plant biology
Plant Biology ISSN 1435-8603

RESEARCH PAPER

Enzymatic scarification of *Anacamptis morio* (Orchidaceae) seed facilitates lignin degradation, water uptake and germination

S. Pierce[1], A. Spada[1], E. Caporali[2], R. M. Ceriani[3] & G. Buffa[4]

1 Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Milan, Italy
2 Department of Biosciences, University of Milan, Milano, Italy
3 The Native Flora Centre (Centro Flora Autoctona; CFA), c/o Parco Monte Barro, Galbiate, Italy
4 Department of Environmental Sciences, Informatics and Statistics, University Ca' Foscari of Venice, Venice, Italy

Keywords
Dormancy; imbibition; laccase; ligninase; orchid; Weisner reaction.

Correspondence
S. Pierce,Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, via G. Celoria 2, I-20133 Milan, Italy.
E-mail: simon.pierce@unimi.it

Editor
J. T. M. Elzenga

Received: 26 July 2017; Accepted: 12 January 2018

doi:10.1111/plb.12694

ABSTRACT

- The seed coat of many species contains hydrophobic lignins, and in soil the action of microbial ligninases may contribute to release from dormancy. Laboratory use of ligninases to stimulate germination is promising because of the specific action on the seed coat, whereas chemical scarification agents may also corrode the embryo. We hypothesised that exposure of *Anacamptis morio* (Orchidaceae) seeds to fungal laccase would stimulate germination, and that the mechanism involves lignin degradation and increased imbibition.
- Germination capacity *in vitro* was quantified with 1 U filter-sterilised laccase added to agar medium following autoclaving, compared to a 10% bleach solution (standard bleach surface sterilisation/scarification method used in orchid seed sowing). Lignin degradation was quantified using an optical method (phloroglucinol-HCl staining) combined with image analysis, following experimental pre-treatments involving immersion in laccase solution, distilled water (negative control) or bleach (positive control). Water uptake after experimental treatments was quantified as the proportion of seeds exhibiting visible uptake of an aqueous fluorochrome under UV excitation.
- Laccase stimulated a doubling of germination *in vitro* with respect to bleach surface sterilisation/scarification alone, from 23.7 to 49.8% ($P = 0.007$). Laccase and bleach methods both significantly decreased the optical signal of phloroglucinol (for laccase, to 79.9 ± 1.3% of controls; ANOVA: $F = 10.333$, $P = 0.002$). Laccase resulted in a modest but highly significant ($P < 0.0001$) increase in water uptake with respect to the control (11.7%; *cf* 99.4% for bleach).
- Laccase scarification can stimulate germination of *A. morio* through a mechanism of targeted seed coat degradation. The results demonstrate the potential of this relatively non-invasive enzymatic scarification technique.

INTRODUCTION

The seed coats of a wide range of Spermatophytes are highly lignified (Schwarz *et al.* 1988; Morrisson *et al.* 1995; Moldes *et al.* 2003; Ma *et al.* 2004; Vasudevan & van Staden 2010; Chen *et al.* 2012; Tobimatsu *et al.* 2013; Ran *et al.* 2015; Sampaio *et al.* 2016). This has a number of implications for our understanding of both dormancy mechanisms and the ecology of dormancy release during germination in soil. Lignins are hydrophobic and extremely resistant to degradation, and thus probably contribute to the capacity of integuments to exclude water (Baskin 2003) and have been directly implicated in 'testa imposed dormancy' (Vasudevan & van Staden 2010). Degradation in nature is usually catalysed by lignin modifying enzymes, or ligninases, a general class of polyphenol oxidases catalysing the oxidation of aromatic compounds. These are produced by soil fungi and bacteria and are involved in the decomposition of organic matter (Zeng *et al.* 2006; McMahon *et al.* 2007). The presence of saprophytic fungi and the availability of woody matter and humus are thought to be crucial sources of energy supporting the establishment of orchid seedlings in nature (Rasmussen *et al.* 2015), and it has also been suggested that ligninases in soil could facilitate seed germination by essentially scarifying the seed coat (Barsberg *et al.* 2013). Indeed, the evolution of ligninolytic peroxidases was one of the fundamental events driving the early co-evolution of fungi and woody plants from the Permian onwards (Martin *et al.* 2016). Thus the use of ligninases by fungi to degrade persistent plant organic matter is an extremely widespread and ancient phenomenon. Indeed, lignin is the second most abundant biomolecule in terrestrial ecosystems after cellulose (Boerjan *et al.* 2003), and contact between seeds and ligninases in soil is probably inevitable wherever humus is present. Ligninases are also produced endogenously during the process of seed maturation, associated with control over seed pigment development and impermeability (Pourcel *et al.* 2005; Cai *et al.* 2006; Aniszewski *et al.* 2008; Wang *et al.* 2014).

The location of lignin as a cell wall component in both inner and outer seed integuments is particularly well studied in a

range of orchid species, and several contrasting methods not only confirm the presence of lignin but also provide a detailed picture of where and when lignin is synthesised, usually during the final stages of seed development resulting in 'browning' of the seeds (Yamazaki & Miyoshi 2006; Barsberg *et al.* 2013; Rao *et al.* 2014; Yang & Lee 2014). Immature orchid seed, still unlignified and white in appearance, has been shown to exhibit higher germination percentages than mature brown seed, with a well-developed 'carapax', when sown *in vitro* (Kitsaki *et al.* 2004; Lee *et al.* 2005; Pierce & Cerabolini 2011), demonstrating that lignin deposition in the integuments and imposition of dormancy represents one of the final stages of seed maturity. The outer integuments of *Dendrobium nobile* Lindl. seeds are lignified whereas the inner integuments are cutinised, which has been suggested as a water exclusion mechanism (Vasudevan & van Staden 2010). Phloroglucinol staining of *Cephalanthera falcata* (Thunb.) Blume seeds has revealed the presence of lignin in both inner and outer integuments (Yamazaki & Miyoshi 2006). Thus, both integuments are potentially lignified, depending on the species, and both form hydrophobic barriers. An additional phenomenon that underlines the importance of lignin and ligninases in the particular case of Orchidaceae is the widely reported dependence of these mycotrophic plants on fungi (*e.g.* Leake 1994; Rasmussen 1995) which, during germination, involves the penetration of fungal hyphae into orchid seeds using a range of enzymes including cellulases and pectinases (Barroso *et al.* 1986; Nieudorp 1972; cited by Rasmussen 1995). Orchid mycorrhizal fungi are known to be capable of degrading lignin as part of their role as decomposers (Holländer 1932; cited by Smith 1966), suggesting the additional involvement of ligninases.

Together, these observations suggest a possible mechanism that could be exploited in the laboratory to stimulate germination by means of enzymatic scarification, whereby ligninases are used to degrade lignin in the seed coat, with concomitant increased water uptake and dormancy release. As lignin is a prevalent and fundamental component of secondary cell walls, but is only a minor element of the primary cell walls characteristic of metabolically active cells such as meristematic cells and parenchyma, ligninases are likely to have a disproportionately strong effect on the lignin-rich cell walls of the seed coat, leaving the meristematic cells of the embryo relatively unscathed.

While a range of ligninases is available commercially, the laccases (copper-containing oxidases) in particular have been isolated on an industrial scale from white rot fungi, particularly *Pleurotus* spp., for wood pulp processing. Thus fungal laccase is readily available at low cost and could potentially be used to stimulate seed germination *via* 'enzymatic scarification'. This would be a useful method because corrosive chemical scarification agents such as acids or the calcium hypochlorite or sodium hypochlorite in bleach (Wilson 1915) must be carefully used; excessive exposure can easily damage the embryo and reduce germination (Alvarez-Pardo *et al.* 2006). A seed scarification method that targets the tissue that imposes dormancy, without inducing damage to the embryo, promises to be a particularly effective technique.

The present study thus investigated two hypotheses: (i) exposure of seeds to fungal laccase stimulates increased germination for a model orchid species, and (ii) increased germination in response to laccase is associated with lignin degradation and increased water uptake as a possible mechanism. Water uptake by seeds is traditionally determined by mass increment but in the case of small seeds this method is complicated by persistent surface water films that can account for a substantial proportion of the measured mass. Here an alternative method is used that relies on the uptake and localisation of aqueous fluorochromes – tracer stains that are excited by UV light and observed under the microscope. Fluorochromes have previously been used to determine changes in seed coat permeability in response to degradation processes for a range of species. In the case of soybean (*Glycine max*) increased fluorochrome uptake for degraded seed coats was associated with increased seed mass, interpreted as higher water uptake (Ma *et al.* 2004). Similar histological observations have been carried out for species in the Proteaceae and Poaceae (Briggs & Morris 2008; Dias *et al.* 2014). Fluorochromes have also revealed that integuments of developing ovules exhibit plasmodesmatal (symplastic) connections, and that after ovule fertilisation fluorochrome movement in integument tissues gradually ceases, denoting closure of plasmodesmata, cessation of symplastic translocation and increasing seed coat impermeability (Stadler *et al.* 2005). Different fluorochromes vary in their capacity to penetrate the mature seed coat; an effect that also depends on the seed coat properties of each species (Salanenka & Taylor 2011). Indeed, the seed coat of tobacco is highly permeable to fluorescein, and the penetration of this fluorochrome into the endosperm is readily observed using fluorescence microscopy (Guan *et al.* 2013). In the case of orchids, 'penetration of the dye [fluorescein diacetate (FDA)] through the testa varies between species' (Pritchard 1985). Indeed, the impermeability of the testa of terrestrial orchids means that when fluorescein is used to conduct embryo viability tests the seed coat must first be removed (Daws *et al.* 2008; see also Thorogood *et al.* (2009) for Orobanchaceae). This implies that fluorescein detection within orchid seeds with the testa present will denote a relatively permeable testa, and if fluorochrome uptake increases in response to scarification it probably denotes seed coat degradation and increased water uptake. This is assumed here.

MATERIAL AND METHODS

Study species and seed collection

The orchid *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase was chosen because it is a relatively common and well-studied species that invests particularly highly in seed production, even with respect to other orchids (Pierce *et al.* 2014; Slaviero *et al.* 2016) and because it is particularly easy to recognise from fruit morphology during seed collection (Redl 1999), meaning that large amounts of seed were available without seed collection having a negative impact on populations. Seed was collected from the Veneto Region of northeast Italy, from the Eastern Lessini Mount, Berici Hills and Euganean Hills Regional Park (45°20'–45°30'N, 11°25'–11°45'E).

Germination

Seed was sown in sterile conditions *in vitro* on Malmgren's (1996) terrestrial orchid medium modified with the addition of 0.5 g l^{-1} peptone and 0.1 mM 6-benzylaminopurine (BAP; see Pierce & Cerabolini 2011) adjusted to pH 6.5. Laccase from

Pleurotus ostreatus was obtained from Sigma-Aldrich (Milan, Italy). The *in vitro* germination experiment required the decontamination and surface sterilisation of seed using sodium hypochlorite solution in the form of bleach: the control treatment thus inevitably included exposure to bleach and represents a standard method for orchid seed sowing (exposure to 10% bleach solution for 10 min; *e.g.* Kitsaki *et al.* 2004; Pierce *et al.* 2015). Initial studies determined that pre-treatment with laccase followed by bleach surface sterilisation inhibited germination (not shown), probably as bleach penetrated to the embryo and caused damage. Thus laccase could not be applied as a pre-treatment prior to *in vitro* sowing. Initial studies also confirmed that application of a sterile laccase solution to 'bathe' seeds previously sown on the surface of the agar medium did not stimulate germination (data not shown), perhaps because of limitations to oxygen diffusion and respiration in embryo tissues. Also, wetting of the agar surface and walls of the Petri dish allowed contamination to enter (data not shown) and this was deemed an inappropriate method of laccase exposure. Thus it was decided to incorporate laccase into the agar medium and the laccase treatment used in the study represented an ongoing background treatment during germination, following an initial bleach surface sterilisation treatment.

As laccase is a heat-labile protein complex, it was added after autoclaving of growth medium (20 min at 121 °C and 1 MPa) *via* filter sterilisation in sterile conditions. Specifically, an infrared thermometer was used to determine when growth medium cooled to 65 °C, and at this point 20 ml laccase solution was forced from a syringe through a cellulose acetate syringe-filter with a pore diameter of 0.2 μm [Permax s.r.l.; Treviglio (BG), Italy] into 480 ml agar medium (a method previously employed to add heat-labile hormones to medium; Pierce *et al.* 2015). The amount of laccase was calculated so that the final activity of the enzyme in each Petri dish was 1 U (the activity of the particular lot of enzyme used, as stated on packaging, was 11.8 U mg, so 1 U = 0.0847 mg per Petri dish, in our experiments).

After sowing, Petri dishes were transferred to a controlled environment chamber [Snijders Economic Deluxe; Thermo-Lab, Codogno (LO), Italy] and kept in the dark for 6 months in a day/night temperature regime of 20/10 °C (for periods of 12 h), with the position of the Petri dishes re-randomised every 2 weeks. Total germination was defined as the percentage of seeds that had developed to produce protocorms with visible rhizoids by the end of this period.

Seed coat lignin degradation

To investigate the possible mechanism of laccase action, degradation of seed coat lignin was investigated using an experiment in which seeds were exposed to either distilled water (negative control) or degradation was forced using a high laccase exposure treatment (600 U for 8 h, overnight) or a strong bleach exposure (10% bleach for 30 min; positive control), at room temperature. A pilot study determined that the short exposure times, measured in minutes, typically used for chemical scarification did not result in seed discolouration when laccase was used, hence the longer exposure times (hours) for the 'laccase scarification method'. Indeed, the experiments should not be seen as comparisons of purely 'bleach' or 'laccase' activity *per se*, but as a comparison of a 'laccase scarification method' against a 'standard chemical scarification method'.

The relative amount of lignin in the seed coat was compared between treatments using a histological method involving the Weisner reaction, whereby acidified phloroglucinol (2% phloroglucinol dissolved in ethanol, mixed with 1% HCl) acted as a red stain in the presence of lignin (Gahan 1984). Photomicrographs were captured using standardised exposure and aperture settings to ensure background colour uniformity between images, and then the relative intensity of red of the seed coat (*i.e.* of both integuments) was quantified from the Red, Green, Blue (RGB) score of image pixels using the free Just Color Picker software (http://annystudio.com). For each individual seed, a mean value was calculated from three measurements across the region of the inner integument (*i.e.* 'looking' through the outer integument at the inner integument; n = 3) and then the mean values for each of six replicate seeds were used to calculate the treatment mean (*i.e.* n = 6, but representing a total of 18 measurements). Replicate values in the control treatment were ranked, and then the intensity of red for both bleach and laccase treatments were also ranked and compared to their respective control values. Thus the relative red intensity of each treatment was expressed as a percentage of the red intensity in the control treatment, with declining red intensity relative to the control representing the relative extent of lignin degradation.

Water uptake

Water uptake was denoted by the entry of an aqueous fluorochrome into embryos of intact seeds. Seeds underwent experimental scarification treatments followed by exposure to fluorochrome solution: treatments included either exposure to 1 ml distilled water for 30 min (negative control), a 10% bleach solution for 30 min (positive control), or 600 U of laccase overnight) in 1.5 ml Eppendorf centrifuge tubes. Tubes were agitated during exposure to experimental treatments. Tubes were then centrifuged at 16,000 rpm for 1 min to gently precipitate seeds and allow the supernatant (*i.e.* treatment solution) to be removed by pipette. Treatment solutions were replaced by distilled water followed by centrifugation, and this procedure was repeated to allow thorough rinsing of seed. Seed in all treatments was then exposed to a 100 ppm (0.01% w/w) solution of fluorescein sodium salt for 10 min, followed by three rinses. Finally, seeds were suspended in distilled water and pipetted onto microscope slides, coverslips were applied, and slides were viewed using a fluorescence microscope (Axio Zoom V16; Carl Zeiss, Milan, Italy) with ultraviolet (UV) excitation. Filters were set to 'Green Fluorescent Protein' for observation of fluorescence at a wavelength of 510 nm (re-emission from fluorescein peaks at around 519 nm).

For each slide, the number of seeds exhibiting fluorescence in the embryo was counted alongside the number of dark seeds, for calculation of the proportion of seeds that had imbibed water (examples of *A. morio* seeds that have absorbed, or not, fluorescein and thus water are visible in Fig. S1). The mean percentage of seeds exhibiting imbibition was calculated and compared between treatments.

Statistical analysis

Statistical analyses (ANOVA, Tukey's multiple comparison procedure and Student's *t*-test) were conducted using Systat 12 statistical software (Systat Software, San Jose, CA, USA).

Percentage data in all experiments were arcsine-transformed prior to statistical analyses.

RESULTS

Germination

Addition of laccase to the growth medium resulted in doubling of germination with respect to the control standard sowing method, *i.e.* from 23.7 ± 1.3% in the control to 49.8 ± 2.1% (Fig. 1): an increase that was highly statistically significant (Student's *t*-test, $P < 0.000$; n = 25).

Seed coat lignin degradation

Bleach scarification decreased the red intensity of the seed coat to 76.0 ± 4.4% of that in the control treatment, and the laccase scarification method also decreased seed coat red intensity to a similar value of 79.9 ± 1.3% (Fig. 2). Indeed, while these mean values were significantly lower than the control (ANOVA: F = 10.333, $P = 0.002$; n = 6), the bleach and laccase scarification methods were not statistically different between themselves (*i.e.* both methods resulted in lignin degradation to the same extent; Fig. 2).

Water uptake

Very few seeds (3.2 ± 0.6%) demonstrated fluorescein and thus water uptake in the control distilled water treatment, while the bleach scarification method resulted in almost all seeds (99.4 ± 0.3%) taking up this fluorochrome (Fig. 3). The laccase scarification method resulted in 15.0 ± 2.1% of seeds taking up water, which was statistically significantly different from both the control and bleach treatments (ANOVA: F = 1471.884, $P < 0.000$; n = 10; Fig. 3).

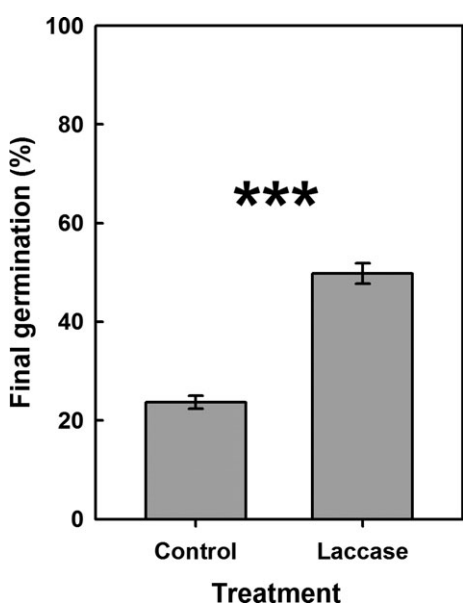

Fig. 1. Final germination of *Anacamptis morio* after 6 months of exposure to 1 U laccase, incorporated directly into the agar substrate (Malmgren's terrestrial orchid medium) *in vitro*, compared to a standard bleach surface sterilisation/scarification treatment (10% bleach for 10 min; control). Values represent means ± 1 SE. Asterisks, ***denote a significant difference between means at $P \leq 0.001$ (Student's *t*-test; n = 25).

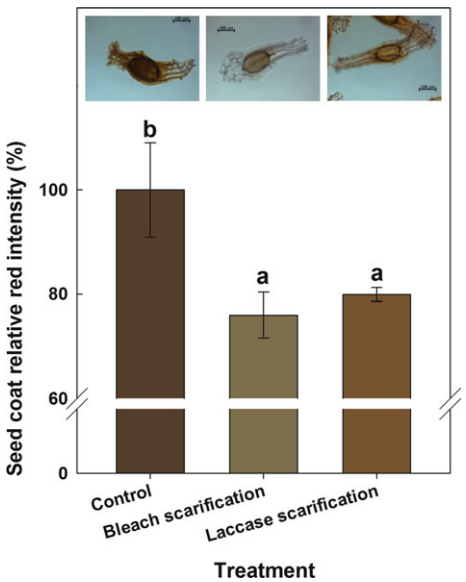

Fig. 2. The intensity of red colouration (following dying with phloroglucinol-HCl) of the testa of *Anacamptis morio* seed pre-treated with distilled water (control), bleach or laccase, expressed as a percentage of the mean control value. Colours represent the actual mean colour (red/green/blue, RGB, value) for each treatment, the value for each replicate seed being calculated as the mean of three values across the carapax region. Different letters denoting significant differences between means (n = 6) ± 1 SE at $P \leq 0.01$ (ANOVA, df = 2, F = 10.333, $P = 0.002$; followed by Tukey's multiple comparison procedure). Photomicrographs, inset, represent examples of typical seed coat colours following each treatment.

DISCUSSION

Exposure of seeds to fungal laccase *in vitro* stimulates germination for *Anacamptis morio*. In separate experiments it was also determined that laccase degraded seed coat lignin and significantly increased water uptake with respect to a control, suggesting a possible mechanism whereby 'testa-imposed dormancy' was broken [to borrow Vasudevan & van Staden's (2010) terminology]. The fact that bleach treatment resulted in much higher water uptake than the laccase treatment and yet was associated with less germination probably reflects the main problem that the new method attempts to address: bleach acts too rapidly and aggressively, extensively facilitating water uptake but concomitantly damaging the embryo. The laccase method stimulates less water uptake (yet significantly more than the control) but is not as reactive as bleach and targets lignin, entailing less risk of damage to the embryo. Hence laccase degrades the seed coat, facilitates some extra water uptake and stimulates germination. The optimum laccase exposure to maximise water uptake and germination was not determined in the present study, but this refinement could form the focus of future research.

Indeed, currently the results relate to a single species and should be seen as an initial trial of a novel methodology: application to other orchid species, and indeed other plant families, is called for. Limited initial investigation for other species was

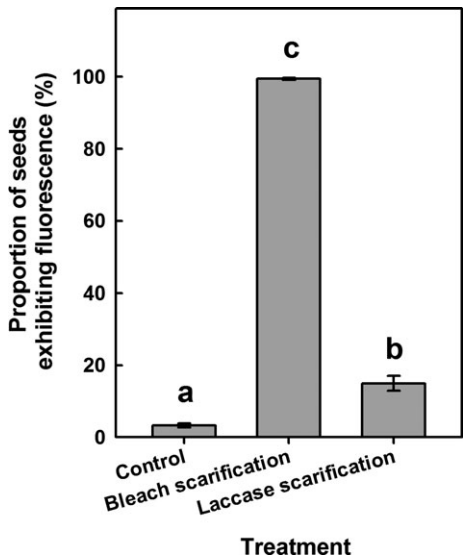

Fig. 3. The proportion of seeds of *Anacamptis morio* exhibiting fluorescence after exposure to water-mobile fluorochrome (*i.e.* exhibiting fluorochrome uptake and thus water uptake) for seed pre-treated with distilled water (negative control), or scarified with bleach (positive control) or laccase. Values represent means ± 1 SE. Different letters denoting significant differences between means at $P \leq 0.001$ (ANOVA: df = 2, F = 1471.884, $P < 0.000$; followed by Tukey's multiple comparison procedure; $n = 10$).

attempted at the time of the present study, but insufficient seed was available for rarer species such as *Himantoglossum adriaticum* H.Baumann and *Cypripedium calceolus* L. Although a statistically significant doubling of germination for *H. adriaticum* was observed in one test, in absolute terms the increase was from 1.3% to just 2.3% (data not shown), which is not a useful amount of germination if the aim is to produce plants for further cultivation, and *C. calceolus* showed no response. Thus the test was deemed a failure for these species. However, observation of the darker seed coats of these taxa suggests that lignin contents vary between species and it is likely that the application of laccase (in terms of activity, number of units and exposure time) must be optimised for each one. Furthermore, if further compounds such as cutin are involved, depending on the species (Vasudevan & van Staden 2010), then additional enzymes may be required.

It is tempting to see laccase application as a 'magic bullet' for seed germination, and indeed laccase is gaining recognition as a potential catalyst for a range of processes, from microbial fuel cells (Schaetzle *et al.* 2008) to food and beverage processing (*e.g.* wine stabilisation; Brijwani *et al.* 2010). However, species-specific responses may mean that this view is optimistic. Realistically, laccase could become another potentially important implement alongside other methods in the toolkit of plant biologists, the main use being for the *ex situ* production of rare or 'difficult' plant species for conservation purposes. For these species the avoidance of the damaging effects of chemical scarification on embryo development, seedling growth and thus fitness are particularly important, and new methods for stimulating germination are most valuable (Pierce *et al.* 2015). In the case of large seeds of non-orchidaceous species, these can present problems of incomplete surface sterilisation, which can easily introduce microbial contamination in sterile conditions (S. Pierce, personal observations), probably because they might present a larger and morphologically more complex seed coat surface where microbial spores shelter. Thus *in vitro* methods are not necessarily appropriate for all species: development of additional methods of seed exposure to laccase even in non-sterile conditions would be useful.

In conclusion, laccase was shown to stimulate a doubling of germination for a model orchid species, associated with degradation of lignin in the seed coat and increased water uptake. This novel method provides an alternative to corrosive chemical scarification methods: chemical scarification is 'quick and corrosive' (as denoted by extensive water uptake yet lower germination) and enzymatic scarification is slightly more complex in preparation but potentially more rewarding. The method could be extended to additional species.

ACKNOWLEDGEMENTS

We thank Elisa Cadei and Simone Masdea for help with laboratory work. Parco Monte Barro hosted the germination experiment and we thank the park for the use of the Laboratory for the Conservation of Biodiversity.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Examples of seeds of *Anacamptis morio* (Orchidaceae) exposed to fluorescein and washed in distilled water, seen under visible light (left column) or UV light (right column), respectively, with water uptake evident for individual seeds as fluorescence within the embryo. (Alberto Spada, Simon Pierce).

REFERENCES

Alvarez-Pardo V.M., Ferreira A.G., Nunes V.F. (2006) Seed disinfestation methods for in vitro cultivation of epiphyte orchids from Southern Brazil. *Horticultura Brasileira*, **24**, 217–220.

Aniszewski T., Lieberei R., Gulewicz K. (2008) Research on catecholases, laccases and cresolases in plants. Recent progress and future needs. *Acta Biologica Cracoviensia Series Botanica*, **50/1**, 7–18.

Barroso J., Chaves Neves H., Pais M.S. (1986) Ultrastructural, cytochemical and biochemical aspects related to the formation of *O. lutea* endomycorrhizae. In: Gianinazzi-Pearson V., Gianinazzi S. (Eds), *Physiological and genetical aspects of mycorrhizae*. INRA, Paris, France, pp. 265–258.

Barsberg S., Rasmussen H.N., Kodahl N. (2013) Composition of *Cypripedium calceolus* (Orchidaceae) seeds analyzed by attenuated total reflectance IR spectroscopy: in search of understanding longevity in the ground. *American Journal of Botany*, **100**, 2066–2073.

Baskin C.C. (2003) Breaking physical dormancy in seeds – focussing on the lens. *New Phytologist*, **158**, 227–238.

Boerjan W., Ralph J., Baucher M. (2003) Lignin biosynthesis. *Annual Review of Plant Biology*, **54**, 519–546.

Briggs C.L., Morris E.C. (2008) Seed-coat dormancy in *Grevillea linearifolia*: little change in permeability to an apoplastic tracer after treatment with smoke and heat. *Annals of Botany*, **101**, 623–632.

Brijwani K., Rigdon A., Vadlani P.V. (2010) Fungal laccases: production, function, and applications in food processing. *Enzyme Research*, 1–10. Article ID 149748

Cai X., Davis E.J., Ballif J., Liang M., Bushman E., Haroldsen V., Torabinejad J., Wu Y. (2006) Mutant identification and characterization of the laccase gene family in *Arabidopsis*. *Journal of Experimental Botany*, **57**, 2563–2569.

Chen F., Tobimatsu Y., Havkin-Frenkel D., Dixon R.A., Ralph J. (2012) A polymer of caffeyl alcohol in

plant seeds. *Proceedings of the National Academy of Sciences, USA*, **109**, 1772–1777.

Daws M.I., Pritchard H.W., Van Staden J. (2008) Butenolide from plant-derived smoke functions as a strigolactone analogue: evidence from parasitic weed seed germination. *South African Journal of Botany*, **74**, 116–120.

Dias M.A.N., Taylor A.G., Cicero A.M. (2014) Uptake of systemic seed treatments by maize evaluated with fluorescent tracers. *Seed Science and Technology*, **42**, 101–107.

Gahan P.B. (1984) *Plant histochemistry and cytochemistry – an introduction*. Academic Press, London, UK.

Guan Y.J., Wang J.C., Hu J., Li Y.P., Ma W.G., Hu W.M., Zhu S.J. (2013) Pathway to keep seed security: the application of fluorescein to identify true and fake pelleted seed in tobacco. *Industrial Crops and Products*, **45**, 367–372.

Holländer S. (1932) *Ernahrungs-physiologische Untersuchung an Pilzen saprophytische lebender Orchideen*. Dissertation, Wurzburg, Germany.

Kitsaki C.K., Zygouraki S., Kintzios S. (2004) *In vitro* germination, protocorm formation and plantlet development of mature versus immature seeds from several *Ophrys* species (Orchidaceae). *Plant Cell Reports*, **23**, 284–290.

Leake J.R. (1994) Tansley review no. 69. The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist*, **127**, 171–216.

Lee Y.I., Lee N., Yeung E.C., Chung M.C. (2005) Embryo development of *Cypripedium formosanum* in relation to seed germination *in vitro*. *Journal of the American Society for Horticultural Science*, **130**, 747–753.

Ma F., Cholewa E., Mohamed T., Petersonmark Gijzen C.A. (2004) Cracks in the palisade cuticle of Soybean seed coats correlate with their permeability to water. *Annals of Botany*, **94**, 213–228.

Malmgren S. (1996) Orchid propagation. Theory and practice. In: Allen C. (Ed.), *North American native terrestrial orchids. Propagation and production. North American Native Terrestrial Orchid Conference Proceedings*. C. Allen, Germantown, MD, USA, pp 63–71.

Martin F., Kohler A., Murat C., Veneault-Fourrey C., Hibbett D.S. (2016) Unearthing the roots of ectomycorrhizal symbioses. *Nature Reviews Microbiology*, **14**, 760–773.

McMahon A.M., Doyle E.M., Brooks S., O'Connor K.E. (2007) Biochemical characterisation of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6. *Enzyme and Microbial Technology*, **40**, 1435–1441.

Moldes D., Gallego P.P., Rodriguez Couto S., Sanroman A. (2003) Grape seeds: the best lignocellulosic waste to produce laccase by solid state cultures of *Trametes hirsuta*. *Biotechnology Letters*, **25**, 491–495.

Morrisson I.M., Asiedu E.A., Stuchbury T., Powell A.A. (1995) Determination of lignin and tannin contents of cowpea seed coats. *Annals of Botany*, **76**, 287–290.

Nieudorp P.J. (1972) Some observations with light and electron microscope on the endotrophic mycorrhiza of orchids. *Acta Botanica Neerlandica*, **21**, 128–144.

Pierce S., Cerabolini B.E.L. (2011) Asymbiotic germination of the white mountain orchid (*Pseudorchis albida*) from immature seed on media enriched with complex organics or phytohormones. *Seed Science and Technology*, **39**, 199–203.

Pierce S., Vagge I., Brusa G., Cerabolini B.E.L. (2014) The intimacy between sexual traits and Grime's CSR strategies for orchids coexisting in semi-natural calcareous grassland at the Olive Lawn. *Plant Ecology*, **215**, 495–505.

Pierce S., Guidi V., Ferrario A., Ceriani R.M., Labra M., Vagge I., Cerabolini B.E.L. (2015) Pea seed extracts stimulate germination of the terrestrial orchid *Ophrys apifera* Huds. during a habitat restoration project. *Plant Biosystems*, **149**, 54–60.

Pourcel L., Routaboul J.-M., Kerhoas L., Caboche M., Lepiniec L., Debeaujon I. (2005) TRANSPARENT TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. *The Plant Cell*, **17**, 2966–2980.

Pritchard H.W. (1985) Determination of orchid seed viability using fluorescein diacetate. *Plant, Cell and Environment*, **8**, 727–730.

Ran X., Wan H., Li J., Liang Y., Xu J. (2015) An investigation of the relationship between lignin content and seed coat traits in yellow-seeded *Brassica napus* L. *Academia Journal of Agricultural Research*, **3**, 1–8.

Rao X., Krom N., Tang Y., Widiez T., Havkin-Frenkel D., Belanger F.C., Dixon R.A., Chen F. (2014) A deep transcriptomic analysis of pod development in the vanilla orchid (*Vanilla planifolia*). *BMC Genomics*, **15**, 964.

Rasmussen H.N. (1995) *Terrestrial orchids: from seed to mycotrophic plant*. Cambridge University Press, Cambridge, UK.

Rasmussen H.N., Dixon K.W., Jersáková J., Těšitelová T. (2015) Germination and seedling establishment in orchids: a complex of requirements. *Annals of Botany*, **116**, 391–402.

Redl K. (1999) *Heimische Orchideen – Identifizierung der Fruchtstände*. Konrad and Kren, Gröbming, Germany (in German).

Salanenka Y.A., Taylor A.G. (2011) Seedcoat permeability: uptake and post-germination transport of applied model tracer compounds. *HortScience*, **46**, 622–626.

Sampaio D.A., Abreu H.S., Augusto L.D.S., da Silva B., Ibanez C.M. (2016) Approach on the integument lignin profile of seeds of *Araucaria angustifolia*. *Bosque*, **37**, 549–555.

Schaetzle O., Barrière F., Schröder U. (2008) An improved microbial fuel cell with laccase as the oxygen reduction catalyst. *Energy and Environmental Science*, **2**, 96–99.

Schwarz P.B., Kunerth W.H., Youngs V.L. (1988) The distribution of lignin and other fiber components within hard red spring wheat bran. *Cereal Chemistry*, **65**, 59–64.

Slaviero A., Del Vecchio S., Pierce S., Fantinato E., Buffa G. (2016) Plant community attributes affect dry grassland orchid establishment. *Plant Ecology*, **217**, 1533–1543.

Smith S. (1966) Physiology and ecology of orchid mycorrhizal fungi with reference to seedling nutrition. *New Phytologist*, **65**, 488–499.

Stadler R., Lauterbach C., Sauer N. (2005) Cell-to-cell movement of green fluorescent protein reveals postphloem transport in the outer integument and identifies symplastic domains in *Arabidopsis* seeds and embryos. *Plant Physiology*, **139**, 701–712.

Thorogood C.J., Rumsey F.J., Hiscock S.J. (2009) Seed viability determination in parasitic broomrapes (*Orobanche* and *Phelipanche*) using fluorescein diacetate staining. *Weed Research*, **49**, 461–468.

Tobimatsu Y., Chen F., Nakashima J., Escamilla-Treviño L.L., Jackson L., Dixon R.A., Ralph J. (2013) Coexistence but independent biosynthesis of catechyl and guaiacyl/syringyl lignin polymers in seed coats. *The Plant Cell*, **25**, 2587–2600.

Vasudevan R., van Staden J. (2010) Fruit harvesting time and corresponding morphological changes of seed integuments influence *in vitro* seed germination of *Dendrobium nobile* Lindl. *Plant Growth Regulation*, **60**, 237–246.

Wang C.-Y., Zhang S., Yu Y., Luo Y.-C., Liu Q., Ju C., Zhang Y.-C., Qu L.-H., Lucas W.J., Wang X., Chen Y.-Q. (2014) MiR397b regulates both lignin content and seed number in *Arabidopsis* via modulating a laccase involved in lignin biosynthesis. *Plant Biotechnology Journal*, **12**, 1132–1142.

Wilson J.K. (1915) Calcium hypochlorite as a seed sterilizer. *American Journal of Botany*, **2**, 420–424.

Yamazaki J., Miyoshi K. (2006) *In vitro* asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). *Annals of Botany*, **98**, 1197–1206.

Yang C.-K., Lee Y.-I. (2014) The seed development of a mycoheterotrophic orchid, *Cyrtosia javanica* Blume. *Botanical Studies*, **55**, 44.

Zeng G.M., Yu H.Y., Huang H.L., Huang D.L., Chen Y.N., Huang G.H., Li J.B. (2006) Laccase activities of a soil fungus *Penicillium simplicissimum* in relation to lignin degradation. *World Journal of Microbiology and Biotechnology*, **22**, 317–324.