

Recalcitrance and morphophysiological dormancy: the role of hormonal balance and polyembryony in the germination of *Psychotria viridis*

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Abstract

Psychotria viridis Ruiz & Pav. is a neotropical shrub of pharmacological relevance whose seeds exhibit slow and irregular germination. This study aimed to evaluate the dormancy mechanisms and the conditions required for seed germination in this species. Pre-germinative treatments were applied, including water imbibition, stratification, chemical scarification, seed coat removal, and embryo excision, followed by *in vitro* and *ex vitro* cultivation. Gibberellic acid (GA₃) and abscisic acid (ABA) were quantified by LC–MS/MS. The seeds were classified as recalcitrant due to a marked loss of viability (from 96% to 19.3%) when moisture content was reduced from 45% to 15%. Dormancy maintenance was associated with seed coat restrictions and elevated ABA levels (38.1 ng g⁻¹), indicating a hormonal imbalance unfavorable to germination. Embryo excision followed by *in vitro* culture on half-strength Murashige and Skoog medium (MS/2) supplemented with 20 µM GA₃ was the most effective treatment to overcome dormancy, resulting in 98.85% germination and 100% seedling formation. A high frequency of polyembryony (78.34%) was observed, with embryos at different developmental stages. The results indicate that *P. viridis* seeds exhibit morphophysiological dormancy characterized by the presence of seed coat barriers, hormonal imbalance, and asynchronous embryo development.

1. Introduction

Psychotria viridis Ruiz & Pav. (Rubiaceae) is a neotropical shrub widely distributed from Central to South America (REFLORA 2025). The species holds significant ethnobotanical importance due to its use in the preparation of ayahuasca, a psychoactive beverage employed by Indigenous peoples and Amazonian religious groups in ritualistic contexts for spiritual and therapeutic purposes (Alexander 2025). In addition, *P. viridis* has attracted considerable therapeutic and pharmacological interest because of the presence of indole alkaloids, particularly N,N-dimethyltryptamine (DMT), which has stimulated investigations focused on its chemical characterization and potential application in the treatment of mental disorders (Costa et al. 2024; De Andrade et al. 2025).

Species within the Rubiaceae family exhibit germination strategies associated with recalcitrance and different classes of dormancy, especially morphophysiological dormancy (Athugala et al. 2016; Baskin and Baskin 2024). These characteristics suggest a synergistic interaction between seed coat constraints and hormonal signaling pathways in the regulation of the germination process (Athugala et al., 2016). In *P. viridis*, the low germination performance (approximately 20%) is characterized by slow and irregular germination kinetics (Corrêa et al. 2007) indicating the presence of dormancy and physiological constraints analogous to those reported for other taxa within the family.

Such germinative traits may favor species persistence under natural conditions by promoting staggered germination and increasing the likelihood of seedling establishment under variable environmental conditions (Baskin and Baskin, 2024). However, they also impose limitations on achieving homogeneous germination and experimental reproducibility, thereby hindering the development of standardized

propagation protocols and the advancement of therapeutic and pharmacological studies that depend on uniform plant material and consistent biological responses (Bewley et al. 2013).

In this context, *in vitro* culture has emerged as an effective strategy for overcoming dormancy and standardizing germination, enabling the exploration of the medicinal potential of the species (Karalija et al. 2025). This approach allows direct manipulation of physicochemical factors and hormonal mechanisms underlying dormancy regulation and germination processes (Homczinski et al. 2023).

The transition from dormancy to germination is primarily governed by the hormonal antagonism between abscisic acid (ABA) and gibberellins (GAs) (Koçak-Şahin et al. 2025). While ABA maintains dormancy and promotes stress tolerance, GAs, particularly gibberellic acid (GA₃), trigger embryo elongation, activate hydrolytic enzymes involved in reserve mobilization, and weaken surrounding tissues, thereby facilitating radicle protrusion (Koçak-Şahin et al. 2025). Consequently, strategies such as the application of plant growth regulators, chemical scarification, and stratification have been employed to modulate these pathways and overcome physiological and seed coat-related restrictions to germination (Fu et al. 2024).

In addition to endogenous factors, the germination environment plays a determinant role in modulating dormancy-associated mechanisms. Comparative studies between *in vitro* and *ex vitro* cultivation systems in taxa such as *Hibiscus* sp., *Rosa persica*, and *Handroanthus* sp. have demonstrated that the abiotic and biological stimuli inherent to each system significantly influence germination kinetics and early seedling development (Hamidou 2009; Apóstolo et al. 2016; Vaziee et al. 2023). Evidence suggests that *in vitro* approaches provide methodological advantages by enabling greater control over environmental variables and isolation of intrinsic seed constraints, thereby facilitating the distinction between physiological responses and external limitations, in addition to improving uniformity and reproducibility of germination processes (Pathak et al. 2023; Souza et al. 2024).

Despite the ethnobotanical and medicinal relevance of *P. viridis*, integrated investigations into the physiological determinants of its germination remain scarce. The present study aimed to elucidate the mechanisms underlying dormancy overcoming and germination in this species through a comparative approach between *in vitro* and *ex vitro* cultivation systems. Seed physiological behavior, the effectiveness of pre-germinative treatments, and endogenous levels of GA₃ and ABA were investigated. Furthermore, polyembryony and embryonic ontogeny were characterized. We hypothesized that germination in *P. viridis* is governed by physiological dormancy regulated by an unfavorable hormonal balance between ABA and GA₃. Furthermore, we expect that responses will differ between cultivation systems due to environmental and microenvironmental constraints imposed by germination conditions.

2. Materials and Methods

2.1 Seed Collection and Storage

Mature fruits of *P. viridis* were collected from randomly selected adult individuals growing in a fragment of Seasonal Semideciduous Forest in the municipality of Mirassolândia, São Paulo, Brazil (Fig. 1). Fruits at the physiological maturity stage, characterized by red pericarp coloration, were selected. The seeds were transported to the laboratory in kraft paper bags and processed within a maximum period of 24 h after collection. Pulp removal was performed manually under running water to eliminate the mesocarp. After drying the surface at room temperature for 24 h, the seeds were visually screened, and only intact seeds without mechanical damage, microbial contamination, and with uniform size and coloration were selected. Seeds intended for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses were immediately stored in an ultra-low temperature (ULT) freezer at -80°C , whereas seeds used in biological assays were maintained in hermetically sealed containers at room temperature.

2.2 Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)

2.2.1 Analytical Standards and Reagents

Analytical standards of GA_3 and ABA (purity $\geq 98\%$; Sigma–Aldrich, Barueri, SP, Brazil) were used for the preparation of standard solutions and recovery assays. Methanol, formic acid, and acetonitrile ($\geq 98\%$, LC–MS grade) were employed for extraction procedures and as mobile phases. Nitrogen used as nebulizing, drying, and collision gas was generated by a dedicated system (Genius NM32LA, Peak Scientific Instruments).

2.2.2 Preparation of Standard Solutions and Calibration Curves

Stock solutions of GA_3 and ABA were prepared in methanol and stored at -20°C under light protection. Working solutions were obtained by serial dilution in methanol to construct analytical calibration curves within the concentration range of $5.7\text{--}50\text{ ng mL}^{-1}$. Linearity was evaluated by linear regression (least squares method), correlating chromatographic peak area with nominal analyte concentrations, using the coefficient of determination (R^2) as the fitting criterion. Limits of detection (LOD) and quantification (LOQ) were calculated based on a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively, and validated using statistical parameters of the calibration curve.

2.2.3 Sample Extraction and Preparation

Approximately 50 mg of seeds were accurately weighed using an analytical balance and subjected to ultrasound-assisted extraction. The plant material was homogenized in 1 mL of an extraction solution consisting of methanol and formic acid (96:4, v/v). Extraction was performed in an ultrasonic bath (42 kHz, 170 W) for 15 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was collected, and the solid residue was re-extracted with 1 mL of the same solution for 10 min under identical conditions. Combined supernatants evaporated to dryness at room temperature. The dry residue was reconstituted in 100 μL of LC–MS grade acetonitrile and centrifuged at 10,000 rpm for

10 min. Finally, the extract was filtered through a 0.22 μm polytetrafluoroethylene (PTFE) membrane filter and transferred to 1.5 mL amber vials for subsequent LC–MS/MS analysis.

2.2.4 Chromatographic Conditions

Analyses were performed using an ultra-high-performance liquid chromatography (UHPLC) system coupled to a triple quadrupole mass spectrometer (Shimadzu LCMS-8045), equipped with a Kinetex XB-C18 column (2.6 μm , 100 $\times$ 2.1 mm; Phenomenex). The column temperature was maintained at 40°C, and the injection volume was 5 μL . Separation was carried out at a flow rate of 0.3 mL min⁻¹ using a mobile phase consisting of (A) water with 0.1% (v/v) formic acid and (B) acetonitrile with 0.1% (v/v) formic acid. The gradient elution program was as follows: 10% B (0 min); linear increase to 55% B (5 min); maintained at 55% B (until 6.5 min); immediate return to 10% B (6.51 min); and column re-equilibration for 1 min (total run time: 7.5 min).

2.2.5 Mass Spectrometry Conditions

Detection was performed using a triple quadrupole mass spectrometer (QqQ) with electrospray ionization (ESI) operating in negative mode under multiple reaction monitoring (MRM). For GA₃ (t_R = 2.92 min), the transition m/z 345.2 $\rightarrow$ 239.1 was used as the quantifier (collision energy, CE: 17 V), with m/z 205.1 (CE: 17 V) and 143.1 (CE: 30 V) as qualifier ions. For ABA (t_R = 4.01 min), the transition m/z 263.2 $\rightarrow$ 153.1 was used as the quantifier (CE: 14 V), with m/z 219.1 (CE: 11 V) and 203.1 (CE: 16 V) as qualifiers. MRM transitions were selected based on fragmentation data reported by (Floková et al. 2014) and optimized for the instrumental conditions employed. The dwell time was set to 100 ms for all transitions. Source conditions were as follows: nebulizing, heating, and drying gas at 3, 10, and 10 L min⁻¹, respectively; interface temperature at 300°C; desolvation temperature at 526°C; DL capillary at 250°C; and heat block at 400°C. High-purity nitrogen was used as the collision gas.

2.2.6 Analytical Performance of the Method

The analytical response was evaluated over a concentration range of 5.7–50 ng mL⁻¹, yielding coefficients of determination (R²) higher than 0.998 for both phytohormones. Precision, expressed as coefficient of variation (CV), remained below 2% across different concentration levels, indicating high analytical repeatability. Recovery assays in spiked samples ranged from 103% to 110%. Limits of detection (LOD) were 1.06 ng mL⁻¹ for GA₃ and 0.78 ng mL⁻¹ for ABA, while limits of quantification (LOQ) were 3.21 ng mL⁻¹ and 2.36 ng mL⁻¹, respectively. These parameters indicate adequate sensitivity for the quantification of ABA and GA₃ in *P. viridis* seeds.

2.3 Moisture content and seed viability after desiccation

Seed water content (WC) was determined by oven-drying at 120°C for 3 h (Hong and Ellis 1996), using five replicates of 50 freshly collected seeds per treatment. Water content was calculated according to the following equation:

$$WC (\%) = \frac{(fresh\ mass - dry\ mass)}{fresh\ mass} \times 100$$

Desiccation tolerance was assessed by gradually reducing seed water content in hermetically sealed containers containing silica gel to reach levels of 20%, 15%, and $\leq 10\%$ (Hong and Ellis, 1996). Seed viability at each hydration level was evaluated using the tetrazolium test (2,3,5-triphenyltetrazolium chloride) at 1.0%. Seeds were pre-soaked in distilled water for 24 h, longitudinally sectioned, and incubated at 30°C in the dark for 4 h. Embryos showing uniform red staining were classified as viable.

Based on storage behavior, seeds were classified as orthodox (maintenance of viability at $WC \leq 10\%$), recalcitrant (severe loss of viability at WC between 15–20%), or intermediate (partial desiccation sensitivity) (Chin et al., 1989).

2.4 Seed Disinfestation for Pre-germinative Treatments

For *in vitro* experiments, seeds were initially washed in a neutral detergent solution under constant agitation for 5 min. Disinfestation was then performed under a laminar flow hood by immersion in 70% (v/v) ethanol for 3 min, followed by treatment with 2.5% (v/v) sodium hypochlorite (NaClO) for 20 min. Subsequently, seeds were rinsed six times with sterile distilled water.

For *ex vitro* assays, seeds were surface sterilized with 70% (v/v) ethanol for 1 min and 1% (v/v) NaClO for 10 min, followed by six successive rinses with autoclaved distilled water.

2.5 Pre-germinative Treatments

Pre-germinative treatments were applied to seeds cultivated under both *in vitro* and *ex vitro* conditions, except for seed coat removal and embryo excision, which were performed exclusively under *in vitro* conditions. All experiments were conducted using five replicates of 50 seeds per treatment.

2.5.1 Water imbibition

Seeds were subjected to water imbibition in distilled water under two thermal regimes: (i) room temperature ($25 \pm 2^\circ\text{C}$) and (ii) initial immersion in water at 50°C followed by cooling. Both treatments were applied for 24 and 48 h, and dry seeds were used as a control.

2.5.2 Stratification

Seeds were kept at $5 \pm 1^\circ\text{C}$ for 15, 30, and 60 days in Petri dishes containing double-layered moistened filter paper (Fig. 4A). Seeds maintained at room temperature served as the control treatment.

2.5.3 Chemical scarification

Seeds were immersed in concentrated sulfuric acid (H_2SO_4 , analytical grade) for 5, 10, 20, and 40 min, in addition to an untreated control. After acid exposure, seeds were rinsed six times with distilled water.

2.5.4 Gibberellic acid (GA_3) modulation

Under *in vitro* conditions, the culture medium (MS/2) was supplemented with GA₃ at concentrations of 2.5, 5.0, 10.0, and 20.0 µM. Under *ex vitro* conditions, seeds were imbibed for 24 h in GA₃ solutions (250, 500, and 1000 mg L⁻¹). For both systems, a control treatment without GA₃ supplementation was included.

2.5.5 Seed coat removal and embryo excision

Manual removal of the seed coat and excision of embryos and endosperm were performed under a stereomicroscope (Lumen XT-3L-B). Zygotic embryos were inoculated onto culture medium (MS/2) supplemented with GA₃ (2.5, 5.0, 10.0, and 20.0 µM), as well as a control treatment without GA₃ addition.

2.6 Culture Medium and Growth Conditions

For *in vitro* experiments, the culture medium consisted of (Murashige and Skoog 1962) basal salt solution at half-strength (MS/2), supplemented with 30 g L⁻¹ sucrose and 5.7 g L⁻¹ agar (Kasvi K25-1800). The pH was measured and adjusted to 5.8 ± 0.1 using 1 N NaOH or HCl. Media were sterilized by autoclaving at 120°C and 1.06 kg cm⁻² for 20 min. When added to the culture medium, GA₃ was sterilized by membrane filtration using a 0.22 µm filter (Millex™). *In vitro* cultures were maintained in test tubes (200 × 100 mm) under controlled conditions at 23 ± 2°C, a 16 h photoperiod, and a photosynthetic photon flux density (PPFD) of 72 µmol m⁻² s⁻¹ provided by white light-emitting diodes (LEDs; 9.5 W, 6000 K, Electrolux).

Ex vitro experiments were conducted in Gerbox® containers (11 cm × 11 cm × 3.5 cm) containing 20 g of fine-grade vermiculite (Glow Cultivo). The containers were incubated in a biochemical oxygen demand (B.O.D.) chamber (LT 320 TFP-I, LIMA TEC) at 25°C under a 12 h photoperiod.

2.7 Polyembryony Characterization and Embryo Ontogeny

After disinfestation, embryos were excised under a stereomicroscope (Lumen XT-3L-B). Polyembryony was quantified using five replicates of 50 seeds and expressed as both percentage and mean number of embryos per seed. Embryos were classified into globular, heart-shaped (cordiform), torpedo, or cotyledonary stages. Three replicates of 50 embryos per developmental stage were cultured on MS/2 medium supplemented with GA₃ (5, 10, 20, and 40 µM). Embryos cultured on hormone-free medium served as controls. Disinfestation procedures, culture medium composition, and growth conditions followed the protocols described above.

2.8 Experimental Design and Statistical Analysis

After 90 days of *in vitro* and *ex vitro* cultivation, germination percentage (G%) and seedling formation percentage (P%) were evaluated. Germination was monitored at three-day intervals to calculate the Germination Speed Index (GSI), according to the equation below, where G_i represents the number of seeds germinated at each count and N_i corresponds to the number of days elapsed since sowing (Maguire 1962):

$$GSI = \sum Gi/Ni$$

The experiment was conducted in a completely randomized design. Germination (G%) and seedling formation (P%) data were analyzed using Generalized Linear Models (GLM) with binomial distribution and logit link function. Continuous variables were assessed for normality and homoscedasticity using Shapiro–Wilk and Levene’s tests, respectively, and analyzed using linear models under Gaussian distribution when assumptions were met. When necessary, data transformations or alternative models were applied. Means were compared using the Bonferroni post-hoc test at a 5% significance level ($p < 0.05$). All analyses and graphs were performed in R software (version 4.4.0).

3. Results

3.1 LC-MS/MS

LC–MS/MS analysis revealed the presence of ABA in *P. viridis* seeds at an average concentration of $38.1 \pm 0.64 \text{ ng g}^{-1}$ fresh weight. The low variability among replicates ($CV = 1.7\%$) highlights the precision and reproducibility of the analytical method for this matrix. In contrast, GA_3 levels remained below the system’s limit of detection ($LOD < 1.94 \text{ ng g}^{-1}$ fresh weight).

3.2 Initial Moisture Content and Post-desiccation Seed Viability

Freshly harvested seeds of *P. viridis* (Fig. 2A) exhibited a mean fresh mass of $0.1254 \pm 0.0211 \text{ g}$ and a dry mass of $0.0689 \pm 0.0187 \text{ g}$, with an initial water content of 45.25% and a viability of 96%. Reduction in seed moisture content resulted in a significant decrease in viability ($p < 0.05$) (Fig. 3), reaching 59.3% at 20% water content and 19.3% at 15%. Desiccation to approximately 10% moisture resulted in a viability of 0.48%. The red staining of embryos, indicating viability during the tetrazolium test, is shown in Fig. 2B.

3.3 Pre-germinative Treatments and *In Vitro* Germination

Water imbibition under different thermal regimes and exposure times, as well as stratification treatments, resulted in low germination percentages ($< 17.0\%$), with seedling formation ranging from 68% to 82% and GSI values between 4.8 and 5.1, with no significant difference compared to the control ($p > 0.05$). Chemical scarification with H_2SO_4 for 5 or 10 min also showed low efficiency, with 4% germination, 69% seedling formation, and a GSI of 4.9, whereas longer exposure periods (20–40 min) completely inhibited germination (Table 1).

In contrast, intact seeds cultured in the presence of GA_3 showed significant increases ($p < 0.05$) in all evaluated parameters, with the 20 μM concentration yielding 60% germination, 98% seedling formation, and a GSI of 6.76.

Seed coat removal combined with culture on MS/2 medium supplemented with 20 and 40 μM GA₃ increased germination to approximately 80%, with high seedling formation percentages ($\approx 98\%$). However, the best results were obtained through embryo excision and culture of zygotic embryos on MS/2 medium containing 20 and 40 μM GA₃, reaching 98.85% and 98.21% germination, respectively. Under these conditions, the highest GSI values (8.52 and 8.44) were also recorded, with 100% seedling formation (Table 1). These treatments resulted in significantly higher germination and germination speed compared to all others ($p < 0.05$).

Table 1

In vitro germination of *Psychotria viridis*: germination percentage (G%), seedling formation percentage (P%), and germination speed index (GSI) under different pre-germinative treatments after 90 days of cultivation.

Experiments	Treatments	G%	P%	GSI
		(mean $\pm$ SD)	(mean $\pm$ SD)	(mean $\pm$ SD)
	Controle	15.36 $\pm$ 1.23 f	80.22 $\pm$ 4.59 d	4.92 $\pm$ 0.54 g
Water imbibition	25°C–24h	24.94 $\pm$ 1.59 e	79.81 $\pm$ 1.89 d	4.98 $\pm$ 0.67 g
	25°C–48h	25.21 $\pm$ 2.13 e	80.52 $\pm$ 4.15 d	5.04 $\pm$ 0.61 g
	50°C–24h	25.38 $\pm$ 1.58 e	82.08 $\pm$ 2.69 d	4.84 $\pm$ 0.48 g
	50°C–48h	26.82 $\pm$ 2.32 e	79.18 $\pm$ 4.87 d	4.88 $\pm$ 0.26 g
Stratification (5°C)	5°C–15d	9.81 $\pm$ 0.94 g	69.3 $\pm$ 3.12 ^e	4.98 $\pm$ 0.76 g
	5°C–30d	10.34 $\pm$ 1.78 g	69.44 $\pm$ 4.22 e	5.08 $\pm$ 0.49 g
	5°C–60d	10.18 $\pm$ 1.49 g	68.98 $\pm$ 2.98 e	5.02 $\pm$ 0.67 g
Chemical scarification (H ₂ SO ₄)	CS5min	4.87 $\pm$ 3.64 h	69.82 $\pm$ 3.15 e	4.96 $\pm$ 0.24 f
	CS10min	4.45 $\pm$ 0.47 h	69.42 $\pm$ 4.56 e	4.98 $\pm$ 0.31 h
	CS20min	0.00 $\pm$ 0.00 ⁱ	0.00 $\pm$ 0.00 ^f	0.00 $\pm$ 0.00 i
	CS40min	0.00 $\pm$ 0.00 ⁱ	0.00 $\pm$ 0.00 ^f	0.00 $\pm$ 0.00 i
Intact seeds cultured with GA ₃	GA ₃ –5uM	19.23 $\pm$ 3.73 f	90.48 $\pm$ 3.26 c	4.92 $\pm$ 0.11 g
	GA ₃ –10uM	49.76 $\pm$ 6.94 d	96.58 $\pm$ 3.48 b	6.18 $\pm$ 0.49 e

Experiments	Treatments	G%	P%	GSI
		(mean $\pm$ SD)	(mean $\pm$ SD)	(mean $\pm$ SD)
	GA ₃ -20uM	60.27 $\pm$ 7.89 c	98.52 $\pm$ 1.21 b	6.76 $\pm$ 0.67 d
	GA ₃ -40uM	58.88 $\pm$ 6.28 c	98.52 $\pm$ 1.10 b	6.86 $\pm$ 0.56 d
Seeds without seed coat cultured with GA ₃	SC+GA ₃ -0uM	24.46 $\pm$ 3.12 e	89.57 $\pm$ 3.97 c	5.01 $\pm$ 0.55 f
	SC+GA ₃ -5uM	26.59 $\pm$ 2.65 e	87.20 $\pm$ 4.25 c	5.42 $\pm$ 0.81 f
	SC+GA ₃ -10uM	61.85 $\pm$ 4.81 c	89.2 $\pm$ 3.65 ^c	6.02 $\pm$ 0.29 d
	SC+GA ₃ -20uM	80.43 $\pm$ 6.12 b	98.2 $\pm$ 0.94 ^b	6.54 $\pm$ 0.67 c
	SC+GA ₃ -40uM	80.62 $\pm$ 5.36 b	98.2 $\pm$ 0.87 ^b	6.68 $\pm$ 0.73 c
Zygotic embryos cultured with GA ₃	ZE+GA ₃ -0uM	59.87 $\pm$ 5.21 c	100 $\pm$ 0.00 ^a	7.19 $\pm$ 0.51 b
	ZE+GA ₃ -5uM	63.65 $\pm$ 4.71 c	100 $\pm$ 0.00 ^a	7.08 $\pm$ 0.44 b
	ZE+GA ₃ -10uM	83.46 $\pm$ 4.86 b	100 $\pm$ 0.00 ^a	7.48 $\pm$ 0.47 b
	ZE+GA ₃ -20uM	98.85 $\pm$ 0.94 a	100 $\pm$ 0.00 ^a	8.52 $\pm$ 0.52 a
	ZE+GA ₃ -40uM	98.21 $\pm$ 1.10 a	100 $\pm$ 0.00 ^a	8.44 $\pm$ 0.18 a

Note: Values are presented as mean $\pm$ standard deviation (n = 5 replicates of 50 seeds per treatment). Different lowercase letters indicate significant differences among treatments according to Bonferroni's test ($p \leq 0.05$). CS = chemical scarification; GA₃ = gibberellic acid; SC = seed coat removal; ZE = zygotic embryo excision.

3.4 Pre-germination treatments and *ex vitro* germination

Pre-soaking in water under different thermal regimes and exposure times, as well as stratification, resulted in germination below 28%, with seedling formation ranging from 51.1% to 82.1% and a GSI ranging from 4.22 to 5.54. Overall, these treatments showed significantly superior performance

compared to the control ($p < 0.05$; Table 2). In contrast, chemical scarification reduced germination (2.47–4.96%), which was associated with the lowest seedling formation rates (33.4–44.6%) and the lowest GSI values (4.35–5.48) ($p < 0.05$; Table 2).

Imbibition in a GA_3 solution at 250 mg L^{-1} promoted the best germinative performance, with 62.55% germination and 81.7% seedling formation, significantly outperforming the other treatments ($p < 0.05$; Table 2). Higher concentrations (500 and 1000 mg L^{-1}) resulted in lower germination percentages (53.04% and 50.32%, respectively), with no significant differences in GSI (7.24–7.86). The initial seedling establishment in the 250 mg L^{-1} GA_3 treatment is evidenced in Figs. 4C and 4D.

Table 2

Ex vitro germination of *Psychotria viridis*: germination percentage (G%), seedling formation percentage (P%), and germination speed index (GSI) under different pre-germination treatments, after 90 days of cultivation.

Experiments	Treatments	G%	P%	GSI
		(mean $\pm$ SD)	(mean $\pm$ SD)	(mean $\pm$ SD)
	Controle	17.88 $\pm$ 3.26 _d	74.42 $\pm$ 5.59 _{ab}	4.22 $\pm$ 1.21 ^c
Water imbibition	25°C–24h	25.96 $\pm$ 4.21 _c	78.49 $\pm$ 8.65 ^a	4.69 $\pm$ 0.84 ^{bc}
	25°C–48h	30.46 $\pm$ 3.95 _c	73.27 $\pm$ 6.48 _{ab}	4.43 $\pm$ 0.67 ^{bc}
	50°C–24h	28.81 $\pm$ 4.36 _c	82.14 $\pm$ 5.77 ^a	4.74 $\pm$ 0.51 ^{bc}
	50°C–48h	29.40 $\pm$ 4.98 _c	75.43 $\pm$ 6.21 _{ab}	5.18 $\pm$ 1.02 ^b
Stratification (5°C)	5°C–15d	19.42 $\pm$ 3.84 _d	51.15 $\pm$ 4.89 ^c	5.26 $\pm$ 0.48 ^b
	5°C–30d	20.50 $\pm$ 3.21 _d	59.87 $\pm$ 8.65 _{bc}	4.81 $\pm$ 0.65 ^{bc}
	5°C–60d	17.45 $\pm$ 4.66 _d	54.21 $\pm$ 7.48 ^c	5.54 $\pm$ 0.49 ^b
Chemical scarification (H ₂ SO ₄)	CS5min	2.47 $\pm$ 1.95 ^e	44.63 $\pm$ 10.4 ^d	5.48 $\pm$ 0.67 ^b
	CS10min	3.60 $\pm$ 1.63 ^e	33.40 $\pm$ 9.98 ^d	4.72 $\pm$ 1.35 ^{bc}
	CS15min	3.83 $\pm$ 1.02 ^e	41.85 $\pm$ 7.48 ^d	4.35 $\pm$ 1.48 ^{bc}
	CS30min	4.96 $\pm$ 0.96 ^e	38.80 $\pm$ 8.33 ^d	4.65 $\pm$ 0.97 ^{bc}
Imbibition in GA ₃ solution	GA ₃ -250mg L ⁻¹	62.55 $\pm$ 7.59 _a	81.76 $\pm$ 5.98 ^a	7.86 $\pm$ 1.68 ^a
	GA ₃ -500mg L ⁻¹	53.04 $\pm$ 9.21 _b	76.49 $\pm$ 6.47 _{ab}	7.45 $\pm$ 1.47 ^a
	GA ₃ -1000mg L ⁻¹	50.32 $\pm$ 8.44 _b	73.48 $\pm$ 7.63 _{ab}	7.24 $\pm$ 1.23 ^a

Notes: Means $\pm$ standard deviation (n = 5 replicates of 50 seeds per treatment). Distinct lowercase letters indicate significant differences between treatments according to Bonferroni's test (p < 0.05). CS = chemical scarification; GA₃ = gibberellic acid.

3.5 Principal Component Analysis (PCA)

The multivariate analysis highlighted the effect of different pre-germination treatments on *P. viridis* seeds cultivated *in vitro* and *ex vitro* (Fig. 5). The first two principal components explained 96.8% of the total data variance. The first principal component (PC1) held the greatest explanatory power (85.9%) and was positively correlated with germination variables and GSI. The second component (PC2) explained 10.9% of the variance, being primarily associated with seedling formation (Fig. 5).

Seeds subjected to pre-germination treatments followed by *in vitro* cultivation clustered on the positive side of PC1, with emphasis on those involving seed coat removal and embryo excision (Fig. 5). In this group, zygotic embryos cultivated in MS/2 medium supplemented with GA₃ (10 and 20 µM) were consolidated as the most effective for overcoming dormancy, showing the highest association with the germination and GSI vectors (Fig. 5).

In contrast, seeds cultivated *ex vitro* exhibited greater dispersion and a shift toward the negative quadrants, indicating a reduction in germinative potential compared to *in vitro* cultivation (Fig. 5). This effect was deleterious in the chemical scarification and stratification treatments, which occupied the opposite end of the germination and seedling development vectors (Fig. 5).

3.6 Polyembryony and zygotic embryo germination

The incidence of polyembryony in *P. viridis* was 78.34% (Fig. 2F), with an average of 3.71 embryos per seed. A predominance of the cotyledonary stage (56.1%) was observed, followed by the torpedo (21.7%), heart (13.5%), and globular (8.7%) stages. Regardless of the initial stage, germinative performance was statistically superior in MS/2 medium supplemented with 20 and 40 µM of GA₃ (Fig. 6).

Embryos at the cotyledonary and torpedo stages showed germination above 50% in the control (without GA₃) and close to 100% from 20 µM GA₃ onwards (Fig. 6). The heart stage exhibited the greatest response to the regulator, with an increase from 35% in the control to 94% at 40 µM. In contrast, embryos at the globular stage presented the lowest germination rates, albeit with a significant increment, rising from 8% in the control to approximately 59% under 40 µM GA₃ (Fig. 6).

4. Discussion

Psychotria viridis seeds exhibited high viability when freshly harvested (96%), but proved to be sensitive to desiccation, with viability decreasing as water content declined, reaching 19.3% when moisture was reduced to 15%. This pattern is characteristic of recalcitrant seeds (Chin et al. 1989), common in tropical species of the Rubiaceae family, where water loss compromises membrane integrity, enzymatic activity, and cellular metabolism, resulting in loss of viability (Sommerville et al. 2021; Ley-López et al. 2023; Escobar et al. 2025). This behavior has direct implications for the storage and propagation of the species, requiring high humidity conditions and immediate use after collection (Lah et al. 2023).

Despite the high initial viability (96%), the seeds showed low germination (< 17%) in the absence of pre-germination treatments, both *in vitro* and *ex vitro*, highlighting the presence of germination blocks even in viable seeds. This pattern indicates that, beyond its recalcitrant nature, there is also the occurrence of dormancy that restricts germination after dispersal (Vaziee et al. 2023). In Rubiaceae, such behavior may represent an adaptive strategy by modulating germination timing in variable environments (Baskin and Baskin 2024), but it also imposes challenges for species management and propagation, especially when seeking greater uniformity in seedling establishment (Bewley et al. 2013).

Despite the increase relative to the control, the low germination *in vitro* (25%) and *ex vitro* (< 30%) after water soaking demonstrated that this treatment was not efficient in overcoming germination restrictions in *P. viridis*. This suggests the presence of structural and/or physiological barriers, such as seed mechanical constraint and the possible action of chemical inhibitors (Awasthi 2023). The low germination rates observed after stratification treatments (*in vitro* < 10%; *ex vitro* < 20%) reinforced the hypothesis of mechanical limitations, since this treatment acted predominantly on metabolic processes internal to the embryo (Bewley et al. 2013). Chemical scarification with H₂SO₄ resulted in low (< 4%) or inhibited germination, possibly due to embryo damage caused by reagent penetration (Awasthi, 2023).

Despite the low germination percentages, satisfactory seedling formation rates ($\approx$ 68–82%) were recorded, indicating that the seeds that germinated exhibited vigor and establishment capacity. This behavior is characteristic of seeds with mechanical constraint that affect germination but not viability or post-germinative performance (Fu et al. 2024). This interpretation was corroborated by the increased germinative responses (59%) observed in the *in vitro* cultivation of zygotic embryos in a medium free of plant growth regulators, showing that once the coat restriction is overcome, the embryo can germinate. This is because the seed coat and adjacent tissues exert mechanical restriction on radicle protrusion (Fu et al., 2024), limiting embryonic elongation, the overcoming of which depends on the removal of these barriers (Lamont and Pausas 2023).

The percentage of germination, seedling formation, and GSI increased significantly in all treatments containing GA₃, both *in vitro* and *ex vitro*, highlighting the role of hormonal regulation in overcoming dormancy in *P. viridis*. The action of GA₃ is associated with increasing the growth potential of the embryo and radicle (Gazzarrini and Tsai 2015) and deactivating mechanisms involved in maintaining dormancy, thus favoring germination (Koçak-Şahin et al. 2025). Physiologically, dormancy is related to high levels of ABA and/or reduced levels of GA, while the relative increase in GA₃ promotes the resumption of embryonic growth and radicle protrusion (Koçak-Şahin et al. 2025). These results indicated the existence of a second level of dormancy control associated with hormonal regulation in *P. viridis*.

Hormonal quantification supported this interpretation by revealing an endogenous balance favoring ABA (38.1 ± 0.64 ng g⁻¹) over GA₃ (< 1.94 ng g⁻¹) in the seeds. These results corroborated the low spontaneous germination of the seeds and reinforced the efficacy of GA₃ supplementation in embryonic development (Koçak-Şahin et al., 2025), increasing turgor pressure and enabling germination (Homczinski et al. 2023). In Rubiaceae, the endosperm has a rigid constitution due to the accumulation

of reserve polysaccharides, such as mannans (Bewley et al. 2013), and GA₃ also acts by inducing hydrolytic enzymes, such as endo-β-mannanase, promoting the degradation of these reserves and reducing the mechanical resistance of the enclosing tissues (Silva et al. 2019). Therefore, dormancy in *P. viridis* was of a complex nature, involving both structural restrictions and physiological components associated with the hormonal imbalance between high ABA levels and low endogenous gibberellin concentrations.

The high incidence of polyembryony and ontogenetic heterogeneity observed in *P. viridis* seeds indicated that, in addition to mechanical constraint and physiological dormancy, there is also a morphological component associated with dormancy. The presence of embryos in the early ontogenetic stages (globular and heart) indicated that the seed was dispersed before all its embryonic axes had completed the development necessary for germination (Bewley et al. 2013). Such a phenomenon is characteristic of other species in the Rubiaceae family, where morphological immaturity has occurred alongside physiological blocks (Baskin and Baskin 2024).

Although cotyledonary embryos showed greater autonomy, GA₃ supplementation was indispensable for uniformizing the maturation of the globular and heart stages. This response indicated that GA₃ accelerated the histological differentiation required to overcome MPD (Athugala et al. 2016). Therefore, the observed heterogeneity not only reflected a taxonomic characteristic of the family (Baskin and Baskin 2024) but also reinforced the need for *in vitro* protocols using gibberellins to rescue the germinative potential of embryos that, under natural conditions, would be restricted by their incomplete development and the imbalance between ABA and GA₃.

Thus, seed dormancy in *P. viridis* can be classified as morphophysiological dormancy (MPD), indicating that the germination process requires both the overcoming of biochemical barriers and an additional period of embryonic growth and differentiation after dispersal. The observed mechanical constraint does not characterize physical dormancy, as there is no impediment to water imbibition, an essential condition for this type of dormancy (Fu et al. 2024). Thus, although the seed coat imposes a barrier to the germination process, its action is associated with the mechanical resistance of the enclosing tissues, rather than the water impermeability typical of physical dormancy (Awasthi 2023).

5. Conclusions

Psychotria viridis presented a germinative system characterized by recalcitrance and morphophysiological dormancy (MPD). Desiccation sensitivity, combined with the seed coat barrier, hormonal imbalance, and the presence of embryos at different ontogenetic stages, confers low germination rates (< 17%) to the species. Overcoming these limitations was enabled by the *in vitro* cultivation of zygotic embryos in MS/2 medium supplemented with 20 µM GA₃. This technique eliminated the coat-imposed restriction, while supplementation with the plant growth regulator simultaneously resolved both physiological and morphological blocks. This occurred because it restored

hormonal balance (compensating for basal GA₃ levels and the ABA surplus) and accelerated the histological differentiation of globular and heart-stage embryos.

Declarations

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

RCVT, LLFR, RFS. Investigation / Methodology: RCVT, LLFR, RFS, DCS. Formal analysis: RCVT. Writing – original draft: RCVT, RFS, DCS, LLFR. Writing – review & editing: RCVT, RFS, DCS, LLFR.

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Figures

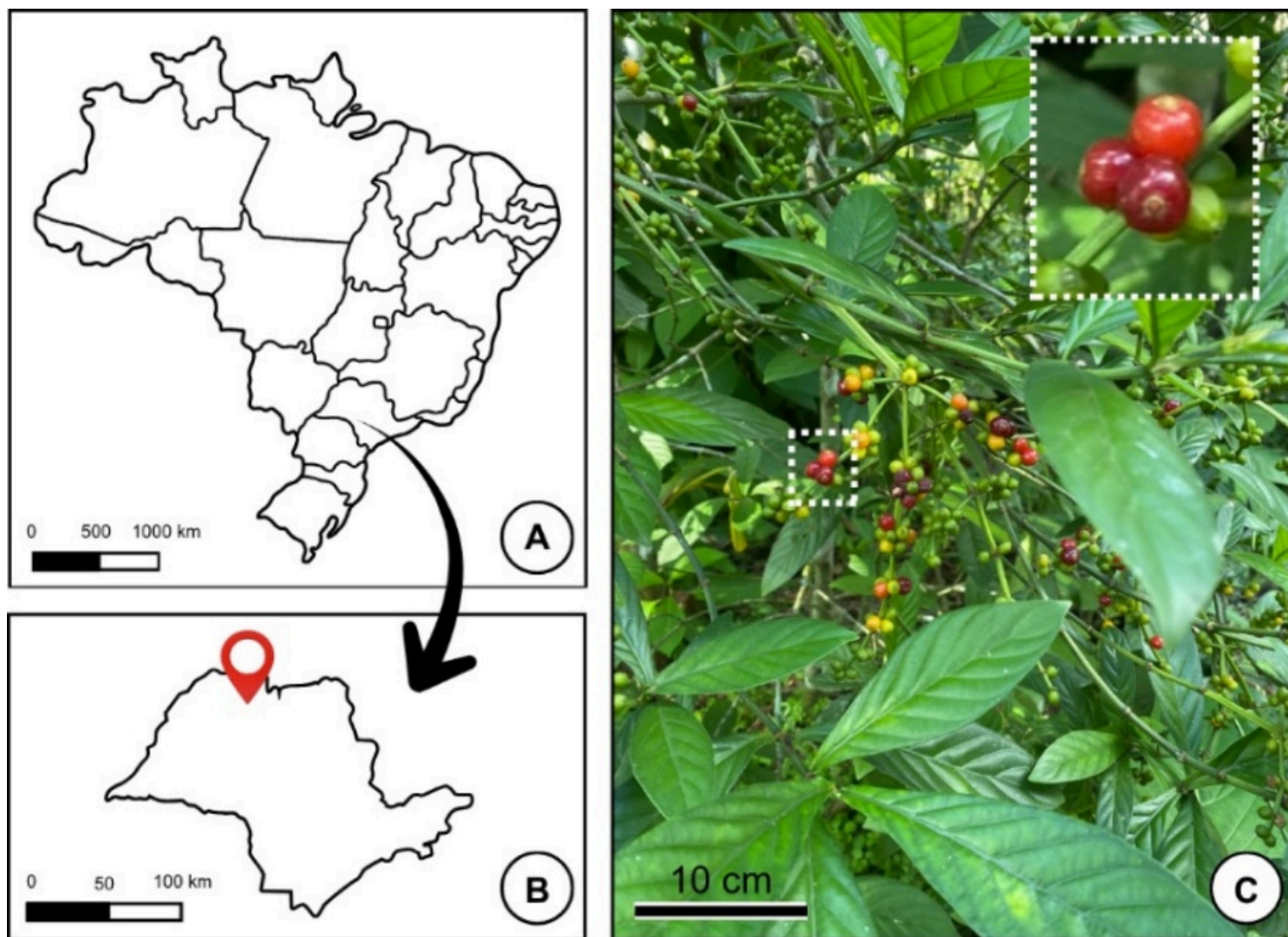


Figure 1

Geographic location of the *Psychotria viridis* mother plants. (A) Map of Brazil highlighting the state of São Paulo; (B) Location of the municipality of Mirassolândia in the state of São Paulo (red dot); (C) Branch of the mother plant showing fruits at different maturation stages, with a close-up of physiologically mature fruits.

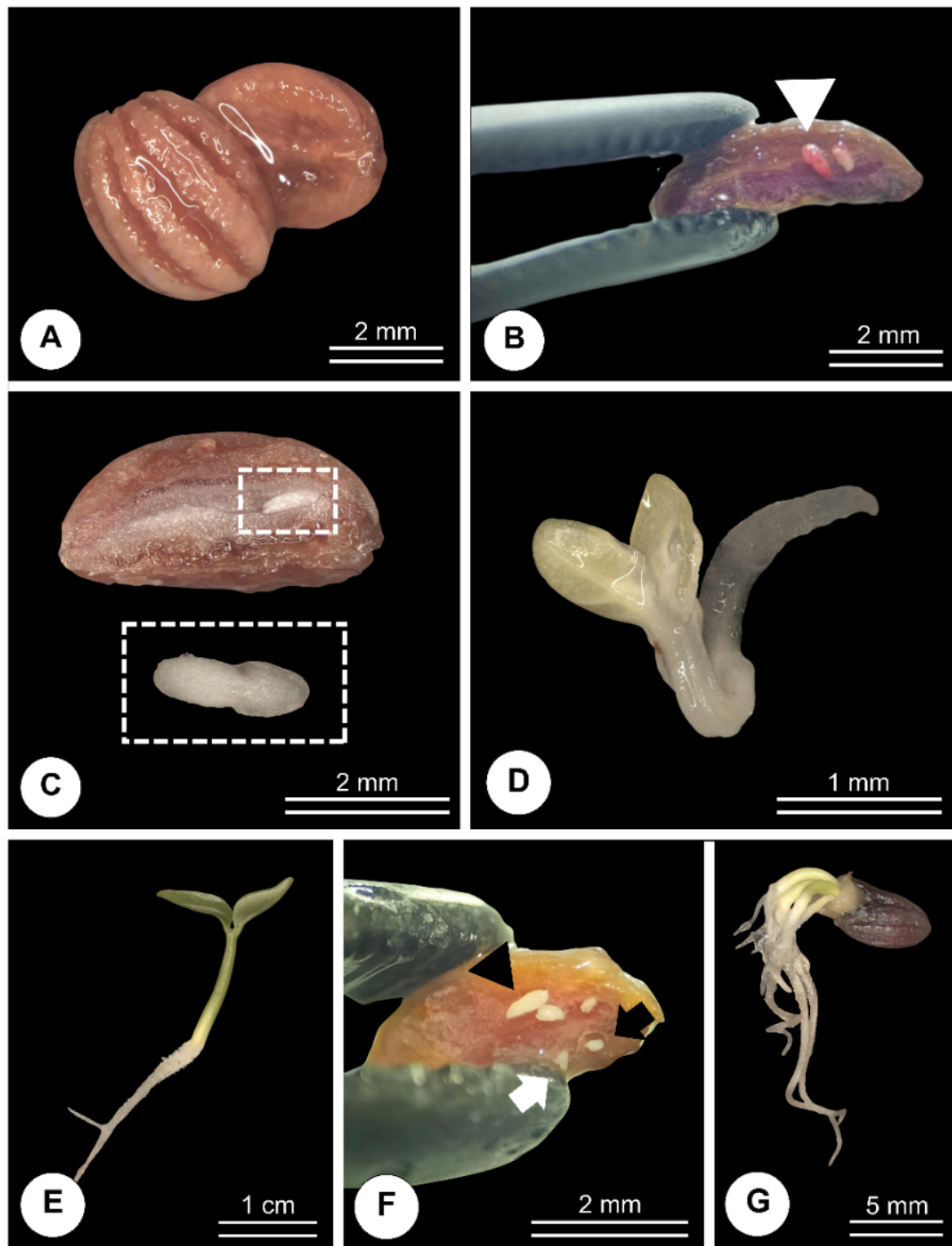


Figure 2

Germination of *Psychotria viridis*. (A) External morphology of seeds. (B) Longitudinal section after viability testing with 2,3,5-triphenyltetrazolium chloride; the white arrowhead indicates the viable embryo (stained red). (C) Longitudinal section showing an embryo at the cotyledonary stage. (D–E) Germination ontogeny of embryos cultured *in vitro* on MS/2 medium (Murashige and Skoog medium with half-strength salts) supplemented with 20 μM gibberellic acid (GA_3) after 7 and 21 days, respectively. (F)

Polyembryony in longitudinal section: arrows indicate embryos at different developmental stages (black arrow: globular; white arrow: cordiform; black arrowhead: torpedo). (G) Phenotypic expression of polyembryony during germination.

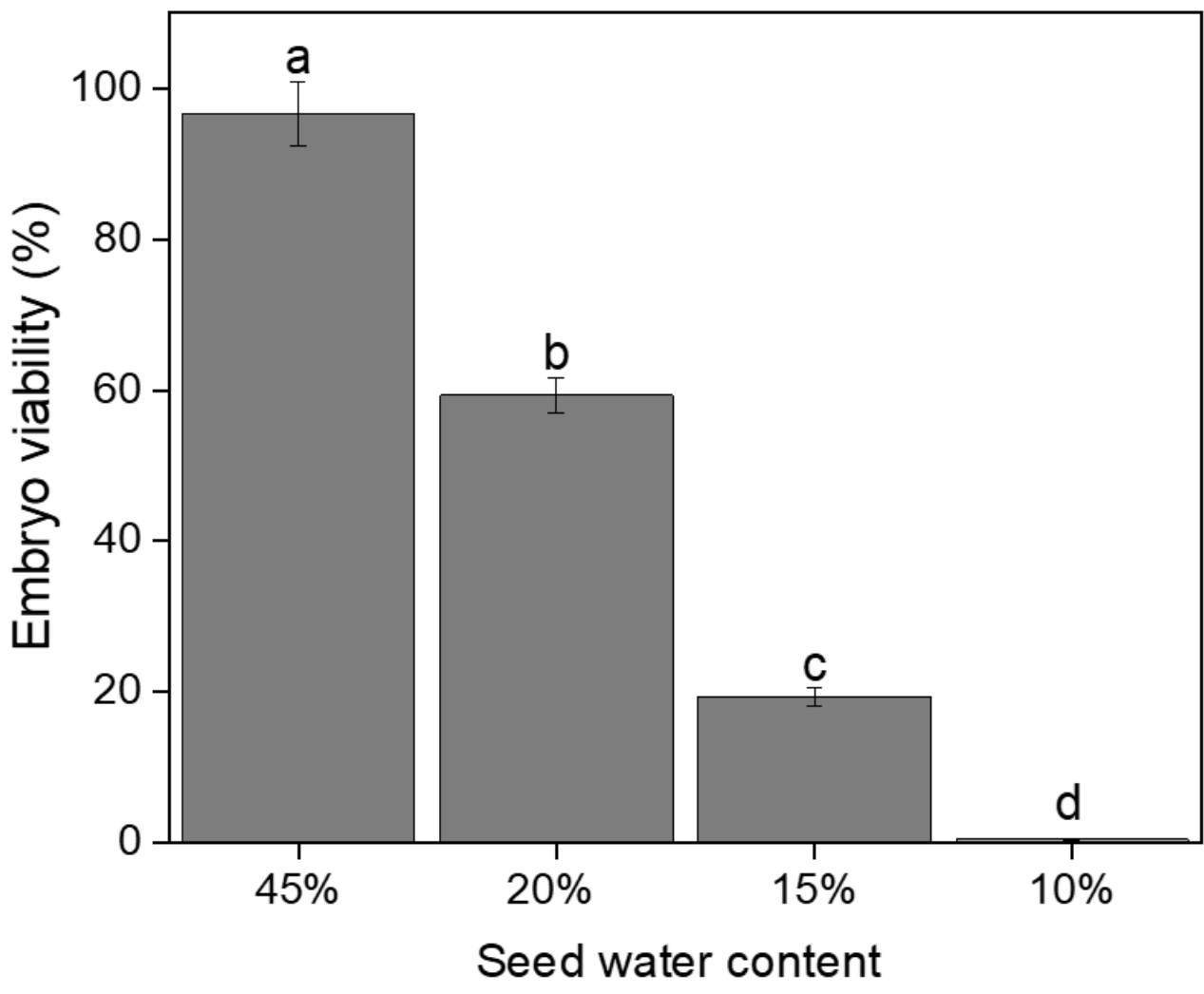


Figure 3

Initial seed moisture content (45%) and the effect of controlled desiccation (20%, 15%, and 10%) on the viability of *Psychotria viridis* seeds (n = 5 replicates of 50 seeds). Different lowercase letters above the standard deviation bars indicate significant differences among treatments according to Bonferroni’s test (p < 0.05).

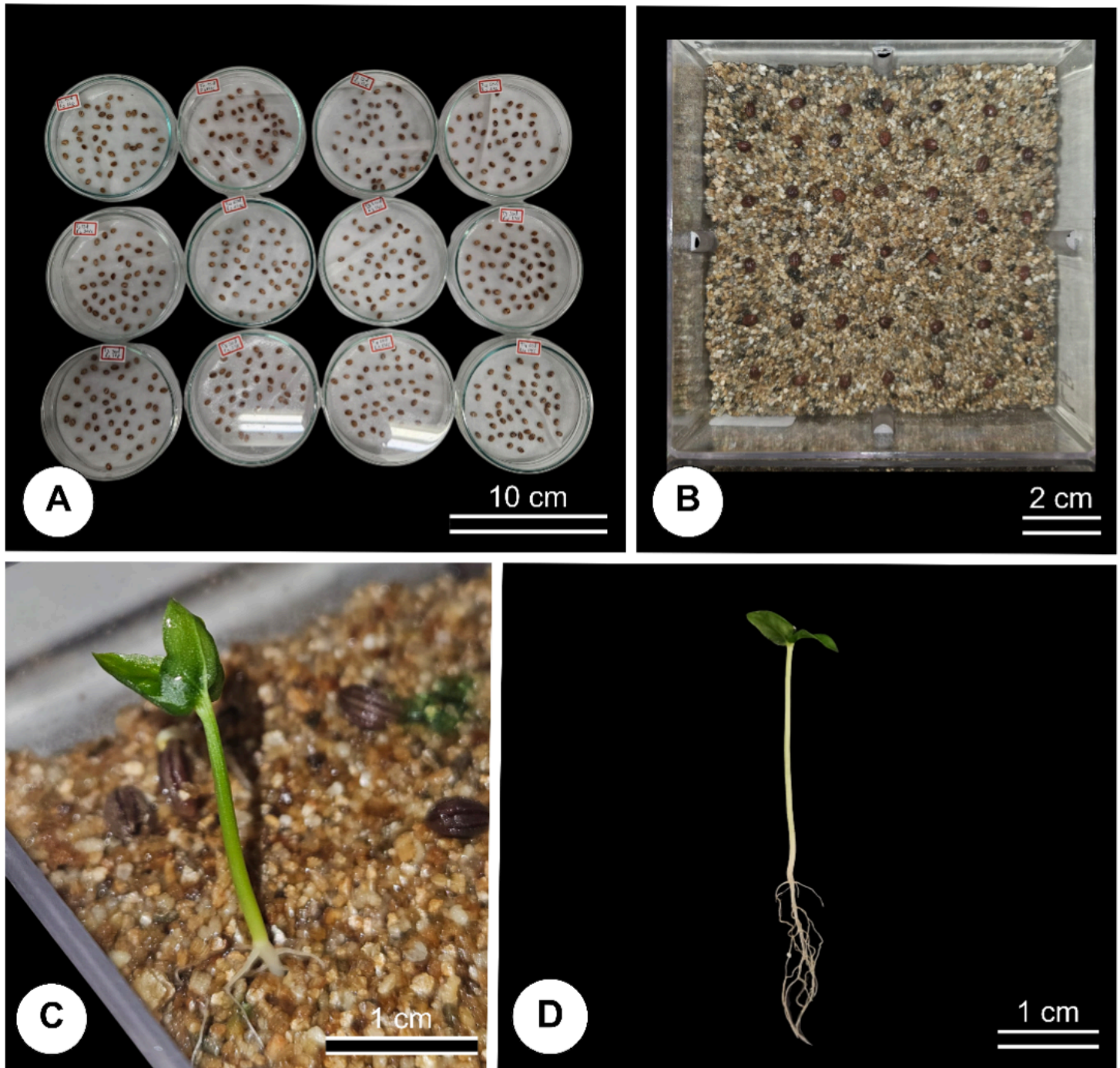
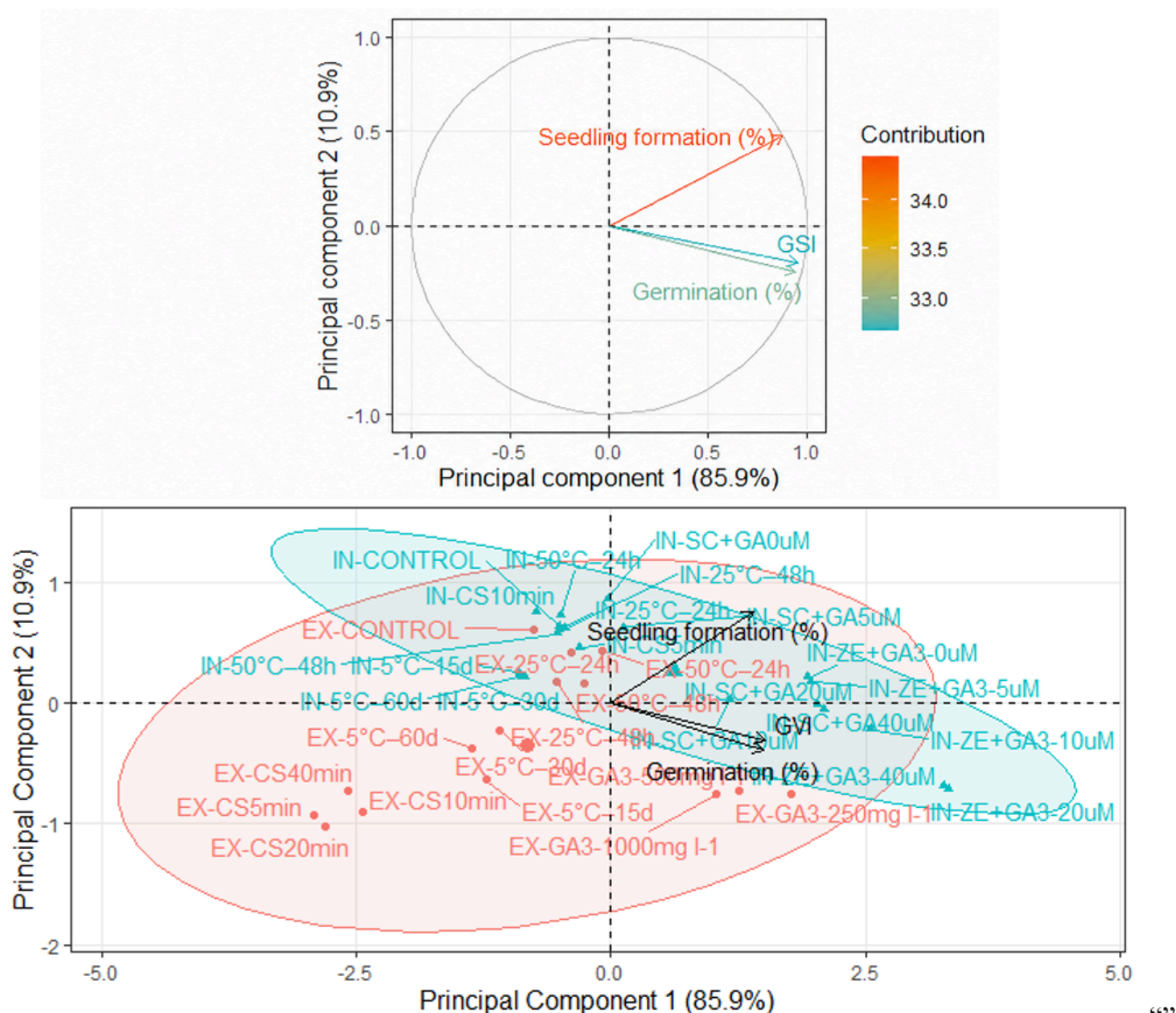


Figure 4

Ex vitro cultivation and initial seedling morphology of *Psychotria viridis*. (A) Arrangement of seeds in Petri dishes on a double layer of moistened filter paper during the cold stratification process at 5 °C. (B) Germination assay conducted in Gerbox®-type containers. (C) Seedling emergence showing initial cotyledon development. (D) Morphological characterization of the established seedling.



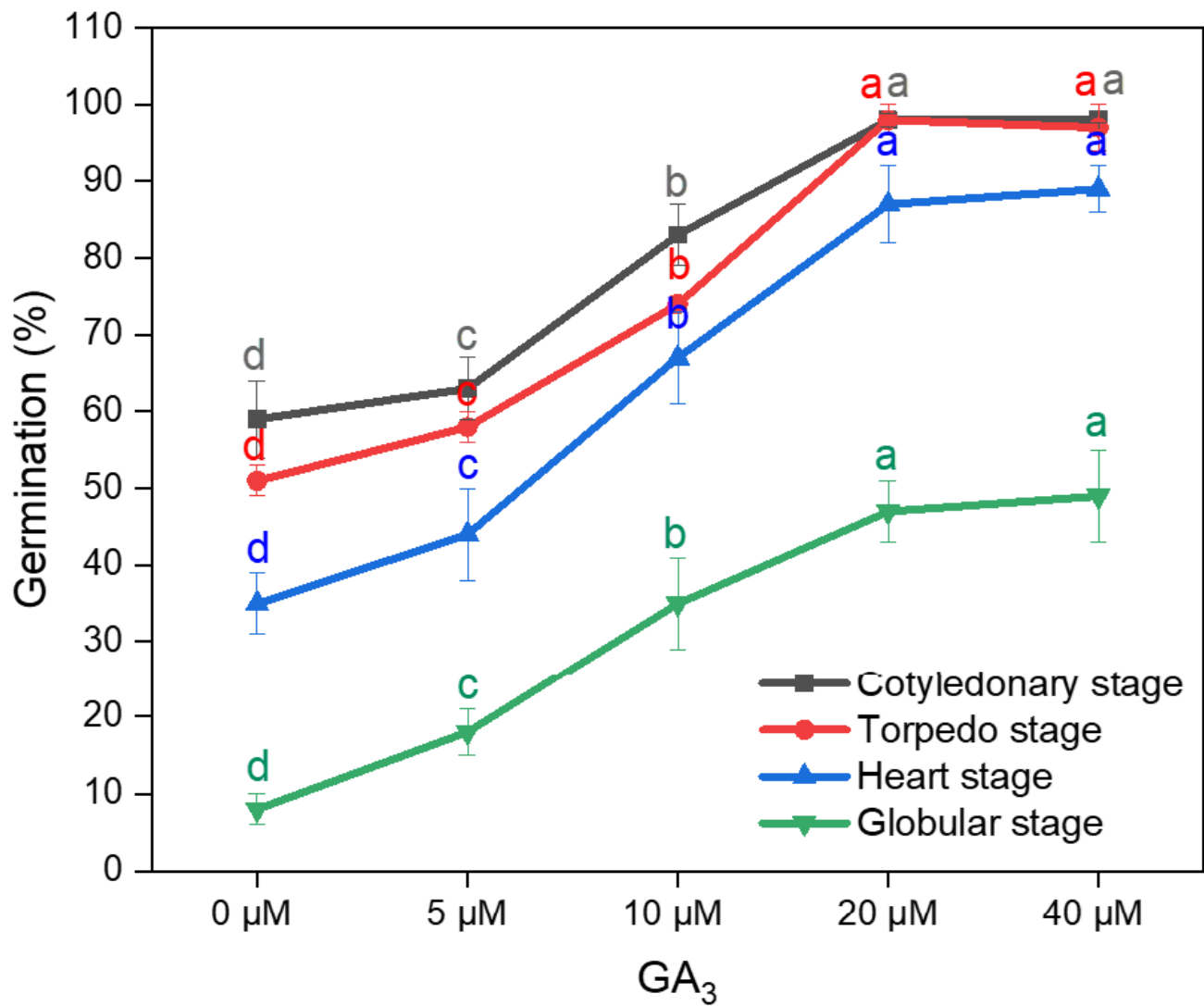


Figure 6

Germination percentage of *Psychotria viridis* zygotic embryos as a function of the ontogenetic stage (globular, heart, torpedo, and cotyledonary) and gibberellic acid (GA₃) supplementation in MS culture medium with half-strength salt concentration (MS/2). Bars represent the mean $\pm$ standard deviation (n = 5 replicates of 50 seeds). Distinct lowercase letters above the bars indicate significant differences in germination percentages according to Bonferroni's test ($p < 0.05$).