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Mycorrhiza

Root soluble carbohydrates of *Afzelia africana* Sm. seedlings and modifications of mycorrhiza establishment in response to the excision of cotyledons

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Abstract. Stem length, number of secondary lateral roots, shoot dry weight and reducing sugar concentrations of root were significantly reduced when translocation of reserves from cotyledons to the roots of *Afzelia africana* seedlings was interrupted by complete or partial cotyledon excision. The sucrose but not the glucose concentration of lateral roots also decreased significantly after complete cotyledon excision. Hartig net development rather than fungal sheath formation was affected after inoculation with the early fungal isolate E1 and by both late-stage fungal isolates L1 and L2 after partial or complete cotyledon excision. However, mycorrhizal colonization by the early fungal isolate E2 was not affected by cotyledonary reserves, suggesting that this fungal isolate has a lower carbohydrate requirement than fungal isolates E1, L1 and L2. The late-stage fungal isolates L1 and L2 induced a hypersensitivity reaction by epidermal cell walls of the host plant after complete cotyledon excision, suggesting they are more dependent than the early fungal isolate E1 on available root carbohydrate substrates for ectomycorrhizal colonization. These results are discussed in the light of the hypothesis that early- and late-stage fungi were different carbohydrate requirements, and that the time sequence of colonization was related to the root carbohydrate status, which increased with time.

Key words: Ectomycorrhiza formation – Root soluble carbohydrates – Cotyledon excision – Hypersensitive reaction – *Afzelia africana*

Introduction

Bâ (1990) showed that ectomycorrhizal colonization by seedlings of the Western African tree *Afzelia africana* (Cesalpinioideae) followed a characteristic time sequence over a 6-month period. Of the fungi present in the natural soil tested, some infect the roots soon after the germination of the seed and before the first leaf is formed, while other mycorrhiza types appear later. This additive time sequence has been reported for other tree species by Dighton and Mason (1985), who referred to "early-stage" and "late-stage" fungi.

In the case of *A. africana,* Bâ et al. (1991) showed that the type of propagule partly determined the sequence of ectomycorrhizal colonization, based on rates of germination in the rhizosphere of the host plant. However, a factor originature in the plant itself might be involved. According to Björkman (1970), Marx et al. (1977) and Dixon et al. (1981), ectomycorrhiza formation and stability is regulated by the soluble carbohydrates of the roots.

The experiments reported in this paper aimed to test the hypothesis that different fungi have different carbohydrate requirements, and that the colonization time sequence is caused by modifications of the root carbohydrate status with time. For this propose, and in order to disociate the carbohydrate status and the time factor, cotyledon excision before leaf formation was used to reduce carbohydrate translocation in young seedlings. This manipulation is easy with the large embryo of *A. africana,* which has two thick, starchy cotyledons and epigeous germination.

Materials and methods

Seedling growth

Seeds of *A. africana* Sm. were surface sterilized as described previously (Bâ and Thoen 1990). Two experiments were set up in a growth cabinet and in a glasshouse. In the growth cabinet, the temperature was 25° C day and 18° C night, daylength was 16 h and light intensity was 107 $\mu Em^{-2}s^{-1}$. Pregerminated seeds were raised in an open plastic contained (200 × 50 × 15 mm) containing a mixture of vermiculite and peat moss (4:1, v:v) moistened with sterile distilled water. In a naturally illuminated glasshouse from July to October 1989 at the INRA center (Nancy,

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France), mean day and night temperatures were 25° C and 10° C, respectively, mean daylength was 12 h and the relative humidity varied between 60% and 80%. Pregerminated seeds were raised as above. Seedlings were watered with a 1/10 diluted nutrient solution (KNO_3, 0.4 g/l^{-1}, $Ca(NO_3)_2$, $4H_2O$ 0.94 g/l^{-1}, NaH_2PO_4, H_2O 0.18 g/l^{-1}, $MgSO_4$, $7H_2O$ 0.37 g/l^{-1}) supplemented with 1 ml of a microelement solution (chelated iron 6 g/l^{-1}, molybdenum 0.27 g/l^{-1}, boron 8.45 g/l^{-1}, manganese 5 g/l^{-1}, copper 0.62 g/l^{-1}, zinc 2.27 g/l^{-1}). The final pH of this nutrient solution was 5.5. Seedlings were watered once each week with 100 ml of this nutrient solution. Lateral roots were sampled for sugar analysis at monthly intervals for 4 months after sowing.

Fungal inocula

Bâ et al. (1991) differentiated two early-stage fungi, designated as ORS. XM001 (E1) and ORS. XM002 (E2), and two late-stage fungi, designated as ORS. XM003 (L1) and ORS. XM004 (L2). These fungi were kept at 25° C on Pachlewski's medium agar (Pachlewski and Pachlewska 1974). For the present work, the mycorrhizal fungi were grown at 25° C for 15 days on absorbent paper cards laid on potato dextrose agar (Chilvers et al. 1986).

Cotyledon excision and mycorrhizal inoculation

In the growth cabinet, large green cotyledons were excised wholly (two cotyledons), partially (one cotyledon) or not at all (control) from 10 to 15-day-old *A. africana* seedlings. For each treatment, inocula of the different fungi, in the form of colonized paper cards, were placed onto a part of the root system. Lateral roots with a fungal sheath were collected 4 days after inoculation.

The effect of cotyledon excision on the growth (stem- and tap root length, number of primary, secondary and tertiary lateral roots, dry weight of shoot and total roots) of noninoculated seedlings was determined. Counting of primary, secondary and tertiary lateral roots was carried out under a binocular microscope.

Carbohydrate analysis of lateral roots

Lateral roots of three noninoculated seedlings of *A. africana* were removed from each cotyledon treatment, washed in tap water, blotted on paper cards to remove excess water and dried immediately under vacuum for 3 days. Lateral roots (50 mg), chopped into 3 to 7-mm pieces, were homogenized in 3 ml sodium acetate buffer (50 mM, pH 4.4) for 5 min using a pestle and mortar. A pinch of fine quartz sand was added to aid homogenization of the tissues. The extract was poured into Eppendorf tubes and centrifuged 15 min at $15000 \times g$ in a cold room (4° C). The supernatant was poured off and the pellet was resuspended in 1 ml of the same buffer and centrifuged as before. The two supernatants were pooled and decanted to separate cell debris and polysaccharides from the soluble carbohydrates. Glucose was estimated using the Sigma glucose diagnostic HK 20 kit. Sucrose was hydrolyzed to glucose and fructose by yeast invertase. For this, a 0.25-ml aliquot of the supernatant was incubated at 30° C with 0.5 ml of yeast invertase solution (0.55 U). Total glucose was estimated as described above. The sucrose concentration was determined from the difference in glucose before and after hydrolysis. Reducing sugar was determined using the dinitrosalicylic acid reagent (Miller 1959).

Experimental design

All experiments were completely randomized with ten replicate plants for the effect of cotyledon excision on growth and three replicate plants for the effect of cotyledon excision on the glucose, sucrose and reducing sugar root concentrations. A further three replicate plants were sampled each month to determine the time course of changes in glucose, sucrose and reducing sugar in glasshouse conditions. The experiments were repeated twice with similar results. The data reported here are means after ANOVA analysis. The mean values were compared with Student's *t*-test.

Tissue processing for microscopy

For each cotyledon treatment and the control, twenty infected lateral roots from three seedlings were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), stored overnight at 4° C and rinsed in the same buffer. Samples were postfixed for 1 h in 2% osmium tetroxide and rinsed in distilled water. They were dehydrated in an acetone series and 3 times in pure acetone, infiltrated in an acetone-Spurr's resin series and then in 100% Spurr's resin. Polymerization took place at 70° C for 48 h. For light microscopy (LM), longitudinal and transverse sections approximately 0.5–1 μm thick were cut from embedded material and stained with 1% toluidine blue in 2.5% Na_2CO_3 (pH 11.6). For transmission electron microscopy (TEM), ultrathin sections (60–80 nm) were mounted on copper grids and contrasted with uranyl acetate for 15 min (Valentines 1961) and with lead citrate for a further 15 min (Reynolds 1963). Some ultrathin sections were mounted on 250-mesh gold grids and stained for polysaccharides (PATAg test) according to the method of Thiéry (1967) using the controls suggested by Courtoy and Simar (1974). For each treatment, at least ten lateral roots were examined using a Nikon microscope (LM) or a Siemens Elmiskop 103 (TEM).

Results

Effects of cotyledon excision on plant growth

Complete cotyledon excision significantly affected stem length, the number of primary, secondary and tertiary lateral roots, shoot and total root dry weight of 10 to 15-day-old *A. africana* seedlings (Fig. 1, Table 1). Partial cotyledon excision affected stem length, the number of secondary lateral roots and shoot dry weight (Fig. 1, Table 1).

Effects of cotyledon excision on root soluble carbohydrate

The concentration of reducing sugars in lateral roots was drastically affected by complete and partial cotyledon excision (Table 2). Root sucrose was significantly reduced after complete but not after partial cotyledon excision (Table 2). Glucose concentration was not significantly affected (Table 2).

Time course of changes in root soluble carbohydrate in glasshouse conditions

The results in Table 3 indicate that the glucose concentration in *A. africana* roots only increased significantly between months 2 and 3. The concentration of reducing sugar in *A. africana* roots increased significantly

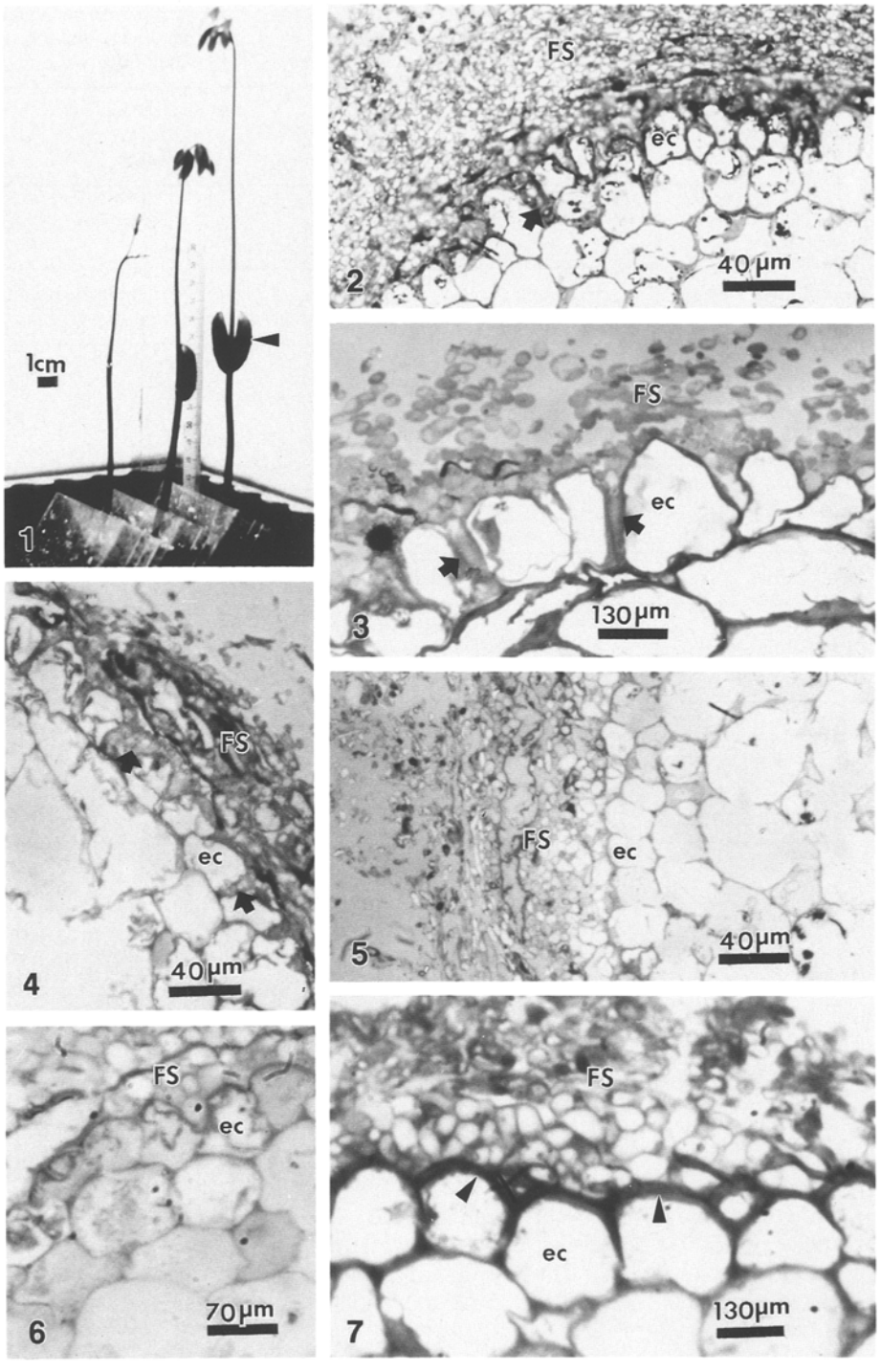

Fig. 1. Effect of cotyledon (*arrowhead*) excision on the growth of *Afzelia africana* seedlings. Abbreviations used in the figures: *fs,* fungal sheath; *ec,* epidermal cell; *ih,* intracellular hyphae; *v,* vacuole; *H,* hyphae

Fig. 2. Fungal sheath formation and Hartig net development (*arrows*) by the late-stage fungal isolate L2 in presence of cotyledons

Fig. 3. Fungal sheath formation and Hartig net development (*arrows*) by the late-stage fungal isolate L1 in presence of cotyledons

Fig. 4. Fungal sheath formation and Hartig net development (*arrows*) by the early-stage fungal isolate E1 in presence of cotyledons

Fig. 5. Fungal sheath formation without Hartig net development by the late-stage fungal isolate L1 after partial cotyledon excision

Fig. 6. Fungal sheath formation without Hartig net development by the early-stage fungal isolate E1 after complete cotyledon excision

Fig. 7. Fungal sheath formation without Hartig net development by the late-stage fungal isolate L2 after complete cotyledon excision. Note the intense toluidine staining of epidermal cell walls of the host plant in contact with fungal hyphae (*arrowhead*)

Table 1. Effects of cotyledon excision on the growth of *Afzelia africana* seedlings after 4 days. Values followed by the same letter are not significantly different (Student's *t*-test, $P=0.05$)

Cotyledons	Stem (cm)	Tap root (cm)	No. of lateral roots			Shoot (g dry wt)	Total root (g dry wt)
			Primary	Secondary	Tertiary		
Nonexcised	32.4 c	11.7 a	149 b	740.8 c	11.4 b	0.594 c	0.178 b
Partially excised	28.82 b	11.48 a	141 b	415.4 b	7.6 b	0.375 b	0.150 b
Wholly excised	19.74 a	11.46 a	100.6 a	202.6 a	0 a	0.259 a	0.128 a

Table 2. Effects of cotyledon excision on root soluble carbohydrate concentrations of lateral roots of *A. africana* seedlings after 4 days. Values followed by the same letter are not significantly different (Student's *t*-test, $P=0.05$)

Cotyledons	Glucose (mg/g dry wt)	Sucrose (mg/g dry wt)	Reducing sugars (mg/g dry wt)
Nonexcised	78.0 a	17.3 b	170.8 c
Partially excised	72.3 a	11.0 b	97.3 b
Wholly excised	51.3 a	1.3 a	51.0 a

Table 3. Time course of root soluble carbohydrate concentrations of *A. africana* seedlings during 4 months. Values followed by the same letter are not significantly different (Student's *t*-test, $P=0.05$)

Time after sowing (months)	Glucose (mg/g dry wt)	Sucrose (mg/g dry wt)	Reducing sugars (mg/g dry wt)
1	17.0 a	13.0 a	31.0 a
2	31.0 a	13.0 a	64.0 b
3	69.0 b	7.5 a	93.0 c
4	79.0 b	1.7 b	102.0 c

Table 4. Influence of cotyledon excision on the mycorrhizal colonization of *A. africana* seedlings after 4 days. + +, Mantle and Hartig net; + − mantle without Hartig net

Cotyledons	Isolate E2	Isolate E1	Isolate L1	Isolate L2
Nonexcised	+ +	+ +	+ +	+ +
Partially excised	+ +	+ −	+ −	+ −
Wholly excised	+ +	+ −	+ −	+ −

between months 1 and 3. In contrast, root sucrose concentration decreased significantly between months 3 and 4.

Effects of cotyledon excision on mycorrhiza formation

All observations were made on the basal second-order lateral roots of *A. africana* seedlings cultivated in a carbohydrate-free substrate. Although variations were observed in each treatment, the features described below were regularly encountered.

In LM, 4 days after inoculation with the fungal isolates L2 (Fig. 2), L1 (Fig. 3), E1 (Fig. 4) and E2 (not shown), mycorrhizal formation occurred before complete development of the first leaf pair. However, mycorrhiza formation by three of these isolates (E1, L1, L2) was incomplete when cotyledons were partially or wholly removed (Table 4, Figs. 5–8). Hartig net was modified whereas fungal sheath formation was not modified by partial or complete cotyledon excision (Table 3, Figs. 5–8). In contrast, mycorrhiza formation by isolate E2 was not affected after partial (not shown) or after complete cotyledon excision (Table 4, Fig. 9). An intense toluidine staining of epidermal cell walls of host plants was induced by two isolates (L1, L2) after complete cotyledon excision (Figs. 7, 8) but not after partial cotyledon excision (Fig. 5, not shown for L2). Intracellular penetration by hyphae of isolate L1 was also seen after complete cotyledon excision (Fig. 8). However, toluidine-stained epidermal cell walls were not observed in the presence of isolates E2 (Fig. 9) or E1 (Fig. 6) after complete cotyledon excision. Intense toluidine staining of epidermal cell walls was not observed in the absence of fungal symbiont after complete cotyledon excision (Fig. 10).

In TEM, the main histological and cytological characteristics induced by the isolates L1 and L2 were comparable after complete or partial cotyledon excision. Therefore, only the significant features observed with isolate L1 are described. After complete cotyledon excision, the intense toluidine staining induced by this isolate observed in LM was correlated with a thickening of the host cell wall seen in TEM, and often occurred together with discrete wall ingrowths. These modifications were seen as electron-transparent materials on the internal face of epidermal cell walls (Fig. 11), which consisted of cell wall depositions reacting positively in the PATAg test (Figs. 12, 14). However, such thickenings were absent after partial cotyledon excision, whereas wall ingrowths were present (Fig. 13). These wall ingrowths were present to the same extent during mycorrhiza establishment (Fig. 15).

Discussion

Cotyledon excision was carried out at the moment of emergence of the epicotyl bearing the first immature pair of leaves of 10 to 15-day-old *A. africana* seedlings. The wounds caused by cotyledon excision did not result in modifications of the stem of the seedlings, but growth was slowed down. Thus, *A. africana* seedlings were dependent on cotyledon reserves during their initial phase of growth, in accordance with the proposed importance of cotyledonary reserves for early growth of woody angiosperms (Marshall and Kozlowski 1976; Mulligan and Patrick 1985).

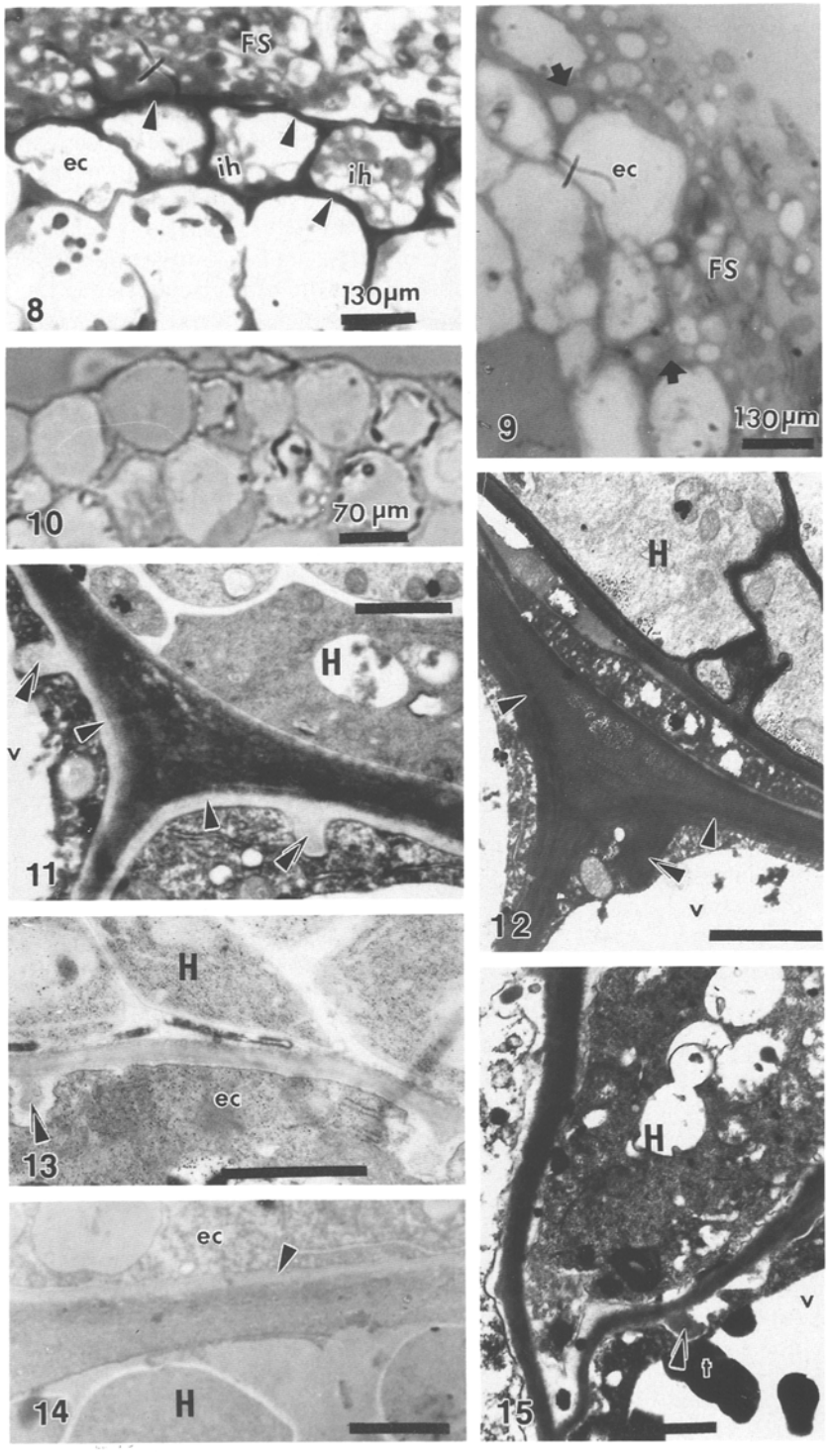

Fig. 8. Fungal sheath formation without Hartig net development by the late-stage fungal isolate L1 after complete cotyledon excision. Note the presence of intracellular hyphae and the intense toluidine staining of epidermal cell walls of the host plant in contact with fungal hyphae (*arrowheads*)

Fig. 9. Fungal sheath formation without Hartig net development (*arrows*) by the early fungal isolate E2 after complete cotyledon excision

Fig. 10. Noncolonized root after complete cotyledon excision

Fig. 11. Deposition of an electron-transparent material (*arrowheads*) and wall ingrowths (*double arrowheads*) on the internal face at the junction of two epidermal cell walls induced by the late stage fungal isolate L1 after complete cotyledon excision; *bar* = 1 µm

Fig. 12. Deposition of electron-transparent material (*arrowheads*) and wall ingrowth (*double arrowheads*) reacting positively to the PATAg test; *bar* = 1 µm

Fig. 13. Electron micrograph of the interface between the late-stage fungal isolate L1 and the host plant after partial cotyledon excision. Note the presence of wall ingrowths (*double arrowheads*) and the absence of electron-transparent material; *bar* = 1 µm

Fig. 14. A negative reaction to Thiéry's procedure in which thiocarbohydrazide was omitted. Note the electron-transparent material (*arrowheads*); *bar* = 1 µm

Fig. 15. Electron micrograph of mycorrhizal initiation by the late-stage fungal isolate L1 in presence of cotyledons. Note the wall ingrowths (*double arrowheads*); *bar* = 1 µm

The reducing sugar concentration of the roots was gradually affected by partial and complete cotyledon excision. Sucrose was only affected after complete cotyledon excision whereas glucose was not affected at all. These results suggest that reserves such as carbohydrates were transferred from the large, green cotyledons to the lateral roots in young *Afzelia* seedlings. Reducing sugar concentrations were generally higher than those of sucrose and glucose in the growth cabinet and in glasshouse conditions. In addition, only root reducing sugar concentration of *A. africana* seedlings increased with age in glasshouse conditions, and lateral roots of *A. africana* contained less sucrose than glucose and reducing sugars. However, our data support the idea that the major carbon source in lateral roots of *A. africana* is reducing sugars, as proposed by Björkman (1970), and that therefore, the factor that influenced susceptibility to ectomycorrhizal colonization of *A. africana* by fungal isolates was a reducing sugar and not a non-reducing sugars such as sucrose. Sucrose

may be converted to glucose and fructose by a plasma membrane invertase, which are then available for the fungus (Bevege et al. 1975). Sucrose is thought to be the main sugar transferred to the mycorrhizal fungus (Lewis and Harley 1965; Lewis 1975; Marx et al. 1977).

Since the root concentration of soluble carbohydrates can affect the development of symbiotic associations (Harley and Smith 1983), the effect of cotyledon excision on ectomycorrhizal infection was also investigated. Reducing sugar concentrations in roots were related to Hartig net formation rather than mantle formation with fungal isolates E1, L1 and L2 after partial or complete cotyledon excision. However, cotyledon reserves did not affect mycorrhizal colonization by isolate E2. This suggests that isolate E2 required a lower carbohydrate level than isolates E1, L1 and L2 for ectomycorrhizal colonization. When translocation of reserves from cotyledons to roots of *A. africana* seedlings was interrupted by cotyledonary excision, the rate of production of carbohydrates was drastically modified. This modification did not affect fungal sheath formation by early- or late-stage fungi. However, the carbohydrate concentrations were apparently not sufficient for Hartig net development, which was highly dependent on sheath formation.

All fungal isolates tested were found to affect the time sequence of ectomycorrhizal colonization of *A. africana* seedlings (Bâ et al. 1991). The isolates E1 and E2 were the first to colonize *A. africana,* with isolates L1 and L2 being much later. Considered separately, the early-stage fungi E1 and E2 each showed a different behaviour. Isolate E2 had the higher density of vegetative propagules in the soil and a lower requirement for carbohydrate substrates. Isolate E1 appears to show intermediate behaviour between the earlier E2 and the late-stage fungi L1 and L2 (Bâ et al. 1991).

Observations made in LM and TEM were correlated. The intense toluidine staining of the epidermal cells in LM in the presence of both late-stage isolates L1 and L2 was correlated with thickening of the epidermal host cell wall seen in TEM. Thickenings of epidermal cell walls were due to the presence of fungi after complete cotyledon excision. However, epidermal cell walls were not affected in noncolonized root after complete cotyledon excision. Thickenings of epidermal cell walls of the sort induced by late-stage fungi L1 and L2 were absent in the presence of the early isolates E1 and E2 after complete cotyledon excision. The increased hypersensitivity of host epidermal cell walls to colonization by both of the late-stage isolates L1 and L2 compared to the early isolates E1 and E2 could be related, at least in part, to reductions in the concentrations of reducing sugar in roots after complete cotyledon excision. Late isolates L1 and L2 triggered a hypersensitive reaction from the host plant presumably in the interests of their greater need for carbohydrates. This suggests that the E2 may have a lower requirement for reducing sugars in roots than L1 or L2. In some circumstances, wall thickenings have been interpreted as a host defense reaction to the fungi related to plant maturity (Tonkin et al. 1989; Bâ et al. 1994) or an indicator of incompatible interactions (Molina 1981; Malajczuk et al. 1982, 1984; Lei et al. 1990).

In conclusion, our results suggest that partial and total cotyledon excision drastically decreases the growth and the root reducing sugar concentrations of *A. africana* seedlings, and thus may determine the ability of fungal isolates to initiate mycorrhizal colonization. The earlier isolate E2 had a lower requirement for root carbohydrate than the other isolates. The late-stage isolates L1 and L2, which induced a hypersensitive reaction by the host plant, could be more dependent than the intermediate isolate E1 on the available root carbohydrate substrates for ectomycorrhizal colonization after complete cotyledon excision. As the concentration of soluble carbohydrates in the roots increased with time, this may be advantageous to isolates E2 and E1 in colonizing the root system of *A. africana* seedlings more quickly than the late-stage isolates L1 and L2 (Bâ et al. 1991). However, the hypothesis than the time sequence reflected changes in root carbohydrate status also needs to be tested during the period of growth of colonized *A. africana* seedlings after the expanding first leaf pair is self sufficient in carbon.

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