Assessing the Influence of Benzylaminopurine (Bap) on *In Vitro* Shoot Proliferation of Pomegranate (*Punica Granatum* L.)

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Abstract

Pomegranate (Punica granatum L.) is an economically important crop, characterized for its nutritious and pharmaceutical applications. However, traditional propagation methods have limitations in producing large quantities of high-quality plantlets. Hence, this research was aimed at developing an efficient tissue culture protocol for the successful establishment and determining the best hormone concentration of Benzyl Amino Purine (BAP) for shoot proliferation of the Sri Lankan pomegranate variety, Cv. Nimali. The explants (axillary nodal segments,1cm long) and shoot tips (1cm long)) were derived from mature healthy mother plants, surface sterilized and introduced into full-strength woody plant media. After two weeks, newly developed clean shoots were transferred into multiplication media in which five different concentration of BAP 0.0 mg/l (T1, control), 0.25mg/l (T2) 0.5mg/l (T3), 1.0mg/l (T4) and 1.5mg/l (T5) were introduced. The cultures were incubated at 26 ± 2ºc under16/8 h light/dark period in the incubation room. The results indicated that the highest shoot bud formation (6.0/plant), along with maximum shoot height of (22.4 mm), the average of leaves (11.4/plant), highest bud multiplication rate of (6.0%), were observed in shoot tip explants treated with 1.5 mg/L BAP (T5) after two-week period. In contrast, axillary nodal segments without BAP (T1) exhibited the lowest values, with shoot bud formation (1.4/plant), shoot height (1.0 mm), number of leaves (1.4/plant), and a bud multiplication rate of (1.4%). These findings demonstrate that the addition of 1.5 mg/L BAP to shoot tip explants significantly enhances shoot proliferation and growth in the Sri Lankan pomegranate variety, Cv. Nimali.

Keywords*: *Axillary nodal segments, Benzyl Amino Purine, Micropropagation, Punica granatum* L., *Shoot tips, WPM media

I. INTRODUCTION

The pomegranate (*Punica granatum* L.), belonging to the family Lythraceae, is an economically important crop of the semi-arid tropics of the world due to its highly nutritious, edible fruits that offer high returns and have great export demand. It also has versatile adaptability, low irrigation water requirement and pharmaceutical and ornamental usage (Chandra *et al.*, 2010). Pomegranate is a rich source of sugars (14-16%) and Vitamin C, along with essential minerals (0.7- 1.0%), iron (0.3-0.7 mg/100g), and other vital nutrients (Pawar *et al.*, 2020). The fruit is highly valued for its antioxidant properties, making it a popular choice both as a table fruit and in the local and export markets. The seeds, along with their arils, are often sun-dried and commercially marketed as condiments, spices, and food flavoring agents. Additionally, pomegranate seed oil has significant market potential due to its immense medicinal properties (Pawar and Singh, 2020).

Pomegranate plants are commercially propagated through stem cutting and air layering. These methods carry pathogens of challenging diseases like nematodes, bacterial blight and wilt, which introduce disease (Saroj and Kumar, 2019).This traditional method of pomegranate propagation is laborious and time-consuming. Its shortcomings include poor success rates, extremely low replication, and a one-year establishing period for obtaining new plants (Besharat *et al.*, 2024). The demand for quality pomegranate planting material is steadily increasing across major cultivation regions worldwide (Chandra and Babu, 2010). However, the available planting material from existing plantations is insufficient to meet this rising demand (Gorad *et al*., 2018).

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To overcome these limitations, tissue culture has emerged as a promising alternative for the mass production of healthy, disease-free pomegranate plants (Kalalbandi *et al.*, 2014). However, achieving large-scale production of high-quality planting material requires the careful selection of optimal explants, appropriate growth media, and the precise combination of plant growth regulators at various stages of micropropagation (Nimavat and Parikh, 2024). Benzylaminopurine (BAP) in micropropagation of woody crops is significant due to its strong influence on shoot induction and multiplication. BAP, a synthetic cytokinin, is widely used to initiate cell division and promote adventitious and axillary shoot proliferation, especially when added singly to the basal medium (Maheswari *et al.*, 2024). Hence, the aims of this study were to establish high throughput commercial tissue culture propagation protocol for shoot proliferation and to assess the ideal level of BAP in multiplication media for the Sri Lankan pomegranate variety *cv*. Nimali.

II. MATERIALS AND METHODS

A. Experimental Location

This study was conducted at the STC Biotech (Pvt) Ltd laboratory, located in Bope, Padukka, Sri Lanka (6°50'3.59" N, 80°05'18.00" E) at an altitude of 44 m above sea level. The area falls within the Low Country Wet Zone (WL1 Agro-ecological zone) and experiences an average annual rainfall of 329.2 mm, a maximum average temperature of 30.8°C, and a daytime relative humidity of 79.71%.

B. Planting material

Mature potted plants of the Sri Lankan pomegranate variety, Cv. Nimali, were obtained from the Fruit Research and Development Institute (FRDI) Kananvila, and maintained in a net house at STC Biotech (Pvt) Ltd. The plants were pruned at middle and sprayed twice weekly with 1% BAP solution (20 ml per plant) to promote new shoot growth(Amelia *et al*., 2020).Axillary nodal segments and shoot tips were collected as explants for initiation in woody plant media (WPM), and after two weeks of incubation at 26 ± 2°C under a 16-hour photoperiod using white fluorescent light, the newly developed shoots were transferred to multiplication media with different BAP concentrations.

C. Establishment of aseptic cultures

The surface sterilization of explants was performed in aseptic conditions to produce contamination-free cultures. Axillary nodal segment and shoot tip explants were initially rinsed in tap water followed by a few drops of T-Pol for 2 minutes and washed under running tap water for 40 minutes to remove the debris and contamination on the surface. Then the explants were immersed in 0.1 % of Carbendazim fungicide solution for one hour ((Prajwala *et al.*, 2022) and in Amoxicillin (200 mg/L) antibiotic solution for 15 minutes and finally washed with distilled water. Explants were then treated with 70 % isopropyl alcohol (IPA) fluid for 1 minute inside the laminar airflow cabinet, then washed five times with autoclaved distilled water. Subsequently, they were dipped in a 4% sodium hypochlorite (NaOCl) solution containing T-Pol for 2 minutes, and finally rinsed again five times with autoclaved distilled water before inoculation onto culture media (Ali et al., 2023). This multi-step surface sterilization ensured maximum reduction of microbial load while preserving explant viability.

D. Culture media preparation

1) Initiation of Media and Culture conditions

Full-strength WPM medium was prepared supplementing with 20 g/L sucrose and solidified with 6.5 g/L agar. The pH of the medium was adjusted to 5.8 ± 0.02 using 0.1 N NaOH (Sodium Hydroxide) or 0.1 N HCl (Hydrochloric Acid) prior to the addition of agar. Then the media were dispensed into 5.12 cm × 8.84 cm autoclaved glass jars as 20 ml per each and autoclaved at 121°C with a pressure of 1.0 kg/cm2 for 20 minutes and sealed with plastic wraps after closing with autoclaved plastic lids. Sterilized axillary nodal segments and shoot tips were trimmed into 1 cm-long sections, each containing one or two nodes, and positioned vertically at the top of the medium. The cultures were incubated at 26 ± 2°C under a 16-hour photo period using white fluorescent light. After two weeks of incubation, newly developed healthy plantlets were transferred to treatment media.

2) Shoot multiplication media

Full strength of WPM media was used for the shoot multiplication media after adding different concentrations of BAP. Newly collected clean shoots from the initiation step were cultured in the

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treatment media as one shoot per jar. The cultures were incubated at 26 ± 2ºC under16 h daily illuminations with white, fluorescent light (50 $\mu molm^{-2}s^{-1}$).

Table 01: Different concentration of BAP on shoot multiplication in *Punica granatum* L.

Treatment Code	(BAP) levels in shoot proliferation media (mg/l)
T1 (Control)	0.00
T2	0.25
T3	0.50
T4	1.00
T5	1.50

BAP: Benzylaminopurine

E. Data Collection

Two weeks after transferring to full strength WPM proliferation media having different concentration of BAP, data collection was conducted based on four main parameters to assess the growth performance of the plantlets. The number of shoot buds and the average number of leaves per plantlet were recorded through direct visual observation of each plantlet. Bud multiplication percentage was evaluated by visually inspecting and calculating the proportion of new shoots formed (Abdalla and Dobránszki, 2024). Shoot lengths were measured accurately by placing the plantlets on millimeter-marked graph paper.

F. Experiment Design and Statistical Analysis

All the experiments were set up in a completely randomized design (CRD) having five treatments with five replicates. Each replicate had five explants. Mean values were subjected to the analysis of variance (ANOVA) using IBM SPSS software version 25. Significantly different means were separated by Tukey's HSD post hoc test at $P \leq 0.05$. Results were represented by means ± Standard Error (SE).

III. RESULTS AND DISCUSSION

A. The Effects of BAP on shoot production in two types of explants

The present study investigated the effect of varying concentrations of BAP on shoot production and bud multiplication percentage in two types of explants: axillary nodal segments and shoot tips. Based on the findings, statistically significant differences in the mean number of shoots were observed among the various concentrations of BAP-supplemented media ($P \leq 0.05$).

The study found that 1.5 mg/L BAP (T5) resulted in the highest shoot proliferation, producing 3.6 ± 0.6 shoots from axillary nodal segments and 6.0 ± 0.3 shoots from shoot tips after two weeks of culturing (Figure 01). In contrast, the control treatment (no BAP) had the lowest shoot numbers, with 1.4 ± 0.2 and 2.8 ± 0.2 shoots from axillary nodal segments and shoot tips, respectively. A dose-dependent increase in shoot numbers was observed as BAP concentrations increased significantly from 0.25 mg/L to 1.5 mg/L (Figure 02 and 03). Increasing BAP beyond 1.5 mg/L may or may not increase shoot numbers and could cause abnormal growth. However, this should be confirmed by further research. Shoot tips consistently produced more shoots than axillary nodal segments across all treatments. Statistical analysis showed that the differences among treatments were significant ($p < 0.05$), confirming that 1.5 mg/L BAP is the optimal concentration for shoot multiplication in both explant types. These findings align with previous research on pomegranate, where MS medium supplemented with 1.5 mg/L BAP in combination with NAA produced the highest shoot proliferation, and higher concentrations either did not improve multiplication or caused abnormal growth, particularly in shoot tip explants(Kumar *et al.*, 2019).

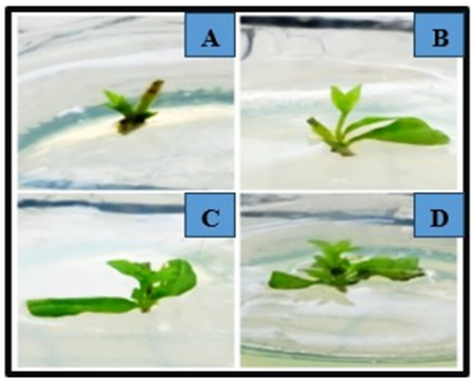

Figure 01: Morphology of induced shoots of *Punica granatum* L derived from axillary nodal segments after two weeks of BAP treatment: Letters A, B, C, and D represent shoots treated with 0.25, 0.50, 1.00, and 1.50 mg/L BAP, respectively.

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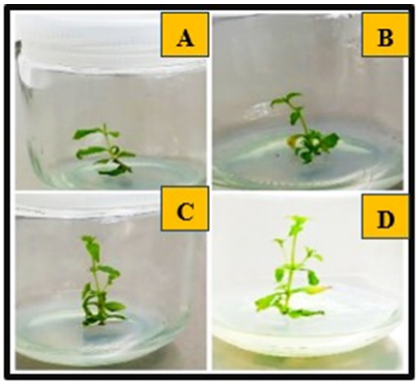

Figure 02: Morphology of induced shoots of *Punica granatum* L derived from shoot tips after two weeks of BAP treatment: Letters A, B, C, and D represent shoots treated with 0.25, 0.50, 1.00, and 1.50 mg/L BAP, respectively.

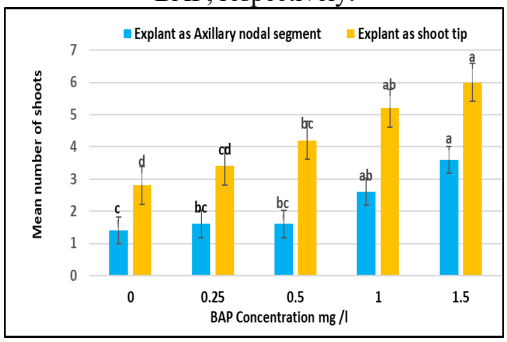

Figure 03: The Effects of BAP in shoot production in two types of explants. Means with the same superscript letter in the same color bar are not significantly different at Tukey's p< 0.05

Table 02. shows that statistical analysis confirmed significant differences (P < 0.05) in bud multiplication % where, the highest bud multiplication and shoot proliferation occurred in treatment T5 (1.5 mg/L BAP), with axillary nodal segments producing 3.6 ± 0.6 shoots and shoot tips 6.0 ± 0.3 shoots after two weeks, identifying 1.5 mg/L BAP as the most effective concentration.

Table 02: Bud multiplication and shoot proliferation percentage of explants

Treatments	Bud multiplication %	
	Axillary Nodal segments	Shoot Tips
T1	1.4 ± 0.2^{c}	2.8 ± 0.2^{d}
T2	1.6 ± 0.2^{bc}	3.4 ± 0.2^{cd}
T3	1.6 ± 0.2^{bc}	4.2 ± 0.2^{bc}
T4	2.6 ± 0.2^{ab}	5.2 ± 0.2^{ab}
T5	3.6 ± 0.2^{a}	6.0 ± 0.3^{a}
P	0.001	0.001
CV (%)	46%	30%

Means with the same superscript letter in a column are not significantly different at Tukey's p< 0.05

B. The Effect of BAP on Shoot Height

The study revealed a significant effect of BAP on shoot height in pomegranate (P ≤ 0.05). The lowest shoot heights were recorded in the control (T1), with axillary nodal segments at 1.0± 0.0 mm and shoot tips at 6.8 ± 0.3 mm. A consistent increase in shoot height was observed with rising BAP concentrations (0.25–1.5 mg/L). The highest shoot heights were recorded in T5 (1.5 mg/L BAP), with axillary nodal segments reaching 4.8 mm and shoot tips 22.4 mm, indicating a promotive effect of BAP on elongation (Figure 04).

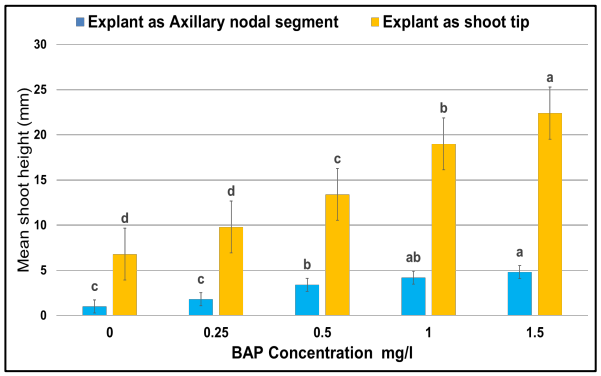

Figure 04: The Effect of BAP in Shoot height (mm). Means with the same superscript letter in the same color bar are not significantly different at Tukey's p< 0.05

C. The Effect of BAP on leaves production

The findings of the study revealed that leaf production per explant was significantly influenced by the concentration of BAP in the culture medium, with statistically significant differences observed among treatments (p ≤ 0.05). The highest mean number of leaves was recorded in treatment T5, which contained 1.5 mg/L BAP, producing 5.6 ± 0.2 leaves in axillary nodal segments and 11.4 ± 0.7 leaves in shoot tips after two weeks of culture followed by (T4), which contained 1.0 mg/L BAP and produced 5.4 ± 0.2 leaves in axillary nodal segments and 10.6 ± 0.2 leaves in shoot tips. In contrast, the control treatment (T1) without BAP showed the lowest leaf numbers, with 1.4 ± 0.2 leaves in axillary nodal segments and 5.2 ± 0.3 in shoot tips. As BAP concentration increased from 0.25 mg/L to 1.5 mg/L, an increasing trend in leaf formation was observed. Specifically, treatments T2, T3, and T4

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resulted in 2.4 ± 0.2, 3.4 ± 0.4, and 5.4 ± 0.2 leaves in axillary nodal segments, and 7.2 ± 0.3, 8.4 ± 0.5, and 10.6 ± 0.2 leaves in shoot tips, respectively. Overall, shoot tips consistently exhibited a higher number of leaves than axillary nodal segments across all treatments, and 1.5 mg/L BAP was identified as the most effective concentration for promoting leaf development in both explant types (Figure 05).

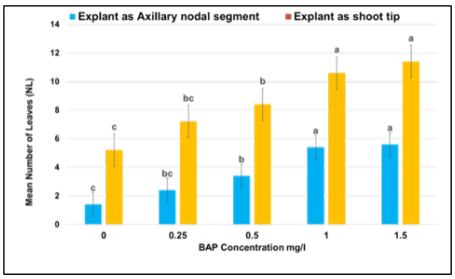

Figure 05: The Effect of BAP on leaves production. Means with the same superscript letter in the same color bar are not significantly different at Tukey's p< 0.05

The results of this study clearly demonstrate that BAP significantly enhances shoot proliferation and growth in the Sri Lankan pomegranate variety, Cv. Nimali. The optimal concentration of BAP for shoot proliferation was found to be 1.5 mg/L. This concentration resulted in the highest shoot bud formation, shoot height, and number of leaves. There are a number of reasons that shoot tip explants perform better than axillary nodal segment explants. Actively dividing meristematic tissues present at the tips of shoots are more receptive to BAP's growth-promoting actions.

The results of this investigation align with previous research on pomegranate micropropagation. According to Kumari et al. (2018), maximum shoot bud induction was achieved when nodal segment and shoot apex explants were cultured on basal medium supplemented with 2.0 mg/l and 2.5 mg/l BAP, respectively. Their findings highlight the potential of BAP in enhancing shoot formation across various woody plant species. The role of BAP has also been substantiated in several other woody crops, including Psidium guajava, Tectona grandis, and Prosopis cineraria, with effective concentrations ranging from 0.5 to 10.0 mg/l depending on the species and type of explant used . (Jayusman et al., 2022). The slightly lower optimal BAP concentration observed in Cv. *Nimali* may be attributed to genotype-specific sensitivity, endogenous cytokinin levels, and the type of explant used, highlighting the importance of tailoring growth regulator concentrations to the specific cultivar for efficient micropropagation. These studies collectively confirm BAP as a critical plant growth regulator for successful micropropagation in woody crops. According to numerous studies, BAP effectively encourages the growth of new shoots in a variety of pomegranate cultivars. However, the cultivar and type of explant employed could influence the optimal BAP concentration.

IV. CONCLUSION

The study demonstrates that 1.5 mg/L BAP significantly enhances *in vitro* shoot proliferation in *Punica granatum* L., resulting in maximum shoot bud formation, height, leaf number, and bud multiplication in shoot tip explants. This concentration proved most effective among all tested, while explants without BAP showed poor growth, confirming the crucial role of cytokinin in shoot organogenesis. Shoot tips outperformed nodal segments across treatments, indicating higher regenerative potential. Thus, 1.5 mg/L BAP and shoot tip explant are recommended for efficient shoot proliferation. To support large-scale production, root induction is necessary, and further trials are required to establish a complete micropropagation protocol with potential applications in the conservation and genetic improvement of *P. granatum* through optimized tissue culture.

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ABBREVIATIONS

- Tc Tissue Culture
- Var Variety
- WPM Woody Plant Basal Medium
- BAP Benzyl Amino Purine
- IPA Isopropyl Alcohol
- NaOCl Sodium hypochlorite
- CRD Completely Randomized Design
- ANOVA Analysis of Variance
- SPSS Statistical Package for the Social Sciences

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