NUTRITIONAL REQUIREMENTS FOR *IN VITRO* SEED GERMINATION OF 12 TERRESTRIAL, LITHOPHYTIC AND EPIPHYTIC ORCHIDS

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NADARAJAN J, WOOD S, MARKS TR, SEATON PT & PRITCHARD HW. 2011. Nutritional requirements for *in vitro* seed germination of 12 terrestrial, lithophytic and epiphytic orchids. Although numerous media have been developed to evaluate both orchid seed germination and further seedling development after storage, few studies have attempted to relate nutritional requirements to life history traits. For a range of terrestrial, lithophytic and epiphytic orchid species, comparisons were made of germination on Knudson C, with and without activated charcoal or banana powder, Norstog and Phytamax™ media to represent variations in available nitrogen. Germination varied, with maximum values ranging from just 9% for *Prosthechea cochleata, Platanthera* sp. and *Spathoglottis paulinae* to 95% for *Phragmapedium longifolium*. Along with *Paphiopedilum delenatii, Paphiopedilum philippinense* and the epiphyte *Guarianthe bowringiana, P. longifolium* germinated well on most media. Germination was significantly higher on Norstog than the other media for four of the six epiphytes tested. Germination was maximum on Knudson C medium with activated charcoal for four of the six terrestrial/lithophyte species. The results indicate a greater preference for nitrogen from amino acids rather than ammonium or nitrate salts in seeds of epiphytes compared with some terrestrial orchid species.

Keywords: Orchidaceae, nitrogen, asymbiotic, *ex situ*, conservation, life history traits

NADARAJAN J, WOODS S, MARKS TR, SEATON PT & PRITCHARD HW. 2011. Keperluan nutrien untuk percambahan biji benih secara *in vitro* bagi 12 orkid daratan, litofit dan epifit. Walaupun pelbagai medium telah dibangunkan untuk menilai percambahan biji benih orkid selepas penyimpanan dan pertumbuhan anak benihnya, hanya beberapa kajian sahaja yang cuba menyelidiki perhubungan keperluan nutrien orkid dengan ciri riwayat hidupnya. Percambahan orkid daratan, litofit dan epifit di atas medium berlainan yang mewakili kandungan nitrogen berlainan dikaji. Medium yang digunakan ialah Knudson C tanpa arang teraktif, Knudson C + arang teraktif, Knudson C + serbuk pisang, Norstog dan Phytamax™. Percambahan maksimum berjulat daripada 9% untuk *Prosthechea cochleata, Platanthera* sp. dan *Spathoglottis paulinae* hingga 95% untuk *Phragmapedium longifolium. Phragmapedium longifolium,* seperti dengan *Paphiopedilum delenatii*, *Paphiopedilum philippinense* dan epifit *Guarianthe bowringiana,* bercambah dengan baik dalam kebanyakan medium. Empat daripada enam orkid epifit yang dikaji menunjukkan percambahan terbaik dalam medium Norstog. Empat daripada enam spesies orkid daratan/litofit menunjukkan percambahan terbaik dalam medium Knudson C + arang teraktif. Keputusan menunjukkan bahawa berbanding spesies orkid daratan yang lain, biji benih epifit lebih menggemari nitrogen dalam bentuk asid amino daripada nitrogen dalam bentuk ammonium atau garam nitrat.

INTRODUCTION

The Orchidaceae family is one of the largest of the plant kingdom, consisting of an estimated 25 000 species and about 8% of all flowering plants (Koopowitz et al. 2003). Orchids are found in a variety of habitats, from tropical rainforests to chalk grasslands with the majority of species found in the tropics and subtropics (Cribb & Whistler 1996). Many orchid species are narrow endemics, and a significant proportion of these are confined to small areas and restricted to narrow altitudinal ranges (Cardelús et al. 2006). Many hot spots of orchid endemism have been indentified such as New Guinea, Madagascar, Colombia, Ecuador, coastal Brazil and Guayana highlands (Pridgeon et al. 2009). Orchids are also often restricted in their habitats by the requirement for specific symbiotic mycorrhizal fungi for growth and a preference for nutrient-poor strata (Rasmussen 1995). High endemism paired with a risky life strategy makes orchids

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particularly vulnerable to environmental change and habitat destruction.

Orchids are of considerable value to the horticultural trade, with commercial activity starting in the 19th century, particularly the transport of orchids from the tropics to Europe. However, the continuing, unsustainable removal of plants from their natural habitat is accelerating the extinction threat (Keel 2007). Orchids are now recognised as an important economic resource for developing countries (Koopowitz et al. 2003, Hossain 2008) and their removal without permission is prohibited by environmental law. All orchid species now appear on the CITES Appendices I and II (CITES 2010). Consequently, there is a need to accelerate conservation programmes for threatened species in this family. An example of this is the Darwin Initiative Project Orchid Seed Stores for Sustainable Use (OSSSU) that is supporting *ex situ* conservation efforts particularly in Central and South America as well as South-East Asia (Seaton & Pritchard 2008).

Most orchid species produce huge quantities of microscopic dust seeds, generally between 0.05 and 6 mm in length and from 0.31 to 24 µg in weight contained within a single capsule (Arditti & Ghani 2000). The seeds are primitive, with ovoid embryos, often with a few hundred cells, surrounded by a testa that is one cell thick. Empty seeds are often present and may have packaging function within the capsule. Full seeds contain relatively few nutrient reserves and tend to be oily, e.g. 30% of seed dry mass in *Cattleya aurantiaca* (Seaton & Pritchard 2002). Orchid seeds require specific germination conditions and their immense diversity is reflected by a variety of nutritional requirements (Hicks 1999). Determining a suitable germination medium for a particular species is an essential prerequisite for any orchid *ex situ* conservation programme as it is important to be able to monitor losses in germinability over time.

Many tropical orchids germinate readily in the presence of moisture, nutrient and suitable temperature. In contrast, some temperate terrestrial species need cold stratification or after-ripening in soil to release dormancy (Stoutamire 1974). Dormancy may also be physical, whereby mechanical removal of the testa can improve germination, e.g. *Aplectrum hyenale* (Lauzer et al. 2007). Chemical scarification with hypochlorite during seed sterilisation can also enhance germination (Rasmussen 1995).

Many orchid seeds develop in association with a compatible mycorrhizal fungus. Although initial germination can occur in their absence, to ensure further development of protocorms and seedlings their presence can be essential (Rasmussen 1995, Weston et al. 2005). This mycorrhizal association was first described by Bernard (1899), whose discovery paved the way for future developments in orchid propagation *in vitro*. Nonetheless, the asymbiotic germination of seed in sterile flasks, pioneered by Knudson (1922), using medium originally intended for corn germination is now known to be successful with a majority of species (Hicks & Lynn 2007). Several modified versions of Knudson's formula have been developed and other plant tissue culture media have been adapted for orchid use. Now there are hundreds of formulations with subtly different nutritional contents that are suggested to be species-specific.

Most studies on orchid seed germination have concentrated on one or a limited number of species, with the objective of improving germination through subtle modifications of the medium (Hicks & Lynn 2007). However, relatively few studies have compared responses of a range of species on different media and sought associations between medium composition and life history traits or habitat. *In vitro* germination of seeds of northern temperate terrestrial orchid species often requires a more complex medium compared with tropical epiphytic orchids, possibly reflecting subtle differences in nitrogen metabolism (Rasmussen 1995). For example, successful asymbiotic germination of terrestrial species may vary with nitrate, ammonium or amino acid concentration. The response can also vary with stage of development. Raghavan and Torrey (1964) showed that ammonium salts were critical for germination and early development in a hybrid of *Cattleya*, but that nitrate salts promoted seedling development along with the parallel appearance of nitrate reductase.

The objective of this study was to compare the efficacy of a range of well-known media of varying nitrogen compositions, with some common generic additives, for orchid seed germination and to establish whether nutritional needs were related to the different growth forms and life history traits of orchids.

MATERIALS AND METHODS

Seed material

Seed collections of the 12 orchid species used in this experiment came from a variety of sources, including private donations, the orchid nursery at the Royal Botanic Gardens, Kew and wild plant collections made under licence (Table 1). Once received at the Millennium Seed Bank, collections were stored in a dry room (operating at 15% relative humidity, 15 °C) in screw-capped glass vials.

Preparation of media

Five media were chosen to reflect nutritional differences. These were designated as Knudson C (Knudson 1946) control (KØ), Knudson C with activated charcoal (KAC), Knudson C with banana powder (KB), Norstog (Ng) (Norstog 1973) and Phytamax™ (Px). Knudson C represents a widely used medium containing both ammonium and nitrate nitrogen (8.76 and 8.46 mM respectively). Knudson C was also formulated with activated charcoal (2 g l^{-1}) or

Table 1 Terrestrial, lithophytic and epiphytic orchid species used in this study with their respective growth habitat and origin

Species	Growth habit	Habitat	Origin
Cattleya maxima	Epiphytic	Tropics, dry forest	Ecuador, N Peru, Colombia
Dendrobium bigibbum	Epiphytic	Tropics, on trees and rocks in a range of habitats from coastal scrub to open forest	New Guinea to NE Australia
Encyclia chloroleuca	Epiphytic	Tropics, on low-land forest trees, needs distinct wet and dry season	Brazil, Colombia, Ecuador, Guyana, Peru, Suriname, Venezuela
Guarianthe bowringiana	Epiphytic	Tropics, on exposed rocks near free-flowing streams, near cliffs between 200–900 m. Can grow as a lithophyte in rocky ravines	Belize, Guatemala
Jumellea sagittata	Epiphytic	Tropics, in mossy montane forest	Madagascar
Prosthechea cochleata (Synonym: *Encyclia cochleata*)	Epiphytic	Tropics, grows on tree or shrub branches, needs distinct wet and dry season	Central America, the West Indies, Colombia, Venezuela, S Florida
Paphiopedilum delenatii	Terrestrial	Tropics, on karst or eroded limestone at 850–1 200 m in open forest	China (SE Sichuan, N Guangxi) to Vietnam
Paphiopedilum philippinense	Terrestrial	Tropics, from sea level in open forest	Philippines to N Borneo
Phragmipedium longifolium	Terrestrial	Tropics, from sea level up to 200 m in almost moist forests, at the edges of stream where they grow on gravel and with moss overgrown rocks and cliffs	Costa Rica, Panama, Colombia, Ecuador, Brazil
Platanthera sp. (Synonym: *Piperia* sp.)	Terrestrial	Temperate, mountainous region	California
Spathoglottis paulinae	Terrestrial	Tropics, moist rainforests	E and SE Asia, New Guinea, Pacific Islands, Australia
Paphiopedilum stonei	Lithophytic	Tropics, clings to limestone rock and cliffs with very little debris covering its roots, prefers alkaline pH	Sarawak, Borneo

Source: Pridgeon et al. (2009), World Checklist of Monocotyledons (2010)

banana powder (100 g l^{-1}). Phytamax™ contains higher concentrations of ammonium and nitrate nitrogen (10.31 and 19.71 mM respectively), activated charcoal (2 g l^{-1}) and peptone (2 g l^{-1}), which itself contains a large concentration of organic nitrogen. Norstog was formulated for immature barley embryos, and is devoid of inorganic nitrogen and contains a mixture of amino acids (total nitrogen is 6.65 mM), the lowest concentration of nitrogen compared with the other media used. While all formulations varied in their composition, each contained 10 g l^{-1} agar and 20 g l^{-1} sucrose. The pH of each medium was adjusted to 5.6 prior to autoclaving.

Surface sterilisation and sowing

A small sample of seeds (approx. 300) was transferred onto filter paper (55 mm circumference), which was then folded into a packet and sealed with a steel staple (Seaton & Ramsay 2005). This ensured that seeds were contained securely during the sterilisation process. Five packets were made (1 per medium) for each orchid species. Packets were sterilised in 10% commercial bleach solution (containing ≤ 1% NaOCl) for 20 min, rinsed three times (1 min each) in sterile deionised water and opened to allow the seeds and paper to dry briefly (5 min) before sowing on three replicated 5 cm-diameter Petri plates for each medium. Plates were sealed with a double layer Nescofilm® and placed in an incubator at 25 ± 2 °C with 12 hours of photoperiod. For terrestrial species, whereby germination might be inhibited by light, plates were double wrapped in aluminium foil before incubation at 25 ± 2 °C.

Germination scoring

Observations were made using a binocular microscope at 25× magnification every two weeks for three months to determine germination. Germination percentages were calculated as the percentage of full seeds (with visible embryos, Figure 1) at the first stage of germination and beyond, defined here as the swelling and growth of the embryo beyond the testa (Figure 2).

Data analysis

All percentage germination data were subjected to logit transformation before ANOVA and Fisher pair-wise comparison tests were performed using MINITAB®.

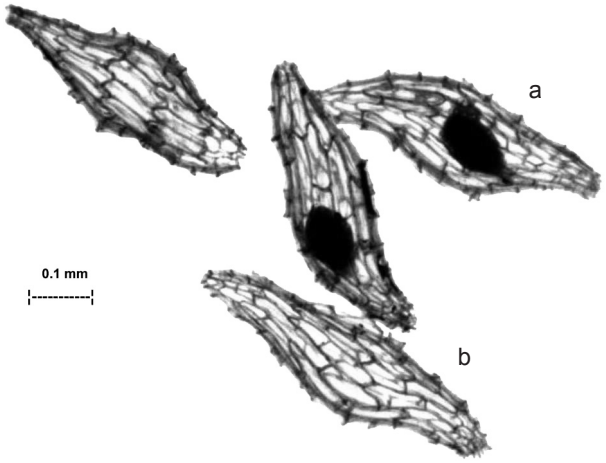


Figure 1 Seeds of *Cattleya maxima* with typical structure for orchids: (a) fusiform in shape with ovoid embryo made from only a few hundred cells, no endosperm and a testa that is one cell layer thick, (b) empty seed

RESULTS

The germination success of the six epiphytic orchids varied greatly between species, independent of medium (Figure 3). *Guarianthe bowringiana* (Figure 3a) seeds germinated well on all five media, with germination ranging from 63 ± 18% on KAC to 85 ± 13% on Px. KB and Px media gave significantly higher germination (80 and 85% respectively) than the other formulae tested ($p \leq 0.005$). In contrast, significantly higher germination was only noted on Ng medium for *C. maxima* (Figure 3b) seeds with germination of 54 ± 11% ($p \leq 0.005$). This species did not perform well on the other media tested, showing around 10% germination.

Prosthechea cochleata (Figure 3c) germinated poorly on all media tested with a maximum germination of 9 ± 4% on Ng medium. No germination was noted for this species on KB and Px media. *Encylcia chloroleuca* (Figure 3d) seeds did slightly better than those of *Prosthechea*, reaching 19 ± 3% on Ng medium. An average of 5–7% germination was noted on KØ, KB and Px media. However, no germination was observed for this species on KAC.

Germination of *Dendrobium bigibbum* was highest on Ng medium and was significantly lower on both KB and Px, while the other two media gave intermediate results (Figure 3e).

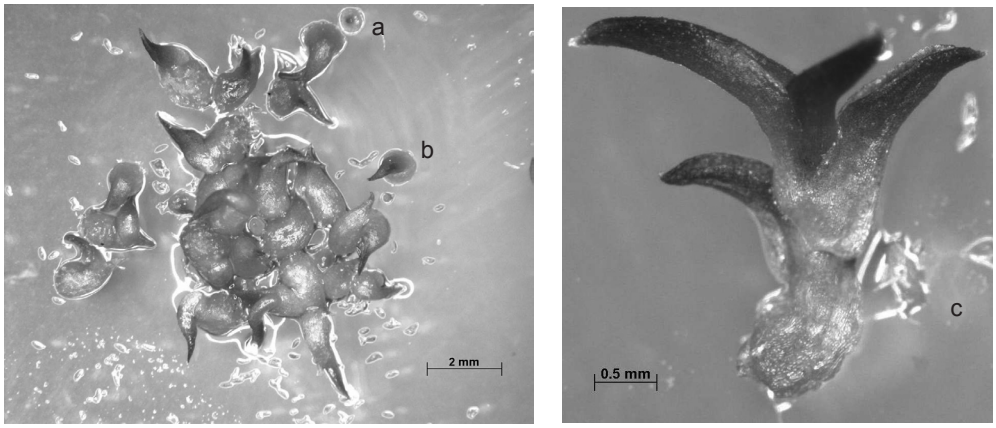


Figure 2 Germinating seeds of the epiphytic species *Encyclia chloroleuca,* demonstrating the various stages of orchid germination: (a) embryo enlarged and burst through testa to produce a spherical protocorm approximately 0.6 mm in diameter, (b) development of first leaves (primordial), (c) complete plantlet

Jumelea sagittata performed poorly across all media other than Ng, whereby germination was 22 ± 5% (Figure 3f).

For the five terrestrial and one lithophytic orchids, germination was also highly variable between species (Figure 4). Germination was low for the *Platanthera* sp. (Figure 4a) and *Spathoglottis paulinae* (Figure 4b) seeds sown on all five media. Maximum germination in both species was significantly higher ($p \leq 0.005$) on KAC (~9%) and Px medium (~10%). *Phragmipedium longifolium*, a lithophyte, germinated very well on all media other than KØ, with mean values ranging from 64 ± 4% on KB to 95 ± 9% on Ng (Figure 4c). Germination of *Paphiopedilum stonei*, *Paphiopedilum philippinense* and *Paphiopedilum delenatii* was highest on KAC medium ($p \leq 0.005$), being 87 ± 10%, 44.8 ± 6.8% and 86.8 ± 9.7% respectively. *Paphiopedilum stonei* (Figure 4d) and *P. philippinense* (Figure 4e) showed relatively poor germination on the two other Knudson formulations and on the activated charcoal containing Px medium. Whilst *P. delenatii* germination was significantly lower on both KB and Ng, overall levels remained high (Figure 4f).

DISCUSSION

Asymbiotic germination has many advantages, including the ability to produce healthy seedlings at a frequency and rate far greater than that achieved in nature, making this approach ideal for use in commercial orchid production and for rapid regeneration from seedbanks. It also has great potential as a propagation method for rare or threatened orchids (Butcher & Marlow 1989). However, maximising the success of asymbiotic germination is dependent upon identifying the most suitable medium and abiotic conditions. Describing the nutritional requirements of most orchids, however, is difficult due to their enormous diversity and complex mycorrhizal interactions that contribute to the nutritional status of the growing seedling (Hicks & Lynn 2007).

A great variability for germination success was observed between the 12 orchid species, independent of medium upon which the seeds were sown. Similarly, within species germination levels varied between media, with most showing significantly improved germination on a particular medium. Firstly, the epiphytic species performed significantly better on Ng than on any other medium, except in *G. bowringiana* where other media were equally effective (Figure 3a). The effectiveness of Ng as an orchid germination medium has been noted before (Butcher & Marlow 1989, Pritchard et al. 1999), and could be explained by the high concentration of amino acids present compared with other media tested. Interestingly, the benefit of amino acids as a nitrogen source is commonly cited for terrestrial species, but there is little knowledge on their effectiveness for tropical species (Hicks & Lynn 2007). All tropical epiphytes tested germinated fairly well on Ng, suggesting that the replacement of ammonium and nitrate salts with amino acids promotes the germination of these species. This may be explained by the absence of nitrate reductase in some germinating seeds, which is instrumental in the conversion of inorganic to organic nitrogen (Raghavan & Torrey 1964).

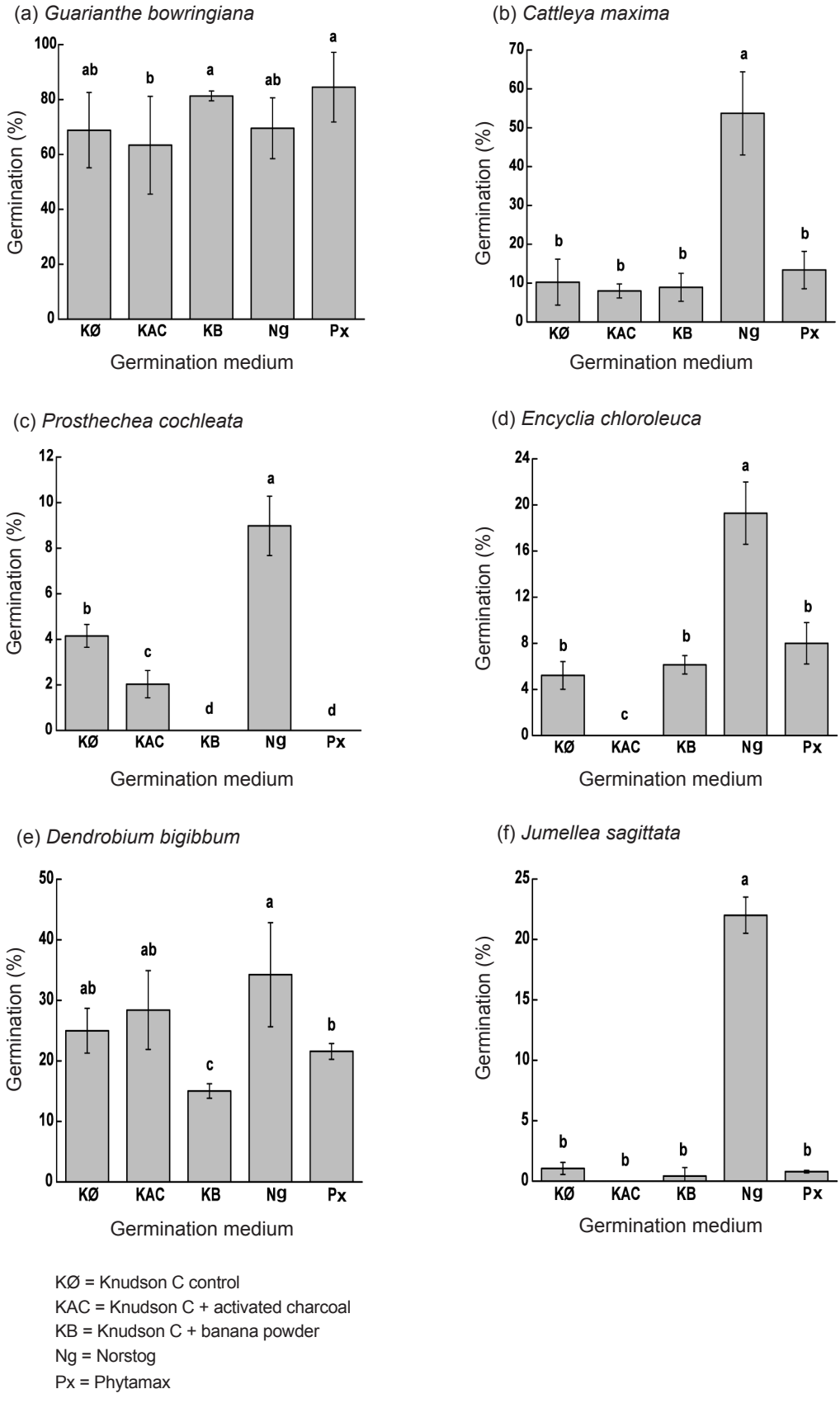
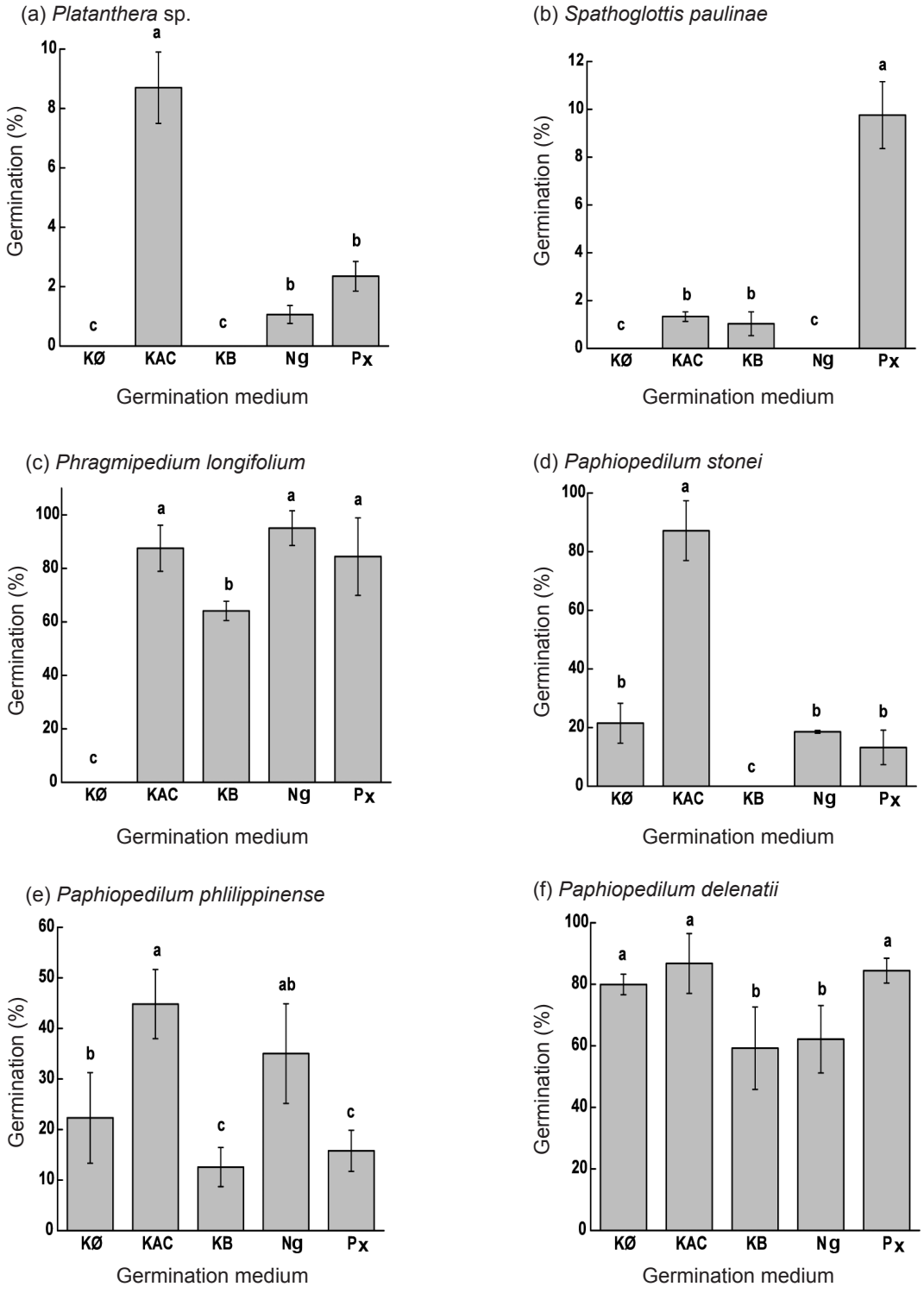


Figure 3 Effects of media on germination of six epiphytic orchid species: (a) *Guarianthe bowringiana*, (b) *Cattleya maxima*, (c) *Prosthechea cochleata*, (d) *Encyclia chloroleuca*, (e) *Dendrobium bigibbum* and (f) *Jumellea sagittata*. Data are means (± SD). Columns with the same letters are not significantly different ($p < 0.005$) using Fisher's pair-wise comparison.



KØ = Knudson C control
KAC = Knudson C + activated charcoal
KB = Khudson C + banana powder
Ng = Norstog
Px = Phytamax

Figure 4 Effects of media on germination of five terrestrial and one lithophytic orchid species: (a) *Platanthera* sp., (b) *Spathoglottis paulinae*, (c) *Phragmipedium longifolium*, (d) *Paphiopedilum stonei*, (e) *Paphiopedilum philippinense* and (f) *Paphiopedilum delenatii*. Data are means of three replicates (± SD). Columns with the same letters are not significantly different ($p < 0.005$) using Fisher's pair-wise comparison.

Knudson C and Px contain both ammonium and nitrate sources of nitrogen, requiring the presence of nitrate reductase for metabolism of these inorganic forms of nitrogen, whereas the amino acids present in Ng may be in a more available form for further assimilation. Amino acids from the breakdown of peptone in Px would also seem to present this opportunity, but the overall higher concentration of nitrogen may be inhibitory to germination. Certainly most orchids evolve in low nutrient habitats, so high ion concentration may be inhibitory (Rasmussen 1995). Preferential uptake of NO_3^- and NH_4^+ ions can also affect the pH of the medium, which may affect germination (Fitter & Hay 1987).

With the exception of *S. paulinae* which showed the highest germination on Px, the response in the other lithophytic and terrestrial species was significantly higher on KAC. Phytamax medium also induced high relative levels of germination in *S. paulinae, P. longifolium* and *P. delenatii*. Phytamax™ and KAC differ from the other media in that they both contain 2 g l^{-1} activated charcoal powder. Previous work has shown that *Paphiopedilum* and *Phalaenopsis* cultures both benefit from the addition of charcoal, possibly by the absorption of inhibitory factors exuded by seedling roots during development such as ethylene or phenolic compounds (Butcher & Marlow 1989, Hicks & Lynn 2007). Terrestrial species may produce more secondary substances (e.g. phenols) than epiphytic species, which explains why they benefit more from the addition of charcoal.

Overall, *P. longifolium* seeds showed high levels of germination *in vitro*, reaching 84–95% on KAC, Ng and Px but germination was reduced to 64 ± 4% on KB. This suggests that the addition of banana powder may have inhibited germination in this species, a phenomenon that has been observed in other species (Hicks & Lynn 2007). In *E. chloroleuca* and *D. bigibbum* it was interesting to note that although germination on KB was low, seedling growth was very good (data not presented). This confirms that the addition of banana to media encourages strong shoots and roots in some species (Butcher & Marlow 1989). Future research should investigate the optimum growth medium for seedlings of these species compared with the optimum germination medium, since observations suggest that the best medium for stimulating germination may not be the best for encouraging seedling growth. The requirement for differential nutrition by later stages of germination and seedling growth have been observed in *Bletia purpurea* (Dutra et al. 2008) and *Calopogon tuberosus* (Kauth et al. 2008), both North American terrestrial species. In contrast, early germination events in *B. purpurea* can be relatively unaffected, such that initial germination varied from 96.3 to 100% on the six media tested (Dutra et al. 2008).

Orchid seeds from temperate regions are thought to have particularly stringent nutritional requirements at different stages of development and some species, e.g. *Cephalanthera falcate,* may benefit additionally from being sown while still immature (Yamazaki & Miyoshi 2006). The *Platanthera* was the only temperate species studied and germination was poor on all media, except for KAC (Figure 4a).

CONCLUSIONS

The tropical epiphytic species performed significantly better on Ng medium. This suggests that nitrogen in the form of amino acids promotes germination of these species. The efficiency of this medium for germination of epiphytic seeds should be assessed for a wider range of species. Meanwhile, lithophyte and terrestrial species benefited from the lower nitrogen concentrations in Knudson C medium and the addition of activated charcoal.

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