In vitro mycorrhization of *Casuarina* and *Allocasuarina* species by *Pisolithus* isolates

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Five *Casuarina* species and five *Allocasuarina* species were inoculated *in vitro* with three isolates of *Pisolithus* sp. (Ors.X004 and Ors.7870 from Senegal, PR86 from Australia) to test their ability to form ectomycorrhizas. The mycorrhiza-forming ability varied between fungal isolates. The greatest differences occurred between *Casuarina* and *Allocasuarina* species. On *Casuarina* species, *Pisolithus* isolates formed only a fungal sheath. However, Ors.X004 induced well-developed ectomycorrhizas on *Casuarina equisetifolia*, whereas PR86 failed to form any fungal sheath on *Casuarina cunninghamiana*. On *Allocasuarina* species, *Pisolithus* isolates formed generally well-developed ectomycorrhizas. In addition, isolates Ors.7870 and PR86 invaded the cortical cells of *Allocasuarina luehmannii* and *Allocasuarina decaisneana*, respectively, thus forming ectendomycorrhizas. Epidermal cells of both *Casuarina* and *Allocasuarina* mycorrhizas showed tannin deposits. In fully developed ectomycorrhizas, the epidermal cells were radially elongated and the Hartig net never developed beyond the epidermal cells. In general, the ability to form ectomycorrhizas was more common with the genus *Allocasuarina* than the genus *Casuarina*.

Key words: *Casuarina*, *Allocasuarina*, *Pisolithus*, ectomycorrhizas.

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Cinq espèces de *Casuarina* et cinq espèces d'*Allocasuarina* ont été inoculées *in vitro* par trois isolats de *Pisolithus* sp. (Ors.X004 et Ors.7870 du Sénégal, PR86 d'Australie) afin de tester leur infectivité et leur aptitude à former des ectomycorhizes. L'infectivité des isolats de *Pisolithus* est variable. Les plus importantes différences d'infectivité ont été observées entre les espèces de *Casuarina* et d'*Allocasuarina*. Chez les *Casuarina*, les isolats de *Pisolithus* forment généralement un manteau fongique et pas de réseau de Hartig. Néanmoins, l'isolat Ors.X004 a formé des ectomycorhizes complètes sur *Casuarina equisetifolia*, tandis que l'isolat PR86 n'a formé aucune mycorhize sur *Casuarina cunninghamiana*. Sur les espèces d'*Allocasuarina*, les isolats de *Pisolithus* forment généralement des ectomycorhizes bien dévelopées. Les isolats Ors.7870 et PR86 ont envahi des cellules corticales respectivement d'*Allocasuarina luehmannii* et d'*Allocasuarina decaisneana*, formant donc des ectendomycorhizes. Les cellules épidermiques des mycorhizes de *Casuarina* et d'*Allocasuarina* sont remplies de tannins. Chez les ectomycorhizes bien dévelopées, le réseau de Hartig est limité aux cellules épidermiques qui sont allongées radialement. L'aptitude à former des ectomycorhizes est plus grande dans le genre *Allocasuarina* que dans le genre *Casuarina*.

Mots clés : *Casuarina*, *Allocasuarina*, *Pisolithus*, ectomycorhizes.

Introduction

The Casuarinaceae comprise about 80 species of shrubs and trees native to the southern hemisphere, mostly to Australia and Malaysia. They occur in tropical, subtropical, and temperate coastal regions as well as in the arid inland (National Research Council 1984). Some species are widely used in afforestation programs of adverse sites.

Among the Casuarinaceae, the genera *Casuarina* and *Allocasuarina* are known to possess ectomycorrhizal species (Tandy 1975; Bamber *et al.* 1980; Warcup 1980; National Research Council 1984; Reddel *et al.* 1986; Ba *et al.* 1987). The fungi involved in the ectomycorrhizas of the Casuarinaceae are, however, not well documented. To our knowledge, the only experimental ectomycorrhizas obtained in the Casuarinaceae with an identified fungus are those of *Allocasuarina distyla* with *Pisolithus tinctorius* (Bamber *et al.* 1980). In Senegal, field observations of sporocarps, rhizomorphs, and mycorrhizas led to the conclusion that *Casuarina equisetifolia* was naturally ectomycorrhizal with *Pisolithus* sp. (Ba *et al.* 1987). According to Reddel *et al.* (1986), ectomycorrhizas are more common in *Allocasuarina* than in *Casuarina*.

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TABLE 1. Infectiveness of three isolates of *Pisolithus* sp. on five *Casuarina* and five *Allocasuarina* species after 2 months growth *in vitro*

Host species	*Pisolithus* isolates[a]		
	Ors.X004	Ors.7870	PR86
C. cristata	(+)	(+)	(+)
C. cunninghamiana	(+)	(+)	0
C. equisetifolia	+	(+)	(+)
C. glauca	(+)	nt	nt
C. obesa	(+)	(+)	(+)
A. campestris	+	+	+
A. decaisneana	+	+	/+/
A. luehmannii	+	/+/	+
A. torulosa	+	+	+
A. verticillata	+	+	+

[a]+, Mycorrhiza with fungal sheath and Hartig net; (+), mycorrhiza with fungal sheath only; /+/, mycorrhiza with fungal sheath and endocellular invasion of cortical cells; 0, no mycorrhiza; nt, isolate not tested.

The objective of this study was to test the ability of three isolates of *Pisolithus* sp. to form mycorrhizas in five species of *Casuarina* and five species of *Allocasuarina*.

Materials and methods

Origin of Pisolithus *isolates*

PR86 was isolated by P. Reddel from sporocarp growing under *Eucalyptus* spp. at Lobethal, South Australia, Ors.X004 was isolated in 1986, from sporocarp fruiting under *Eucalyptus camaldulensis* Dehn, at Djibélor, Senegal, and Ors.7870 was isolated in 1987, from sporocarp fruiting under *Racosperma holosericea* (Cunn. ex G. Don) Pedley (syn. *Acacia holosericea* Cunn. ex G. Don), at Sangalkam, Senegal. The isolates Ors.X004, Ors.7870, and PR86 have been deposited at the B.S.S.F.T. (Nogent-sur-Marne, France).

The *Pisolithus* strains were isolated on MNM agar (Marx 1969) and subcultured on the same medium before inoculating the host trees. They originated from sporocarps, which differed from *Pisolithus tinctorius* (Pers.) Coker & Couch (syn. *P. arhizus* (Pers.) Rausch) by the straight ornamentation of the spores and by the white colour of the peridium of young sporocarps (Demoulin and Dring 1974; Thoen 1985). As far as is known, these forms of *Pisolithus* are confined to tropical regions where they mainly grow under introduced *Eucalyptus* spp. (Thoen 1985).

Origin of the seeds

Certified seeds of *Casuarina cristata* F. Muell. ex Miq., *C. cunninghamiana* Miq., *C. glauca* Sieb ex Spreng., *C. obesa* Miq., *Allocasuarina campestris* (Diels) L. Johnson, *A. decaisneana* (F. Muell.) L. Johnson, *A. luehmannii* (R.T. Bak.) L. Johnson, *A. torulosa* (Ait.) L. Johnson, and *A. verticillata* (Lam.) L. Johnson were provided by Kimberely seeds Pty Ltd (51 King Edward Road, Osborne Park, W.A., 6017 Australia). The seeds of *Casuarina equisetifolia* Forst. were collected at Kayar (Senegal) in May 1986 (86/1398/ISRA/CNRF).

Infectivity studies

Seeds were surface sterilized by immersion for 3 min in concentrated sulfuric acid and rinsed five times in sterile distilled water. The seeds were allowed to germinate in the dark at 30°C for 5 days in Petri dishes filled with water agar (8 g/L). The germinants were transferred to 125 × 4 cm test tubes filled with 90 mL of a perlite, peat, and water mixture (90:5:17.5, v/v/v) previously autoclaved twice at 120°C for 20 min.

Seedlings were placed for 5–6 weeks in a growth chamber (28°C day, 18°C night; day length 16 h; light intensity 107 E m^{-2} s^{-1}). Before inoculation, 50 mL of sterile liquid MNM medium was added to the tubes. Agar plugs, 8 mm in diameter, of actively growing *Pisolithus* sp. were put on fresh agar MNM medium for 4 days to stimulate the regeneration of the hyphal tips. Three plugs were then transferred to each test tube. Inoculations were replicated three times. Two months after inoculation, the seedlings were carefully removed from the test tubes and gently washed under running tap water. Root tips were examined under a dissecting microscope for fungal sheath formation. Root tips showing a fungal sheath were hand sectioned, cleared in 15% sodium hypochloride, rinsed in distilled water, and stained with 0.5% Congo red. Root sections were examined under a differential interference contrast microscope. Tannin deposits in epidermal and cortical cells were located by examining uncleared root sections under a stereoscopic microscope.

Results

Table 1 shows the degree of mycorrhiza formation 2 months after inoculation of five *Casuarina* and five *Allocasuarina* species with three *Pisolithus* isolates which behaved differently on *Casuarina* than on *Allocasuarina* species. On *C. equisetifolia*, only isolate Ors.X004 formed well-developed ectomycorrhizas, viz. with fungal sheath and Hartig net (Fig. 1*a*), whereas Ors.7870 and PR86 formed only a fungal sheath. On the other *Casuarina* species, the three *Pisolithus* isolates achieved a well-developed fungal sheath but no Hartig net, except for PR86, which failed to form any sheath on *C. cunninghamiana*.

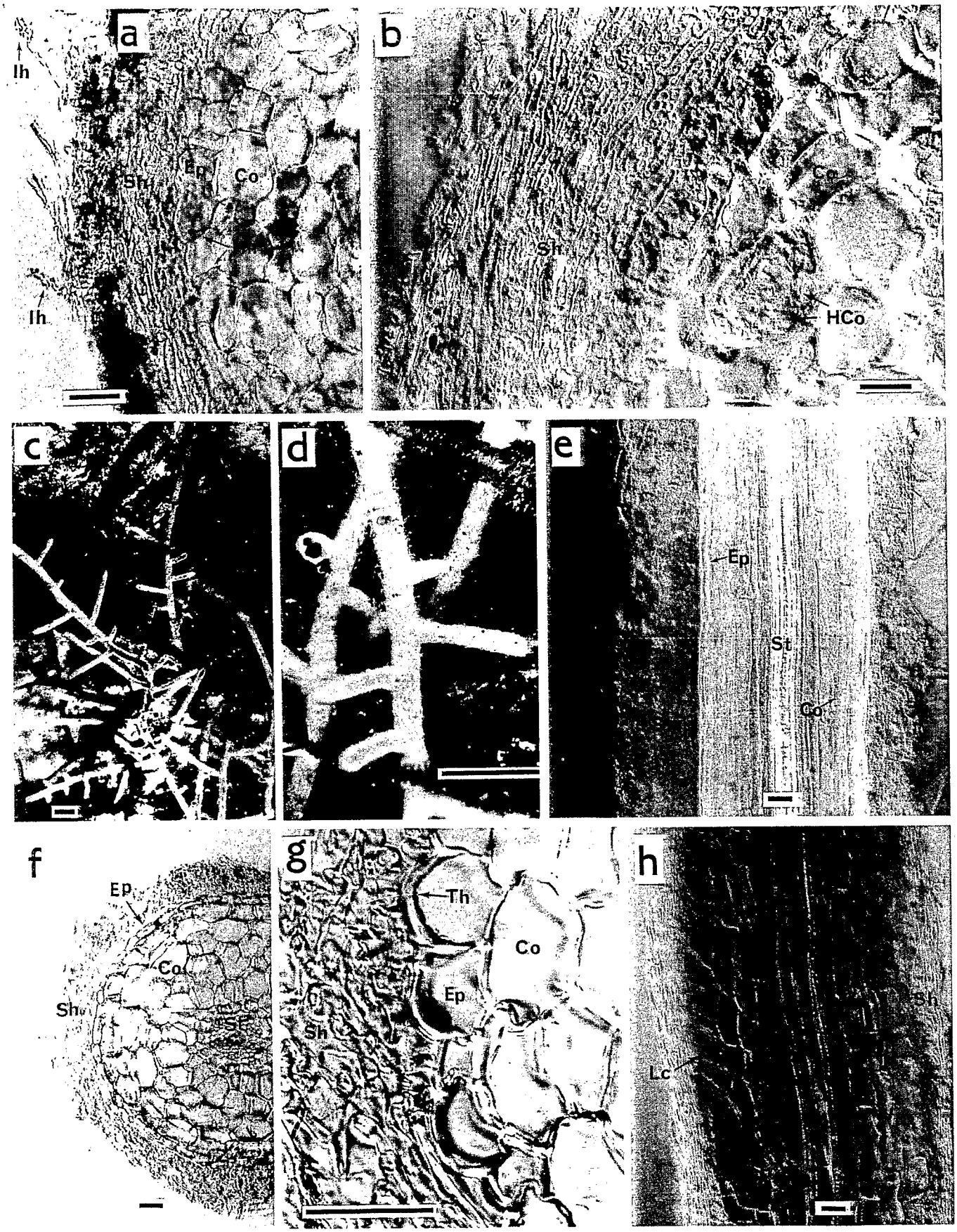
On the five *Allocasuarina* species, all *Pisolithus* isolates formed well-developed ectomycorrhizas. Isolates Ors.7870 and PR86 invaded some cortical cells of *A. luehmannii* and *A. decaisneana* to form ectendomycorrhizas (Fig. 1*b*).

Ectomycorrhizas occurred on single root tips as well as on ramified roots (Fig. 1*c*). The surface of the fungal sheath was yellow, felty, with numerous emanating hyphae (Fig. 1*d*).

Table 2 describes the main anatomical characteristics of *Casuarina* and *Allocasuarina* mycorrhizas. The values of the table are established on single observations and the mean values of columns A–E are only indicative. Sections of mycorrhizas are illustrated in Fig. 1 and 2. Epidermal cells of mycorrhizas devoid of Hartig net were not radially elongated (Figs. 1*e*–1*g*) whereas those of mycorrhizas with Hartig net were more or less radially elongated (Figs. 1*h*, 2), sometimes forming a palisade layer (Figs. 2*a*–2*d*). The radial penetration of the Hartig net never exceeded the epidermal cells and ranged from 10 to 29 μm. Thickening of the epidermal cell wall in direct contact with the fungal sheath was observed in some *Casuarina* (Fig. 1*g*) as well as in some *Allocasuarina* mycorrhizas. Tannin deposits were present in the epidermal cells of

ABBREVIATIONS: T.S., transverse section; L.S., longitudinal section; Sh, sheath; Ih, incrusted hyphae; Hn, Hartig net; Lc, labyrinthine cells of Hartig net; Hco, intracellular hyphae in cortical cells; Ep, epidermal cells; Co, cortical cells; Th, wall thickening; St, stele.

FIG. 1. Synthesized mycorrhizas of *Casuarina* and *Allocasuarina* species with *Pisolithus* isolates: (*a*) T.S., *C. equisetifolia* + Ors.X004; (*b*) T.S., *A. decaisneana* + PR86; (*c*) and (*d*) stereomicrographs of *A. verticillata* + Ors.X004; (*e*) L.S., *C. glauca* + Ors.X004; (*f*) T.S., *C. cristata* + Ors.7870; (*g*) T.S., *C. obesa* + Ors.X004; (*h*) L.S., *A. verticillata* + Ors.X004. Bars = 20 μm, except for (*c*) and (*d*) where bars = 1 mm.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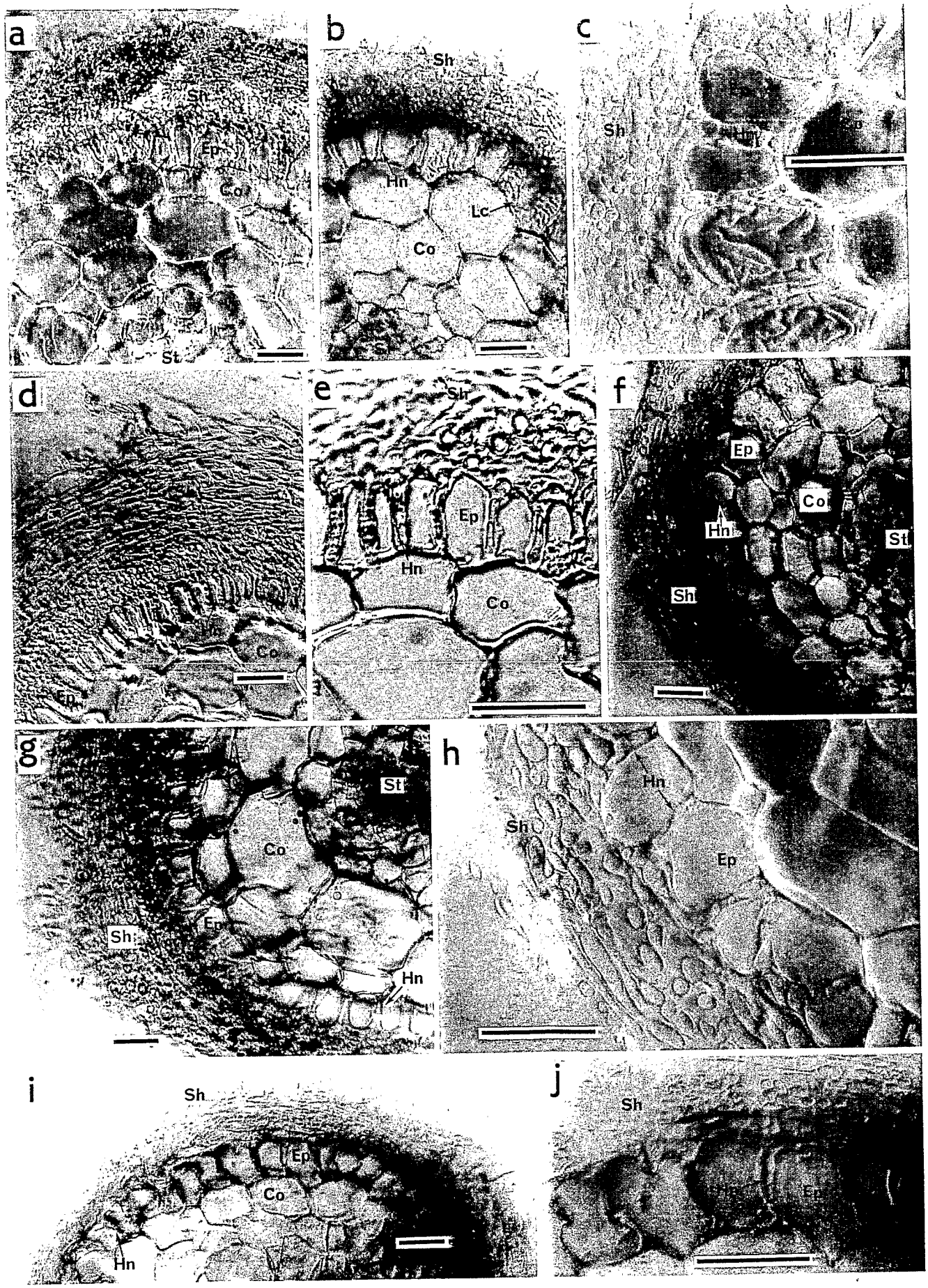
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TABLE 2. Main anatomical characteristics noted on cross sections of synthesized mycorrhizas of *Casuarina* and *Allocasuarina* species with three isolates of *Pisolithus* sp.

Host species	Isolate[a]	Anatomical characteristics[b]							
		A	B	C	D	E	F	G	H
C. cristata	1	280	57	65	0	60	3	++	+
	2	300	41	47	0	72	3	++	+
	3	180	24	46	0	46	3	++	−
C. cunninghamiana	1	280	38	46	0	68	2	++	+
	2	280	44	53	0	48	2	++	−
C. equisetifolia	1	340	45	46	13	80	3	++	−
	2	440	42	34	0	100	3	++	−
	3	250	34	47	0	69	3	++	−
C. glauca	1	260	52	64	0	60	3	++	−
C. obesa	1	240	32	46	0	64	3	++	+
	2	230	32	48	0	70	3	++	−
	3	280	26	34	0	80	3	++	−
Mean value		280.0	38.9	48.2		68.1			
SEM		64.0	9.9	9.9		14.7			
A. campestris	1	368	50	47	16	96	3	+	−
	2	370	60	54	19	90	3	+	−
	3	376	54	49	17	100	3	+	−
A. decaisneana	1	240	45	61	14	82	3	+	−
	2	270	36	46	18	76	3	+	−
	3	640	90	48	16	96	3	+	+
A. luehmannii	1	340	62	68	15	64	2	++	−
	2	290	22	28	0	64	3	++	+
	3	260	54	66	16	60	2	+	−
A. torulosa	1	210	24	40	16	60	3	+	+
	2	240	24	36	12	80	3	+	−
	3	280	28	36	18	50	2	+	−
A. verticillata	1	220	24	39	25	64	4	++	−
	2	334	55	55	10	90	4	++	+
	3	320	52	54	29	95	4	+	+
Mean value		317.2	46.9	48.5	17.2	77.8			
SEM		105.0	18.8	11.5	4.8	16.4			
General mean		300.7	42.5	48.3	16.9	73.5			
SEM		89.6	15.8	10.5	(4.8)	16.1			

[a]Isolates: 1, Ors.X004; 2, Ors.7870; 3, PR86.

[b]A, diameter of mycorrhiza (μm); B, fungal sheath thickness (μm); C, percent of cross-sectional area of the fungal sheath; D, radial depth of Hartig net (μm); E, stele diameter (μm); F, number of xylem poles; G, tannin deposit in epidermis (+) or in epidermis and cortical cells (++); H, presence (+) or absence (−) of wall thickening of epidermal cells.

mycorrhizas of both genera. Tannins were also observed in cortical cells of all *Casuarina* species and of *A. luehmannii* and *A. verticillata*. The fungal sheath was prosenchymatous (Figs. 1, 2) with radiating hyphae (Figs. 1*a*, 1*e*). In *C. equisetifolia* mycorrhizas, the emerging hyphae of the sheath were incrusted by small crystals of calcium oxalate (Fig. 1*a*). The sheath thickness varied considerably on the same host tree according to the fungal isolate (e.g., from 24 to 57 μm in *C. cristata* and from 36 to 90 μm in *A. decaisneana*). The diameter of the mycorrhizas varied, but the mean was close to 300 μm in both host genera. The mean percent of cross-sectional area of the sheath reached 48% for both *Casuarina* and *Allocasuarina* mycorrhizas. In *Allocasuarina* ectomycorrhizas, the mean depth of the Hartig net was 17.2 μm, which is approximately the third of the mean thickness of the fungal sheath (46.9 μm). The number of xylem poles varied from two to three in *Casuarina* and from two to four in *Allocasuarina*. It was rather constant within species and might be a specific characteristic.

Discussion

The results confirmed field observations by Reddel *et al.* (1986) showing that *Allocasuarina* species formed ectomycorrhizas more commonly than did *Casuarina* species.

Pisolithus sp. has a broad host range, since in addition to *Casuarina* and *Allocasuarina*, it is also associated in Senegal with the genera *Eucalyptus*, *Melaleuca*, and *Racosperma* (Thoen and Ducousso 1990).

Tannin deposits were observed in the epidermal cells of all the synthesized mycorrhizas. Such deposits are common in natural ectomycorrhizas in the tropics (e.g., Alexander and Högberg 1986; Thoen *et al.* 1990; Thoen and Ducousso 1990). Tannins also occurred in cortical cells of some synthesized

FIG. 2. Transverse sections of synthesized ectomycorrhizas of *Allocasuarina* species with *Pisolithus* isolates: (*a*) *A. campestris* + PR86; (*b*) and (*c*) *A. verticillata* + Ors.X004; (*d*) and (*e*) *A. campestris* + Ors.7870; (*f*) *A. decaisneana* + Ors.7870, (*g*) *A. luehmannii* + Ors.X004; (*h*) *A. torulosa* + Ors.X004; (*i*) and (*j*) *A. torulosa* + PR86. Bars = 20 μm.

mycorrhizas. The role of tannins in mycorrhizal roots is not yet fully elucidated (Molina and Trappe 1982). Tannin deposits are host reactions allowing, presumably, control of fungal aggressiveness. Thickening of the epidermal wall close to the fungal sheath was observed in some mycorrhizas. Host cell wall thickening may indicate incompatibility between host and fungus but occurs also in well-developed ectomycorrhizas (Molina and Trappe 1982). Thickening of host cell wall might be another mechanism allowing to control penetration of the fungus.

The artificial conditions of our experimental design did not show beneficial influence of the fungal isolates on seedling growth, because the substrate was not deficient in phosphorus or other nonmobile nutrients. Beneficial influences of endomycorrhizas on nodulation and growth of *C. equisetifolia* have been demonstrated (Diem and Gauthier 1982; Gauthier *et al.* 1983). Further research is planned to study the ability of ectomycorrhizal *Pisolithus* isolates to promote growth, phosphorus uptake, and nodulaton of Casuarinaceae. The effectiveness of mycorrhizas lacking a Hartig net also needs to be compared with that of fully developed ectomycorrhizas.

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