# Final Report to the Australian Flora Foundation on the Project

# Mycorrhizal associations of *Gastrolobium vestitum*

Photos by MD Crisp

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June 2009

# Identification of fungal mycorrhizal species associated with increased plant growth of the rare Fabaceae species *Gastrolobium vestitum*

## Introduction

*Gastrolobium* belongs to the plant family Fabaceae and mostly occurs in Western Australia. Gastrolobiums are small shrubs ranging from prostrate in habit to about 2 metres. Around 30 of the *Gastrolobium* species are known to be poisonous and therefore, little attention has been given to *Gastrolobium* cultivation. An exception is *Gastrolobium celsianum*, previously placed in the genus *Brachysema*, which is the most commonly cultivated species, often under its older names of *Brachysema lanceolatum* and *Brachysema celsianum.*

Some *Gastrolobium* species has a narrow distribution in nature and are considered at risk. One such spe cies is *Gastrolobium vestitum* whereas *Gastrolobium rubrum* is a common species with no apparent survival constraints, both occurring in the Stirling Ranges, Western Australia. Apart from the difference in their distribution, there are many similarities between the species. The species are closely related and belong to the *G. celsianum* group (formerly *Nemcia*), which has red flowers that are putatively modified for bird-pollination and both species grow on skeletal soils in heathland. *G. vestitum* occurs at higher altitudes in the Stirling Ranges and is considered rare, but not at risk, whereas *G. rubrum* can only be found at lower altitudes where it occurs commonly.

Apart from altitudinal differences, the distribution of the *G. vestitum* and *G. rubrum* is likely to be affected by mycorrhizal associations as found in other plant systems (van der Heijden et al., 2003; Hartnett and Wilson, 1999). The relative abundance or occurrence of mycorrhizal types, could influence plant distributions, particularly if the quantity or diversity of the inoculum of one type becomes limited. Mycorrhizal fungi are known to be important mutualists because they receive nutrition and structural refuge from the host, while the host receives benefits through increased resistance to herbivores, pathogens, drought and flooding stress, enhanced competitive abilities, and importantly, they facilitate enhanced nutrient uptake. However, although ectomycorrhizal fungi have been identified on *Gastrolobium* in Australia, little is known on the species of mycorrhizal fungi diversity present, nor about their quantitative contribution to plant growth and survival. Many different species of mycorrhizal fungi can coexist on host plants, but all may not contribute equally to plant growth and health.

Species composition of mycorrhizal fungi may be as important to plant performance as the level of mycorrhizal colonization. Species of mycorrhizal fungi vary in their carbohydrate requirements (Durral et al., 1994), tolerance of environmental extremes (Bunn and Zabinski, 2003), enzymatic capabilities (Finlay et al., 1992), ability to transport water and nutrients (Mitchell et al., 1984), and in their dispersal and colonization strategies (Jones et al., 2003). In a study on birch seedlings, it was shown that the diversity of the ectomycorrhizal community had a larger impact than increased root colonization on nutrient uptake (Baxter and Dighton, 2001). The species composition and diversity may therefore also play a role in the distribution and abundance of *G. vestitum* and *G. rubrum.*

The aims of this study were to identify the ectomycorrhizal (EM) species diversity in *G. vestitum* and *G. rubrum* in natural host populations. This would indicate whether mycorrhizal associations are specific for *G. vestitum* and/or *G. rubrum*, and whether mycorrhizal diversity is associated with host abundance. Secondly we aim to identify which mycorrhizal species contributes most to plant performance. Cross-inoculation experiments were conducted to determine whether mycorrhizal fungi fr om the common species (*G. rubrum*) can enhance growth of the rare species

and thus enable the rare species to be cultivated. Unfortunately the second aim could not be achieved as the inoculation-growth trials were destroyed during a hailstorm in 2007. Therefore, only preliminary results of the growth trials are presented.

Fungal mycorrhizae have been associated with *Gastrolobium* species but mostly include arbuscular mycorrhiza (AM), and to a much lesser extent ectomycorrhizae (EM). Identification of fungal mycorrhiza species associated with Fabaceae has been limited and generally no attempt has been made for species identification. This lack of species identification is partly due to the fact that AM fungal species cannot be cultured on artificial media and they also appear morphologically very similar on roots. The only means of identification of these fungal species is by molecular methods, eg. sequencing. On the other hand, EM can be divided into morphotypes as they usually are morphologically distinguishable on the roots, and some of them are culturable. Nevertheless, sequencing will be invaluable to group these fungi into different taxa. Most fungal mycorrhizal species have not been identified to species level, and thus this project will significantly improve our knowledge of fungal mycorrhizal species/taxa associated with *Gastrolobiums* in Western Australia.

## Materials and Methods

*Sampling of mycorrhizal associations*

Since *G. vestitum* only occurs on Mt Toolbrunnup and Mt Hassell (Western Australia), both sites were included in the survey. *G. rubrum* occurring closest to plants of *G. vestitum* were sampled. In both cases, plants were separated by no more than 1 km with *G. vestitum* present on the higher slopes of the mountains, whereas *G. rubrum* occurred lower down and into the valleys. Roots of ten plants of each species in each of the locations were sampled and inspected for the presence of mycorrhizae with a compound microscope. Two soil core samples were sampled in the rhizosphere (including roots) of each plant, taken from opposite sides and taking care not to disturb the plants.

*Sequence identification ofEM associations*

The ectomycorrhizal root tips from each sample were sorted by morphology and the same morphotypes from the same samples were combined and lyophilized for DNA extraction with the DNeasy Plant Mini DNA kit (Qiagen). Primers ITS 1F and ITS4B (Gardes and Bruns, 1993) were used to PCR amplify rDNA. Amplification products were electrophoresed on 1% agarose gels to verify the amplification of fragments of the appropriate length. Most samples in which mycorrhizal roots was observed with a compound microscope yielded fungal PCR products except for *G. rubrum* samples from Mt Hassell. PCR-cloning were conducted with a pGEM-T cloning kit (Promega) and rDNA fragments were sequenced bi-directionally with ABI PRISM BigDye Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A). The DNA samples were sequenced with an ABI-3 100 automated sequencer. DNA sequences were edited manually with Sequencher 4.5 (Gene Codes Corporation, Ann. Arbor, MI, USA). Sequence alignment was completed in Geneious 4.6.4. Individual sequences were compared to a sequence database on GenBank using a BLASTN search (Basic Local Alignment Search Tool Nucleotide; NCBI, Bethesda, MD).

*Inoculation studies*

Seed of *G. vestitum* and *G. rubrum* were collected from Mt Toolbrunnup, germinated and grown in a sand based potting medium free of mycorrhiza. Seven-month-old seedlings were transplanted to a potting mix containing soil and root segments from either the same species (eg. *G. vestitum* seedlings planted in pots containing *G. vestitum* soil) or congeneric species (eg. *G. vestitum* seedlings planted in pots containing *G. rubrum* soil), or potting medium amended with sterilized soil collected from either *G. rubrum* or *G. vestitum* (no mycorrhizae). Twenty plants per

treatment were used. Plant height and length of each side branch of each plant was measured at the onset of the experiment. After 3.5 months' growth, plant height and length of side branches were measured to determine the possible positive effects of mycorrhizal additions. After 5 month's growth, it was planned that each plant be measured again. It was also planned to sort EM roots into morphotypes, quantify each morphotype, and subsequently identify morphotypes with sequence analyses. Morphotypes were also to be cultivated *in vitro* to use as inoculum in future cultivation studies. Unfortunately, 4 months into this experiment, the greenhouses were destroyed by a hail storm, plants were damaged and knocked out of their pots, rendering completion of this experiment impossible.

Statistical analyses of stem length differences were conducted in Systat. Stem lengths after 3.5 months were calculated as the difference between stem lengths after 3.5 months and those at the onset of the experiment.

## Results

*Sequence identification ofEMfungi*

Various ectomycorrhizal (EM) fungi could be identified from *G. vestitum* and *G. rubrum* roots collected from Mt Hassell and Mt Toolbrunnup in the Stirling Ranges, Western Australia (Table 1). Roots from ten plants in each location from each host was sampled. All plants displayed EM fungi on roots based on a microscopic examination. The EM fungi were identified with direct sequencing of mycorrhizal infected roots. Based on a GenBank BLAST search, the EM fungi were identified as species of *Cortinarius, Laccaria, Russula, Boletus, Inocybe, Nolanea, Xerocomus, Tylopilus, Thaxterogaster* and *Tomentella* (Table 1., Fig 1). None of these species were shared among hosts or sampling location (Table 1, Fig 1). When present, EM fungi belonging to *Cortinarius, Laccaria* and *Russula* were the dominant fungi on a particular plant (Table 2). EM diversity was high, with at least 11 EM associations identified from 10 plants of *G. vestitum* examined at each of Mt Toolbrunnup and Mt Hassell (Table 1).

Microscopic examination of *G. rubrum* samples from Mt Hassell revealed at least four morphotypes of EM present. PCR amplification of these samples with ITS 1F and ITS4B, however, did not produce any fragments of the expected size.

*Growth trial*

Unfortunately the growth trial could not be completed due to a hailstorm destroying glasshouses and plants. Growth measurements taken 3.5 months after seedlings were tra nsplanted into treatment potting mixes were taken and results presented in Figs 2 and 3. No significant differences in stem length (growth) could be obtained for *G. vestitum* seedlings transplanted into a pots amended with soil collected from *G. vestitum* or *G. rubrum,* or sterilized *G. rubrum* soil, or pots amended with roots of EM colonised *G. vestitum* plants. Stem lengths of *G. vestitum* plants grown in a pots amended with soil collected from *G. vestitum* in WA that has been sterilized, was significantly ($P < 0.05$) longer than stems of *G. vestitum* plants of other treatments except plants grown in pots amended with sterilized soil collected from *G. rubrum* plants in WA (Fig 2). Similarly, no significant differences in stem lengths of *G. rubrum* seedlings transplanted into treatment mixes could be observed, except for seedlings grown in pots amended with sterilized soil collected from *G. rubrum* plants in the Stirling Ranges, WA, which performed significantly ($P < 0.05$) worse than any other treatment (Fig 3).

## Discussion

All the fungi identified from EM colonized *Gastrolobium* roots are known mycorrhizae of a variety of plants, although it is the first time that they have been associated with *Gastrolobium*. However, although preliminary species identification has been achieved with the GenBank searches, phylogenetic studies will be essential to identify the fungi to species level, as GenBank can be limited in the species information available. Also, incorrect species assignment of sequences in GenBank has been identified as a significant problem (Harris, 2003; Vilgalys, 2003).

Even though EM morphotypes were observed on *G. rubrum* samples from Mt Hassell, no PCR products could be obtained with the ITS primers used in this study. Although the primers we used are reported to amplify the ITS region of most fungi, other primers annealing at slightly different positions could be used to amplify these samples (Shefferson et al., 2007). The lack of PCR products for these sample therefore does not imply a lack of EM fungi, but rather that these plants were colonised by a different group of EM fungi that could not be amplified with ITS 1F and ITS4B.

EM species were not shared between *G. vestitum* and *G. rubrum*, indicating that the two *Gastrolobium* species have their own suite of mycorrhizal associations. However, mycorrhizal species were also not shared between sites, indicating that *G. vestitum* is capable of associating with many different EM species, pending availability. Mycorrhizal specificity, defined as the phylogenetic breadth of fungi that a plant associate with, is generally broad in most plants other than orchids (Molina et al., 1992; Hoeksema 1999; Massicotte et al., 1999). Indeed, from only 10 plants of *G. vestitum* examined at each of Mt Toolbrunnup and Mt Hassell, at least 11 EM associations were identified. This is encouraging for the survival and/or cultivation of *G. vestitum*, as rare mycorrhizal associations would not appear to pose a limiting factor for its growth. It is still unclear though whether EM fungi of *G. rubrum* would be able to associate with *G. vestitum* roots, as the experiment testing for cross-specificity was terminated due to a hailstorm. It is also likely that the suit of EM associations of *G. rubrum* is equally broad, since failed PCRs of the Mt Hassell collection indicate a different set of EM fungi present.

The only EM genus shared among sites and *Gastrolobium* species was *Cortinarius*. Approximately 50 species of *Cortinarius* are described from Australia. The closest *Cortinarius* matches found on GenBank, however, are not reported to be from Australia (May and Wood, 1997). This is not surprising as very few Australian EM fungi have been sequenced and therefore their sequences will not be available on GenBank. Also, Australian fungi are grossly under described and it is likely that the number of e.g. *Cortinarius* species exceeds the reported 50 species.

It is clear from this study that a large number of EM fungi are associated with the two *Gastrolobium* species studied. As it was shown that EM diversity (species composition) plays an important role in plant diversity and performance (Durral et al., 1994; Bunn and Zabinski, 2003; Mitchell et al., 1984; Baxter and Dighton, 2001), it is encouraging to see a large number of EM associations with the *Gastrolobiu m* species studied. Whether these EM species are host specific and differ in their ability to promote plant growth, however, still needs to be examined.

**References**


Baxter J. W., Dighton J. 2001. Ectomycorrhizal diversity alters growth and nutrient acquisition of grey birch (*Betula populifolia*) seedlings in host-symbiotic culture conditions. *New Phytol* 152: 139Ð149.

Bunn R. A., Zabinski C. A. 2003. Arbuscular mycorrhizae in thermal-influenced soils in Yellowstone National Park. *West NAm Nat* 63: 409–415

Durall D. M., Marshall J. D., Jones M. D., Crawford R, Trappe J. M. 1994. Morphological changes and photosynthate allocation in aging *Hebeloma crustuliniforme* (Bull.) Quel. And *Laccaria bicolor* (Maire) Orton mycorrhizas of *Pinus ponderosa* Dougl. ex Laws. *New Phytol* 127: 719–724.

Finlay R. D, FrostegŒrd A, Sonnerfeldt A-M. 1992. Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougl. ex Loud. *New Phytol* 120: 105–115

Gardes M., Bruns T. D. 1993. ITS primers with enhanced specificity for basidiomycetes Ð application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2: 113-118.

Harris, D. J. 2003. Can you bank on GenBank? *Trends Ecol. Evol.* 18: 317–319.

Hartnett D. C, Wilson G. W. T. 1999. Mycorrhizae influence plant community structure and diversity in tallgrass prairie. *Ecology* 80: 1187–1195.

Hoeksema, J. D. 1999. Investigating the disparity in host specificity between AM and EM fungi: lessons from theory and better-studied systems. *Oikos* 84:327Ð332.

Jones M. D., Durall D. M., Cairney J. W. G. 2003. Ectomycorrhizal fungal communities in young forest stands regenerating after clearcut logging. *New Phytol* 157: 399Ð422.

May, T. W., and Wood, A., E. 1997. Catalogue and bibliography of Australian macrofungi 1. Basidiomycota p.p. Fungi of Australia volume 2A. Australian Biological Resources Study, Canberra.

Massicotte, H. B., R. Molina, L. E. Tackaberry, J. E. Smith, M. P. Amaranthus. 1999. Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by five host species. *Can. J. Bot.* 77: 1053–1076.

Mitchell R. J., Cox G. S., Dixon R. K., Garrett H. E., Sander I. L. 1984. Inoculation of three *Quercus* species with eleven isolates of ectomycorrhizal fungi. II. Foliar nutrient content and isolate effectiveness. *For Sci* 30: 563Ð572.

Molina, R., H. Massicotte, J. M. Trappe. 1992. Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. Pp. 357–423 in M. F. Allen, ed. Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall, New York.

Mauricio, R., 2007. The evolutionary history of mycorrhizal specificity among lady's slipper orchids. Evolution 61, 1380-1390.

van der Heijden M. G. A., Boller T, Wiemken A, Sanders I. R. 2003. Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occurring plants. *New Phytol* 157: 569 –578.

Vilgalys, R. 2003. Taxonomic Misidentification in Public DNA Databases *New Phytol* 160: 4-5.