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Transformation Efficiency and Plant Proliferation through Direct Organogenesis from Leaf-Derived Callus in Ginger (Zingiber officinale Rosc.)

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ABSTRACT

This research aims to optimize in vitro propagation techniques for ginger (Zingiber officinale Rosc.) by investigating the effects of plant growth regulators (PGRs) on shoot induction, root formation, and callus proliferation. Ginger is an economically valuable crop with medicinal, culinary, and industrial applications, but its conventional propagation through rhizomes is slow, prone to disease, and limits large-scale production, necessitating improved tissue culture techniques. The highest shoot induction (4.9 shoots) occurred at 3.0 mg·L^{-1} BAP, while optimal shoot length (5.9 cm) was observed at 2.0 mg·L^{-1} BAP. Root induction was most effective at 3.0 mg·L^{-1} IAA, producing the highest root count (7.1), with the longest root length (5.3 cm) achieved at 4.0 mg·L^{-1} IAA. Callus formation was optimized using 1.0 mg·L^{-1} 2,4-D combined with 0.5 mg·L^{-1} Kinetin and 0.750 mg·L^{-1} IAA, leading to the quickest callus initiation (10 days), highest fresh weight (3.5 g), and largest diameter (2.3 cm). Acclimatization trials demonstrated high survival rates, with 93% in the growth chamber, 78% in the shade house, and 81% in open conditions. This study provides an efficient and reproducible tissue culture protocol for the mass production of disease-free, genetically uniform ginger plants, offering a sustainable alternative to traditional propagation methods and significantly improving cultivation practices, ensuring higher yields, better plant quality, and increased availability of superior planting material for agricultural and commercial applications.

Introduction

Ginger (Zingiber officinale Roscoe) belongs to family Zingiberaceae is a perennial herb, monocotyledonous, renowned for its aromatic rhizomes. The plant is characterized by linear, sessile leaves, yellowish-green flowers, and cylindrical spikes that bear oblong, capsule-shaped fruits. The rhizomes, which are the most economically significant part of the plant, their color varies from white to yellowish-brown. They have a flattened shape, irregularly branched, and exhibit fibrous vascular bundles at the cut ends (Dhanik et al., 2017). Ginger is among the most commonly utilized spices worldwide, with India is the largest producer, consumer and exporter. It is available in various forms, including raw ginger, dried ginger, ginger powder, ginger oil, and

ginger-based beverages, used in both culinary (Kumara et al., 2017) and industrial applications (Dhanik et al., 2017), (Brooks et al., 2009).

Despite its global significance, ginger cultivation faces numerous challenges that hinder its production and quality. Previously propagated through rhizomes are plagued by several limitations. Rhizome-based propagation is highly prone to soil-borne diseases such as bacterial wilt, Fusarium rot, and rhizome rot, which significantly reduce yield and quality (Mohapatra et al., 2018; Meenu & Jebasingh, 2020). These diseases are spread by poor soil health and inadequate farming practices. Moreover, biotic stressors like viruses, fungi, nematodes, and insects for instance, bacterial pathogens cause wilt and soft rot, while fungal infections lead to rhizome rot and yellows disease. Nematodes, produce root-knot disease, and viruses cause mosaic and chlorotic fleck, that reduce the overall yield and quality of ginger crops (Meenu & Jebasingh, 2020). Abiotic factors such as high light intensity, which causes sunburn, and excessive liming in soil, which leads to lime-induced chlorosis, also contribute to crop losses (Meenu & Jebasingh, 2020).

Direct organogenesis process involves the regeneration of plants from callus tissue derived from leaf explants. This approach not only facilitates rapid plant proliferation but also ensures the production of disease-free, genetically stable plants (Kambaska & Santilata, 2009). Direct organogenesis bypasses the need for intermediate callus formation, reducing risk of somaclonal variation and ensuring production of cloned plants (Mehaboob et al., 2019), (Higgins et al., 2020).

The present study focuses on optimizing transformation efficiency and plant regeneration through direct organogenesis callus in ginger. Callus induction, shoot proliferation, and root formation, this research aims to design a robust protocol for the large-scale cultivation of premium-quality and quantity of ginger plants for farmers.

Methodology

Plant Material

For this study, I used ginger (Zingiber officinale Rosc.) as the primary plant material. To obtain the explants, I sourced fresh ginger rhizomes from a local market in Hyderabad.

Selection of explant

Once the rhizomes had sprouted, carefully separated shoots from the rhizomes using a scalpel. Then selected only the healthiest and most vigorous shoots.



Figure 1: Fresh ginger rhizomes



Figure 2: Healthy ginger sprouts (1–2 cm) Culture



Figure 3: Ginger explant inoculation.

Surface Sterilization

The leaf explants underwent surface sterilization to remove any microbial contaminants treated with a 0.1% mercuric chloride solution for 10 minutes and then washed with sterilized distilled water. Subsequently, the explants were dipped in 70% ethanol for 1 minute to enhance surface disinfection. They were then sterilized in a 2% sodium hypochlorite solution, which included a drop of Tween-20, for 20 minutes, the explants were carefully washed three times with sterilized distilled water. All procedures were conducted under aseptic conditions within a laminar airflow cabinet.

Media Preparation

MS basal medium was used for callus formation, shoot regeneration, and root growth. The MS medium was prepared by mixing required quantities of nutrient solutions and different concentrations of growth regulators as necessary. Once all ingredients were fully dissolved, the pH was carefully adjusted to a range of 5.6–5.8 using a digital pH meter. To solidify the medium, 0.8% agar was added. The prepared solution was heated on a magnetic stirrer until fully liquefied, after medium was added into sterile culture jars. These jars were sealed with sanitized polythene sheets and subjected to autoclave. Then culture media was allowed to cool and kept at room temperature until further use.

Inoculation

The sterilized explants were carefully transferred to medium under sterile conditions inside a laminar airflow cabinet. All glassware, tools and culture media were sterilized through autoclaving. Throughout the process, instruments were kept in a beaker containing 70% ethanol and regularly sterilized by passing them through a flame using a spirit burner. At this stage, the explants were carefully trimmed, and unwanted outer layers were removed using scalpel. As get an appropriate length of 1.5–2 cm, the explants were placed in culture bottles. After positioning the explants vertically in the culture bottles. Each bottle was then labeled with essential information, before transferred to growth room.

Incubation

After following inoculation, the culture bottles were arranged on racks and kept in a controlled environment to support growth. The cultures were incubated at 25±2°C, with sufficient light intensity. Growth conditions were regulated with a 14-hour light and 10- hour dark photoperiod.

Callus induction

For callus induction, the outer layers of the shoots were removed to expose the young, tender leaves. After selection, the leaves were gently washed under running tap water to eliminate any surface impurities. The sterilized leaf explants were inoculated onto the prepared MS medium containing different concentrations 2,4-D in combination of kinetin and IAA and placed in a growth chamber. The cultures were kept at a stable temperature of 25 ± 2°C with a 16-hour light and 8-hour dark cycle. Callus cultures were monitored routinely, and sub-culturing was carried out every 3–4 weeks onto fresh medium.

Shoot proliferation

Once the shoots had successfully regenerated from the callus cultures, they were transferred to a medium specifically designed for shoot elongation. This medium was based on the (MS) with varying concentrations of BAP and IAA. The cultures remained under the same controlled conditions as before. The shoots were cultivated for 4–6 weeks, during which they were routinely observed for elongation and overall health.

Root induction

The rooting medium consisted of MS basal medium containing varying concentrations of IAA. The optimal concentration for root induction was determined through preliminary experiments. The cultures were maintained under the same environmental conditions as the shoot elongation phase. Roots began to emerge within 2–3 weeks, and the plantlets were nurtured until they developed a strong and well-formed root system.

Acclimatization

The plantlets had transplanted into small pots filled with a sterile substrate composed of soil, sand, and organic compost in equal proportions (1:1:1). The plantlets need high-humidity environment by covering pots with transparent plastic bags for 2–3 weeks. Then humidity was gradually lowered by puncturing small holes in plastic bags. The plantlets were watered regularly with sterile distilled water to maintain moisture levels. After a period of 3–4 weeks, the plantlets successfully acclimatized and shifted to greenhouse, under controlled conditions.

Experimental design

The experiments will be conducted using a Completely Randomized Design (CRD), with each treatment replicated three times. The collected data were analyzed statistically using (ANOVA) method. Statistic 8.1 software of analysis was used to identify significant differences.

Results

Shoot induction and growth

The results were demonstrated that BAP is crucial for shoot induction, affecting the number of shoots, their length, and the formation of leaves. The highest mean number of shoots (4.9) was observed at 3.0 mg·L^{-1} BAP, while the control treatment (0.00 mg·L^{-1} BAP) exhibited the lowest mean number of shoots (1.2). However, at 4.0 mg·L^{-1} BAP, the number of shoots decreased (3.1), indicating that excessive BAP may have an inhibitory effect on shoot multiplication. Similarly, shoot length was highest (5.9) at 2.0 mg·L^{-1} BAP, the shortest shoot length (3.6 cm) was recorded in the control treatment, A decline in shoot length (4.2 cm) was observed at 4.0 mg·L^{-1} BAP.

The number of days required for shoot initiation was reduced with increasing BAP concentrations (13 days) at 3.0 mg·L^{-1} BAP. The control treatment required (17 days), highlighting BAP for accelerating shoot initiation. A slight delay in shoot initiation (16 days) was observed at 4.0 mg·L^{-1} BAP. The highest number of leaves (5.2) was observed at 2.0 mg·L^{-1} BAP, while the lowest (2.5 ± 0.4) was recorded in the control treatment. A decrease in leaf number (3.5) was recorded at 4.0 mg·L^{-1} BAP. The data indicate that BAP is a critical growth regulator for shoot induction, with its effects being concentration-dependent



Figure 4: ginger plantlets in tissue culture jar



Figure 5: shoot proliferation of Zingiber officinale Rosc

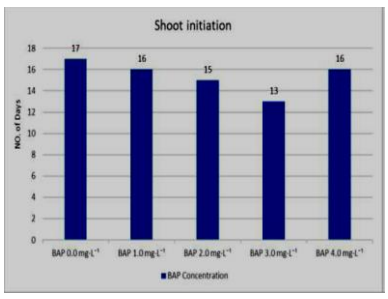


Figure 6: No. of days to shoot initiation

Root induction and growth

The study revealed a strong correlation between IAA concentration and root induction. The highest number of roots (7.1) at 3.0 mg·L^{-1} IAA, whereas the control treatment (0.00 mg·L^{-1} IAA) exhibited the lowest number of roots (3.2). At 4.0 mg·L^{-1} IAA, the number of roots slightly decreased (5.7), indicating that excessive IAA may suppress root initiation.

Root length was maximized (5.3) at 4.0 mg·L^{-1} IAA, indicating that root elongation. The control treatment resulted in the shortest root length (2.5 cm). However, a slight reduction in root length (3.9) was observed at 3.0 mg·L^{-1} IAA, indicating that an optimal concentration is required for balancing root initiation and elongation. The number of days required for root initiation decreased with increasing IAA concentrations (6 days) observed at 4.0 mg·L^{-1} IAA. The control treatment exhibited the longest duration (12 days), emphasizing the necessity of exogenous auxin for rapid root initiation. It is important to note that excessively high concentrations of IAA may lead to abnormal root morphology or reduced plant vigor, emphasizing the need for optimal concentration selection.

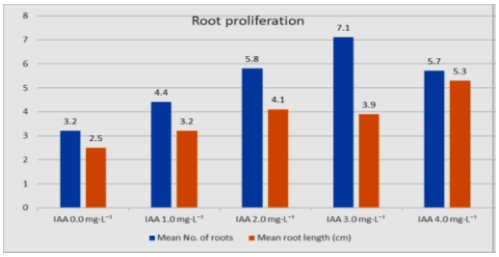


Figure 7: root proliferation

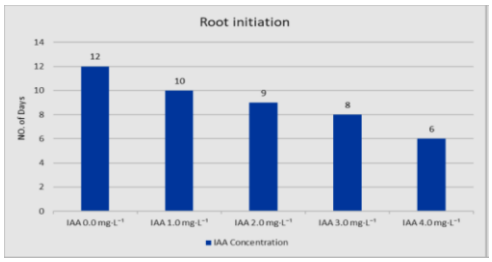


Figure 8: no. of days to root initiation

Callus formation

Callus induction were observed based on different concentrations of 2,4-D in combination with Kinetin and IAA. The shortest duration for callus initiation (10 days) was observed at 1.0 mg·L^{-1} 2,4-D with 0.5 mg·L^{-1} Kinetin and 0.750 mg·L^{-1} IAA, which also resulted in the highest callus fresh weight (3.5 g) and the largest callus diameter (2.3 cm). The control treatment exhibited the longest time (20 days), the lowest fresh weight (0.5 g), and the smallest diameter (0.9 cm).

Callus initiation time was observed with higher 2,4-D concentrations. At 2.0 mg·L^{-1} 2,4-D, callus initiation was delayed (13 days), with a fresh weight of (2.7 g) and a diameter of (1.9) cm. At 3.0 mg·L^{-1} 2,4-D, the callus initiation period further increased to (19 days), and the fresh weight reduced to (1.2 g), with a smaller callus diameter (1.1 cm), indicating a shift toward compact morphology. At the highest concentration of 2.4-D (4.0 mg·L^{-1}), callus initiation required (17 days), with a fresh weight of (2.1 g) and a callus diameter of (1.7 cm).

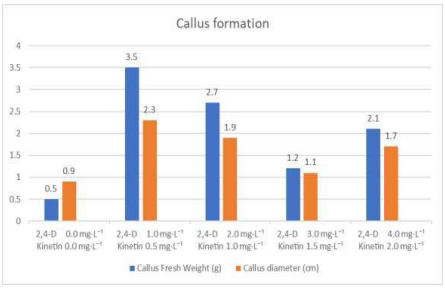


Figure 9: callus formation

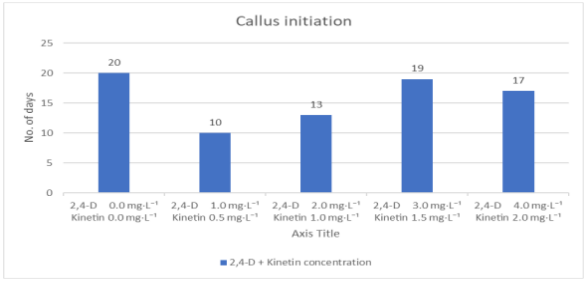


Figure 10: no. of days to callus initiation

Acclimatization and plant survival

The acclimatization of plantlets was conducted under 3 different conditions: growth chamber, shade house, and pot culture. In the growth chamber, 15 plantlets were transplanted and observed for 7 days. Out of these, 14 plantlets survived, resulting in a survival rate of 93% (Table 4.1). Following this, the plantlets were moved to a shade house, where they were gradually exposed to reduced humidity and indirect sunlight. To retain moisture, the pots were covered with transparent plastic sheets. They were maintained at room temperature for 14 days, with irrigation provided every two days. Under these conditions, 11 out of 14 plantlets survived, yielding a survival rate of 78%. After 3 weeks, the healthy plantlets were carefully removed from their pots and transplanted into larger containers. Regular watering was maintained, and mulch was applied to the soil surface as needed. When exposed to open environmental conditions, 9 out of 11 plantlets survived, achieving a survival rate of 81%.

Table 1: Survival rate of plants

Acclimatization Condition	No. of Plants Transplanted	Duration of Observation	No. of Plants Survived	Survival Rate (%)
In Growth Chamber	15	7 days	14	93
In Shade House	14	14 days	11	78
In Pot	11	14 days	9	81

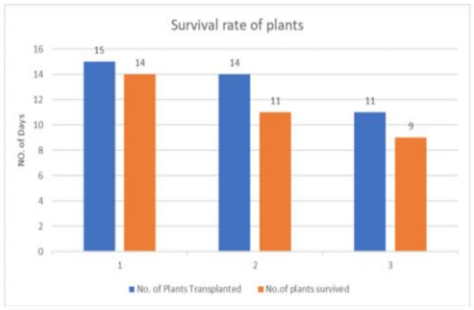


Figure 11: Survival rate of plants

Discussion

Somatic embryogenesis and plant regeneration are used on large-scale propagation of Zingiber officinale Rosc. (ginger) crop. Recent advancements in plant biotechnology, including the use of PGRs like 2,4-D, BAP, IAA, and kinetin, have improved tissue culture protocols. These advancements, combined with Agrobacterium mediated gene transfer, offer opportunities for developing genetically modified ginger plants with desirable traits. This study focuses on optimizing PGR combinations and concentrations to enhance shoot induction, (Sharma & Singh, 1995; George et al., 2008) root induction, and callus formation. The objective is to establish a reliable and optimized protocol for efficient plant regeneration and gene transformation in Zingiber officinale Rosc., ultimately improving ginger varieties for agricultural and commercial use.

These results provide valuable insights into the optimization of conditions for plant regeneration and transformation, particularly in the context of using PGRs such as 2,4-D, BAP, IAA, and kinetin. The comparison of treatments and their outcomes reveals interesting trends and provides context for interpreting efficacy of these findings are interpreted in the context of previous research in plant biotechnology and tissue culture.

Conclusion

The present study demonstrates that the application of different concentrations of BAP, IAA, and 2,4-D in combination with Kinetin and IAA significantly influences shoot induction, root formation, and callus proliferation in Zingiber officinale Rosc. The optimal concentration of BAP effectively enhances shoot proliferation and elongation, while excessive levels may have inhibitory effects. Callus induction and biomass accumulation are significantly influenced by the concentration of 2,4-D in combination with Kinetin and IAA, with lower concentrations producing highly friable callus with higher fresh weight, while higher concentrations result in compact callus with reduced proliferation. These findings provide valuable insights into optimizing in vitro propagation protocols for Zingiber officinale, which may serve as an effective method for large-scale propagation and conservation of elite germplasm.

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