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Mycorrhizal Fungal Structures are Stimulated in Wildtype Peas and in Isogenic Mycorrhiza-Resistant Mutants by Tri-Iodo-Benzoic Acid (TIBA), an Auxin-Transport-Inhibitor

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Abstract

Roots of pea (*Pisum sativum* L. cv. Frisson and Finale) wildtype and mycorrhiza-resistant mutant plants (P2, P6, DK 10) were treated with the auxin transport inhibitor tri-iodo-benzoic acid (TIBA) and grown in the presence of mycorrhizal inocula. In wildtype plants, mycorrhizal infection was considerably enhanced upon treatment with TIBA. In roots of mycorrhiza-resistant pea mutants, a two-(DK 10) to tenfold (P2, P6) increase of appressoria formation could be observed upon addition of TIBA. In roots of P2 and P6 treated with TIBA, length of intraradical hyphae was nearly 1 cm m^{-1} root length as compared to less than 1 mm m-1 in control roots. In mutant P2, this enhancement of mycorrhiza formation was correlated to an enhancement of growth. These results indicate that the mycorrhiza-resistant phenotype of these mutants can be partially reverted by alterations of the phytohormonal balance of the root.

Keywords: Arbuscular mycorrhiza, nodulation mutants, phytohormones, root development, symbiosis

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1. Introduction

More than 80% of the species of higher plants form root borne associations with obligate biotrophic zygomycetes of the order *Glomales* (Smith and Read, 1997). These associations are called (vesicular-)arbuscular mycorrhizae (AM) or endomycorrhizae. This interaction is considered to be mutualistic. The fungal partner translocates nutrients, mainly phosphate, from the soil to the plant (Smith and Gianinazzi-Pearson, 1988) and receives assimilated carbon necessary for growth from the plant host (Wright et al., 1998). This nutrient exchange is of great interest for agricultural applications and has been reviewed extensively (Hall, 1988). Recent findings suggest that diversity of communities of non-agricultural plants is determined by the diversity of mycorrhizal fungi (van der Heijden et al., 1998a).

The molecular and genetic bases of the processes involved in establishing mycorrhizal symbiosis are still unclear. So far, mycorrhiza-resistant mutants have been found upon screening legumes for non-nodulating mutants (e.g. in *Pisum sativum*, see Duc et al., 1989; Sagan et al., 1993; in *Medicago truncatula*, see Sagan et al., 1995; in *Phaseolus vulgaris*, see Shirtliffe and Vessey, 1996). Very recently, a non-mycorrhizal mutants has also been described for a non-legume, namely for tomato (Barker et al., 1998). In mycorrhiza-resistant pea mutants, fungal growth seems to be arrested soon after the formation of colonizing structures, the so-called appressoria at the root surface by local defence reactions (Gianinazzi-Pearson et al., 1996). A link between mycorrhiza and nodule formation has not become evident only as a consequence of the mutant screens quoted above. Very interestingly, external applications of lipooligosaccharides secreted by rhizobia in order to initiate nodule formation (nodulation factors) stimulate mycorrhiza formation (Xie et al., 1995). External applications of the auxin-transport-inhibitor tri-iodo-benzoic acid (TIBA) not only promotes formation of pseudonodules (Hirsch et al., 1989; Xie et al., 1998), but also stimulates mycorrhizal colonization in the legume *Lablab purpureus* (Xie et al., 1998). Here, data are presented showing that addition of TIBA is able to promote the formation of appressoria and intraradical mycelium in mycorrhiza-resistant pea mutants thus partially reverting their phenotype.

2. Material and Methods

Seeds of pea (*Pisum sativum* L.) mycorrhiza-resistant mutants (P2, P6 and DK 10; Sagan et al., 1993) and their corresponding wildtypes (cv. Frisson for P2 and P6; cv. Finale for DK 10) were kindly provided by Dr. Duc (INRA, Dijon, France). All seeds were surface sterilised with 30% H_20_2 for 20 min followed by washing with sterile tap-water and incubated at room temperature on 1%

water agar plates during four days for germination. Then, seedlings were transferred to fire-clay pots (8 cm diameter; cleaned and sterilised at 180° for 12 h) filled with 1/3 (v/v) vermiculite and 2/3 (v/v) sand. This substrate was mixed either with an inoculum of *Glomus mosseae* (BEG No.19) produced on *Lotus corniculatus* (soil with propagules, infected roots) in a 2:1-ratio or with a commercial inoculum of *Sclerocystis* sp. (a commercial inoculum produced by Dr. B. Blal, Biorhize Inc., Dijon, France) in a 9:1-ratio. Experiments with uninfected plants were performed using only vermiculite as substrate.

Plants were grown in a climate chamber during 6 weeks (16 h day at 220 $\mu E/m^2/sec$ and 22°C; night at 19°C; 60 to 70% rH). Plants were watered daily with demineralised water and once per week with 50 ml of a commercial nutrient solution (Biorhize, Dijon, France) providing 5 µg KH_2PO_4 and 50 mg KNO_3. Uninfected plants were fertilised as described, but with a nutrient solution containing 50 mg phosphate. Where indicated, 5 µmoles tri-iodobenzoic acid (TIBA) were added once, namely one week after planting, to the pots. Plants were harvested after 5 weeks. For each wildtype and mutant, three plants were treated with TIBA, and three plants were left untreated as a control.

Root and shoot fresh weights (fw) were determined and roots were treated in order to determine mycorrhizal infection as described (Müller and Dulieu, 1998). Roots were bleached by incubation for 30 min in KOH (10% w/v), followed by extensive washing with tap water and staining with trypan blue (see Xie et al., 1995). In wildtype plants, mycorrhizal infection was determined using a microscope (100×) according to McGonigle et al. (1990) with a slight modification. If, per intersection, mycorrhizal structures, i.e. hyphae, arbuscules or vesicles, were present at both sides of the central cylinder, they were counted twice. Thus, per 1000 intersections, 2000 structures could be theoretically counted in fully infected roots. In these roots, the number of appressoria could not been evaluated correctly and was therefore not considered. In mutant plants, the length of an aliquot of the root system and the length of intraradical mycelium present therein was measured using a microscope (100×) with a graduated ocular and the number of appressoria was counted.

Statistical tests were performed using the software SigmaStat (Jandel Scientific, San Rafael, CA, USA).

3. Results

Effects of TIBA on infection of pea wildtype plants by Glomus mosseae

Pea plants (cv. Frisson and Finale) with a normal mycorrhiza formation were

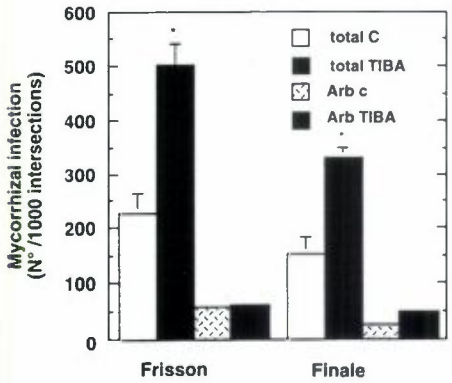

Fig. 1.

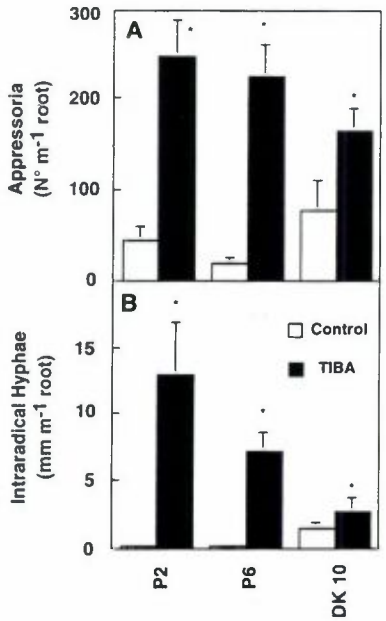

Fig. 2.

Figure 1. Mycorrhizal infection of pea plants. Pea wildtype plants (*Pisum sativum* L. cv. Frisson and cv. Finale) were grown for 5 weeks in presence of a mycorrhizal inoculum (*Glomus mosseae*). Plants were either untreated (control; white bars) or grown in presence of TIBA (5 µmoles per pot; black bars). Mean values±SD of total mycorrhizal infection and of formation of arbuscules are given for three plants per treatment. Number of vesicles was too small to be correctly represented (1 to 2 per 1000 intersections). *, differences between control and TIBA-treated plants are significantly different (t-test, $p<0.05$).

Figure 2. Formation of fungal structures on roots of mycorrhiza-resistant pea plants. Pea (*Pisum sativum* L. cv. Frisson and cv. Finale) mycorrhiza resistant mutants (P2, P6 and DK 10) were grown for 5 weeks in presence of a mycorrhizal inoculum (*Glomus mosseae*). Plants were either untreated (control; white bars) or grown in presence of TIBA (5 µmoles per pot; black bars). Mean values±SD are given for three plants per treatment. *, differences between control and TIBA-treated plants are significantly different (t-test, $p<0.05$). A, formation of appressoria; B, length of intraradical hyphae.

cultivated in presence of *Glomus mosseae*. To some plants, TIBA was added. After 5 weeks, in TIBA-treated roots, total mycorrhizal infection was more than twice as high as in untreated roots. In plants of cv. Frisson, formation of arbuscules was not affected by TIBA (60 per 1000 intersections in mean in both treatments). In plants of cv. Finale, a significant increase of arbuscules from 27

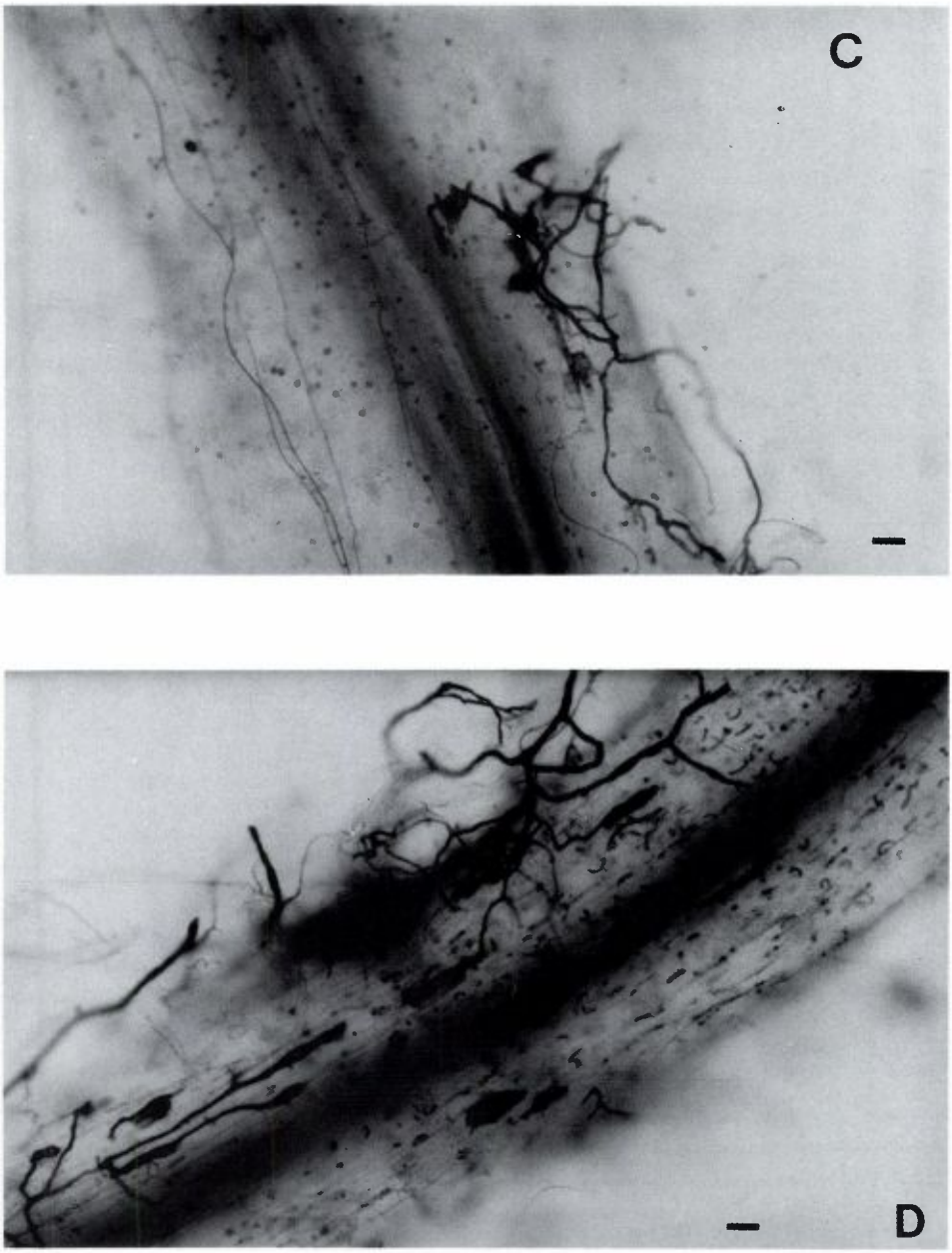

Figure 2. Formation of fungal structures on roots of mycorrhiza-resistant pea plants. C, photograph of mutant P2 treated with TIBA; D, photograph of mutant P6 treated with TIBA (bar represents 50 μm).

to 50 per 1000 intersections in average was observed ($p<0.05$; t-test; Fig. 1). The number of vesicles was so low with this inoculum that a correct evaluation was not possible (1 to 2 per 1000 intersections).

Effects of TIBA on infection of mycorrhiza-resistant pea mutants by Glomus mosseae

The mycorrhiza-resistant mutants P2 and P6 (cv. Frisson) and DK 10 (cv. Finale) were cultivated in presence of a mycorrhizal inoculum (*G. mosseae*) with or without TIBA for 5 weeks and formation of appressoria and growth of intraradical mycelium was measured. In all mutants, a significant increase of appressoria formation due to TIBA could be observed. This increase was more than tenfold in case of the mutant P6 (Fig. 2A). Furthermore, the growth of intraradical structures, namely thick, short hyphae (Fig. 2C and D) was considerably enhanced in the mutants P2 and P6, but not in DK 10 (Fig. 2B). Formation of typical intraradical structures like arbuscules and vesicles could, however, not been observed.

Effects of TIBA on mycorrhizal infection and growth in presence of a commercial inoculum (Sclerocystis)

In a second experiment, plants of the mycorrhiza-resistant mutant P2 and the wildtype cultivar Frisson were grown in presence of a commercial inoculum (*Sclerocystis* sp.) with a high infective potential (Müller and Dulieu, 1998) and harvested after 5 weeks.

In a preliminary experiment, it was investigated whether TIBA had any negative growth effects on pea wildtype (cv. Frisson) and mutant (P2) plants. Here, no significant effects on shoot and root growth could be detected (Table 1). Upon mycorrhizal infection, no significant effects of TIBA on growth neither of shoots nor roots could be observed in wildtype plants. P2 mutants, however, had significantly enhanced shoot and root biomasses (Table 2).

As expected, mycorrhizal infection was much higher with *Sclerocystis* than with *G. mosseae*. Even here, one treatment with TIBA could considerably enhance mycorrhizal infection. Furthermore, the number of vesicles was more than twice as high in TIBA-treated roots as compared to control roots. As observed with *G. mosseae*, the number of arbuscules was not affected by TIBA (Fig. 3). As observed with *G. mosseae*, TIBA had dramatic effects on the formation of appressoria and intraradical structures in roots of the mycorrhiza-resistant mutant P2 infected by *Sclerocystis*. Appressoria formation was increased from ca. 30 per m root length to 170 in average. As observed for mutant P2, length of intraradical hyphae was strongly stimulated from less than 1 mm per m root length to more than 8 mm (Fig. 4).

Table 1. Biomasses of shoots and roots of axenically grown pea plants. Pea wildtype plants (*Pisum sativum* L. cv. Frisson) and mycorrhiza resistant mutants (P2) were grown for 4 weeks. Plants were either untreated (control) or grown in presence of TIBA (5 µmoles per pot). Mean values±SD are given for three plants per treatment. Biomasses were not significantly different between treatments (t-test, $p>0.2$).

Plant	Treatment	Shoot (g fresh weight)	Root (g fresh weight)
Wildtype	Control	11.5 ± 3.5	9.9 ± 2.8
	TIBA	9.8 ± 2.3	12.3 ± 4.7
P2	Control	12.1 ± 2.7	10.2 ± 1.8
	TIBA	10.9 ± 3.1	12.6 ± 3.4

Table 2. Biomasses of shoots and roots of mycorrhizal pea plants. Pea wildtype plants (*Pisum sativum* L. cv. Frisson) and mycorrhiza resistant mutants (P2) were grown for 5 weeks in presence of a mycorrhizal inoculum (*Sclerocystis* sp.). Plants were either untreated (control) or grown in presence of TIBA (5 µmoles per pot). Mean values±SD are given for three plants per treatment. *, differences between control and TIBA-treated plants are significantly different (t-test, $p<0.05$).

Plant	Treatment	Shoot (g fresh weight)	Root (g fresh weight)
Wildtype	Control	8.4 ± 2.4	6.6 ± 2.6
	TIBA	5.5 ± 1.1	8.1 ± 2.7
P2	Control	2.1 ± 0.2	3.9 ± 1.2
	TIBA	3.4 ± 0.4*	7.3 ± 0.4*

4. Discussion

These experiments have shown that an external application of TIBA stimulates mycorrhizal infection of wildtype pea plants. This confirms results obtained with *Lablab purpureus* (Xie et al., 1998). Formation of appressoria and growth of intraradical mycelium is considerably enhanced in mutants which are normally considered as mycorrhiza resistant. Thus, this mycorrhiza-resistant phenotype can be partially reverted by addition of TIBA. Moreover, in the case of the mutant P2, this increase of mycorrhizal structures is even correlated to a growth enhancement. In uninfected plants, addition of TIBA has never yielded any positive growth effects (see also Xie et al., 1998). This

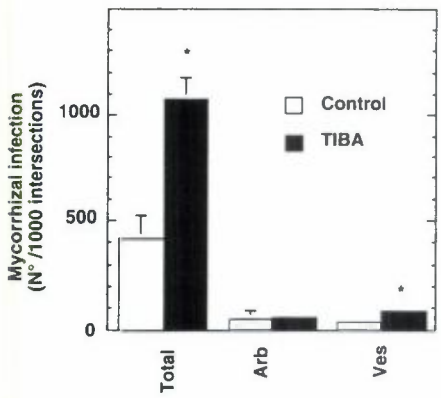

Fig. 3.

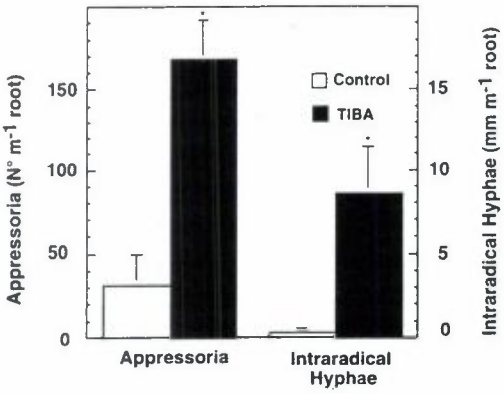

Fig. 4.

Figure 3. Mycorrhizal infection of pea plants. Pea wildtype plants (*Pisum sativum* L. cv. Frisson) were grown for 5 weeks in presence of a mycorrhizal inoculum (*Sclerocystis* sp.). Plants were either untreated (control) or grown in presence of TIBA (5 µmoles per pot). Mean values±SD are given for total infection, arbuscules and vesicle formation for three plants per treatment. *, differences between control and TIBA-treated plants are significantly different (t-test, $p<0.05$).

Figure 4. Formation of fungal structures (appressoria, intraradical hyphae) on roots of mycorrhiza-resistant pea plants. Pea mycorrhiza resistant mutant (P2) plants were grown for 5 weeks in presence of a mycorrhizal inoculum (*Sclerocystis* sp.). Plants were either untreated (control) or grown in presence of TIBA (5 µmoles per pot). *, differences between control and TIBA-treated plants are significantly different (t-test, $p<0.05$).

growth enhancement could be the result of a transfer of nutrients (phosphorous?) from the fungus to the root through intraradical structures formed upon TIBA treatment. In general, it is thought that nutrient exchange occurs at the surface of arbuscules (Smith and Read, 1997). In a recent study, it has been shown, however, that growth of a mycorrhiza-dependent plant (*Hieracium pilosella*) is strongly stimulated upon colonization by fungi not producing arbuscules (in this case *Glomus laccatum*, van der Heijden et al., 1998b).

How can the stimulation of mycorrhizal infection by TIBA be explained? At the level of preinfection and infection, it could be possible that TIBA just stimulates infection and growth of the germinating hyphae. This hypothesis could not have been tested, so far, since axenical germination of spores from fungi used in this study is nearly impossible to obtain. Moreover, TIBA-treated roots might produce exudates stimulating germination of fungal spores, growth

of infective hyphae and formation of appressoria (Nair et al., 1991). These effects could account for the higher number of appressoria in the roots of TIBA-treated mutants. The strong enhancement of growth of intraradical structures could be explained by a better supply with assimilates from the host or by a release of down-regulating defence mechanisms. Both phenomena can be directed by a modification of the phytohormone balance in the root cortex. Mycorrhizal roots have been shown to produce cytokinins (Dixon et al., 1988) and auxins (Ludwig-Müller et al., 1997) in the root cortex. A local accumulation of auxins induced by TIBA could create a sink for assimilates in the vicinity of fungal infection structures (Xie et al., 1998). This influx of assimilates could stimulate fungal growth and result in the formation of more reserve structures (vesicles in the case of wildtype pea infected with *Sclerocystis*). In *Lablab* infected with *G. mosseae*, addition of TIBA, Nod factors and cytokinin strongly stimulates growth of extraradical mycelium and sporocarp formation (Xie et al., 1998). Moreover, production of phytohormones by mycorrhizal fungi could be involved in downregulation of plant defence reactions (see Kapulnik et al., 1996) like cell wall appositions (Gollotte et al., 1995; Gianinazzi-Pearson et al., 1996) or local induction of PR-proteins could be reduced thus allowing a better fungal growth. These findings are in good agreement with the observation that in tobacco leaf disks, expression of glucanase and chitinase activities are downregulated by auxin and kinetin (Rezzonico et al., 1998). In a study more relevant for the situation in mycorrhiza, it has been shown that responses to elicitors derived from ectomycorrhizal fungi, e.g. the secretion of peroxidases, are reduced in the presence of auxins (Mensen et al., 1998). Taken together, it could be postulated that mycorrhiza formation is subject to autoregulation by negative feed-back involving phytohormones similar to nodulation (see Hirsch and Fang, 1994). By altering the phytohormone balance upon addition of TIBA or Nod factors (Xie et al., 1995; Xie et al., 1998), downregulation of mycorrhiza formation is altered thus leading to a higher degree of mycorrhiza formation not only in wildtype, but also in mutant roots where normally any intraradical structures are formed. Further experiments using a split-root-system will provide more information.

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