Mycorrhiza (1994) 4:247–250

Mycorrhiza

IAA-overproducer mutants of *Hebeloma cylindrosporum* Romagnesi mycorrhizal with *Pinus pinaster* (Ait.) Sol. and *P. sylvestris* L. in hydroponic culture

Jan-Erik Nylund[1], **Håkan Wallander**[1], **Björn Sundberg**[2], **Gilles Gay**[3]

[1] Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, S-75007 Uppsala, Sweden
[2] Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S-90183 Umeå, Sweden
[3] Unité d'Ecologie Microbienne, Bât. 405, 43, Université Claude-Bernard Lyon 1, Boulevard du 11 Novembre, F-69622 Villeurbanne Cedex, France

Abstract. Indole-3-acetic acid (IAA) is thought to play a role in the regulation of ectomycorrhiza development, and vigorous mycorrhiza formers such as *Pisolithus* and *Laccaria* have previously been shown to accumulate large amounts of IAA in the culture medium in vitro, particularly in the presence of tryptophan. Recently, 5-fluoroindole-resistant and IAA-overproducing mutant strains of *Hebeloma cylindrosporum* Romagnesi have been developed and described by Durand et al. (1992). We have used some of these and corresponding wild-type strains as mycobionts on seedlings of *Pinus pinaster* (Ait.) Sol. and *P. sylvestris* L. in semi-hydroponic culture in an attempt to study IAA effects independent of species-specific differences. However, no significant differences between strains were found in host growth rate, shoot carbohydrate concentration, root morphology, root IAA concentration or mycorrhizal biomass. Since previous work showed a stimulation by these and other mutants and strains on mycorrhiza formation in Petri dish and test tube cultures, we assume that a semi-hydroponic culture system prevents the build up of tryptophan of fungal origin, which is most likely a precondition for enhanced IAA production.

Key words: IAA overproducers – Ectomycorrhiza – *Hebeloma cylindrosporum*

Introduction

The possible role of the plant hormone auxin, or indole-3-acetic acid (IAA), in the regulation of the ectomycorrhizal symbiosis has been discussed in a review article by Nylund (1988), and was further developed in papers by Wallander et al. (1992, 1994) and Durand et al. (1992). According to the hormone theory as formulated by Slankis (1974), IAA of fungal origin induces the morphological changes characteristic of ectomycorrhizal roots and, more important creates a carbohydrate sink effect leading to carbohydrate flow to the root and further to the fungus. This is comparable to the well-known effect of external application of auxin to plant parts which leads to sugar accumulation in the treated tissue. Thus increased IAA release by the fungus would stimulate carbohydrate flow and lead to stronger development of the fungus and a corresponding drain of host resources.

Such a drain is hard to observe in pot or field experiments, where the beneficial effects of improved nutrient uptake through mycorrhiza more than compensate for the carbohydrate cost of maintaining the fungus. However, in steady-state culture systems such as that used by us in this and previous studies, where mineral nutrition is not limited and the mycorrhiza supposedly confers no advantage to the host, the production of fungal biomass estimated by the ergosterol method and, more vaguely, fungal "vigour" have consistently been negatively correlated with host growth and shoot carbohydrate pools (Nylund and Wallander 1989). Thus the semi-hydroponic system appeared to be a useful tool to investigate IAA effects on mycorrhizal systems.

The isolation of IAA overproducers independent of added tryptophan has opened up new opportunities to study the role of IAA in the regulation of ectomycorrhiza development. The present study aimed to examine the effects of such mutants on host growth and carbohydrate pools and on fungal biomass in a controlled system previously used for studies of fungal species and mineral nutrition effects on mycorrhiza formation (Nylund and Wallander 1989; Wallander and Nylund 1992; Wallander et al. 1994). Here we report on the initial results; questions arising from the results are being addressed by on-going research.

Materials and methods

The study was based on the wild type and tryptophan- and IAA-overproducing mutants of *Hebeloma cylindrosporum* Romagnesi, isolated by Gay and coworkers in Lyon. The mutant isolation and

Correspondence to: J.-E. Nylund

biochemical characterization have been described by Durand et al. (1992) and a first round of screening tests has been described by Gea et al. (1994). The strains used in the present study were HC1 (the parental wild type), D1 (wild-type dikaryon produced by two monospore progenies of HC1 designated h1 and h6), h1 FIR4 (the original monokaryotic mutant), and two dikaryotic strains from compatible fusions of IAA-overproducing monospore progeny of h1 FIR4 paired with h6, designated h1 FIR4 F1 331*h1FIR4 F199 and 331*233.

The experiments were conducted in a growth chamber using a semi-hydroponic setup for maintaining approximately constant relative growth rates, as described by Nylund and Wallander (1989), but with an 8-h dark period. The seedling material consisted of *Pinus pinaster* (Ait) Sol., the natural host of *H. cylindrosporum*, and *P. sylvestris* L., 6 weeks old upon inoculation and transplanting. The fungal inoculum was grown in liquid MMN (100 ml in 250-ml bottles) for 6 weeks without shaking, and briefly homogenized in a blender after rinsing in distilled water. The seedlings, initially grown in Leca brick pellets, were shaken free of pellets, weighed, and dipped into the fungal slurry. The growing time was 42 days for *P. pinaster* and 55 days for *P. sylvestris*. The N concentration of the medium was maintained at 20–25 mg l^{-1}, which is the highest N concentration that caused no inhibition of mycorrhiza development with the closely related species *H. crustuliniforme* (Wallander and Nylund 1992). The medium was replaced each week, and the tray positions shifted to prevent border effects.

At harvesting, the seedlings were briefly rinsed, weighed, divided into root and shoot and pooled into three batches of seven seedlings each, frozen in liquid N, and freeze dried. A few short roots from each sample were not frozen but fixed in 4% buffered formalin for microscopy. After dry weight determination, ball mill-ground root material was sampled and used for ergosterol assay of fungal biomass (Nylund and Wallander 1992) and IAA determination (Sundberg 1990). The remaining pellet medium was also extracted and assayed for ergosterol to obtain an estimate of extramatrical mycelium biomass. With this type of culture, we did not find it meaningful to count mycorrhizal root tips etc., since all short roots in the upper part of the pots were converted into mycorrhiza, while the roots in the very wet bottom part were mainly long and white and always without mycorrhiza, regardless of strain. Shoot samples were used for enzymatic determination of glucose, fructose, sucrose and starch (by the Government Laboratory for Agricultural Chemistry, using routine procedures).

Besides the two reported experiments, partial replication experiments were conducted with both host species but only on a few of the strains, with different growing times and, for reasons of expense, incomplete sets of analyses.

Results

All data referred to in the text are shown in Table 1.

Performance of the fungal strains

All strains grew submerged in liquid culture, the wild-type strain D1 most vigorously. On agar, unless the medium was very thick, staling (arrested growth and pigmentation of the central part of the colony) occurred when the colony had grown about 1 cm. In an introductory assay using 3-week-old mycelium grown in liquid MMN as above (no tryptophan added), wild-type culture filtrate contained very little IAA, and the mutant filtrates approximately 100 times more. Considering the instability of IAA, these data can not be taken as quantitative estimates of IAA production by the strains. However, we consider that the wide differences in concentration establish clearly that there are indeed very large differences in IAA production capacity between the strains. This view is also supported by the data published by Durand et al. (1992). As mycorrhiza formers, D1 and the dikaryotic mutant 331*99 appeared to be the most vigorous.

Table 1. Effects of *Hebeloma cylindrosporum* strains on host and mycorrhiza. LSD, Least significant difference, RGR, relative growth rate

Strain	Control	Wild types		Overproducer mutants			LSD
		HCl	D1	h1Fir4	331*99	331*233	
Pinus sylvestris grown for 55 days after transplanting							
Total wt (mg)	821	690	758	818	946	937	143
RGR, % per day	5.30	5.16	5.07	5.34	5.84	5.67	0.30
Root, %	18.5	26.3	25.4	22.7	23.3	20.6	4.9
Ergosterol (μg/g root)	7.0	97.9	122.2	49.6	64.3	53.8	18.5
Sugars, % of shoot wt	5.8	4.7	5.3	5.3	5.8	5.8	0.7
Starch, % of shoot wt	14.6	8.3	9.5	12.0	10.5	14.3	3.0
Sugars+starch	20.4	13.0	14.8	17.3	16.3	20.1	3.7
IAA (ng/g root)	73	71	71	64	58	55	22
Pinus pinaster grown for 43 days after transplanting							
Total wt (mg)	405.5	339.3	331.0	324.2	280.2	348.7	62.8
RGR, % per day	5.84	4.94	4.88	4.88	4.65	4.84	0.47
Root, %	17.7	18.4	20.8	20.0	21.0	17.6	3.1
Ergosterol (μg/g root)	3.3	68.2	75.4	60.3	77.9	59.5	7.1
Sugars, % of shoot wt	5.9	6.7	6.3	6.4	6.3	6.4	0.8
Starch, % of shoot wt	5.0	8.5	7.6	7.2	5.5	9.9	1.6
Sugars+starch	10.9	15.2	13.9	13.6	11.8	16.3	2.4
IAA (ng/g root)	657	369	453	436	395	482	92

Effects on host development

Compared to the non-mycorrhizal control, the growth rate and final weight of mycorrhizal *P. pinaster* were reduced to extents comparable to those found by Nylund and Wallander (1989) in *P. sylvestris* with *Laccaria bicolor* and *H. crustuliniforme.* Mutant strain 331*99 depressed the dry weight more than the other mutants, but taken as a whole, no consistent or significant differences to the wild types were found. In *P. sylvestris*, the growth depression was less pronounced with the wild-type strains; with the dikaryotic mutants, a small but significant increase in relative growth rate was observed.

In *P. pinaster*, the growth reducing effect was approximately equal in root and shoot, and the differences in root percentage insignificant, again with the exception of the mutant strain 331*99, which had significantly more root than the control and 331*223. However, the special position of strain 331*99 was not maintained in the partial replication of the main experiment. In *P. sylvestris,* both root absolute weight and root percentage were higher in mycorrhizal seedlings than in the control, but no consistent differences between wild types and mutants were noted. *P. pinaster* contained less dry matter than *P. sylvestris,* but the treatments did not differ from each other in either species.

Regarding shoot carbohydrate pools, the *P. sylvestris* data showed a reduction for all mycorrhizal strains compared to the uninfected control. This is in agreement with several of our previous experiments (Nylund and Wallander 1989; Wallander and Nylund 1992) and also with data from Rieger et al. (1992). Again, no consistent differences between wild types and mutants were found. In *P. pinaster*, the control carbohydrate concentration was, surprisingly, significantly lower than with any of the mycorrhiza strains, with strain 331*99 again being significantly lower than the other two, paralleling the growth performance. Since there was no corresponding relationship between control growth and carbohydrate concentration, and the carbohydrate reduction was well established with *P. sylvestris*, the low sugar content in the control may be spurious.

The two host species also differed in the proportions of sugar and starch. The sugar pool size was similar in the two species, but the amount of starch was higher in *P. sylvestris*, with the result that the total pool was larger in this species.

Mycorrhiza development

In both host species, mycorrhiza caused a major modification in root structure. The root systems of uninfected controls were made up of a number of fairly thin, long roots with sparse branches and scattered short roots, as is typical for hydroponic culture pines. All mycorrhizal seedlings had short, compact root systems, where large numbers of clustered mycorrhizal short roots emerged from a few long roots. It was striking to see that both wild-type and mutant mycorrhizas displayed traits characteristic of strong IAA action, despite the low IAA accumulation in wild-type culture filtrates.

The ergosterol assays confirmed the visual impression. The data were comparable to that recorded with *Laccaria bicolor* and *H. crustuliniforme* in previous studies (Wallander and Nylund 1991, 1992). Ergosterol assay of the pellet medium after extraction showed that very little mycelium remained after extraction of the roots and these data have been omitted. Although statistically significant differences were found among the *P. pinaster* treatments, they reveal no consistent differences between wild type and mutant strains. The high value of mutant 331*99 corresponds to the largest growth reduction (discussed above), but the almost as high value for D1 is not reflected in any negative growth effect. In *P. sylvestris*, the ergosterol content of the wild-type roots was significantly elevated, while in the mutant treatments it was approximately the same as in both wild-type and mutant treatments of *P. pinaster.*

Auxin assays

The values and variability of the auxin concentrations in *P. pinaster* root systems were as expected from the parallel work on *Laccaria* and *Hebeloma* on *P. sylvestris* (Wallander et al. 1992, 1994). Again, mycorrhizal root systems had lower values than the control. In the *P. sylvestris* samples, the concentrations observed were one order of magnitude smaller, and the control did not differ from the mycorrhizal samples. The reason for this is not understood; several replicates were made to ensure that handling or assay errors did not cause this deviation from other assays of *P. sylvestris* roots. We assume, however, that the assay results shold not be taken as representative of *P. sylvestris* mycorrhiza with *H. cylindrosporum.*

Discussion

Mycorrhiza inoculation, generally speaking, caused the same effects as in previous experiments with *P. sylvestris* in hydroponic culture (Nylund and Wallander 1989; Wallander and Nylund 1991, 1992). There were, however, a few differences. First, the expected reduction in shoot carbohydrate concentration in *P. pinaster* was absent in spite of vigorous fungal development and reduced host growth. Our previous work has concentrated on *P. sylvestris* and this may well be a species-dependent difference. Furthermore, in *P. sylvestris*, the wild types depressed the host growth less than they did in *P. pinaster*, and the mutants caused a small but statistically significant growth stimulation. Such a stimulation has not been noted with any fungal species in Scots pine, while Norway spruce is sometimes stimulated even in the hydroponic system. This indicates

that the host may benefit from the symbiosis by effects other than improved nutrition and water uptake.

The close agreement between the present IAA concentration in *P. pinaster* and those previously published (Wallander et al. 1992, 1994) make us confident that the mycorrhiza-induced IAA reductions indicate a general phenomenon to be further investigated. The consequence of these findings for the hormone theory of Slankis (1974 and previous publications), which predicted increased, levels, will be discussed in a coming paper.

The mutants were expected to create a stronger sink for carbohydrate through their assumed higher IAA production. This should have resulted in reduced host growth, lower shoot carbohydrate concentrations, higher fungal biomass and more and denser mycorrhizal short roots, but none of these effects could be demonstrated. However, clear mutant effects on mycorrhiza development could be seen in Petri dish systems (Gea et al. 1994). Rudawska (personal communication) also demonstrated similar stimulation of mycorrhiza development using a range of (wild type) *Paxillus involutus* strains selected for extremes in IAA excretion.

Trying to interpret our findings, we note that the mutants have an altered tryptophan metabolism, lacking the anthranilate synthase feedback regulation (Veal and Casselton 1985; Gay et al. 1989). When the mutants are grown in a closed system, tryptophan is likely to accumulate in the medium and in the apoplastic space of the roots. Eventually, the tryptophan concentration will be high enough to induce a substantial production of IAA (cf. Gay et al. 1989). Without tryptophan accumulation, not even the mutants will produce an excess of IAA.

Working with an open system such as ours, this accumulation of tryptophan probably did not take place, and even the feedback downregulation of tryptophan synthesis in the wild types may not have occurred. Furthermore, in preliminary short-term (4-day) time-course experiments, we could detect no significant differences in IAA excretion into the medium between the wild type, h1, and the best performing mutant, 331*233, either with no added tryptophan or at a low (10^{-5} M) tryptophan concentration, suggesting that rather high amounts are needed to induce downregulation of tryptophan synthesis in the wild types. In contrast, the orginal studies of the mutants (Durand et al. 1992) examined the IAA accumulation in the medium and in mycelia after 6 weeks of liquid culture.

The observations, and the unexplained fact that mycorrhizal roots have similar or lower bulk concentrations of endogenous IAA than uninfected plants (Wallander et al. 1992, 1994) (see above), call for a more detailed investigation of the metabolism and behaviour of fungal IAA in ectomycorrhiza. The capacity of the mycobiont to synthesize IAA at tryptophan concentrations similar to the quantities available in the root and rhizosphere region, and the relative importance of the fungal and root pools of tryptophan from need to be investigated. Furthermore, there is still no compelling evidence that IAA of fungal origin is taken up by the host in amounts large enough to affect the normal IAA homeostasis in the plant.

Acknowledgements. This project was financed by the Swedish Forestry and Agriculture Research Council.

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