Research Article

Effective Hormone Combinations for Enhanced Shoot and Root Development in Pomegranate

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ABSTRACT

The study investigates the effects of various concentrations of Indole-3-Acetic Acid (IAA) and Benzyl adenine (BA) on the proliferation of shoots from pomegranate (*Punica granatum* L.) 'Ganesh' cultivar explants. The experiment evaluated the number of shoots produced per explant, the percentage of explants showing a response, and the shoot length. The optimal concentration for shoot proliferation was found to be 0.8 mg/L IAA and 1.6 mg/L BA, resulting in the highest shoot production (6 ± 1 shoots per explant), maximum response rate (99 ± 1%), and greatest shoot length (1.9 ± 0.7 cm). Lower concentrations showed minimal proliferation, while higher concentrations led to reduced shoot production, indicating potential inhibitory effects at elevated hormone levels. Additionally, the effects of Sodium Nitrate ($NaNO_3$) and Thidiazuron (TDZ) on shoot proliferation were examined. The optimal NaNO3 concentration (0.2 mg/L) achieved the highest shoot production (7 ± 1 shoots per explant) and response rate (98 ± 1%), while higher concentrations resulted in decreased proliferation efficiency. For TDZ, 0.4 mg/L was the most effective, yielding the highest shoot production (6 ± 1 shoots per explant) and response rate (98 ± 1%), with longer shoot lengths. The combined application of α-NAD and IAA significantly enhanced root formation, with the best results observed at 1.5 mg/L α-NAD and 2.0 mg/L IAA, producing the highest number of roots (8 ± 1 per explant), highest response rate (98 ± 1%), and longest roots (3.3 ± 0.6 cm). The study also explored the combined effects of BA and IAA on leaf proliferation, identifying the optimal combination as 0.4 mg/L BA and 0.5 mg/L IAA, which resulted in the highest leaf production, response rate, and shoot length. These findings highlight the importance of optimizing hormone concentrations to improve the efficiency of In vitro propagation protocols. The results provide a foundation for refining tissue culture techniques to enhance shoot and root proliferation, potentially benefiting the large-scale propagation of pomegranate and other plant species.

Keywords: Indole-3-Acetic Acid (IAA), Benzyl adenine (BA), Pomegranate (*Punica granatum* L.), Tissue Culture.

INTRODUCTION

Pomegranate (*Punica granatum L.),* belonging to the family Punicaceae, is a significant fruit tree cultivated primarily for its delicious fruits and diverse applications in pharmaceuticals and ornamentals[1]. Native to regions spanning from Iran and Afghanistan to Northern India's Himalayan foothills, it has been extensively cultivated in tropical and subtropical regions worldwide, including Southeast Asia, tropical Africa, and India[2]. In India, commercial cultivation is predominant in Maharashtra, with smaller plantations in Gujarat, Rajasthan, Karnataka, Tamil Nadu, Andhra Pradesh, Uttar Pradesh, Punjab, and Haryana[3]. Approximately 100,000 hectares are devoted to pomegranate cultivation in India, yielding around 0.45 million tons of fruit annually.

Pomegranate fruit juice is rich in sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols, and iron, making it a valuable nutritional resource. Additionally, various parts of the pomegranate tree, such as leaves, immature fruits, fruit rind, and flower buds, have been traditionally used for their medicinal properties and in leather tanning. The wild pomegranate, although too acidic for direct consumption, is utilized as a souring agent known as Anar dana. Double-flowered varieties, prized for their ornamental red flowers, are cultivated in parks and gardens[4].

Vegetative propagation of pomegranate traditionally involves rooting of hardwood cuttings, which is time-consuming and requires a year for plant establishment. In vitro propagation offers a viable alternative, facilitating rapid and mass production of true-to-type plants from selected cultivars[5]. Consequently, extensive research has been conducted on In vitro propagation techniques for P. granatum L., focusing on proliferation through organogenesis from various explants such as leaf segments, cotyledons, anthers, and embryogenesis from seedling explants and zygotic embryos[4-7]. Recent studies have demonstrated that optimizing hormone concentrations can significantly enhance the efficiency of In vitro propagation. For instance, Kshirsagar Ashwinikumar *et al*. (2023) highlighted the efficacy of Benzyl adenine (BA) in promoting shoot initiation and structural development in pomegranate explants. This underscores the critical role of growth regulators in improving plant tissue culture outcomes.

This study aimed to develop effective In vitro propagation protocols for the 'Ganesh' cultivar of pomegranate by optimizing hormone combinations for shoot and root development. Specifically, the research evaluated the efficacy of Indole-3-acetic acid (IAA) and Benzyl adenine (BA) for shoot proliferation, and α-naphthyl acetic acid (α-NAD) in combination with IAA for root formation. The optimal concentrations of IAA (0.8 mg/L) and BA (1.6 mg/L) were found to maximize shoot production, explant

response, and shoot length, while the best results for root formation were achieved with 1.5 mg/L α-NAD and 2.0 mg/L IAA. These findings provided a refined In vitro propagation protocol that enhanced the efficiency and success rates of plant tissue culture, facilitating rapid and mass production of true-to-type pomegranate plants.

MATERIALS AND METHODS

The details of various material and methods were adopted during the course of present investigation which was narrated in this chapter under suitable sub-heads. This work was conducted at the Department of Plant Breeding and Molecular Genetics, Institute of Biosciences and Technology, MGM University, Chatrapati Sambhajinagar, Maharashtra, India, to establish In vitro propagation protocol for the prominent pomegranate cultivar 'Ganesh' grown in India.

Explant collection and Inoculation:

Pomegranate plants were collected from ICAR-National Research Centre on Pomegranate, Kegaon, Solapur-413 255, Maharashtra, India. Healthy nodal segments, approximately 2 to 3 cm long, were excised from mature trees of the 'Ganesh' cultivar using sterile tools. These segments were first rinsed in distilled water, then surface-sterilized with 70% ethanol for 1 minute, followed by 1% sodium hypochlorite for 10 minutes. After thorough rinsing with sterile water, the explants were processed in a sterile laminar flow hood.

The isolated nodal segments were cleaned under running tap water for approximately 15 to 20 minutes. Subsequently, they were treated with an antioxidant solution (150 mg/L ascorbic acid and 100 mg/L citric acid) for 20 minutes each under a laminar airflow hood, followed by three rinses in sterile distilled water. The nodal segments were then immersed in a fungicide (M-45) solution (1 mg/L) for 45 minutes and washed again with sterile distilled water. Explants were also treated with streptomycin solution (100 mg/L) for 20 minutes and subsequently washed with sterile distilled water. Finally, complete sterilization of the nodal explants was achieved by treating them with a 1 g/L mercuric chloride solution for 10 minutes, followed by three additional rinses with sterile distilled water.

Culture media:

The Linsmaier and Skoog (LS) media were prepared as basal media supplemented with organic acids and vitamins. Sucrose was added at 30.0 g/L and myo-inositol at 0.1 g/L. The pH of the prepared media was adjusted to between 5.6 and 5.8, and agar-agar (Hi-Media) was added at 8.0 g/L for solidification.

For the establishment stage, the media were supplemented with 0.2 to 2.0 mg/L Benzyl adenine (BA), 0.1 to 1.0 mg/L Indole-3-Acetic Acid (IAA), 0.5 to 2.5 mg/L sodium nitrate, and 10 to 50 mg/L Thidiazuron (TDZ). During the proliferation stage, 0.1 to 0.5 mg/L Benzyl adenine (BA) and 0.1 to 0.5 mg/L IAA were tested. For the rooting stage, two different auxins, α-naphthalene acetamide (α-NAD) and Indole-3-Acetic Acid (IAA), were tested at concentrations of 0.0, 0.25, and 0.5 mg/L on media at full strength.

RESULTS

Shoot Proliferation: The influence of various concentrations of Indole-3-Acetic Acid (IAA) and Benzyl adenine (BA) on shoot proliferation was evaluated. The study assessed the number of shoots produced per explant, the percentage of explants showing a response, and shoot length. The results are summarized in Table 1.

Table 1: Effect of IAA and BA Concentrations on Shoot Proliferation

Concentration IAA+ BA (mg/L)	Shoots Produced / Explant	Explants Showing Response (%)	Shoot Length (cm)
0.2+1.0	4 ± 1	36 ± 1	0.7 ± 0.2
0.4+1.2	5 ± 1	41 ± 1	1.0 ± 0.3
0.6+1.4	5 ± 1	86 ± 1	1.7 ± 0.9
0.8+1.6	6 ± 1	99 ± 1	1.9 ± 0.7
2.0+1.8	5 ± 1	84 ± 1	1.5 ± 0.3

At low concentrations (IAA 0.2 + BA 1.0 mg/L), shoot proliferation was minimal, with only 4 ± 1 shoots produced per explant, 36 ± 1% of explants showing a response, and an average shoot length of 0.7 ± 0.2 cm. These results suggest that this concentration is insufficient for optimal shoot induction and growth. Increasing the concentration to 0.4 mg/L IAA and 1.2 mg/L BA led to a modest improvement in shoot proliferation, with 5 ± 1 shoots per explant, 41 ± 1% response rate, and an average shoot length of 1.0 ± 0.3 cm, indicating a beneficial but still limited effect. At higher concentrations (IAA 0.6 + BA 1.4 mg/L), there was a significant enhancement in proliferation parameters, with 5 ± 1 shoots per explant, a dramatic increase in response rate to 86 ± 1%, and an average shoot length of 1.7 ± 0.9 cm, demonstrating a positive trend with higher hormone concentrations. The optimal concentration (IAA 0.8 + BA 1.6 mg/L) proved to be the most effective, with 6 ± 1 shoots per explant, 99 ± 1% of explants showing a response, and the greatest average shoot length of 1.9 ± 0.7 cm, suggesting that this concentration maximizes the regenerative potential of the explants. Although excessive concentrations (IAA 2.0 + BA 1.8 mg/L) still supported shoot proliferation, they were less effective than the optimal concentration, with 5 ± 1 shoots per explant, 84 ± 1%

response rate, and an average shoot length of 1.5 ± 0.3 cm, indicating that higher concentrations do not offer significant improvements over the optimal levels and may even result in reduced shoot production.

The study demonstrates that the combination of IAA and BA significantly influences shoot proliferation in plant tissue culture. The optimal results were achieved with 0.8 mg/L IAA and 1.6 mg/L BA, which provided the highest shoot production, maximum response rate, and longest shoot length. This concentration should be used to maximize the efficiency of In vitro propagation protocols. Higher concentrations did not improve outcomes further and may not be advantageous, suggesting a balance must be struck for optimal plant proliferation.

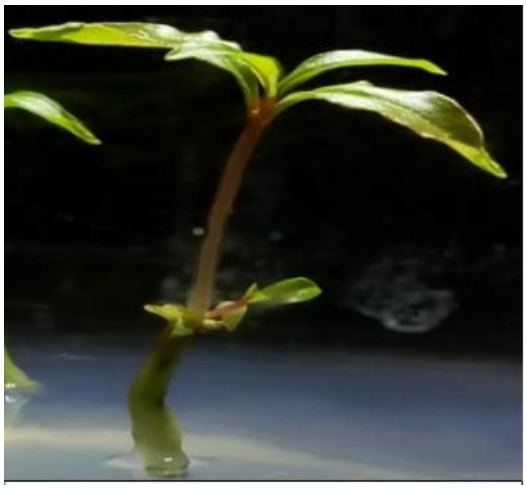

Figure 1: Combination of IAA and BA significantly influences shoot proliferation

Figure 2: *In vitro* shoot Multiplication

Table 2: Effect of sodium nitrate and Thidiazuron in MS medium on the rates of nodal explants regenerating shoots for establishment stage.

Concentrations (mg/L)	Shoot produced / Explant	Explant Response (%)	Shoot length (cm)
$NaNO_3$-0.1	5±1	86±1	1.8±0.9
$NaNO_3$-0.2	7±1	98±1	2.4±1.3
$NaNO_3$-0.3	5±1	82±1	1.5±0.5
$NaNO_3$-0.4	4±1	70±1	0.8±0.3
$NaNO_3$-0.5	3±1	65±1	0.6±0.1
TDZ-0.2	4±1	73±1	0.5±0.2
TDZ-0.3	5±1	97±1	1.1±0.6
TDZ-0.4	6±1	98±1	2.6±0.8
TDZ-0.5	5±1	84±1	0.8±0.3
TDZ-0.6	4±1	70±1	0.4±0.1

Figure 3: *Punica granatum* L. Mother Plant

Figure 4: Excised node explant

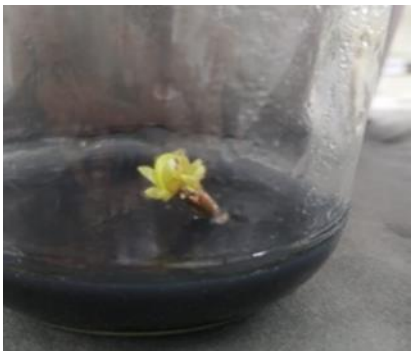

Figure 5: Shoot induction

Effect of Sodium Nitrate and IAA on proliferation of shoots

Effect of Sodium Nitrate ($NaNO_3$):

The highest shoot production per explant (7 ± 1) and explant response (98 ± 1%) were observed at a concentration of 0.2 mg/L NaNO3. The corresponding shoot length at this concentration was 2.4 ± 1.3 cm, indicating robust shoot development. As the concentration of NaNO3 increased beyond 0.2 mg/L, there was a notable decline in both shoot production and explant response. For example, at 0.5 mg/L NaNO3, shoot production dropped to 3 ± 1 shoots per explant, and the explant response decreased to 65 ± 1%. The shoot length at this concentration also reduced to 0.6 ± 0.1 cm. The lowest performance was observed at 0.5 mg/L NaNO3, suggesting that higher concentrations may have a toxic effect or inhibit shoot proliferation.

Effect of Thidiazuron (TDZ):

For TDZ, the optimal concentration was found to be 0.4 mg/L, which resulted in the highest shoot production (6 ± 1) and explant response (98 ± 1%). The shoot length at this concentration was 2.6 ± 0.8 cm, indicating a strong positive effect of TDZ on shoot elongation. Similar to NaNO3, increasing the concentration of TDZ beyond the optimal level resulted in reduced shoot production and explant response. At 0.6 mg/L TDZ, shoot production was 4 ± 1 shoots per explant, the explant response was 70 ± 1%, and the shoot length was 0.4 ± 0.1 cm. The lowest performance was at 0.6 mg/L TDZ, indicating that excessive TDZ can also have inhibitory effects on shoot proliferation.

The findings suggest that for efficient shoot proliferation in pomegranate tissue culture, careful optimization of NaNO3 and TDZ concentrations is crucial. Future research could explore the combined effects of these chemicals and their long-term impacts on plant development.

Leaf proliferation

Effect of Benzyl adenine (BA) and Indole-3-Acetic Acid (IAA) on Leaf Proliferation

This section explores the combined effect of different concentrations of Benzyl adenine (BA) and Indole-3-Acetic Acid (IAA) on leaf proliferation, focusing on the number of leaves produced per explant, the percentage of explants showing a response, and the shoot length.

Table 3: Effect of Benzyl adenine (BA) and Indole-3-Acetic Acid (IAA) on Leaf Proliferation.

Concentration (mg/L)	Leaves Produced / Explant	Explants Showing Response (%)	Shoot Length (cm)
BA-0.2 + IAA-0.3	13 ± 1	62 ± 1	1.2 ± 0.3
BA-0.3 + IAA-0.4	16 ± 1	82 ± 1	1.4 ± 0.4
BA-0.4 + IAA-0.5	20 ± 1	98 ± 1	2.3 ± 0.5
BA-0.5 + IAA-0.6	18 ± 1	92 ± 1	2.0 ± 0.4
BA-0.6 + IAA-0.7	17 ± 1	89 ± 1	1.7 ± 0.4

The results indicate that combining BA and IAA at specific concentrations leads to varied responses in terms of leaf production, shoot length, and the percentage of explants showing a response. The combination of BA at 0.2 mg/L and IAA at 0.3 mg/L resulted in the production of 13 leaves per explant, with 62% of explants showing a response and an average shoot length of 1.2 cm. Increasing the concentration to BA at 0.3 mg/L and IAA at 0.4 mg/L produced 16 leaves per explant, with 82% of explants showing a response and an average shoot length of 1.4 cm. The combination of BA at 0.4 mg/L and IAA at 0.5 mg/L yielded the highest number of leaves at 20 per explant, the highest response rate at 98%, and the longest shoot length at 2.3 cm, indicating a synergistic effect. At BA 0.5 mg/L and IAA 0.6 mg/L, there were 18 leaves per explant, with 92% of explants showing a response and an average shoot length of 2.0 cm. Finally, the concentrations of BA at 0.6 mg/L and IAA at 0.7 mg/L resulted in 17 leaves per explant, with an 89% response rate and an average shoot length of 1.7 cm.

The combined analysis of BA and IAA shows that their synergistic effects significantly enhance leaf proliferation in pomegranate nodal explants. The optimal combination was BA at 0.4 mg/L and IAA at 0.5 mg/L, which resulted in the highest number of leaves, the highest response rate, and the longest shoot length.

Figure 6: Synergistic effects significantly enhance leaf proliferation

These results suggest that the specific combinations of BA and IAA can be used to optimize tissue culture protocols for pomegranate nodal explants, enhancing the efficiency and

success of the In vitro propagation process. Further studies could explore other combinations to maximize the regenerative potential of the explants.

Root formation

The number of roots per explant increases with higher concentrations of both α-NAD and IAA, with the combination of 1.5 mg/L α-NAD and 2.0 mg/L IAA producing the highest number of roots (8 ± 1) per explant. At lower concentrations, such as 0.5 mg/L α-NAD and 1.0 mg/L IAA, the number of roots is significantly lower (2 ± 1). The response percentage also improves with increased concentrations of α-NAD and IAA, with the highest response rate (98 ± 1%) observed at both 0.5 mg/L α-NAD and 2.0 mg/L IAA, and at 1.5 mg/L α-NAD and 2.0 mg/L IAA. The lowest response rate is observed with 0.5 mg/L α-NAD and 1.0 mg/L IAA (19 ± 1%). Root length is positively correlated with the concentration of both hormones, with the longest roots (3.3 ± 0.6 cm) observed at 0.5 mg/L α-NAD and 2.0 mg/L IAA, and at 1.5 mg/L α-NAD and 2.0 mg/L IAA. The shortest roots (0.2 ± 0.3 cm) are seen with 0.5 mg/L α-NAD and 1.0 mg/L IAA. The results indicate that the combined application of α-NAD and IAA significantly enhances root formation in plant tissue cultures. The optimal concentrations for maximizing root number, response rate, and root length are 1.5 mg/L α-NAD and 2.0 mg/L IAA. The data suggests a synergistic effect between α-NAD and IAA, where the combination of these hormones at higher concentrations improves root induction and growth more effectively than either hormone alone.

Table 4: Combined Effect of α-Naphthaleneacetic Acid (α-NAD) and Indole-3-Acetic Acid (IAA) on Root Formation.

Concentration α-NAD + IAA (mg/L)	Number of Roots / Explant	Explants Showing Response (%)	Root Length (cm)
0.5+1.0	2 ± 1	19 ± 1	0.2 ± 0.3
0.5+1.5	4 ± 1	81 ± 1	1.6 ± 0.3
0.5+2.0	5 ± 1	98 ± 1	3.3 ± 0.6
1.0+1.0	5 ± 1	83 ± 1	1.6 ± 0.3
1.0+1.5	6 ± 1	88 ± 1	2.0 ± 0.4
1.0+2.0	7 ± 1	95 ± 1	2.9 ± 0.5
1.5+1.0	6 ± 1	75 ± 1	1.8 ± 0.4
1.5+1.5	7 ± 1	85 ± 1	2.5 ± 0.5
1.5+2.0	8 ± 1	98 ± 1	3.3 ± 0.6

These findings can be utilized to optimize tissue culture protocols for plant In vitro propagation, improving root formation and overall plant development. Further research could explore additional concentrations or combinations to refine these results and optimize the rooting process for various plant species.

DISCUSSION

The results of this study demonstrate the significant impact of varying concentrations of Indole-3-Acetic Acid (IAA) and Benzyl adenine (BA) on the proliferation of shoots from explants. These findings are consistent with previous research indicating the critical role of phytohormones in plant tissue culture and *In vitro* propagation protocols.

Effect of IAA and BA on Shoot Proliferation

The combination of IAA and BA significantly influenced shoot proliferation, with optimal results observed at 0.8 mg/L IAA and 1.6 mg/L BA. This concentration yielded the highest shoot production (6 ± 1 shoots per explant), the maximum percentage of explants showing a response (99 ± 1%), and the greatest shoot length (1.9 ± 0.7 cm). These results suggest a synergistic effect of IAA and BA at this concentration, enhancing the regenerative potential of the explants. This finding is consistent with earlier studies that have shown the importance of optimizing hormone concentrations for improved shoot proliferation.

In-vitro propagation of pomegranate through auxiliary proliferation from nodal explant was studied. For this purpose, the explants fortified with various concentrations of Benzyl adenine (BA) were employed. Benzyl adenine at the concentration of 10.0 µM was found superior for shoot initiation, number of leaves and number of shoots per explant. The treatment with silicea (60µl/l) resulted into remarkable structural development of the explants [8].

At lower concentrations (0.2 + 1.0 mg/L IAA + BA), the shoot proliferation was minimal, indicating that these levels are insufficient for optimal shoot induction and growth. Moderate concentrations (0.4 + 1.2 mg/L IAA + BA) led to a modest improvement, while higher concentrations (0.6 + 1.4 mg/L IAA + BA) showed significant enhancements in proliferation parameters. However, excessively high concentrations (2.0 + 1.8 mg/L IAA + BA) did not offer significant improvements over the optimal levels and even resulted in reduced shoot production, suggesting potential inhibitory effects at higher hormone levels. This aligns with the findings of previous research which highlights that overly high concentrations of plant growth regulators can have detrimental effects on tissue culture responses[9].

Effect of Sodium Nitrate and IAA on Proliferation of Shoots: The study also evaluated the effect of Sodium Nitrate () and IAA on shoot proliferation. The highest shoot production per explant (7 ± 1) and explant response (98 ± 1%) were observed at a concentration of 0.2 mg/L, with a corresponding shoot length of 2.4 ± 1.3 cm. As the concentration of increased, there was a notable decline in both shoot production and explant response, with the lowest performance at 0.5 mg/L. These results suggest that higher concentrations of may have a toxic effect or inhibit shoot proliferation, which is consistent with earlier studies on the effects of nitrate on plant tissue culture[10].

Effect of Thidiazuron (TDZ): The optimal concentration of Thidiazuron (TDZ) for shoot proliferation was identified as 0.4 mg/L. At this concentration, the highest shoot production was achieved, with an average of 6 ± 1 shoots per explant and a notable explant response rate of 98 ± 1%. Additionally, this concentration resulted in the longest shoot length of 2.6 ± 0.8 cm, demonstrating a substantial positive effect on shoot elongation. In contrast, higher TDZ concentrations, specifically 0.6 mg/L, led to decreased shoot production and explant response. This outcome aligns with previous research indicating that excessive cytokinin levels can inhibit shoot proliferation[11]. The study focused on optimizing TDZ concentrations to enhance shoot proliferation in plant tissue cultures. By evaluating various TDZ levels, the research pinpointed specific concentrations that significantly improved shoot induction, proliferation rate, and overall shoot quality. These findings highlight the potential of TDZ as a crucial component in refining tissue culture protocols for better shoot proliferation. The study also provides insights into the mechanisms by which TDZ influences shoot development, offering practical guidelines for its effective application in plant tissue culture systems [12].

Effect of BA and IAA on Leaf Proliferation

The combined effect of BA and IAA on leaf proliferation also showed varied responses. The optimal combination (0.4 mg/L BA + 0.5 mg/L IAA) resulted in the highest number of leaves (20 per explant), the highest response rate (98%), and the longest shoot length (2.3 cm). These results suggest that specific combinations of BA and IAA can be used to optimize tissue culture protocols for leaf proliferation, enhancing the efficiency and success of the In vitro propagation process. This observation is in line with studies that have demonstrated the importance of fine-tuning hormone combinations for maximizing proliferation potential in various plant species[13, 14].

Effect of α-NAD and IAA on Root Formation

The combined application of α-NAD and IAA significantly enhanced root formation. The optimal concentrations for maximizing root number, response rate, and root length were 1.5 mg/L α-NAD and 2.0 mg/L IAA, which produced the highest number of roots (8 ± 1) per explant, the highest response rate (98 ± 1%), and the longest roots (3.3 ± 0.6 cm). These results indicate a synergistic effect between α-NAD and IAA, improving root induction and growth more effectively than either hormone alone. This synergistic interaction has been reported in other studies involving plant tissue cultures[15].

CONCLUSION

The findings of this study underscore the importance of optimizing hormone concentrations for efficient shoot and root proliferation in plant tissue culture. The optimal concentrations of IAA and BA (0.8 mg/L and 1.6 mg/L, respectively) maximized shoot production, explant response, and shoot length. Similarly, the best results for root formation were achieved with 1.5 mg/L α-NAD and 2.0 mg/L IAA. These results can be utilized to refine In vitro propagation protocols, improving the efficiency and success rates of plant tissue culture.

Future research could explore the combined effects of different chemicals and their long-term impacts on plant development, as well as the optimization of hormone combinations for other plant species. Additionally, understanding the underlying mechanisms of hormone interactions could further enhance tissue culture methodologies and outcomes.

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