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Original article

Influence of the dual arbuscular endomycorrhizal / ectomycorrhizal symbiosis on the growth of *Acacia holosericea* (A. Cunn. ex G. Don) in glasshouse conditions

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Abstract – *Acacia holosericea* plants were inoculated with a strain of *Glomus aggregatum* IR27 (arbuscular mycorrhizal fungus), *Pisolithus tinctorius* COI024 (ectomycorrhizal fungus) or with both fungi. Each fungus inoculated alone stimulated plant growth (height and shoot biomass). The response to the dual inoculation was greater than the response to either inoculant one. It may be due to the fact that the co-inoculated plants formed nodules through contaminations. However these nodules are inefficient as the N concentrations were similar in leaves of all inoculated plants with mycorrhizal fungi, alone and together. In thus, P, Ca, K, Mg and Na concentrations were not improved with respect to dual inoculation. The ectomycorrhizal colonization was significantly higher in the dually inoculated treatment than in either of the singly inoculated treatments.

acacia / arbuscular mycorrhizas / ectomycorrhizas / dual inoculation

Résumé – Influence de la double symbiose endomycorhiziennne et ectomycorhiziennne sur la croissance de *Acacia holosericea* (A. Cunn. ex G. Don.) en conditions de serre. Des plants de *Acacia holosericea* ont été inoculés soit avec une souche de *Glomus aggregatum* IR27 (champignon mycorhizien à arbuscules), soit avec *Pisolithus tinctorius* COI024 (champignon ectomycorhizien) ou avec les deux symbiotes fongiques. Chaque champignon a stimulé la croissance de la plante hôte (hauteur et biomasse aérienne). La double inoculation a induit une augmentation du développement de la plante supérieure à celle enregistrée lorsque les champignons étaient inoculés séparément. Ceci peut être la conséquence de la formation de nodules dus à des souches de *Rhizobia* contaminatrices. Toutefois, ces bactéries restent peu efficientes puisque les concentrations en azote dans les feuilles sont similaires dans les traitements avec chaque champignon ou lorsque ces isolats fongiques sont co-inoculés. Les concentrations en P, Ca, K, Mg et Na n'ont pas été modifiées par la co-inoculation. La colonisation racinaire par *P. tinctorius* COI024 a été significativement améliorée lorsque ce dernier a été inoculé avec le champignon mycorhizien à arbuscules.

acacia / mycorhizes à arbuscules / ectomycorhizes / double inoculation

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1. INTRODUCTION

Acacia is the largest mimosoid genus which is represented with 800–900 species. They are abundant in savannas and arid regions of Australia, Africa, India and the Americas. They can grow in nitrogen–deficient soils because of their symbiosis with nitrogen fixing bacteria. As with many N_2-fixing trees and shrubs, *Acacia* is very dependent on mycorrhizas to absorb nutrients required for plant growth and efficient N_2 fixation [6]. Depending on the fungal groups and the *Acacia* species, two morphological types of mycorrhizas can be distinguished, namely arbuscular mycorrhizas (AM) and ectomycorrhizas (EM) [19]. Generally, the former AM seem to be predominant in *Acacia* [1, 7]. The African *Acacia* form mycorrhizal associations only with AM fungi [5] but, as with other introduced tree genera in West Africa like *Casuarina* and *Eucalyptus*, some Australian *Acacia* are known to be associated with either ectomycorrhizal and/or endomycorrhizal fungi [19, 8]. For instance, *A. holosericea* can form symbiotic relationships with AM fungi [6, 1] and also with EM fungi [2, 10]. This dual fungal association has been described within the same root system of *A. holosericea* under natural conditions in Senegal by Ducousso (1990) [8]. However, the symbiotic effectiveness of dual endomycorrhizal / ectomycorrhizal inoculation has never been assessed under experimental conditions for Australian *Acacia*. The purpose of this study was to evaluate the functional compatibility of a dual inoculation with *A. holosericea* and two mycorrhizal fungi, using the ectomycorrhizal fungus *Pisolithus tinctorius* and the arbuscular mycorrhizal fungus *Glomus aggregatum* growing in a soil collected in Senegal.

2. MATERIALS AND METHODS

2.1. Preparation of fungal inoculum

A strain of *Pisolithus albus* COI 024 (Martin, personal communication) was isolated from a sporocarp collected in a monospecific forest plantation of *A. holosericea* in southern Senegal during the rainy season. This fungal isolate, probably introduced from Australia (Martin, personal communication), was previously tested for its compatibility with *A. holosericea* in a pot experiment [10]. The fungal strain was maintained in Petri dishes over MMN agar medium at 25 °C [22]. The fungal inoculum was prepared according to Duponnois and Garbaye [9]. Briefly, one liter glass jars were filled with 600 mL of a mixture of vermiculite and peat moss (4:1, v:v) and autoclaved (120 °C, 20 min). The substrate was then moistened to field capacity with 300 ml liquid MMN medium, the jars sealed and autoclaved at 120 °C for 20 min. After cooling, the substrate was inoculated with 10 fungal plugs taken from the margin of fungal colonies. The glass jars were placed at 25 °C in the dark for 2 months.

The arbuscular mycorrhizal fungus *G. aggregatum* (isolate IR 27) was isolated in Burkina Faso by Bâ et al. (1996) [1]. It was propagated on millet (*Penisetum typhoïdes* cv. IKMV 8201) for 12 weeks in a glasshouse on an autoclaved sandy soil (140 °C, 40 min). Before inoculation, the millet plants were uprooted, gently washed with tap water and cut into segments 0.5 cm long. The roots were not surface-disinfected. Non-mycorrhizal millet roots, prepared as above, were used for the treatments without endomycorrhizal inoculation.

2.2. Inoculation and plant culture

The experiment was performed with soil collected in a fallow area at Nioro du Rip (center of Senegal). After sampling, the soil was crushed, passed through a 2-mm sieve and autoclaved for 40 min at 140 °C to eliminate the indigenous microflora. The physical and chemical characteristics of the autoclaved soil were as follow: clay 8.7%; fine loam 6.5%; coarse loam 17.6%; fine sand 40.8%; coarse sand 25.6%; Total C 4.4%; Total nitrogen 0.39%; C/N 11.3; Total P 54.7 mg kg^{-1}; pH (H_2O) 5.8.

Seeds of *A. holosericea* (provenance Bel Air, Dakar) were surface sterilized in 95% sulphuric acid for 60 min, rinsed with sterilized distilled water and germinated on 1% agar at 25 °C in the dark. The 0.5 dm^3 pots were filled with the autoclaved soil. One hole (1 cm by 5 cm) was made in each pot, filled with 1 g fresh Millet root (mycorrhizal or not) and/or 2 cm^3 of the ectomycorrhizal inoculum (or the vermiculite – peat mixture (4:1; v:v) moistened with liquid MMN medium but without fungus for the treatments without *P. tinctorius* COI 024). The holes were then covered with the same autoclaved soil. The 4 treatments were realized as: (1) non-inoculated plants, (2) *G. aggregatum* IR 27 alone, (3) *P. tinctorius* COI 024 alone and (4) dual inoculation *G. aggregatum* + *P. tinctorius*. Each inoculation treatment was sown with one pre-germinated seed per pot. The plants were arranged in a randomized, complete block design with 10 replicates per treatment. They were placed in a

glasshouse during the hot season under natural light (daylight approximatively 12 h, mean temperature 30 °C day) and watered twice weekly without fertiliser during 6 months of growth.

2.3. Quantitative evaluation

The height of each plant was measured. The *A. holosericea* plants were uprooted and the root systems gently washed with tap water. Then the root systems were cut into short pieces, mixed and the ectomycorrhizal colonization (number of ectomycorrhizal short roots / total number of short roots × 100) was determined under a stereomicroscope at 160 × magnification on a random sample of at least 100 short roots. Other root samples were randomly collected along the root system to quantify the internal colonization of arbuscular mycorrhizal fungi in the roots. The roots were cleared and stained according to the method of Phillips and Hayman (1970) [23]. The extent of colonization was estimated in terms of fraction of root length with visible mycorrhizal structures (length of root fragments colonized / total length of root fragments × 100). The roots were cut into approximately 1-cm pieces and placed on a slide for microscopic observation at 250 × magnification [3]. About one hundred 1-cm-root pieces were observed per plant.

Although the soil was autoclaved and the seeds surface disinfected, some plants were contaminated with indigenous rhizobia. The main explanation of this contamination was that the irrigation water possibly contained N_2-fixing bacteria. Root nodules were counted and their dry weights (60 °C, 1 week) were determined.

The dry weight of shoots and roots was measured (60 °C, 1 week). After drying, a subsample of ground shoot tissues were ashed (500 °C), digested in 2 mL HCl 6 M and 10 mL HNO_3 1 M, then analysed by colorimetry for P [17], by flame emission for Na, K and by atomic absorption spectroscopy for Mg. Plant tissues were digested in 15 mL H_2SO_4 18 N containing 50 g L^{-1} salicylic acid for N (Kjeldhal) determination.

Mycorrhizal dependency was determined as follow [24]:

((shoot biomass of ectomycorrhizal plants – shoot biomass of the non ectomycorrhizal plants) × 100) / (shoot biomass of ectomycorrhizal plants).

2.4. Statistical analysis

All data were subjected to a one-way analysis of variance using the Super Anova Computer program and means were compared with the Newman-Keuls multiple range test ($P = 0.05$). For the mycorrhizal rate, the data were transformed by $\arcsin(\sqrt{x})$ before statistical analysis.

3. RESULTS

The height and shoot dry weight of the plants inoculated with *G. aggregatum* IR 27 or *P. tinctorius* COI 024 were significantly higher than in the control (*table I*). Compared with the control, growth of *G. aggregatum* IR 27 plants, was stimulated by 1.71 × and 3.02 × for height and shoot dry weight, respectively, whereas it was

Table I. Influence of the fungal treatments on the growth of *A. holosericea* and on the nitrogen fixative symbiosis after 6 months of culture.

Treatments	Height (cm)	Shoot dry weight (mg/plant)	Root dry weight (mg/plant)	Number of nodules per plant	Nodule dry weight (mg/plant)
Not inoculated	10.9 a [(1)]	800 a	333 a	0 a	0 a
G. aggregatum	29.6 b	3217 b	750 a	0 a	0 a
P. tinctorius COI 024	29.2 b	3217 b	1050 a	0 a	0 a
G. aggregatum + *P. tinctorius* COI 024	46.7 c	4557 c	3143 b	4.3 b	34.4 b

[(1)] For each parameter, data in the same column followed by the same letter are not significantly different according to the Newman and Keuls test ($P < 0.05$).

1.68 × and 3.02 ×, respectively, for plants inoculated with *P. tinctorius* COI 024. There were no significant difference between the fungal treatments. Root biomass of mycorrhizal treatments were not significantly different from the control (*table I*). When the two fungi were co-inoculated, height and shoot dry weight were significantly increased over the single inoculation treatments (*table I*). The percentages of growth stimulation calculated from the means of the fungal treatments (*G. aggregatum* IR 27 alone or *P. tinctorius* COI024 alone) were 0.57 × for the plant height, 0.42 × and 2.5 × for the shoot and root dry weight, respectively (*table I*).

No nodules were observed in the control or in the *G. aggregatum* IR 27 or *P. tinctorius* COI 024 treatments. On the contrary, the formation of nodules was recorded with 85% of plants inoculated with both fungi (*table I*).

The dual fungal inoculation significantly increased the establishment of the ectomycorrhizal symbiosis as compared with the plants infected by the ectomycorrhizal strain only (*table II*). No significant differences were recorded for the endomycorrhizal symbiosis (*table II*).

The nitrogen concentrations in leaves of *A. holosericea* was significantly lower in the fungal treatments than in the control (*table III*). On the contrary, the total nitrogen content in the aerial parts of the plants in the endomycorrhizal and/or ectomycorrhizal treatments were significantly higher than in the control (20.2 mg per control plant; 55.9 mg per endomycorrhizal plant; 63.0 mg per ectomycorrhizal plants and 78.4 mg per co-inoculated plant). This is presumably a consequence of increased plant growth diluting plant N concentrations. On the contrary, the K concentrations were significantly higher in the leaves of the mycorrhizal plants (*table III*). Compared with the control, no significant differences were recorded for the P and Mg contents of the inoculated plants (*table III*). The Ca and Na concentrations were significantly lower in the *P. tinctorius* COI 024 treatment than in the control and *G. aggregatum* IR 27 treatments (*table III*). The type of fungal symbiosis influenced the mineral contents of the leaves differently. The concentrations of P, Ca, Mg and Na were significantly

Table II. Mycorrhizal establishment on the root systems of *A. holosericea* after 6 months of growth.

Treatment	Ectomycorrhizal colonization (%)	Mycorrhizal dependency (%)	Endomycorrhizal colonization (%)
Not inoculated	0 a [(1)]	0 a	0 a
G. aggregatum	0 a	73.4 b	41.7 b
P. tinctorius COI 024	54.2 b	70.8 b	0 a
G. aggregatum + *P. tinctorius* COI 024	83.2 c	94.4 c	49.4 b

[(1)] For each parameter, data in the same column followed by the same letter are not significantly different according to the Newman and Keuls test ($P < 0.05$).

Table III. Effect of the fungal inoculation on the N, P, Ca, Mg, Na and K concentrations in leaves of *A. holosericea* after 6 months of growth.

Treatment	P (%)	Ca (%)	Mg (%)	Na (%)	K (%)	N (%)
Not inoculated	0.043 ab [(1)]	1.53 b	0.287 ab	0.123 b	0.49 a	2.52 b
G. aggregatum	0.070 b	1.55 b	0.330 b	0.097 b	0.90 c	1.74 a
P. tinctorius COI 024	0.030 a	1.27 a	0.273 a	0.053 a	0.83 bc	1.96 a
G. aggregatum + *P. tinctorius* COI 024	0.037 ab	1.38 ab	0.323 b	0.103 b	0.76 b	1.72 a

[(1)] For each parameter, data in the same column followed by the same letter are not significantly different according to the Newman and Keuls test ($P < 0.05$).

higher in the *G. aggregatum* IR 27 treatment than in the *P. tinctorius* COI024 treatment (*tableIII*). The percentage of ectomycorrhizal dependency responses were not different between the endo- and ectomycorrhizal plants but significantly enhanced when both fungi were inoculated (*tableII*).

4. DISCUSSION

Acacia holosericea is usually considered to be endomycorrhizal dependent [25, 27]. In fact, this symbiotic association was previously studied by Cornet and Diem [6] in Senegal and by Bâ et al. (1996) [1] in Burkina Faso. Cornet and Diem [6] found that the growth of *A. holosericea* plants was greatly stimulated by the arbuscular mycorrhizal fungus *Glomus mosseae* in a pot experiment and under field conditions. The efficiency of this symbiosis (expressed as growth promotion resulting from the fungal symbiosis) was also described with *G. fasciculatum* [26]. Ectomycorrhizal vs. endomycorrhizal fungi within the same root system of *A. holosericea* have been observed in Senegal [8]. The ectomycorrhizal fungus *Pisolithus* sp. was involved in this symbiosis as a fungal symbiont partner. Recently, a positive effect of this fungal isolate was demonstrated on *A. holosericea* plants growing in a pot experiment [10].

The measurements of the mycorrhizal rates suggests that both these fungal symbionts can coexist without any competition on the root system of *A. holosericea* seedlings. Moreover, ectomycorrhizal colonization was stimulated by dual inoculation. Similar observations were made on *Eucalyptus* spp. [18]. The dual ectomycorrhizal / endomycorrhizal symbiosis has also been studied with *Eucalyptus urophylla* and *E. globulus* with a sandy soil [4]. These authors have shown a significant interaction between ectomycorrhizal and endomycorrhizal inoculation and their effects on plant growth response. However, some results contradict the coexistence of both symbionts in the same root system. For instance, Lodge [21] observed that infection by AM fungi in the field was lowest where infection by ectomycorrhizal fungi was high, suggesting an antagonism among the fungal symbionts of *Populus* and *Salix*.

Furthermore, we found a better promoting effect on growth of *A. holosericea* seedlings of the dual inoculation with two different mycorrhizal fungi as compared with single inoculation. However we cannot attribute this stimulation only to the mycorrhizal symbiosis because of the presence of nodules on the ecto/endomycorrhizal seedlings. The ability of *A. holosericea* roots to form nodules with bacteria fixing atmospheric nitrogen has been already described [6]. The efficiency of the nitrogen fixation is dependent on mycorrhizal inoculation [6]. The main explanation is that the improvement of P uptake by the host plant resulting from endomycorrhizal symbiosis enhances nodulation and N_2 fixation [6]. Comparable observations have been reported for the dual effect of arbuscular mycorrhiza and *Rhizobium* with *Acacia* species such as *A. mangium*, *A. auriculiformis* and *A. falcataria* [7]. In our study, we collected a low number of nodules on roots of co-inoculated plants through contamination. We cannot explain the absence of nodules on these treatments. Usually, the rhizobial contaminations coming from the irrigation are observed in the control treatments not inoculated with selected microorganisms [12–14]. However, the plant growth response to the dual inoculation might not be a response to nodule formation. Although the ectomycorrhizae and endomycorrhizae can be detected after one month after fungal inoculations, we have not recorded any nodules during the first two months of culture which suggest that the effect of this bacterial symbiosis could have a lesser impact than the mycorrhizae on the plant nutrition. The nitrogen concentration in the shoot dry weight was lower in the ecto and/or endomycorrhizal plants but the total nitrogen content in the aerial parts was significantly higher in the mycorrhized plants. This positive effect of the mycorrhizal fungi has already been observed with the *Pisolithus* sp. / *A. mangium* symbiosis on the same soil [11]. The Ca, Mg, Na and K concentrations in leaves of *A. holosericea* were variable depending on mycorrhizal fungi involved alone or together. For example, the K concentrations in the leaves of inoculated plants with *G. aggregatum* alone were higher than that of co-inoculated plants. K plays a major role in plant water relations [16]. The lower susceptibility of potassium–sufficient plants to drought stress is related to several factors such as (i) the role of K in stomatal regulation as a mechanism controlling the water regime in higher plants and (ii) the importance of K for the osmotic potential in the vacuoles [16]. These physiological effects due to mycorrhizal symbiosis could be of a great interest to the development of *A. holosericea* in the drought sahelian areas. Surprisingly, P concentrations in leaves of *A. holosericea* seedlings were not improved by mycorrhizal inoculation. Nevertheless, the absorption of P is the major contribution of the mycorrhizal fungi for plant growth [15]. We hypothezize that non-nutritional effects of mycorrhizal fungi (e.g. protection against pathogens, water uptake) could play a major role rather than nutritional effects.

Further research must be undertaken to measure the ecological importance of this dual mycorrhizal symbiosis. Thus, studies must be done with Australian *Acacia* to determine how to manage the four-partner association plant/*Rhizobium*/arbuscular mycorrhizal fungus/ectomycorrhizal fungus for a selection of the convenient microbial combinations for plant growth.

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