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NOTES AND COMMENTS

Short-term response of arbuscular mycorrhizal association to spider mite herbivory

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Abstract We examined effects of aboveground herbivory by spider mites (*Tetranychus urticae*) on colonization and activity of arbuscular mycorrhizal fungi (AMF; *Gigaspora margarita*) using potted plants (*Lotus japonicus*). We evaluated changes in arbuscular mycorrhizal (AM) association two ways: (1) conventional trypan blue staining of mycorrhizal hyphae to examine AMF biomass in roots (mycorrhizal colonization) and (2) vital staining for a mycorrhizal enzyme (succinate dehydrogenase, SDH) to examine mycorrhizal activity (SDH activity). Mycorrhizal colonization and SDH activity started to increase 4 days after aboveground herbivory, and returned to the initial levels in the absence of mite herbivory in 7 and 12 days, respectively. These results suggest that the change in AM association in response to mite herbivory is a short-term response.

Keywords Aboveground–belowground interaction · Arbuscular mycorrhizal fungi · Indirect effect · *Lotus japonicus*

Introduction

Nutrient intake of most terrestrial plants largely depends on arbuscular mycorrhizal fungi (AMF) (Hodge 2000). AMF improve plant growth and nutrient conditions through increasing the acquisition of soil nutrients such as phosphate and nitrate (Tobar et al. 1994; Smith and Read 1997). AMF may also positively affect plant characteristics (Newsham et al. 1995a), such as enhancement of drought resistance (Davies et al. 1992), tolerance to heavy metals (Gildon and Tinker 1983), and resistance to fungal pathogens (Newsham et al. 1995b).

Previous herbivory alters plant traits and results in induced defenses and plant regrowth (Karban and Baldwin 1997; Ohgushi 2005). These changes in plant traits influence performance and preference of herbivorous insects, and therefore alter interactions between plants and herbivorous insects (Ohgushi 2005). Recently, there has been increasing appreciation that herbivorous insects are indirectly influenced by belowground organisms (Wardle 2002). In this context, AMF can positively or negatively affect performance of aboveground herbivorous insects through changes in plant traits (Rabin and Pacovsky 1985; Gange and West 1994; Gange and Nice 1997; Gange 2001; Gange et al. 1999, 2002a; Wamberg et al. 2003; Gange 2007). Gange and West (1994) showed that a reduction in mycorrhizal colonization of ribwort plantain, *Plantago lanceolata*, resulted in increased damage by insect chewers, and a laboratory experiment demonstrated that the reduction in mycorrhizal colonization increased growth rate and food consumption of the generalist chewer insect *Arctia caja*. In contrast, Gange et al. (1999) showed that AMF increased growth rate and fecundity of the aphids *Myzus persicae* and *Myzus ascalonicus*, suggesting a positive effect of AMF on the aphids.

Although less attention has been paid to effects of aboveground insect herbivory on AMF (Gange 2007), a few studies have shown indirect effects of insect herbivory on colonization of AMF through changes in plant

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traits (Gange et al. 2002b; Wamberg et al. 2003; Kula et al. 2005). However, these studies did not examine the temporal pattern of responses in the relationship between the plant and AMF following insect herbivory. Plants often increase nutrient uptake immediately following aboveground herbivory (Chapin and Slack 1979; McNaughton and Chapin 1985; Ruess 1988). Thus, the insect herbivory may temporally enhance the association between plants and AMF because AMF provide nutrients to host plants. To understand the effects of aboveground herbivory on AMF, it is necessary to examine when the effects of aboveground herbivory on the arbuscular mycorrhizal (AM) association occur and how long the effects continue.

This study examined temporal changes in the AM association with *Lotus japonicus* after aboveground herbivory by spider mites. We used two different staining methods: nonvital staining with trypan blue and vital staining of a mycorrhizal enzyme. Previous studies have mostly used nonvital staining with trypan blue as described by Phillips and Hayman (1970) to examine the effects of herbivory on AM association (Gange et al. 2002b; Wamberg et al. 2003; Kula et al. 2005). Although this method can detect biomass of mycorrhizal hyphae in roots, it is difficult to detect metabolic functions of AMF. In this context, the vital staining of a mycorrhizal enzyme has provided a good method to detect the effect of biotic and abiotic factors on metabolic function (Saito et al. 1993; Tisserant et al. 1993). The vital staining of succinate dehydrogenase (SDH) has been recently used in detection of the activity and efficiency of AM association (Vivas et al. 2003) because this enzyme plays an important role in mycorrhizal nutrient efficiency (Smith and Dickson 1991; Guillemin et al. 1995).

Methods

Plant, herbivore and AMF

We used a leguminous perennial, bird's foot trefoil (*L. japonicus*), a spider mite (*Tetranychus urticae*), and AMF (*Gigaspora margarita*) for our experiments. *Lotus japonicus* seeds were obtained from the National Bioresource Project (Miyazaki, Japan). *Tetranychus urticae* is an herbivorous spider mite, a typical generalist feeding on some 900 plant species (Navajas 1998). The spider mites were reared on lima bean plants (*Phaseolus lunatus* L.) in a climate-controlled greenhouse (25 ± 2°C, 50–70% relative humidity, and natural lighting conditions). *Gigaspora margarita* was provided by Sun Green (Tokyo, Japan).

Plant growth conditions and experimental design

On 10 March 2007, 500 *L. japonicus* seeds were surface-sterilized for 3 min in saturated 1% sodium hypochlorite solution and germinated on moist vermiculite. Five-day-old seedlings were transplanted individually into plastic pots (5 cm in diameter, 7 cm in height) filled with 300 ml autoclaved soil with a 1:1:1 mixture of sand, brunizem, and peat. Approximately 50 spores of *G. margarita* were placed per pot under the seedling roots. Seedlings were grown in a climate-controlled greenhouse (25 ± 2°C, 50–70% relative humidity, and natural lighting conditions) for 70 days. During the plant growing period in the greenhouse, seedlings were watered every day and fertilized once a week with 25 ml basal nutrient solution (PO_4^{3-}: 1 mM, NO_3^-: 9 mM, K^+: 7 mM, Ca^{2+}: 2 mM, Mg^{2+}: 5 mM, SO_4^{2-}: 5 mM). Seventy days after transplanting, the seedlings were randomly assigned to herbivory and nonherbivory treatments. In the herbivory treatments, 15 female mites were inoculated into each seedling pot (day 1), and the seedlings were exposed to mite herbivory for 3 days (day 1 to day 3). On day 4, 3 days after mite introduction, the spider mites were removed by spraying with a miticide (Sankyo Agro, Tokyo, Japan). Seedlings in the nonherbivory treatment were only sprayed with the miticide. The mean number of damaged leaves of plants in the herbivory treatments was 20 ± 34 (mean ± SE) and the mean herbivory rate, the proportion of damaged to total leaves, was 16 ± 39%. No damaged leaves were observed in the nonherbivory treatment. Roots of eight seedlings from each treatment were randomly collected on day 0, 1 day before the mite introduction, and 4, 7, 10, and 16 days after mite introduction.

Measuring AMF colonization and SDH activity

Within 30 min after sampling, the roots were brought to a laboratory on ice to prevent inactivating mycorrhizal enzymes. The roots were cut into approximately 5-mm-long sections. To measure mycorrhizal colonization and SDH activity, two sets of approximately 30-mg root sections were taken from each root sample, and other root sections were used for measurement of dry mass. Root sections for measuring mycorrhizal colonization were cleared in 10% potassium hydroxide for 20 min at 90°C and stained using 0.5% trypan blue for 15 min at 90°C (Phillips and Hayman 1970). Root sections for measuring SDH activity were saturated with a solution of nitroblue tetrazolium chloride (NBT) for 5 h at room temperature. NBT solution (1 mg/ml) was prepared in 50 mM Tris/HCl buffer (pH 7.4) containing 0.1 M sodium succinate and 0.5 mM $MgCl_2$ (MacDonald and Lewis 1978). Samples for the measurement of root dry mass were oven-dried for 48 h

at 60°C and weighed. Mycorrhizal colonization and SDH activity were quantified using the magnified interactions method (McGonigle et al. 1990) at 200× magnification. Mycorrhizal colonization and SDH activity were calculated as percentages as follows:

$$\text{Mycorrhizal colonization rate} = (\text{number of colonized AMF})/(\text{total number of observations}) \times 100$$

$$\text{SDH activity} = (\text{number of active hyphae})/(\text{total number of observations}) \times 100$$

A two-way factorial ANOVA was performed using three dependent variables: mycorrhizal colonization, SDH activity, and root dry mass. The independent variables were herbivory treatment and time after mite introduction. Significant differences in mycorrhizal colonization and SDH activity between herbivory and nonherbivory treatment were determined by Welch's *t*-test at each sampling date after mite introduction. Significant differences in mycorrhizal colonization and SDH activity before herbivory treatment (day 0) and at each date after herbivory treatment (days 4, 7, 10 and 16) were determined using a Dunnett test to compare herbivory and nonherbivory treatments. Prior to analysis, all percentage data were arcsine square root-transformed. All statistical analyses were performed by JMP Version 5.0 software (SAS, Cary, NC).

Results

Mycorrhizal colonization was significantly influenced by herbivory, time after mite introduction, and the interaction of these two factors (Table 1). Similarly, SDH activity was significantly influenced by herbivory, and by the interaction of herbivory and time after mite introduction. Root dry mass was unaffected by any of these factors.

Both mycorrhizal colonization and SDH activity increased shortly after the start of mite herbivory. The mycorrhizal colonization rate of plants subjected to herbivory 4 and 7 days after mite introduction was significantly higher than that of the control plants not subjected to herbivory (Fig. 1: Welch's t test, $P < 0.05$). Mycorrhizal colonization of damaged plants was altered during the experimental period, although this effect was not observed in undamaged plants. Mycorrhizal colonization rates at days 4 and 7 were greater than that at day 0, before mite introduction (Fig. 1: Dunnett test, $P < 0.05$). However, the enhanced mycorrhizal colonization returned to the initial level (the level at day 0) 10 days after mite introduction.

Succinate dehydrogenase activity of plants subjected to herbivory at 4, 7, and 10 days after mite introduction was significantly higher than that of control plants (Fig. 2: Welch's t test, $P < 0.05$). SDH activity of damaged plants, but not undamaged plants, was altered during the experimental period. SDH activity significantly increased on days 4, 7, and 10 relative to that at day 0 (Fig. 2: Dunnett test, $P < 0.05$). However, the enhanced SDH activity returned to the initial level 16 days after mite introduction.

Discussion

We evaluated AM association using two different staining methods. Mycorrhizal colonization estimated by the trypan blue staining is an indicator of mycorrhizal biomass, because trypan blue can detect total mycorrhizal hyphae regardless of mycorrhizal activity (Guillemin et al. 1995). On the other hand, SDH activity by vital staining can be used to estimate surface area for nutrient exchange between a living symbiont and host plant and is an indicator of mycorrhizal function such as plant nutrient acquisition (Smith and Dickson 1991; Guillemin et al. 1995). Using both staining methods, we observed that the mycorrhizal colonization rate in plants subjected to herbivory was 13 and 24% higher than in control plants on days 4 and 7, respectively. Moreover, the plants subjected to herbivory had 23% higher SDH activity than control plants on day 4. These results showed that mite herbivory alters biomass and metabolite function of AMF, and suggested that previous studies that examined only mycorrhizal colonization

Table 1 Effects of mite herbivory and time after herbivory on mycorrhizal colonization and SDH activity ratio and root biomass (two-way ANOVA)

Factors	*df*	Mycorrhizal colonization		SDH activity		Root biomass	
		F	*P*	*F*	*P*	*F*	*P*
Herbivory	1	5.10	<0.05	11.98	<0.01	1.53	0.22
Time	4	14.98	<0.01	2.15	0.083	1.19	0.32
Herbivory × time	4	8.80	<0.01	3.02	<0.05	1.46	0.22

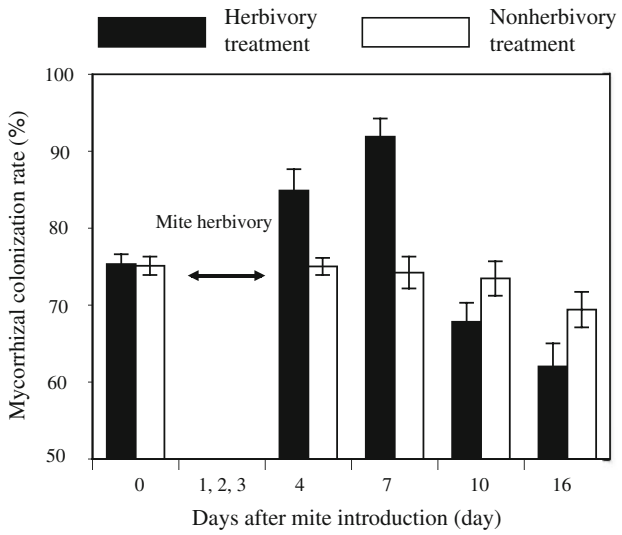

Fig. 1 Temporal changes in the mycorrhizal colonization rate following a 3-day exposure to spider mite herbivory. *Filled and open bars* represent mycorrhizal colonization in the presence and absence of spider mite herbivory, respectively. *X*-axis labels represent days after mite introduction. Mycorrhizal colonization of plants with herbivory on days 4 and 7 was significantly higher than that of plants without herbivory (Welch's *t* test, $P < 0.05$). Mycorrhizal colonization of plants with spider mite herbivory increased on days 4 and 7, compared to that on day 0, before the mite introduction (Dunnett test, $P < 0.05$)

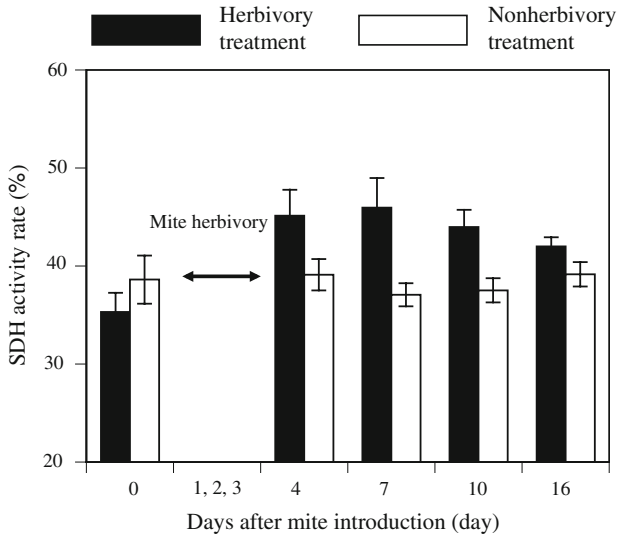

Fig. 2 Temporal changes in the succinate dehydrogenase (*SDH*) activity rate after a 3-day exposure to spider mite herbivory. *Filled and open bars* represent SDH activity in the presence and absence of spider mite herbivory, respectively. *X*-axis labels represent days after mite introduction. The SDH activity of plants with herbivory on days 4, 7, and 10 was significantly higher than that of plants without the herbivory (Welch's *t* test, $P < 0.05$). The SDH activity of plants with spider mite herbivory increased on days 4, 7, and 10 following the mite herbivory compared to that on day 0, before the mite introduction (Dunnett test, $P < 0.05$)

would have underestimated the effects of aboveground herbivory on AM association.

In our study, the AM association immediately enhanced following aboveground herbivory. Previous studies reported responses of AM association to insect herbivory (Gange et al. 2002b; Wamberg et al. 2003; Kula et al. 2005). Gange et al. (2002b) found that foliar herbivory by *A. caja* reduced mycorrhizal colonization of *P. lanceolata* after 15 weeks. Also, mycorrhizal colonization of *Senecio jacobaea* decreased 20 days after herbivory by larvae of the cabbage moth *Phlogophora meticulosa*. Kula et al. (2005) showed that grasshopper herbivory of tallgrass increased mycorrhizal colonization after 2 months. Wamberg et al. (2003) examined effects of leaf beetle (*Stona lineatus*) on mycorrhizal colonization of pea plant (*Pisum sativum*). The mycorrhizal colonization was altered 10 days after herbivory. Also, in the present study, the enhanced AM association returned to the initial level within a few days. Although Gange et al. (2002b) reported that it took several weeks for the reduced mycorrhizal colonization to recover in the absence of herbivory, there were few studies examining the temporal changes in AM association.

The rapid response in AM association observed in our study would be caused by changes in plant nutrient demands. Several studies have shown that aboveground herbivory increases nutrient uptake within a few days (Chapin and Slack 1979; McNaughton and Chapin 1985; Ruess 1988). Ruess (1988) showed that nitrogen and phosphorus uptake in a C_4 grass (*Sporobolus kentrophyllus*) increased within 24 h after aboveground herbivory. Also, colonization of AMF increased in low soil nutrient conditions, in which plant nutrient demand increased (Smith and Read 1997). On the other hand, rapid recovery of AM association within a few days would depend on the carbon burden of AM association. Plants provide large amounts of photosynthates, approximately 10–20% of total primary production, to maintain AMF association (Jakobsen et al. 2002). Plants with AMF association are likely to suffer from carbon limitation (Gange et al. 2002b). Thus, plants would lose so as to avoid the carbon costs of supplying excess nutrients to AMF, and AMF associations would return to the initial levels in several days. Therefore, a short-term response of AM association to previous herbivory would be due to rapid changes in plant nutrient demands.

The rapid temporal changes in AM association may benefit host plants. Nutrient uptake of damaged plants enhances induced responses to herbivory such as compensation regrowth (Bazley and Jefferies 1985; Paige and Whitham 1987; Karban and Baldwin 1997). Since enhanced mycorrhizal colonization and activity increase plant nutrient acquisition (Smith and Dickson 1991; Smith and Read 1997), the increase in AM association following herbivory could result in induced plant responses to

herbivory. Actually, AMF-colonized *Lotus corniculatus* can more rapidly show induced resistance against aboveground herbivory than uncolonized plants (T. Nishida, unpublished data). Also, some studies have reported that AMF enhances plant regrowth following insect herbivory (Kula et al. 2005; Bennett and Bever 2007).

To better understand how aboveground herbivory influences AM association with host plants, we need to examine the underlying mechanisms responsible for the temporal patterns in AM association following herbivory.

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