**Original Research Article**

**Optimizing *In Vitro* callus induction from *Spathiphyllum* leaf and petiole explants**

**Abstract**

Spathiphyllum species are globally renowned ornamental houseplants, appreciated for their elegant foliage and air-purifying properties. Conventional methods of propagation such as rhizome division are labor-intensive, slow and inadequate for meeting the commercial demand for uniform and disease-free plant material. To address these limitations the present study developed an efficient in vitro regeneration protocol using leaf and petiole explants derived from cultured Spathiphyllum plantlets. These explants were selected due to their non-destructive nature, sterility and regenerative capability. Explant viability remained consistently high (>90%) across treatments. Notably, callus formation—a crucial intermediate stage in indirect plant regeneration—was significantly influenced by the type and concentration of plant growth regulators (PGRs). The highest frequency of callus induction (53.80%) was recorded on MS medium enriched with 3.0 mg L⁻¹ BAP and 1.0 mg L⁻¹ 2,4-D (Treatment C5), with visible callus emerging within 151.40 days. Interestingly, leaf explants exhibited no callus initiation under any tested condition, although they maintained high survival rates. Remarkably, a novel regeneration pathway was developed wherein petiole explants produced somatic embryos under continuous dark incubation on MS medium containing TDZ and 2,4-D which later transitioned to light for embryo maturation. A unique aspect of this protocol is the extended (3–4 months) dark incubation period without subculturing, which may induce somatic embryogenesis by activating stress-related developmental cues. The results confirm the potential of this method as a reliable platform for large-scale propagation of Spathiphyllum, enabling the production of uniform, commercially viable plants.

**Key words:** TDZ, 2,4-D, Micropropagation, Callus induction, Somatic embryogenesis, Spathiphyllum, Leaf, Petiole.

**Introduction**

*Spathiphyllum* spp., commonly referred to as peace lily, snow flower, or spathe flower, is a prominent ornamental plant in global floriculture. Its lush green leaves, striking white inflorescences, and exceptional ability to thrive in indoor environments contribute to its popularity both as a potted display and in floral arrangements. Native to Central and South America, the genus comprises approximately 41 species identified across diverse tropical regions (Chen *et al*., 2003). Beyond its visual appeal, *Spathiphyllum* is notable for its capacity to remove volatile organic pollutants such as benzene, formaldehyde, and carbon monoxide from indoor air—a property confirmed by NASA’s Clean Air Study (Chen *et al*., 2003; Rout *et al*., 2006). The combination of aesthetic value and air-purification capacity substantially drives its widespread demand.

Traditionally, *Spathiphyllum* is propagated through rhizome division. However, this conventional approach is labor-intensive, exhibits slow multiplication rates, and falls short of meeting growing market requirements, both domestically and internationally. It also does not comply with modern phytosanitary standards or the commercial need for uniformity in plant material (Chen and Henny, 2006). In contrast, micropropagation has emerged as a reliable and efficient alternative, offering year-round production of genetically uniform and disease-free plants through advanced in vitro techniques (Chen and Henny, 2008). This method is particularly advantageous for propagating rare or recalcitrant ornamental species, including high-value taxa like *Spathiphyllum*.

The current study aims to evaluate the regenerative capacity of in vitro-derived leaf and petiole explants of *Spathiphyllum*. Utilizing explants from in vitro-grown plantlets presents several benefits: they are abundantly available, inherently sterile, and reduce the need for surface sterilization associated with field-collected material. Furthermore, using leaf and petiole tissues is non-destructive, allowing donor plantlets to remain viable for future sampling. These tissues also possess totipotent potential, which makes them ideal for initiating various regeneration pathways such as somatic embryogenesis or organogenesis through dedifferentiation (Park, 2021). To establish a standardized and efficient protocol for micropropagating *Spathiphyllum*, this research investigates the influence of specific plant growth regulator (PGR) combinations and environmental conditions. Developing a dependable and reproducible method is essential to meet the increasing global demand for high-quality, genetically stable *Spathiphyllum* plants (Zhao *et al*., 2012).

**Materials and methods**

Source of Explants

Leaf and petiole tissues were excised aseptically from *in vitro*-cultured *Spathiphyllum* plantlets that had developed 4 to 5 leaves derived from shoot tip cultures. Dissection of explants was carried out under sterile conditions inside a laminar airflow chamber.

Culture Media and Growth Conditions

All experimental treatments utilized Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962), supplemented with 4% (w/v) sucrose and 7.5 g L⁻¹ of agar as the gelling agent. The medium’s pH value was adjusted to 5.8 using 1 N NaOH or 1 N HCl before sterilization in an autoclave at 121.8°C for 30 minutes. Cultured explants were incubated in a growth chamber maintained at 24–28°C under a 16-hour light/8-hour dark photoperiod. Illumination was provided by cool white fluorescent lights spaced appropriately for uniform intensity.

Callus Induction Protocol

Leaf segments (1.0–2.0 cm in length) and petiole sections (1.0–1.5 cm) were obtained from healthy *in vitro* plantlets and inoculated onto MS medium differing by plant growth regulator combinations. Treatments included varying concentrations of benzylaminopurine (BAP; 0.5, 1.0, 2.0, and 3.0 mg L⁻¹) in combination with 1.0 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D), as well as thidiazuron (TDZ) as a single supplement at 0.1, 0.5, and 1.0 mg L⁻¹. A control group was maintained using hormone-free MS medium. The cultures were sealed with Parafilm™ and maintained in complete darkness to stimulate callus initiation.

Somatic Embryo Induction

For somatic embryogenesis, leaf explants were oriented adaxial side upward, and petiole explants were laid horizontally across the culture surface, following the protocol described by Nazari *et al*. (2016). Each petri plate contained 12–15 explants, with four replicate dishes per treatment. Cultures were maintained at 25°C in dark conditions. Callus formation frequency was assessed after four weeks of incubation.

**Results**

This experiment evaluated the influence of various plant growth regulators (PGRs) on *Spathiphyllum* explant viability, callus formation rates, and the duration required for induction. Across all treatments, explant survival was notably high, ranging between 90.43% and 96.29%, indicating consistent initial viability regardless of the PGR type employed.

Distinct differences were observed in callus initiation across hormone treatments. The formulation designated C5, comprising MS medium enriched with 3.0 mg L⁻¹ BAP and 1.0 mg L⁻¹ 2,4-D, led to the highest callus formation rate (53.80%). In contrast, the control group (C1) lacking growth regulators exhibited minimal callus development (1.33%). Within the thidiazuron (TDZ) group, the concentration of 0.1 mg L⁻¹ (C6) resulted in the most favorable callus induction (32.94%) (Table 1). Treatments combining BAP and 2,4-D outperformed TDZ used alone, particularly at elevated concentrations of BAP, suggesting a synergistic effect between the cytokinin and auxin. Comparable trends were reported in Gerbera (Kumar and Kanwar, 2006; Nazari et al., 2016) and Anthurium (Oo *et al*., 2019; Prakasha *et al*., 2017; Rachmawati *et al*., 2020), reinforcing the effectiveness of such combinations in promoting callogenesis.

Regarding induction timing, C5 required 151.40 days for visible callus emergence, while the hormone-free treatment (C1) was markedly delayed, requiring 231.09 days—highlighting the pivotal role of exogenous PGRs in accelerating morphogenic responses (Table 2). These outcomes also align with findings by Yu *et al*. (2009) in *Gerbera* using a similar BAP + 2,4-D combination for enhanced callus production.

Notably, leaf explants exhibited no callus formation (0%), regardless of the hormonal treatment, including BAP, 2,4-D or TDZ applications. Despite this, their survival remained high, ranging between 88.73% and 93.84%, suggesting that although leaf tissues are metabolically active and viable, they may lack the responsiveness or competence for callogenesis under the tested conditions (Table 3).

**Discussion**

The micropropagation protocol developed for *Spathiphyllum* demonstrated consistently high explant survival across all test conditions, with rates ranging between 90.43% and 96.29%. These outcomes reflect the inherent viability of the petiole explants and the effectiveness of the culture conditions. In contrast, notable variation was observed in callus formation, which was strongly dependent on the type and concentration of applied plant growth regulators (PGRs). The absence of hormonal supplementation, as seen in the control (C1), resulted in minimal callus development (1.33%) and the longest induction duration (231.09 days), underscoring the importance of exogenous hormones in initiating morphogenesis.

**Effectiveness of BAP and 2,4-D in Callus Induction**

The combination of benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) was highly efficient in promoting callogenesis. As BAP concentrations increased from 0.5 to 3.0 mg L⁻¹ (while keeping 2,4-D constant at 1.0 mg L⁻¹), callus induction rates rose significantly, peaking at 53.80% in treatment C5. This response occurred within 151.40 days, demonstrating both effectiveness and timeliness. The synergy between auxin and cytokinin at optimal ratios is critical for activating cellular dedifferentiation and tissue proliferation (Raad *et al*., 2012; Ebrahimzadeh *et al*., 2022).

**Dual Role of TDZ in Callus Initiation**

Thidiazuron (TDZ), known for its strong cytokinin-like effects, also promoted callus formation, though its efficacy was concentration-sensitive. The highest callus induction (32.94%) was recorded at 0.1 mg L⁻¹ TDZ (C6), with response initiation at 158.59 days. At concentrations exceeding this threshold, both the frequency and the speed of callus formation declined sharply. This behavior aligns with known characteristics of TDZ, where excessive levels can inhibit growth or induce abnormal morphogenetic responses due to its high activity (Chen *et al.,* 2024; Ozel, 2018). Despite its potency, TDZ alone was less effective than the BAP + 2,4-D combination for callus production in this study.

**Somatic Embryogenesis and the Role of Extended Darkness**

A key achievement in this protocol was the successful induction of somatic embryos from petiole explants under specific conditions. Culturing explants in complete darkness on MS medium containing TDZ with 2,4-D or BAP initiated embryogenic development, which continued upon shifting cultures to a lighted environment. TDZ, when combined with either 2,4-D or naphthaleneacetic acid (NAA), has been previously reported to induce embryogenesis due to its unique dual activity as both an auxin and cytokinin mimic (Hou *et al*., 2020). In the current study, globular embryos appeared only after a prolonged dark incubation of 3 to 4 months without subculturing, emphasizing the physiological significance of extended darkness in triggering stress-related embryogenic pathways. Similar findings were documented in other species such as *Begonia* (Nada *et al*., 2012) and *Lilium* (Tang *et al*., 2010). The role of stress signalling in embryogenic transition has been also supported at the molecular level, particularly involving auxin signalling and pH changes (Pasternak *et al*., 2002; Zhang *et al.,* 2011), possibly explaining the delayed, but successful, somatic embryo development observed in *Spathiphyllum*.

**Conclusion**

The present investigation established a reliable and efficient protocol for *in vitro* regeneration of *Spathiphyllum* using leaf and petiole-derived explants. The explants consistently exhibited high survival rates across treatments, confirming their suitability for culture initiation. Callus formation was strongly influenced by plant growth regulator combinations, with the optimal response—53.80% induction within 151.40 days—achieved on MS medium supplemented with 3.0 mg L⁻¹ BAP and 1.0 mg L⁻¹ 2,4-D (Treatment C5). Although thidiazuron (TDZ) also promoted callogenesis, its effectiveness diminished at higher doses, demonstrating its concentration-sensitive activity. Interestingly, leaf explants, while maintaining high viability, failed to induce callus under any hormonal condition. A significant outcome of the study was the development of a somatic embryogenesis system using petiole explants, wherein embryos were induced after a 3–4 month dark incubation without subculturing. This dark-phase–triggered response suggests the involvement of stress-induced developmental pathways. Overall, this protocol presents a repeatable and scalable strategy for producing high-quality, genetically uniform *Spathiphyllum* plants, which can substantially benefit its commercial propagation.

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| **Treatments** | **Percentage survival** | **Percent callus induction** |
| **C1** | MS+ devoid of growth regulators | 90.43 | 1.33 |
| **C2** | MS + BAP 0.5 mg L-1 + 2,4-D1.0 mg L-1 | 91.07 |  9.82 |
| **C3** | MS + BAP 1.0 mg L-1 + 2,4-D1.0 mg L-1 | 91.40 | 16.20 |
| **C4** | MS + BAP 2.0 mg L-1 + 2,4-D1.0 mg L-1 | 94.04 | 37.17 |
| **C5** | MS + BAP 3.0 mg L-1 + 2,4-D1.0 mg L-1 | 95.23 | 53.80 |
| **C6** | MS + TDZ 0.1 mg L-1 | 96.29 | 32.94 |
| **C7** | MS + TDZ 0.5 mg L-1 | 95.05 | 28.13 |
| **C8** | MS + TDZ 1.0 mg L-1 | 94.09 | 11.31 |
| **SE(m)** | 1.70 | 0.92 |
| **CD** | NS | 2.77 |

**Table 1:** Influence of BAP, 2,4-D and TDZ on *in vitro* petiole explant survival andcallus formation

|  |  |
| --- | --- |
| **Treatments** | **Number of days taken for callus induction** |
| **C1** | MS+ devoid of growth regulators | 231.09 |
| **C2** | MS + BAP 0.5 mg L-1 + 2,4-D1.0 mg L-1 | 192.20 |
| **C3** | MS + BAP 1.0 mg L-1 + 2,4-D1.0 mg L-1 | 175.60 |
| **C4** | MS + BAP 2.0 mg L-1 + 2,4-D1.0 mg L-1 | 166.60 |
| **C5** | MS + BAP 3.0 mg L-1 + 2,4-D1.0 mg L-1 | 151.40 |
| **C6** | MS + TDZ 0.1 mg L-1 | 158.59 |
| **C7** | MS + TDZ 0.5 mg L-1 | 162.19 |
| **C8** | MS + TDZ 1.0 mg L-1 | 171.80 |
| **SE(m)** | 1.29 |
| **CD** | 3.86 |

 **Table 2:** Influence of BAP, 2,4-D and TDZ on number of days taken for callus induction of *in vitro* petiole

|  |  |  |
| --- | --- | --- |
| **Treatments** | **Percentage survival** | **Percent callus induction** |
| **C1** | MS+ devoid of growth regulators | 88.73 | 0 |
| **C2** | MS + BAP 0.5 mg L-1 + 2,4-D1.0 mg L-1 | 92.16 | 0 |
| **C3** | MS + BAP 1.0 mg L-1 + 2,4-D1.0 mg L-1 | 93.84 | 0 |
| **C4** | MS + BAP 2.0 mg L-1 + 2,4-D1.0 mg L-1 | 92.84 | 0 |
| **C5** | MS + BAP 3.0 mg L-1 + 2,4-D1.0 mg L-1 | 91.21 | 0 |
| **C6** | MS + TDZ 0.1 mg L-1 | 90.82 | 0 |
| **C7** | MS + TDZ 0.5 mg L-1 | 91.89 | 0 |
| **C8** | MS + TDZ 1.0 mg L-1 | 91.05 | 0 |
| **SE(m)** | 1.27 | 0 |
| **CD** | 3.81 | 0 |

 **Table 3:** Influence of BAP, 2,4-D and TDZ on *in vitro* leaf explant survival andcallus formation

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**Figure 1:** Sequential development of callus from *Spathiphyllum* petiole explants as influenced by BAP and 2,4-D

**COMPETING INTERESTS DISCLAIMER:**

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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