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ROLE OF SMOKE ON ORCHID SEED GERMINATION : RECENT UPDATES AND PROSPECTIVES

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This review paper highlights the influence of smoke saturated water (SSW) on asymbiotic seed germination and an early differentiation of protocorms and plant regeneration of *Pholidota pallida* Lindl and *Vanda parviflora*. High percentage germination (85%) and high percentage of plantlet recovery (71%) was achieved by culturing seeds on Mitra *et al.* basal medium supplemented with 10% (v/v) SSW. The application of smoke and aqueous smoke extracts to improve seed germination has been shown in a wide range of plants from many families, irrespective of their fire sensitivity. Therefore, from the above results it is clear that active compound(s) within SSW play a regulatory role in plant development. As all these physiological effects are in part controlled by plant growth regulators (PGRs), indications are that the smoke extracts interact in same way with endogenous PGRs. In addition to impacts on germination performance, butenolide applied as a germination treatment, has been reported to have positive impacts on seedling growth in orchids.

Keywords: Butenolide; Germination; Micropropagation; Native orchid; Western Ghat forests

Introduction

The increased popularity of orchids has lead to a major increase in production and sales. With increased popularity a potential need exists for the introduction of new commercially valuable orchids[1-10]. Very recently commercial production and cultivation of native orchid production have slowly increased. A major obstacle to native orchid production is the difficulty in seed germination. Native orchids are produced through seed germination, but seedling development can be a long process and flowering plants are often produced only after 3-5 years of growth. Orchid seeds are often named as *'dust seeds'* as they are very minute, tiny and contain few food reserves. In nature they may germinate but will not grow unless infected by a mycorrhizal fungus, which supplies the young plants with all the sugars and nutrients they need until the plants are large enough to produce food on their own[1-10]. Once the seed has germinated it produces a fairly undifferentiated mass of cells called a protocorm in orchids. All being well this protocorm will continue to grow for many weeks, months or even years depending on species, until large enough to produce leaves and roots. In case of terrestrial orchids it is vitally important that the orchid/fungus relationship is maintained during the early stages of the plants life, as the protocorm is subterranean and can not produce any food of its own. In epiphytic orchids the protocorms are often green, and thus can produce some food of their own[1-10]. In symbiotic seed germination, the seeds are sown with a small piece of an appropriate mycorrhizal fungus. This fungus then grows over the media, colonizes the germinating seeds and a symbiotic relationship is formed which presumably will sustain the protocorm until it produces leaves and becomes autotrophic[1-10]. This technique is widely used for the propagation of temperate terrestrial orchids. It has the advantage that media used is very simple (consisting of only powdered oats with a little yeast extract), and the resultant mycorrhizal plants are often stronger and more resistant to fungal infection than some of their asymbiotically propagated counterparts. It has the disadvantages that you need the correct strain of mycorrhizal fungus, or the symbiosis will not develop or

might become parasitic and the seedlings die. Asymbiotic germination is commonly used in the propagation of tropical orchids, which tend to be easier to grow than their temperate relatives. The media used for asymiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the orchid without the fungus intermediary[1-10].

However, the greatest threat to orchid diversity is habitat loss; for orchids this may occur on a very small scale because a single tropical tree may bear hundreds of epiphytic orchid species. The scale of threat to orchid diversity then reaches frightening proportions as millions of hectares of habitat are lost annually to ranching, monocrop agriculture, mining, logging, and urban development[1-10]. Orchids are badly affected by habitat destruction and their unabated collection. Because orchids are the most evolved of all flowering plants, they are very site-specific and need optimum conditions to thrive in a given ecosystem. If orchids are present in an ecosystem, this is a good indicator of a healthy, functioning ecosystem. Additionally, many orchid flowers and their habitats are beautiful, and provide pleasure to those who seek out these unique members of the plant kingdom[1-10]. Once discovered, the places where these rare, beautiful plants grow can become popular sites for naturalists, and photographers. Obviously, poaching destroys natural populations of orchids, but other detrimental effects may be less obvious. Secondly, since orchid habitats are so sensitive, they typically die several years after being transplanted into a garden. Not only does this kill the individual plant, it also destroys its chances for reproduction, challenging to grow, some are relatively easy. Although orchid sales are quickly rising, production and sales of native orchids, at best are slowly increasing. Production of native orchids has not been fully commercialized, but is centralized within hobby growers and small, specialized nurseries. These nurseries only offer a small selection of showy genera and species. Conventional vegetative propagation is beset by a slow multiplication rate, and does not provide sufficient clones within a short timeframe. Therefore, it is essential to take immediate measures for the micropropagation of many orchids using *in vitro* culture techniques. Plant tissue culture methods have played an important role in the micropropagation of several commercially important orchids to meet the demands of a growing market throughout the world. Therefore, there is an urgent need to develop *in vitro* propagation protocols for the conservation of many native orchid species[1-10].

Effect of smoke on in vitro seed germination of orchid-

Smoke has been shown to stimulate germination of numerous species from a range of fireprone environments worldwide including Australian kwongan, Californian chaparral, Western Cape fynbos, and the Mediterranean basin. In a survey of 301 South African fynbos species, it was found that 49.8% of species, ranging from annual herbs to geophytes to trees, had a positive germination response to smoke. However, a smoke germination response is not limited to species from fire-prone environments as smoke stimulates germination in species such as lettuce, red rice, wild oats and native orchid germination of *Vanda parviflora and Pholidota pallida*[11]. An increase in percentage germination as well as early differentiation of protocorms into seedlings was observed on 10% (v/v) SSW-supplemented Mitra et al. basal medium compared to control in *Pholidota pallida*[11]. Maximum percentage germination (85%) was observed on 10% (v/v) and seed germination percentage was greatly inhibited at higher concentrations of SSW (15 and 20%) compared to the control and most seeds turned brown without germination. Therefore, the presence of SSW at 10% (v/v) in basal medium resulted in faster differentiation of protocorms to form plantlets (i.e. leaves and roots) than the control[11]. On the other hand, another study related to orchid seed germination revealed that 10% (v/v) SSW-supplemented basal medium formed plants during hardening that were normal and showed healthy growth with a 90% survival rate, i.e. SSW at 10% (v/v) aids in rapid regeneration of *V. parviflora*[11].

Smoke; Background - It was of interest that a wide range of sources of plant material produced smoke that stimulated the germination of *T. triandra* seeds and Grand Rapids lettuce seeds. Of particular importance was the finding that smoke from burned paper, or even an extract prepared from heated agar or cellulose, could stimulate the germination of Grand Rapids lettuce seeds. Results from these studies demonstrated that the germination-promoting compound(s) were produced from commonly occurring plant constituents[12-20]. Prior to the isolation and identification of the active butenolide from smoke, it was known that the germination cue(s) were water soluble chemical (s) that were thermostable, long-lasting in solution, and highly active at very low concentrations[21-22]. Baldwin[23] identified 71 compounds in active fractions of smoke by GC-MS and atomic absorption (AA) spectrometry, and tested a total of 233 compounds using seeds of *Nicotiana attenuata.* None of these compounds, however, promoted germination. In this study, they also demonstrated that germination activity could be obtained from smoke produced from burned cellulose and it was

estimated that less than 1 pg of the active chemical is needed per seed. Thus, the difficulty in isolating the active component(s) from aqueous smoke extracts was partly due to the large number of compounds present in the smoke extract, possibly up to several thousand, and partly due to the very low concentration of the active compound(s) relative to the other components present in the smoke. They also demonstrated that ethylene was probably not responsible for the observed germination activity, because it is unlikely that it would still be present in the active fractions following the various chromatographic procedures[24-59].

South African research group headed by Prof Johannes van Staden conducted chromatographic separation of two different smoke extracts, from burned fynbos material or burned *Themeda triandra* (climax grass). One major peak of germination activity, with a similar retention time from the non-polar fraction, was found in both extracts although; some other fractions also had limited positive effects on germination. Germination activity was tested using achenes of *Syncarpha vestita* (a fynbos species) and caryopses of *T. triandra.* Similar results were also observed using achenes of Grand Rapids lettuce. The lettuce seeds, which germinated within 24 h, proved to be more suitable for bioactivity-guided fractionation than the *S. vestita* achenes, which required 20 days or longer showing an optimal response[85-91]. Subsequently, South African research group identified seven compounds present in both *Passerina vulgaris* and *T. triandra* smoke extracts. Four of the compounds (available commercially) were tested in the Grand Rapids lettuce seed bioassay at concentrations from 10^{-4} to 10^{-15} M. However, none were found to be active. Chromatographic separation of these two extracts, using thin layer chromatography (TLC), semi-preparative high-performance liquid chromatography (HPLC), and analytical HPLC, indicated that the compound with germination activity was present in the same fractions[72-85]. Although bioactivity-guided fractionation led to one major peak of activity, there was some chromatographic evidence indicating that there may be more than one active component in smoke that promotes seed germination. Furthermore, chromatographic purification of aqueous smoke extracts from fynbos material and *T. triandra,* as well as a commercial food-flavourant, supported the notion of a common active compound. Prior to the identification of the active compound in smoke, it was also concluded that nitrogen oxides, and nitric oxide (NO) in smoke were most likely responsible for stimulating seed germination. However, a study by South African Research group examined the effects of two NO releasing compounds, N-tert-butyl-α-phenylnitrone (PBN) and sodium nitroprusside (SNP), on the germination of Grand Rapids lettuce seeds. In contrast to smoke application, neither PBN nor SNP stimulated the germination of Grand Rapids lettuce seeds in the dark. Additionally, the NO-specific scavenger 2-(4-carboxyphenyl)- 4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium (c-PTIO) was unable to reduce the germination response observed with smoke solutions. These results suggested that NO was unlikely to be responsible for the enhanced germination of Grand Rapids lettuce seeds by smoke solutions. In another study it was also confirmed the absence of NO^{2-} in aqueous smoke solutions derived from burned cellulose or wood, although these solutions effectively promoted germination of *Emmenanthe penduliflora* and *N. attenuata.* Continued efforts at isolating the compound by both the South African and Australian research groups culminated in the characterization of a highly active butenolide compound, 3- methyl-2H-furo[2,3-c]pyran-2-one, from plant-derived smoke and burned cellulose respectively. The compound has become commonly referred to as 'butenolide' in several studies, although strictly speaking this name refers to the type/class of compound, and in a recent article, it has been referred to as 'karrikinolide' (KAR1)[40- 93]. It was found that the compound promoted the germination of certain seeds over a wide range of concentrations, and at concentrations as low as 10^{-9} M for Grand Rapids lettuce seeds, and in the region of 10^{-7} M for *Conostylis aculeata* and *Stylidium affine.* Similarly, it was showed that activity in Grand Rapids lettuce seeds from 10^{-4} M down to 10^{-9} M. Further experiments by the South African research group showed that 3-methyl-2H-furo [2,3-c]pyran-2-one could also be formed during Maillard reactions between sugars and amino acids. Heating proteins or amino acids with sugars at 180°C for 30 min produced water-soluble extracts that promoted the germination of Grand Rapids lettuce seeds in the dark. Using HPLC fractionation, it was demonstrated that the active compound(s) formed during these reactions co-eluted with the active fraction from the smoke extract. Further analysis using GC-MS showed that the active constituent was identical to the active compound isolated from plant-derived smoke[58-90]. Thus, the study confirmed that germination promoting compounds, including 3- methyl-2H-furo [2,3-c]pyran-2-one, could indeed be formed by heating ubiquitously occurring organic compounds. In particular, extracts prepared from reactions between d-xylose or dribose (aldopentose sugars) with the amino acids arginine,

asparagine, aspartic acid, glycine, serine, tyrosine or valine, gave the greatest germination response[54-90]. The butenolide was tested by Verschaeve for possible mutagenic and genotoxic effects using the VITOTOX® test and the Ames assay. Importantly, the results indicated that the compound is not toxic nor genotoxic at the levels tested (1×10^{-4} to 3×10^{-10} M), which raises the possibility of wide scale usage of the compound as both a germination stimulant and in a field setting. Following the identification of the active butenolide from smoke, there have been several groups who have successfully synthesised the active butenolide, as well as several analogues[68-92]. The Australian research group, who originally reported the active compound, described the synthesis of 3-methyl-2H-furo [2,3-c]pyran-2-one from pyromeconic acid and the preparation and activity of several derivatives. It was also described that the synthesis of the compound, and several analogous compounds from d-xylose. Interestingly, the butenolide was found to be a product formed during a heating reaction between d-xylose and glycine. Two more recent publications by other research groups also describe the synthesis of the compound. Butenolide not only enhances germination percentage and rate but can also widen the environmental window over which germination can occur. Thus, for a range of Australian ephemeral Asteraceae species, it was reported that, like GA_3, butenolide was able to partially or fully substitute for a light requirement for germination, as observed with Grand Rapids lettuce seeds. Butenolide at 10^{-7} M can also affect the temperature range for germination/seedling development[27-92].

Role and function of smoke in germination - Butenolide certainly holds potential for field-scale use in; for example, weed control and re-vegetation of degraded areas. However, for effective use in the natural environment, it is important to know (1) how long butenolide persists in the soil, (2) natural concentrations in fire-prone environments, and (3) potential impacts on soil microbes. However, these topics have received scant attention. The only study to date that has addressed any of these issues investigated the movement of butenolide down a soil (white silica sand) profile following simulated rainfall events ranging from 4 to 16 mm. Therefore it was found that application of butenolide, at a rate equivalent to 2 g/ha, resulted in butenolide, at germination active concentrations, moving down the profile to depths ranging from 8.5 to 18.3 cm (following simulated rainfall events of 4 and 16 mm, respectively)[80-91]. Thus, butenolide is mobile in soil and retains bioactivity, at least in the short-term. However, we still know neither the effects of soil type on the activity of butenolide nor the half-life of butenolide in soil. Such questions could partly be addressed using different soil types in the bioassay approach. However, it would also be of value to determine actual field level concentrations of butenolide in post-fire environments and the distribution of butenolide down soil profiles. The mode of action of smoke, and hence by implication butenolide, has been ascribed to an interaction with the gibberellin pathway in seeds[90-93]. For example, smoke has a similar effect to GA_3 in substituting for red light (640 nm) in the stimulation of Grand Rapids lettuce germination. Similarly, butenolide has been reported to have similar effects on germination as GA_3 by both stimulating germination and substituting for light in the germination of Australian Asteraceae, and stimulating germination in arable weeds. It was reported that a significant relationship, across the study species, between the germination response to butenolide and GA_3. However, overall, butenolide was the most effective and did not result in the elongated internodes that are typically associated with GA_3. Consequently, butenolide is likely to be of greater value than GA_3 for germination testing on diverse species since the resulting seedlings are more likely to be morphologically 'normal'. Other studies have also indicated that smoke affects endogenous GA synthesis and ABA content. However, the effect of butenolide on levels of endogenous plant hormones has not been fully investigated. While there are clear similarities in the responses of seeds to butenolide/smoke and GA_3, there are few obvious similarities between the chemical structures of the two compounds. There are, however, structural similarities between butenolide and the strigolactones, which stimulate germination in parasitic plant species such as *Orobanche* and *Striga*[45-92]. Recently, in another report it was revealed that butenolide can substitute for strigolactones in stimulating germination of parasitic weeds (including *Striga* and *Orobanche*) suggesting that butenolide may function in the same way as strigolactones. This proposition is further supported by structure-activity relationship studies of strigolactones, using synthetic analogues of strigol, which have shown that the lactone-enol ether is primarily responsible for the biological activity of these compounds[38-92]. Auxins play an important role in embryogenesis and seedling development and are important for 'normal' development in *in vitro* cultures by providing positional information for the coordination of correct cellular patterning from the globular stage onwards. It was found that butenolide may function in a similar way to auxins being able to substitute for 2,4-D (a synthetic auxin) in somatic

embryogenesis of *B. tetraphyllum*. However, very little is known about what role auxins play during seed germination although a relationship between IAA, dormancy and pre-harvest sprouting of wheat has been reported[35-90]. Butenolides that are structurally related to 3-methyl-2H-furo [2,3-c]pyran-2-one in smoke are produced by a range of microorganisms. For example, *Fusarium* sp. produces a 'butenolide' that functions as a mycotoxin with a mode of action resulting from an impact on the intracellular redox environment. Since oxidative stress has been proposed to have a signalling role in germination, this area may also be worth pursuing in relation to the role of butenolide in germination[29-92]. Thus, studies suggest potential similarities between butenolide and gibberellins, auxin and strigolactones. However, it is perhaps not surprising that a single molecule can appear to have analogous properties to a range of plant growth regulating compounds since plants have signaling proteins that can function in several pathways[65-91]. For example, a key integrating factor is BIG which is necessary for auxin transport, cytokinin, GA, ABA, ethylene and brassinosteroid signaling. To date, however, only one study has been published which aimed at elucidating the regulation of germination in the presence of butenolide at a molecular level, though additional studies are no doubt in progress. Recently a study of differential display during tomato seed germination, reported the up-regulation of genes encoding expansins in the presence of butenolide. Expansin genes are highly conserved and most have been proposed to be involved in cell expansion during tissue growth. They are thought to function by disrupting the hydrogen bonds between cellulose and hemicellulose polymers thereby allowing cells to expand. Expansions have been reported previously in seed germination, playing a role in both endosperm cap weakening and embryo growth in tomato[46-82]. Germination is, in essence, cell expansion and/or elongation culminating in visible radicle emergence through the testa. If up-regulation of expansins by butenolide/ smoke is widespread it also provides a mechanism to explain the enhancement in seed germination rate even in seedlots that germinate to 100% in the absence of butenolide[46-91]. It was reported that germination associated expansins in tomato are under the control of GA, further reinforcing the likely cross-talk between butenolide and endogenous plant growth regulators. Recent study investigated that the sensitivity of seven Australian *Asteraceae* from non-fire-prone environments to butenolide and found that it was an effective germination stimulant that could also overcome the light requirement for germination[33-82]. They also speculated that the release of butenolide from soil surface layers following disturbance may be a mechanism to explain this apparent ecological anomaly and the large-scale emergence of these *Asteraceae* following soil disturbance. This premise remains to be tested, however, and a simpler or partial explanation for germination following disturbance in these species may be due to short-term exposure of these light sensitive seeds to irradiance. Another important aspect related to plant growth in natural or agricultural environments is the presence of arbuscular mycorrhizal (AM) fungi which form symbiotic relationships with plant roots[38-90]. Such fungi supply the plant host with nutrients, such as phosphate and obtain photosynthates from the host plant[36-90]. Plant roots secrete a 'branching factor', which stimulates branching of the fungal hyphae that penetrate plant roots. Recently the strigolactone 5-deoxy-strigol has been isolated from *Lotus japonicus* root exudates and identified as a branching factor. AM fungal spores can germinate in the absence of a host, but hyphae exhibit limited branching and development[34-87]. Strigol and the synthetic strigol analogue GR24 can also induce extensive hyphal branching in *Gigaspora margarita* suggesting that parasitic plants find their hosts by detecting the same chemical signals that AM fungi use for host recognition[35-72]. Since butenolide can also stimulate the germination of parasitic weeds (i.e. it can function as a strigolactone analogue) this raises the question of what impact butenolide may have on the growth and morphology of AM fungi if applied as a soil treatment. For example, would soil application of butenolide result in stimulation of hyphal growth and branching in the absence of suitable plant hosts? Such concerns are further reinforced by the mycotoxic effects of similar 'butenolides' produced by other microbes[46-90]. While the natural occurrence of butenolide in fire-prone environments may appear to negate these arguments, there is evidence that fire reduces the levels of AM fungal colonization of roots, with the mechanisms causing these negative impacts unclear[42-87]. However, several knowledge gaps remain, not least the persistence of butenolide and its wider effects in the rhizosphere, before this potential can be fully realized. Finally, opportunities now present themselves to elucidate the mode of action of butenolide, whether 'butenolides' should be recognized as a new class of plant growth regulators and likely cross-talk with endogenous growth regulators using molecular biology techniques, such as microarrays and other developing technologies. As knowledge of the intricacies of seed germination and plant signalling become better understood, we will be able to piece together the

fundamental mechanisms of this fascinating phenomenon[29-92]

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