Arbuscular mycorrhizal fungi persist in dying *Euphorbia ingens* trees

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Highlights

- Arbuscular mycorrhizal (AM) fungi are associated with *Euphorbia ingens* trees.
- AM abundance was influenced by site specific properties and not by *E. ingens* health.
- Soil NO_3^- was related to AM colonisation in roots.
- Soil texture appears to be correlated with AM spore numbers

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, 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: 23º17'21.39''S, 29º55'27.93''E and Capricorn: 23º21'54.6''S, 29º44'37.3''E), and one in the North West Province (Enzelsberg: 25º22'58.05''S, 26º16'4.21''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 utilization 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 ºC 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 ºC 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 ºC 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 (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 sec. 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 & 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 with the response variables (Šmilauer and Lepš, 2014). All response variables were centred and standardized due to their varying measurement units. These statistics were calculated in CANOCO 5 (Ter Braak and Šmilauer, 2012).

3. Results

3.1. AM fungal assessment

AM fungi were strongly associated with *E. ingens* trees, since 46 out of 48 sampled trees were colonised (Fig. 1). AM root colonisation and spore numbers significantly differed between the three sites (Table 1). Specifically, the frequency of AM fungi in the root system (F%) was significantly higher in Last Post when compared to Capricorn and Enzelsberg (Fig. 2a). AM spores were lowest in the more sandy Capricorn soils (Fig. 2c). Interestingly, the change in tree health status only affected the spore numbers (Table 1). Categories indicating varying levels of grey discoloration only affected the number of spores in the surrounding soil (Fig. 2d), and there was no difference in the frequency of AM fungi in the root system (F%; Fig. 2b).

3.2. Soil properties

Soil properties differed between the study sites (Table 2). The sites at Last Post and Enzelsberg had sandy loam soils, which were both markedly rockier than the loamy sand soils found at Capricorn. The Capricorn site also had the lowest P and NH_4^+ content. The site at Last Post had the lowest amount of NO_3^-. The pH of soils varied less among sites (ranged between 6.04 and 6.69), with Capricorn having the least acidic soils on average.

3.3. Relationship between soil properties and AM fungal colonisation of the roots and AM spores

Only the level of soil NO_3^- and soil texture significantly influenced AM colonisation (%F, %M, %m) of the roots and the number of AM spores recorded (Fig. 3). However, AM colonisation of the roots and AM spores appeared to respond along distinct soil gradients. Higher frequencies of AM fungal colonization of the roots were more directly

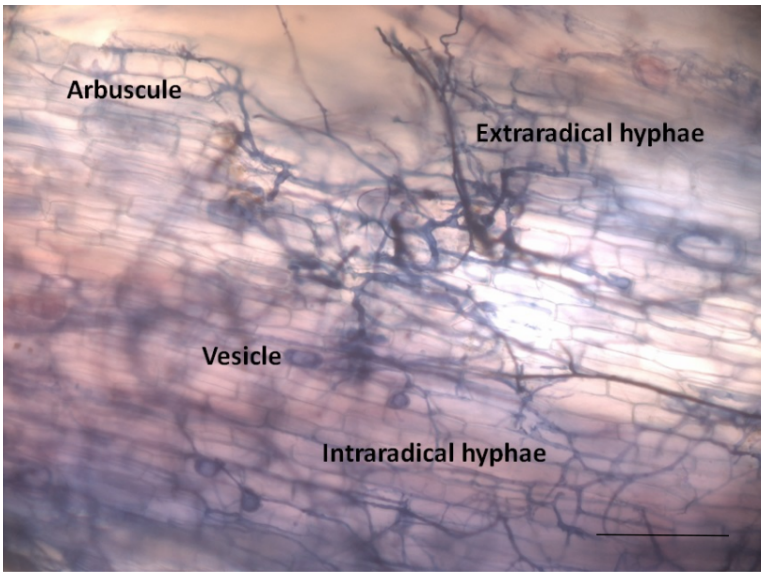

Fig. 1. Arbuscular mycorrhiza hyphae's in a root fragment of a *Euphorbia ingens* tree. Scale bar = 100 μm

Table 1. Results of the ANOVA for the analysis of the AM fungal colonisation of *Euphorbia ingens* roots and the number of spores found in 100 g of soil around each *E. ingens* tree. Bold *P* values indicate $P < 0.05$.

	F%			M%			m%			# AM spores		
	df	F	*P*-value	*df*	F	*P*-value	*df*	F	*P*-value	*df*	F	*P*-value
Site	2	12.53	**< 0.001**	2	1.11	0.336	2	0.06	0.940	2	6.66	**< 0.01**
Grey Discoloration	3	1.47	0.227	3	1.31	0.278	3	1.07	0.366	3	3.09	**< 0.05**
Aspect (N/S)	1	0.56	0.456	1	0.11	0.742	1	0.16	0.688	1	0.61	0.435
Site × Grey Discoloration	6	1.70	0.131	6	1.56	0.170	6	1.03	0.410	6	1.34	0.250
Residuals	80			80			80			80		

*F%: Frequency of mycorrhiza in the root system; M%: Intensity of the mycorrhizal colonisation in the root system; m%: Intensity of the mycorrhizal colonisation in the root fragments.

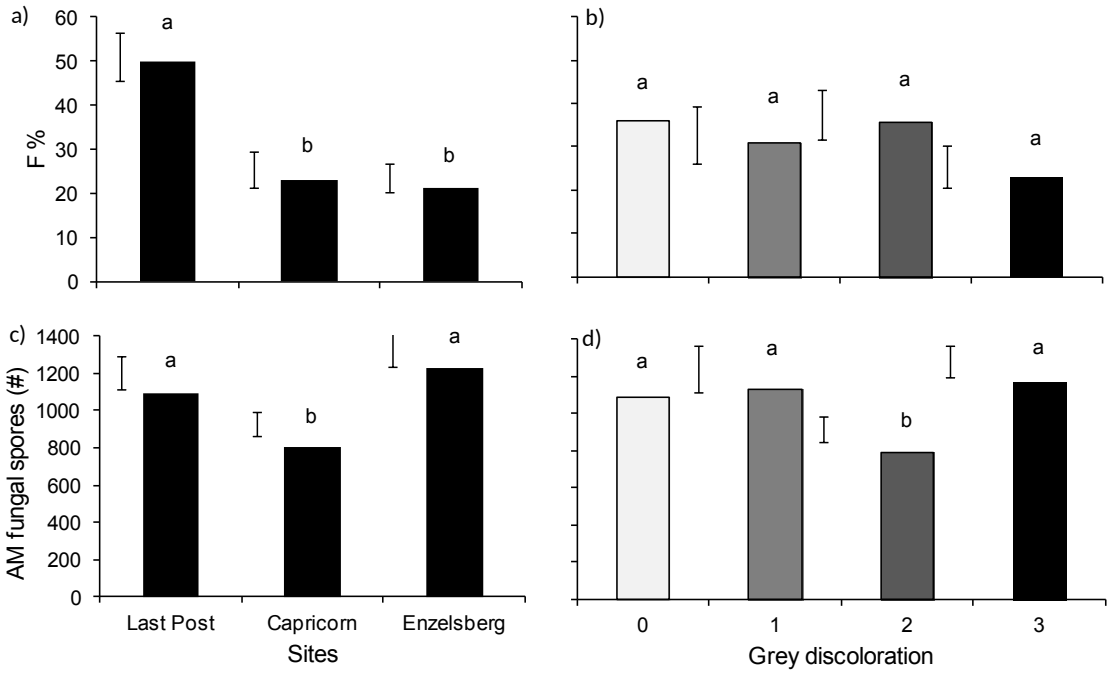

Fig. 2. Frequency of AM fungi in the roots (F%), and AM spore numbers in 100 g of soil, in the study sites (a and c) and between the grey discoloration (b and d) of *E. ingens* trees. Bars show standard errors and different letters indicate significance differences ($P < 0.05$).

Table 2. Soil characteristics surrounding *Euphorbia ingens* trees at each studied site. Means (±1SE) are shown and different letters indicate significant differences between the study sites ($P < 0.05$).

Soil properties	Study sites		
	Last Post	Capricorn	Enzelsberg
Soil texture	Sandy Loam*	Loamy Sand	Sandy Loam*
P (mg/kg)	17.81 ± 3.44 a	5.30 ± 0.85 b	14.41 ± 1.65 a
NH_4^+ (mg/kg)	4.44 ± 0.35 a	2.71 ± 0.21 b	4.11 ± 0.71 a
NO_3^- (mg/kg)	3.96 ± 0.43 b	4.81 ± 0.39 b	7.19 ± 0.66 a
pH	6.06 ± 0.09 b	6.63 ± 0.19 a	6.34 ± 0.09 ab

*Sites with sandy loam soils were also noticeably rockier (higher stoniness).

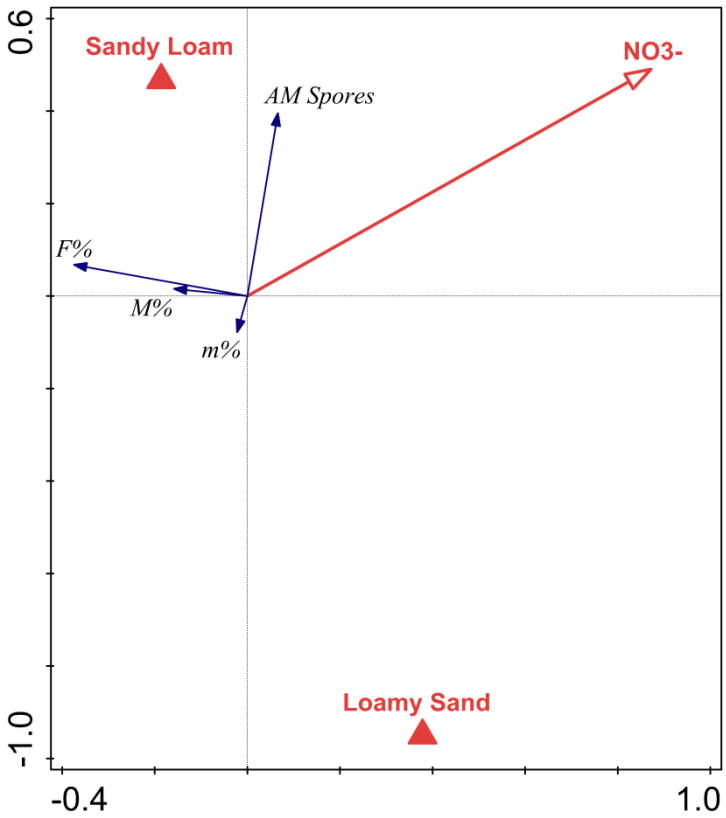

Fig. 3. Redundancy analysis (RDA; test on all axes, P = 0.004) depicting AM fungal colonisation in *Euphorbia ingens* roots and AM spore abundance in relation to the forward selected, significant explanatory soil variables. NO_3^-: Pseudo-F = 3.9, *P* = 0.017; Loamy Sand: Pseudo-F = 4.0, *P* = 0.015; Sandy Loam Pseudo-F = 4.0, *P* = 0.016). F%: Frequency of mycorrhiza in the root system; M%: Intensity of the mycorrhizal colonisation in the root system; m%: Intensity of the mycorrhizal colonisation in the root fragments.

related to soils with lower NO_3^- concentration. Soil texture appears to be more directly correlated with AM spore numbers. In particular, sandy loam soils were significantly associated with higher numbers of AM spores.

4. Discussion

4.1. AM fungal assessment

AM fungi are strongly associated with *E. ingens* in South Africa, as colonisation was recorded in 96% of root samples. Surprisingly, the frequency of AM colonisation in the root system (F%) was not affected by varying levels of tree health. Thus, although the greying of succulent branches is the main characteristic of *E. ingens* decline (van der Linde et al., 2012) it appears that photosynthesis and carbon allocation to the AM fungus is not limiting. This contrasts with other findings where mycorrhiza colonisation was higher in healthy rather than declining trees (Gehring and Whitham, 1992; Power and Ashmore, 1996; Kovacs et al., 2000; Corcobado et al., 2014). This lack of a tree-health influence in our study could be explained by the unique life-history strategy of this xerophytic tree species, and current assumptions about carbon allocation and storage in plants.

Tree die-back is generally calculated as a percentage of crown defoliation. Defoliation decreases photosynthetic activity, which in turn reduces carbon allocation to the roots and thus to mycorrhizas (Finlay and Söderström, 1992; Kuikka et al., 2003). However, *E. ingens* is a succulent, non-foliar tree that evolved from a non-succulent woody ancestor to facilitate living in xeric or seasonally dry environments (Horn et al., 2012). In fact, using stable carbon isotopes, Codron et al. (2005) showed how *E. ingens* had

carbon fractionation values similar to C_4 plants, such as grasses. This suggests that *E. ingens* should have distinct C-dynamics compared to 'normal' C_3 savanna trees.

Compared to C_3 photosynthesizing trees, *E. ingens* utilizes a markedly different photosynthetic pathway, Crassulacean acid metabolism (CAM). CAM allows plants to fix CO_2 at night when it is most available, and thus also limiting excessive water loss (Pearcy et al., 1987; Keeley and Rundel, 2003). Apart from being water-use efficient, another advantage of plants using CAM is the ability to maintain carbohydrate stocks to maintain functioning when being water stressed (Dodd et al., 2002; Winter and Holtum, 2015). The resource efficiency of *E. ingens* individuals may lead to more efficient utilization of carbon stocks during distressed periods, maintaining some payment of the 'cost' of preserving AM fungal benefits. Indeed, after disturbances such as branch die-back, some plants shift carbon allocation to be prioritized to maintain reserve stocks (non-structural carbohydrates), instead of plant growth (Wiley et al., 2016). Given that CAM plants are already photosynthetically efficient in drylands, the complementary role of non-structural carbon reserves being allocated to maintain crucial symbioses after environmental shocks needs further exploration.

4.2. Relationship between soil properties and AM fungal colonisation of the roots and AM spores

Site characteristics influenced AM ecology. However, AM root colonisation appeared to respond along a different soil gradient than AM spores. The frequency of AM colonisation is more likely determined by soil chemistry while the number of AM spores is related to soil texture. In particular, the frequency of AM colonisation in the root system was negatively correlated with the NO_3^-. In general, a higher N availability

in the soil negatively affects fine root quality (Pregitzer et al., 1995), which could decrease the habitat for AM fungi (Treseder and Allen, 2000). Our findings support reports that AM colonisation should be higher in N-limited environments (Blanke et al., 2005). However, to our knowledge there is no defined N threshold inhibiting AM colonisation. Some studies argue that regulation of AM colonisation based on soil N involves complex mechanisms, making it difficult to generalize any observed trends (Treseder and Allen, 2002; Johnson et al., 2003; Staddon et al., 2004; Treseder, 2004). One way forward is to determine which type of N, e.g., NH_4^+ or NO_3^-, would be more likely to influence AM fungal presence and abundance.

The lowest number of AM spores was predominantly associated with sandy soils. Sandy textures favours water infiltration and low nutrient retention (Lehmann and Schroth, 2003). Thus, sandy soils might have favoured lower spore retention in comparison with the more loamy soils. Furthermore, the AM spore abundances observed in our study was considerably higher in comparison to a study conducted in the same region of our Enzelsberg study site (averaging only 80 spores/100 g of soil, Straker *et al.*, 2007). However, it should be noted that the lower AM spore abundance in the latter study is most likely determined by the study system, since it was done in slime dams of gold mines, where soil degradation is arguably much higher than the livestock farming at our sites. Degradation is thus another element that might affect AM spore numbers. Nonetheless, there is increasing evidence that, in general, AM fungal communities primarily respond to prevailing habitat conditions, in particular soil heterogeneity, than to land-use conditions *per se* (de Carvalho et al., 2012; Hazard et al., 2013; Jansa et al., 2014; Cheeke et al., 2015). Of course, in some cases the intensity of disturbance would also influence AM fungal presence (Oehl et al., 2010).

4.3. Conclusions

Euphorbia ingens trees are symbiotically associated with AM fungi, but unexpectedly AM abundance was not influenced by levels of tree die-back. *E. ingens* maintains carbon allocation to roots and AM fungal structures, and in turn, the persistence of AM fungi would remain to supply nutrient benefits. This persistence could slow down tree die-back, but clearly does not prevent die-off, as perpetual loss of branches would inevitably decrease carbohydrate supply beyond critical levels (Galiano et al., 2011). Eventually, both tree and fungus will die. However, persistence of AM fungi suggests that carbon reserves could aid host-recovery should the landscape disturbance fall short of killing the entire *E. ingens* population (Wiley et al., 2016). We further highlight why all efforts should be directed to mitigate large-scale declines of *E. ingens* populations (van der Linde et al., 2017).

The fact that there is a lack of association between AM root colonisation and the number of spores found in the adjacent soil matrix is not unexpected (López-Sánchez and Honrubia, 1992; Uhlmann et al., 2006). The number of spores obtained by sieving methods detects only those species which produce spores and not all spores observed are necessarily viable. Thus, it is conceivable that with our study design the number of AM spores could be an artefact of soil texture. This finding is important for future sampling designs and biological inference. Seasonal samplings may also provide a clearer understanding of the symbiosis. Nonetheless, for these tree-fungal interactions, local site effects most likely determined AM presence and abundance, in particular NO_3^- for the frequency of AM colonisation, and more loamy soils for AM spores.

AM colonisation in grasses decreased in overgrazed xeric savannas in Namibia (Uhlmann et al., 2006). Necessary future studies of *E. ingens* die-offs should also incorporate land-use intensity and plant host combinations to assess AM fungal ecology. Also, increased nitrogen deposition due to agricultural activities may decrease AM populations. This is worrying in times of dramatic CO_2 increases, as these fungi are crucial for carbon sequestration (Treseder and Allen, 2000). Finally, future studies based on molecular identification techniques will help to understand whether AM fungal species richness and diversity are indeed declining with *E. ingens*.

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References

Allen, C.D., Macalady, A.K., Chenchouni, H., Bachelet, D., McDowell, N., Vennetier, M., Kitzberger, T., Rigling, A., Breshears, D.D., Hogg, E.H., Gonzalez, P., Fensham, R., Zhang, Z., Castro, J., Demidova, N., Lim, J-H., Allard, G., Running, S., Semerci, A., Cobb, N., 2010. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. Forest Ecology and Management 259, 660-684.

Anderegg, W.R.L., Hicke, J.A., Fisher, R.A., Allen, C.D., Aukema, J., Bentz, B., Hood, S., Lichstein, J.W., Macalady, A.K., McDowell, N., Pan, Y., Raffa, K., Sala, A., Shaw, J.D., Stephenson, N., Tague, C., Zeppel, M., 2015. Tree mortality from drought, insects, and their interactions in a changing climate. New Phytologist 208, 674-683.

Anderegg, W.R.L., Plavcová, L., Anderegg, L.D.L., Hacke, U.G., Berry, J.A., Field, C.B., 2013. Drought's legacy: multiyear hydraulic deterioration underlies widespread aspen forest die-off and portends increased future risk. Global Change Biology 19, 1188-1196.

Berendsen, R.L., Pieterse, C.M.J., Bakker, P.A.H.M., 2012. The rhizosphere microbiome and plant health. Trends in Plant Science 17, 478-486.

Blanke, V., Renker, C., Wagner, M., Fullner, K., Held, M., Kuhn, A.J., Buscot, F., 2005. Nitrogen supply affects arbuscular mycorrhizal colonization of *Artemisia vulgaris* in a phosphate-polluted field site. New Phytologist 166, 981-992.

Bray, R.H., Kurtz, L.T., 1945. Determination of total organic and available forms of phosphorus in soils. Soil Science 59, 39-45.

Brundrett, M.C., 2009. Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving

conflicting information and developing reliable means of diagnosis. Plant and Soil 320, 37-77.

Codron, D., Codron, J., Lee-Thorp, J.A., Sponheimer, M., De Ruiter, D., 2005. Animal diets in the Waterberg based on stable isotopic composition of faeces. South African Journal of Wildlife Research 35, 43-52.

Corcobado, T., Vivas, M., Moreno, G., Solla, A., 2014. Ectomycorrhizal symbiosis in declining and non-declining *Quercus ilex* trees infected with or free of *Phytophthora cinnamomi*. Forest Ecology and Management 324, 72-80.

Cheeke, T.E., Schütte, U.M., Hemmerich, C.M., Cruzan, M.B., Rosenstiel, T.N., Bever, J.D., 2015. Spatial soil heterogeneity has a greater effect on symbiotic arbuscular mycorrhizal fungal communities and plant growth than genetic modification with *Bacillus thuringiensis* toxin genes. Molecular Ecology 24, 2580-2593.

De Carvalho, F., De Souza, F.A., Carrenho, R., Moreira, F.M., Jesus, E.C., Fernandes, G.W., 2012. The mosaic of habitats in the high-altitude Brazilian rupestrian fields is a hotspot for arbuscular mycorrhizal fungi. Applied Soil Ecology 52, 9-19.

Desprez-Loustau, M.L., Marçais, B., Nageleisen, L.M., Piou, D., Vannini, A., 2006. Interactive effects of drought and pathogens in forest trees. Annals of Forest Science 63, 597-612.

Dodd, A.N., Borland, A.M., Haslam, R.P., Griffiths, H., Maxwell, K., 2002. Crassulacean acid metabolism: plastic, fantastic. Journal of Experimental Botany 53, 569-580.

Druva-Lusite, I., Ievinsh, G., 2010. Diversity of arbuscular mycorrhizal symbiosis in plants from coastal habitats. Environmental and Experimental Biology 8, 17-34.

Finlay, R.D., Söderström, B., 1992. Mycorrhiza and carbon flow to the soil, in: Allen, M., (Ed.), Mycorrhiza Functioning. Chapman & Hall, London, pp. 134-160.

Galiano, L., Martinez-Vilalta, J., Lloret, F., 2011. Carbon reserves and canopy defoliation determine the recovery of Scots pine 4 yr after a drought episode. New Phytologist 190, 750-759.

Gehring, C., Whitham, T., 1992. Reduced mycorrhizae on *Juniperus monosperma* with mistletoe: the influence of environmental stress and tree gender on a plant parasite and a plant-fungal mutualism. Oecologia 89, 298-303.

Harley, J.L., Harley, E.L., 1987. A check-list of mycorrhiza in the British flora. New Phytologist 107, 741-749.

Hazard, C., Gosling, P., van der Gast, C.J., Mitchell, D.T., Doohan, F.M., Bending, G.D., 2013. The role of local environment and geographical distance in determining community composition of arbuscular mycorrhizal fungi at the landscape scale. The ISME Journal 7, 498-508.

Horn, J.W., van Ee, B.W., Morawetz, J.J., Riina, R., Steinmann, V.W., Berry, P.E., Wurdack, K.J., 2012. Phylogenetics and the evolution of major structural characters in the giant genus *Euphorbia* L. (Euphorbiaceae). Molecular Phylogenetics and Evolution 63, 305-326.

Jansa, J., Erb, A., Oberholzer, H-R., Šmilauer, P., Egli, S., 2014. Soil and geography are more important determinants of indigenous arbuscular mycorrhizal communities than management practices in Swiss agricultural soils. Molecular Ecology 23, 2118-2135.

Jansa, J., Mozafar, A., Anken, T., Ruh, R., Sanders, I.R., Frossard, E., 2002. Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12, 225-234.

Johnson, N.C., Rowland, D.L., Corkidi, L., Egerton-Warburton, L.M., Allen, E.B., 2003. Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. Ecology 84, 1895-1908.

Keeley, J.E., Rundel, P.W., 2003. Evolution of CAM and C_4 Carbon - Concentrating mechanisms. International Journal of Plant Sciences 164, S55-S77.

Keeney, D.R., Nelson, D.W., 1982. Nitrogen-inorganic forms, in: Page, A.L. (Ed.), Methods of soil analysis. Part 2. American Society of Agronomy, Madison, Wisconsin, pp. 643-693.

Koske, R.E., Gemma, J.N., 1989. A modified procedure for staining roots to detect VA mycorrhizas. Mycological Research 92, 486-488.

Kovacs, G., Pausch, M., Urban, A., 2000. Diversity of ectomycorrhizal morphotypes and oak decline. Phyton-Annales Rei Botanicae 40, 109-116.

Kuikka, K., Härmä, E., Markkola, A., Rautio, P., Roitto, M., Saikkonen, K., Ahonen-Jonnarth, U., Finlay, R., Tuomi, J., 2003. Severe defoliation of Scots pine reduces reproductive investment by ectomycorrhizal symbionts. Ecology 84, 2051-2061.

Lehmann, J., Schroth, G., 2003. Nutrient leaching, in: Schroth, G., Sinclair, F., (Eds.), Trees, Crops and Soil fertility - Concepts and Research methods. CAB International, Wallingford, UK, pp. 151-166.

López-Sánchez, M.E., Honrubia, M., 1992. Seasonal variation of vesicular-arbuscular mycorrhizae in eroded soils from southern Spain. Mycorrhiza 2, 33-39.

Lumini, E., Orgiazzi, A., Borriello, R., Bonfante, P., Bianciotto, V., 2010. Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach. Environmental Microbiology 12, 2165-2179.

McDowell, N.G., Beerling, D.J., Breshears, D.D., Fisher, R.A., Raffa, K.F., Stitt, M., 2011. The interdependence of mechanisms underlying climate-driven vegetation mortality. Trends in Ecology and Evolution 26, 523-532.

Oehl, F., Laczko, E., Bogenrieder, A., Stahr, K., Bösch, R., van der Heijden, M., Sieverding, E., 2010. Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. Soil Biology and Biochemistry 42, 724-738.

Pagano, M.C., Zandavalli, R.B., Araújo, F.S., 2013. Biodiversity of arbuscular mycorrhizas in three vegetational types from the semiarid of Ceará State, Brazil. Applied Soil Ecology 67, 37-46.

Palgrave, K., Drummond, R., Moll, E., Palgrave, M., 2002. *Euphorbia L*, in: Moll, E.J. (Ed.) Trees of Southern Africa. Struik, Cape Town, pp. 523-535.

Pearcy, R.W., Björkman, O., Caldwell, M.M., Keeley, J.E., Monson, R.K., Strain, B.R., 1987. Carbon gain by plants in natural environments: carbon assimilation analysis provides an understanding of how plants function in diverse environments. BioScience 37, 21-29.

Power, S.A., Ashmore, M.R., 1996. Nutrient relations and root mycorrhizal status of healthy and declining beech (*Fagus sylvatica* L.) in Southern Britain. Water, Air, and Soil Pollution 86, 317-333.

Pregitzer, K.S., Zak, D.R., Curtis, P.S., Kubiske, M.E., Teeri, J.A., Vogel, C.S., 1995. Atmospheric CO_2, soil nitrogen and turnover of fine roots. New Phytologist 129, 579-585.

R Core Team 2014. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Roux. J., Malan, R., Howitt, M., Six, D., Wingfield, M.J., 2008. Discovery of new fungi associated with the decline and death of *Euphorbia ingens* in the Limpopo Province of South Africa. South African Journal of Botany 74, 377-378.

Schenck, N.C., 1982. Methods and Principles of Mycorrhizal Research, American Phytopathological Society.

Šmilauer, P., Lepš, J., 2014. Multivariate analysis of ecological data using CANOCO 5, Cambridge University Press, UK.

Smith, S., Dickson, S., 1997. VA Mycorrhizas: Basic Research Techniques, CRC Soil & Land Management, Adelaide.

Staddon, P.L., Jakobsen, I., Blum, H., 2004. Nitrogen input mediates the effect of -air CO_2 free enrichment on mycorrhizal fungal abundance. Global Change Biology 10, 1678-1688.

Straker, C.J., Weiersbye, I.M., Witkowski, E.T.F., 2007. Arbuscular mycorrhiza status of gold and uranium tailings and surrounding soils of South Africa's deep level gold mines: I. Root colonization and spore levels. South African Journal of Botany 73, 218-225.

Tao, L., Jianping, L., Zhiwei, Z., 2004. Arbuscular mycorrhizas in a valley-type savanna in southwest China. Mycorrhiza 14, 323-327.

Ter Braak, C.J.F., Šmilauer, P., 2012. Canoco reference manual and user's guide: software for ordination, version 5.0. Microcomputer Power, Ithaca, USA.

Trejo, D., Barois, I., Sangabriel-Conde, W., 2016. Disturbance and land use effect on functional diversity of the arbuscular mycorrhizal fungi. Agroforestry Systems 90, 265-279.

Treseder, K.K., 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO_2 in field studies. New Phytologist 164, 347-355.

Treseder, K.K., Allen, M.F., 2000. Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO_2 and nitrogen deposition. New Phytologist 147, 189-200.

Treseder, K.K., Allen, M.F., 2002. Direct nitrogen and phosphorus limitation of arbuscular mycorrhizal fungi: a model and field test. New Phytologist 155, 507-515.

Uhlmann, E., Görke, C., Petersen, A., Oberwinkler, F., 2006. Arbuscular mycorrhizae from arid parts of Namibia. Journal of Arid Environments 64, 221-237.

Vályi, K., Rillig, M.C., Hempel, S., 2015. Land-use intensity and host plant identity interactively shape communities of arbuscular mycorrhizal fungi in roots of grassland plants. New Phytologist 205, 1577-1586.

van der Heijden, M.G., Bardgett, R.D., van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296-310.

van der Heijden, M.G.A., Martin, F.M., Selosse, M-A., Sanders, I.R., 2015. Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytologist 205, 1406-1423.

van der Linde, J.A., Roux, J., Wingfield, M.J., Six, D.L., 2012. Die-off of giant *Euphorbia* trees in South Africa: Symptoms and relationships to climate. South African Journal of Botany 83, 172-185.

van der Linde, J.A., Wingfield, M., Crous, C., Six, D., Roux, J., 2017. Landscape degradation may contribute to large-scale die-offs of *Euphorbia ingens* in South Africa. South African Journal of Botany 111, 144-152.

Wehner, J., Antunes, P.M., Powell, J.R., Mazukatow, J., Rillig, M.C., 2010. Plant pathogen protection by arbuscular mycorrhizas: A role for fungal diversity? Pedobiologia 53, 197-201.

Wiley, E., Casper, B.B., Helliker, B.R., 2016. Recovery following defoliation involves shifts in allocation that favour storage and reproduction over radial growth in black oak. Journal of Ecology 105,412-424.

Winter, K., Holtum, J.A.M., 2015. Cryptic crassulacean acid metabolism (CAM) in *Jatropha curcas*. Functional Plant Biology 42, 711-717.