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Integrated assessment of seed desiccation, storage, and viability of *Grammatophyllum speciosum* for ex-situ conservation

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Abstract. *Puspitaningtyas DM, Diantina S, Yuniati R, Handini E, Aprilianti P, Lestari R. 2026. Integrated assessment of seed desiccation, storage, and viability of* Grammatophyllum speciosum *for ex-situ conservation. Biodiversitas 27 (3): d270323. https://doi.org/10.13057/biodiv/d270323.* Orchid seed banking is recognized as an efficient ex-situ conservation strategy. However, standardized protocols for seed drying, storage, and viability assessment are still lacking for many tropical species. This study evaluated the effects of desiccation duration, frozen storage at -20°C, and tetrazolium chloride (TTC) staining to develop a practical conservation protocol for *Grammatophyllum speciosum*. Fresh seeds were dried over silica gel for 3-9 days and germinated asymbiotically on several basal media. Seed viability after one year of storage was assessed using eight TTC pre-treatments that combined NaOCl scarification, sucrose pre-conditioning, and two TTC concentrations. Germination was significantly influenced by drying duration, and a significant interaction between medium and drying period was detected (ANOVA, $p<0.01$). The highest germination (74.82%±6.01) was obtained after three days of drying on Vacin & Went (VW) medium, whereas prolonged drying (6-9 days) reduced germination to 55-65%. After one year of storage at -20°C, seeds retained high viability (71.87-72.98%), with no significant decline compared to fresh seeds, indicating tolerance to both desiccation and subzero conditions. TTC-based viability assessment revealed significant differences among pre-treatments (ANOVA, $p<0.001$). Combined NaOCl scarification and sucrose pre-conditioning improved staining clarity, producing the highest viability values (up to 73.57%±5.16). No significant difference was observed between 0.1% and 1% TTC concentrations, demonstrating that the lower concentration was sufficient for reliable viability estimation. TTC-based viability closely matched germination results, indicating the consistency of direct and indirect assessment methods. Overall, short-term desiccation (3 days), storage at -20°C, and optimized TTC pre-treatment provide a scalable and resource-efficient strategy for orchid seed banking. This protocol supports the long-term preservation of genetic diversity and contributes to conservation management of threatened epiphytic orchids.

Keywords: Asymbiotic germination, desiccation, orchid conservation, seed storage, tetrazolium test

INTRODUCTION

The genus *Grammatophyllum* comprises approximately twelve orchid species, one of which is *Grammatophyllum speciosum* Blume, commonly known as the giant orchid. This large epiphytic orchid is widely distributed from Indochina to the Malesian region, including Laos, Myanmar, Thailand, Vietnam, Peninsular Malaysia, Sumatra, Borneo, Java, Sulawesi, the Philippines, and Papua New Guinea (POWO 2025). Despite its wide distribution, natural populations of *G. speciosum* are increasingly threatened by habitat loss, logging, and overcollection for ornamental purposes. Since all orchid species are listed under Appendix II of CITES, the development of effective ex situ conservation strategies is essential to safeguard their genetic resources and support long-term species persistence. For vulnerable species such as *G. speciosum*, seed banking supports genetic diversity conservation and reduces pressure on wild populations.

Orchid seed banking offers a practical ex situ conservation strategy because a single capsule may contain hundreds of thousands to millions of minute seeds that can be stored in minimal space (Swartz and Dixon 2017). In many orchid species, seed longevity can be extended by reducing seed moisture content and storing seeds at low temperatures, as most orchid species exhibit orthodox storage behavior (Seaton et al. 2013; Merritt et al. 2014). However, the degree of desiccation tolerance and storage performance varies among taxa, highlighting the need for species-specific investigations to establish reliable seed-banking protocols. Importantly, integrated protocols linking drying, storage, and post-storage viability assessment remain undefined for *G. speciosum*.

Assessing seed quality before and during storage is essential in seed banking. Viability can be evaluated using direct methods, such as asymbiotic in vitro germination, or indirect biochemical assays that estimate metabolic activity. Among indirect methods, the 2,3,5-triphenyl tetrazolium chloride (TTC) test is widely used because it is rapid and strongly correlated with germination performance (Hosomi et al. 2011, 2012; França-Neto and Krzyzanowski 2019). However, effective TTC staining in orchid seeds often requires pre-treatment to enhance reagent penetration. These methods include sucrose pre-conditioning (Hosomi et al. 2011, 2012), sodium hypochlorite (NaOCl) scarification (Dowling and Jusaitis 2012; Bae et al. 2013; Custódio et al. 2016), or vacuum suction (Diantina et al. 2020), which have been applied to improve staining efficiency. Although other assays such as fluorescein diacetate (FDA) and Evans blue (EB) have also been used (Srivastava et al. 2015; Pradhan et al. 2022), TTC remains the most widely adopted method for routine conservation monitoring. Moreover, TTC-based viability estimates have not been validated against post-storage germination in *G. speciosum*.

Previous studies on orchid seed viability have mainly focused on optimizing germination media or refining TTC protocols in selected genera, such as *Cattleya*, *Epidendrum*, and *Phalaenopsis* (Hosomi et al. 2011, 2012; Srivastava et al. 2015; Mercado et al. 2019). In *G. speciosum*, research has largely concentrated on asymbiotic germination and micropropagation, whereas integrated evaluations of seed drying, storage behavior, germination performance, and TTC-based viability assessment remain limited. In particular, the relationship between TTC-derived viability estimates and post-storage germination performance has not been systematically examined for this species. This lack of integration limits conservation programs, as seed banks require clear protocols linking pre-storage handling, storage conditions, and rapid viability testing for effective germplasm management.

Biological characteristics of large epiphytic orchids with dust-like seeds may contribute to variability in storage and germination responses, as differences in embryo structure and nutrient reserves influence tolerance to desiccation and storage. Consequently, protocols developed for other orchids cannot be directly applied to *G. speciosum* without species-specific validation. Therefore, species-specific evaluation is essential to determine whether moderate desiccation and frozen storage can maintain viability and whether TTC testing reliably supports routine seed-bank monitoring.

Therefore, this study aimed to evaluate seed storage performance and viability assessment strategies for *G. speciosum* to support ex situ conservation. Specifically, we investigated: (i) the effect of drying duration on germination, (ii) the suitability of different asymbiotic media for germination; (iii) the impact of storage at −20°C on seed viability, and (iv) the optimization of TTC testing using NaOCl scarification and sucrose pre-conditioning. By integrating germination assays with optimized TTC testing, this study provides a practical framework for rapid viability assessment and long-term seed banking of *G. speciosum*.

MATERIALS AND METHODS

Plant material

Flowers of *Grammatophyllum speciosum* from the living collection of Bogor Botanic Gardens (Indonesia) were naturally pollinated under garden conditions. Although several capsules developed, their maturation stages were not uniform. Therefore, only one capsule showing uniform physiological maturity was selected as the seed source for all experiments. Capsules were harvested approximately nine months after pollination at physiological maturity, prior to full dehiscence. After harvest, the pods were placed on filter paper at room temperature for 48 hours (h) until natural dehiscence occurred (Mercado and Manrique 2015).

Seed drying and storage

Fresh seeds were released from naturally dehiscent capsules, cleaned using a fine-mesh tea strainer to remove debris, and spread on Petri dishes. Seeds were dried over silica gel in a sealed desiccator for 3, 6, or 9 days. For storage, dried seeds were placed in 2 mL cryogenic vials, and then placed inside sealed glass jars (300 mL) containing orange silica gel sachets. During storage at -20°C, the Relative Humidity (RH) inside the storage containers was monitored using a digital hygrometer (Tinytag View 2, UK), with the silica gel sachets serving as humidity indicators (Seaton and Pritchard 2003; Seaton et al. 2018). Seed moisture content was not measured directly using a gravimetric method but was inferred indirectly from the equilibrium relative humidity within the sealed storage system. This indirect estimation approach is commonly applied in orchid seed banking; however, minor variation in moisture content may occur depending on silica gel efficiency and container sealing conditions. Germination tests were conducted in triplicate immediately after drying (fresh seeds) and repeated in triplicate after one year of storage.

Seed disinfection and germination

Dried seeds from each drying treatment (3, 6, and 9 days) were surface-sterilized by soaking in 10% sodium hypochlorite solution (0.525% NaOCl) for 10 min, followed by 5% sodium hypochlorite (0.2625% NaOCl) for 5 min, and rinsed three times with sterile distilled water. During the final rinse, seeds were aseptically sown onto Petri dishes containing various germination media used as treatments. Germination percentage was calculated based on counts of approximately 100-200 seeds per Petri dish observed under a stereomicroscope. Seed number per dish was estimated by counting seeds within a defined microscopic field of view and extrapolating the count to the total sown area of the Petri dish. This subsampling approach is commonly applied for minute orchid seeds that are difficult to count individually under microscopic observation. To reduce potential counting error, multiple microscopic fields were observed for each Petri dish and the counts were averaged prior to extrapolation. Each germination treatment was conducted in triplicate. Each replicate consisted of an independent Petri dish containing seeds from the same seed lot and was treated as an experimental unit for statistical analysis. Petri dishes were sealed with plastic film to maintain sterility and prevent contamination (Puspitaningtyas and Handini 2014, 2020, 2021).

Asymbiotic germination media

Four basal media were evaluated for asymbiotic germination following established protocols (Puspitaningtyas and Handini 2014, 2020, 2021). These consisted of: (i) KC, A modified Knudson C supplemented with 150 g L^{-1} mung bean sprout (*Vigna radiata*) extract and 150 mL L^{-1} young coconut water. (ii) VW, A modified Vacin and Went supplemented with 100 g L^{-1} mung bean sprout extract, 100 g L^{-1} tomato extract, and 150 mL L^{-1} young coconut water. (iii) HS, A foliar fertilizer-based medium (25:5:20, N:P:K) supplemented with 2 g L^{-1} peptone and 40 g L^{-1} potato. (iv) GM, A foliar fertilizer-based medium (32:10:10, N:P:K) supplemented with 2 g L^{-1} peptone and 40 g L^{-1} potato.

All media were supplemented with 2% (w/v) sucrose, 1 g L^{-1} activated charcoal, and solidified with 0.8% (w/v) agar, and adjusted to pH 5.6-5.8 using 1 N NaOH or HCl prior to autoclaving. Based on germination performance of fresh seeds, only the best-performing medium was used for germination tests after one year of storage.

Tetrazolium viability assay

Seed viability after one year of storage at -20°C was evaluated using 2,3,5-triphenyl tetrazolium chloride (TTC) staining. To enhance TTC imbibition, seeds were subjected to vacuum suction for one hour (h) prior to staining (Diantina et al. 2020). Pre-treatments included sodium hypochlorite (NaOCl) scarification (Dowling and Jusaitis 2012; Bae et al. 2013; Custódio et al. 2016) and sucrose pre-conditioning (Hosomi et al. 2011, 2012). A combined treatment of NaOCl scarification and sucrose pre-conditioning, previously tested on other orchid species (Mercado et al. 2019, 2020a, 2020b), was newly applied to *G. speciosum* in this study.

Seeds were incubated in 1% (w/v) TTC solution for 24 h or in 0.1% (w/v) TTC solution for 48 h, in the dark at room temperature. These TTC concentrations were selected based on protocols previously applied in orchid seed viability studies, where 1% TTC is commonly used for embryo staining, while lower concentrations (around 0.1%) have also been tested to optimize staining clarity in minute orchid seeds (Hosomi et al. 2011, 2012; Mercado et al. 2019). The two concentrations were therefore evaluated to determine suitable staining conditions for *G. speciosum*. Following incubation, embryos exhibiting pink to red staining were scored as viable, whereas unstained embryos were classified as non-viable. Seeds lacking visible embryos were excluded from the analysis (Hosomi et al. 2017). Each TTC treatment was assessed in triplicate. Observations were made under a binocular light microscope at 40× magnification. The combinations of pre-treatments and TTC concentrations are summarized in Table 1.

Observation

Seed germination was monitored daily to weekly until no further increase was observed. Germination was defined as testa rupture followed by embryo swelling and the formation of a protocorm-like body (PLB). Non-developing embryos were classified as ungerminated. Germination percentage was calculated based on counts of approximately 100-200 seeds per Petri dish observed under a binocular light microscope at 40× magnification:

$$\text{Seed germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds observed } (\pm 100 - 200 \text{ seeds})} \times 100\%$$

Seed viability following TTC staining was assessed microscopically. Embryos exhibiting pink to red staining were considered viable, whereas unstained embryos were considered non-viable. Viability percentage was calculated based on the number of stained embryos relative to the total number of embryos observed:

$$\text{Seed viability (\%)} = \frac{\text{Number of pink to red} - \text{stained embryos}}{\text{Total number of embryos observed } (\pm 100 - 200 \text{ seeds})} \times 100\%$$

Statistical analysis

Germination and viability data were analyzed using IBM SPSS Statistics 22. Analysis of Variance (ANOVA) was applied to evaluate the effects of culture medium, drying duration, and storage time on seed germination and TTC-based viability, including their interactions. Data transformation was applied only when required to satisfy ANOVA assumptions. The results are presented as mean±SD (standard deviation) based on three independent replicates (n = 3). The null hypothesis (H_0) assumed no differences among treatments, whereas the alternative hypothesis (H_1) stated that at least one treatment mean differed.

Prior to ANOVA, the assumptions of normality and homogeneity of variances were examined using the Shapiro-Wilk test and Levene's test, respectively. Only data meeting these assumptions were analyzed. When significant effects were detected ($p<0.05$), differences among treatment means were compared using Tukey's Honestly Significant Difference (HSD) test at a significance level of $\alpha = 0.05$.

Table 1. Combination of pre-treatments and TTC concentrations used for seed viability assay of *Grammatophyllum speciosum*

Treatment	NaOCl scarification	Sucrose pre-conditioning (18 h)	TTC concentration (w/v)	Incubation time
T1	-	-	1%	24 h
T2	-	+	1%	24 h
T3	+	-	1%	24 h
T4	+	+	1%	24 h
T5	-	-	0.1%	48 h
T6	-	+	0.1%	48 h
T7	+	-	0.1%	48 h
T8	+	+	0.1%	48 h

RESULTS AND DISCUSSION

Plant material and seed source

The reproductive material used in this study originated from mature plants of *G. speciosum* maintained in the living collection of Bogor Botanic Gardens. Representative images of the flowering plant and its growth habit are shown in Figure 1.

Capsules were harvested at physiological maturity prior to natural dehiscence. The seeds exhibited the typical dust-like structure characteristic of epiphytic orchids, with a thin testa and minute embryo, consistent with their rapid moisture equilibration and asymbiotic germination behavior. The morphology of the mature capsule (Figure 2.A) and seeds (Figure 2.B) used in this study are shown in Figure 2.

Assessment of asymbiotic seed germination media

The effects of media on seed germination

Fresh seeds of *G. speciosum* germinated on all four basal media tested (KC, VW, HS, and GM) supplemented with organic additives. Germination was first observed approximately eight weeks after sowing, following seed imbibition. Viable seeds showed embryo swelling and rupture of the testa, leading to the formation of protocorm-like bodies (PLBs), consistent with germination patterns reported for other orchids (Samala et al. 2014; Udomdee et al. 2014).

Data were normally distributed for most treatment combinations based on the Shapiro-Wilk test ($p>0.05$), and homogeneity of variances was confirmed by Levene's test ($F_{11,24} = 2.113$, $p = 0.061$). Two-Way ANOVA showed that the overall model was significant ($F_{11,24} = 4.737$, $p = 0.001$), with the model explaining 68.5% of the total variation in germination percentage ($R^2 = 0.685$). Seed drying duration significantly affected germination ($F_{2,24} = 9.090$, $p = 0.001$), whereas the main effect of media was not significant ($F_{3,24} = 2.832$, $p = 0.060$). However, a significant interaction between media and drying duration was detected ($F_{6,24} = 4.239$, $p = 0.005$), indicating that the effect of drying duration on germination depended on the culture medium used. In general, seeds dried for 3 days tended to show higher germination than those dried for 6 or 9 days, although the magnitude of this effect varied among media.

Seed germination of *G. speciosum* differed significantly among treatment combinations (Figure 3). After three days of drying, seeds cultured on VW and KC media showed the highest germination percentages and did not differ significantly from those on GM and HS media. At six days of drying, germination on HS and VW media remained relatively high, whereas GM and KC tended to show lower values. In contrast, after nine days of drying, germination on VW medium declined markedly and was significantly lower than that on the other media, which did not differ significantly from one another.

These results indicate that the effect of culture medium on seed germination in *G. speciosum* is strongly influenced by drying duration. Short-term desiccation (3 days) supported high germination across all media, suggesting that seeds retained sufficient physiological integrity under mild drying conditions. However, as drying duration increased, germination declined and differences among media became more pronounced. VW medium exhibited the greatest reduction after prolonged drying, whereas GM, HS, and KC maintained comparatively more stable germination under extended desiccation. Together, these findings highlight the importance of matching post-harvest drying duration with an appropriate germination medium to ensure reliable seedling establishment in *G. speciosum*.

Among the four media tested, nitrogen availability can be considered highest in GM medium, followed by HS, whereas KC and VW contain comparatively lower levels of inorganic nitrogen. VW medium, in particular, is characterized by low ionic strength and a relatively limited nitrogen supply. Media with higher nitrogen availability, such as GM and HS, are likely to provide stronger nutritional support during post-desiccation recovery. Following prolonged drying, orchid embryos may experience reduced metabolic activity and require additional nitrogen to support protein synthesis and cellular repair during early germination. This could explain the relatively stable germination observed on GM and HS media after extended drying periods.

Figure 1. The plant morphology of *Grammatophyllum speciosum*. A. Inflorescences, B. Single flower, and C. Growth habit on host

Figure 2. Fruit pod and orchid seeds of *Grammatophyllum speciosum* Blume: A. Mature fruit/pod, bar: 1 cm, B. Orchid seeds, observed under light microscopy (40 × magnification), bar: 0.5 mm

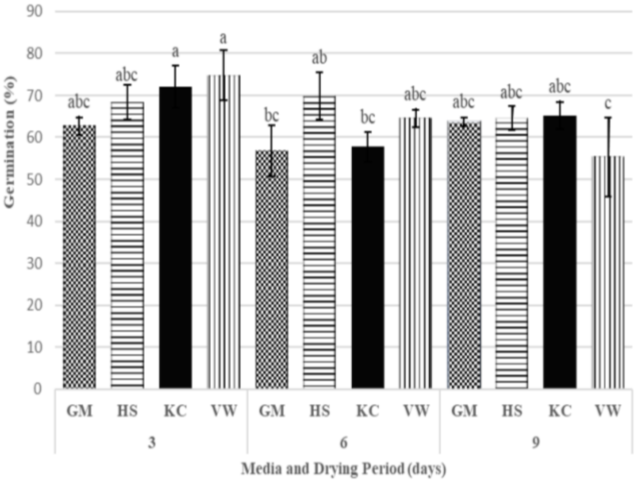

Figure 3. Germination percentage of fresh *Grammatophyllum speciosum* seeds on different germination media (GM, HS, KC, VW) after 3, 6, and 9 days of drying in a desiccator. Bars represent mean±SD. Different letters above bars indicate significant differences among treatments based on Tukey's Honestly Significant Difference (HSD) at α = 0.05

Seed germination patterns varied with increasing desiccation duration, revealing differential responses among the tested media. All media showed increased germination after a brief drying period of three days, with VW and KC promoting the best early development, indicating that short-term desiccation did not compromise seed physiological integrity. As drying time extended to 6 and 9 days, germination generally declined, and differences among media became more evident. VW exhibited the most pronounced reduction after prolonged drying, suggesting greater sensitivity of VW-supported germination to extended desiccation. In contrast, HS and KC maintained comparatively stable germination across drying treatments, implying a more resilient nutritional environment under desiccation stress. GM medium consistently yielded lower germination percentages regardless of drying duration, indicating limited suitability for promoting early protocorm development in *G. speciosum*.

Seed germination and development of protocorm-like bodies (PLBs) of *G. speciosum* on the four media are shown in Figure 4. Although germination percentages did not differ significantly among media, qualitative visual observation indicated that PLBs formed on VW and KC tended to be larger, greener, and bore more rhizoids (absorbing hairs) than those on HS or GM (Figure 4). Although HS and GM media supported relatively stable seed germination, subsequent development of protocorm-like bodies into plantlets appeared to be more successful on KC and VW media. This pattern suggests that *G. speciosum* has different nutritional requirements during the germination phase and later stages of development. Media with higher nitrogen availability may promote embryo swelling and early metabolic activation, which would favor germination; however, they might not offer the ideal ionic balance needed for organized protocorm differentiation and plantlet formation. On the other hand, coordinated morphogenesis during post-germination development appeared to be better supported by KC and VW media, which provide lower but more balanced inorganic nutrients.

Similar responses have been reported in other orchid species. For example, VW and KC media promoted superior protocorm development in *Cymbidium finlaysonianum* Lindl. (Puspitaningtyas and Handini 2020), likely due to their more complete macro- and micronutrient profiles compared with HS and GM media (Puspitaningtyas and Handini 2014). Conversely, HS medium resulted in the highest germination of *C. finlaysonianum* and *Phalaenopsis amabilis* (L.) Blume (Puspitaningtyas and Handini 2020, 2021), highlighting species-specific preferences for germination media. Notably, HS and GM are fertilizer-based media formulated primarily for shoot multiplication, with relatively high nitrogen contents (N:P:K ratios of 25:5:20 and 32:10:10, respectively), which may explain their effectiveness during early germination but not during subsequent developmental transitions.

Previous studies have shown that *G. speciosum* responds positively to a range of nutrient formulations. Samala et al. (2014) reported successful germination on several media, including ½ Gamborg's B5, New Dogashima (ND), and ½ MS. However, protocorm proliferation and early PLB development were most vigorous on ½ MS supplemented with growth regulators and organic additives, indicating that hormonal balance becomes increasingly important at later developmental stages. Sopalun et al. (2010) similarly demonstrated enhanced plantlet regeneration following transfer to hormone-enriched media. In contrast, the present study found that simpler basal formulations such as VW and KC, without exogenous growth regulators, were sufficient to promote uniform protocorm initiation, particularly after short desiccation treatments. Collectively, these findings suggest that while MS-based media supplemented with hormonal regulators remain advantageous for advanced developmental stages, simpler media with balanced nutrient availability can adequately support germination and early PLB formation when pre-culture seed moisture is appropriately managed.

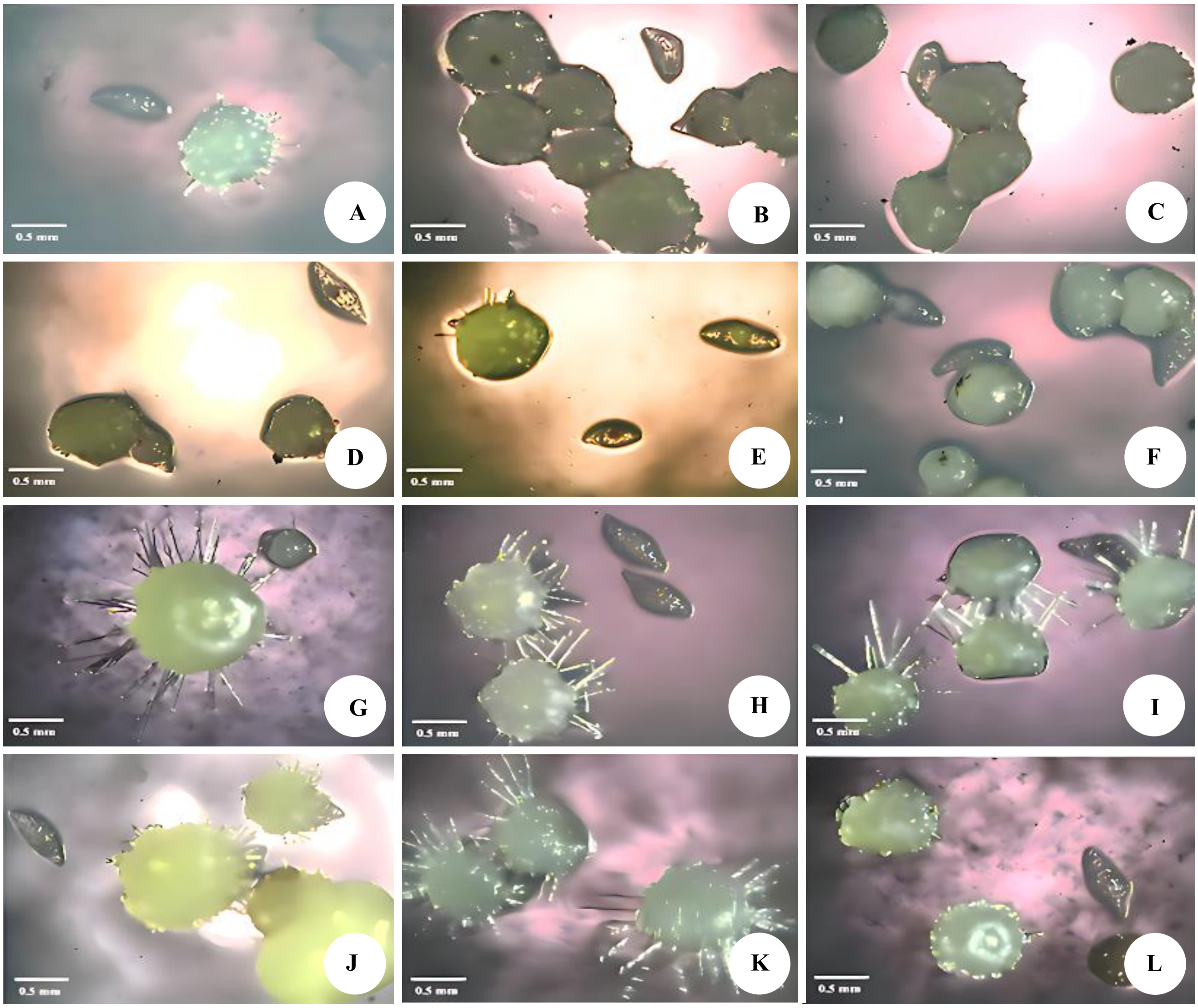

Figure 4. Protocorm-like bodies (PLBs) development of *Grammatophyllum speciosum* formed 8 weeks after planting on four culture media (columns) following three desiccation periods (rows). A. GM: 3 days drying, B. GM: 6 days drying, C. GM: 9 days drying, D. HS: 3 days drying, E. HS: 6 days drying, F. HS: 9 days drying, G. KC: 3 days drying, H. KC: 6 days drying, I. KC: 9 days drying, J. VW: 3 days drying, K. VW: 6 days drying, L. VW: 9 days drying, respectively. Scale bar: 0.5 mm

The superior performance of VW medium in supporting early germination and uniform protocorm development can be attributed to its balanced macronutrient composition and moderate inorganic nitrate levels, which favor cell expansion and chlorophyll development (Vacin and Went 1949). VW medium, originally developed for *Vanda* species, contains lower total nitrogen than Murashige and Skoog (MS) or Gamborg's B5, minimizing the risk of osmotic stress. In addition, enrichment with organic additives such as coconut water and tomato extract provides natural cytokinins, amino acids, vitamins, and readily available carbon sources that stimulate cell division and promote differentiation (Yong et al. 2009; Gnasekaran et al. 2012; Utami and Hariyanto 2020). These organic components may partially substitute for the regulatory functions normally supplied by mycorrhizal fungi during symbiotic germination.

Comparable responses to organic supplementation and reduced inorganic salt concentration have been reported in several tropical epiphytic orchids (Gnasekaran et al. 2012; Shekarriz et al. 2014; Pradhan et al. 2013; Zakiyah et al. 2025), supporting the physiological relevance of these mechanisms across related taxa.

On the other hand, HS and GM media, which contain higher total nitrogen content and ionic strength due to elevated concentrations of inorganic nitrate and ammonium, tended to be less suitable for *G. speciosum* germination in our study. High levels of inorganic nitrogen or salts have been shown to inhibit seed germination or protocorm development in some orchids, probably because many species are adapted to low-nutrient microsites and respond better to organic nitrogen sources or diluted salts. Recent asymbiotic germination studies report reduced germination or poorer protocorm growth when media are dominated by high inorganic N or high ionic strength. Orchid seeds often respond negatively to high nitrate/salt environments and instead benefit from media providing organic nitrogen or lower total salt concentration (Park et al. 2023; Kauth et al. 2008). In *Paphiopedilum wardii* Summerh., reduced germination and difficulty in protocorm formation were reported on undiluted, high-salt formulations compared with more dilute or modified media (Zeng et al. 2012). Previous work on epiphytic orchids has shown that elevated concentrations of inorganic nitrogen and salts can inhibit protocorm differentiation and pigment formation (Sopalun et al. 2010; Custódio et al. 2016; Puspitaningtyas and Handini 2021). Accordingly, many epiphytic species with dust-like seeds, including *Grammatophyllum* and *C. finlaysonianum*, germinate better on formulations with lower salt concentration and moderate organic supplementation such as VW medium (Puspitaningtyas and Handini 2020).

KC medium, although less effective than VW, consistently supported moderate germination of *G. speciosum* seeds. Its relatively simple mineral composition, together with organic supplementation such as coconut water, provides amino acids, vitamins, and natural cytokinins that favor embryo swelling and early protocorm-like body (PLB) initiation, and for this reason KC is commonly used as a starter medium in orchid propagation protocols (Kauth et al. 2006). These results indicate that media characterized by low ionic strength and organic additives are particularly suitable for the early developmental stages of *G. speciosum*, a pattern frequently observed among large epiphytic orchids. Accordingly, both VW and KC media can support early seed and protocorm development, although VW generally promotes more uniform and vigorous protocorm growth under the conditions tested.

From a physiological perspective, the observed responses reflect the limited internal reserves of orchid seeds, which lack endosperm and therefore depend entirely on externally supplied carbon and nitrogen for respiration and biosynthesis. Because orchid seeds contain only minimal nutrient reserves, successful germination depends heavily on the composition of the external medium. The source and concentration of nitrogen strongly influence germination performance (Stewart and Kane 2006; Park et al. 2023), and media with balanced nutrient composition together with moderate osmotic potential can more effectively promote metabolic reactivation, enzymatic processes, and early protocorm differentiation. In contrast, excessive salt or nitrogen concentrations may impose osmotic or ionic stress that constrains embryo development. The superior performance of VW medium suggests that its nutrient balance is particularly well suited to the metabolic requirements of *G. speciosum* embryos during early development.

From a conservation perspective, optimizing germination media is fundamental for assessing seed quality and supporting ex situ propagation systems. Reliable germination and viability testing are essential for orchid seed banks, as they enable routine monitoring of stored accessions and support the regeneration of plant material for conservation collections or potential restoration programs. In this context, the high and consistent germination obtained on VW medium provides a reliable baseline for evaluating post-storage viability in seed-banking studies and for regenerating stored accessions of *G. speciosum*. Thus, this medium can serve as a practical benchmark for testing stored seeds and supporting regeneration of conservation collections and related taxa.

Storage and desiccation effects

Fresh seeds of *Grammatophyllum speciosum* exhibited 100% equilibrium-Relative Humidity (e-RH) immediately after harvest, reflecting high moisture content at physiological maturity. For long-term conservation, it is essential to reduce this moisture level to ensure safe storage. Desiccation over silica gel for 3 to 9 days gradually reduced e-RH from 54.6% to 50.6% corresponding to estimated moisture contents of 15.0%, 14.7%, and 14.4%, respectively (Table 2), although differences among treatments were not statistically significant. Such a gradual decline indicates that orchid seeds, which possess thin, non-lignified testa and air-permeable cells, can equilibrate rapidly with the drying environment (Kauth et al. 2008; Hay et al. 2023). Under these conditions, seeds dried for three days showed the highest germination, suggesting that moderate moisture reduction before storage helped maintain seed viability. Previous studies have demonstrated that controlled desiccation using silica gel can effectively lower orchid seed moisture to levels compatible with medium- to long-term storage and cryopreservation (Vendrame et al. 2007; Merritt et al. 2014). Maintaining seed eRH below approximately 55% corresponds to the low-moisture "Region II" zone, where water is loosely bound and metabolic activity remains minimal (Ellis et al. 1989; Hay et al. 2023), thereby supporting the maintenance of desiccation tolerance and viability in many epiphytic orchids during storage (Pritchard and Seaton 1993; Seaton et al. 2013; Pradhan et al. 2022). These values therefore fall within the moisture range considered safe for medium- to long-term storage of desiccation-tolerant orchid seeds.

Since PLB development of *G. speciosum* was highest on VW and KC media (Figure 4), these two media were selected for the subsequent germination test following one year of storage at -20°C. After one year of frozen storage, seed germination of *G. speciosum* remained comparable to freshly harvested seeds, indicating no significant loss of viability (Table 3). This result is consistent with the orthodox storage behavior reported for many orchid species, whereby longevity can be extended through moderate moisture reduction combined with low temperature storage, under seed-bank conditions (Seaton and Pritchard 2011; Seaton et al. 2013; Merritt et al. 2014), suggesting that the seeds of *G. speciosum* may behave similarly to orthodox seeds under the storage conditions tested. Although cellular stability was not directly examined in this study, the maintenance of germination after freezing is consistent with the presence of protective mechanisms commonly reported for desiccation-tolerant orchid seeds.

Table 2. Equilibrium Relative Humidity (eRH) and estimated moisture content of *Grammatophyllum speciosum* seeds after desiccation over silica gel for different durations

Duration in desiccator (days)	**eRH (%)**	**a_t (=eRH/100)**	**$\log_{10}(a_t)$**	**MC (%) $\approx 20 + 19\times\log_{10}(a_t)$**
3	54.60±1.31	0.546	-0.262	$20 + 19\times(-0.262) = 15.0\%$
6	52.83±1.58	0.528	-0.278	$20 + 19\times(-0.278) = 14.7\%$
9	50.57±3.11	0.506	-0.296	$20 + 19\times(-0.296) = 14.4\%$

Table 3. Seed germination of *Grammatophyllum speciosum* before and after storage under different desiccation durations

Storage time (years)	**Media**	**Germination (%)**		
		Duration of drying in a desiccator		
		3 days (± SD)	**6 days (± SD)**	**9 days (± SD)**
0 year (fresh)	KC	72.00±5.05	57.74±3.48	65.11±3.24
	VW	74.82±6.01	64.53±2.13	55.29±9.50
1 year	KC	71.87±2.36	58.59±8.21	64.08±3.35
	VW	72.98±9.08	67.63±6.25	63.39±1.86
Mean±SD		72.92±1.36 a	62.12±4.75 b	61.97±4.51 b

Note: The mean±SD followed by different letters indicate significant differences among the desiccation durations (3 days, 6 days, 9 days), based on Tukey's Honestly Significant Difference (HSD) at α = 0.05

Statistical analysis indicated that the data met the assumptions of normality (Shapiro-Wilk test, $p>0.05$ for most groups) and homogeneity of variances (Levene's test, $F_{11,24} = 2.002$, $p = 0.075$). Seed germination percentage (0 and 1 year of storage) was significantly influenced by desiccation duration (ANOVA, $F_{2,24} = 14.614$, $p<0.001$), whereas neither culture medium ($F_{3,24} = 0.671$, $p = 0.578$) nor the interaction between desiccation and medium ($F_{6,24} = 1.739$, $p = 0.155$) showed significant effects. The overall model was significant ($F_{11,24} = 3.789$, $p = 0.003$), explaining 63.5% of the total variation in germination percentage ($R^2 = 0.635$). No significant differences were observed between KC and VW media and in their interaction with storage duration (0 and 1 year of storage).

Post hoc analysis (Tukey's HSD) indicated that desiccation duration significantly influenced germination, with short-term drying (3 days) consistently producing the highest germination both before and after storage (72.92±1.36%), while extended drying (6-9 days) slightly reduced germination. No significant difference was observed between 6 and 9 days (Table 3). These results suggest that excessive drying may reduce seed performance, even in species showing tolerance to desiccation and freezing. This finding is also consistent with previous reports showing that moderate desiccation preserves cellular function in orchid seeds without inducing structural damage (Seaton et al. 2013; Hay et al. 2023).

These findings suggest that a short drying period (3 days) optimally balances moisture reduction and viability preservation, supporting an efficient seed storage strategy for *G. speciosum*. Therefore, although *G. speciosum* exhibits orthodox behavior, longer-term storage studies are also needed to determine the limits of seed viability under conventional seed storage conditions.

In physiological terms, these patterns suggest that moderate moisture reduction stabilizes cellular structures prior to freezing, thereby reducing the risk of desiccation-induced damage during storage. Although *G. speciosum* seeds tolerated both drying and freezing, desiccation tolerance alone does not guarantee survival during prolonged sub-zero conditions. Previous studies have shown that prolonged drying can be detrimental to orchid embryos, particularly in species exhibiting intermediate responses between orthodox and recalcitrant storage behavior (Machado-Neto and Custódio 2005).

Short-term desiccation improved seed viability in *G. speciosum*, whereas prolonged drying slightly reduced germination after storage. This pattern suggests that moderate moisture reduction may help maintain cellular stability during early imbibition without inducing damage associated with excessive dehydration. Similar responses have been reported in orthodox seeds, where moderate moisture reduction may contribute to the maintenance of membrane integrity and macromolecular stability, while excessive dehydration may lead to structural damage (Vertucci and Roos 1990; Walters 1998). In the present study, seeds equilibrated at approximately 50% eRH, corresponding to moderate moisture levels, which appear sufficient to support storage without imposing severe desiccation stress. According to the current findings, *G. speciosum* seeds have a comparatively small window for moisture tolerance, during which time moderate desiccation promotes long-term viability while prolonged drying may become harmful. From a conservation perspective, moisture reduction alone is insufficient to ensure long-term stability; rather, moderate drying combined with low-temperature storage provides a more reliable strategy for maintaining orchid seed viability (Seaton and Pritchard 1993). For successful ex situ conservation, these results emphasize the importance of balancing temperature management and moisture reduction. In this study, short drying (3 days) prior to storage resulted in the most consistent germination after one year at -20°C.

An indirect method using the tetrazolium viability assay

An indirect viability test using 2,3,5-triphenyl tetrazolium chloride (TTC) staining was conducted on *G. speciosum* seeds after one year of storage using eight pre-treatment combinations (Table 1). Sodium hypochlorite (NaOCl) scarification is widely used to weaken the seed coat to improve TTC penetration, while sucrose pre-conditioning has been shown to enhance staining clarity and intensity in orchid embryos.

In this study, no significant difference was observed between 0.1% and 1% tetrazolium in terms of embryo staining intensity (Figure 5), indicating that the lower concentration of tetrazolium (0.1%) successfully stained the embryos of *G. speciosum* seeds. The effectiveness of low TTC concentration is consistent with previous studies on orchid seeds (Hosomi et al. 2011), who also recommended using a low concentration of 0.1% tetrazolium for seed viability testing, especially with an exposure time longer than 12 hours. On the other hand, Mercado et al. (2019) found that pre-treatment with 1% sodium hypochlorite (NaOCl) for 10 minutes enhanced the seed viability of *Epidendrum barbaricum* seeds when using a concentration of 0.25% or 1% TTC for 24 hours of exposure. This pre-treatment improved embryo staining by scarifying the seed coat. Their findings align with the results of this study, suggesting that 0.1% tetrazolium is as effective as 1% for testing seed viability in *G. speciosum*, and a lower concentration may suffice for effective embryo staining. Extended exposure at reduced concentration allows adequate enzyme-mediated reduction.

According to Hosomi et al. (2011), pre-conditioning seeds in a 10% sucrose solution improves staining clarity during TTC assays. When seeds are immersed in a colorless TTC solution, the compound penetrates seed tissues and is reduced by active dehydrogenase enzymes in metabolically living cells, producing a red formazan pigment. Microscopic analysis (Figure 5) confirmed that red or pink embryos indicate viable seeds, whereas unstained embryos are non-viable. The TTC method has proven effective for estimating seed viability in numerous orchid species and provides a much faster alternative to conventional germination tests. While germination tests may take at least 32 days, the TTC test can be completed in approximately three days, including time for pre-conditioning, staining, and viability scoring (Hosomi et al. 2011).

Data met the assumptions of normality and homogeneity of variances (Shapiro-Wilk, p>0.05; Levene's test: $F_{7,16}$ = 1.916, p = 0.133). One-way ANOVA showed that pre-treatment combinations significantly affected TTC-based seed viability ($F_{7,16}$ = 9.415, p<0.001). Post hoc Tukey's HSD test indicated that treatments 4 (73.57%±5.16) and 8 (69.27%±6.22) produced the highest viability and were not significantly different from each other, but both were significantly higher than treatment 3, which showed the lowest viability (40.01%). The remaining treatments (1, 2, 5, 6, and 7) showed intermediate viability values (43-61%) and overlapped statistically with both higher and lower groups (Figure 6).

Based on this result, pre-treatment combining scarification and sucrose pre-conditioning significantly affected seed viability outcomes. Results showed that treatments 4 and 8 (10% and 5% NaOCl + 10% sucrose, 1% and 0.1% TTC respectively) produced the highest seed viability, significantly exceeding most other treatments. In contrast, NaOCl alone resulted in lower viability values (approximately 40%). Treatments without combined pre-treatment showed intermediate responses (between 40-60%). The data suggest that pre-treatment combinations of NaOCl scarification and sucrose pre-conditioning enhance TTC staining efficiency and improve the accuracy of viability estimation, likely by improving seed coat permeability and metabolic activation prior to staining. This finding is consistent with earlier observations in other orchid species (Hosomi et al. 2017; Mercado et al. 2019).

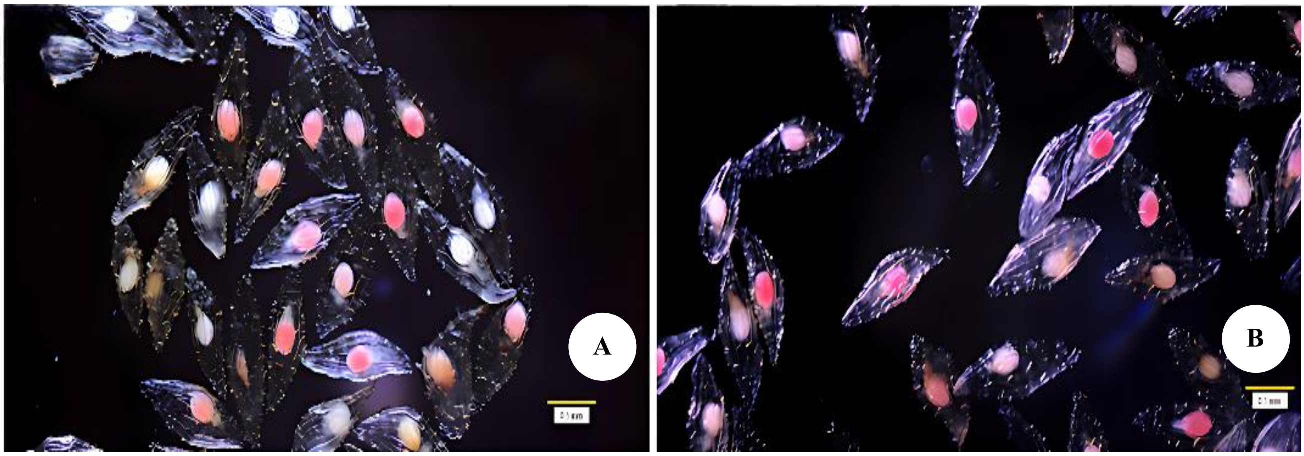

Figure 5. Microscopic observations of *Grammatophyllum speciosum* seeds subjected to TTC staining: A. TTC 1%, and B. TTC 0.1%. Red or pink embryos indicate viable seeds, while unstained embryos are non-viable. Scale bar: 0.5 mm

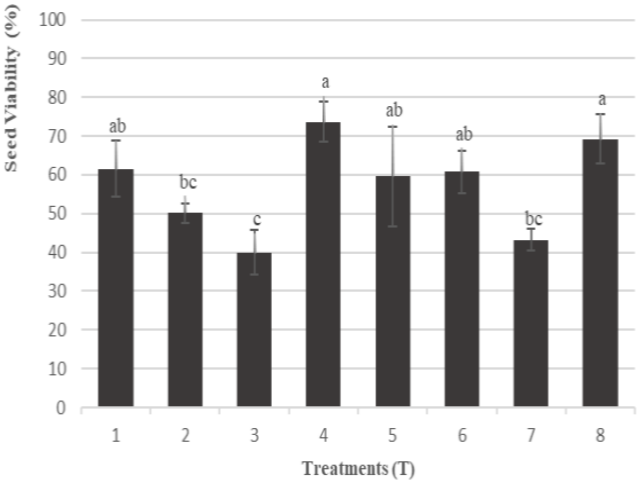

Figure 6. Seed viability (%) of *Grammatophyllum speciosum* evaluated using 2,3,5-triphenyl tetrazolium chloride (TTC) staining at two concentrations (1% for 24 h and 0.1% for 48 h) under different pre-treatments: no pre-treatment, sucrose pre-conditioning (10% sucrose, 18 h), NaOCl scarification (10% NaOCl for 10 min followed by 5% NaOCl for 5 min), or their combination (see Table 1 for details). Values are presented as mean±Standard Deviation (SD). Different letters above bars indicate significant differences among treatments according to Tukey's HSD test at $\alpha = 0.05$

In this study, TTC staining results were consistent with asymbiotic germination tests, which showed no significant differences in viability estimates. The highest TTC-based viability after one year of storage (73.57±5.15) closely matched the highest germination percentage (72.98±9.08). This close numerical agreement indicates a strong correspondence between TTC viability estimates and germination outcomes in *G. speciosum* seeds, suggesting that both direct (germination test) and indirect (TTC staining) methods provide reliable assessments of seed viability. However, improper selection of scarification methods for breaking dormancy and inappropriate selection of asymbiotic media for evaluating storage behavior can affect germination response and interpretation of seed quality (Dowling and Jusaitis 2012; Magrini and De Vitis 2016; Popova et al. 2016). There are significant benefits to using TTC assays in seed-bank monitoring from a conservation standpoint. Because large epiphytic orchids like *Grammatophyllum* can lose their natural habitats rapidly, accurate viability testing is crucial for figuring out when stored accessions need to be regenerated in order to preserve genetic diversity. Because it takes little equipment and produces data in a matter of days, the improved TTC methodology presented here is practical for routine monitoring, even in laboratories with limited resources. Coordinated ex-situ conservation initiatives across botanic gardens and seed banks are supported by the establishment of uniform viability benchmarks, which also facilitates data comparability between institutions.

In the broader context of orchid conservation, integrating both germination tests and biochemical assays offers a robust framework for monitoring seed quality across storage intervals. For many tropical epiphytic orchids, viability can fluctuate due to inherent variation in seed maturity, capsule position, and maternal plant condition. Therefore, relying on a single viability test may occasionally lead to over- or underestimation of seed performance. By combining asymbiotic germination with optimized TTC pre-treatments, this study demonstrates a complementary system that captures both physiological and metabolic aspects of seed viability. Such dual-method approaches are increasingly recommended for seed banks handling species of high conservation concern, ensuring that accessions meet the standards required for future multiplication, reintroduction, or genetic management programs.

In conclusion, this study established a practical protocol for preserving mature seeds of *G. speciosum*. Short desiccation for three days maintained the highest germination before and after one year of storage at -20°C, suggesting that the seeds show storage characteristics consistent with orthodox behavior under the conditions tested. Both asymbiotic germination and optimized TTC assays yielded comparable viability results, demonstrating that direct and indirect methods can reliably assess seed quality. The combined protocol three-day desiccation, frozen storage, and TTC pre-treatment with NaOCl scarification and sucrose pre-conditioning provides a rapid and scalable framework for orchid seed banking. Although seed viability was maintained after one year of storage, the present study evaluated only a relatively short storage period. Longer-term evaluations will therefore be required to determine the maximum storage longevity of *G. speciosum* seeds under conventional seed bank conditions. Overall, this protocol contributes to strengthening ex situ conservation strategies for *G. speciosum* and other threatened epiphytic orchids by supporting seed banking, viability monitoring, and the long-term preservation of orchid genetic resources.

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