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Arbuscular mycorrhizal fungi persist in dying *Euphorbia ingens* trees

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ABSTRACT

Forest declines have been reported with increasing regularity during the last decade and are expected to increase due to the ongoing environmental changes. During adverse environmental conditions, plant symbioses with mycorrhizas can help to reduce plant stress. Mycorrhizas are symbiotic associations between fungi and roots of living plants. Plants offer carbohydrates to the fungus and the fungus improves the acquisition of nutrients and water to the plant. Specifically, arbuscular mycorrhizal (AM) fungi are the most abundant mycorrhizas. In South Africa, there are increasing reports describing the decline of native *Euphorbia ingens* trees. This study analysed the presence and abundance of AM fungal colonisation in the roots of *E. ingens* trees, and the number of AM fungal spores in the surrounding soil, with the aim to improve the understanding of the rapid decline of these trees. AM fungal colonisation and spores in relation to the soil properties were also analysed. Soil and root samples were collected from different rates of declining *E. ingens* trees at three sites in South Africa. AM fungal colonisation of the roots was assessed and fungal spores in the surrounding soil were enumerated. Soil phosphorus, mineral nitrogen and pH were analysed from the soil samples. The results showed that AM fungi are associated with *E. ingens* trees. AM abundance was influenced by site specific properties and not by *E. ingens* health. Moreover, the level of soil NO_3^- and soil texture significantly influenced AM colonisation in roots and the number of spores enumerated. These preliminary findings provide background information for further research into the large-scale decline of *E. ingens* populations in South Africa.

1. Introduction

Large-scale forest declines have been reported the last decade, and are expected to increase due to the ongoing environmental changes (Allen et al., 2010; Anderegg et al., 2013). Climatic extremes trigger tree die-back due to physiological stresses, yet individuals are most likely finally killed-off by coinciding pest and pathogen outbreaks (Desprez-Loustau et al., 2006; McDowell et al., 2011; Anderegg et al., 2015). During adverse environmental conditions, plant symbioses with soil microbes in the rhizosphere can help to reduce plant stress (Berendsen et al., 2012). Mycorrhizas are symbiotic fungi that establish associations with the roots of living plants. They improve the acquisition of nutrients for plant growth, receiving carbohydrates in return (van der Heijden et al., 2008). Mycorrhiza also directly influence the drought-tolerance of plants during dry periods (Pagano et al., 2013), and can benefit plant health by outcompeting root pathogenic fungi for niche space (Wehner et al., 2010). Tree-mycorrhiza symbioses could thus help to maintain healthy plant development and ultimately improve forest adaptation to adverse abiotic and biotic conditions in the landscape (van der Heijden et al., 2015).

In South Africa there are increasing reports of large-scale die-back of *Euphorbia ingens* populations (Euphorbiaceae; van der Linde et al., 2012; van der Linde et al., 2017). These succulent trees are native to Africa, but most densely populated and charismatic in the xeric northern parts of South Africa (Palgrave et al., 2002). The most common symptoms of *E. ingens* die-back are greying and subsequent death of the succulent branches, browning and rotting of the tissues of the branches, blue stain of the main stem, and high levels of insect infestation (Roux et al., 2008). To date, it is generally accepted that *E. ingens* die-back is triggered by poor rangeland management and climatic variation, with subsequent kill-off caused by secondary pests and pathogens (van der Linde et al., 2017).

The most common and abundant type of mycorrhizas are arbuscular mycorrhizal (AM) fungi, present in *c.* 74% of flora (Brundrett, 2009). The presence of AM fungal communities depends mainly on host plant affinity to such symbioses, but also on the environmental conditions surrounding the host, e.g. soil types and land-use practices (Jansa et al., 2002; Lumini et al., 2010; Oehl et al., 2010; Vályi et al., 2015; Trejo et al., 2016). In some cases, AM presence and abundance can even be influenced by plant–plant interactions. For example,

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Gehring and Whitham (1992) showed that AM fungi in *Juniperus* roots were significantly lower when heavily parasitized by mistletoe. Although AM associations have been reported in the genus *Euphorbia* (Harley and Harley, 1987; Tao et al., 2004; Druva-Lusite and Ievinsh, 2010), there exist no records that these fungi are associated with *E. ingens*. There is also no information regarding the die-off of this unique savanna tree species in relation to its AM fungal symbionts. Analysis of the presence and abundance of AM association in roots of declining *E. ingens* populations could help improve our understanding of the cumulative losses associated with forest decline.

This study aimed to establish the variation in AM fungal colonisation in the roots of *E. ingens* trees that vary in health status. AM fungal spores extracted from soil surrounding the sampled *E. ingens* roots were also enumerated. Finally, key soil properties and their relationship to AM fungal colonisation in the roots and AM spores were analysed.

2. Material and methods

2.1. Study sites

The study was conducted in South Africa at three xeric savanna sites (<600 mm/year) in which *E. ingens* trees were abundant. Study sites included three previously sampled by van der Linde et al. (2012), where we had permission to conduct field studies. Two sites were in the Limpopo province (Last Post: 23°17′21.39″S, 29°55′27.93″E and Capricorn: 23°21′54.6"S, 29°44′37.3″E), and one in the North West Province (Enzelsberg: 25°22′58.05″S, 26°16′4.21″E). Tree individuals were selected using a tree-health indicator based on the grey discoloration and subsequent die-back of the succulent branches (see van der Linde et al., 2012 for more details): 0 = no discoloration; 1 = primary tier braches discoloured; 2 = primary and secondary tier branches discoloured; and 3 = primary, secondary, and tertiary tier branches discoloured. Primary branches represent the lowest, oldest branches. In total, 48 *E. ingens* individuals were selected across the three studied sites, i.e. 16 trees per site (3 sites × 4 grey discoloration categories × 4 trees).

2.2. Sample collection

Samples were collected the first week of August 2015, overlapping with the growing season of *E. ingens* trees that spans from July till the end of October (Palgrave et al., 2002). The growing season represents a period when trees need mycorrhizal fungi due to a higher utilisation of resources (López-Sánchez and Honrubia, 1992). To evaluate AM fungal colonisation of the roots, ~20 fine roots (<1 mm diameter and >20 cm length) were collected per tree. Roots were obtained by excavating from the trunk to the lateral root system on the north and south side of each tree. The root samples were stored in 50% ethanol until analyses were conducted. For the quantification of AM fungal spores and soil characteristics, two soil samples per tree were dug, on the north and south side of each tree. The soil samples were collected from the top 20 cm of the rhizospheric soil with a soil core of 7 cm in diameter. The soil samples were air-dried and stored at 4 °C until they were analysed.

2.3. AM fungal assessment

2.3.1. AM fungal colonisation of the roots

To assess AM colonisation, root samples were washed, cleared and stained according to a modified method of Koske and Gemma (1989). Roots were rinsed and cut into 1–3 cm fragments. The fragments were cleared in 5% KOH at 90 °C for 30 min, then bleached in alkaline H_2O_2 for 10 min. The roots were acidified with 0.1 M HCl for 2 h and stained in lactoglycerol (lactic acid, glycerol, water 13:12:16 (v/v/v)) containing 0.05% trypan blue, at 90 °C for 30 min. Finally, the roots were destained in lactoglycerol for 12 h. Twenty root fragments were randomly selected from each tree, these were mounted on a microscope slide and examined using a light microscope (Zeiss Axioskop 2 Plus, Oberkochen, Germany).

The percentage of AM colonisation was estimated according to Trouvelot et al. (1986), and the following parameters were recorded: frequency of AM in the root system (F%); intensity of AM colonisation in the root system (M%); intensity of AM colonisation in root fragments (m%). These parameters were calculated with Mycocalc, a free mycorrhiza measuring programme (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

2.3.2. AM fungal spores

To measure AM spore numbers, spores from the soil samples were extracted using a wet sieving and decanting method followed by sucrose centrifugation (Schenck, 1982; Smith and Dickson, 1997). To extract the spores, soil samples were sieved through a 2 mm mesh. The sieved soil (100 g) was stirred with 200 ml of water for 5 min and settled for 15 s. The supernatant obtained was decanted through a nest of soil sieves (425 μm, 250 μm, 125 μm, 50 μm), and the remaining debris per soil sieve was collected into centrifuge tubes with water. To purify the spores, the aqueous suspension was centrifuged (1900*g* for 5 min) and the supernatant discarded. The debris was resuspended in 60% sucrose and centrifuged for 5 min (1900*g*). The supernatant obtained was decanted onto the 50 μm sieve and rinsed. The spores decanted onto the sieve were transferred to a filter paper using a Buchner funnel. The number of total spores was counted per sample, with spore abundance expressed as the number of spores per 100 g dry soil.

2.4. Soil properties

Soil phosphorus (P), mineral nitrogen and pH were analysed from a composite soil sample from the north and south hemisphere of each tree. Soil P was extracted from the soil samples according to the P-bray method (Bray and Kurtz, 1945) and determined by automatic colorimetric analysis. Mineral nitrogen defined as ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 1 M KCl (SSSA, 1977) and determined by the Kjeldahl method, using Devarda alloy to reduce NO_3^- to NH_4^+ (Keeney and Nelson, 1982). Soil pH was determined after dilution at a ratio of 1:2.5 soil:water (v/v) using a digital pH meter. The soil texture at Last Post and Enzelsberg were previously classified as sandy loam and were very rocky, whereas the soils at Capricorn are loamy sand and markedly less rocky (van der Linde et al., 2017). All analyses were performed at the Department of Plant and Soil Sciences at the University of Pretoria.

2.5. Data analyses

The influence of tree health status (grey discoloration), the site and their interaction on the AM colonisation (F%, M%, m%) of the roots were analysed using linear models. The number of AM spores was similarly analysed. To account for micro-environmental variations within the tree, the models also included the co-variation with root hemisphere (north- or south-side). Variables indicating AM colonisation were square root transformed prior to analysis to conform to normality. Model validity was also tested by visual examination of residual plots and by assessment of dispersion parameters. When an explanatory variable was significant, individual means were compared by Fisher's least significant difference (LSD) test. The 'agricolae' package of the R software (R Core Team, 2014) was used for linear models.

To examine if soil texture (sandy loam or loamy sand), pH, NH_4^+, NO_3^-, and/or P was significantly associated with observed AM colonisation (F%, M%, m%) of the roots and AM spores, a redundancy analysis (RDA) was used. The significance of the overall ordination (*test on all axes*) was tested using 9999 permutations. A forward selection of variables was used to rank the most important soil properties associated