Hypaphorine from the Ectomycorrhizal Fungus *Pisolithus tinctorius* Counteracts Activities of Indole-3-Acetic Acid and Ethylene but Not Synthetic Auxins in Eucalypt Seedlings

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Very little is known about the molecules regulating the interaction between plants and ectomycorrhizal fungi during root colonization. The role of fungal auxin in ectomycorrhiza has repeatedly been suggested and questioned, suggesting that, if fungal auxin controls some steps of colonized root development, its activity might be tightly controlled in time and in space by plant and/or fungal regulatory mechanisms. We demonstrate that fungal hypaphorine, the betaine of tryptophan, counteracts the activity of indole-3-acetic acid (IAA) on eucalypt tap root elongation but does not affect the activity of the IAA analogs 2,4-D ((2,4-dichlorophenoxy)acetic acid) or NAA (1-naphthaleneacetic acid). These data suggest that IAA and hypaphorine interact during the very early steps of the IAA perception or signal transduction pathway. Furthermore, while seedling treatment with 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, results in formation of a hypocotyl apical hook, hypaphorine application as well as root colonization by *Pisolithus tinctorius*, a hypaphorine-accumulating ectomycorrhizal fungus, stimulated hook opening. Hypaphorine counteraction with ACC is likely a consequence of hypaphorine interaction with IAA. In most plant-microbe interactions studied, the interactions result in increased auxin synthesis or auxin accumulation in plant tissues. The *P. tinctorius* / eucalypt interaction is intriguing because in this interaction the microbe down-regulates the auxin activity in the host plant. Hypaphorine might be the first specific IAA antagonist identified.

During ectomycorrhizal development, the plant and fungus undergo profound reorientation of their development due to the interaction between both symbionts (Dexheimer and Paragne 1991; Martin et al. 1997). This interaction must be controlled by signal molecules exchanged very early between the symbionts, but so far very little is known about these molecules. Much attention has been focused on auxin. Indeed, most ectomycorrhizal fungi are able to synthesize auxin (Beyrle

1995), and auxin applied on roots mimics some of the features seen after colonization of roots by ectomycorrhizal fungi (Gogala 1991). The addition of 2,3,5 triiodobenzoic acid (TIBA), an inhibitor of auxin transport, in the culture medium blocked the colonization of the Norway spruce main root cortex and the Hartig net formation by *Laccaria bicolor* (Karabaghli-Degron et al. 1998). Studies of fluorindole-resistant mutants of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* that overproduce tryptophan and indole-3-acetic acid (IAA) support the idea that fungal IAA controls major anatomical features of pine ectomycorrhizae (i.e., enhanced lateral root formation and an increased number of ectomycorrhizal tips were recorded), but IAA overproduction also induced an abnormal proliferation of the intercellular network of hyphae and intracellular penetration of hyphae (Gay et al. 1994; Gea et al. 1994). The role of fungal auxins in ectomycorrhizal development has been challenged repeatedly (Harley and Smith 1983; Wallander et al. 1992). Ectopic expression of the T-DNA IAA-biosynthetic gene in transgenic hybrid aspen roots did not result in any obvious change in mycorrhizal morphology (Hampp et al. 1996). According to Horan (1991), while treatment of *Eucalyptus* spp. seedling roots with micromolar concentrations of exogenous IAA induced the formation of slow-growing, swollen, and highly branched roots that were superficially similar to ectomycorrhizae, these roots exhibited anatomical alterations that are not observed in ectomycorrhizae, including an increased number of cortical cell layers and an increased number of vascular bundles in the stele. This literature survey would suggest that if fungal auxins control some steps of ectomycorrhizal development, auxin activity might be tightly controlled in time and in space by plant and/or fungal regulatory mechanisms.

Recently, we purified hypaphorine (or tryptophan betaine) the major indole alkaloid in *Pisolithus tinctorius* mycelium and exudates (Béguiristain et al. 1995). The accumulation of hypaphorine increases fourfold soon after the hyphae come in contact with the host plant root surface (Béguiristain and Lapeyrie 1997). Fungal hypaphorine has a morphogenetic effect by reducing root hair elongation (Béguiristain and Lapeyrie 1997), but elongation of hypaphorine-inhibited-root hairs could be restored by an exogenous IAA application (F. A. Ditengou and F. Lapeyrie, unpublished data). Furthermore,

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hypaphorine regulates the expression of a plant symbiosis regulated (SR) gene, the *EgHypar* gene, exhibiting a high degree of homology with plant auxin-induced glutathion-S-transferase (GST) (Nehls et al. 1998). Some of these GSTs would be involved in auxin metabolism and transport regulation (Marrs 1996). Therefore, among numerous hypotheses, we suggested that fungal hypaphorine could regulate auxin activity during ectomycorrhizal symbiosis development.

Here we demonstrate that fungal hypaphorine counteracts the activity of IAA in host plant organs. While there are numerous examples of plant-microbe interactions that lead to increased auxin accumulation in plant tissues (Morris 1995; Mathesius et al. 1998), this is, as far as we know, the first reported case in which the microbe down-regulates the auxin activity in the host plant organs.

RESULTS

Interaction between hypaphorine and exogenous auxins.

Eucalyptus globulus subsp. *bicostata* seedlings were grown on a cellophane film over agar in petri dishes maintained vertically. Compared with control plants, the addition of 1 μ M IAA in the nutrient medium resulted in a 60% inhibition of tap root elongation (Figs. 1A and 2). The addition of hypaphorine at 100 μ M (Figs. 1A and 2) and up to 1 mM (data not shown) had no effect on tap root elongation. In the presence of IAA at 1 μ M, normal tap root elongation, equal to that of the control, could be restored by the simultaneous addition of 100 μ M hypaphorine (Figs. 1A and 2).

Hypaphorine and tryptophan activities were compared in the presence of 1 μ M IAA (Fig. 1A). Tryptophan at 100 μ M slightly inhibits tap root elongation, but 100 μ M tryptophan is able to stimulate the elongation of the tap root in the presence of 1 μ M IAA, compared with roots treated with IAA alone. However, 100 μ M tryptophan does not restore normal growth, compared with control.

The activities of IAA and two synthetic auxins on root growth were compared. Inhibition of tap root elongation could be induced with both synthetic auxins, 1 μ M 2,4-D ((2,4-dichlorophenoxy)acetic acid) (Fig. 1B) and 50 μ M NAA (1-naphthaleneacetic acid) (Fig. 1C). While 100 μ M hypaphorine counteracts the activity of IAA (Fig. 1B,C), as previously observed, it did not counteract the activities of the synthetic auxin (Fig. 1B,C) at any of the concentrations applied, up to 1 mM (data not shown).

Interaction between hypaphorine and exogenous ACC.

Incorporation of the immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) at 10 μ M in the agar medium enhanced the formation of an apical hook on *E.*

globulus hypocotyl (Fig. 3) and delayed hook opening and cotyledon expansion (Fig. 4), as described for numerous plant species (Smalle and Van Der Straeten 1997). After 7 days of incubation in a growth chamber, 100% of the hypocotyls of control seedlings were elongated, while in the presence of

Fig. 1. Interaction between hypaphorine and indole-3-acetic acid (IAA) controlling tap root elongation. *Eucalyptus globulus* subsp. *bicostata* seedlings were cultivated for 7 days over agar nutrient medium (Control) supplemented with 100 μ M hypaphorine (HYP. 100), 1 μ M IAA (IAA 1), 100 μ M tryptophan (Trp 100), 1 μ M 2,4-D ((2,4-dichlorophenoxy)acetic acid) (2,4-D 1), 50 μ M NAA (1-naphthaleneacetic acid) (NAA 50), or a combination of some of these molecules. Mean values were recorded on at least 30 seedlings $\pm$ SD, then compared among each experimental set (1a, 1b, 1c) by analysis of variance and Fisher's protected least significant difference test, $P = 0.05$.

ACC, 100% of the seedlings displayed an apical hook. It took 15 days for 75% of the ACC-treated seedlings to elongate and, after 20 days of incubation, 5% of the hooks had not yet opened (Figs. 3 and 4).

The incorporation of hypaphorine at 100 or 500 μM in the nutrient medium does not affect shoot development, compared with control seedlings (data not shown).

When 500 μM hypaphorine was added to the agar medium together with 10 μM ACC, seedlings elongated much earlier than those treated with ACC alone (Figs. 3 and 4). Around 50% of the hypocotyls were elongated after 7 days, 90% after 15 days, and 100% after 20 days (Fig. 4). The same trend was recorded, with a lower intensity, when hypaphorine at 100 μM or even 10 μM (Fig. 4) was added with 10 μM ACC. While elongation of the tap root was inhibited as well by 10 μM ACC, the addition of hypaphorine did not always allow this inhibition to be overcome (Fig. 3). The activity of 100 μM tryptophan was not significantly different from that of hypaphorine at the same concentration (Fig. 4).

ACC activity in host plants colonized by *P. tinctorius*.

While the results above suggest that the hypaphorine producing fungus, *P. tinctorius*, could counteract the IAA-induced inhibition of tap root elongation, it was not possible to test such a hypothesis because root elongation and ramification are strongly altered by hyphal colonization as well. On the other hand, such a comparison between the exogenous hypaphorine supply and the activity of hypaphorine-producing strains on shoot elongation in the presence of ACC could be made. Indeed the site of fungal infection, the root, and the site of symptom development, the shoot, are then distinct.

The respective developments of control seedlings and seedlings germinated on 10 μM ACC agar medium were closely related to those described above (Figs. 3 and 5). Seedling root colonization by *P. tinctorius* strain 2144, on media containing 10 μM ACC, stimulated hypocotyl elongation, as did 500 μM hypaphorine (Figs. 3 and 5). After 7 days, approximately 50%

of hypocotyls were elongated; after 10 days, 90% were elongated; and after 15 days, all hooks were opened. With a delay, a similar trend was recorded when roots were colonized by *P. tinctorius* strain 441, a less aggressive strain toward *Eucalyptus* spp. roots (Fig. 5). To rule out the objection that roots were isolated from the agar medium by colonizing hyphae, preventing the absorption of ACC, the same experiment was conducted again, but some seeds were purposely placed further away from the fungal colonies. Consequently, most of the root length, bearing root hairs, was still clearly in contact with the agar surface and only the root tip was colonized by *P. tinctorius* strain 2144 (Fig. 3, lower row); however, these seedlings responded to hyphal colonization as previously described. Then, the hypaphorine concentration in the agar medium under *P. tinctorius* strain 2144 colonies was still very low (highest concentration recorded: 2.5 μM) and not significantly different whether the agar medium was supplemented or not with ACC.

Carbon dioxide can be an ethylene antagonist (John 1997). To rule out the objection that hypocotyl elongation could be mediated by the CO_2 resulting from fungal respiration in sealed petri dishes, the same experiment was conducted in sealed or unsealed petri dishes, with or without contact between roots and hyphae. The same conclusion was reached regarding the role of hypaphorine (Fig. 6). Indeed, after 10 days, less than 25% of the hypocotyls were elongated among ACC-treated seedlings (Fig. 6A), while 96% of the hypocotyls were elongated following *P. tinctorius* colonization whether the petri dishes were sealed (Fig. 6B), or unsealed (Fig. 6C). Furthermore, when, in sealed dishes, the hyphae were grown without direct contact with the seedling roots, both organisms being separated by a trench in the agar, *P. tinctorius* activity was strongly reduced, as only 50% of the hypocotyls were elongated (Fig. 6D). This residual activity, which could be attributed to fungal CO_2 , was slightly lower when the dishes were maintained unsealed, as expected if a volatile compound was involved (Fig. 6E).

Fig. 2. Development of 7-day-old *Eucalyptus globulus* subsp. *bicostata* seedlings cultivated over agar nutrient medium (Control) supplemented with 100 μM hypaphorine, 1 μM indole-3-acetic acid (IAA), or a combination of 1 μM IAA and 100 μM hypaphorine.

Since ACC could have been metabolized by the hyphae, the concentration of ACC in the agar medium at the end of the experiment under fungal colonies was accessed, and was found to be not significantly different from the initial concentration ($10.8 \pm 0.1 \mu\text{M}$). Furthermore, at the end of the experiment, the cellophane films were removed together with seedlings and fungal colonies, to allow fresh seeds to be laid over the agar surface. Following germination, the new seedlings suffered the same symptoms as the first rotation seedlings (data not shown), confirming that ACC was still present in the nutrient medium and that hypaphorine did not accumulate significantly in the agar medium.

DISCUSSION

The aim of this study was to determine whether fungal hypaphorine could regulate auxin activity during host plant root colonization.

The results presented demonstrate that hypaphorine is able to antagonize exogenous IAA activity on root elongation. In contrast to known molecules inhibiting auxin-regulated growth, hypaphorine probably does not directly control the

activity of secondary messengers (Augur et al. 1992) or the basic machinery of cell elongation (Hoson et al. 1992; Morré et al. 1995) because hypaphorine alone has no activity on root elongation, and because hypaphorine does not restore root elongation following 2,4-D or NAA treatment. While the mechanism of IAA/hypaphorine antagonism has not yet been elucidated, four major steps could be involved. Hypaphorine could regulate IAA transport, induce a cellular, IAA-specific detoxification mechanism, compete with IAA for some receptor, or interfere with IAA signal transduction.

So far the only auxin activity regulators identified are clofibric acid (PCIB) (Tsai and Arteca 1984) and anion-channel blockers (Thomine et al. 1997). We cannot exclude that hypaphorine regulates anion channels and consequently signal transduction. However, our results clearly contrast with those of Thomine et al. (1997) on elongating *Arabidopsis* hypocotyl. Indeed, anion-channel blockers counteract NAA and 2,4-D as well as IAA whereas hypaphorine counteracts exclusively IAA activity. Furthermore, while anion-channel blockers attenuated only partially auxin-induced responses, hypaphorine fully restored root elongation. Regarding PCIB, binding of 2,4-D to the auxin binding protein ABP40 was completely

Fig. 3. Development of 10-day-old *Eucalyptus globulus* subsp. *bicostata* seedlings cultivated over agar nutrient medium (Control) supplemented with 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC), or a combination of ACC and 500 μM hypaphorine. Seedlings treated with 10 μM ACC were inoculated as well with the hypaphorine-producing fungus *Pisolithus tinctorius* strain 2144. To visualize the contact zone between roots and the agar medium, both photographs in the lower row were taken through the agar medium.

Fig. 4. Interaction between hypaphorine and 1-aminocyclopropane-1-carboxylic acid (ACC) controlling hypocotyl elongation. *Eucalyptus globulus* subsp. *bicostata* seedlings were cultivated over agar nutrient medium (Control) supplemented with 10 µM ACC, a combination of ACC and hypaphorine (10, 100, 500 µM), or ACC and tryptophan (100 µM). The kinetics of the hypocotyl elongation were followed over 20 days. Percentages of elongated hypocotyls were recorded among at least 30 seedlings per treatment.

Fig. 5. Regulation of hypocotyl elongation by ectomycorrhizal fungi in the presence of 1-aminocyclopropane-1-carboxylic acid (ACC). *Eucalyptus globulus* subsp. *bicostata* seedlings were cultivated over agar nutrient medium (Control) supplemented with 10 µM ACC, or a combination of ACC and 500 µM hypaphorine. Seedlings treated with 10 µM ACC were inoculated as well with two different strains of the hypaphorine-producing fungus *Pisolithus tinctorius*, an aggressive strain (441) and a very aggressive strain (2144). The kinetics of hypocotyl elongation were followed over 20 days. Percentages of elongated hypocotyls were recorded among at least 30 seedlings per treatment.

inhibited by PCIB (Sugaya and Sakai 1996), while our results recorded on elongating tap root suggest that hypaphorine and 2,4-D do not compete.

Hypaphorine probably does not interfere with IAA transport. Indeed, since NAA and 2,4-D are assumed to share either the influx or the efflux carrier with IAA (Delbarre et al. 1996; Yamamoto and Yamamoto 1998), hypaphorine would be expected to counteract as well with at least one of these two non-indolic auxins.

Here we assume that IAA and hypaphorine counteract during the very early steps of IAA perception or IAA signal transduction. Indeed, to a lesser extent, tryptophan antagonizes IAA activity as does hypaphorine. This suggests that the indol ring is a major active component of the hypaphorine molecule and therefore that both indolic molecules, IAA and hypaphorine, could be competing on a receptor involving the indol ring. The fact that both non-indolic auxins are not antagonized by hypaphorine would suggest that IAA is not perceived in the present case by the same receptors as those involved with 2,4-D and NAA.

The curvature of the apical hook in response to treatment with ACC, the precursor of ethylene, would result from accelerated growth of cells on the outer side of the hook, while hook opening under light would be due to accelerated growth of cells on the inner side thought to result from a reduction of ethylene biosynthesis or sensitivity (Goeschl et al. 1967; Smalle and Van Der Straeten 1997). Hypaphorine applied on roots antagonizes ACC activity in eucalypt seedling shoots.

Fig. 6. *Pisolithus tinctorius* requires direct contact with roots to fully regulate hypocotyl elongation in the presence of 1-aminocyclopropane-1-carboxylic acid (ACC). *Eucalyptus globulus* subsp. *bicostata* hypocotyl elongation was recorded after 10 days of culture over agar nutrient medium supplemented with 10 µM ACC in (A, B, D) sealed or (C, E) unsealed petri dishes. The hypaphorine-producing fungus *Pisolithus tinctorius* strain 2144 was introduced as well in the same petri dishes (B, C, D, E), either (B, C) in contact with the seedling roots or (D, E) separated from the roots by a trench into the agar medium. Percentages of elongated hypocotyls were recorded among at least 30 seedlings per treatment.

Both observations, hypaphorine interaction with IAA and ACC, tend to confirm that ethylene and IAA activities are linked somewhere to control the apical hook, as previously reported by Lehman et al. (1996). Indeed, while overexpression of the *HLS1* gene in Arabidopsis results in constitutive hook curvature, treatment of such seedlings with 2-[1-naphthalenylamino]carbonylbenzoic acid (NPA), an inhibitor of auxin transport, gives rise to *hls1* phenocopies in which hook curvature cannot even be induced by exogenous ethylene (Lehman et al. 1996). Therefore, here in eucalypt seedlings, by analogy with the above role of NPA and because hypaphorine is an indol alkaloid counteracting with IAA, we postulate that hypaphorine counteraction with ACC is a consequence of hypaphorine interaction with IAA.

The significance during root colonization of such hypaphorine interaction with IAA has been addressed. Because root elongation and ramification are strongly altered during fungal infection, it would have been hopeless to attempt to visualize IAA and fungal hypaphorine antagonism during root colonization by *P. tinctorius*. However, we could demonstrate that hypaphorine-producing fungal strains can mimic exogenous hypaphorine activity on shoot elongation in the presence of ACC. Considering the above postulate that hypaphorine counteraction with ACC is a consequence of hypaphorine interaction with IAA, this result suggests that the fungus is down-regulating the IAA activity in some plant tissues. As far as we know this is the first reported case of IAA activity in a plant being down-regulated by a microorganism. On the other hand, there are numerous cases in which microorganism colonization resulted in auxin over-accumulation in plant organs due to either massive microbial auxin delivery (Clark et al. 1989), host plant synthesis of auxin following microbe genome integration (Morris 1995), or auxin transport disruption (Mathesius et al. 1998).

Root colonization by *P. tinctorius* promotes hook opening, as does exogenous hypaphorine application. *P. tinctorius* activity is similar to the activities of hypaphorine concentrations ranging from 100 to 500 μM . Such hypaphorine concentrations are not unrealistic, in view of the high hypaphorine concentrations detected in mycelial mats of various *P. tinctorius* strains grown in pure culture on mineral nutrient media, ranging from 0.29 to 6.02 $\mu\text{mol g}^{-1}$ FW (Béguiristain et al. 1995). For comparison, the IAA concentration in *P. tinctorius* strain 441 hyphae, in the same experimental conditions, was 5.62 nmol g^{-1} FW while the hypaphorine concentration was 1,000 times higher, 5.14 $\mu\text{mol g}^{-1}$ FW (Béguiristain et al. 1995). The concentration of free tryptophan in *P. tinctorius* hyphae is at least 10-fold lower than the hypaphorine concentration and therefore tryptophan should not interfere significantly.

The flux of hypaphorine delivered to the root by colonizing hyphae still needs to be quantified. The fact, that after removal of the mycelium, hypocotyls again exhibited apical hooks when a second rotation of seedlings was grown over the ACC containing medium, suggests that fungal hypaphorine is not simply released into the nutrient medium where it accumulates before being absorbed by the roots. Accordingly, very low concentrations of hypaphorine were detected in the agar medium at the end of the experiment. Instead, hypaphorine must be transferred directly from hyphae to root cortex, i.e., through the Hartig net, the specialized transfer cells. There-

fore, the hypaphorine flux must be highly dependent on strain aggressiveness, its ability to rapidly colonize the root and develop a typical Hartig net. Here, the comparison of strains Pt 441 and Pt 2144 supports this idea. Indeed, among numerous strains compared in vitro or in a glasshouse, strain 2144 is one of the most aggressive toward *Eucalyptus* spp. seedlings (Albrecht et al. 1994; Burgess et al. 1994); accordingly, Pt 2144 is much more active on hook opening than is Pt 441. This is true despite the fact that both strains share rather similar hypaphorine concentrations in pure culture (4.98 and 5.14 $\mu\text{mol g}^{-1}$ FW, respectively).

While in the present experimental conditions hypaphorine acts as an IAA antagonist, we reported previously the induction of *EgHypar* gene expression in eucalypt seedlings treated by IAA as well as hypaphorine (Nehls et al. 1998). As it is widely accepted that IAA is perceived by different types of receptors in the cell, this apparent contradiction would suggest that IAA and hypaphorine interact differently with different receptors.

Therefore, while the ectomycorrhizal fungus *P. tinctorius* is producing auxin (Beyrle 1995; Béguiristain et al. 1995), it is also accumulating and releasing an auxin antagonist, hypaphorine, whose concentration in hyphae increases fourfold during root tip colonization (Béguiristain and Lapeyrie 1997). It is not yet understood how, when, and where fungal and plant IAA on the one hand and fungal hypaphorine on the other interact during ectomycorrhiza development. We assume that such a subtle balance between active and counteractive molecules would regulate some critical developmental steps.

Regulation of IAA activity in host plant tissues by *P. tinctorius* hypaphorine could be a significant pathway of the symbiotic interaction; however, generalization of such a conclusion to other fungal species is still under investigation. Indeed, while hypaphorine has been detected so far only in *P. tinctorius* strains (Béguiristain et al. 1995), it cannot be excluded that other indole alkaloids, among some 1,200 described in plant and fungi, play a similar role in mycorrhizae involving non-hypaphorine-accumulating species.

MATERIALS AND METHODS

Biological material.

The host plant was *Eucalyptus globulus* subsp. *bicostata* (Maid. et al.) Kirkp. Seeds were collected at Hill Top via Wee Jasper NSW, Australia and purchased from Kylisa Seeds (Australian Capital Territory, Australia). The ectomycorrhizal fungi were two strains of *Pisolithus tinctorius* Coker & Couch. These strains differ in their ability to colonize the root and to induce defense reactions (Albrecht et al. 1994). Strain 441, less aggressive, and strain 2144, more aggressive, toward *Eucalyptus* spp. were isolated from sporocarps collected respectively under *Eucalyptus citriodora* in Brazil and a *Eucalyptus* sp. in Australia.

Chemicals.

Hypaphorine was synthesized in vitro from tryptophan (Romburgh & Barger 1911). The purity of the product was determined by nuclear magnetic resonance (NMR) and high-pressure liquid chromatography (HPLC) analysis (Béguiristain et al. 1995). Tryptophan, ACC, IAA, 2,4-D, and NAA were purchased from Sigma (St. Louis, MO).

Eucalypt seedling growth conditions and treatments.

Eucalypt seedlings were germinated in petri dishes (140 mm diameter) on cellophane laid over modified Pachlewski's medium (7.3 mM KH_2PO_4 , 2.7 mM diammonium tartrate, 7.3 mM MgSO_4 , 2.9 μM thiamine-HCl, 1 ml of trace element stock solution [Kanieltra, St. Pierre des Corps, France] and 20 g of agar per liter) supplemented or not with IAA (1 μM), ACC (10 μM), 2,4-D (1 μM), NAA (50 μM), tryptophan (100 μM), or hypaphorine (10, 100, 500 μM). When fungi were grown together with seedlings, and in comparative treatments, glucose (5 mM) was added to the nutrient medium. Petri dishes were "sealed" with Parafilm along 90% of their circumference. Where specified, to increase gas exchange during ACC treatment, "unsealed" petri dishes were used. Plates were maintained vertically with a slight angle to allow full elongation of the roots on the agar surface, in a controlled environment growth chamber (25°C) with 16 h light (200 $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) and 8 h dark. Tap root length was recorded 7 days after germination; shoot elongation was monitored between 7 and 20 days after germination. At least 30 replicate seedlings were analyzed for all presented data. Each observation was confirmed by at least three independent replicate experiments.

Eucalyptus root colonization by *P. tinctorius* hyphae.

Petri dishes prepared as described above were inoculated with 9 fungal plugs distributed in the lower part of the plates. Colonies were grown for 2 weeks at 25°C in dark before eucalypt seeds were introduced as previously indicated. Then it took less than 3 days for the emerging roots to come in contact with the fungal colony.

Split-plate experiments.

When specified, to separate hyphae from roots inside the same plate, the medium was divided vertically into two compartments by removing a 5-mm-wide slice of agar.

Quantification of hypaphorine and ACC.

To assess the ACC and hypaphorine concentrations in the medium at the end of the experiment, agar samples were frozen, thawed, and centrifuged. The supernatant was analyzed by gas chromatography according to Lizada and Yang (1979) for ACC, and by HPLC according to Béguiristain and Lapeyrie (1997) for hypaphorine.

Statistical analysis.

Data from replicates were analyzed with StatView Student software (Abacus Concepts, Berkeley, CA) (analysis of variance followed by Fisher's protected least significant difference test, $P = 0.05$).

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LITERATURE CITED

Albrecht, C., Burgess, T., Dell, B., and Lapeyrie, F. 1994. Chitinase and peroxidase activities are induced in eucalyptus roots according to ag-

- gressiveness of Australian ectomycorrhizal strains of *Pisolithus* sp. New Phytol. 127:217-222.
- Augur, C., Lu, Y., Sakai, K., Ogawa, T., Sinaý, P., Darvill, A. G., and Albersheim, P. 1992. Further studies of the ability of xyloglucan oligosaccharides to inhibit auxin-stimulated growth. Plant Physiol. 99: 180-185.
- Béguiristain, T., Côté, R., Rubini, P., Jay-Allemand, C., and Lapeyrie, F. 1995. Hypaphorine accumulation in hyphae of the ectomycorrhizal fungus *Pisolithus tinctorius*. Phytochemistry 40:1089-1091.
- Béguiristain, T., and Lapeyrie, F. 1997. Host plant stimulates hypaphorine accumulation in *Pisolithus tinctorius* hyphae during ectomycorrhizal infection while excreted fungal hypaphorine controls root hair development. New Phytol. 136:525-532.
- Beyle, H. 1995. The role of phytohormones in the function and biology of mycorrhizas. Pages 365-390 in: Mycorrhiza: Structure, Molecular Biology and Function. A. K. Varma and B. Hock, eds. Springer-Verlag, Berlin.
- Burgess, T., Dell, B., and Malajczuk, N. 1994. Variation in mycorrhizal development and growth stimulation by 20 *Pisolithus* isolates inoculated on to *Eucalyptus grandis* W. Hill ex Maiden. New Phytol. 127: 731-739.
- Clark, E., Vigodsky-Haas, H., and Gafni, Y. 1989. Characteristics in tissue culture of hyperplasia induced by *Erwinia herbicola* pathovar *gypsophylae*. Physiol. Mol. Plant Pathol. 35:383-390.
- Delbarre, A., Muller, P., Imhoff, V., and Guern, J. 1996. Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198:532-541.
- Dexheimer, J., and Pargney, J. C. 1991. Comparative anatomy of the host-fungus interface in mycorrhizas. Experientia 47:312-320.
- Gay, G., Normand, L., Marneise, R., Sotta, B., and Debaud, J. C. 1994. Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. New Phytol. 128:645-657.
- Gea, L., Normand, L., Vian, B., and Gay, G. 1994. Structural aspects of ectomycorrhiza of *Pinus pinaster* (Ait.) Sol. formed by an IAA-overproducer mutant of *Hebeloma cylindrosporum* Romagnési. New Phytol. 128:659-670.
- Goeschl, J. D., Pratt, H. K., and Bonner, B. A. 1967. An effect of light on the production of ethylene and the growth of the plumular portion of etiolated pea seedlings. Plant Physiol. 42:1077-1080.
- Gogala, N. 1991. Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. Experientia 47:331-340.
- Hampp, R., Ecker, M., Schaeffer, C., Wallenda, T., Winkler, A., Kottke, I., and Sundberg, B. 1996. Axenic mycorrhization of wild type and transgenic hybrid aspen expressing T-DNA indoleacetic acid-biosynthetic genes. Trees 11:59-64.
- Harley, J. L., and Smith S. E. 1983. Mycorrhizal Symbiosis. Academic Press, London.
- Horan, D. P. 1991. The infection process in eucalypt mycorrhizas. Ph.D. thesis. Australian National University, Canberra, Australia.
- Hoson, T., Masuda, Y., and Nevins, D. J. 1992. Comparison of the outer and inner epidermis inhibition of auxin-induced elongation of maize coleoptiles by glucan antibodies. Plant Physiol. 98:1298-1303.
- John, P. 1997. Ethylene biosynthesis: The role of 1-aminocyclopropane-1-carboxylate (ACC) oxidase, and its possible evolutionary origin. Physiol. Plant. 100:583-592.
- Karabaghli-Degron, C., Sotta, B., Bonnet, M., Gay, G., and Le Tacon, F. 1998. The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) inhibits the stimulation of in vitro lateral root formation and the colonization of the tap-root cortex of Norway spruce (*Picea abies*) seedlings by the ectomycorrhizal fungus *Laccaria bicolor*. New Phytol. 140:723-733.
- Lehman, A., Black, R., and Ecker, J. R. 1996. HOOKLESS1, an ethylene response gene, is required, for differential cell elongation in the *Arabidopsis* hypocotyl. Cell 85:183-194.
- Lizada, M. A. C., and Yang, S. F. 1979. A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Anal. Biochem. 100:140-145.
- Marrs, K. A. 1996. The functions and regulation of glutathion S-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 127-158.
- Martin, F., Lapeyrie, F., and Tagu, D. 1997. Altered gene expression during ectomycorrhizal development. Pages 223-242 in: The Mycota. Vol. V part A. G. Carroll and P. Tudzynski, eds. Springer-Verlag, Berlin.

- Mathesius, U., Schalman, H. R. M., Spaink, H. P., Sautter, C., Rolfe, B. G., and Djordjevic, M. A. 1998. Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoides and derivatives of chitin oligosaccharides. *Plant J.* 14:23-34.
- Morré, D. J., Brightman, A. O., Hidalgo, A., and Navas, P. 1995. Selective inhibition of auxin-stimulated NADH oxidase activity and elongation growth of soybean hypocotyls by thiols reagents. *Plant Physiol.* 107:1285-1291.
- Morris, R. O. 1995. Genes specifying auxin and cytokinin biosynthesis in procaryotes. Pages 318-339 in: *Plant Hormones*. P. J. Davies, ed. Kluwer Academic Pub., Dordrecht, The Netherlands.
- Nehls, U., Béguiristain, T., Ditengou, F., Lapeyrie F., and Martin, F. 1998. The expression of a symbiosis-regulated gene in eucalypt roots is regulated by auxins and hypaphorine, the tryptophan betaine of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. *Planta* 207:296-302.
- Romburgh, P. V., and Barger, G. 1911. Preparation of the betaine of tryptophan and its identity with the alkaloid hypaphorine. *J. Am. Chem. Soc.* 99:2068-2070.
- Smalle, J., and Van Der Straeten, D. 1997. Ethylene and vegetative development. *Physiol. Plant.* 100:593-605.
- Sugaya, S., and Sakai, S. 1996. Identification of a soluble auxin-binding protein as a glutathione-dependent formaldehyde dehydrogenase. *Plant Sci.* 114:1-9.
- Thomine, S., Lelievre, F., Boufflet, M., Guern, J., and Barbier-Brygoo, H. 1997. Anion-channel blockers interfere with auxin response in dark-grown *Arabidopsis* hypocotyls. *Plant Physiol.* 115:533-542.
- Tsai, D. S., and Arteca, R. N. 1984. Inhibition of IAA-induced ethylene production in etiolated mung bean hypocotyl segments by 2,3,5-triiodobenzoic acid and 2-(p-chlorophenoxy)-2-methyl propionic acid. *Physiol. Plant.* 62:448-452.
- Wallander, H., Nylund, J. E., and Sundberg, B. 1992. Ectomycorrhiza and nitrogen effects on root IAA: Results contrary to current theory. *Mycorrhiza* 1:91-92.
- Yamamoto, M., and Yamamoto, K. T. 1998. Differential effects of 1-naphtaleneacetic acid, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid on the gravitropic response of roots in an auxin-resistant mutant of *Arabidopsis*, *aux1*. *Plant Cell Physiol.* 39:660-664.