NOTAS CIENTÍFICAS

MYCORRHIZAL COLONIZATION AND PHENOLIC COMPOUNDS ACCUMULATION ON ROOTS OF *EUCALYPTUS DUNNII* MAIDEN INOCULATED WITH ECTOMYCORRHIZAL FUNGI[1]

EDUARDO LUIZ VOIGT[2], VETÚRIA LOPES DE OLIVEIRA[3] and ÁUREA MARIA RANDI[4]

ABSTRACT - Compatibility between *Eucalyptus dunnii* and the ectomycorrhizal fungi *Hysterangium gardneri* and *Pisolithus* sp. − from *Eucalyptus* spp. −, *Rhizopogon nigrescens* and *Suillus cothurnatus* − from *Pinus* spp.−, was studied *in vitro*. *Pisolithus* sp., *H. gardneri* and *S. cothurnatus* colonized the roots. *Pisolithus* sp. mycorrhizas presented mantle and Hartig net, while *H. gardneri* and *S. cothurnatus* mycorrhizas presented only mantle. *S. cothurnatus* increased phenolics level on roots. *Pisolithus* sp. and *R. nigrescens* decreased the level of these substances. The isolates from *Eucalyptus* seem to be more compatible towards *E. dunnii* than those from *Pinus*. The mechanisms involved could be related, at least in the cases of *Pisolithus* and *Suillus*, to the concentration of phenolics in roots.

COLONIZAÇÃO E ACUMULAÇÃO DE COMPOSTOS FENÓLICOS EM RAÍZES DE *EUCALYPTUS DUNNII* MAIDEN INFECTADAS COM FUNGUS ECTOMICORRÍZICOS

RESUMO - Estudou-se a compatibilidade entre *Eucalyptus dunnii* e os fungos ectomicorrízicos *Hysterangium gardneri* e *Pisolithus* sp. − isolados de *Eucalyptus* spp.−, *Rhizopogon nigrescens* e *Suillus cothurnatus* − isolados de *Pinus* spp.−, *in vitro*. *Pisolithus* sp., *H. gardneri* e *S. cothurnatus* colonizaram as raízes. As micorrizas de *Pisolithus* sp. apresentaram manto e rede de Hartig; as de *H. gardneri* e *S. cothurnatus* apresentaram apenas manto. *S. cothurnatus* provocou aumento de fenóis nas raízes; *Pisolithus* sp. e *R. nigrescens* provocaram diminuição dessas substâncias. Os fungos isolados de *Eucalyptus* parecem mais compatíveis em relação a *E. dunnii* do que os de *Pinus*. A concentração de fenóis nas raízes parece estar envolvida nesse fenômeno, particularmente em relação a *Pisolithus* sp. e *S. cothurnatus*.

---

[1] Accepted for publication on December 27, 1999.

Part of B.Sc. thesis presented by the senior author to Universidade Federal de Santa Catarina (UFSC).

[2] Biologist, Dep. de Microbiologia e Parasitologia, Centro de Ciências Biológicas, UFSC, Caixa Postal 476, CEP 88040-900 Florianópolis, SC, Brazil. CAPES scholar. E-mail: elvoigt@garoupa.bio.ufpr.br

[3] Agronomist, Doct., Dep. de Microbiologia e Parasitologia, Centro de Ciências Biológicas, UFSC. E-mail: veturia@mbox1.ufsc.br

[4] Biologist, Ph.D., Associate Professor, Dep. de Botânica, Centro de Ciências Biológicas, UFSC. E-mail: bot@ccb.ufsc.br

Specificity between ectomycorrhizal (ECM) fungi and host plants has been observed both in field (Molina & Trappe, 1982; Molina et al., 1992) and controlled conditions (Malajczuk et al., 1982, 1984; Oliveira et al., 1994). Knowledge of the mechanisms controlling this phenomenon is important to understand mycorrhizal functioning and to guide the selection of isolates for inoculation programmes.

Malajczuk et al. (1982, 1984) reported that ECM fungi from eucalypts were unable to colonize *Pinus radiata*, while those from conifers did not colonize eucalypts. In incompatible pairings, phenolics accumulated in roots as a result of a hypersensitive reaction. In mycorrhizas of *Picea abies*, *Larix decidua* and *Pinus sylvestris*, a lower concentration of soluble and cell wall bound phenolics than in uninoculated roots was observed (Münzenberger et al., 1990, 1995, 1996). Later, they observed that laccase and peroxidase activities differed between mycorrhizas and uninoculated roots of *P. abies* and *L. decidua* (Münzenberger et al., 1997). In both species, mycorrhizas contained the highest laccase activity and the lowest peroxidase activity. The high laccase activity could induce the polymerisation of soluble phenolics contributing to their decreasing. The low peroxidase activity would inhibit oxidative rigidification of cell wall. These reactions would favour root colonization by ECM fungi.

In Southern Brazil, *Eucalyptus* spp. and *Pinus* spp., even when planted in the same sites, seldom have the same fungal symbionts. This difference in fungal diversity could possibly be related to fungus-host specificity. In this sense, this phenomenon deserves further consideration.

Thus, this study was carried out with four ectomycorrhizal fungi well known by their specific occurrence in *Eucalyptus* or *Pinus* stands in Santa Catarina, Southern Brazil. The aim was to determine fungal infectivity towards *Eucalyptus dunnii* Maiden and its relationship with phenolic compounds accumulation on roots.

Seeds of *E. dunnii* were disinfected in 70% ethanol (30 seconds) and surface sterilized in 1% sodium hypochloride (20 minutes). They were placed on the surface of Modified Melin Norkrans agar (MMN) (Marx, 1969) and germinated at 25±2ºC under 16 hours photoperiod, during two weeks.

Fungal inocula were obtained from MMN agar-cultures of *Hysterangium gardneri* Fisher (UFSC-Hg93) and *Pisolithus* sp. Alb. & Schwein (UFSC-Pt44) – isolated from *Eucalyptus* spp. plantations –; *Rhizopogon nigrescens* Coker & Couch (UFSC-Rh95) and *Suillus cothurnatus* Sing. (UFSC-Su94) – isolated from *Pinus* spp. stands – both in Southern Brazil. Ten mm-diameter discs were cut from the edge of four week-old colonies and placed centrally in Petri dishes (∅=150 mm) containing MMN agar overlaid by a sterile cellophane film. Mycelial growth took place at 25±2ºC in the dark for two weeks.

For mycorrhizal synthesis, five seedlings were placed concentrically on the surface of the cellophane film with their roots in contact with the fungus (Burgess et al., 1995) and kept at the same conditions described for the germination. Uninoculated controls were prepared similarly, except for the absence of fungal colony. There were ten replicates (dishes) per treatment (50 seedlings).

Five weeks later, plants were carefully removed, shoots were eliminated, and roots were placed in distilled water. Roots were observed under stereomicroscope (30 x) to determine the number of colonized root tips per plant. After that, they were divided into two parts, one for microscope observations and another for extraction of phenolic compounds.

For microscope observations roots were fixed in FAA (formalin-aceto-alcohol, 1:1:9, v/v/v) (Kormanik & McGraw, 1982) for 48 hours, cut in a cryo-microtome in 30 μm sections and mounted with lactophenol-cotton blue.

Three samples of 100 mg of fresh roots were used per treatment for the extraction of total phenolics. Each sample was grinded in 2 mL of 70% ethanol and kept in a water bath at 60ºC before being centrifuged for 2 minutes at 700 g. The pellet was dissolved and extracted twice again by the same procedure (Phillips & Henshaw, 1977). The three extracts of the same sample were combined in order to obtain a final extract of 6 mL.

Phenolic compounds were quantified in three aliquots of 0.5 mL from each sample (three samples/treatment), according to the Folin-Denis technique (Swain & Hills, 1959). The extract was dried at 50ºC for 24 hours. The pellet was redissolved in 1 mL of distilled water and 0.5 mL of Folin-Denis reagent and 1 mL of saturated calcium carbonate solution were added. The final volume was adjusted to 10 mL with distilled water. The solution was kept at room temperature for 45 minutes. Optical density was measured at 725 nm. The results were compared with a standard curve obtained with tanic acid at different concentrations (0, 20, 40, 60, 80 and 100 μg/mL).

Data on number of colonized root tips and total phenolic compounds per plant were submitted to variance analysis and the averages compared by the t test.

Pisolithus sp., *H. gardneri* and *S. cothurnatus* colonized *E. dunnii* roots, whereas no colonization was observed in plants inoculated with *R. nigrescens*. *Pisolithus* sp. formed typical mycorrhizas (Fig. 1B), with a well developed mantle and a Hartig net limited to the first layer of cortical cells. *H. gardneri* and *S. cothurnatus* mycorrhizas (Figs. 1A and 1D), although presenting a well developed mantle, showed no discernible Hartig net besides a few hyphae dispersed in epidermal intercellular spaces. No mantle nor Hartig net were observed on roots inoculated with *R nigrescens* (Fig. 1C).

According to statistical analysis, *Pisolithus* sp. and *H. gardneri* showed a higher infectivity in relation to *E. dunnii* – with 15.8 and 2.8 colonized root tips per plant, respectively – than *S. cothurnatus* and *R. nigrescens* which colonized only 1.4 and 0.0 root tips, in this order (Table 1).

Smith & Read (1997) consider that mantle and mainly Hartig net are indicative of effective establishment of ectomycorrhizas. In this way, *Pisolithus* sp. (UFSC-Pt44) presents higher compatibility towards *E. dunnii* than the other fungi, because this fungus formed typical mantle and Hartig net structures on roots. In comparison, *H. gardneri* and *S. cothurnatus* mycorrhizas although presenting a well developed mantle, had no typical Hartig net, presenting a superficial colonization. However, other authors consider that an ectomycorrhiza is characterized by any case of a fungus forming a mantle, with or without Hartig net (Warcup, 1980). Superficial mycorrhizas in

FIG. 1. Cross sections of *Eucalyptus dunnii* roots inoculated with different ectomycorrhizal fungi. A. *Hysterangium gardneri*. B. *Pisolithus* sp. C. *Rhizopogon nigrescens*. D. *Suillus cothurnatus*. All figures x 750; m = mantle; h = Hartig net; c = cortical cell.

TABLE 1. Mycorrhizal colonization and total phenolic compounds accumulation in *Eucalyptus dunnii* roots inoculated with ectomycorrhizal fungi after five weeks of incubation[1].

Treatment	Mycorrhizal colonization (root tips/plant)[2]	Phenolic compounds (μg/mg fr. wg.)[3]
Pisolithus sp. (UFSC-Pt44)	15.8a	8.8c
Hysterangium gardneri (UFSC-Hg93)	2.8b	11.1b
Suillus cothurnatus (UFSC-Su94)	1.4c	12.3a
Rhizopogon nigrescens (UFSC-Rh95)	0.0d	9.1c
Uninoculated control	0.0d	10.2b

[1] Values in the same column followed by different letters are significantly different according to t test (P=0.05).
[2] Values are the average of 50 replicates per treatment.
[3] Values are the average of nine replicates per treatment.

Eucalyptus spp. formed by *Hysterangium* spp. have previously been described by Warcup (1980) and Malajczuk et al. (1987). The former author demonstrated plant growth stimulation by this type of mycorrhizas. In this sense, *H. gardneri* and *S. cothurnatus* superficial colonizations have been considered as ectomycorrhizas as well.

Data on number of mycorrhizal root tips show that *Pisolithus* sp. and *H. gardneri* were more infective towards *E. dunnii* roots than *S. cothurnatus* and *R. nigrescens*, suggesting that the isolates from *Eucalyptus* spp. are more compatible in relation to this plant than those from *Pinus* spp. Species of *Suillus* and *Rhizopogon* spp. are well known by associating specifically with certain plant genera mainly of conifers (Garbaye, 1990).

Plants inoculated with *S. cothurnatus* presented a higher accumulation of phenolics in roots, with an average of 12.3 μg/mg of fresh weight (Table 1). Those inoculated with *H. gardneri* did not differ from controls, with an average of 11.1 and 10.2 μg/mg of fresh weight, in this order. Conversely, roots inoculated with *Pisolithus* sp. and *R. nigrescens* presented a lower level of these substances, 9.1 and 8.8 μg/mg of fresh weight, respectively.

Métraux (1994) related phenolic accumulation in plants to defence mechanisms against pathogenes. Malajczuk et al. (1982, 1984) related this phenomenon also to plant reaction to incompatible ECM fungi. In this study, *S. cothurnatus* induced a significant accumulation of phenolics in roots which coincided with a lower infectivity compared to *Pisolithus* sp. and *H. gardneri*. Roots inoculated with *H. gardneri* did not significantly increase the production of these substances, whereas those inoculated with *Pisolithus* sp. and *R. nigrescens* presented a lower level of phenolics than uninoculated roots. These observations indicate that only *S. cothurnatus*, isolated from *Pinus* sp., stimulated a hypersensitive reaction on *E. dunnii* roots. Conversely, *R. nigrescens*, also from *Pinus*, did not induce this reaction, but was unable to colonize roots. The incompatibility between this fungus and *E. dunnii* must be related to other mechanisms, unless the total absence of infection had prevented accumulation of phenolics.

The results suggest that the specific occurrence of *Pisolithus* sp. and *H. gardneri* and the absence of *S. cothurnatus* and *R. nigrescens* in *Eucalyptus* plantations could be related to their compatibility/incompatibility towards these plants. Nevertheless, these results refer only to four isolates hence the need for more studies in order to establish a full explanation for the fungus-host specificity observed in Southern Brazil plantations.

REFERENCES

BURGESS, T.; LAURENT, P.; DELL, B.; MALAJCZUK, N.; MARTIN, F. Effect of fungal-isolate aggressivity on the biosynthesis of symbiosis-related polypeptides in differentiating eucalypt ectomycorrhizas. **Planta**, Berlin, v.195, p.408-417, 1995.

GARBAYE, J. Utilisation des mycorhizes en sylviculture. In: STRULLU, D.G. (Ed.). **Les mycorhizes des arbres et plantes cultivées**. Paris : Lavoisier, 1990. p.197-250.

KORMANIK, P.P.; McGRAW, A.C. Quantification of vesicular-arbuscular mycorrhizae in plant roots. In*:* SCHENCK, N.C. (Ed.). **Methods and principles of mycorrhizal research**. St. Paul : American Phytopathological Society, 1982. p.37-45.

MALAJCZUK, N.; DELL, B.; BOUGHER, N.L. Ectomycorrhiza formation in *Eucalyptus*. III. Superficial ectomycorrhizas initiated by *Hysterangium* and *Cortinarius* species. **New Phytologist**, Cambridge, Great Britain, v.105, p.421-428, 1987.

MALAJCZUK, N.; MOLINA, R.; TRAPPE, J.M. Ectomycorrhiza formation in *Eucalyptus*. I. Pure culture synthesis, host specificity and mycorrhizal compatibility with *Pinus radiata*. **New Phytologist**, Cambridge, Great Britain, v.91, p.467-482, 1982.

MALAJCZUK, N.; MOLINA, R.; TRAPPE, J.M. Ectomycorrhiza formation in *Eucalyptus*. II. The ultrastructure of compatible and incompatible mycorrhizal fungi and associated roots. **New Phytologist**, Cambridge, Great Britain, v.96, p.43-53, 1984.

MARX, D.H. The influence of ectotrophic mycorrhizal fungi on resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. **Phytopathology**, St. Paul, v.59, p.153-163, 1969.

MÉTRAUX, J.P. Plants. In*:* TURNER, R.J. (Ed.). **Immunology**: a comparative approach. Chichester : J. Wiley, 1994. p.1-27.

MOLINA, R.; MASSICOTTE, H.; TRAPPE, J.M. Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: ALLEN, M.F. (Ed.). **Mycorrhizal functioning**: an integrative plant-fungal process. London : Chapman & Hall, 1992. p.357-423.

MOLINA, R.; TRAPPE, J.M. Patterns of ectomycorrhizal host specificity and potential among pacific northwest conifers and fungi. **Forest Science**, Bethesda, v.28, p.423-458, 1982.

MÜNZENBERGER, B.; HEILEMANN, J.; STRACK, D.; KOTKE, I.; OBERWINKLER, F. Phenolics of mycorrhizal and non-mycorrhizal roots of Norway spruce. **Planta**, Berlin, v.182, p.142-148, 1990.

MÜNZENBERGER, B.; KOTKE, I.; OBERWINKLER, F. Reduction of phenolics in mycorrhizas of forest trees. In: AZCON-AGUILAR, C.; BAREA, J.M. (Ed.). **Mycorrhizas in integrated systems**: from genes to plant development. Brussels : European Commission, 1996. p.248-251.

MÜNZENBERGER, B.; KOTKE, I.; OBERWINKLER, F. Reduction of phenolics in mycorrhizas of *Larix decidua* Mill. **Tree Physiology**, Victoria, v.15, p.191-196, 1995.

MÜNZENBERGER, B.; OTTER, T.; WÜSTRICH, D.; POLLE, A. Peroxidase and laccase activities in mycorrhizal and non-mycorrhizal fine roots of Norway spruce (*Picea abies*) and larch (*Larix decidua*). **Canadian Journal of Botany**, Ottawa, v.78, p.932-938, 1997.

OLIVEIRA, V.L.; SCHMIDT, V.D.B.; GOMES, N.C.; MAIA, D.C. Spécificité de champignons ectomycorhiziens vis-à-vis d'*Eucalyptus viminalis* Labill et *E. dunnii* Maiden. **Agronomie**, Paris, v.14, p.57-62, 1994.

PHILLIPS, R.; HENSHAW, G.G. The regulation of synthesis of phenolics in stationary phase cell cultures of *Acer pseudoplatanus* L. **Journal of Experimental Botany**, Oxford, v.35, p.108-114, 1977.

SMITH, S.E.; READ, D.J. **Mycorrhizal symbiosis**. London : Academic, 1997. 605p.

SWAIN, T.; HILLS, W.E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. **Journal of the Science of Food and Agriculture**, Chichester, v.10, p.63-68, 1959.

WARCUP, J.H. Ectomycorrhizal association of Australian indigenous plants. **New Phytologist**, Cambridge, Great Britain, v.81, p.531-535, 1980.