REVIEW ARTICLE

The challenges of growing orchids from seeds for conservation: An assessment of asymbiotic techniques

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This article is part of the special issue "Meeting the Challenge of Exceptional Plant Conservation: Technologies and Approaches."

Abstract

Lewis Knudson first successfully germinated orchid seeds asymbiotically on artificial medium in 1922. While many orchid species have since been grown asymbiotically, the tremendous variation in how species respond to artificial medium and growth conditions ex situ has also become apparent in the past century. In this study, we reviewed published journal articles on asymbiotic orchid seed germination to provide a summary of techniques used and to evaluate if these differ between terrestrial and epiphytic species, to identify areas where additional research is needed, and to evaluate whether asymbiotic germination could be used more often in ex situ conservation. We found articles reporting successful asymbiotic germination of 270 species and 20 cultivars across Orchidaceae. Researchers often used different techniques with epiphytic versus terrestrial species, but species-specific responses to growth media and conditions were common, indicating that individualized protocols will be necessary for most species. The widespread success in generating seedlings on artificial media suggests that asymbiotic techniques should be another tool for the conservation of rare orchid species. Further advances are needed in understanding how to introduce mycorrhizae to axenically grown orchids and to maximize the viability of seedlings reintroduced into natural habitats to fully utilize these methods for conservation.

KEYWORDS

asymbiotic, disinfection, ex situ, germination, growth medium, Orchidaceae, seed, stratification

Orchidaceae is at the forefront of conservation as it has higher numbers of threatened taxa than any other plant family (Swarts and Dixon, 2009; Fay, 2018; Phillips et al., 2020). With an estimated 25,000–30,000 species, making up 8% of all vascular plant species, Orchidaceae contributes significantly to the earth's biodiversity (Chase et al., 2015; Givnish et al., 2015; Hassler, 2022) across all continents except Antarctica and over a diverse range of tropical and temperate habitats (Givnish et al., 2015). Wild orchid populations have been a primary source of plants for cultural and commercial anthropogenic uses, diminishing already localized, small populations (Roberts and Dixon, 2008; Dulić et al., 2020). The degradation of populations by over-harvesting and illegal trade is compounded by the

multifaceted issues stemming from climate change, resulting in a high projected future loss of orchid species (Swarts and Dixon, 2009; Seaton et al., 2013; Fay, 2018).

Conservation efforts surrounding orchids commonly focus on propagation with the intent to decrease harvesting from wild populations, reintroduce plants into wild populations, add seeds to the global network of orchid seed banks, and secure plants from extirpated habitats (Seaton et al., 2013, 2018; Dulić et al., 2020; North American Orchid Conservation Center, 2022). The propagation of orchids from seed is difficult due to their small seeds (0.1–6 mm), mycorrhizal dependency, and germination and development nutritional requirements (Arditti, 1967; Arditti and Ghani, 2000; Kauth et al., 2008). Whereas methods for

commercial propagation from seed of some orchid genera are well developed (Teixeira da Silva et al., 2015; Zeng et al., 2016), ex situ propagation has not been widely applied to conserve native species across temperate and tropical habitats.

Known as dust seeds, orchid seeds are minute, produced in abundance by each flower, and adapted for wind dispersal (Eriksson and Kainulainen, 2011). The number of seeds per capsule may range from as high as 4,000,000 to only 20–50 (Arditti and Ghani, 2000). An orchid seed contains an undifferentiated embryo, few reserves, and air spaces covered by a testa, characteristics thought to aid in longer periods of dispersal time (Arditti and Ghani, 2000; Prutsch et al., 2000; Bewley and Black, 2014; Yeung, 2017). Readers are referred to Arditti and Ghani (2000) for a thorough review of the physical characteristics of orchid seeds and their biological functions. Species-specific seed traits, such as the size of the embryo relative to the air space, the presence of a carapace around the embryo, and the presence of plant growth regulators and other compounds, influence germination and seed banking potential (Pritchard and Seaton, 1993; Arditti and Ghani, 2000; Prasongsom et al., 2017). For example, air space between the embryo and testa enhances buoyancy on water during dispersal while a lignified testa can prevent seeds from getting wet (Arditti and Ghani, 2000). These characteristics may contribute to the dormancy observed in orchids occurring in seasonal climates (Arditti and Ghani, 2000).

Under natural conditions, orchid seeds rely on a symbiotic relationship with mycorrhizae to germinate (Rasmussen, 1992; Yoder et al., 2000). Mycorrhizal dependency is the largest hurdle to overcome in orchid propagation as the mycorrhizae provide most of the minerals, nutrients, vitamins, and water needed for germination and seedling development (Herrera et al., 2019) and vary in the specificity of their relationship with their orchid hosts (McCormick et al., 2018; Li et al., 2021). In vitro symbiotic germination has been used for orchid propagation (Pujasatria et al., 2020), however, successful propagation requires extensive knowledge of the specific orchid–mycorrhizal interaction, as well as the ability to isolate and grow the fungus, which can take considerable time and effort. Mycorrhizae isolated from the roots of mature plants may not be suitable for inducing seed germination (McCormick et al., 2021; Zhao et al., 2021), and many orchid mycorrhizal fungi are unculturable axenically, making symbiotic germination impossible (Li et al., 2021). By contrast, germination of orchid seeds on artificial medium without mycorrhizae, or asymbiotic germination, could be a more time- and cost-efficient means of propagating orchids.

Asymbiotic seed germination is a process in which organic nutrients are provided to seeds through a nutrient medium (Knudson, 1922, 1946). By replacing the role of mycorrhizae with organic nutrients, propagation efforts are not inhibited by the obstacle of orchid–fungal specificity. Although asymbiotic germination may allow for a more efficient process to grow orchids from seed, such techniques are not foolproof. The developmental requirements of orchids vary drastically across the family, especially between species in tropical and temperate regions, necessitating the use of diverse methodologies (Diantina et al., 2020). With little known about the precise developmental requirements of most orchid species, many germination efforts are done by trial and error, which complicates the widespread use of these techniques for orchid conservation.

Several authors have produced syntheses of methodologies in asymbiotic germination, but many of these are focused on a particular geographic area (e.g., Dixon, 1994; Herrera et al., 2019) or genus (Teixeira da Silva et al., 2015; Zeng et al., 2016). Kauth et al. (2008) provided an extensive review of many aspects of asymbiotic and symbiotic orchid seed germination, but this was not an exhaustive examination of empirical studies. Designing effective experiments to germinate orchid seeds asymbiotically can still be difficult for a researcher entering this field, may lead to low rates of propagation success for a focal species, and can easily result in wasted seed.

In this review, we analyzed and summarized publications in botany and horticulture journals spanning more than 70 years to search for commonality in techniques used to successfully germinate orchid seeds on artificial media. Approximately 70% of orchid species are epiphytic/lithophytic, 25% are terrestrial, and 5% exhibit variation in their growth habit (Arditti, 1992). Most epiphytic orchids occur in tropical forests, and most terrestrial orchids are found in North America, Europe, and cooler areas of Asia (Givnish et al., 2015), with a few terrestrial genera spanning temperate and tropical latitudes. Given the strong association of growth habit with climatic zone, we expect that researchers have used different techniques in epiphytic versus terrestrial species. For example, chilling seeds may be used more often and show greater effectiveness for germination of terrestrial species as they often experience seasonal temperature fluctuations, whereas epiphytic species have more likely evolved under stable temperatures (Rasmussen, 1995). Exposure of seeds to chemical agents prior to sowing can enhance germination of terrestrial species because these agents aid in breaking the harder testa common in terrestrial species and may reduce seed dormancy (Kauth et al., 2008). Terrestrial species may exhibit higher germination rates in dark environments while epiphytic species may benefit from light environments due to the differences in light conditions where these species naturally germinate (Rasmussen, 1995).

Our focus in this review is not to restate the requirements for asymbiotic germination or to explain the physiology of this process. Instead, we aim to report on what has been used successfully by researchers in controlled experimental studies of asymbiotic germination. Several excellent papers by Malmgren (1996), Kauth et al. (2008), Park and Yeung (2018), Yeung et al. (2018), and Utami and Hariyanto (2020) provide details of specific techniques and the benefits and consequences of individual media and supplements. We hope that our review contributes to this vast body of literature on propagating orchids from seed and further stimulates botanists to engage in experiments

that determine optimal conditions for asymbiotic germination of native orchids, particularly for those species that face severe threats to their continued existence due to loss of habitat, herbivory, low reproduction, and illegal collection.

METHODS

We identified relevant peer-reviewed journal articles on asymbiotic germination techniques through searches in Web of Science with the following search terms: Orchid AND asymbiotic, Orchid AND asymbiotic AND seed germination, Orchid AND ex situ AND asymbiotic, Orchid AND axenic AND seed AND germination, Orchid AND in vitro AND asymbiotic AND germination, Orchid AND in vitro AND seed AND germination. All searches included papers from 1900 to May 2021. Results of asymbiotic seed germination in orchids have also been published outside of scientific journals, but these are difficult to comprehensively identify and to verify that they are original, peer-reviewed publications. Thus, while we acknowledge that all relevant pieces of literature have likely not been considered in this review, the Web of Science database is a respected resource among scientists and widely used in other reviews (Li et al., 2018). We nevertheless recognize the value of comprehensive works on orchid seed germination that may not report original results or may not appear in journals. Table 1 includes a list of suggested references that were not considered for data collection in our study but that readers may find helpful in comparison to the assessment of methods reported in our review.

We removed papers that were not focused on orchids before conducting further review of 315 journal articles. Of these, 85 additional articles were removed from further consideration because they were reviews ($n = 9$), unrelated to our study objectives ($n = 61$), duplicate results already in the data set ($n = 4$), did not report asymbiotic experiments in sufficient detail or clarity ($n = 7$), or could not be readily accessed ($n = 4$). Removed articles included those with a primary focus on cryopreservation of seeds; although these articles often reported germination trials, they were frequently done only to verify if seeds remained alive after cold storage, rather than as species-specific methods for asymbiotic germination. Cryopreservation is a sophisticated technique that has clear advantages for preserving seeds, particularly of rare species, and readers are referred to other literature on cryo-techniques (e.g., Seaton et al., 2018; Kaur, 2019). From the 230 remaining articles, we extracted the following data: species studied, Orchidaceae subfamily in which the focal species is assigned, growth habit as terrestrial or epiphytic, whether seeds were stored or stratified prior to sowing and the respective method(s) and duration, the focal unit for disinfection (i.e., capsule, seeds, both, or none), methods and duration of disinfection, methods of seed scarification, artificial media and supplements used in germination trials, medium pH, light environment(s) used during germination trials (i.e., continuous light, continuous dark, cyclic, or multiple environments) and the photoperiod if the light environment was cyclic, the temperature at which germination trials were conducted, the number of times seedlings/plantlets were transplanted to new containers, growth media and supplements used for transplants, whether plants with leaves and roots were obtained in the experiment, and whether seed age was examined in the context of germination rates. When subfamily or growth habit were not explicitly stated in an article, we conducted an external search to determine these data points. When more than one species was examined in a paper, we treated each species as a separate study. Additionally, many papers reported multiple experiments involving disinfecting procedures, growth media, supplements, and light environments for both seed germination and development of young plants. For these papers, we collected data on each

TABLE 1 Select references that are useful starting points for initiating studies on asymbiotic germination of orchids. They provide greater detail of the unique attributes of the orchid life history and efforts to conserve them through propagation techniques.

Reference[a]	Description
Allen, 1996	Conference proceedings detailing individual efforts of propagation of many North American orchid species through in vitro techniques.
Kauth et al., 2008	Comprehensive review article that covers asymbiotic and symbiotic germination of orchid seeds, with topics ranging from seed collection to acclimatization of plants grown in vitro.
Rasmussen, 1995	A book providing a comprehensive treatment of the stages in the orchid life cycle, with a strong emphasis on orchid–mycorrhizal symbioses from germination to adult plants.
Rasmussen et al., 2015	A review article detailing the complex requirements for orchid seed germination in natural conditions and how that knowledge is important to conservation considerations.
Seaton et al., 2018	A book chapter that provides an overview of best practices in collecting seeds and their long-term storage.
Swarts and Dixon, 2007	A review article focusing on threats to terrestrial orchids of the Southwest Australian Floristic Region and methods that have been implemented or tested for their conservation.
Utami and Hariyanto, 2020	A review article covering media and supplements and their potential benefits to orchid seeds.
Yam and Arditti, 2009	An article that provides a historical account of advances in biotechnology of plant propagation, focused on orchids.

[a]Full citations of references are in the References section.

unique experiment conducted with each species and considered each of these as a separate/standalone/individual study. During data collection, we identified much inconsistency in how techniques were described across studies. Thus, Appendix S1 provides a table of key terms and the definition considered in this review. A list of the papers examined for this study is available in Appendix S2.

Data were summarized to evaluate the variety of methods employed in pre-treatment of capsules and seeds, disinfection, media, and growth conditions. For these analyses, we excluded 20 studies of cultivars because they are not naturally occurring. G-tests of independence (Wilks, 1935) were conducted to evaluate if techniques for seed storage, seed chilling, disinfection, and light environment varied between terrestrial and epiphytic species. A t-test was used to evaluate if the number of media tested per study differed between epiphytic and terrestrial species. All statistical analyses were conducted in SPSS version 27 (IBM Corporation, 2020) and a P value < 0.05 was used to indicate a significant result.

RESULTS

The 230 papers examined for this study yielded 384 studies, as many papers contained more than one relevant experiment. The papers were published from 1949–2021, with most occurring since 2008 (Figure 1). The mean number of publications per year in the past decade is 14.9. Among all examined studies ($n = 384$), 270 unique species and 20 cultivars were represented. (Two studies are excluded in these counts because only the genus was provided in the publication.) Across the taxa studied, 113 species are terrestrial, whereas 157 are primarily epiphytes. More than one-half of the taxa studied are within the largest subfamily, Epidendroideae ($n = 157$), followed by Orchidoideae ($n = 90$), then Cypripedioideae ($n = 24$), and Vanilloideae ($n = 1$).

Most studies represent the only study of asymbiotic germination for the focal species, but 60 species have been the focus of multiple independent studies (Box 1).

We identified several aspects, from seed collection to growth conditions, that vary between epiphytic and terrestrial species. Surprisingly, only 45 studies reported testing the impact of seed age on germination rate, although there was tremendous variation in the age of seeds used in germination trials, from as little as one month after pollination to two years or longer. The studies testing seed age were heavily biased toward members of Cypripedioideae and Epidendroideae, thus we did not conduct statistical tests on these data. Nevertheless, these studies suggest that the seed age for optimal germination is complex and determined by species-specific physiology and pre-treatments that could influence seed dormancy.

Techniques involving seed storage, chilling or stratification, disinfection, seed scarification, and light environment also varied across studies. For most studies (58%), seeds were not stored for an extended time after collection. Stratification, or specifically a cold, moist treatment (Baskin and Baskin, 2014), was only mentioned in three studies, but 76 studies reported chilling seeds before germination (i.e., exposure to a temperature between −25°C and 8°C). Among 23 studies that explicitly included chilling as an experimental variable, this was often found to increase germination. If seeds were not used immediately for germination, then they were stored until use for periods ranging from a few days up to five years, although chilled seeds were generally used within a year after collection. Seeds of terrestrial species were more often stored ($n = 359$ studies, $G = 24.213$, df = 1, $P < 0.001$) than seeds of epiphytic species, but there was no difference between these groups in whether seeds were chilled ($n = 140$ studies, $G = 1.657$, df = 1, $P = 0.2$; Figure 2). Many studies reported that seeds were kept in storage until used, without specifying time periods, which makes it difficult to improve our knowledge of how different
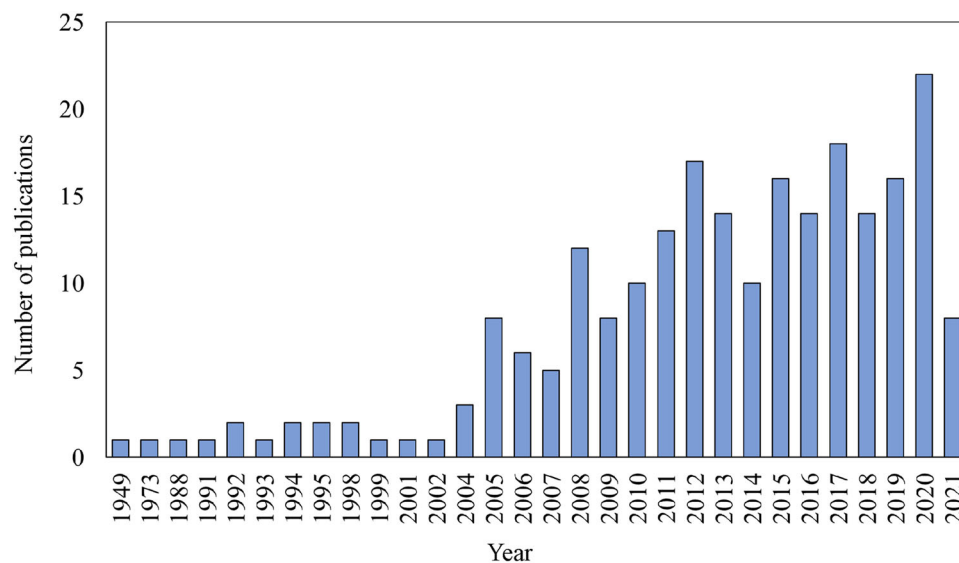

FIGURE 1 Number of articles on asymbiotic germination of orchid seeds published by year that were reviewed for this study. Data for 2021 are through May 26, 2021.

BOX 1. Orchid species that were the subject of multiple studies on asymbiotic germination of their seeds.

Subfamily Cypripedioideae

Cypripedium calceolus L.

Cypripedium candidum Muhl. ex Willd.

Cypripedium macranthos Sw.

Cypripedium reginae Walter

Paphiopedilum armeniacum S. C. Chen & F. Y. Liu

Paphiopedilum delenatii Guillaumin

Paphiopedilum insigne (Wall. ex Lindl.) Pfitzer

Paphiopedilum spicerianum (Rchb. f.) Pfitzer

Paphiopedilum villosum (Lindl.) Stein

Subfamily Epidendroideae

Acianthera prolifera (Herb. ex Lindl.) Pridgeon & M. W. Chase

Ansellia africana Lindl.

Bletia purpurea (Lam.) A. DC.

Bletilla striata (Thunb.) Rchb. f.

Calanthe discolor Lindl.

Calanthe tricarinata Lindl.

Calopogon tuberosus (L.) Britton, Sterns & Poggenb.

Cattleya intermedia Graham

Cattleya walkeriana Gardner

Cephalanthera falcata (Thunb.) Blume

Cymbidium finlaysonianum Lindl.

Cymbidium aloifolium (L.) Sw.

Cyrtopodium glutiniferum Raddi

Cyrtopodium saintlegerianum Rchb. f.

Dendrobium bigibbum Lindl.

Dendrobium nobile Lindl.

Dendrobrium officinale Kimura & Migo

Epidendrum secundum Jacq.

Epipactis flava Seidenf.

Epipactis helleborine (L.) Crantz

Gastrochilus matsuran (Makino) Schltr.

Limodorum abortivum (L.) Sw.

Papilionanthe teres (Roxb.) Schltr.

Spathoglottis plicata Blume

Vanda stangeana Rchb. f.

Subfamily Orchidoideae

Anacamptis laxiflora (Lam.) R. M. Bateman, Pridgeon & M. W. Chase

Chloraea crispa Lindl.

Anacamptis morio (L.) R. M. Bateman, Pridgeon & M. W. Chase

Anoectochilus formosanus Hayata

Caladenia huegelii Rchb. f.

Caladenia latifolia R. Br.

Chloraea crispa Lindl.

Chloraea gavilu Lindl.

Dactylorhiza fuchsii (Druce) Soó

Dactylorhiza maculata (L.) Soó

Dactylorhiza maculata (L.) Soó

Disa cooperi Rchb. f.

Gymnadenia conopsea (L.) R. Br.

Habenaria macroceratitis Willd.

Herorchis coriophora (L.) D. Tyteca & E. Klein

Himantoglossum adriaticum H. Baumann

Himantoglossum robertianum (Loisel.) P. Delforge

Microtis media R. Br.

Ophrys apifera Huds.

Ophrys sphegodes Mill.

Orchiodes discolor (Ker Gawl.) Kuntze

Orchis mascula (L.) L.

Platanthera bifolia (L.) Rich.

Pseudorchis albida (L.) Á. Löve & D. Löve

Serapias vomeracea (Burm. f.) Briq.

Subfamily Vanilloideae
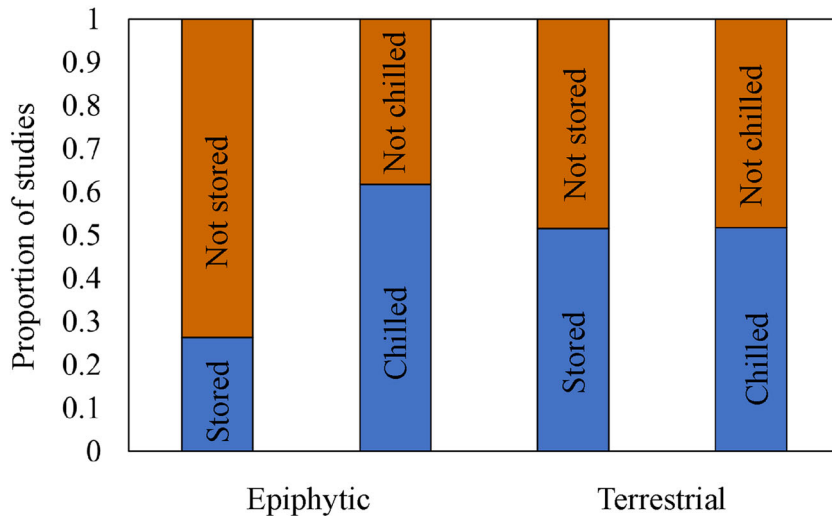
Vanilla planifolia Andrews

FIGURE 2 Variation between epiphytic and terrestrial species in storage and chilling of seeds prior to sowing. Studies reporting use of a stratified treatment were included as chilled.

lengths at cool temperatures impact seed viability and germination across Orchidaceae.

Although most methodologies included a chemical or mechanical treatment applied to capsules or seeds prior to sowing, details were often not reported as to whether the intent of the treatment was for disinfection or seed scarification. When a treatment is applied only to capsules, then it is likely that this served only for disinfection, whereas treatments to seeds could serve to disinfect and chemically scarify them. We only assessed whether such pre-treatments were applied to capsules, seeds, or both across the studies examined. For epiphytic species, capsules were more often treated, whereas seeds were the primary target for terrestrial species ($n = 379$ studies, $G = 26.433$, df = 3, $P < 0.001$; Figure 3A). Additionally, we noted 23 studies in which capsules and then seeds were treated and 27 studies that did not pre-treat seeds before germination. The most common chemical agents used were ethanol and a form of hypochlorite (i.e., commercial bleach, calcium hypochlorite, or sodium hypochlorite). These chemicals were often used together successively (note that ethanol should never be mixed with hypochlorite as it produces a toxic gas) or in combination with other chemical and mechanical treatments of capsules and seeds. Chemicals used on capsules and seeds differed between epiphytic and terrestrial species (capsules: $n = 152$ studies, $G = 25.915$, df = 6, $P < 0.001$; seeds: $n = 240$ studies, $G = 19.463$, df = 6, $P = 0.005$; Figure 3B). Ethanol was rarely used alone on seeds, whereas hypochlorite was the most frequent chemical used on seeds. Terrestrial and epiphytic seeds were treated in hypochlorite for similar amounts of time (epiphytic: 1–90 min, terrestrial: 1–120 min). The use of hypochlorite did increase germination compared to seeds not exposed to hypochlorite, but few studies mentioned specific impacts to seeds from such treatment. Agitation, vacuum pressure, detergents, antimicrobial solutions, mercuric chloride, sulfuric acid, hydrogen peroxide, sucrose, and flaming of ethanol-dipped capsules and seeds were also mentioned in some studies. Tween was regularly used as a wetting agent. Finally, scarification by enzymes and ultraviolet irradiation were mentioned in one study each.

Light environment varied across studies and between epiphytic and terrestrial species. When only a single light environment was used, most studies exposed seeds to either cyclic light/dark periods (56% of cases) or continuous darkness (34% of cases) (Figure 4). Continuous light was used in only a small percentage (6%) of studies. Variation in the environment was associated with growth habit ($n = 377$ studies, $G = 133.81$, df = 3, $P < 0.001$). A greater number of studies on terrestrial species used continuous darkness, whereas cyclic light environments were most frequently used on epiphytic species. When a cyclic light/dark period was used, a photoperiod of 16 h was most common, followed by 12, 14, or 8 h. Experimentation on the effect of the light environment on seed germination was uncommon but more frequently reported in studies of terrestrial species. Seed germination of some species was unaffected by light conditions, but for other species longer light exposure (e.g., 12- or 16-h photoperiod) or complete darkness resulted in maximal germination rates.

There was little experimentation with media pH or germination temperature. The reported media pH ranged from 4.8 to 6.5 but was between 5.5 and 6 for most studies. If multiple media were tested in a study, then researchers often prepared media at different pH levels. The germination temperature ranged from 15°C to 28°C, but most seeds were germinated at 25°C. In some studies, seeds were exposed to cooler temperatures at night and warmer temperatures during the day.

Screens of many different formulations of artificial media and supplements were common in germination trials of both epiphytic and terrestrial species. Additionally, researchers often varied media between germination and transplantation of seedlings. The five most frequently reported artificial media for germination were Murashige and Skoog (1962) basal medium in concentrations of one-tenth to full strength, Knudson C medium (Knudson, 1946) in concentrations of one-fourth to full strength, Vacin and Went (1949) in concentrations of one-half to full strength, Malmgren
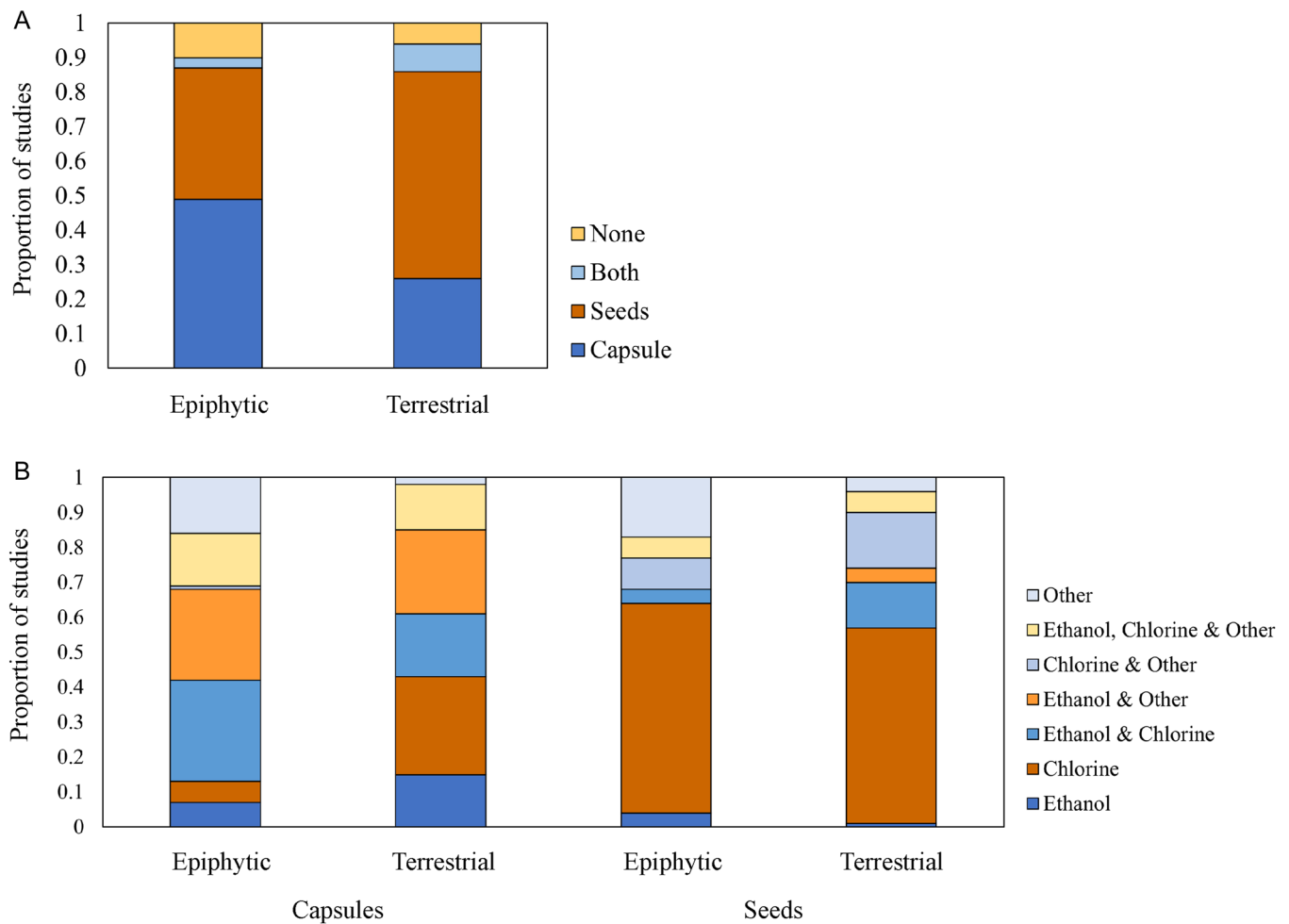
FIGURE 3 Variation between epiphytic and terrestrial species in (A) disinfection unit (capsules or seeds) and (B) disinfection method used. Hypochlorite includes the use of calcium hypochlorite, sodium hypochlorite, and commercial bleach. See text for other disinfecting agents that were used.

FIGURE 4 Variation between epiphytic and terrestrial species in the light environment used for germination.
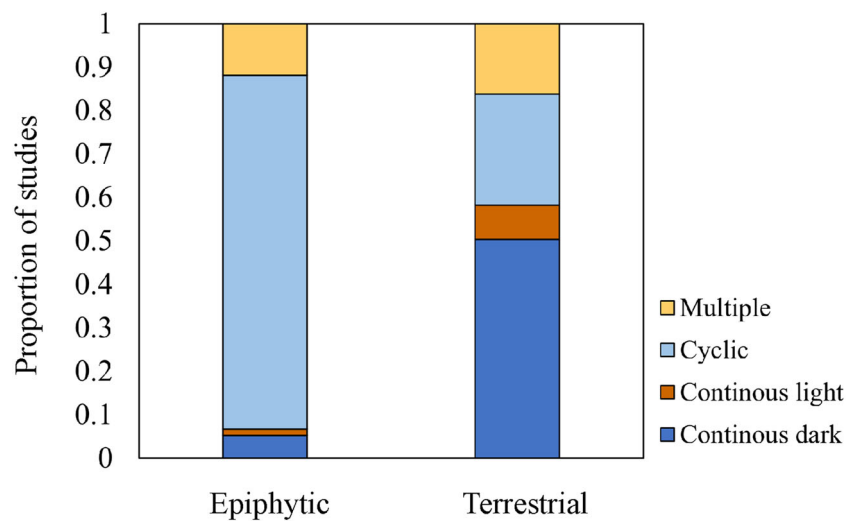
(1996), and BM-1 or BM-2 (PhytoTech Laboratories, Lenexa, Kansas, USA) (Figure 5A). For most studies, the medium was solidified by a gelling agent, such as agar. We also found that researchers have experimented with many variations on the original published compositions of media, which vary in their macronutrients, micronutrients, and other organic compounds. Many artificial media were developed for specific types of orchids
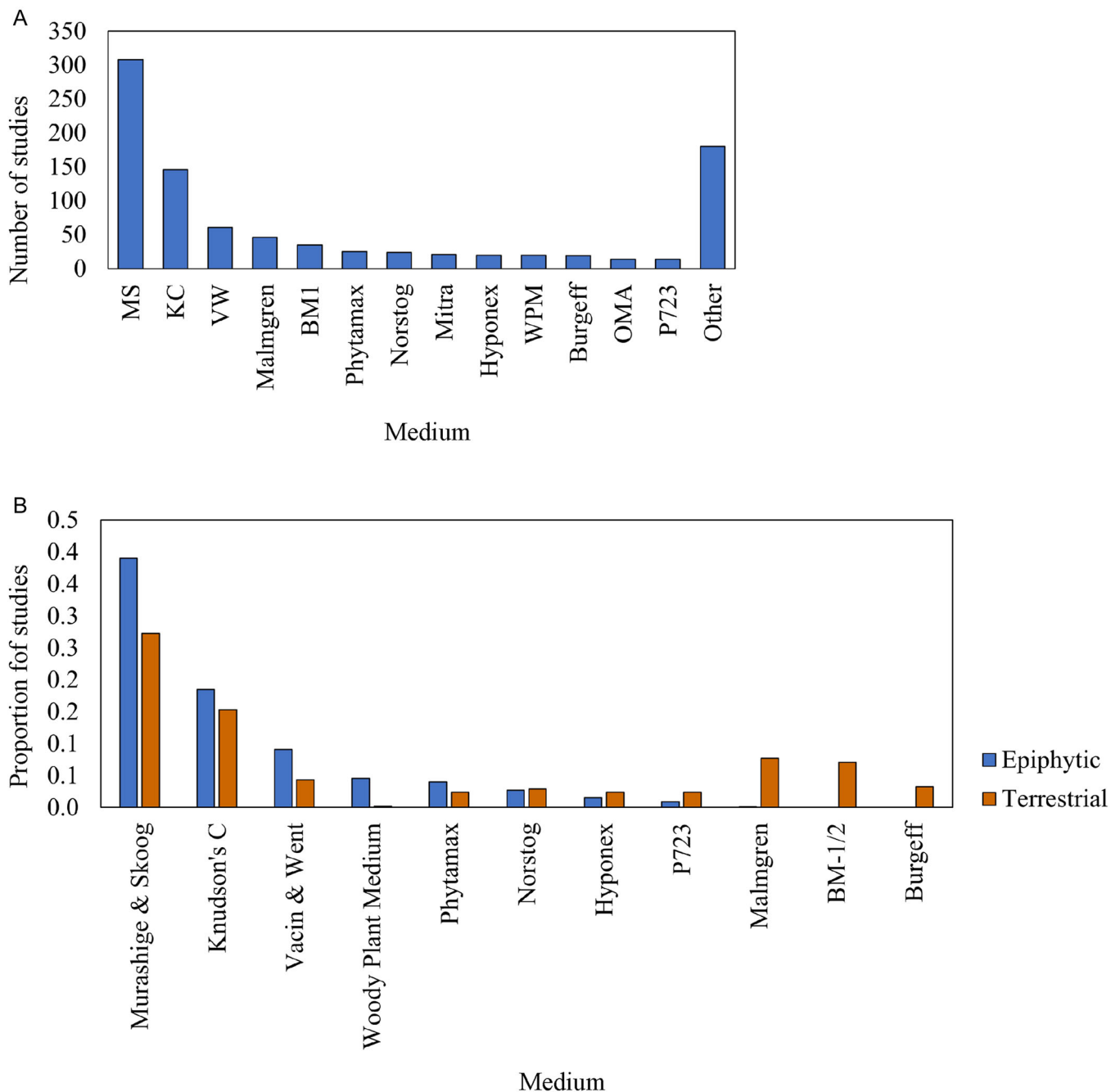
FIGURE 5 (A) Media reported across all studies reviewed and (B) differences in media reported for at least 2% of studies of epiphytes or terrestrial species. BM-1 and BM-2 are very similar media and were counted together for this figure.

(Table 2) and affect the germination and development of species differently. We did not find significant differences in the media that are most frequently used in germination of epiphytic versus terrestrial species (i.e., Murashige and Skoog and Knudson C medium), but the rank of lesser-used media differed between the two groups (Figure 5B). Multiple media were often tested on species (mean = 2.07 for terrestrial species and 2.12 for epiphytic species), but the average number of media tested did not differ significantly between epiphytes and terrestrial species ($t = 0.277$, $P = 0.8$). While multiple media were often found to support

germination, the success and rate of development to protocorm and later stages varied. There was wide variation in how results of germination were reported across studies, which makes it difficult to identify clear trends in which media consistently promote each stage of germination and seedling development.

The addition of supplemental carbohydrates, nitrogen, plant growth regulators, other organic substances, and activated charcoal to media is widely used (ca. 80% of studies) and may underlie some of the variation in the performance of different media to support germination and

TABLE 2 Characteristics of the most frequently reported artificial media used for asymbiotic germination of orchid seeds and seedling growth.

Medium[a]	Description	Additional considerations
Knudson C medium (Knudson, 1946)	This was one of the first media developed specifically for germinating orchid seeds. It has a high ammonium content, which may be more beneficial to germinating seeds than nitrates. Contains no organics other than sucrose, thus it is often used in conjunction with various supplements.	Modern formulations contain charcoal, sucrose, and banana powder. Often used with Morel's (1965) modification of iron as chelate of EDTA.
Vacin and Went (1949)	This was developed as an alternative to Knudson C medium because it reportedly had a better buffering effect through the addition of tomato juice. This was a simple mixture of tomato juice, sucrose, agar, and 10% hydrolyzed casein. Modern formulations contain potassium nitrate and ammonium sulphate as sources of nitrogen and may be beneficial to support germination and development of protocorms.	Sucrose and thiamine are commonly added.
Murashige and Skoog (1962)	Originally developed and widely used for plant tissue culture, this medium is often used in reduced concentration for orchid seed germination because it contains high levels of nitrate and supplements such as sugars, vitamins, and growth regulators.	
Hyponex (Kano, 1965)	When originally developed, it contained a simple composition of nitrogen, phosphorus, and potassium. This medium may be more appropriate for transplanted protocorms where it can promote development of roots and shoots.	It is often supplemented with coconut water, charcoal, peptone, and sucrose to provide other essential components when used for seed germination.
Mitra (Mitra et al., 1976)	This medium was developed for the propagation of the epiphytic and tropical terrestrial orchids. It contains both ammonium and nitrate sources of nitrogen, charcoal, and is supplemented with sucrose. Used extensively for the propagation of species of *Vanda*, *Dendrobium*, and *Cymbidium*.	A common supplement is sucrose.
Malmgren (1996)	Originally developed for germination of *Cypripedium* seeds, it has been used for other species of subfamily Cypripedioideae and terrestrial species in other subfamilies.	Modified versions are low in mineral salts and contain glycine as the sole nitrogen source, pineapple powder, and sucrose. Coconut water often enhances germination.
BM-1/BM-2	Developed for the germination of terrestrial orchid species, especially *Paphiopedilum* and *Phragmipedium*. BM-2 differs only in the addition of the cytokinin 6-benzylaminopurine (BA).	Coconut water is commonly added to BM-1 media to enhance germination.
Phytamax (Sigma Aldrich)	Formulated as a multiplication and maintenance medium, the former contains the auxin α-naphthaleneacetic acid (NAA).	Charcoal and banana powder are recommended supplements to enhance germination.

[a]For individual chemical components and concentrations, readers are referred to the included references, as well as Kauth et al. (2008), Yam and Arditti (2009), and manufacturer's websites.

subsequent development that we found across studies. Many of the frequently used supplements have been shown to support critical aspects of seed germination and protocorm development, but reasons for the use of more obscure supplements are less clear (Table 3). Among supplemental carbohydrates, sucrose was used more often than any other form. Common nitrogen supplements included peptone, thiamine, glycine, and tryptone. Among the plant growth regulators reported, cytokinins and auxins were used more frequently than gibberellic acid, jasmonate, and karrikins. Many studies used complex mixtures derived from a variety of plant sources, including coconut endosperm, banana, pineapple, potato, and tomato. These mixtures contain carbohydrates, nitrogen, vitamins and minerals, and plant growth regulators, but the exact concentrations can vary by source and are often not known when added to an artificial medium. Finally, it should also be noted that supplements

were often added to commercially prepared media that often have substantial macro- and micronutrients, thus providing seeds with an extremely rich and potentially inhibitory germination environment.

We found that only 34% of studies of terrestrial species and 51% of studies of epiphytic species reported transplanting seedlings to the same or new media. Most studies reported transplanting seedlings one or two times, while a few studies transplanted three or more times. Transplant media and supplements were diverse, although they were generally stepwise from an agar-based medium to a potting mix as plantlets matured. Among agar-based media, Murashige and Skoog (1962) basal medium was most frequently used, followed by other media routinely reported for seed germination, including Knudson C medium (Knudson, 1946), Vacin and Went (1949), Malmgren (1996), Phytamax (Sigma-Aldrich, St. Louis, Missouri,

TABLE 3 Reported supplements that have been used in artificial media to support the germination of orchid seeds and their presumed function in germination and seedling growth.

Category	Sample types used[a]	Function
Activated charcoal	NA	Absorption of inhibitory compounds and decrease of toxic metabolites.
Carbohydrates	Sucrose (79%), myo-inositol (6%), glucose (2%), mannitol (2%), fructose (2%), others (9%)	Source of energy used by the developing embryo prior to photosynthesis.
Nitrogen	Peptone (36%), glycine (19%), tryptone (17%), $NaNO_3$ (8%), yeast extract (8%), others (12%)	Supplies nitrogen directly to developing cells or serves as a precursor to the formation of other compounds important to development.
Plant growth regulator	Cytokinins (58%), auxins (35%), gibberellic acid (5%), others (2%)	Important for breaking dormancy and promoting cell division and development.
Other substances	B vitamins (69%), chitosan (5%), others (26%)	Variety of purposes to support primary growth.
Plant-derived mixture	Coconut water (57%), banana powder or homogenate (22%), pineapple (9%), potato homogenate (6%), others (6%)	Mixtures of carbohydrates, vitamins, nitrogen, and plant growth regulators with similar functions to those listed above.

Note: NA = not applicable.

[a]Percent values indicate the frequency within each category among the studies reviewed.

USA), Hyponex (Kano, 1965), and Mitra et al. (1976). Fewer supplements overall were reported in transplant media, although these included similar compounds to those used in germination media. Activated charcoal, sucrose, plant growth regulators, coconut water, banana powder, and potato homogenate were often used in transplant media, whereas supplemental nitrogen was used less frequently. Potting mixes used for transplanting seedlings include bark and wood chips, charcoal, coconut fibers, sphagnum moss, sand, potting soil, compost, tree fern, vermiculite, and perlite. Approximately 40% of studies reported the development of photosynthetic (or otherwise mature plants in the case of heterotrophic species) plants with leaves and roots. This included 70 studies of epiphytes, 72 of terrestrial species, and one for which only the genus was reported (i.e., therefore we did not assign a growth habit). These results suggest different rates of success in growing orchids to this stage, as 100% of studies involving Vanilloideae, 58% involving Cypripedioideae, and 49% involving Epidendroideae species reported the attainment of photosynthetic plants, whereas only 16% of studies involving Orchidoideae reported this result.

DISCUSSION

Orchid populations are declining in their natural habitats at an astonishing rate (Kull and Hutchings, 2006; Štípková and Kindlmann, 2021), which has led to a designation of threatened status for most species that have been assessed by the International Union for Conservation of Nature (IUCN, 2021). Meeting the challenges of orchid conservation will require the creation of new habitats, transplantation, and ex situ conservation in seed banks and living collections (Swarts and Dixon, 2009; Seaton et al., 2013; Reiter et al., 2016; Fay, 2018; Phillips et al., 2020; Wraith

et al., 2020). The use of asymbiotic orchid seed germination has developed extensively in the past century since Knudson (1922) published Knudson B medium and demonstrated that orchids could be grown from seed in the absence of mycorrhizae. The research directions have broadened to include more studies of terrestrial and uncultivated species, the development of new media, and the introduction of diverse organic supplements to support germination and development of orchids through multiple developmental stages. Given this progress, asymbiotic germination may be useful for the conservation of orchids. Asymbiotic growth can yield many more viable seedlings than in situ germination. As many orchid populations decline, transplanted seedlings could enhance population sizes and reproduction in the future. Axenically produced seedlings may also reduce the need for harvesting from natural populations for commercial needs, thereby reducing threats from illegal collecting and overharvesting from wild populations.

Asymbiotic germination can also reduce the vulnerability of young orchid seedlings because they can be cultured in a low-stress environment. If healthy plants can be cultivated prior to introducing them into natural habitats, then the chances of individuals reaching reproductive maturity may be more favorable than supplementing wild populations by artificial pollination or seeds alone. Orchid–mycorrhizal interactions are complex, and it is possible to establish asymbiotically germinated orchids into wild populations where they develop mycorrhizal associations (Fay et al., 2018). Even so, it is important to also consider how introductions of asymbiotically produced seedlings could alter the evolution of wild populations. There is strong selection on early life history stages of orchids, given the disproportionate number of seeds produced versus seedlings that survive to reproductive maturity (Eriksson and Kainulainen, 2011). The introduction of novel genotypic variants that might not

otherwise germinate and survive in the wild could influence interactions between orchids and other community members. The most notable of these are with mycorrhizae, which are important drivers of biogeographic patterns (Jacquemyn et al., 2017), local abundance and distribution of orchids (McCormick et al., 2016, 2018; Li et al., 2021), and community dynamics (McCormick and Jacquemyn, 2014). Given that variation exists in the specificity of orchid–mycorrhizal associations (Jacquemyn et al., 2017), it is difficult to predict the evolutionary consequences of orchid introductions without extensive knowledge of the local mycorrhizal community. Yet, despite the concern that introductions could alter evolutionary processes in wild populations, asymbiotic germination may be necessary to buffer the negative effects of decreased reproduction and declining population size for certain orchid species. The evolutionary consequences of artificial introductions might be lessened by considering the genetic diversity of seeds selected for asymbiotic germination and seedlings selected for introduction into wild populations to ensure that it reflects natural patterns of diversity as closely as possible.

This review corroborates other studies in demonstrating that while many conditions can support asymbiotic germination of orchid seeds, species vary in the conditions that maximize their germination and protocorm development (Dixon, 1994; Kauth et al., 2008; Rasmussen et al., 2015; Teixeira da Silva et al., 2015; Zeng et al., 2016; Herrera et al., 2019; Zale et al., 2022a, b). In addition to variation among species, there is also potential for variation at the intraspecific level due to population responses to environmental heterogeneity (Rasmussen et al., 2015). For example, Kauth et al. (2011) demonstrated variable responses to media, supplements, temperature, and light environment for seeds originating from *Calopogon tuberosus* (L.) Britton, Sterns & Poggenb. populations from three locations in the United States. Studies that incorporate intraspecific variation in seed source as an experimental variable are rare in the literature, but such variable responses are not unexpected, particularly for species with wide geographic distributions or mixed growth habits. The following discussion summarizes the trends we identified in this literature review that may be of use in advancing asymbiotic germination as a more accessible tool for conservation of Orchidaceae.

Seed age

Orchid seeds have a wide range of maturation times (Sauleda, 1976). For example, mature fruits occur in as little as 12 weeks after pollination in *Cypripedium* L. (De Pauw and Remphrey, 1993), whereas *Laelia speciosa* (Kunth) Schltr. requires 7–9 months to mature (Ávila-Díaz et al., 2009). As noted here and in other studies, the age of seeds can have a strong effect on germination (Tsuchiya, 1954; Balilashaki et al., 2015; Zhang et al., 2015; Kendon et al., 2017). Seeds harvested too early may germinate but then fail to develop further, whereas seeds

harvested when fully mature can have reduced germination rates. The higher germination rates often seen in immature seeds have been associated with greater permeability of the testa, which allows for imbibition by the embryo (van Waes and Debergh, 1986), and the absence of chemical inhibitors (van der Kinderen, 1987; Lee et al., 2007, 2015). Seeds of terrestrial species may remain in the soil for months or years (Rasmussen and Whigham, 1993; Whigham et al., 2006), thus premature harvesting may reduce the development of physiological dormancy but alter development after germination. By contrast, seeds of epiphytic species may lack extensive dormancy (Machado Neto and Custódio, 2005), and the longer developmental period of their capsules may enhance their ability to germinate and grow quickly when given nutrients. For example, the pretreatment of mature seeds with hypochlorite can increase germination, as reported in some studies (e.g., Miyoshi and Mii, 1998; Bae et al., 2010; Fu et al., 2016).

For species whose dormancy mechanisms are unknown, it can be difficult to determine the optimal harvest time. Capsule dehiscence usually indicates the presence of mature seeds, but does not necessarily indicate the optimal time to maximize seed germination in an asymbiotic environment. Post-harvest storage conditions, whether for mature or immature seeds, also impact seed viability and germination rate (Machado Neto and Custódio, 2005). Furthermore, other factors to consider with the use of both mature and immature seeds include the ease of disinfecting capsules versus seeds and the potential loss of seed viability due to exposure to chemical treatments that may damage the embryo. Thus, when starting research on a new species, we suggest that incorporating seeds of varying age is ideal to explore how different pre-treatments, media, and growth conditions may influence germination as well as protocorm and seedling development. Testing viability across seeds (Pradhan et al., 2022) of varying ages may be a cost-effective means of identifying the optimal age of seeds for each developmental stage from germination to protocorms to photosynthetic plants (Hosomi et al., 2012; Seaton et al., 2018).

Seed chilling

We expected that chilling seeds after harvest would enhance germination of temperate species but have little effect on epiphytic species due to adaptive differences related to climate seasonality. Cold temperatures are important for many plant species to break dormancy as they slow metabolic processes that inhibit germination and enhance processes that promote cell division (Bewley, 1997; Bewley and Black, 2014). Additionally, seed moisture can influence germination (Baskin and Baskin, 2014). In a natural environment, both temperature and precipitation likely influence seed germination, thus stratified treatments (i.e., cold and moist) are often suggested for terrestrial species (Kauth et al., 2008; Poff et al., 2016). Thus, it was surprising to find that few studies indicated the use of a stratified

treatment on seeds. Many more studies included exposure of seeds to cold, dry conditions, and this was more often seen in studies of terrestrial species than epiphytes. Although there were only a small number of these studies, those that experimented with chilling often reported an increase in germination over non-chilled seeds. Seed age can affect response to cold treatments, and the ideal length of cold treatment, as well as whether seeds are exposed to moisture during cold storage (i.e., stratified), is species-specific (Kauth et al., 2008). These factors may underlie the variability in the degree of coldness used across the studies we examined. If one is studying an orchid species that is subject to seasonal temperature variation, then it may be advantageous to expose seeds to a cold treatment prior to sowing seeds. Chilling seeds versus cold stratification should be considered in the context of the species under study and the age of seeds. The use of chemical agents during disinfection may also reduce inhibitory compounds and could aid in overcoming dormancy as well, but caution is necessary as these chemicals can also damage the embryo (Ponert et al, 2011; Sen et al., 2013).

Chemical treatment of capsules and seeds

A preferred manner of sowing seeds on media is from an intact, clean capsule because the seeds then have minimum exposure to airborne and surface microbes. However, if seeds are separated from a capsule, as they often are for storage or because of capsule dehiscence, then they can be exposed to surface contaminants. We found that it is common practice to use a chemical treatment prior to sowing seeds. For epiphytes, the disinfection of capsules by ethanol with or without flaming is common, whereas the seeds of terrestrial species are frequently treated with a hypochlorite solution. Hypochlorite solutions may be preferred for terrestrial species because these solutions can also disrupt the testa and enhance seed germinability, thereby providing a means for both disinfection and scarification of the seeds. Exposure of seeds to both ethanol and hypochlorite may also be beneficial for germination (Ponert et al., 2011; Acemi and Özen, 2019) as ethanol is effective in reducing suberin and other waxy compounds on the testa. Some studies indicated soaking seeds in hypochlorite solutions for ≥60 min, but the mean time for exposure of seeds to a hypochlorite solution was 12.8 min for both terrestrial and epiphytic species. When using stronger hypochlorite solutions (e.g., >5%), the exposure time should be reduced to minimize damage to the embryo. For example, Zale et al. (2022a) found that seeds of *Spiranthes* Rich. lost germination ability with a 10-min exposure to 10% NaOCl solution made from commercial bleach. The properties of the testa should also be considered when determining an optimal concentration and exposure time for chemical treatments as prolonged exposure of seeds to ethanol can be detrimental to

germination (Ponert et al., 2011; Sen et al., 2013). Thus, ethanol is frequently used first and for no more than 1–2 min. Although less frequently reported in the reviewed studies, other chemical agents that may warrant consideration are mercuric chloride, sulfuric acid, and hydrogen peroxide, and the physical agitation of seeds should also be considered.

Temperature and light conditions

The temperature at which seeds are sown on artificial medium is often selected without explanation or consideration of temperatures in natural habitats. Based on the range of temperatures used in the studies reviewed (15–28°C), we suggest that seeds may be robust to the temperature of the germination environment that can sustain homeostasis of growing cells. Nevertheless, temperature is an important environmental cue for plant development, and studies have supported its role in influencing germination (Yan and Chen, 2020). It is important to consider that while seeds may be capable of germinating at a range of temperatures, maximal germination is often achieved within a smaller temperature range, from 23–27°C (Kauth et al., 2008). Given the seasonality of temperatures encountered by orchid species and their mycorrhizae in natural environments and increasing global temperatures associated with climate change, experiments that test the effects of varying temperatures on orchid seed germination would be beneficial.

Light is also an important environmental cue for germination in many plant species, and their response to light is often species-specific (Kauth et al., 2008). We found a similar result in that the light environment varied for epiphytic and terrestrial species, and germination response varied under light and dark conditions. Cyclic light environments were commonly used for seeds of epiphytic species, whereas seeds of terrestrial species were placed in continuous darkness for germination. Higher germination of terrestrial species in dark conditions may be attributable to a mechanism that breaks dormancy (Rasmussen and Rasmussen, 1991), which could reflect an adaptation by many terrestrial species for living in shaded environments (Rasmussen, 1995), or it may indicate that burial of seeds is necessary before germination can begin. For those terrestrial species occurring in open, sunny habitats, exposure to complete darkness may enhance germination rates but retard the development of protocorms to advanced stages (Kauth et al., 2006). Given the variation in germination response to the light environment across plants generally, we recommend that the native environment of a species is an important consideration when conducting asymbiotic seed germination. Additionally, further experimentation with the impact of variable light environments would be helpful for understanding how epiphytic and terrestrial orchid seeds respond to light conditions in their natural environments.

Artificial medium

Since Knudson's (1922) publication of Knudson B medium, there have been refinements of this medium and entirely new media developed for asymbiotic germination of orchids. Many of these are now commercially available, making it easy to obtain standardized formulations and increase efficiency, particularly in larger labs. Additionally, commercially prepared media are often marketed for use with certain genera, as well as for epiphytic or terrestrial species (Table 2). These media vary in ingredients as well as component concentrations, which may explain frequent reports of differential effects on germination and growth across species and across developmental stages within species. Such differences are expected to reflect variation in how orchids metabolize nutrients sourced from mycorrhizal partners or how they respond to the germination environment more generally. Other studies have provided thorough discussions of the roles that carbohydrates, nitrogen, plant growth regulators, and other organic substances play in orchid seed germination and development (Kauth et al., 2008; Park and Yeung, 2018). Thus, we will not restate their conclusions, and will instead discuss how different forms of critical nutrients may affect orchid seed germination.

Simple forms of carbohydrates are important energy sources for young embryos prior to becoming photosynthetic. While some orchid seeds may contain negligible amounts of carbohydrates, most carbohydrates are supplied to a germinating seed by mycorrhizae. Many commercially prepared media contain sucrose, and other carbohydrates are often added as supplements. Different carbohydrate sources may differentially affect germination and development. For example, Knudson (1922) produced green seedlings with the addition of fructose but not with glucose. More complex carbohydrates can be inhibitory for germination of some species (Smith, 1973; Ernst and Arditti, 1990). Sucrose has frequently been used and may be beneficial because it is a primary form of carbon transmitted from maternal plants to seeds (Morley-Smith et al., 2008). Including some form of carbohydrate seems essential for the asymbiotic germination of orchid seeds, although many simple sugars may be interchangeable in how they are metabolized by orchid seeds (Stewart and Kane, 2010). As many commercially prepared media contain sucrose, it is advisable to evaluate whether supplemental carbohydrates, either of a higher concentration or different form, aid in germination and growth.

Nitrogen, used in the formation of proteins, nucleic acids, and enzymes, is transferred to orchids via mycorrhizae in both organic and inorganic forms (Cameron et al., 2007; Kuga et al., 2014). Diverse nitrogen metabolic pathways in orchids (Spoerl and Curtis, 1948) may explain why some species germinate well with media containing ammonium (Stenberg and Kane, 1998; Kauth et al., 2006), whereas for other species germination is improved with organic nitrogen (Stewart and Kane, 2006). Nitrates can reduce or prevent germination (Ponert et al., 2013; Figura et al., 2020), thus variation in how species metabolize nitrogen sources should be considered when choosing an artificial medium. For example, Knudson C medium (Knudson, 1946) and Vacin and Went (1949) media have high ammonium:nitrate ratios, whereas Malmgren's (1996) medium uses only glycine. Complex mixtures of polypeptides, such as those found in peptone, tryptone, and yeast extract, may more effectively provide diverse nitrogen sources that can be used by orchid seeds.

Plant growth regulators (PGR) serve important regulatory and signaling functions in cell division and development, but the use of these compounds is not always beneficial for germinating seeds. We found that cytokinins (57%) and auxins (36%) were more frequently used than gibberellins (7%) for orchid seed germination. Auxins and cytokinins are well-known promoters of cell division and growth in plants, and they are often used to stimulate germination of a variety of seed types (Kucera et al., 2005). While gibberellins can also promote seed germination through dormancy release, these are often more important for post-germination development and maturation. The optimal quantity of PGR can be difficult to determine yet appears to be important for germination. Very small quantities of auxins and cytokinins promoted germination in several orchid species, whereas higher concentrations had no effect or sometimes inhibited germination (De Pauw et al., 1995; Stewart and Kane, 2006). Different forms of artificial PGRs also influence germination differently (De Pauw et al., 1995). Given that orchid seed responses to PGRs appear to be species-specific, we recommend that both the form and concentration should be considered for use in germination media and later developmental stages.

Activated charcoal was frequently added as a supplement and is routinely used in plant culture media for its ability to adsorb inhibitory compounds produced by developing seedlings, to stimulate growth, especially of the roots, and to promote uptake of nutrients from the medium (Thomas, 2008). Complex plant mixtures from coconut, banana, pineapple, and potato were also frequently added to media. Among these, coconut water was the most used supplement and appears to have a beneficial effect on germination. Coconut water is part of the endosperm tissue and thus contains beneficial nutrients and PGRs that have a positive effect on germination. The mechanism of action of the other plant mixtures is less clear but may be beneficial to certain species.

Given the species-specific responses found across artificial media, it may be beneficial to screen media and supplements to identify optimal nutrient conditions that not only support but also maximize germination for the focal orchid species. Understanding nutrient conditions in the natural environment should aid in selecting optimal media that closely match germination conditions. Media used by others for related taxa may be a good starting point when selecting media to test. Nevertheless, it will also be helpful to screen artificial media that are outside of these boundaries,

as seed germination in vitro may require a unique set of conditions in the absence of mycorrhizae. Given that media can yield differences in germination rates and subsequent development of protocorms, we also recommend that experiments be planned to track development across multiple growth stages.

Transplant and acclimatization of seedlings

Less than one-half of the studies we reviewed reported transplanting seedlings to new media or containers. Transplant media and supplements were diverse, although they were generally stepwise from an agar-based or liquid medium to a potting mix containing wood chips, sphagnum or peat moss, or other substances. Among those that did conduct transplants, mature photosynthetic plants with leaves and roots were often produced, indicating that asymbiotic techniques can be used with a variety of orchid species in vitro. The low incidence of transplants in species of Orchidoideae may suggest less success in generating plants from this subfamily for reintroductions. Epidendroids, many of which are epiphytic, may be better suited to survive on artificial media because epiphytic orchids may have less specific mycorrhizal relationships than terrestrial orchids (Liu et al., 2010). Additional research is needed to fully understand if transplantation in vitro is less successful in Orchidoideae and, if so, the reasons underlying this difference in comparison to Epidendroideae.

Although mature plants with leaves and roots were often produced in the studies we reviewed, not all demonstrated that plants were acclimatized or grown in semi-natural conditions. For those that did indicate such attempts, acclimatization was usually conducted in a greenhouse setting where stressors are likely to be fewer than in a natural environment. Successful reintroductions into wild habitats are dependent on the establishment of mycorrhizal symbioses (Fay et al., 2018), and suitable fungal partners can be patchy within populations (McCormick et al., 2018). Disparities in reintroduction success rates among species may reflect differences in the nature of mycorrhizal symbioses across orchids as well as the local site conditions (Downing et al., 2017, 2020). Clearly, additional studies that focus on effective means of establishing mycorrhizae in orchid roots before or after introduction into a natural environment are needed. For example, Zale et al. (2022b) recently documented in a novel experiment successful introduction of mycorrhizae into asymbiotically germinated seedlings of two temperate orchid species within a sphagnum-based potting mix. Additionally, some seedlings survived explanting into natural habitats. This study demonstrates the great potential in combining asymbiotic and symbiotic techniques to grow native orchids from seeds, but, as noted by the authors, environmental and horticultural conditions must be considered for both orchids and fungi to establish long-lasting symbioses important to orchid health. Finally, there is also a need to better understand turnover in mycorrhizal partners as mycorrhizae of protocorms and adult orchids can vary and may influence how young seedlings respond to environmental conditions (McCormick et al., 2021; Zhao et al., 2021).

Standardization in reporting methods and results and areas of future study

Arditti (1967) commented on the lack of consistency and completeness in the published literature on orchid seed germination techniques. More than half a century later, we find that this issue persists. Identifying the appropriate references needed to develop effective experiments for germinating orchid seeds of a focal species can be daunting because relevant information spans journals, books, and gray literature in print and digital forms. This review focused on empirical studies published in journals and that were accessible through searches of certain keywords. There likely are studies that were missed in our searches because they were not in peer-reviewed journals indexed by Web of Science or did not contain our search terms in the title, abstract, or keywords. We do not expect such missed records to be extensive or indicative of different patterns than those identified here. Nonetheless, we encourage others to expand upon the synthesis presented here by considering references included in other databases in future reviews and in their own research on orchid seed germination.

When initiating new studies on orchid germination, it is important to review diverse references that are relevant to the focal taxon. The use of standardized terminology would also aid in uniting this body of literature and increasing access for readers. We hope that Appendix S1 aids readers in understanding key terminology used in the literature on orchid seed germination. The definitions provided are commonly used, albeit not ubiquitous, interpretations of these terms. We hope that this reference can contribute to use of a more standardized terminology in future papers on orchid seed germination. Another source of difficulty concerns the names used for media. Some manufacturers use identical or very similar names for media of different formulations, and some media have been reported in the literature under different names. Many researchers use commercially prepared media, and there can be slight differences in formulation among manufacturers. When reporting media in articles, it is important to provide the commercial source and reference of each medium used or concentrations of individual ingredients if media are customized. Additionally, we also encourage clear reporting of the source and age of seeds; how they are stored; all stratification, disinfection, and scarification treatments; number of replicates; temperature; light environment; means of assessing germination and growth; and observations of contamination. Because studies often incorporate multiple experiments, it is important that each unique experiment is clearly distinguished and described in sufficient detail, particularly when these involve the

transplant of seedlings. These aspects were often the most difficult to decipher in the articles we reviewed.

While we suggest that asymbiotic germination of orchid seeds could be a useful conservation tool, we have also identified several areas that warrant additional research. Most studies involved seeds from a single source population, thus it is unclear how seeds from ecologically variable populations respond to artificial media or varying growth conditions (Kauth et al., 2008, 2011), an important consideration for managing variation for reintroductions. We expect that terrestrial species, particularly those with wide latitudinal distribution, are most likely to exhibit intraspecific variation in asymbiotic germination and would be ideal subjects for such studies. Second, studies that focus on the impacts of macronutrients across species could aid in understanding how orchids have evolved to utilize individual nutrients, developing media that more accurately reflect natural soil and mycorrhizal environments, and improving asymbiotic germination rates. If asymbiotic germination is to be a successful tool for conservation, then acclimatized photosynthetic plants that can survive in wild habitats must be produced. Reintroductions of orchids grown from seed have not been met with great success, but there also are few studies on techniques that can promote successful mycorrhizal uptake by axenically grown orchids. Thus, we also see a great need for research on the development of mycorrhizal symbioses with orchids after their introduction to wild habitats or as part of the acclimatization process. Relative to the diversity of Orchidaceae, less than 2% of species have been studied for their ability to be grown on an artificial medium. We encourage continued study of diverse species from all subfamilies and geographic areas and emphasize the need to test these techniques with some of the rarest species. Such measures could be a last resort to preserving these species before wild populations become extinct. Finally, it is important to note that this review has revealed to us the tremendous variation that exists in species' germination ability, particularly involving seed age, storage conditions, scarification, and artificial media. Experimental studies that develop best practices for individual species will be needed to maximize the success of asymbiotic germination as a tool for restoring native orchids. We hope that this review spurs additional research on novel species and new techniques that eventually contribute to making asymbiotic germination a regular tool in the conservationist's toolbox.

AUTHOR CONTRIBUTIONS

D.J. collected data, contributed to the design of the project, conducted data analyses, wrote portions of the manuscript, and edited drafts. M.I.B., A.H., and P.N. collected data, contributed to the design of the project, and edited drafts of the manuscript. N.T. conceived of the project, collected data, contributed to the design of the project, and edited drafts of the manuscript. L.E.W. conceived of the project, collected data, conducted data analyses, wrote portions of the manuscript, and edited drafts. All authors have read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The full list of references used for data collection to support this review is available as supplemental information in Appendix S2.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

APPENDIX S1. Key terms and their definitions as used in this review. All terms are used in the context of orchid germination and growth.

APPENDIX S2. Reference list of papers from which data analyzed for this review were extracted.