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RESEARCH ARTICLE

Embryo Rescue Techniques in Intersubgeneric Hybrid Ornamental Waterlilies (Subg. *Anecphya* x Subg. *Lotos*)

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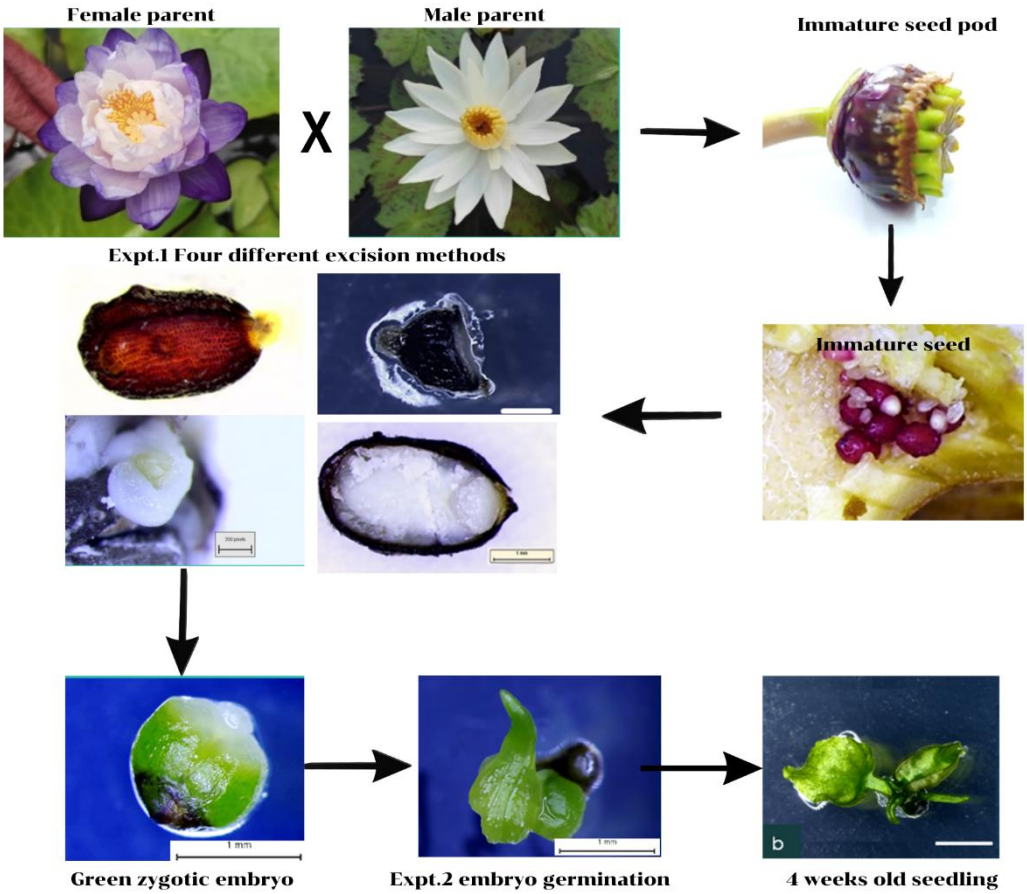
GRAPHICAL ABSTRACT



ABSTRACT

The global floriculture and ornamental plant industry relies heavily on continuously introducing novel varieties with unique aesthetic and commercial value. Consumer preferences drive the demand for new and unique floral traits. Intersubgeneric hybridization of ornamental waterlilies (*Nymphaea* spp.) plays a significant role in creating novel cultivars with desirable traits. Still, post-zygotic reproductive barriers, particularly embryo abortion, often impede the success of hybridization efforts. This study aimed to enhance embryo survival and germination rates in intersubgeneric hybrid waterlilies using *in vitro* embryo rescue techniques with plant growth regulators (PGRs). Immature seed pods from a cross between the subgenus *Anecphya* and *Lotos* were collected and subjected to various excision methods. The direct embryo excision method resulted in the highest survival rate (75%), with embryos greening in the shortest time (3.75 days). The germination rate was further enhanced when embryos were cultured on semi-solid MS (Murashige and Skoog) medium supplemented with 0.1 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), resulting in 95% germination and a reduced germination time of 5 days. These findings provide a foundation for advancing hybridization techniques in ornamental waterlilies and enhancing genetic diversity through embryo culture.
Keywords: Embryo rescue, Intersubgeneric hybridization, Waterlily, Callus induction, Tissue culture

1. INTRODUCTION

Hybridization plays a crucial role in the genetic improvement of ornamental waterlilies. Traditional breeding programs for ornamental waterlilies primarily focus on improving flower traits, including color, size, fragrance, blooming duration, and adaptability to diverse aquatic environments (Gaurav and Agnihotri, 2017). The most widely used technique for developing new cultivars is crossing, particularly for hand pollination techniques, which involve controlled pollination between different species or cultivars to introduce desirable genetic traits (Priyadarshan, 2019; Gepts and Pfeiffer 2017; Nicholas, 1996). However, crossing often requires overcoming physiological and genetic barriers, such as fertilization incompatibility, embryo abortion, and seed dormancy. Since waterlilies exhibit a degree of reproductive isolation and genetic barriers, successful hybridization often requires precise techniques to ensure effective fertilization and seed production (Sargar *et al*., 2023; Sun *et al*., 2015; Sun *et al*., 2018)

Intersubgeneric hybridization, a novel technique which involves crosses between different subgenera (e.g., between subgenus *Anecphya* and subg. *Lotos*), have led to the development of novel cultivars with unique floral and growth characteristics (Yamaguchi *et al*., 2018; Singh and Nelson, 2015). Notable hybrids such as *N*. 'Srichon' an intersubgeneric hybrid between *N. atrans* (subg. *Anecphya*) and *N. colorata* (subg. *Brachyceras*), highlights the significant role of transmitting distinct floral traits from different subgenus to its progeny, including vibrant petal and stamen coloration, long-lasting blooms, and fragrance (Rodboot *et al*., 2024). One of the primary challenges in intersubgeneric hybridization is genetic incompatibility, which can lead to low seed set, high embryo abortion rates, or complete sterility in offspring. When distantly related *Nymphaea* species are crossed, differences in chromosome number, genetic structure, or reproductive mechanisms may hinder normal fertilization and embryo development (Zhou *et al*., 2024; Sun *et al*., 2018; Sun *et al*., 2015). In many cases, hybrid embryos fail to mature due to physiological barriers that prevent proper seed formation, resulting in poor germination rates or

non-viable seeds. Even when hybrid seeds are produced, the resulting plants often exhibit partial or complete sterility, making it difficult to propagate them through conventional seed-based methods (Doran *et al*., 2004). This sterility significantly limits the ability to establish stable hybrid lines through successive generations.

Many crosses among different Subgenera and intersubgenera result in embryo abortion due to the failure of endosperm development. To address these issues, embryo rescue (ER) techniques have been successfully employed to rescue and develop immature or non-viable embryos into viable plants (Rogo *et al*., 2023; Eeckhaut *et al*., 2007). This approach is particularly useful for overcoming hybridization barriers, preserving endangered species, and accelerating breeding programs (Liu *et al*., 2007). Plant growth regulators (PGRs), such as 2,4-D, dicamba, and BAP, have been shown to significantly enhance embryo development in tissue culture systems. The auxin-like activity of 2,4-D promotes the formation of a nutritive callus-like structure around the isolated embryos, which may act as a substitute for endosperm development, thereby facilitating embryo survival and subsequent growth (Agung *et al*., 2023; Raghavan, 2004).

In this study, immature seed pods were harvested at 14 days after pollination (DAP) based on preliminary observations that zygotic embryos at this stage are sufficiently developed to be excised but have not yet undergone deterioration due to embryo desiccation, followed by surface sterilization and dissection to obtain immature zygotic embryos. Due to the intersubgeneric hybrid waterlilies, seed development is often impaired, resulting from genetic incompatibility, leading to malformed or weak seed structures that inhibit germination (Sun *et al*., 2018). Four different excision methods, whole seed, one-third vertical excision, half-horizontal excision, and direct embryo excision, were tested to determine the most effective method for promoting embryo survival and germination. The embryos were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with varying concentrations of 2,4-D for germination studies. This study aims to provide insights into the optimal conditions for embryo rescue in ornamental waterlilies, contributing to developing more effective hybridization techniques that could potentially overcome reproductive barriers and expand the genetic diversity of waterlily cultivars.

2. MATERIALS AND METHODS

2.1 Parent Plant Preparation

The female and male parent plants were grown under field conditions in 14-inch pots filled with nutrient-rich clay soil. The pots were then placed into large black plastic tubs (60 cm × 50 cm), which were filled with water to submerge the pots. A balanced fertilizer mix (15-15-15 and 8-24-24) was applied at a rate of 15 grams every 15 days to ensure adequate nutrition. The plants were left to grow for approximately 70 days, at which point flowering began. To prevent inadvertent cross-pollination, both female and male flowers were covered with mesh bags. Pollination was carried out by collecting stigmatic fluid from the female flowers using a dropper and transferring it into a tube. Pollen from the male flowers was collected using a clean brush and mixed until a golden-yellow mixture was achieved. This mixture was then applied to the female stigma. The pollinated flowers were re-covered with mesh bags, and the cross-pollination pair and date were recorded on a label attached to the flower stem.

After successful pollination, the female flowers closed within 1-2 hours. Approximately 3-4 days later, the flower stalks bent, causing the fertilized flowers to submerge underwater, where seed pod development commenced. The seed pods were monitored daily for development. Embryo abortion was observed as early as 10 days post-pollination, with brown discoloration and

eventual rotting near the stalk. The seed pods continued to develop for 17-19 days post-pollination, after which the pods were harvested. In this study, seed pods 14 days post-pollination were selected for dissection, as they contained immature embryo that were of an appropriate size and developmental stage for excision, with sufficient firmness for dissection without being too soft.

2.2 Explant preparation

Immature seed pods were collected 14 days after pollination (DAP) from a cross between two different subgenera, subgenus *Anecphya* and subgenus *Lotos* of *Nymphaea* (**Fig. 1**). The immature hybrid seed pods (**Fig. 2A**) with the consistency of herbaceous tissue were subjected to surface sterilization. Initially, the seed pods were soaked in running tap water for 2 hrs., followed by immersion in 70% ethanol with gentle shaking for 1 min. To further sterilize, the seed pods were treated with a 15% Clorox solution containing sodium hypochlorite (NaOCl) as the active ingredient, containing 2–3 drops of Tween-20 for 20 min, ensuring proper sterilization of the outer surfaces. The sterilized pods were then rinsed thoroughly three times with sterilized distilled water, each rinse lasting 5 min to remove residual sterilizing agents. To remove excess moisture, the seed pods were placed on sterile filter paper in a sterile Petri dish. Under a stereomicroscope, the seed pods were carefully dissected (**Fig. 2B**) to extract the soft seeds using forceps. The extracted seeds (**Fig. 2C**) were placed in sterilized distilled water to prevent desiccation before further processing for embryo dissection.

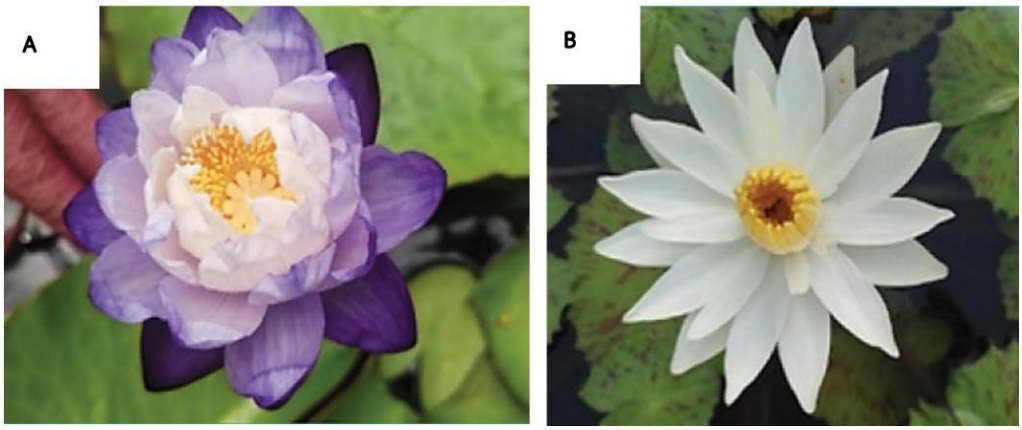


Figure 1 Preparation of female (*Nymphaea gigantea*) and male (*N. pubescent*) parent plants prior to hybridization (A) *N. gigantea* on its first day of bloom, with the floral center fully open and the presence of stigmatic fluid, indicating readiness for fertilization, (B) *N. pubescent* on its second day of bloom, used as the pollen donor

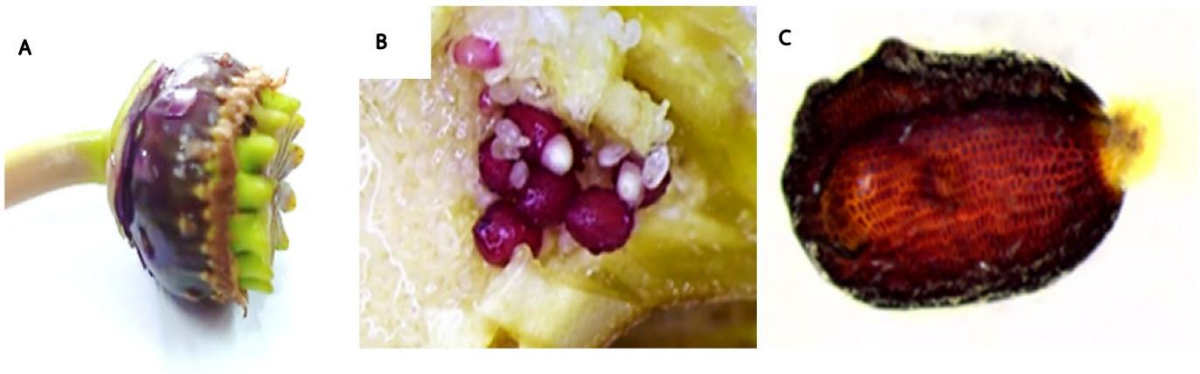


Figure 2 Morphological features of successful fertilized seed pot and immature seed development after 14 days of pollination, (A) External view of the fertilized seed pod showing characteristic enlargement and purplish coloration, indicating early seed development, (B) longitudinal section of the pod revealing immature seeds embedded in soft, mucilaginous placental tissue which the seeds exhibit a mixture of pale and reddish pigmentation, typical of developing hybrid embryos and (C) Isolated immature seed with a reddish testa and distinct oval shape, suitable for embryo excision and *in vitro* culture

2.3 Effect of different immature seed excision methods on zygotic embryo survival

Four different seed excision techniques were tested to evaluate their effect on the survival rate and germination time of zygotic embryos. The methods evaluated included: entire seed excision, one-third vertical excision, half-horizontal excision, and zygotic embryo excision. Each seed was excised according to the method described above and transferred onto semi-solid MS (Murashige and Skoog, 1962) medium containing 0.05 mg/L of 2,4-D. The medium was dispensed in 20 mL per 90 × 16 mm Petri dish. The experiment was carried out in three replicates, each replicate containing 5 Petri dishes with 10 explants per dish. The germination percentage, time to first germination, and visible characteristics of the embryos were recorded after two weeks of culture.

2.4 Effect of different concentrations of 2,4-D on immature zygotic embryo germination

The excised embryos that survived the initial culture were transferred to an embryo germination medium, consisting of semi-solid MS medium supplemented with various concentrations of 2,4-D (0, 0.1, 0.3, 0.5, 1.0, and 2.0 mg/L). The experiment was conducted in three replicates, with each replicate consisting of 5 Petri dishes, each containing 5 explants per dish. The germination percentage, time to first germination, normal shoot development, and browning were recorded after four weeks of culture.

2.5 Culture Conditions

All experiments were carried out using Murashige and Skoog (MS) basal medium supplemented with 30 g/L sucrose. The pH of the medium was adjusted to 5.7 using 1 N NaOH or HCl before autoclaving at 121°C for 15 minutes. The media (20 mL) were dispensed into 90 × 16 mm Petri dishes. The cultures were incubated in a plant incubator (Sanyo, MLS-3750) with a temperature of 26 ± 2 °C, under a 10/14 h day/night photoperiod. Light intensity was set at 60 µmol m^{-2} s^{-1} using fluorescent lamps.

2.6 Statistical Analysis

The data from all experiments were arranged in a completely randomized design (CRD). Each treatment was conducted with three replicates, and each replicate included 5 Petri dishes, with 10 explants per dish. Data were analyzed using Analysis of Variance (ANOVA), and the mean values were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ using SPSS 17.0 software.

3. RESULTS

3.1 Effect of different immature seed excision methods on zygotic embryo survival rate

The study compared four different immature seed excision techniques to assess their effectiveness in promoting zygotic embryo survival rate and reducing the requirement for embryo germination. The methods tested included whole seed excision, longitudinal excision, one-third horizontal excision, and direct embryo excision. To undergo induction of embryo germination, four different excised methods of seeds were embedded with the cut surface in contact with the semi-solid culture medium for efficient nutrient absorption. Among the excision techniques, embryo excision resulted in the highest survival rate, with 75% of embryos becoming green subsequent germination within an average of 3.75 days (**Table 1**). In contrast, the whole seed excision method showed a much lower survival rate of only 10%, with embryos taking significantly longer (22.65 days) to start recovering and green. The longitudinal excision method also showed a relatively higher survival rate of 30%, but with a delay in the time to recover and green (16.55 days), accompanied by abnormal embryo development and browning (**Fig. 3**). The one-third horizontal excision method yielded no response, as all seeds displayed browning with no viable germination (**Fig. 4**).

Table 1 Effect of different immature seed excision methods on survival rate of zygotic embryo after culture for 2 weeks

Excision methods	Zygotic embryo germination (%)	Time required for first germination (days)	Visible characteristics
Entire seed	10	22.65	Seed swelling, funiculus softening, and small shoot germination
1/3 horizontal excision	0	0	Unresponsive seed, browning observed
Haft longitudinal excision	30	16.55	Embryo swelling, endosperm decomposition, abnormal shoot germination, and browning occur
Embryo excision	75	3.75	Embryo rapidly swells with a light-green color

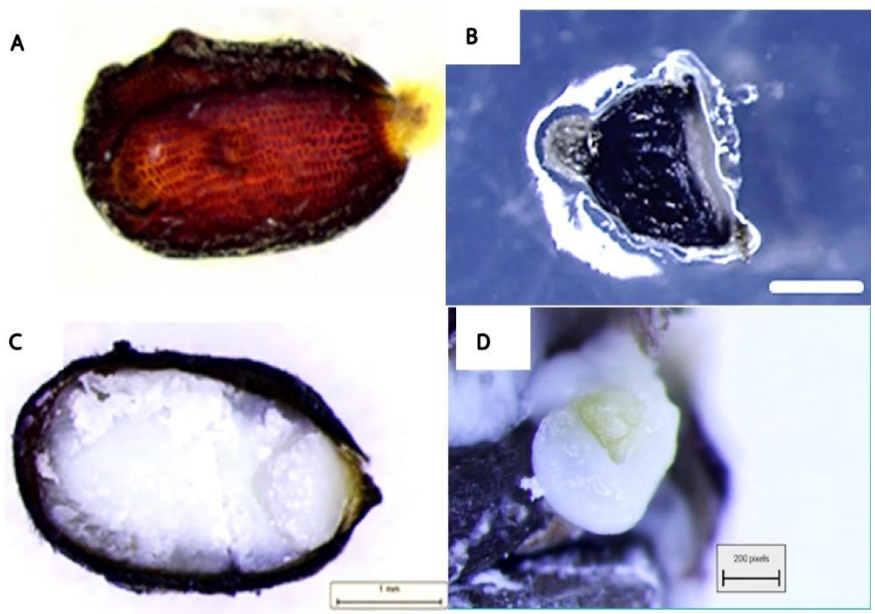


Figure 3 Seed excision techniques for evaluating zygotic embryo survival and germination in Intersubgeneric *Nymphaea* hybrid at 14 DAP, (A) entire seed excision: whole immature seed cultured intact without dissection, (B) one-third vertical excision: transverse cut removes one-third of the seed, exposing the inner endosperm, (C) half-horizontal excision: longitudinal cut removal of the upper half of the seed to partially expose the embryo and (D) zygotic embryo excision: isolated embryo carefully extracted from the seed coat for direct culture

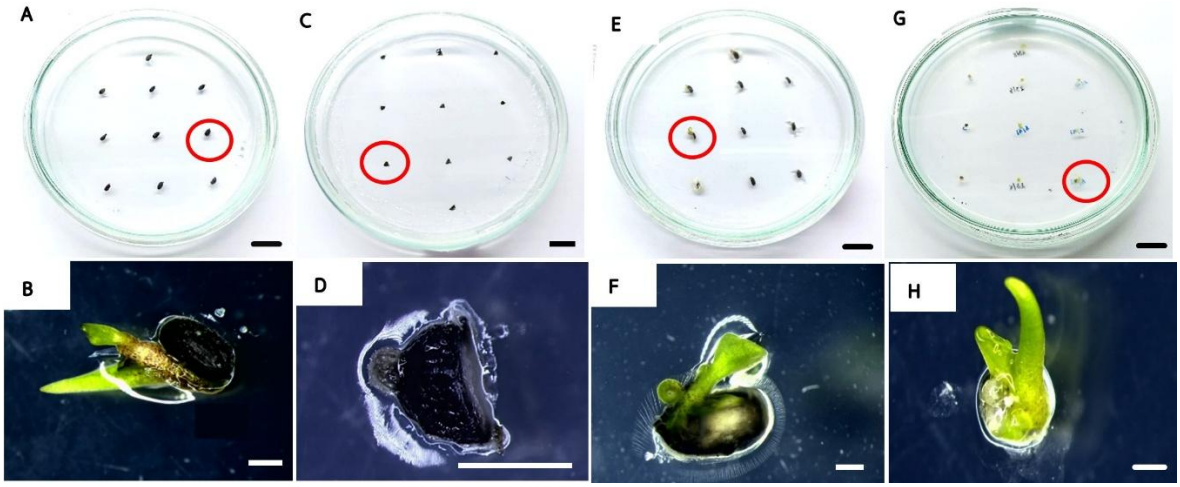


Figure 4 Zygotic embryo responses following different immature seed excision techniques cultured on MS medium supplemented with 0.05 mg/L 2,4-D after 14 days of culture. (A, B) Entire seed culture resulted in delayed and limited germination, with most embryos showing healthy growth, (C, D) one-third vertical excision: transverse cut removes one-third of the seed, exposing the inner endosperm, embryos showing poor germination rate, (E, F) half-horizontal excision: longitudinal cut removal of the another half of the seed to partially expose the embryo, embryos showing delayed in germinate and (G, H) Zygotic embryo excision: isolated embryo, with most embryos showing healthy growth

3.2 Effect of different concentrations of 2,4-D on immature zygotic embryo germination

The application of various concentrations of 2,4-D significantly influenced the germination of immature zygotic embryos. The embryos cultured on MS medium without 2,4-D (control) showed a germination rate of 40%, with the first germination occurring at 3.75 days. The addition of 0.1 mg/L 2,4-D resulted in a marked improvement in germination, increasing the rate to 80%, with germination occurring within 5 days. As the concentration of 2,4-D increased, germination rates gradually decreased. At 0.3 mg/L 2,4-D, the germination rate was reduced to 60%, and the time required for the first germination increased to 7.00 days. Further increases in 2,4-D concentration resulted in a continued decline in both germination rate and speed. At 0.5 mg/L 2,4-D, the germination rate dropped to 50%, with 8.75 days required for the first germination. At higher concentrations of 1.0 mg/L and 2.0 mg/L, the germination rates were significantly lower, with only 20% and 10% germination, respectively. Additionally, the germination time was delayed considerably, reaching 15.00 days at 1.0 mg/L and 17.00 days at 2.0 mg/L (**Table 2**)

The normal shoot development was highest in control and 0.1 mg/L 2,4-D treatments, both showing 60% normal shoot development, while the higher concentrations of 2,4-D (0.5 mg/L and above) led to a reduction in the percentage of normal shoot development, particularly at concentrations above 1.0 mg/L, where shoot development was impaired (**Fig. 5**). These results suggest that lower concentrations of 2,4-D (0.1 mg/L) are optimal for promoting zygotic embryo germination and normal shoot development, while higher concentrations inhibit germination and cause abnormal growth.

Table 2 Effect of different concentrations of 2,4-D on immature zygotic embryo germination after culture for 4 weeks

2,4-D (mg/L)	Zygotic embryo germination (%)	Time required for first germination (days)	Normal shoot development (%)	Browning (%)
0 (control)	40	3.75±0.70d	60	10
0.1	80	5.00±0.63cd	60	15
0.3	60	7.00±1.58bc	50	20
0.5	50	8.75±1.50b	45	50
1.0	20	15.00±1.54a	20	90
2.0	10	17.00±1.41a	0	90
F-test		**		
C.V. (%)		15.56		

Different letters were significantly different by DMRT; ** = significantly different at P≤0.01

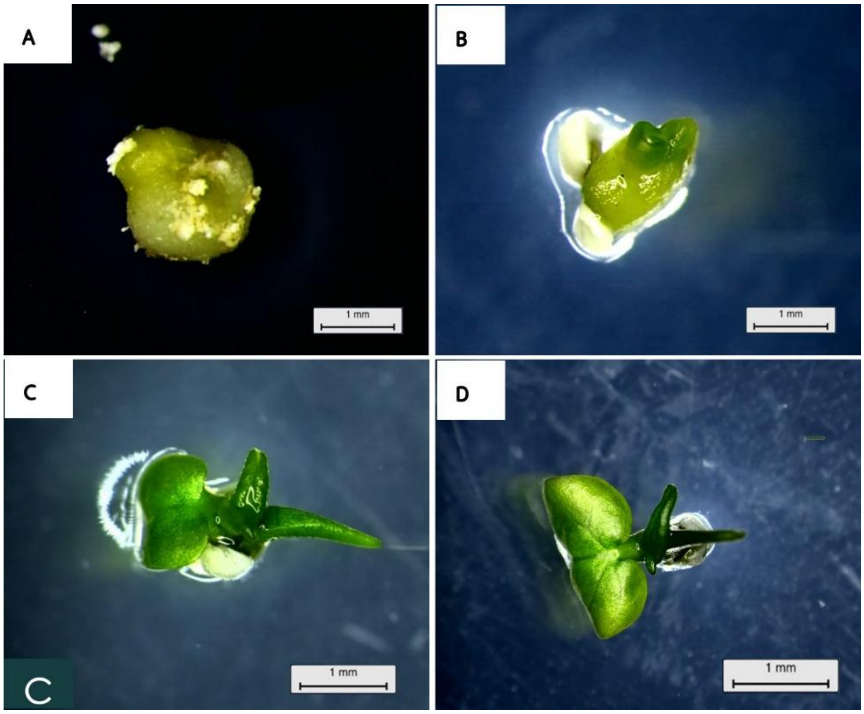


Figure 5 The developmental progression of immature zygotic embryo to seedling culture on semi-solid MS medium fortified with 0.1 mg/L 2,4-D after 4 weeks, (A) swollen and green zygotic embryo after culture for 1 weeks, (B) the first foliage emergent after culture for 2 weeks, (C) expansion of the first foliage and emergence of a new leaf after 3 weeks of culture and (D) fully expanded first foliage after 4 weeks of culture

4. DISCUSSION

4.1 Effect of different immature seed excision methods on zygotic embryo survival rate

In this study, four different seed excision techniques were tested to evaluate their effectiveness in promoting zygote survival and germination success. Among these methods, direct embryo excision demonstrated the highest success, with 75% of embryos becoming green and germinating within an average of 3.75 days (**Table 1**). These results are consistent with findings from Houghton *et al*. (2022), which reported that embryo excision significantly enhanced germination rates by breaking seed dormancy, yielding the highest germination rate of 78% in *Phacelia argillacea*. Both studies underscore the effectiveness of embryo excision as a technique for improving embryo survival and germination.

The whole seed excision method in this study resulted in a significantly lower germination rate (10%), with embryos taking much longer (22.65 days) to recover and turn green. This is in contrast with Houghton *et al*. (2022), which found that excising the embryo from the seed led to a more efficient germination process. The longitudinal excision method showed moderate success (30% survival), but the delay in germination (16.55 days), accompanied by abnormal development and browning, aligns with previous studies indicating that improper excision can damage the embryo and hinder proper growth. Similarly, the one-third horizontal excision method, which showed no viable germination, highlights a key limitation in the excision technique—embryo damage, which is often a result of improper handling or inadequate exposure to nutrients.

Houghton *et al*. (2022) also emphasized the importance of endosperm exposure in their research. They found that when the endosperm was direct contact with the nutrient medium, germination success was enhanced. This is consistent with the results of our study, where whole seed excision, longitudinal excision, and one-third horizontal excision methods—despite exposing some of the endosperm—failed to produce successful germination, further supporting the notion that endosperm contact alone does not guarantee success. In contrast, direct embryo excision, where the embryo was removed directly and placed into a nutrient medium, showed optimal germination, suggesting that the isolation of the embryo from the endosperm may be crucial for promoting embryo survival and development.

Furthermore, the research by Tzec-Simá *et al*. (2006) reported that embryo isolation without the endosperm can result in higher germination rates. Their work found 100% germination success when isolated embryos from *Bactris major* Jacq. and *Desmoncus orthacanthos* Mart. were cultured *in vitro*. This result reinforces the importance of precise excision techniques and suggests that some species, particularly those with hard seed coats or seed dormancy issues, may benefit more from direct embryo excision than from partial seed excision methods.

4.2 Effect of different concentrations of 2,4-D on immature zygotic embryo germination

The application of 2,4-D at various concentrations has a significant effect on the germination of immature zygotic embryos, with distinct patterns observed as the concentration of this plant growth regulator (PGR) increases. In this study, embryos cultured on MS medium without 2,4-D (control) exhibited a 40% germination rate, with germination occurring at 3.75 days. This is consistent with previous studies showing that low levels of growth regulators can have a beneficial effect on embryo germination by stimulating the necessary physiological responses for seedling establishment (Gaj, 2004; Jiménez, 2005; Hong *et al*., 2022). However, the introduction of 0.1 mg/L 2,4-D led to a marked improvement in germination, which increased to 80% and occurred within 5 days, suggesting that this concentration promotes optimal growth without detrimental effects. This finding aligns with other research where low concentrations of 2,4-D are shown to be effective in improving embryo development and germination rates in various plant species (Mok and Mok, 2001). Bronsema *et al*. (2001) reported that 2,4-D is necessary for callus induction in zygotic embryos, but higher concentrations inhibit germination. At 0.2 mg/L, embryos of maize form callus without germinating, while lower concentrations (0.002 mg/L) promote germination instead of callus formation. In *Ranunculus kazusensis*, a low concentration of 0.1 mg/L 2,4-D resulted in an 84.9% callus formation rate, but higher concentrations reduced this frequency (Min *et al*., 2007). Similarly, in *Arabidopsis thaliana*, 2,4-D treatment induced early-stage somatic embryos, with subsequent transfer to auxin-free medium promoting maturation (Raghavan, 2005).

As the concentration of 2,4-D increased, germination rates from immature zygotic embryos gradually decreased. At 0.3 mg/L, the germination rate declined to 60%, with delayed germination onset (7.00 days), suggesting that elevated levels of this PGR disrupt physiological processes critical for embryo development. Similar inhibitory effects have been documented across species. de Morais Oliveira *et al*. (2023) observed that 2,4-D concentrations above 0.2 mg/L suppressed germination in *Coffea arabica* zygotic embryos, while recent studies attribute this to auxin-induced oxidative stress and disrupted auxin signaling pathways (Tarigan *et al.,* 2024). The delay may stem from 2,4-D interference with cellular differentiation, as high concentrations (1.0 mg/L)

can induce callus proliferation at the expense of organized growth or trigger abnormal cell division (Alhussein and Almasoody, 2023; Dwitara *et al*., 2023).

At concentrations of 0.5 mg/L 2,4-D and higher, germination rates continued to decline significantly, with 50% germination at 0.5 mg/L, 20% at 1.0 mg/L, and only 10% at 2.0 mg/L, accompanied by a substantial delay in germination time. These results are consistent with other studies showing that excessively high concentrations of 2,4-D can inhibit germination by causing physiological stress and disrupting the normal development of the embryo. For instance, 2,4-D has been reported to cause growth inhibition and abnormal seedling development when used in high concentrations, as it may alter the hormonal balance and suppress the activity of endogenous auxins necessary for the proper development of the plant (Karami *et al*., 2023; Petrášek and Zažímalová, 2006). The observed increase in browning percentage with higher concentrations of 2,4-D can be attributed to several physiological mechanisms. When 2,4-D concentrations exceed optimal levels, they induce oxidative stress through the accumulation of reactive oxygen species (ROS), which damage cellular membranes and disrupt normal plant metabolism. This oxidative stress triggers enzymatic browning reactions mediated by polyphenol oxidase (PPO) and peroxidase (POD) enzymes that catalyze the oxidation of phenolic compounds to form brown-colored quinones (Jones and Saxena, 2013). According to, 2,4-D at higher concentrations can also stimulate phenylalanine ammonia lyase (PAL) activity, the first enzyme in the phenylpropanoid pathway, increasing the production of phenolic compounds that become substrates for browning reactions. It can be demonstrated that tissue injury combined with high auxin concentrations creates cellular conditions where phenolics stored in vacuoles contact oxidative enzymes, accelerating browning formation. (Alagarsamy *et al*., 2018; Liu *et al*., 2024)

Normal shoot development was highest in control and 0.1 mg/L 2,4-D treatments, with 60% normal shoot development, suggesting that these concentrations promote proper development. However, higher concentrations of 2,4-D, particularly 1.0 mg/L and above, resulted in a reduction in the percentage of normal shoot development, with shoot development impaired at higher concentrations. This finding is in agreement with several studies showing that 2,4-D, particularly at concentrations exceeding 0.5 mg/L, can lead to shoot malformation, root inhibition, and abnormal plant growth (Garcia *et al*., 2019). The observed impairment in shoot development may be due to the overaccumulation of auxin-like effects at higher concentrations, which can lead to disrupted cellular patterns and inhibited morphogenesis.

5. CONCLUSION

This study demonstrated the significant effects of seed excision techniques and 2,4-D concentrations on zygotic embryo survival, germination success, and callus induction in intersubgeneric hybrid ornamental waterlilies. Among the excision techniques, direct embryo excision was the most effective, achieving the highest zygote survival rate (75%) and the fastest germination time (3.75 days). The optimal 2,4-D concentration (0.1 mg/L) resulted in 80% germination and rapid embryo recovery, while higher concentrations inhibited germination. These findings emphasize the importance of optimizing PGR concentrations and excision methods for successful embryo rescue. Further research is needed to refine these techniques and explore their application across different plant subgenera.

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