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Mycorrhiza

The effect of carbohydrate on the development of a *Cattleya* hybrid in association with its mycorrhizal fungus

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Abstract. To investigate beneficial effects of mycorrhizal fungi to advanced leafy orchids, growth studies on the development of symbiotic seedlings of the orchid *Cattleya* (*aclandiae* × *schoeffeldiana*) × *aclandiae* were conducted in vitro over a period of 18 months using split plates with minerals and carbohydrates on one side and water agar on the other. Mycorrhizal infection and shoot and root growth of seedlings on the nutrient side were compared to growth on the water agar side with nutrient uptake by the orchid only possible via external mycorrhizal hyphae. Seed germination was followed by mycorrhizal infection and rapid development of protocorms on both nutrient and non-nutrient sides of the plates. With 0.5% starch, development of protocorms was sustained for a least 12 weeks, compared to only 6 weeks with 0.1% starch. Advanced protocorms with two small leaves and a smoll root were transferred at week 22 to new fungal plates. When harvested at week 43, plantlets on 0.5% starch (both nutrient and water agar sides) had 2.7 times the dry weight of plantlets on 0.1% starch. Shoot-root ratios were higher on the lower level of carbon. In all plantlets, mycorrhizal infection involved less than 5% of the root length. With zero, 0.1% or 0.5% starch, the roots were re-infected on transfer to fresh fungal plates but young roots that developed following the transfer stayed free of infection, Plantlets on 0.5% starch (nutrient and water agar side) after 18 months had longer roots than plantlets grown in the absence of starch or on 0.1% starch. Shoots were small but significantly larger on the nutrient side than on the water agar side, independent of the carbohydrate level. The shoot-root ratio was highest on the nutrient side with no starch present. In this latter case, plantlet development was steady but plantlets on the non-nutrient side developed slowly; thus there was little evidence of nutrient translocation by the mycorrhizal fungus from the nutrient to the non-nutrient side in the absence of carbohydrates. Mycorrhizal infection is discussed as a mechanism for heterotrophic carbon assimilation. In advanced leafy orchids of *Cattleya,* external carbon resulted in increased root growth, decreased shoot/root ratio and sometimes yellowish-green plantlets.

Key words: Carbohydrate – *Cattleya* – Orchid mycorrhiza

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Introduction

Orchids completely depend on mycorrhizal fungi for seed germination and early growth in their natural habitats. This was established by the early work of Bernard (1899) and many experimental studies have been subsequently conducted on primary infection, seedling development, nutrient transfer and plant/fungus relationships using the early seedling stages of orchids (protocorms). Asymbiotic studies on seed germination showed that an extracellular source of carbohydrate could substitute for the symbiont in *Cattleya* and many other epiphytic orchids (Knudson 1922). Seeds of *Cattleya*, which can turn green 8 to 10 days after sowing on sucrose media, are not capable of photosynthesis before the initiation of leaves 30 days later (e.g. *C. aurantiaca*, Harrison and Arditti 1978). Furthermore, the ability of *Cattleya* to assimilate nitrate develops only 60 days later (Raghavan and Torrey 1964). Other mainly terrestrial orchids also rely on extracellular sources of amino acids and vitamins (Schaffstein 1941). The interaction with mycorrhizal fungi is nutritionally important, with translocation of carbohydrates (Smith 1966, 1967) and minerals (Alexander et al. 1984) into the protocorms occurring via external mycelium. However, the mycorrhizal interaction is also delicately balanced, with infection shifting readily into a parasitic state in which the fungus either kills its host or is eliminated from the orchid by defence reactions (Hadley 1982).

The degree of dependency of adult green orchids on the mycorrhizal interaction is very variable. Whereas symbiotic infection of protocorms seems obligate with respect to carbohydrate assimilation in all orchids, most species soon develop leaves and are then supposed to be-

come autotrophic for carbon. In some leafy species, mycorrhizal fungi are absent from adult plants but other, especially terrestrial, species have heavily infected roots throughout their life. Infection of wild epiphytic orchids can also be very extensive (Alexander 1987; Benzig 1982; Benzig and Friedman 1981; Hadley and Williamson 1971; Mejstrik 1970). The importance of the mycorrhizal fungi at this stage is not yet understood. The only experimental studies on advanced seedlings and adult plants of orchids are those on the terrestrial orchid *Goodyera repens* with respect to translocation of carbohydrates (Alexander and Hadley 1985) and phosphate (Alexander et al. 1984). This work provided evidence that carbon is supplied via external mycorrhizal mycelium to advanced but not yet mature plants of *G. repens* possessing three or four leaves. However, in similar experiments using mature plants of *G. repens* (possessing five to seven leaves), no detectable ^{14}C was transported to the plant (Alexander 1987). It thus appears that *G. repens* eventually becomes fully autotrophic for carbon and the same may be true for *Dactylorhiza majalis* (experiments by Hadley, see Alexander 1987). In contrast, phosphate transfer to the plant via *Rhizoctonia repens* occurred in both young and older plants and was greatly reduced when the activity of this fungus was restricted by application of a fungicide. The mycorrhizal system of adult orchids therefore appears similar to other mycorrhizal systems of ecto, vesicular-arbuscular mycorrhizal and ericoid plants with respect to P nutrition. However, no evidence for carbohydrate transfer from photosynthetic orchids to associated symbiotic fungi has been obtained (Alexander and Hadley 1985; Purves and Hadley 1975; Smith et al. 1969). The present study was conducted to investigate the growth responses of a leafy epiphytic orchid (a *Cattleya* hybrid) to carbohydrate supplies when grown in symbiosis. External carbon may be beneficial to epiphytic orchids as was shown in the terrestrial orchid *G. repens*.

Materials and methods

Symbiotic fungus

The fungus used in this investigation was *Tulasnella asymetrica* (Isolate 0632) supplied by J. H. Warcup, Kingswood, South Australia. The fungus was maintained in culture on Czapek Dox (minerals diluted six-fold).

Seed material

Ripened seeds of the orchid *Cattleya* (*aclandiae* × *schoeffeldiana*) × *aclandiae* were supplied by Kenntner Orchideenzucht, Steinheim/Sontheim St., Germany. Seeds were sterilised for 2 to 3 min in 5% (w/v) sodium hypochloride on a vacuum frit. After removal of the solution by suction, the seeds were washed with sterile distilled water.

Culture media

Nutrient media were based on Burgeff Sb medium (Burgeff 1936), supplemented with 0.02% yeast extract and NaEDTA (100 μM) and ferrous sulphate (100 μM) as iron source and solidified with 1% DIFCO agar. Water agar medium consisted of 1% DIFCO agar in distilled water. All media were adjusted to pH 5.7 and autoclaved at 121 °C for 20 min.

Experimental design

Small groups of surface-sterilised seeds were dispersed on split plates (90 × 14 mm) with a barrier dividing them into two equal halves (side A and side B). Side A (nutrient side) was inoculated with a mycorrhizal fungus under sterile conditions 14 days before the start of the experiments to enable the fungus to grow over the barrier and colonise side B (water agar side). The media on side A contained different starch levels: minerals and no starch (A0), minerals and 0.1% starch (A1), minerals and 0.5% starch (A5) and minerals and 0.5% starch without yeast extract (A5-Y).

The experiment was conducted in three stages. In stage 1 (0–22 weeks), germination of seeds and protocorm development on side A (A1 and A5) was compared to the development on side B. In stage 2 (22–43 weeks), protocorms originating from treatment A5 were subcultured in week 22 on to new fungal plates, with groups of 8 protocorms each on side A (A1 or A5) and side B. In stage 3 (43–78 weeks), protocorms originating again from treatment A5 were subcultured in week 43 as single plantlets on either side A (A0, A1, A5 or A5-Y) or side B.

Culture conditions

Cultures were maintained in a tissue culture room and illuminated for 16 h daily with artificial light (White L7, Thorne EMI, 40 μmol $m^{-2}s^{-1}$) in addition to natural day ligth (80 μmol $m^{-2}s^{-1}$). The temperature throughout the experiments was 25 ± 1 °C.

Data collection

Because the most advanced and comparable plantlets from one stage were used for subsequent stages, the number of replicate plates varied between treatments and at different stages during the investigation. In the first and second stages, each treatment consisted of 10 replicate plates (with the exception of A5, which consisted of 5 replicate plates). In the third stage, each treatment consisted of 8 replicate plates. Data on dry weight were obtained at the end of each stage by drying the plantlets in an oven for 48 h (80 °C) and calculating the mean dry weight of protocorms. Germination of seeds and growth of protocorms and plantlets was observed under a dissecting microscope at regular intervals during the experiment. In the third stage, the lengths of leaves and roots were marked on the plastic petri dishes (immediately after subculturing at week 43) and new growth recorded by measurement. Infection of roots was estimated under the dissecting microscope as percent root length colonised by the fungus. An analysis of variance was conducted on the data for average dry weight of protocorms and plantlets and a student's *t*-test was performed to find significantly different means with $P < 0.05$.

Results

Development of Cattleya

Stage 1: germination and protocorm development. Germination of seeds of *Cattleya* on side A (0.5% starch) was apparent by day 6 as enlargement of the embryos.

Table 1. Time taken (days) to reach the different stages of developmental of *Cattleya* on different media during stage 1 of the experiment. The medium on side A of split plates contained starch at the concentrations shown. The medium on side B contained water agar only

Position on split plate	0.1% starch		0.5% starch	
	A	B	A	B
Enlargement of protocorms	6	7	6	7
Development of chlorophyll	10	13	10	13
Emerging rhizoids	14	12	12	14
Heart shape	22	24	23	23
Leaf initials	33	33	33	35

Table 2. Mean dry weight (mg) of protocorms ± standard errors of the means after 22 weeks growth on side A of split plates (minerals plus starch at 0.1% or 0.5%) or side B (water agar only). Values marked with an asterisk are significantly different from A1 with $P<0.05$

A1	B1	A5	B5
0.27±0.06	0.22±0.05	0.72±0.15*	0.65±0.10*

Table 3. Mean dry weight (mg) of plantlets ± standard errors of the means after 43 weeks growth on side A of split plates (minerals plus starch at 0.1% or 0.5%) or side B (water agar only). Values marked with a asterisk are significantly different from A1 with $P<0.05$

	A1	B1	A5	B5
Shoots	1.07±0.10	1.00±0.09	2.41±0.39*	1.80±0.22*
Roots	2.63±0.27	2.46±0.32	7.59±1.35*	7.47±1.29*
Shoot/root ratio	0.41±0.06	0.41±0.07	0.33±0.09*	0.25±0.07*

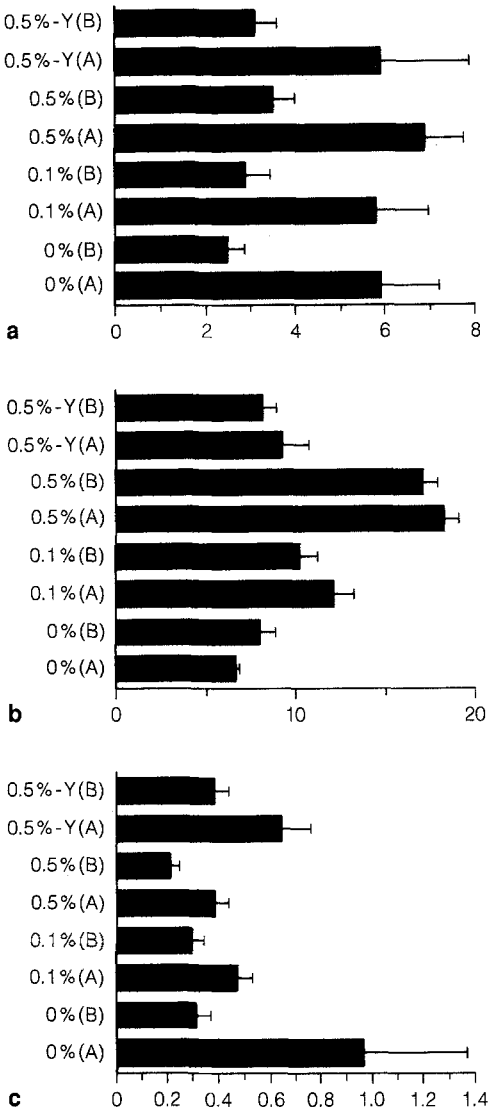

Fig. 1. The mean dry weights (mg) of **(a)** shoots and **(b)** roots of plantlets reached after 78 weeks growth on either side A or side B of split plates (see Experimental design). **(c)** The shoot/root ratios of plantlets after 18 months. *Y*, Yeast extract

Subsequent development consisted of greening (day 10), emergence of rhizoids (day 12), change from ovoid to heart shape (day 23) and the initiation of the first leaf (day 33). Only small differences were observed with the other experimental conditions (see Table 1). Growth on side B was similar to the growth on the corresponding nutrient agar. Totals of 29 to 56 plantlets, some more advanced than others and some merely developed protocorms, were counted in symbiotic cultures. Observations on the growth of protocorms indicated that on side A (0.1% starch) growth slowed down after 6 weeks but on side A (0.5% starch) it continued until 12 weeks. The dry weigth after week 22 was highest in cultures with 0.5% starch present (Table 2). The average plantlet on 0.5% starch agar (side A and B) had two small leaves and a small root.

Stage 2: development of 22- to 43-week-old plantlets. After 22 weeks, protocorms were subcultured to preinoculated split plates and new growth was apparent within 3 days with the emerging of new root hairs (rhizoids) near the root tips and the subsequent elongation of roots. New shoot growth was first observed after 1 week. Root growth continued for longer on 0.5% starch (side A and B) and reached a higher dry weight compared to 0.1% starch (side A and B) (Table 3); shoot growth was slower on A5 than on A1 but continuous. Plantlets harvested at week 43 again had the highest dry weight with 0.5% starch (side A) (Table 3). Symbiotic plantlets had several long roots but shoots were small and the root/shoot ratios (Table 3) were higher with 0.1% starch than with 0.5% starch.

Stage 3: development of 43 to 78 weeks old plantlets. After 43 weeks, plantlets were subcultured as single individuals per plate. The data for dry weight of shoots, roots and shoot/root ratios are given in Fig. 1a,b,c. Shoot growth is given in Fig. 2 and root growth is given in Fig. 3, as measured during the following weeks. Shoot growth on side A was greater than that on side B, especially with 0.5% starch. However, root growth on

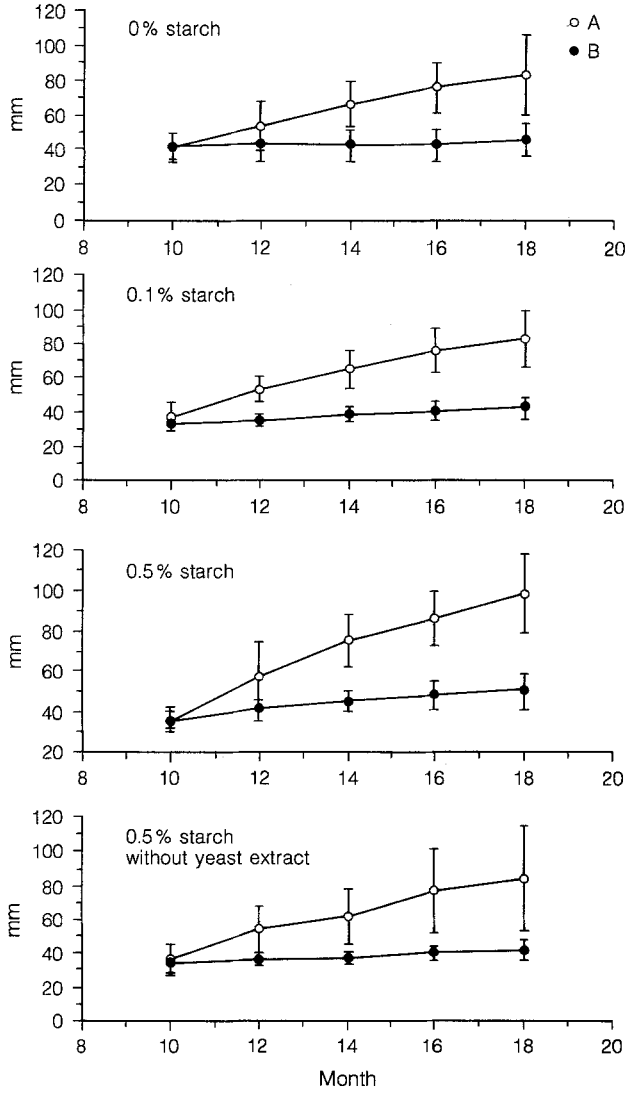

Fig. 2. The change in mean shoot length with time of all shoots present per plantlet on media containing starch at different concentrations. *A*, Side A; *B*, side B

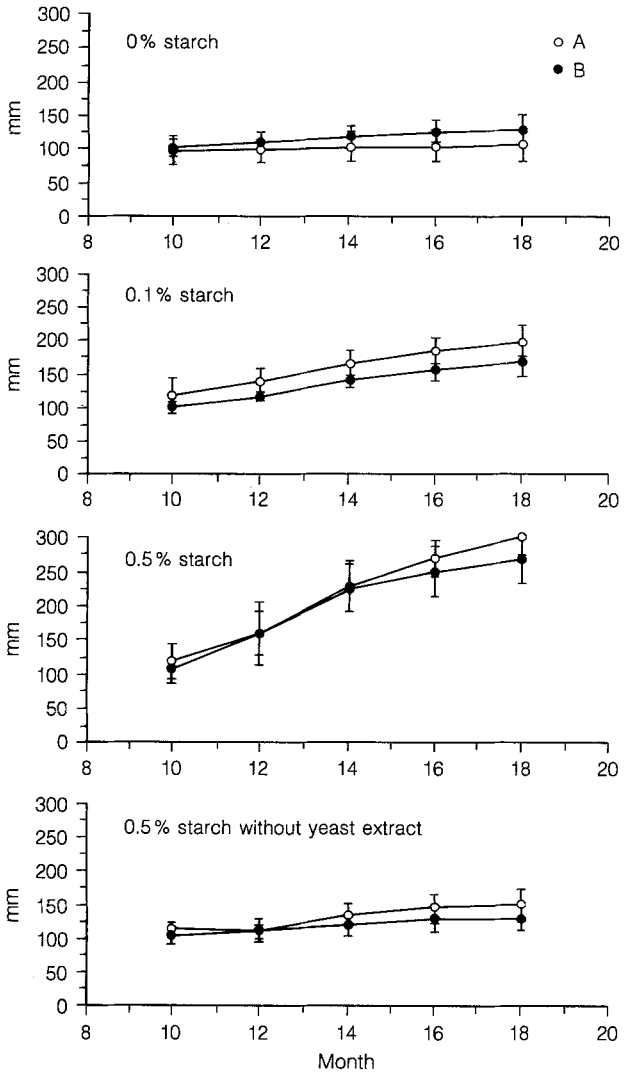

Fig. 3. The change in mean root length with time of all roots present per plantlet on media containing starch at different concentrations. *A*, Side A; *B*, side B

side A was only slightly greater than that on side B. Roots developed more prolifically with 0.5% than with 0.1% starch. Plantlets with 0.5% starch grown on side B had the lowest shoot/root ratio and had very long, yellowish-green roots with a comparatively small yellowish-green shoot. Shoots were dark green with zero starch, but slightly yellowish on side B when 0.1% starch was present.

Infection of roots

Symbiotic plantlets were examined for the presence of the mycorrhizal fungus in the roots and the following pattern was observed. At week 22 (first harvest), protocorms were strongly infected, but contained mainly digested coils with few hyphae present and the small roots were rarely infected. Examination of roots after 43 weeks showed many patches of infection 1 to 4 mm long. The hyphal coils were mainly digested and restricted to the older roots. Recently grown roots were usually free of infections. Infections were prevalent in roots attached to the agar, but were also observed in roots growing along the lid of petri dishes. At this time, less than 5% of the root length was infected in all treatments. Examination at the end of the experiment showed that all roots present at the time of transferring from one stage to another became infected on the new fungal plates, but roots developing after transfer stayed free of infection. Infection percentages were variable and usually higher on starch agar compared to starch-free agar, with no obvious differences between sides A and B. None of the plantlets examined was free of infection and all contained at least a few intact and undigested hyphae.

Discussion

The symbiotic method of raising orchid protocorms is well established for many terrestrial and epiphytic orchids (Clements 1987; Clements and Ellyard 1979; Clements et al. 1986; Warcup 1973), but this is the first experimental study of the development of leafy symbiotic plantlets over a prolonged period. The present study showed that the amount of carbon available to the symbiotic fungus determined the growth of the mycorrhizal orchid. Carbohydrates were important for early growth of green, leafless protocorms but had little effect on further development of leaves when leaves were already present. However, development of roots showed a different pattern with carbohydrates promoting a more extensive root system independent of the plantlet position on the plate (side A or B). The overall result was differences in shoot/root ratios, these being greatest in the complete absence of carbohydrates.

Harvais and Hadley (1967) showed that in nongreen protocorms of the terrestrial orchid *Dactylorhiza purpurella* growth was renewed by drip feeding of dextrose or fructose after growth in old symbiotic cultures had ceased. The same result was obtained with cellulose (Hadley and Williamson 1971). Further experiments by Beyrle et al. (1991) showed that the amount of carbohydrate available to the system is, within certain limits, proportional to the heterotrophic growth of the orchid, with 0.5% starch sustaining growth for 12 days (day 4 to day 16) and 1% starch sustaining growth for 20 days (day 4 to day 24). Here we have shown this growth promoting effect of external carbon in *Cattleya* in all three stages (up to 78 weeks), although in stage 3 it was restricted to root growth. The fact that there was no difference between side A and B suggests that translocation of carbon was effective. Photosynthesis is unlikely to have been important in stage 1 but may have begun to play a role in carbon nutrition by the end of stage 2. A different picture emerges from stage 3, with plantlets on side A growing well in the absence of external carbon. Plantlets obtained from asymbiotic cultures on Knudson C (Knudson 1946) (unpublished work) had shoot/root ratios greater than one, indicating that the shoot/root ratio is not genetically fixed in this *Cattleya* hybrid but is related to nutrition. Furthermore, root development in *Cattleya* was poor in the presence of starch, when the activity of the mycorrhizal fungus was reduced by the omission of yeast extract, indicating that the observed developmental pattern was due to mycorrhizal infection. The ability of the orchid to absorb nutrients from its mycorrhizal fungus is possibly restricted. Minerals directly available to roots (side A) significantly increased the development of leaves, especially with advanced plantlets in stage 3, indicating direct nutrient uptake via roots. In the absence of external carbon, leafy advanced plantlets of *Cattleya* showed good development on side A where minerals were directly available to the roots. Development on side B, however, was poor in the absence of external carbon. This suggests that carbon is necessary for translocation of minerals via mycorrhizal hyphae. As the direct availability of minerals for epiphytic orchid roots in the natural habitat is very limited (Curtis 1946), external carbon may be important for growth and development in nature, even for adult orchids, at least as long as carbon is not supplied to the mycorrhizal fungus by the photosynthetic orchid.

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