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Original article

Ectomycorrhization of *Acacia holosericea* A. Cunn. ex G. Don by *Pisolithus* spp. in Senegal: Effect on plant growth and on the root-knot nematode *Meloidogyne javanica*

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Summary – The ectomycorrhization of *Acacia holosericea* with fungi isolated in Senegal (belonging to the *Pisolithus* genus) and their effect on *Meloidogyne javanica* have been studied. In 100 tree plantations of *Eucalyptus camaldulensis, Casuarina equisetifolia, Acacia mangium* and *A. holosericea,* 33 fruiting bodies of the ectomycorrhizal fungus *Pisolithus* were collected and cultured under axenic conditions. Only four fungal isolates have induced the formation of typical ectomycorrhizae with *A. holosericea* under axenic conditions. One of these, COI 024, increased plant development under glasshouse conditions and decreased the multiplication of the root-knot nematode *Meloidogyne javanica.* The mechanisms involved in these interactions and the potential use of the ectomycorrhizal symbiosis in agroforestry programs are being discussed.

***Pisolithus* / *Meloidogyne* / ectomycorrhizae / leguminous trees / Senegal**

Résumé – Ectomycorhization de *Acacia holosericea* A. Cunn. ex G. Don par *Pisolithus* spp. au Sénégal : effet sur le développement de la plante et sur la multiplication du nématode à galles *Meloidogyne javanica.* L'effet de symbiotes fongiques ectomycorhiziens, appartenant au genre *Pisolithus* et isolés au Sénégal, a été étudié sur la croissance de plants d'*Acacia holosericea* et sur le développement d'un peuplement de nématodes phytoparasites *Meloidogyne javanica.* Une enquête a été réalisée sur 100 arbres choisis dans différentes plantations de *Eucalyptus camaldulensis, Casuarina equisetifolia, Acacia mangium* et *A. holosericea* où trente trois carpophores de *Pisolithus* spp. ont été prélevés. Le mycélium issu de chaque carpophore a été cultivé en conditions axéniques et chaque souche a été testée pour sa compatibilité avec des plants d'*A. holosericea.* Seulement 4 isolats ont induit la formation d'ectomycorhizes en conditions axéniques. Dans une expérience conduite en serre avec *A. holosericea,* une souche, COI 024, a stimulé la croissance de la plante hôte et inhibé la multiplication de *Meloidogyne javanica.* Les mécanismes susceptibles d'être à l'origine de ces phénomènes sont discutés et les effets bénéfiques potentiels du recours à la mycorhization contrôlée dans les programmes agroforestiers sont exposés.

***Pisolithus* / *Meloidogyne* / ectomycorhize / legumineuse / Sénégal**

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1. INTRODUCTION

The population explosion in sahelian areas of West Africa has led to a restructuring of traditional agricultural systems using more intensive but unsustainable systems over-exploiting the natural resources. The decrease in soil fertility in Sub-Saharan Africa, particulary with deficiencies of N and P, remains the most significant result of this practice.

It is well known that trees can potentially improve soil characteristics through a number of processes, such as nitrogen fixation, maintenance of soil organic matter, etc. These influences are well studied in agroforestry systems, such as alley cropping [20] or parklands [3].

Nitrogen-fixing legumes and leguminous tree species, such as *Acacia,* play a major role in environmental protection and in the local economies of dry and semi-arid tropical Africa [8, 9, 17]. The *Acacia* species remain very abundant in savannas and arid regions of Australia, Africa, India and the Americas. They generally are dependent on mycorrhizae for the absorption of nutrients required for their growth and for nitrogen fixation [4, 6, 7, 18]. One of the fast-growing leguminous trees, brought from Australia and introduced in Western Africa, *Acacia holosericea,* has been studied in Senegal. This species forms endomycorrhizae and/or ectomycorhizae [1, 7, 10]. This tree species and its bacterial or fungal symbionts appears to be well adapted to the Senegal climatic conditions [7]. However, it has recently been shown that *A. holosericea* was very susceptible to root-knot nematodes (*Meloidogyne* spp.) [13] which are major pests of vegetable crops in tropical areas. Therefore, *Acacia* species may increase the occurrence and abundance of root knot nematodes in agroforestry systems and decrease the potential benefits (for plantation trees and adjacent crops) of this agronomical practice.

As it is known that mycorrhizal fungi can reduce the effects of root-knot nematodes [12], the present study was initiated to investigate the association of *A. holosericea* with ectomycorrhizal fungi with emphasis on the effect of this fungal symbiosis on the plant development, and the relationships between the root-knot nematode *M. javanica* (Treub) Chitwood and an ectomycorrhizal fungus (*Pisolithus* sp.) on *A. holosericea.*

2. MATERIALS AND METHODS

2.1. Isolating fungi into axenic conditions

The occurrence of fruit bodies of the ectomycorrhizal fungus *Pisolithus* was investigated from 100 tree plantations of *Eucalyptus camaldulensis, Casuarina equisetifolia, A. mangium* and *A. holosericea.* Sporocarps were detected in thirty three of these tree plantations. Sporocarps were brushed free of adhering soil and fractured carefully in a laminar flow hood. A small amount of tissue was then removed with a fine forceps and placed on MNM agar medium in a Petri dish (Melin and Norkrans modified by Marx) [21]. The fungal cultures were incubated at 25 °C in the dark and subcultured until all contaminating microorganisms were eliminated. Pure fungal cultures were initiated with 21 fruiting bodies.

2.2. Preliminary compatibility testing

2.2.1. Axenic culture of A. holosericea *seedlings*

The compatibility between the fungal isolates and *A. holosericea* was tested using the paper-sandwich technique [5]. Seeds of *A. holosericea* were surface – sterilized with concentrated sulphuric acid (36 N) for 60 min. The acid solution was then decanted off and the seeds rinsed and imbibed for 12 h in four rinses of sterile distilled water. Seeds were then transferred aseptically in Petri dishes filled with 1% (w:v) water agar medium. These plates were incubated for 1 week at 25 °C. When the radicles had grown to 1 cm, the seedlings were transferred into large (15-cm-diameter) plastic Petri dishes. These plates were filled with a mineral salt agar medium [11, 24]. Its composition per litre was as follows: $MgSO_4$, $7H_2O$: 150 mg; $(NH_4)_2HPO_4$: 125 mg; $CaCl_2.2H_2O$: 50 mg; KCl: 108 mg; agar: 20 g; distilled water: 1 litre. The micronutrients (Fe, Mo, B, Mn, Cu and Zn) were added together as 0.1 ml of a concentrated commercial solution: Kanieltra (COFAZ, BP 198-08, 75261 Paris Cedex, France). The surface of the agar medium was covered with an autoclaved Whatman (120 °C, 20 min) No. 1 filter paper (125-mm-diameter). Young seedlings (three per plate) were then placed on the filter paper near the edge of the plate. Another sterile paper filter, cut across the top, was laid over the radicule but not the colyledons. The plates were sealed with plastic adhesive tape and placed upside down at a 45° angle in a climate-controlled growth chamber with a constant 16 h photoperiod with 240 $\mu E.m^{-2}.s^{-1}$ at 25 °C.

2.2.2. Preparation of fungal inoculum and axenic mycorrhizal synthesis

The fungal isolate was maintained on MNM agar medium. After one month culturing at 25 °C in the dark, about five fungal plugs, taken from the margin of the colonies, were placed on an autoclaved square (6 × 6 cm) of paper card laid over the MNM agar medium in 9-cm-diameter Petri dishes. These plates were then incubated at

25 °C in the dark in order to achieve a good coverage of the paper card.

The filter paper covering the roots was removed and the mycelium colonizing the paper card was placed on the root. Subsequently the plates were incubated under the same conditions as described above. There were three replicates per fungal treatment. After 2 weeks culturing, the Petri dishes were opened and the root systems were observed under a stereomicroscope (magnification ×120) to confirm the presence of ectomycorrhizas.

2.2.3. Preparation of root samples for microscopy observations

Ectomycorrhizae were confirmed by a microscopic study to check formation of a Hartig net between epidermal cells. For each treatment, 10 lateral roots were fixed overnight in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C and rinsed in the same buffer. The roots were post-fixed for 1 h in 2% osmium tetroxide and rinsed in distilled water. They were dehydrated through an acetone series and three times in pure acetone. They were then infiltrated by an acetone-Spurr's resin series and embedded in 100% Spurr's resin. Transverse sections (0.5 – 1 µm thick) were cut from embedded samples and stained with 0.05% toluidine blue in 1% sodium tetraborate.

2.3. Effects of the fungal isolates on plant growth and on *M. javanica* development

2.3.1. Fungal inoculum

The ectomycorrhizal fungus *Pisolithus* sp., isolate COI 024, was maintained on MNM agar medium [21]. This fungal strain was choosen because of its high growth rate in axenic conditions. The solid inoculum was prepared in 1.6 liter glass jars containing 1.3 liter vermiculite-peat mixture (4:1, v:v) moistened with liquid MNM medium. This substrate was inoculated with fungal plugs taken from the margin of the fungal colonies. The jars were then sealed and incubated for 6 weeks at 28 °C in the dark.

2.3.2. Glasshouse experiment

Seeds of *A. holosericea* were from provenance Bel Air (Dakar, Senegal) and desinfected as described before (see preliminary compatibility testing). The germinated seeds were individually grown in 0.5 dm^3 polythene bags (5-cm-diameter) filled with autoclaved soil (140 °C, 40 min). The physicochemical characteristics of the soil were as follows: pH H_2O 6.5; clay 3.5%; fine silt 7.4%; coarse silt 25.4%; fine sand 36.6%; coarse sand 21.5%; total carbon 0.54%; total nitrogen 0.06% and Olsen phosphorus 0.88%. This soil was mixed with 10% (v:v) fungal inoculum or 10% vermiculite-peat mixture (4:1, v:v) for the control treatments without the fungus. The seedlings were placed in a glasshouse during the hot season (35 °C day, 30 °C night, 12 h photoperiod) and watered twice a week without fertiliser. The pots were placed in a randomized, complete block design with ten replicates per treatment.

After one month culture, the seedlings were inoculated with 5 ml suspensions of 0, 300 and 700 second stage juveniles (J2) of *M. javanica.* In a preliminary trial, it has been demonstrated that these 2 inoculum rates could reduce the growth of A. holosericea seedlings [13]. The inoculum of *M. javanica* was multiplied on tomato (*Lycopersicon esculentum* Mill.) cv. Roma. After 2 month culturing, the tomato roots were harvested, cut into short lengths and placed in a mist chamber for 1 week to enable the nematode eggs to hatch [22].

Two months after nematode inoculation, when the damage associated with the different inoculum densities was observed, the plants were uprooted. The oven dried (1 week at 65 °C) weight of the shoot was measured. After drying, plant tissues were ashed (500 °C), digested in 2 ml HCL 6N and 10 ml HNO_3 N, then analysed by colorimetry for P [19], by flame emission for Na, K and Ca and by atomic absorption for Cu, Mg, Fe. Plant tissues were digested in 15 ml H_2SO_4 36 N containing 50 $g.l^{-1}$ salicylic acid for N (Kjeldhal) determination. The root systems were washed, cut into short pieces, mixed and the percentage of ectomycorrhizal short roots (ectomycorrhizal rate: (number of ectomycorrhizal short roots/total number of short roots) × 100) was determined under a stereomicroscope (magnification: × 40) on a random sample of at least 100 short roots. Root pieces were then placed in a mist chamber for 2 weeks to recover hatched juveniles [23]. The roots were oven dried (65 °C, 1 week) and weighed. The data were analyzed with a one-way analysis of variance. Mean values were compared using Student's t-test ($P < 0.05$). Nematode numbers were $\log_{10}(x + 1)$ transformed before statistical analysis. For mycorrhizal rate, data were previously transformed by Arcsinsqrt (x).

3. RESULTS

Most of the fruiting bodies were found in the *E. camaldulensis* plantations whereas one was associated with *A. mangium,* one with *Casuarina equisetifolia* and two with *A. holosericea.* Compatibility tests performed under axenic conditions between *A. holosericea* and different

Table I. Effect of *Meloidogyne javanica* inoculum density and inoculation with Pisolithus sp. COI 024 on growth and ectomycorrhizal colonization of *Acacia holosericea.*

Mycorrhizal treatments	Number of inoculated nematodes	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Mycorrhizal rate (%)
Without COI 024	0 (control)	1095 c [1]	897 b	0
	300	667 d	694 b	0
	700	586 d	926 b	0
With COI 024	0	2646 a	1060 b	42.3 a
	300	1501 b	1945 a	45.3 a
	700	1346 bc	1741 a	39.8 a

[1] : Values (means of ten replicates) in the same column followed by the same letter are not significantly different according to the Student "t" test ($P < 0.05$).

Table II. Effect of *Meloidogyne javanica* inoculum density on the number of nematodes per plant and the leaf mineral concentrations.

Mycorrhizal treatments	Number of nematodes inoculated	Number of nematodes per plant	Mineral concentrations (% of dry matter)							
			N	P	K	Ca	Na	Cu	Mg	Fe
Without COI 024	0 (control)	0	ND [2]	0.027 b	0.694 b	0.896 c	0.076 b	0.001 a	0.307 c	0.022 b
	300	4185 a [1]	ND	0.021 b	0.526 b	1.012 bc	0.124 a	0.0009 a	0.278 c	0.024 b
	700	3905 a	ND	0.034 b	0.641 b	1.386 ab	0.111 a	0.0009 a	0.363 bc	0.027 ab
With COI 024	0	0	1.82 a	0.056 ab	1.078 a	1.022 bc	0.049 b	0.001 a	0.454 ab	0.026 b
	300	1384 b	1.55 a	0.087 a	1.130 a	1.461 a	0.069 b	0.001 a	0.515 a	0.029 ab
	700	1088 b	2.01 a	0.062 ab	1.415 a	0.893 c	0.059 b	0.001 a	0.420 ab	0.039 a

[1] : Values (mean of ten replicates) in the same column followed by the same letter are not significantly different according to the Student "t" test ($P < 0.05$). (2) ND: not determined.

fungal isolates showed that only 4 *Pisolithus* isolates (COI 007, COI 024, COI 029 and COI 032) could induce the formation of ectomycorrhizae with a yellow mantle and a Hartig net.

In the glasshouse experiment, shoot growth was significantly increased ($P < 0.05$) by the fungal isolate COI 024 inoculated alone (*table I*). No fungal effects were recorded on the root biomass (*table I*). In the absence of the nematodes, mycorrhizal symbiosis increased the leaf mineral K and Mg concentrations (*table II*).

All *M. javanica* inoculum densities had significantly reduced shoot growth regardless of ectomycorrhizal fungus treatment (*table I*). Root growth of the seedlings without *Pisolithus* was not affected by the nematode (*table I*). In contrast, root biomass of the mycorrhizal plants was significantly higher ($P < 0,05$) in plants inoculated with nematodes (*table I*). Element concentrations in leaves of seedlings parasitized by *M. javanica* were not significantly different from the uninoculated controls (without nematodes), except for Ca which was increased with the inoculum 700 and 300 J2 in the treatments without and with COI 024, respectively and for Na which was increased in the treatments with nematodes but without COI 024 (*table II*).

The ectomycorrhizal symbiosis reflected by the mycorrhizal rate was not influenced by the root-knot nematodes. In contrast, nematode reproduction was significantly decreased by the ectomycorrhizal fungus (*table I*).

4. DISCUSSION

One of the *Pisolithus* isolates, COI 024 dramatically promoted plant growth (+ 142% in shoot biomass). It is well known that ectomycorrhizal fungi improve plant productivity in low fertility soils producing better mineral nutrient concentrations [2]. This biological effect was demonstrated for other Australian acacia species such as

A. mangium [16]. The mean P concentration in leaves of *Acacia* mycorrhized with COI 024 and infected or not by *M. javanica* is about 2.5 fold higher than in the control plants with or without *M. javanica* which could suggest that this provenance of *A. holosericea* is ectomycorrhizal-dependent.

The benefical effects of COI 024 on plant growth have clearly been demonstrated throughout this experiment. However, this type of symbiosis can be involved in other beneficial processes, such as a protecting effect against soil borne pathogens [20].

In West Africa, several investigations have pointed out that the root-knot nematode *M. javanica* may affect the benefits resulting from growing *A. holosericea* [13–15], especially in agroforestry systems, to the detriment of adjacent susceptible crops. Our results indicate that the ectomycorrhizal symbiosis with COI 024 suppresses the development of this nematode and minimizes its pathogenic effect. It is well documented that endomycorrhizal fungi could inhibit the development of root-knot nematodes [12] but no data was available so far for the antagonistic effect of ectomycorrhizae against *M. javanica.* The mechanism of this antagonism remains unknown. Two types of fungal activity could explain this effect: (i) physical and (ii) chemical. In the first case, the fungal mantle which forms around the short roots could act as a mechanical barrier preventing the penetration of the juvenile nematodes. In the second, some ectomycorrhizal fungi, such as *Pisolithus* produce large quantities of polyphenolic compounds which could decrease the viability of eggs and juveniles. This second aspect has been studied by Senghor [23] with 32 isolates of *Pisolithus* collected from Australia and West Africa. Results indicate that most of the fungal strains (30 isolates) inhibited the eggs. The identification of these toxic compounds is currently being undertaken.

In conclusion, this paper reports for the first time in West Africa, a positive effect of the ectomycorrhizal symbiosis with one strain of *Pisolithus* sp. COI 024 on *A. holosericea.* The use of the fungal strain COI 024, isolated in Senegal and therefore well adapted to climatic conditions in Senegal, competitive against the indigenous microflora, could contribute to the growth of *A. holosericea* seedlings under nursery conditions. However, further investigations should be made to measure this fungal effect under field conditions and to optimize this ectomycorrhizal symbiosis (screening of efficient fungal strains; investigating mycorrhizal-dependence of different provenances of *A. holosericea,* dual inoculation with efficient strains of rhizobia).

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