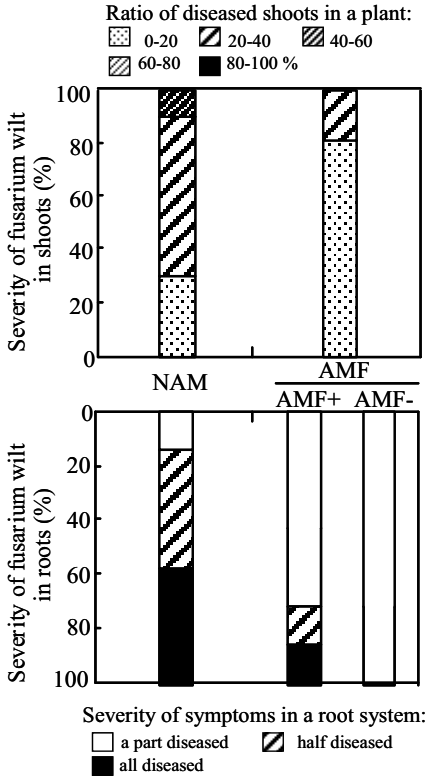

Fig. 3 Severity of fusarium wilt in strawberry plants with split root system. NAM, noninoculated; AMF, *Glomus mosseae*-inoculated plants; AMF+, AMF-inoculated roots; AMF-, AMF-noninoculated roots.

increase SOD activity in petioles; a reduction of DPPH in plant crown, and a reduction of SOD and polyphenols in main roots. Mycorrhized plants challenged with Fof showed a significant increase in 3 out of 4 antioxidative composts in the crown (DPPH, polyphenols, and ascorbic acid), but not in the petioles. In the main roots of these plants, SOD and DPPH activities and polyphenols were restored to the levels detected in the healthy plants, whereas ascorbic acid was not imbalanced.

SOD plays a primary role in defensive reactions and detoxify superoxide (O_2^-) among the antioxidative enzymes, thus, SOD activity is considered as the most important key enzyme in antioxidative abilities in plants (Fridovich 1986). Garmendia *et al*. (2006) reported that tolerance to *Verticillium dahliae* and increase in SOD activity occurred in mycorrhizal pepper plants. Moghaddam *et al*. (2006) and Sahoo *et al*. (2007) mentioned that resistant cultivar to *Mycosphaerella fragariae* and *Phytophthora colocasiae* showed higher levels in SOD activity than a susceptible one in strawberry and taro, respectively. The results in this study had similar patterns with the findings as the increase in SOD activity related to the disease tolerance.

Previously, Pozo *et al*. (2002) demonstrated a relationship between the tolerance to *Phytophthora parasitica* of mycorrhizal tomato plants and SOD activity in both non-

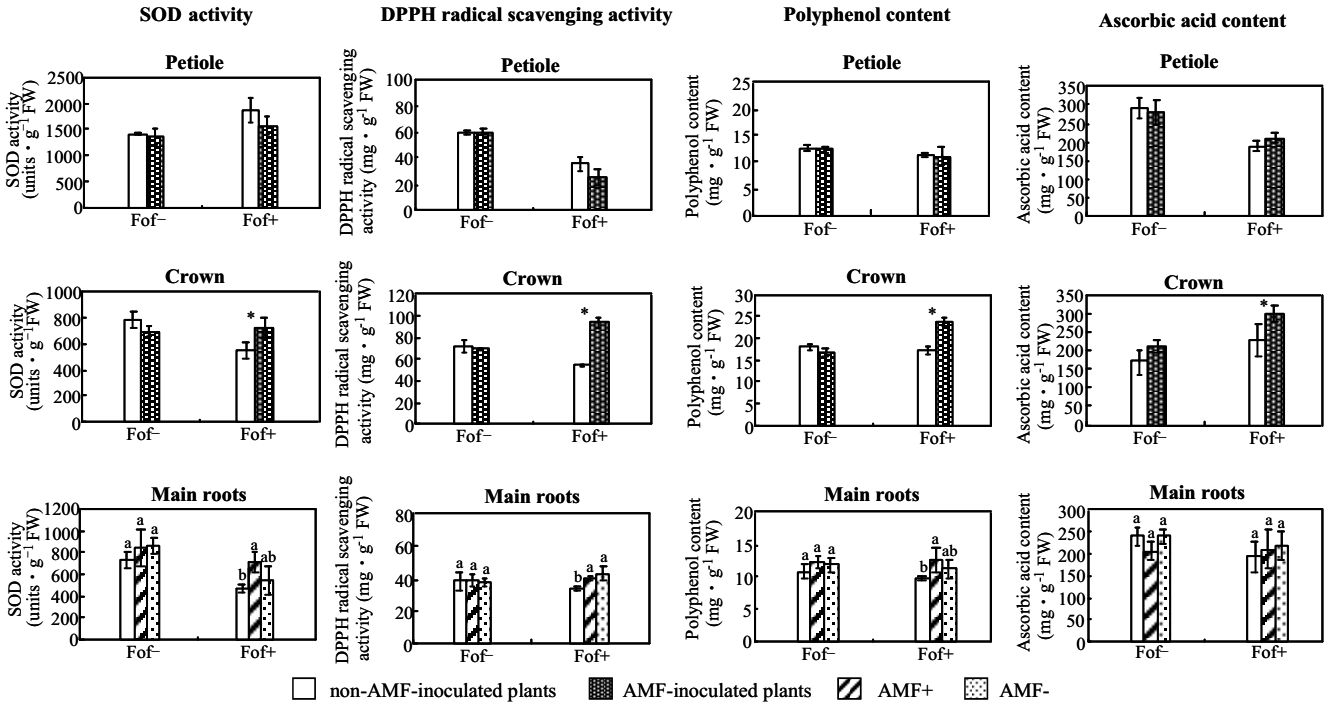

Fig. 4 Antioxidative ability in mycorrhizal strawberry plants. Fof-, Fof+, see **Fig. 1**. AMF+ and AMF-, see **Fig. 3**. Bars represent standard errors. *Data significantly different following *t*-test, *P*=0.05. Columns denoted by different letters indicate significant difference according to Tukey's multiple range test (*P*=0.05).

AMF inoculated and AMF-inoculated roots in split root system. Li *et al*. (2008) reported that tolerance to high temperature stress and the increase in activities of SOD and APX under the stress condition occurred in strawberry (cv. 'Nohime') plants inoculated with *Glomus mosseae*, though the antioxidative enzyme activities differed little between AMF and NAM plants before high temperature stress condition. Hence, the increase in antioxidative ability might be induced especially under the stress conditions in mycorrhizal strawberry plants, resulted in the tolerance to Fusarium wilt in this study.

Some other reports indicate that the mycorrhizal colonization itself induces temporal increases in antioxidative enzymes (APX, CAT), H_2O_2, flavonoid content, suggesting that colonization might act as a temporal stress for host plants (Volpin *et al*. 1995; Salzer *et al*. 1999; Blilou *et al*. 2000). In this study, *G. mosseae* colonization did not cause changes in antioxidative ability. However, the levels of most antioxidative factors varied in mycorrhizal plants after Fof inoculation rather than those before the inoculation. Following our results, antioxidative abilities may play a partial or a secondary role in induced tolerance to Fof in mycorrhizal strawberry plants. Further investigation would be needed to clarify the relationship between induced tolerance and antioxidative ability in mycorrhizal plants.

In the present study, AMF symbiosis promoted the growth of strawberry plants, and reduced the severity of Fusarium wilt symptoms. Opposite conclusions were drown by others: no relationship were found between AMF colonization level and: tolerance to *P. fragariae* in strawberry (Mark and Cassells 1996), growth promotion and tolerance to *Phytophthora capsici* in pepper (Ozgonen and Erkilic 2007), SOD activity and drought tolerance in lettuce and shrub (Lozano *et al*. 1996; Roldan *et al*. 2008).

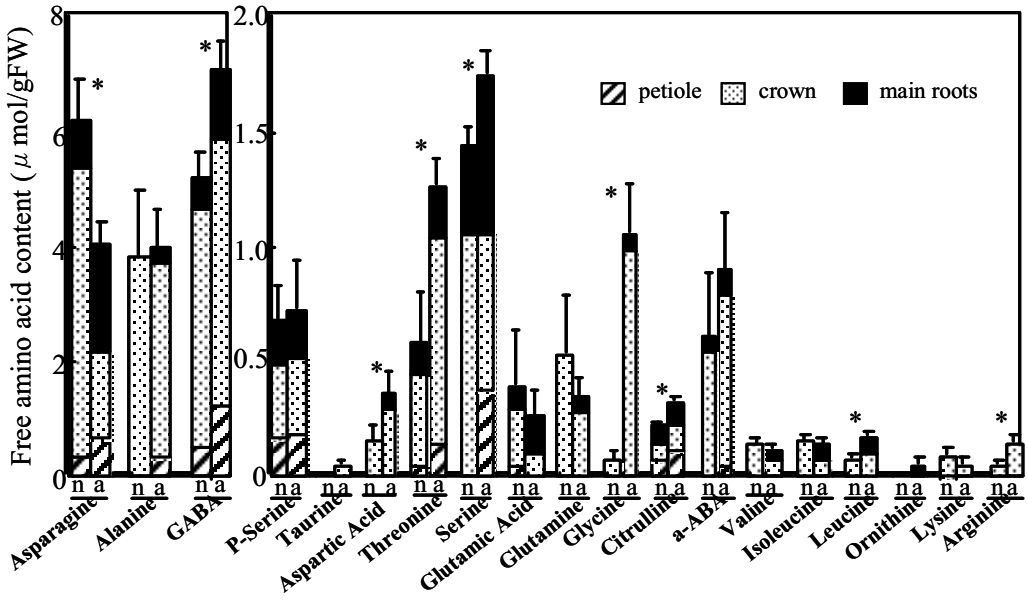

Fig. 5 Influence of AMF colonization on free amino acid content in strawberry plants. n, non-AMF-inoculated plants; a, AMF-inoculated. Bars represent standard errors for total contents. *Data significantly different following *t*-test, *P*=0.05.

Changes in free amino acid content and influence of several amino acids on Fof propagation

In the present study, as for free amino acid content, GABA, aspartic acid, threonine, serine, glycine, citrulline, leucine and arginine increased in AMF plants more than in NAM ones; the effect differed among plant portions (**Fig. 5**). In addition, some of the increased amino acids in mycorrhizal plants showed suppression effect on the index of Fof propagation (**Fig. 6**). Sood (2003) reported increases in glutamic acid, glycine, alanine and leucine in mycorrhizal tomato seedlings, and Fattah and Mohamedin (2000) reported glutamic acid and serine increases in mycorrhial sorghum plants. On the other hand, Baltruschat and Schonbeck (1975) demonstrated that in tobacco plants, an increase in both arginine and citrulline occurred in mycorrhizal plants. The results in this study havesimilar points as those reported for tomato, sorghum and tobacco.

Fattah and Mohamedin (2000) 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 (1973) 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 this study, amino acids and AMF colonization were investigated only 8 weeks after inoculation, so that it was difficult to estimate the fluctuations.

Baltruschat and Schonbeck (1975) 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 (1999) 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 strawberry was confirmed. From these findings, tolerance to Fusarium wilt occurred through symbiosis with AMF in strawberry plants and the increase in free amino acid content in mycorrhizal plants would be also associated with disease tolerance as a direct factor to Fof. On the other hand, Dehne and Schonbeck (1979) reported that the lignification in the endodermis and the stele enhanced by AMF colonization suppressed Fusarium wilt in tomato plants. Matsubara *et al*. (2003) reported that pectic substances in asparagus roots were 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.

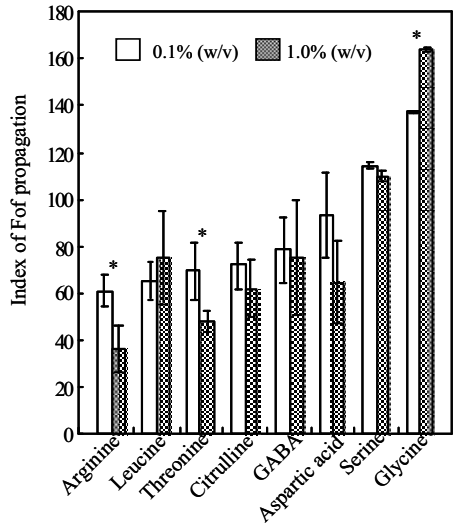

Fig. 6 Influence of several amino acids on propagation of Fof. Bars represent standard errors. *Data significantly different following *t*-test, *P*=0.05.

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