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Occurrence of arbuscular mycorrhiza in *Castanospermum australe* A. Cunn. & C. Fraser and effects on growth and production of castanospermine

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Abstract *Castanospermum australe* A. Cunn. & C. Fraser is the only species of the genus *Castanospermum* (the Moreton Bay chestnut or black bean) native to NE Australia. One constituent of the plant, castanospermine, can inhibit the AIDS virus. The present study investigated possible symbioses between its roots and arbuscular mycorrhizal (AM) fungi. The effects of mycorrhizal fungi on the growth of the plant and yield of alkaloid castanospermine were also studied. The mycorrhizosphere soil and roots of *C. australe* collected from various sites in and around Sydney, Australia showed AM symbiotic associations with roots, with arbuscules and vesicles in the root cortices. Wet sieving and decanting yielded AM fungal spores, mainly *Glomus* spp. A positive correlation was found between AM fungal infection and the castanospermine content of seeds of field-grown trees. Field study results were confirmed by growing seedlings under greenhouse conditions and inoculating them with *Glomus intraradices* Schenck and Smith (INVAM isolate KS906) and *Gigaspora margarita* Becker & Hall (INVAM isolate BR444–2). The AM fungi increased the growth and P contents of plants and the yield of castanospermine in the leaves, irrespective of the P treatment. No correlation was found between the alkaloid contents of leaves from mycorrhizal seedlings and from non-mycorrhizal plants which received P. No significant difference in the production of castanospermine was found between P treatments when *G. margarita* was used as inoculum.

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Introduction

The importance of plants in medicine and a growing interest in the potential medicinal properties of Australian trees has led to an increase in research programs to identify and evaluate such trees (Lassak and McCarthy 1983). Castanospermine, an alkaloid of the indolizidine type (Hohnenchutz et al. 1981), is synthesized by the monotypic Australian rainforest and riverine tree species. *Castanospermum australe* A. Cunn. & C. Fraser and is effective against AIDS (Hadwiger et al. 1986), Pompe's disease (Reichmann et al. 1987), diabetes (Rhinerhart et al. 1990) and cancer (Spearman et at. 1991). Most studies of this plant have concerned the medicinal values of castanospermine and very few were carried out in Australia. These studies were mostly limited to veterinary and botanical aspects (Reichmann et al. 1987; Herwitz 1993, Leishman and Westoby 1994).

Medicinal plants in India were originally reported to be non-mycorrhizal, probably due to the presence of various secondary metabolites (Mohankumar and Mahadeven 1984). However, roots of field-grown garlic were found to be colonized by arbuscular mycorrhizal (AM) fungi (Shuja and Khan 1977) and this observation has more recertly been supported by many workers from Asia who found the roots of various medicinal plants to be mycorrhizal (Laksman and Raghavendra 1990; Sullia and Sampath 1990; Sharma and Roy 1991; Ueda et al. 1992; Burni et al. 1994; Srivastava and Basu 1995; Ratti and Janardhanan 1995). The occurrence, biodiversity and taxonomy of AM fungi in native Australian medicinal plants in general, and *C. australe* in particular, have not been described, except in a passing reference by Reddell and Theodorou (1990). The present study is the first detailed report of AM in this native species (Abu-Zeyad et al. 1993; Abu-Zeyad and Khan 1995).

The present report concerns the occurrence of AM infection in roots and AM fungal propagules in the rhizosphere of field-grown trees of *C. australe* in relation to the castanospermine contents of leaves and seeds. The effects of AM infection on growth and castanospermine yield of seedlings grown from field-collected seeds was also studied under green house conditions.

Materials and methods

Field collection sites

Mycorrhizosphere samples and plant material including roots were collected from three sites in and around Sydney on a monthly basis for 12 months. The sites were selected according to legal and physical access and knowledge of the presence of this plant in the area. The study sites were Sydney Botanic Gardens (trees grown in rain forest conditions), Cook Road in Kensington and Werona Road in Gordon, where the trees were growing by the roadside.

Field material collected

Seeds, roots, leaves and rhizosphere soil of field-grown *C. australe* trees were collected from the study sites. Using an auger, five rhizosphere samples were collected at each site from around the tree at a depth of 10–30 cm and about 1 m away from the tree. The five 10-cm diameter × 20-cm deep core samples collected from each tree were mixed to form one composite sample for further study. The roots were separated, washed with tap water to remove attached soil and fixed in 70% ethanol. Seeds were separated from pods and stored at 4 °C for further treatment.

Mycorrhizosphere soil characteristics and AM fungal infection of roots

The pH, moisture, and P contents of the rhizosphere soil samples were measured after 1 day storage at 4 °C. Total P (Blackmore et al. 1987) and available P (Colwell 1963) were determined.

The wet sieving and decanting method adopted from Brundrett et al. (1996) was employed for AM fungal spore extraction from the soil samples. The roots fixed in 70% ethanol were stained with the non-vital stain 0.05% trypan blue (Phillips and Hayman 1970) and the percentage of roots colonized by AM fungi determined by the root slide method of Giovannetti and Mosse (1980). Field-collected fresh root samples were also stained with the vital stain di-Na succinate-$6H_2O_2$.at 2.5 M, as proposed by Hamel et al. (1990) and modified by Schaffer and Peterson (1993).

Castanospermine content

Castanospermine was extracted from leaves and seeds of *C. australe* and analyzed according to Hohenschutz et al. (1981) and Nash et al. (1986). The GC separation was performed on a DANI 8500 machine equipped with a flame-ionization detector, and a 30 m × 0.53 mm × 3.0 μm film thickness HP-1 (cross-linked methyl silicon gum) fused silica capillary column. Data was acquired with a DAPA (Data Acquisition and Plotting Analysis) system on an Epson 386 computer. The trimethylsily derivative of castanospermine was used because the alkaloid is too polar and insufficiently volatile to be successfully chromatographed.

AM infection of seedlings

Seeds of uniform shape, size and weight were selected from the field-collected material of *C. australe*, surface sterilized with 3% H_2O_2 for 15 min followed by 3–4 washings with sterilized distilled water, and germinated on moistened filter paper in a Petri dish at 25 °C for 2 weeks. Uniform germinated seeds were planted in 17 cm × 15 cm pots filled with sterilized sand with one seedling per pot. Half the pots were each inoculated with 1 g of a sand culture of the AM fungus consisting of sand, AM fungal spores and AM root segments added to the planting hole before insertion of the seed. The seed was then placed in the hole with the emerging radical facing down. The AM fungi, *Glomus intraradices* Schenck and Smith (INVAM isolate KS906) and *Gigaspora margarita* Becker & Hall (INVAM isolate BR444-2) were used for infection. The control pots received AM-spore free soil leachate. The pots were placed in a greenhouse shaded with green shade cloth (50 μm) and subjected to 16/8 h light/dark cycle with a photosynthetic photon flux density of 500 $\mu mol/m^{-2}\ s^{-1}$ (Osram HQI 250 W/D). The temperature was maintained at 25–27 °C by a Braemar (SE A 200 GY) air cooler. Hoagland's solution (minus P or supplemented with 50 mg/l^{-1} H_3PO_4) was used as plant nutrient. About 50 ml of the respective nutrient solution was added to each pot every 10 days. A NETAFIM irrigation system was used with a water flow rate of 25 ml/h^{-1}. The pots were arranged in a completely randomized block with treatments in factorial combination in five replicates per treatment.

After 24 weeks, seedlings were harvested for dry weight analyses by drying in an oven at 100 °C to constant weight. P in the leaves of seedlings was determined by digestion of plant tissue with HCl and analysis of the digest on a Shimadzu UV-160 UV-Visible Recording Spectrophotometer. The leaves were also analyzed for castanospermine using the method described above.

The data collected from the field and the greenhouse study were analyzed by Minitab. The data were subjected to one-way ANOVAR and c^2 test.

Results

Field soil characteristics

Soil collected from all three sites had low pH values, ranging from 4.6 (Cook Road and Werona Roadd) to 5.3 (Sydney Botanic Gardens). The moisture content of the soil collected from the Sydney Botanic Gardens was higher (23%) than that from the roadside areas (4.8–5.3%). The available P level was significantly ($P>0.05$) higher (17.6 ppm) in the soil samples from the Sydney Botanic Gardens than the other two sites (6.8 ppm Cook Road and 4.2 ppm Weronora Road). Total P was also significantly higher (25.2 ± 4 ppm) in the soil from the Sydney Botanic Gardens than that from Cook Road (12.4 ± 3 ppm) and Werona Road (9.8 ± 3 ppm).

AM fungal spores in the mycorrhizosphere of field trees

Spores of *Glomus* spp. (70–155 per 100 g soil), ranging in size from small (150–200 μm) to large (200–450 μm), with thick, brown walls and subtending hyphal attachments, were recoveredfrom the mycorrhizospheres of all trees from the three sites. The rhizospheres of trees

at the Sydney Botanical Gardens also contained aggregated spores or sporocarps, mainly of *Glomus* spp.

AM fungal infection of roots from field trees

The roots of all trees examined were found to possess dual symbioses of arbuscular mycorrhizaes and N-fixing nodules. The mycorrhizal roots were heavily colonized with both intra- and intercellular AM fungal hyphae and vesicles, indicating that AM fungal infection of secondary metabolite-producing *Castanospermum australe* is possible under field conditions (Table 1).

The cortical cells of roots from all three sites harbored arbuscules at various stages of development. Septate hyphae were also observed in root samples from Werona Road. No significant seasonal fluctuations in percentage of AM infection were noted for the field trees ($P>0.05$). All root segments infected by AM fungi contained both vital and non-vital fungal hyphae and vesicles.

AM fungal infection and pH and moisture contents were positively correlated in the samples from the Botanic Garden and Cook Road, but poorly correlated for the Werona Road samples. P contents of the rhizosphere and percentage AM fungal infection in the root cortices were negatively correlated (Table 2).

Castanospermine content

Castanospermine contents of seeds collected from mature trees in the Sydney Botanical Gardens were significantly higher than in those collected from the roadside sites (Table 1). No significant difference in the castanospermine content of seeds collected from the two roadside sites was found. Mature leaves from trees growing at all three sites contained lower amounts of castanospermine than their respective seeds ($P<0.05$) (Table 1).

AM fungal infection of roots and castanospermine contents of leaves collected from the Sydney Botanic Gardens were highly correlated ($r=0.567$). There was also a significant correlation between castanospermine content and AM fungal infection in the Cook Road ($r=0.423$) and Werona Road ($r=0.463$) samples.

Greenhouse experiment

All the inoculated seedlings became mycorrhizal and percentage AM infection did not differ significantly in roots with or without P (Table 3). A few arbuscules were seen in stained roots of inoculated seedlings grown in the greenhouse. Staining of the infection structures, ie. hyphae, arbuscules and vesicles, showed them to be non-viable, whereas staining of the AM fungal hyphae showed them to be metabolically active.

Effect of AM infection on seedling growth and castanospermine biosynthesis

The total dry weights were significantly higher ($P<0.05$) for inoculated seedlings than for the controls where no P was added (Table 3). However, these differences were eliminated when 50 mg/l^{-1} of P was added to the pots. The dry weights of seedlings inoculated with *Glomus intraradices* were significantly higher ($P<0.05$)) than those of seedlings inoculated with *Gigaspora margarita* when no P was added (Table 3). Addition of 50 mg/l^{-1} H_3PO_4 to the pots, however, reduced the difference in dry weights of seedlings inoculated with the two AM fungi.

The P content of leaves from mycorrhizal plants in the greenhouse, inoculated with *Gigaspora margarita*

Table 1 AM infection in roots and castanospermine contents of field-grown trees of *Castanospermum australe* from three different sites during the summer month of February. Values are means of 3 replicates. Mean values in a column followed by different letters are significantly different at $P<0.05$ ($n=10$)

Study site	% Root length colarization (mean ± SD)	Castanospermine in seeds (mg/100 g)	Castanospermine in leaves (mg/100 g)
Sydney Botanical Gardens	$81^a \pm 8$	$20^a \pm 3$	$15^a \pm 6$
Werona Road	$70^b \pm 1$	$15^b \pm 1$	$14^a \pm 2$
Cook Road	$72^b \pm 5$	$17^b \pm 4$	$13^a \pm 1$

Table 2 The correlation coefficients (r) values between AM fungal infection percentages, soil pH, soil moisture contents and P in the mycorrhizosphere soil samples from three study sites. Values are means of 3 replicates

Variable	Sydney Botanic Gardens	Cook Road	Werona Road
AM infection versus pH	0.385	0.557	0.192
AM infection versus moisture content	0.508	0.654	0.050
AM infection versus P	−0.062	−0.368	−0.074

Table 3 AM fungi infection, leaf P contents and castanospermine contents in the leaves of greenhouse-grown *C. australe* seedlings inoculated with AM fungi and cultivated with or without 50 mg/l H_3PO_4 every 10 days. Values are means of three replicates ($n=10$). Mean values in a column followed by different letters are significantly different at $P<0.05$

Mycorrhizal treatment	P treatment (mg/l)	Dry weight (g per plant)	Leaf P (μg per g dry wt.)	Castanospermine (mg per 100 g leaves)	Root Length colonization (%)
Control	0	$0.4^{a}\pm0.05$	$0.3^{a}\pm0.07$	$1.2^{a}\pm0.3$	0.0
Control	50	$1.1^{b}\pm0.41$	$1.3^{b}\pm0.4$	$1.6^{b}\pm0.5$	0.0
Mycorrhizal *Glomus intraradices*	0	$1.0^{b}\pm0.15$	$1.5^{b}\pm0.4$	$2.0^{b}\pm0.4$	$28^{a}\pm7$
Mycorrhizal *Glomus intraradices*	50	$1.2^{b}\pm0.2$	$2.4^{c}\pm0.9$	$3.5^{c}\pm0.6$	$29^{a}\pm6$
Mycorrhizal *Gigaspora margarita*	0	$0.7^{c}\pm0.15$	$1.9^{b}\pm0.6$	$2.6^{b}\pm0.4$	$26^{a}\pm8$
Mycorrhizal *Gigaspora margarita*	50	$1.2^{b}\pm0.3$	$3.4^{d}\pm0.8$	$2.9^{c}\pm0.5$	$32^{a}\pm5$

or *Glomus intraradices* were significantly higher ($P<0.05$) than those of control seedlings with no P treatment (Table 3). However, when P was supplied to the seedlings, no significant dry weight differences between the non-mycorrhizal and mycorrhizal seedlings were observed (Table 3).

Leaf P contents were significantly ($P<0.05$) higher in mycorrhizal seedlings than in the controls, irrespective of the P treatment (Table 3). Addition of AM fungal inoculum to pots without added P did not increase P in seedlings. However, seedlings inoculated with *Gigaspora margarita* and supplied with P contained significantly higher ($P<0.05$) P in leaves than those inoculated with *Glomus intraradices* and treated with P (Table 3).

Mycorrhizal plants contained higher amounts of castanospermine in their leaves than non-mycorrhizal plants, irrespective of P treatment (Table 3). A differential effect of AM fungal inocula and P treatment was, however, noted. Seedlings inoculated with *Glomus intraradices* and supplied with P contained significantly higher amounts of castanospermine than those inoculated with *Gigaspora margarita*. No significant difference in content of castanospermine was recorded between P-minus and P-plus treatments when *Gigaspora margarita* was used as inoculum (Table 3).

AM fungal infection of roots and castanospermine contents of leaves of greenhouse-grown seedlings were more highly correlated in seedlings inoculated with *Glomus intraradices* ($r=0.72$) than in seedlings inoculated with *Gigaspora margarita* ($r=0.58$). No significant correlation was found between the alkaloid contents of leaves from mycorrhizal seedlings and those from the control treatment which received P.

Discussion

These observations of AM fungal associations are the first detailed account for *C. australe* and confirm the passing reference of Reddell and Theodorou (1990). They are also consistent with those of earlier investigators (Ueda et al. 1992; Ratti and Janardhanan 1995; Srivasta and Basu 1995), who reported AM fungal infections in roots of many medicinal plants and AM fungal propagules in their rhizospheres. Although AM infection levels in the roots of *C. australe* varied with location, the roots harbored AM fungal hyphae, vesicles and arbuscules, indicating that the secondary metabolite, castanopsermine, did not prevent AM fungal colonization. Mohankumar and Mahadevan (1984) regarded the absence of AM fungal infection in the roots of medicinal plants as being due to the secondary metabolites produced by the plants. Our results, however, revealed high vesicular and hyphal AM fungal infection with fewer arbuscules. These findings are consistent with those of Selvaraj and Subramanian (1990) and Ratti and Janardhanan (1995), who also reported mainly vesicular infections in the medicinal plants of India. Ueda et al. (1992) reported both vesicles and arbuscules in the roots of Japanese medicinal plants. Some workers report that AM infection significantly changes the chemical composition of plant tissues. Baltruschat and Schonbeck (1975) reported that AM infection increases arginine accumulation in tobacco. Morandi and co-workers (Morandi et al. 1984; Morandi and Gianinazzi-Pearson 1986) found an increase in the concentration of isoflavonoid compounds in AM compared with non-AM soybeans. Flavonoid contents of alfalfa roots were increased by AM infection (Volpin et al. 1994). Peipp et al. (1996) suggested a correlation between the continuous accumulation of secondary metabolites in the roots of some Poaceae members and the establishment of mycorrhizal fungi in these plants.

Staining methods to distinguish metabolically active and inactive AM fungal structures showed that the roots of *C. australe* contain both viable and non-viable AM fungal structures. However, Vierheilig and Ocampo (1991) suggested that the amount of living root which stains for SDH activity is not an indicator of the efficiency of AM symbiosis for plant growth. Schaffer and Peterson (1993) claimed that fresh AM fungus-colonized root incubated in nitro-blue tetrazolium always contains both viable and non-viable fungal structures. Zhao et al. (1996) found that the production of viable AM fungal mycelium depended on the host plant.

Our mycorrhizosphere soil analyses for *C. australe*, a leguminous tree, showed the soil pH to be mildly acidic. This is consistent with results of Ueda et al. (1992), who recorded AM fungal infection in medicinal plants of the Leguminoseae from three sites in Japan and found pH values of 4.2–5.9 in the mycorrhizosphere soils. We found a positive correlation between soil acidity and AM fungal infection in field grown-roots of *C. australis*. These observations contrast with those of Khaliel (1988), who concluded that there is no correlation between AM fungal infection and soil pH in three Indian forest trees. Other workers (Mosse 1972, 1973; Sheikh et al. 1975) noted differences among strains of AM fungi in their tolerance of acidity. Soedarjo and Habte (1993) claimed that AM colonization in the leguminous *Acacia mangium* increased as soil pH rose from 4.3 to 5, but colonization was not significantly influenced by further increase in pH.

Another factor affecting AM fungal colonization of tree roots in the present study was the moisture content: AM fungal infection decreased with increase in moisture content of the rhizosphere. Khan (1993) also noted a positive correlation between redox potential values and AM infection levels in the aquatic trees of Australia. Negative correlations between AM fungal infection and P level in the present study is consistent with observations by other workers (Khan 1972; Allsopp and Stock 1994; Chandrashekara et al. 1995; Rathore and Singh 1995; Asif et al. 1999), who found AM infection percentage to decrease with increasing P. Crush (1974) claimed that the most important factor limiting the performance of tropical legumes is P, since it stimulates nodulation in the legume roots.

Most of the AM fungal spores recovered from mycorrhizospheres of the trees studied belonged to the *Glomus* genus, whereas researchers elsewhere have recovered AM propagules belonging to many different genera in the rhizospheres of medicinal plants (Sullia and Sampath 1990; Ueda et al. 1992; Ratti and Janardhanan 1995).

Castanospermine contents of field-collected seeds and leaves were found to be positively related to AM fungal infection in the roots of *C. australe*. Various authors have reported increased alkaloid, flavonoid, essential oils and other secondary metabolites in AM plants compared with non-mycorrhizal controls (Kape et al. 1992; Volpin et al. 1994).

In the present study, *C. australe* seedlings formed arbuscular mycorrhiza after inoculation with two AM fungal species, confirming the field observations that this plant forms AM mycorrhiza. The differential effects of the AM fungi noted are consistent with the observations of other workers. Plants inoculated with *Glomus* spp. also showed a greater growth response than those inoculated with *Gigaspora margarita* (Medina-Gonzales et al. 1987; Vasanthakrishna and Bagyaraj 1993; Ahiabor and Hirata 1994). Our results also showed higher AM infection percentages in seedlings inoculated with *Glomus intraradices* than with *Gigaspora margarita*. These results are consistent with those of Raverkar and Tilak (1983), who found that soybean plants inoculated with *Glomus* spp. showed more efficiency and AM fungal infection than plants inoculated with *Gigaspora margarita*.

Differences in castanospermine contents of leaves from greenhouse-grown seedlings of *C. australe* inoculated with AM fungal endophytes supported our field observations, i.e. highly mycorrhizal seedlings had higher castanospermine contents, whatever the P treatment. There was a significant difference in the amount of castanospermine extracted from leaves between seedlings inoculated with *Glomus intraradices* and those inoculated with *Gigaspora margarita*. These findings support those of Volpin et al. (1994), who found higher amounts of flavonoid in the leaves of mycorrhizal than in non-mycorrhizal alfalfa. Subhashini and Krishnamurty (1995) reported an increase in nicotine content of tobacco inoculated with *Glomus fasciculatum*. Janardhanan and Abdul-Khaliq (1995) found an increased amount of the essential oil chamomile in mycorrhizal *Matricaria chamomilla*. A positive effect on the growth of some medicinal plants belonging to the family Apocyanaceae of inoculation with AM fungi was reported by Prasad and Sailaja (1995), but no data on secondary metabolite content where provided. Ours is the first correlation study showing a positive effect of AM infection in the roots on the accumulation of castanospermine in the leaves.

Our results also revealed that the castanospermine content of mature leaves collected from the field was higher than those of young leaves of inoculated seedlings in the greenhouse. These observations support those of other researchers (Nash et al. 1988; Donaldson et al. 1990). The two AM fungi used as inoculum differed in their effects on the growth and the alkaloid contents of inoculated seedlings in the present study. AM fungal species are known to have differential effects on the growth of plants (Medina-Gonzales et al. 1987; Vasanthakrishna and Bagyaraj 1993; Ahiabor and Hirata 1994; Asif et al. 1995, 1998), but no comparative studies of AM fungi on biosynthesis of secondary metabolites were found in the literature.

Our data suggest that castanospermine accumulation in plants does not inhibit the ability of the AM fungus to infect the roots. Ridrugo et al. (1989), however, reported that castanospermine inhibited the growth of the fungus *Candida albicans* by inhibition of exoglucanases.

Extraction of castanospermine from roots of *C. australe* was not attempted in the present study and no references to the presence of castanospermine in roots were found, but it would be interesting to conduct such a study. Secondary metabolites are generally synthesized in the roots and translocated to the aerial parts of plants (Waller and Nowacki 1978). Khanam (1999), however, has recently reported biosynthesis of tropane alkaloids in the shoots of *Duboisia myoporoides* generated from calli without pre-cursor feeding and before

root formation, suggesting the complete expression of the biosynthetic pathway in aerial parts.

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