Many triggerplants (*Stylidium* spp.; Stylidiaceae) form arbuscular mycorrhizal associations

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Introduction

Triggerplants (*Stylidium* spp.; Stylidiaceae) grow in Australia and a few areas to its north, though >95% of the >300 species in the genus *Stylidium* are endemic to Australia (Darnowski 2002). Their name comes from the extremely rapid, active, and resettable pollination mechanism which they use. In addition, they have been recently recognized as possessing at least some of the traits of carnivorous plants, specifically the ability to trap and digest prey insects (Darnowski *et al*. 2006), as well as the more recently-demonstrated ability to transport and, therefore, benefit, from nutrients obtained in that way (Darnowski 2017). Generally, they trap prey which are much smaller than their pollinators. Triggerplants typically are found in extremely nutrient poor conditions where genera such as *Drosera* and *Utricularia* are usually found in the same places.

In these nutrient-poor conditions, associations between plants and arbuscular-mycorrhizal fungi are common. Such mycorrhizal associations are widespread and considered to be classic examples of a mutualism between plants and fungi (Taiz & Zeiger 2010). The plants provide all the carbon to support fungal growth. In turn, arbuscular mycorrhizae (AM)-fungal hyphae penetrate plant root cells and extend into the soil matrix, effectively increasing root surface area and mineral uptake potential. It is well known, for example, that mycorrhizal inoculation of trees can greatly enhance survival and post-inoculation growth after transplantation (Taiz & Zeiger 2010). The carbon demands of the fungus can be high, taking up as much as 20% of the assimilate fixed by the plant.

Thus, mycorrhizae are most persistent when plant nutrient demand is high and photosynthesis can be carried out efficiently. Under shade or higher nutrient conditions, plants may shed mycorrhizae and the relationship becomes facultative (Watson *et al*. 2001). Little is known about whether triggerplants form mycorrhizae, but the conditions which they prefer, bright light and low nutrients, favor both AM formation and carnivory.

The authors examined ten species of triggerplants grown in the greenhouse and not specifically inoculated with AM-fungal spores. These species were selected to give a range of species 1) from different sections of the genus *Stylidium* and 2) from different geographic regions of Australia, in order to assess whether formation of mycorrhizae is likely to be a general phenomenon.

Materials and methods

Plants were greenhouse grown (Darnowski 2003) in Corydon, Indiana, USA. They were started in the US either from seed or from greenhouse-grown stems which lacked roots at the start of the experiment, so any fungi associated with the plants would have come from either the soil mix used (not autoclaved) or from sources within the greenhouse. Ten species, all grown for >1 year in a 1:1 mix of silica sand and sphagnum peat were examined in total (see Table 2 for names). All subsequent steps were undertaken at room temperature (approximately 20-25ºC). Roots were harvested and fixed in 95% ethanol for a minimum of 24 hr. To detect arbuscular mycorrhizal fungi (AMF), roots were rinsed in deionized water and stained using a mixture of 80% (by volume) solution A, and 20% (by volume) solution B. Solution A was 0.3% (by mass) aniline blue in 90% ethanol. Solution B was Lactophenol Blue Solution (all solutions from FLUKA, Fuchs, Switzerland; Marx 1982; Nemec 1982; Ruzin 1999).

Three 1 cm sections of root were selected randomly from each species and used for analysis. Sections were stained for approximately 30 min. then washed in deionized water for approximately 5 min. to remove excess stain. Roots were examined using a student-grade Nikon compound microscope, first at 100× total magnification and then at 1000× for scoring. Fields of view for scoring were chosen randomly by moving the travel controls on the microscope randomly and then scoring the image found as long as root tissue filled the field of view. Images were taken using a Nikon EOS Digital Rebel. Images were cropped and adjusted for levels and brightness and contrast using Adobe Photoshop (San Jose, California, USA).

Fungal colonization was measured using a numerical scale as defined in Table 1, and each of the three samples per species showed similar staining for each of the species examined. The number reported in Table 2 is a typical one for the species, chosen as being either the same as three identical scores or as the midpoint among the three scores if they differed. Ecto- and endomycorrhizae (AM) were distinguished by using the microscope fine focus, with ectomycorrhizae (not counting towards the scores in Table 2) being exclusively on the outside of the root, not penetrating any cells.

Table 1. Definitions for scoring of levels of mycorrhiza formation.

Mycorrhiza Formation Score	Definition, per 3 cm of root
0	No staining or staining structures other than mycorrhizae.
1	1-3 cells in field of view with mycorrhizal infection
2	4-5 cells in field of view with several AM structures per cell or a similar total number of AM structures in a smaller number of cells
3	6-8 cells in field of view with several AM structures per cell or a similar total number of AM structures in a smaller number of cells
4	9-10 cells in field of view with several AM structures per cell or a similar total number of AM structures in a smaller number of cells
5-10	Several clusters of cells per field of view, each scoring from 1-4

*Note that more elaborately detailed or larger mycorrhizal clusters in a given cell raised the score for a field of view by 1.

Table 2. Mycorrhiza-formation scores for various species of triggerplants grown in the greenhouse. Subgenera from Mildbraed (1908).

Stylidium Species	Subgenus, Section of Genus *Stylidium*	Eastern, Western	Typical Score
adnatum R. Br.	*Nitrangium*, *Rhynchangium*	Western	4
brunonianum Benth.	*Tolypangium*, *Echinospermum*	Western	0
bulbiferum Benth.	*Nitrangium*, *Thyrsiformes*	Western	4
caespitosum R. Br.	*Tolypangium*, *Lineares=Stylidium*	Eastern	4
debile F. Muell.	*Tolypangium, Debiles*	Eastern	4
graminifolium Sw.	*Tolypangium*, *Lineares=Stylidium*	Eastern	3
lineares Sw. ex. Willd.	*Tolypangium*, *Lineares=Stylidium*	Eastern	3
scandens R. Br.	*Tolypangium*, *Verticillatae*	Western	10
soboliferum F. Muell.	*Tolypangium*, *Lineares=Stylidium*	Eastern	2
uniflorum Sond.	*Nitrangium*, *Thyrsiformes*	Western	10

Results

From Table 2, it can be seen that all but one of the nine species examined, the exception being *S. brunonianum*, formed mycorrhizae when grown in the greenhouse, with wide variation from species to species in the extent of the mycorrhizae formed. Some, including *S. graminifolium* and *S. scandens*, formed very extensive networks, while other species, such as *S. soboliferum*, had much more modest colonization. Examples of highly infected cells and tissues can be seen in Figure 1.

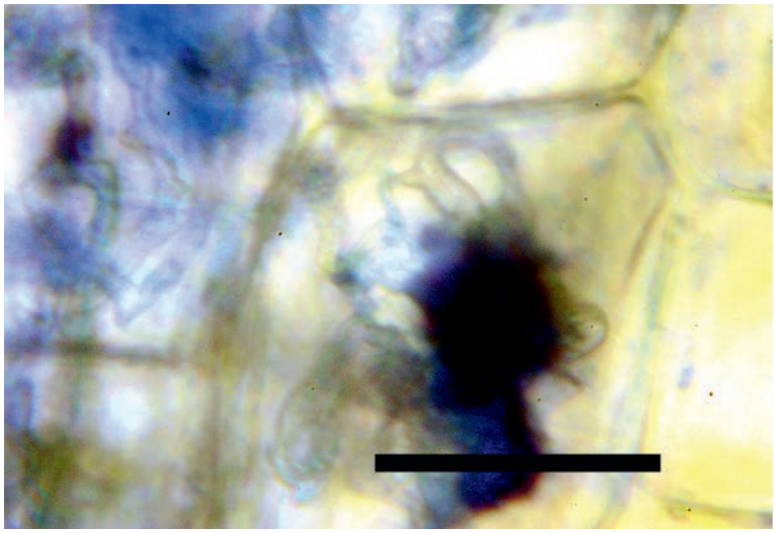

Figure 1: Roots from *Stylidium scandens* grown in the greenhouse in Corydon, Indiana, USA, colonized by mycorrhizal fungi (sample scored 7). The roughly rectangular structures are the plant cells, and the curved and coiled structures within them are the AM mycorrhizal hyphae. Bar = 5 µm.

Discussion

From the observations presented, many species of *Stylidium*, from at least two of six subgenera, including the largest subgenus of *Stylidium* (*Tolypangium*) and a total of six sections, are able to form mycorrhizae. These plants come from temperate regions in both eastern and western Australia. This greatly expands on the meager existing literature on mycorrhizae in *Stylidium*, which simply states that *S. graminifolium* could form synthesized associations with the mycorrhizal fungus *Labryinthomyces* sp. (Kope & Warcup 1986) and that *S. soboliferum* and *S. perpusillum* formed mycorrhizae in the field (Warcup 1980). While *S. graminifolium* and *S. soboliferum* are also considered in this work, providing a useful confirmation of Warcup's (1980) data, *S. perpusillum* adds an ephemeral, temperate species from yet another subgenus, *Centridium*.

In addition, data obtained in other experiments on tropical *S. fimbriatum* (Subgenus *Andersoniana*) show that that species also forms abundant mycorrhizae, at a score of about 9 (Table 1; data not shown). Thus, triggerplants from all areas of Australia, including both tropical and temperate places, are capable of forming mycorrhizae.

Even more interestingly, Kope & Warcup (1986) and Warcup (1980) only reported ectomycorrhizae, while the authors of this paper found endomycorrhizae. There is precedent for this finding, as has been seen *in vivo* and/or *in vitro* for the woody genera *Acacia* and *Eucalyptus* (Boudarga *et al*. 1990; Founoune *et al*. 2002; dos Santos *et al*. 2001). These two genera are both very successful in the flora of Australia, as is *Stylidium*. In both of these woody genera, early associations favored endomycorrhizae, with a shift to ectomycorrhizal associations in older plants; it would be instructive to follow AM formation in *Stylidium* for a longer period of time to determine if this pattern is repeated by *Stylidium*.

The numbers presented here are based on plants grown in the greenhouse, not the field, and all of them were grown from seed in North America. For this reason, these numbers do not rule out that a particular species, here *S. brunonianum*, can form mycorrhizae, and the relative abundance of mycorrhizae in the field in Australia will probably vary from the data shown here since different species of mycorrhizal fungi and different numbers of spores will probably be available.

It is also interesting to note that, overall, the eastern species examined, which come from more fertile soils, scored lower (2-4) than the western species scored (4-10), which live on older and more depleted soils. This could be a genetically-determined featured which would makes sense given that western species need more of the nutrients which AM help to provide.

Nevertheless, the fact that triggerplants can form mycorrhizae adds an extra dimension to their ecological interactions. Given the existence of important parts of the carnivorous syndrome in triggerplants (Darnowski *et al*. 2006; Darnowski 2017) and their unusual pollination mechanism, there are a range of combined ecological interactions to be studied in these plants, such as the use of nutrients from prey organisms by mycorrhizae or the reliance on mycorrhizae for nutrients which are important for processes which enhance the attraction of pollinators. Some of these experiments are already underway.

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