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THE EFFECT OF SMOKE DERIVATIVES AND CARBON UTILISATION ON SYMBIOTIC GERMINATION OF THE ENDANGERED *PTEROSTYLIS DESPECTANS* (ORCHIDACEAE)

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Abstract. Orchids are highly dependent on exogenous nutritional sources and mycorrhizal associations to survive, particularly when challenged by extreme environmental stress such as bushfires that contribute significantly to its decline in nature. In this study, the effect of smoke derivatives and carbon utilisation was explored to improve germination and seedling establishment of an Australian endangered orchid, *Pterostylis despectans* (Lowly Greenhood) and its mycorrhizal fungi. Stored seeds were germinated *in vitro* with peloton-isolated fungal isolates with varying concentrations of smoke water (0–1.0 mL L^{-1}) to simulate fire and sucrose as the carbon source (10 g L^{-1}). Smoke water significantly increased germination, with advanced protocorms and robust seedlings produced. Sucrose inhibited germination such that protocorms and leafing was absent with sucrose inclusion. Fungal isolates were highly variable on its germination efficacy and tolerance to smoke water, highlighting the importance of fungal diversity and supports research-based conservation strategies to circumvent environmental challenges.

Key words: *in vitro* culture, mycorrhizae, smoke water, symbiotic germination

Introduction. Orchids form minute dust-like seeds that are ideal for wind dispersal. However, they are unable to store nutrients in the embryo and often rely on fungi (predominantly imperfect *Rhizoctonia* spp.) in order to germinate (Arditti & Ghani 2000, Brundrett *et al.* 2003, Rasmussen 1995). When fungi colonise orchid seeds, they grow as intracellular tightly coiled hyphal pelotons and a symbiotic relationship is established (Huynh *et al.* 2004). In this symbiotic relationship, fungi supply the orchid with nutrients including nitrogen (Girlanda *et al.* 2011) and phosphorus (Cameron *et al.* 2007) while the orchid supply carbon to the fungus (Cameron, Leake & Read 2006, Látalová & Baláž 2010). Carbon utilisation by mycorrhizal fungi vary with some clades from the same *Rhizoctonia* species inhibited by sucrose (Wright *et al.* 2011) resulting in suboptimal seed germination (Huynh *et al.* 2004, Nikabadi *et al.* 2014, Wright *et al.* 2009).

Some *in vitro* studies have successfully germinated orchid seeds asymbiotically (without fungi) using specific stimulants such as growth hormones to promote germination (Huynh *et al.* 2004, Nikabadi *et al.* 2014). Despite the germination success of asymbiotic plants, symbiotically germinated orchids established in soil better in the long term than those without fungi (Batty *et al.* 2001, Rasmussen 1995) which suggests fungal superiority and importance to orchid conservation particularly for plants that reside in depleted nutrient habitats.

Fungal specificity of orchids is highly variable between species and different fungi are not equally effective in seed germination or growth (Phillips *et al.* 2011). Australian orchids generally have higher specificity for symbiotic fungi compared to species from other continents (Batty *et al.* 2001, Pandey *et al.* 2013, Phillips *et al.* 2011, Wright *et al.* 2009). Moreover, *Rhizoctonia* diversity in Australia is lower compared to other continents (Brundrett *et al.* 2003). Patchy fungal distribution in the soil, high fungal-host specificity and the preference for same-site specific fungal selections (Wright *et al.* 2011) can lead to orchid rarity (Phillips *et al.* 2011) and is a considerable barrier for the conservation of endangered species, for example *Caladenia huegelii* (Swarts *et al.* 2010) and some other *Caladenia* spp. (Wright *et al.* 2010) but not others (Bailarote, Lievens & Jacquemyn 2012).

Australian orchids reside in fire-prone regions and respond to fire differently ranging from destructive for some species whilst stimulatory for others (Brundrett 2007, Duncan & Coates 2010, Janes, Vaillancourt &

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Steane 2008, Jasinge, Huynh & Lawrie 2018a,b). Smoke water is a byproduct of fire and has been investigated for its ability to increase the germination and development of some orchids (Papenfus *et al.* 2016, Mulgund *et al*. 2012, Malabadi *et al*. 2011) but not others (Teixeira da Silva 2013). Since smoke residues can be fungistatic (Jasinge *et al.* 2018a) or fungicidal (Jasinge 2014, Lin *et al.* 2012, Parmeter & Uhrenholdt 1975, Zagory & Parmeter 1984) due to compounds such as phenolics, imidazole (Chumpookam *et al.* 2012), karrikinolide and trimethylbutenolide (Papenfus *et al.* 2016), this could affect the ability for fungi to assist in seed germination and development and negate the benefits of smoke water.

Pterostylis R.Br has over 400 species that are spread across Australasia (Phillips *et al.* 2014). Many of these species were described only recently and their identification remains difficult due to repeated taxonomic revisions as well as rare flowering (Janes *et al.* 2008). One representative, *Pterostylis despectans* (Nicholls) M. A. Clem. & D. L. Jones (Lowly Greenhood) is critically endangered (Bickerton & Robertson 2000, Duncan, Pritchard & Coates 2005, Janes *et al.* 2008, Marsh 2011), restricted to south east Australia (NSW Government 2018) and conservation efforts are required to mitigate its decline. This study investigated factors to improve germination for reintroduction based on success from other orchid genera. The aim was to determine the usefulness of smoke water or sucrose utilisation on germination; the impact of fungal diversity and smoke water on protocorm development; and the effect of smoke water on fungal growth.

Materials and methods

Seed collection and preparation.— Germination was performed on 8 year old dried seeds of *P. despectans* collected in Talbot (Victoria) on January 2006 from seed capsules of six random plants. The seeds were surface sterilised for 1 min in 0.5% NaOCl with one drop of Tween 20 (Fisher BioReagents®). Seeds were spun at 13,000 rpm for 30 s and the supernatant was removed with a sterile glass pipette. Seeds were trice rinsed with sterile milliQ water and the supernatant removed.

Fungal isolation.— Three collars were collected *in situ* during the growing season in July 2013 from a population in Bung Bong state forest (Victoria). The collars were cleaned under running tap water, surface sterilized with 1% NaOCl for 3 minutes and rinsed trice with sterilized MilliQ water in a laminar flow cabinet. The collars were sliced into 1 mm longitudinal sections in sterile MilliQ water under sterile conditions. The pelotons were observed with a dissecting microscope, scraped out, dispensed in sterile MilliQ water and droplets containing pelotons were plated onto fungal isolation medium (FIM 0.3 g L^{-1} sodium nitrate, 0.2 g L^{-1} potassium dihydrogen orthophosphate, 0.1 g L^{-1} magnesium sulphate, 0.1 g L^{-1} potassium chloride, 0.1 g L^{-1} yeast extract, 5 g L^{-1} sucrose, 10 g L^{-1} agar, prepared to 1 L with deionized (DI) water, pH adjusted to 6.8 before autoclaving (20 min at 121ºC, 105 kg cm^{-2}) (Clements 1981). Isolated pelotons were grown for 48 h at room temperature and scored as 1) *Rhizoctonia*-like fungi, 2) bacteria, 3) other fungi and 4) no growth (Huynh *et al*. 2009).

Fungal growth and smoke water.— *Rhizoctonia*-like fungi were transferred onto malt agar medium (MAM) with three smoke water concentrations (0, 0.1 and 1 mL L^{-1}) and labelled as collar number (1–3) and a letter representing separate fungal isolates from each collar. Each plate contained triplicate plugs from the same isolate. The fungi were incubated at 25°C in darkness and their growth was measured using a digital calliper at the same time point (five days) to test the effect of smoke water. When fungal colonies reached optimal growth on MAM plates, three agar blocks from control plates (no smoke water) were used to inoculate the symbiotic germination plates containing autoclaved oatmeal agar (OMA).

Symbiotic germination.— OMA (2.5 g L^{-1} finely ground rolled oats, 0.1 g L^{-1} yeast extract, 8 g L^{-1} agar, pH adjusted to 5.3–6.0) (Nikabadi *et al*. 2014) was prepared in sterile petri dishes with three concentrations (0, 0.1 and 1 mL L^{-1}) of smoke water (Regen 2000® Smokemaster, Australia) in the absence or presence (10 g L^{-1}) of sucrose (Sigma Aldrich) before sterilization. One cm^2 squares of sterile Miracloth (Calbiochem, USA) were placed onto set OMA. One droplet of surface sterilised seeds was released onto each Miracloth square and a fungal square was placed in the middle of each plate. Nine fungal isolates were used for each of the six media types. One plate per treatment was not inoculated and was used as a control. The plates were sealed with Parafilm® (Sigma Aldrich) and incubated for six weeks at 25°C

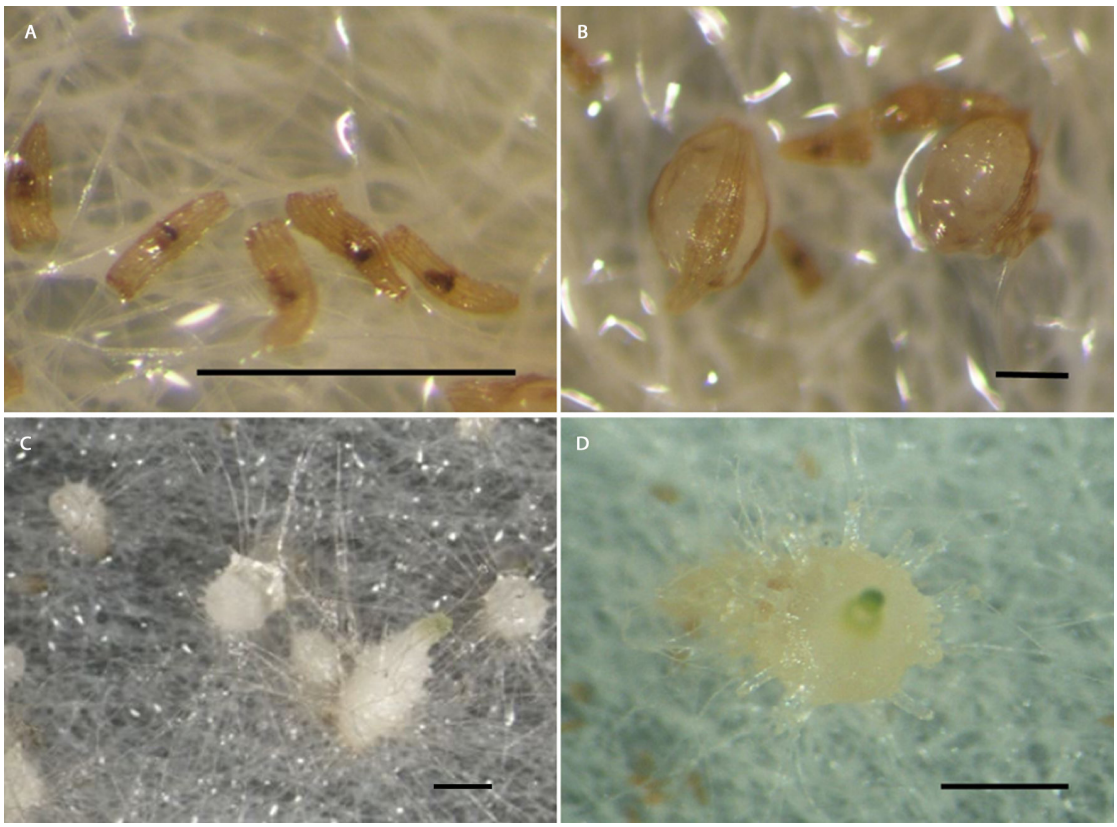

Figure 1. *Pterostylis despectans* seeds at different stages of germination. A. Dormant. B. Swollen. C. Protocorm. D. Protocorm with green leaf primordium. Bars (right bottom corner) = 1 mm.

in darkness and 1 week in a growth room under 16 h light cycles (NEC TRI-phosphor 30 watts fluorescent light FL). Seed changes were scored as 1) dormant - unchanged, 2) swollen, 3) protocorm and 4) protocorm with green leaf primordium (Fig. 1).

Data analysis.— Data was tested for normality and homogeneity. Normality was tested using Shapiro-Wilk analyses at $p \geq 0.05$. Homogeneity of data was tested using Levene's test for equality of variance and was considered homogeneous at $p \geq 0.05$. Data not meeting the above assumptions were transformed to normality. Normal data was analysed for statistical differences by ANOVA or t-test. Tukey HSD test was used as a post-hoc test for homogeneous data and Games-Howell post-hoc for non-homogeneous data. Abnormal data was tested using non-parametric Kruskal-Wallis test. All tests were performed at significance of $p \leq 0.05$ using IBM SPSS statistical software (version 23). Fisher's family error test was performed using Minitab (version 17).

Results

Effect of smoke water and sucrose on germination.— Symbiotic germination for *P. despectans* was low with the majority (94–99%) of seeds unchanged and categorised as dormant (Fig. 2). Smoke water had a positive effect on seed germination produced more than double the number of primordia stages. The highest germination was observed in media without sucrose and both smoke water concentrations (0.1 and 1.0 mL L^{-1}) significantly increased germination when compared to controls (without smoke water), particularly for protocorm and primordia stages. Although swollen seeds were noticeably changed from dormant seeds, they were not significantly affected by smoke water ($p \geq 0.05$, Tukey HSD test).

There were significant differences in protocorm numbers that increased for smoke water concentrations from 0.0 to 0.1 mL L^{-1} but decreased from 0.1 to 1.0 mL L^{-1} ($p \leq 0.05$, Games-Howell test) (Fig. 2). No

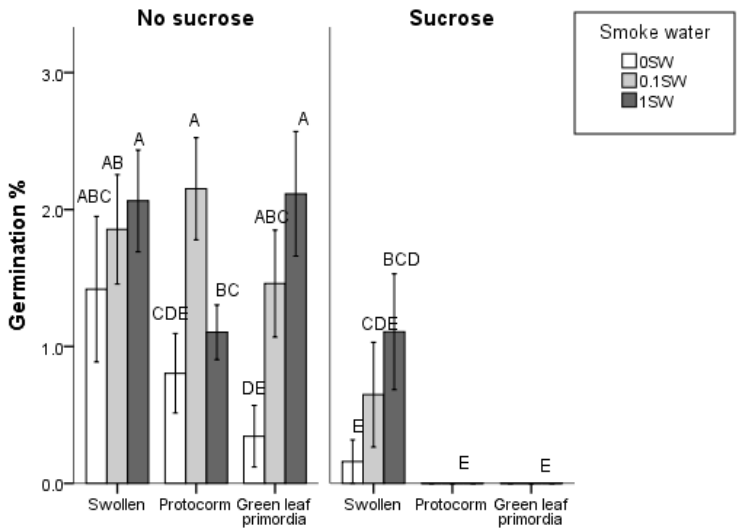

Figure 2. The effect of sucrose and smoke water on *Pterostylis despectans* symbiotic seed germination. Data are mean % (± 1SE) of germination stages reached (swollen, protocorms and leafing) in following treatments: ± sucrose in three concentrations of smoke water 0SW=0.0 mL L^{-1}, 0.1SW=0.1 mL L^{-1}, 1SW=1.0 mL L^{-1}. Means that do not share a letter are significantly different using Fisher method grouping at p≤0.05 on arcsine transformed data. Dormancy scores (>95%) were excluded from the graph.

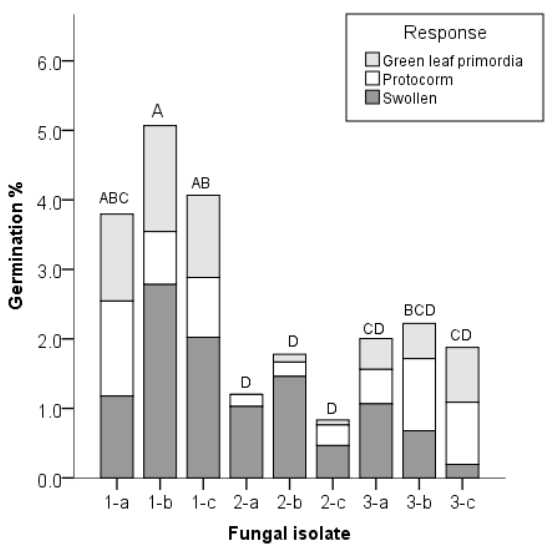

Figure 3. *Pterostylis despectans* fungal isolates' effectiveness (%) to germinate *P. despectans* seed (± 1SE). Fungal isolate abbreviations: numbers indicated a different *P. despectans* plant and letters indicated a different fungal isolate. Means that do not share a letter are significantly different using Fisher method grouping at p≤0.05 on arcsine transformed data. Dormancy scores (>95%) were excluded from the graph.

significant difference was found between smoke water concentrations of 0.0 and 1.0 mL L^{-1} (p>0.05, Games-Howell test). Smoke water significantly increased the number of seedlings with green leaf primordia (p≤0.05, Games-Howell test) and even though there was more leafing for the higher smoke water concentration, this was not significant (p=0.523, Games-Howell test) (Fig. 2). Sucrose had a significantly negative effect on all seed development stages from swelling to germination and leafing (p≤0.001, t-test and Kolmogorov-Smirnov tests) (Fig. 2) such that no seed reached protocorm or leafing stages.

Effect of fungal variability and smoke water on germination stages reached.— Control (without fungal inoculum) was absent of germination. This was significantly different to symbiotic germination and the efficacy for each fungal isolate to promote germination to different stages of germination were significant (p<0.05, Fisher's post-hoc test). There were significant differences in total germination between and within each replicate plant and isolate, with plant 1 isolate b initiating the highest overall and individual stages of germination. Even though there was a noticeable variation on all stages of germination, the efficacy was

not significantly different if the fungus was isolated from the same plant (Fig. 3). The effectiveness of fungal isolates from different plants was variable with the best isolates from plant 1 and the worst isolates from plant 2 with varying germination within.

Effect of smoke water on fungal growth.— Smoke water had a significant effect on fungal growth ($p<0.05$, Fisher's post-hoc test) however the impact of smoke water presence and the concentration on fungal growth greatly varied between fungal isolates (Fig. 4). The greatest overall radial growth was in isolates from plant 3 and the least from plant 2. These patterns did not reflect germination, with the best germination from plant 1 that had middle range radial growth.

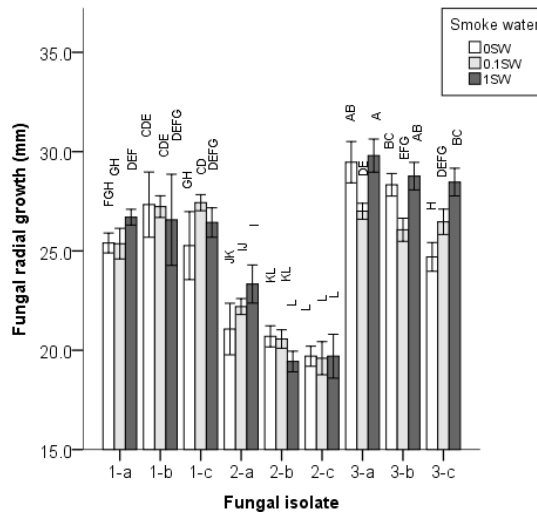

FIGURE 4. The effect of smoke water concentrations on fungal radial growth (mm) of nine isolates from *P. despectans* in three smoke water concentrations (0SW=0.0 mL L^{-1}, 0.1SW=0.1 mL L^{-1}, 1SW=1.0 mL L^{-1}). Data are means (± 1SE). Fungal isolate abbreviations: numbers indicated a different *P. despectans* plant and letters indicated a different fungal isolate. Means that do not share a letter are significantly different using Fisher method grouping at $p\leq0.05$.

Discussion

Germination.— *Pterostylis despectans* seed in this study had very low germination success compared to other orchid genera. For example, other Australian orchids showed high germination that reached up to 100%, including more common *Pterostylis* species (Batty *et al*. 2006, Huynh *et al*. 2004, Nikabadi *et al*. 2014). This low germinability may indicate requirements for additional stimulants and be a contributing factor to its rarity and consequent endangered status of *P. despectans*. There are several other reasons that cause low germination numbers: seed age, post-harvest seed storage conditions and fungal specificity. *Pterostylis despectans* seed were collected 8 years prior to the experiment and may be too long for the optimum viability to be maintained. Studies on other plants have shown that time affects seed viability (Merritt *et al*. 2003) with high orchid seed germination achieved when seed material was obtained within a year before germination (Batty *et al*. 2001, Nikabadi *et al*. 2014). Seed viability of Australian plants was affected by relative humidity and temperature during storage (Merritt *et al*. 2003). *Pterostylis sanguinea* had higher germination in 15–20°C than in 25°C (Nikabadi *et al*. 2014), and the higher incubation temperature used in this experiment at 25°C may have restricted the full germination potential of the species.

Australian orchids are often colonised with more than one taxa of endophytic fungi (Dixon & Tremblay 2009, Rasmussen *et al.* 2015) which explains why fungi isolated from three different plants had significantly different effect on seeds (Fig. 3). Other studies found no correlation between fungal taxonomy with germination efficacy (Wright *et al.* 2010) and could indicate other factors contributing to varying seed responses. Our findings also imply that the fungal diversity in *Pterostylis* orchid species varies between the plants rather than within one plant despite morphological and genetic similarities (Huynh *et al*. 2009). Complex comparisons such as gene-environment interactions and metabolomic studies may provide more useful answers and direct future conservation efforts such as *in situ* inoculations to rejuvenate fungal diversity to improve germination and growth.

Symbiotic fungi were essential for *P. despectans* seed germination but highlighted that other factors may be important for improved germination and survivorship of this species. High and successful asymbiotic *in vitro* germination can be achieved to rival or exceed symbiotic germinations beyond 93% (Bustam, Dixon & Bunn 2014) and may be the only alternative for similar endangered orchids like *P. despectans* that have fastidious requirements for both the fungal partner and seed.

The effect of smoke water on germination.— Smoke water is beneficial for germination of Australian orchids' seeds. The presence of smoke water had a positive effect on *P. despectans* seed germination (Fig. 3). There are limited studies on smoke water effects on orchid seed germination (Jasinge 2014, Papenfus *et al.* 2016). Other studies have found that smoke released the dormancy of non-orchid Australian native plants (Bradshaw *et al.* 2011, Dixon *et al.* 2009, Flematti *et al.* 2004). On the other hand, heat was found to be more important in seed germination initiation of some plants in Western Australia than smoke (Tieu *et al.* 2001) and this is a possible research opportunity to investigate fire and smoke derivatives to improve major orchid life-cycle events particularly for recalcitrant species.

Even though smoke water significantly increased the germination of *P. despectans*, the results were still suboptimal with germination not exceeding 5%. This result translated into an ecological conservation context would mean that copious volumes of viable seed is required to replace existing populations and even more for the expansion of populations which is not sustainable for the longevity of the species without human intervention. The only other study to use smoke water as a stimulant also resulted in low germination rates on an African orchid, *Ansellia africana* (Papenfus *et al.* 2016) with <19% at stages 4–5 of development, equivalent to the leafing stages categorised in this study. The significance of smoke water on seed germination and the lack of published studies is an opportunity for future research, especially highly endangered species that have low germinability.

The effect of sucrose on germination.— Sucrose had an adverse negative effect on *P. despectans* seed germination. Similarly, the addition of sucrose decreased the germination of *Caladenia* species (Wright 2007, Wright *et al*. 2011) resulting in the omission of sucrose in other germination studies (Nikabadi *et al*. 2014). On the other hand, some other orchid species (*Microtis parviflora, Caladenia formosa*) grew better with the presence of sucrose (Huynh *al*. 2004, Wright *et al*. 2009) especially those germinated asymbiotically (Huh *et al*. 2016) so there is a need for individualised ingredients to cater for the preferences of both orchid and fungus.

In the presence of sucrose, fungi dominated and outcompeted seeds for nutrients and thus negatively affected seed germination. The fungi in sucrose media were morphologically different with more vigorous dense growth and covered orchid seeds. Similarly, it was observed that high concentrations of sucrose encouraged parasitic fungal growth whereas lower concentrations of sucrose promoted symbiotic associations in *Dendrobium chrysanthum* (Hajong, Kumaria & Tandon 2013). In contrast, other studies on *C. tentaculata* found no changes in hyphal growth or dominance regardless of sucrose presence (Wright *et al.* 2011). They found that individual fungal isolates responded to different carbon sources differently, likely due to fungal isolates belonging to different taxa (Wright *et al.* 2011). Fungal isolates from *P. despectans* may also have different requirements and responses to carbon sources that reflect the complexity of fungal carbon utilisation and assimilation. This may also stimulate or exacerbate fungal functionality *in situ* under extreme environmental stress such as fire events.

The effect of smoke water on fungal growth.— These findings are contrary to previous studies, where smoke or smoke water had significantly inhibited fungal growth (Jasinge 2014, Zagory & Parmeter 1984). Smoke water contains phenolic compounds that are toxic to fungi and inhibit fungal growth by altering the fungal cell walls (Chumpookam *et al*. 2012) suggesting that fungi from *P. despectans* had higher tolerance to phenolic compounds. The varying fungal tolerance to phenolic compounds is thought to be due to the quantity or quality of enzymes (laccases) that metabolise these toxins (Jasinge 2014, Zagory & Parmeter 1984) and have significant consequences on plants that rely on symbiotic fungi.

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