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Pea seed extracts stimulate germination of the terrestrial orchid *Ophrys apifera* Huds. during a habitat restoration project

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Abstract
Novel methods are required to break the seed dormancy of temperate-zone orchids and aid the conservation of rare species. Zeatin is produced in increasing concentrations during the development of pea (*Pisum sativum*) seeds. We hypothesised that hot water extracts of pea seeds stimulate germination of the orchid *Ophrys apifera in vitro*, particularly for extractions made during later pea development. Pea seeds, exposed to 0, 3, 6, 9, 12 or 15 days of periodic wetting, were extracted in hot water and the extracts were added to Malmgren's growth medium. Germination of *O. apifera* on this medium was quantified after 7 months, stimulated by a range of pure hormones. Pea seed extract collected later in pea development (at 6–15 days) inhibited germination of *O. apifera*. However, extracts taken at 0 and 3 days significantly increased germination from 3.8 ± 0.32% in the control to 9.1 ± 1.84% and 7.6 ± 0.79%, respectively: increases comparable to the most effective of the pure hormones. Dried peas therefore provide an economical alternative source of germination stimulants for orchids. We briefly report how sufficient mature plants of *O. apifera* were produced to allow a population to be multiplied to 15 times its original size during a habitat restoration project.

Keywords: *Asymbiotic germination, bee orchid, coconut milk, cytokinin, in vitro, Orchidaceae, zeatin*

Introduction

Habitat restoration may involve the sowing of seed mixtures or wild hayseed to improve the species richness of degraded vegetation, but these methods sometimes neglect the rarest elements of the flora. The rarest species must be propagated, usually from seed, and planted out to reinforce wild populations or to reintroduce locally extinct plant species. Furthermore, populations of some species, particularly temperate terrestrial orchids, may be naturally small even where the habitat is ideal. It is these species that stand to benefit most from successful *ex situ* seed germination, cultivation and population reinforcement (Pierce et al. 2006, 2010). However, many rare or endangered species have particular, and unknown, germination requirements, and one of the critical steps in their conservation is often understanding germination requirements *ex situ* (e.g. Lantieri et al. 2012; Mattana et al. 2012; Pradhan & Badola 2012) or techniques for successful *in vitro* micropropagation (e.g. Panda & Hazra 2012).

Orchids are characterised by an extremely specialised reproductive ecology, particularly for temperate-zone species that remain dormant for a significant period of the year, and seed dormancy breaking *ex situ* is often problematic and requires specialist *in vitro* techniques (e.g. Butcher & Marlow 1989; Lauzer et al. 2007). The symbiotic fungi that stimulate germination in the wild (see Perotto et al. 2013) are time-consuming and expensive to use and unreliable in the laboratory, and are not necessarily those that are isolated from root tissues of adult plants (Xu & Mu 1990; Pierce et al. 2010). Indeed,

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most orchid production for commercial or conservation purposes relies on asymbiotic techniques (Butcher & Marlow 1989). Orchids produced asymbiotically and reintroduced into the wild form normal mycorrhizal relationships within weeks (Sgarbi et al. 2001). Thus one of the main aims of orchid conservation is the discovery of novel asymbiotic methods for seed germination, which is the main objective of this study.

The vast numbers of seeds produced by orchids (Darwin 1862) mean that even small improvements in percentage germination translate into the production of hundreds of extra plants. The asymbiotic germination of terrestrial orchids has been described extensively previously (e.g. Hadley & Harvais 1968; Butcher & Marlow 1989; Kitsaki et al. 2004; Lauzer et al. 2007; Dutra et al. 2008, 2009; Sgarbi et al. 2009; Pierce 2011), but it is worthwhile to reiterate the key concept of the use of phytohormones to stimulate germination and subsequent development. This may take the form of either pure research-grade materials (e.g. De Pauw et al. 1995; Pierce et al. 2010; Pierce & Cerabolini 2011) or alternatively, and more economically, "complex organic media" (COM; Kitsaki et al. 2004), such as seed extracts or fruit juices that contain a mixture of these compounds (e.g. banana homogenate; Kaur & Bhutani 2013). Coconut water is a liquid endosperm that contains the hormones zeatin and, to a lesser extent, kinetin and other substances, that stimulate orchid growth, such as sugars, a range of amino acids (which orchids prefer as a nitrogen source over inorganic compounds; Malmgren 1996) and vitamins (Yong et al. 2009). Coconut milk (a mixture of coconut water and coconut flesh) contains a range of compounds that can be used by the developing orchid, and so actually stimulates a greater degree of germination and stronger subsequent growth than pure hormones alone (Hadley & Harvais 1968). Novel sources of biologically active COM, containing phytohormones such as zeatin alongside amino acids and sugars, provide potential alternatives for the stimulation of orchid seed germination. These could rival or supersede hormones or coconut-enriched medium (CEM).

While coconut milk has become a staple of propagation methods for terrestrial orchids (e.g. Kitsaki et al. 2004) and for general plant tissue culture (e.g. Ibrahim et al. 2009; Mujib et al. 2013), the main active ingredient, zeatin, is abundant in a range of readily available seeds. For example, Hahn et al. (1974) found that dried pea (*Pisum sativum* L.) seeds contained a cytokinin and also that the concentration of this increased over a 2-week period as the pea seeds germinate and develope. It was later found (Stirk et al. 2008) that the radicle in pea seeds produces zeatin, and that over the first few hours and days of pea seed germination the root produces increasing quantities, peaking at around 6 days after germination with the initiation of secondary root production and continuing for at least 2 weeks. Thus pea seeds are a promising source of economical and readily available zeatin, and zeatin production is potentially greatest after the first week of development.

Zeatin is only moderately soluble in water, and organic solvents, alcohol or sodium hydroxide are typically used when extracting zeatin for isolation and purification purposes (Hahn et al. 1974); i.e. when precision is required. However, the aqueous extraction of hormones from plant tissues avoids the complications of extracting other metabolites that are soluble in alcohol or organic solvents (Jones 1968), and this is an important consideration when the extracts will then be used directly to induce seed germination and toxic effects are undesirable. Direct extraction and application of aqueous extracts also has the advantages of rapidity, ease of application and economy, compared to the purification of particular hormones (Jones 1968). These are important considerations for workers seeking simple and inexpensive methods of dormancy breaking for the large-scale production and conservation of rare plants in an austere economic climate.

Ophrys apifera Huds. (Orchidaceae) is one of the rarest orchid species in northern Italy (Ferlinghetti 2001) and exhibits highly dispersed populations that are small even where suitable habitat is available. Individuals of *O. apifera* forming a population of 25 individuals at Parco Monte Barro (a small regional park in northern Italy; see Pierce et al. 2006) were found to have *in vitro* germination rates of just 4.1% on standard terrestrial orchid growth media (Pierce et al. 2010). The principal hypotheses of this study were (1) extracts of pea seeds stimulate significantly greater germination of *Ophrys apifera* with respect to a control treatment and (2) extracts of developing pea seeds have a greater capacity to stimulate germination than un-germinated (dried) pea seeds. Furthermore, baseline data on the germination of *O. apifera* in response to various pure hormones and COM such as CEM are reported here, alongside information on how to use the plants that are produced during practical habitat restoration and population reinforcement activities.

Materials and methods

Mature seeds were collected from a population of 25 plants growing in mesobromion calcareous grassland vegetation (EU habitat 6210⋆) at Parco Monte Barro, Lecco, Italy, on either 22/06/2007 or 29/06/2009. Seed was collected in 20 ml capacity glass bottles and stored for 2 months in the drying

room of a seed bank, maintained at 15% relative humidity and ambient temperature (~22°C). For seed sowing, seed sub-samples were transferred to 1.5 ml Eppendorf tubes and surface sterilised using 5% (v/v) bleach solution (equivalent to 0.4% NaOCl or 0.25% active chlorine) containing 0.1% Tween surfactant, as a wetting agent, for 25 min, followed by six rinses in sterilised, distilled water, in a sterile environment. Seeds were sown using a sterilised stainless steel spatula on agar media contained in Petri dishes subsequently sealed using laboratory film (Parafilm).

The basal culture medium consisted of modified Malmgren's orchid medium (Malmgren 1996; detailed in Pierce et al. 2010), containing $6\,g\,l^{-1}$ agar, $0.5\,g\,l^{-1}$ activated charcoal powder, $10\,g\,l^{-1}$ sucrose, $75\,mg\,l^{-1}$ $MgSO_4{\cdot}7H_2O$, $75\,mg\,l^{-1}$ $(Ca)_3PO_4$, $75\,mg\,l^{-1}$ KH_2PO_4, $100\,mg\,l^{-1}$ NH_4NO_3, $150\,mg\,l^{-1}$ $NH_4H_2PO_4$. Modifications of this basal, control medium included the exclusion of amino acids, which cannot be added precisely in the same manner as Malmgren's (1996) original medium as an unspecified mixture of amino acids was employed, and amino acids have been found to inhibit the germination of a number of *Ophrys* species, including *O. apifera*; Ferrario 2008). An additional modification was the addition of either $50\,ml\,l^{-1}$ coconut milk (5%, v/v), 5% (w/w) yeast extract or 5% (w/w) banana powder (Micropoli, Milan, Italy), added to the medium immediately prior to autoclaving at 0.1 MPa and 121°C for 20 min. Coconut milk, yeast extract and banana powder were added to the growth media prior to autoclaving because this is the typical manner in which they are used for orchid germination; after heat treatment (e.g. canning, autoclaving) coconut milk retains the ability to stimulate orchid germination (Kitsaki et al. 2004; Pierce et al. 2010). A range of phytohormones are typically tested when germination requirements are unknown (e.g. Seglie et al. 2012). Because phytohormones can lose some activity following heat treatment and their precise concentrations were required, 6-benzylaminopurine (6BAP), gibberellic acid (GA_3), kinetin, thidiazuron (TDZ) and zeatin (Micropoli, Milan, Italy) were added to sterilised basal growth medium when this had cooled to 65°C (the temperature of the growth medium within conical flasks was measured from a distance of approximately 3 cm using an infrared thermometer). Hormone solution was added via filter sterilisation in sterile conditions, in which 20 ml of a hormone 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 of sterile, autoclaved agar medium; the final concentration of each hormone being 0.1 mM, in 500 ml of medium (each 500 ml conical flask of medium is sufficient to produce approximately 50 agar plates in 6-cm diameter Petri dishes). In this experiment, eight agar plates were produced for each treatment, but due to low levels of contamination only seven replicate agar plates per treatment were used in the final analysis.

Forty grams of dried, viable pea (*Pisum sativum* "Progress No. 9") seeds were washed with water every 12 h over a 15-day period, and further 40 g samples were washed subsequently every 3 days. This provided, on the 15th day, samples of seeds at the following stages of development: 0, 3, 6, 9, 12 and 15 days. Washing was performed by containing each pea sample in a separate glass jar closed with screen-door fly-mesh that allowed water to be poured in and to be drained away whilst retaining the seeds: seeds were not left to stand in water at any time. At the end of the final day, the seed samples were each boiled for 20 min in 400 ml of distilled water, and the cooled extract solution was then used to produce the agar medium via the addition of Malmgren's basal medium salts, made up to a 500 ml volume, which was then used to produce agar plates. Eight replicate agar plates were produced for each treatment.

The use of agar plates in Petri dishes for seed germination, rather than flasks, allowed the use of a stereomicroscope to count the number of seeds containing embryos and the number of embryos that developed to form white, swollen protocorms with rhizoids. Agar plates were stacked and double-wrapped in aluminium foil (germination of *Ophrys* spp. is strongly inhibited by light; Mead and Bulard 1975) and placed in a growth chamber (Snijders Economic Deluxe; Thermo-Lab, Codogno (LO), Italy) at 20°C. Agar plates were removed regularly and seeds checked qualitatively for the presence of protocorms, taking care to minimise the exposure of seeds to light. A low light intensity was used whenever agar plates were viewed under the microscope. (Note that high germination percentages have been recorded for other species of *Ophrys* using this method in our laboratory (Pierce et al. 2010) and it is unlikely that short exposure to low light intensities inhibited germination in this study.) The position of plates within the growth chamber was then re-randomised to minimise the possible effects of local temperature variation. Final percentage germination was quantified for each treatment when no further germination was observed, at 7 months after sowing.

Statistics

Systat 12 statistical software (SPSS, Inc., Chicago, IL, USA) was used to perform analysis of variance (ANOVA) and Tukey's *post hoc* multiple comparison procedure to compare means between different treatments. Standard errors of proportion data were calculated via the method of Fowler et al. (1998).

Results

COM and hormones increased the final percentage germination of *O. apifera in vitro* that were highly significant (ANOVA, df = 7, $F = 4.235$, $P = 0.001$; Figure 1). However, different COM and hormones produced different results: only coconut milk and 6BAP significantly increased the percentage germination from $0.5 \pm 0.12\%$ in the control to $12.1 \pm 2.99\%$ (coconut milk) and $12.3 \pm 1.77\%$ (6BAP; Figure 1). No other complex organic medium (banana powder or yeast extract) or pure hormone (GA_3, kinetin, TDZ, zeatin) resulted in a significant increase in germination, despite apparent positive trends, particularly for banana ($3.3 \pm 0.70\%$ germination) and kinetin ($7.6 \pm 1.06\%$ germination; Figure 1).

Hot water extracts of pea seeds had significant effects on the final percentage germination of *O. apifera* (ANOVA, df = 5, $F = 19.181$, $P < 0.0001$; Figure 2). Extracts of dried pea seeds (at 0 days of development) and pea seeds at 3 days of development resulted in significant increases in final percentage germination of *O. apifera* of approximately twice than that of the control (i.e. control = $3.8 \pm 0.32\%$; 0 days = $9.1 \pm 1.84\%$; 3 days = $7.6 \pm 0.79\%$; Figure 2). However, from 6 days of development onwards pea seed extract completely inhibited the germination of *O. apifera* (i.e. the final germination percentage after 7 months in these treatments was precisely zero in all replicates of these treatments; Figure 2). The final germination percentage in the zeatin treatment was intermediate between the effect of the control treatment and the pea extracts at 0 and 3 days, and was not significantly different from the effects of any of these treatments at the $P \leq 0.05$ level (Figure 2).

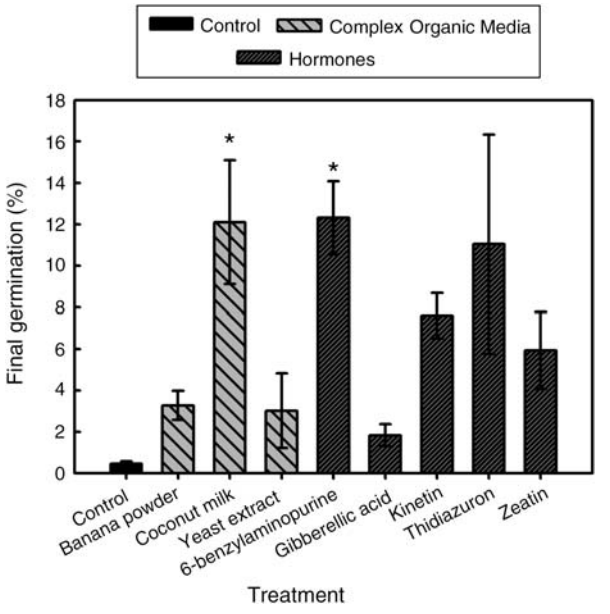

Figure 1. The final percentage germination of *Ophrys apifera* sown on either Malmgren's medium (control) or Malmgren's medium plus COM (banana powder (5%, w/w), coconut milk (5%, v/v) or yeast extract (5%, w/w)) or cold-filtered phytohormones at a concentration of 0.1 mM (6BAP, GA_3, kinetin, TDZ or zeatin). Values represent the mean ± 1 SE of seven replicate agar plates for each treatment. ANOVA determined that treatments had significant effects (df = 7; $F = 4.235$; $P = 0.001$) and Tukey's *post hoc* pairwise comparison procedure then determined which treatment means were significantly different from the control treatment mean at the $P \leq 0.05$ level, denoted by asterisks (*).

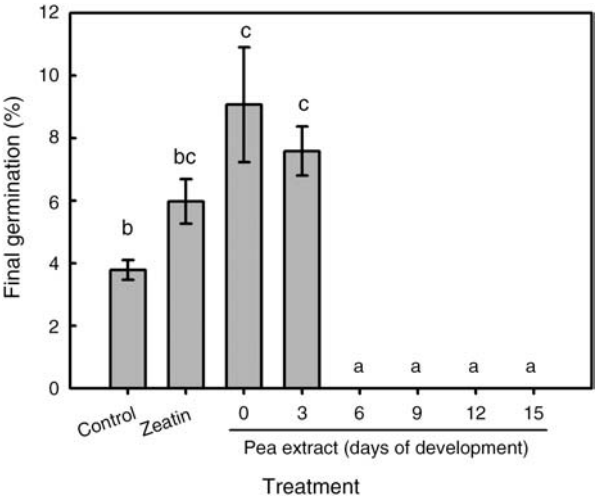

Figure 2. The final percentage germination of *Ophrys apifera* sown on either Malmgren's medium (control) or Malmgren's medium plus 0.1 mM Zeatin, or hot-water extracts of *Pisum sativum* "Progress No. 9" at 0, 3, 6, 9, 12 and 15 days of development. Values represent the mean ± 1 SE of eight replicate agar plates for each treatment. ANOVA determined that treatments had significant effects (df = 5; $F = 19.181$; $P < 0.000$) and different letters represent significantly different means at the $P \leq 0.05$ level as determined by Tukey's *post hoc* pairwise comparison procedure.

Discussion

The hypothesis that developing pea seeds, which have greater zeatin contents compared to dried peas, stimulate germination for the orchid *Ophrys apifera* was proved to be incorrect in this study, but dried pea seeds and seeds in the earliest stages of germination did significantly increase the final germination rate by a factor of approximately two. The resulting percentage germination was similar to that attained by standard methodologies using coconut milk or a high concentration of 6BAP and, as detailed below, allowed the production of hundreds of extra plants that were used to reinforce the original population. We can thus conclude that the use of hot water extracts of dried pea seeds provides an economical and readily available alternative to pure hormones or coconut milk. Research-grade hormones are not in themselves expensive, but they can only be obtained from specialist chemical companies and shipping costs can be several times more than the price of the

hormone. This means that in practise hormones are typically bought in bulk or alongside other chemicals, with associated extra costs and bureaucracy. In comparison, dried peas can be obtained as and when required from virtually any supermarket, at a real cost an order of magnitude less than the hormones. Thus dried peas provide an economical, useful extra weapon in the arsenal of the plant conservationist.

The present germination study was not simply a hollow exercise – the plants that were produced were subsequently cultivated and ultimately almost 400 large, healthy tubers were returned to the wild, and at the date of writing have produced fruits and seeds. Figure 3 demonstrates this additional output of the study. Seven months after sowing, the seedlings consisted of protocorms with rhizoids (Figure 3A) and at 8 months of growth in the dark protocorms produced the first green leaf (Figure 3B), denoting readiness for transfer into upright De Wit tubes in the light (Figure 3C). Growth in the light failed if plants were not supplied with 5 or 10% (v/v) coconut milk, and the concentration of sucrose in the growth medium was increased to $20\,g\,l^{-1}$. Otherwise the growth medium was the same as the basal medium used for seed germination. At around 26 months after sowing, the plantlets produced tubers and the leaves died (Figure 3D) – it was found that tubers could be transplanted into soil in pots in a temperate greenhouse, or tubers kept *in vitro* would sprout a fresh rosette and initiate a new tuber. ("Soil" consisted of a mixture of potting compost, sphagnum moss, fine silver sand and perlite in approximately the ratio 5:3:2:1, respectively). All plants transferred to soil during the growth phase (i.e. with green leaves and white roots) died, but when dormant tubers were transferred fresh rosettes were produced in the subsequent autumn and grew throughout the winter (Figure 3E). Four years after sowing, 391 dormant tubers or tubers with senescent leaves and roots were transplanted into the wild during the summer of 2011 (Figure 3F–H), these produced fresh rosettes of leaves (Figure 3I and J) and 17 plants flowered at the end of May/start of June 2012 (5 years after sowing), several of which produced fruits by self-pollination (Figure 3K and L).

Figure 3. Propagation *in vitro*, cultivation and population reinforcement of *Ophrys apifera*: (A) a seedling (protocorm) with rhizoids 7 months after sowing *in vitro* on Malmgren's medium plus 5% (v/v) coconut milk, (B) plantlets with the first green leaf and rhizoids growing in the dark at 8 months after sowing, ready for transplantation to De Wit tubes, (C) plantlets at 11 months after sowing, growing in De Wit tubes on Malmgren's medium plus 10% (v/v) coconut milk in the light, (D) tuber production *in vitro* 26 months after sowing, (E) 3-year old plants in cultivation in a temperate greenhouse, (F–H) three of the 391 tubers used during population reinforcement at Parco Monte Barro, Lecco, Italy, in 2011, shown at 4-year after sowing, in the summer resting phase when plants can be handled and transplanted, (I, J) rosettes of the tubers shown in G and F, respectively, in the spring of the fifth year (i.e. 8 months after transplantation to the wild), and (K, L) some of the 17 plants in flower and in fruit later in the fifth year (both photos taken on 1/6/2012). Photos by Simon Pierce.

With an original population size of 25 plants, this represents a more than 15-fold increase in population size brought about by this intervention. This demonstrates that, when germination is maximised by optimising *in vitro* techniques, sufficient plants may be produced that a large population may ultimately be established in which individual orchid plants survive to complete the entire life-cycle.

To conclude, hot water extracts of dried pea seeds are an effective and economical alternative to pure research-grade hormones or coconut milk for the germination of *O. apifera*, as studied here, and thus potentially for other orchid species. Despite the increase in zeatin content over the first 2 weeks of pea development recorded by Hahn et al. (1974), this study found that extracts of peas that have developed past the first week strongly inhibited germination of this orchid: only dried or recently germinated peas can be recommended. The concentrations of the hormones used in the study were moderately high, the aim being to use concentrations that are typically employed in orchid propagation work and that are known to be sufficient to potentially stimulate germination (e.g. Pierce & Cerabolini 2011). Pea seed extracts were also tested at just one concentration. Unfortunately seed of this rare orchid species is extremely scarce, meaning that we did not have sufficient seed to run a large number of experiments and thoroughly optimise the method over a range of

hormone/pea extract concentrations (the entire seed production of the population in 2 years was used). Thus we could only check whether the hypothesis of stimulation by pea seed extracts was correct or not. Future work could define the optimum amount of pea seeds required per volume of growth medium for a range of orchid species. A number of legumes and cereals such as *Zea mays* L. and *Triticum vulgare* L. are also known to produce zeatin and other cytokinins (Papet et al. 1990; Martin et al. 1993), and this study suggests that these may also be potential economical sources of germination stimulants for orchid *ex situ* conservation.

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