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Research Article

In-Vitro Shoot Induction Of *Z. armatum* In High Proportion Of Cytokinin Containing MS Media

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Abstract

Background: *Zanthoxylum armatum* is a medicinally important plant facing threats due to over-exploitation and habitat destruction. In vitro propagation provides a sustainable solution for its conservation and large-scale production.

Objective: This study aimed to optimize the regeneration, proliferation, and multiplication protocols for *Z. armatum* using tissue culture techniques.

Methods: Explants of *Z. armatum* were cultured on Murashige and Skoog (MS) medium supplemented with varying concentrations of plant growth regulators (PGRs) such as benzyl amino purine (BAP), indole-3-butyric acid (IBA), and kinetin. Shoot regeneration, proliferation, and elongation were evaluated.

Results: The optimal shoot regeneration was observed with 3 mg/L BAP and 2 mg/L IBA, achieving a 60% response rate, 3.46 shoots per explant, and 4.78 mm average shoot length. Maximum shoot proliferation was obtained with 4 mg/L BAP and 0.5 mg/L kinetin, resulting in 9.42 shoots per explant and a shoot length of 5.06 mm. Statistical analysis confirmed significant variations in growth parameters ($p < 0.05$).

Conclusion: This study establishes an efficient protocol for the in vitro propagation of *Z. armatum*, supporting its conservation and commercial cultivation for pharmaceutical and ethnobotanical applications.

Key-words: *Zanthoxylum armatum*, Benzyl amino purine, Indole-3-butyric acid, Regeneration

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1. Introduction

1.1. Overview of *Zanthoxylum armatum*

Zanthoxylum armatum, commonly known as the Indian prickly ash or Timur, is a deciduous shrub or small tree belonging to the Rutaceae family. Native to the Himalayan region, this plant is valued for its wide range of medicinal and culinary uses. The fruits and bark are traditionally used for treating digestive disorders, respiratory ailments, and skin conditions. Its phytochemical composition includes essential oils, alkaloids, and flavonoids, making it a vital resource in pharmacology and ethnomedicine (Gupta et al., 2016).

1.2. Need for Plant Regeneration

Regeneration of *Z. armatum* through in vitro techniques has gained prominence due to the challenges associated with its natural propagation. Over-exploitation and habitat destruction threaten its survival, necessitating the development of effective propagation methods. Tissue culture techniques, particularly regeneration, serve as a sustainable solution by producing disease-free, genetically stable, and high-quality plantlets (Murashige & Skoog, 1962).

1.3. Proliferation and Multiplication through Tissue Culture

Proliferation and multiplication of shoots in *Z. armatum* are critical for large-scale propagation. Utilizing plant growth regulators (PGRs) such as 6-benzylaminopurine (BAP), kinetin, and indole-3-butyric acid (IBA) in Murashige and Skoog (MS) medium has proven effective. Optimal concentrations of BAP (3 mg/L) and IBA (2 mg/L) have been shown to yield the highest shoot regeneration rates, with significant differences observed in shoot length and the number of shoots per explant. Moreover, shoot multiplication experiments reveal that a combination of 4 mg/L BAP and 0.5 mg/L kinetin significantly enhances shoot elongation and proliferation rates. These results highlight the importance of optimizing media composition and growth regulator combinations for efficient shoot culture (Singh et al., 2018).

1.4. Significance of In Vitro Propagation

In vitro propagation methods offer a rapid, reliable, and resource-efficient approach to conserve *Z. armatum* and ensure its sustainable use. Through systematic regeneration and proliferation studies, this plant can be cultivated at a commercial scale to meet the demands of the pharmaceutical and agricultural industries while contributing to biodiversity conservation (Sharma et al., 2020).

2. Literature Review

2.1. Medicinal Importance of *Zanthoxylum armatum*

Zanthoxylum armatum, commonly known as Indian prickly ash or Timur, is a valuable medicinal plant native to the Himalayan region. It is widely utilized for its therapeutic properties, particularly in treating digestive, respiratory, and skin ailments. Its bioactive compounds, including essential oils, flavonoids, and alkaloids, make it a significant resource in

pharmacology and traditional medicine (Gupta et al., 2016). Conservation challenges due to habitat destruction and overexploitation have spurred interest in developing sustainable propagation methods.

2.2. In Vitro Propagation as a Conservation Strategy

In vitro propagation techniques offer a sustainable solution to address the declining natural populations of *Z. armatum*. Tissue culture methods facilitate the production of disease-free, genetically stable, and high-quality plantlets, thereby supporting both conservation and commercial cultivation efforts (Murashige & Skoog, 1962). Such methods are particularly beneficial for medicinal plants like *Z. armatum* that face high demand in pharmaceutical and ethnobotanical applications.

2.3. Role of Plant Growth Regulators (PGRs)

Plant growth regulators (PGRs) play a pivotal role in the regeneration and proliferation of shoots in tissue culture systems. Cytokinins like benzyl amino purine (BAP) and auxins such as indole-3-butyric acid (IBA) have been shown to significantly influence shoot induction and elongation. Studies demonstrate that optimal concentrations of BAP (3 mg/L) and IBA (2 mg/L) result in the highest shoot regeneration rates, achieving a 60% response rate, 3.46 shoots per explant, and an average shoot length of 4.78 mm (Singh et al., 2018).

2.4. Optimization of Culture Conditions

The Murashige and Skoog (MS) medium, enriched with appropriate PGRs, has been identified as an effective substrate for shoot culture. Adjusting the concentrations of BAP and kinetin has been critical in optimizing shoot multiplication. The combination of 4 mg/L BAP and 0.5 mg/L kinetin yielded the highest proliferation rates, with 9.42 shoots per explant and an average shoot length of 5.06 mm. However, supra-optimal concentrations led to inhibitory effects, emphasizing the need for precise optimization (Sharma et al., 2020).

2.5. Significance of the Study

The findings of this study not only establish an efficient protocol for the in vitro propagation of *Z. armatum* but also contribute to its conservation and sustainable use. The optimized techniques can support large-scale propagation, catering to the demands of various industries while reducing the pressure on natural populations. This aligns with broader goals of biodiversity preservation and sustainable utilization of medicinal plants (Gupta et al., 2016; Sharma et al., 2020).

2. Methodology

This chapter provides a detailed description of the experimental design, materials, procedures, and techniques employed to conduct the research. Accordingly, in the present investigation, we tried to compare the phytochemical activity, biological activity,

and essential oil estimation of wild and *In vitro* propagated *Z armatum*.

The details of the materials and methods used in these experiments were thoroughly described and explained in the following sections, providing a comprehensive understanding of the procedures and techniques employed during the investigation:

3.1. Experimental location

The study was specifically conducted in the laboratory of Department of Basic Science, Shri Guru Ram Rai University, Dehradun, (Uttarakhand). Dehradun is situated at a latitude of approximately 30.3165° North and a longitude of 78.0322° East. Being located in the foothills of the Himalayas, Dehradun enjoys a pleasant and moderate climate throughout the year. Summers are generally mild and winters can be cool, with occasional snowfall in the higher elevations.

3.2. Materials and Equipment

All the chemicals utilized in the study, including folin-Ciocalteu phenol reagent, gallic acid, quercetin, anhydrous sodium carbonate, methanol, deionized water, hexane, benzene, ethanol, sodium nitrite, aluminum trichloride, and sodium hydroxide, were of analytical grade and obtained commercially (Harborne, 1998). The Auxin chemicals, specifically indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA), and cytokinins, such as 6-benzyl amino purine (BAP), were procured from Sigma-Aldrich Co. LLC, Mumbai. The solvents methanol and hexane were obtained from Loba Chemie Pvt. Ltd., Mumbai (India).

3.2.1. Selection and collection of plant:

The most crucial element of this work is the careful selection of a specific plant. To identify the desired plant, researchers traditionally rely on the field of ethnobotany, which involves studying plants that hold significance for indigenous communities of Sariyatal, Nainital, Uttarakhand (Edeoga et al., 2005). Specifically, ethnobotany focuses on plants that are important to primitive or traditional cultures. For this study, the plant chosen is *Z armatum*, and specimens of this species were collected from the Uttarakhand region in northern India.

3.2.2. Authentication of plant:

The plant species *Z armatum* was undergo authentication at the Botanical Survey of India (B.S.I) in Dehradun. To ensure its accuracy and validity, a herbarium copy was carefully prepared and subsequently submitted for verification (Adams, 2007).

3.3. In vitro Propagation of Z. armatum

3.3.1. Plant material and surface disinfection

The plant material was collected from wild with non destructive approach.

3.3.1.1. Washing of explants

The explants were initially rinsed under a stream of running tap water to eliminate any dust, dirt, and unwanted debris. Subsequently, they were subjected to a detergent solution (Labolenne) containing 2-3 drops of a wetting agent (Tween 20). This was followed by another rinse with tap water to eliminate the detergent. To eliminate potential fungal contamination (Harborne, 1973), the explants were treated with 2g/l Dithane M-45 and 1g/l Bavistin for 15-20 minutes with intermittent shaking. They were then washed with running tap water to remove any remaining traces of the fungicides. Finally, the explants were rinsed 2-3 times with distilled water.

3.3.1.2. Surface sterilization

For varying amounts of time, the tissue specimens had been sterilized using a newly made 0.1 percent HgCl₂ mixture. In order to eliminate any remaining residues of the sterilant, the surface-sterilized explants were then cleaned five or six times using autoclaved water that had been double-distilled. After being sterilized, the explants were placed on petri dishes that had been pre autoclaved, sliced to the appropriate size, and then aseptically injected into culture medium that had been enhanced with a variety of developmental factors (Murashige and Skoog, 1962). About 10–20 extracts were used in each medication, and every study was triple-replicated. Using refreshing white florescent tubes (Phillips, India), the cultures were cultured in a culture chamber at 25±2°C for 16 hours while being exposed to light intensities ranging from 3K lx to 5K lx.

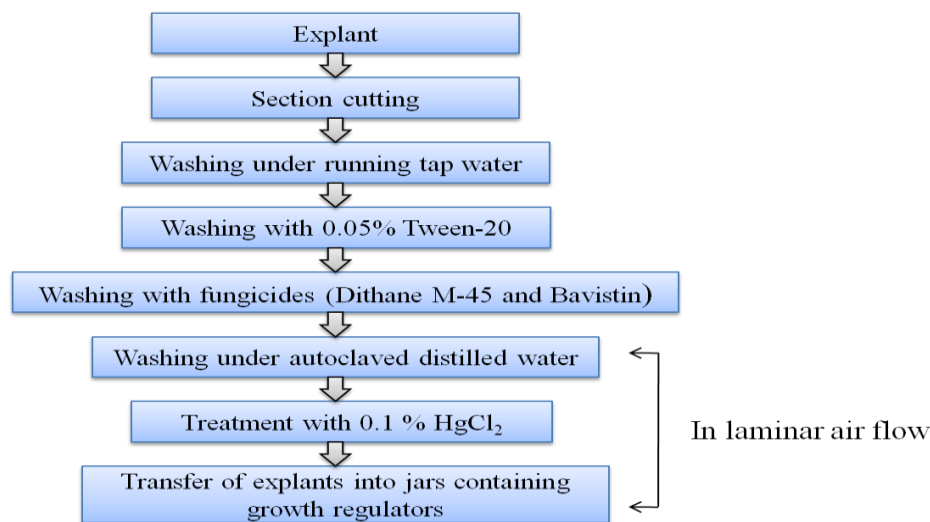


Figure 1. Preparation and culturing of explants

3.3.2. Culture medium and condition

3.3.2.1. Media composition

The success of a tissue culture protocol relies on the appropriate formulation of the growth medium. In this study, the composition of the medium was based on the Murashige and Skoog (1962) medium, commonly referred to as MS medium. All the necessary chemicals, including agar powder, growth hormones, and sucrose, were procured from Himedia Laboratories, located in Mumbai, India.

The concentrations of macro elements, micro elements, vitamins, salts, organic and inorganic constituents of

the MS medium were determined according to the specifications listed in Table 3.1. Stock solutions of inorganic nutrients, vitamins, plant growth regulators (PGRs), and $\text{Na}_2\text{EDTA} \cdot \text{FeSO}_4$ were prepared separately and stored in a refrigerator until further use. Fresh stock solutions of vitamins and plant growth regulators were prepared as needed based on the requirements of the experiment. The chemicals used were of analytical grade and were obtained from Himedia.

Table 1. MS media composition

Ingredients	mg/L
MACROELEMENTS	
Ammonium nitrate	1650.000
Magnesium sulphate	180.690
Potassium nitrate	1900.000
Potassium phosphate monobasic	170.000
MICROELEMENTS	
Boric acid	6.200
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	0.025
EDTA disodium salt dihydrate	37.300
Ferrous sulphate heptahydrate	27.800
Manganese sulphate monohydrate	16.900
Molybdic acid (sodium salt)	0.213
Potassium Iodide	0.830
Zinc sulphate heptahydrate	8.600
VITAMINS	
myo-Inositol	100.000
Nicotinic acid (free acid)	0.500
Pyridoxine HCl	0.500
Thiamine hydrochloride	0.100
AMINO ACID	
Glycine	2.000
Total(gms/litre)	4.1

To prepare a specific amount of MS substrate, a known volume of water that had been double-distilled was mixed with the MS basal medium powder (4.42g/l), which contains macro nutrients, micronutrients as vitamins, and an iron source. The powder was purchased from HiMedia Industries in Mumbai, India. 0.1 N NaOH or 0.1 N HCl were used to bring the medium's pH down to between 5.6 and 5.8. In varying amounts (Zu et al., 2006), growth-promoting substances such BAP, IAA, and IBA were introduced. This solution had been boiling until the agar was completely dissolved after adding 30 g/l sucrose and 0.8 g/l agar. Following chilling, around 50 milliliters of this medium were poured into 250 milliliter Borosil conical flasks, which were securely sealed with sterile, non-absorbent cotton plugs. These culture flasks with nutrient media were then autoclaved for 15 to 20 minutes at 121°C and 15 psi of pressure to steam sanitize them. Before being used for inoculation, these semisolid medium-filled culture pots were stored in a dark place at the ambient temperature (25±2°C) for a week.

3.3.2.2. Growth regulators

Auxins, namely indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA), along with cytokinins, specifically 6-benzyl amino purine (BAP), were the primary phytohormones employed in different concentrations and combinations within various media for the initiation and development of callus, shoots, roots, and *in vitro* propagation of *Zanthoxylum armatum*. To facilitate their usage, auxins were dissolved in a 0.5N NaOH solution, while cytokinins were dissolved in a dilute solution of 0.1N HCl or NaOH. The experiment employed an MS basal medium containing various components (Chang et al., 2002).

3.3.2.3. Culture condition

In test tubes with full-strength MS media supplemented with the necessary PGRs, the explants, obtained from the plants, were prepared as previously mentioned and were vertically implanted. The test tubes were put in a culture environment that was kept at a constant temperature of 25±1°C (McDonald et al., 2001). The culture room used a 8-hour dark cycle and an 16-hour light cycle, with low irradiance outside the culture tubes (42 IEm-2 s-1). Philips 40 W cool white fluorescent bulbs served as the lighting source. In every 35 to 42 days, the explants were regularly subculture.

3.4. Shoot induction, multiplication, rooting and acclimatization of plantlets

For the induction and proliferation of shoots, both half-strength and full-strength MS basal medium were utilized. Furthermore, MS medium was supplemented with varying concentrations of BAP, IAA, and IBA to enhance shoot development. To facilitate rooting of the *in vitro* shoots, a half-strength MS medium was employed, supplemented with 2 mg/L of both IAA and IBA. When the shoots reached a length of 2 cm or longer, they were transferred to the rooting medium.

3.4.1. Shoot proliferation and elongation

Surface-sterilized nodal explants were placed on two types of culture media: Murashige and Skoog's (MS) medium alone or MS medium supplemented with varying concentrations of BAP, IBA, and IAA. The objective was to induce the formation of shoots or shoot buds. After four weeks of culture, the nodal explants exhibited swelling and had elongated shoots, along with a large number of shoot buds when cultured on the optimal shoot induction medium, which was MS medium supplemented with 2.0 mg/l of BAP. These nodal explants showing promising growth were further subcultured by transverse cutting. This step aimed to facilitate shoot proliferation and elongation in subsequent culture stages (Nithianantham et al., 2011).

3.5. Statistical analysis

The statistical analysis was carried out using SPSS 26.0 for Windows. The results of this study's data collection were shown as mean and standard deviation. One-way ANOVA with Tukey's multiple comparisons test was employed for data analysis. A t-test was also used for the comparison between the samples. Statistical significance was defined as a p-value of 0.05 or less (Van Buren and Robinson, 1969; Amakura et al., 2000).

4. Result

4.1. Shoot regeneration

The effects of different Benzyl amino purine (BAP) and Indole-3-butyric acid (IBA) concentrations in Murashige and Skoog (MS) medium on the induction of shoots in *Zanthoxylum armatum* are shown in the table. The response rate, the number of shoots per explant, and the average length of shoots are among the characteristics that are evaluated.

Table 2. Effects of various concentration of BAP and IBA in MS media on *Zanthoxylum armatum* shoot induction

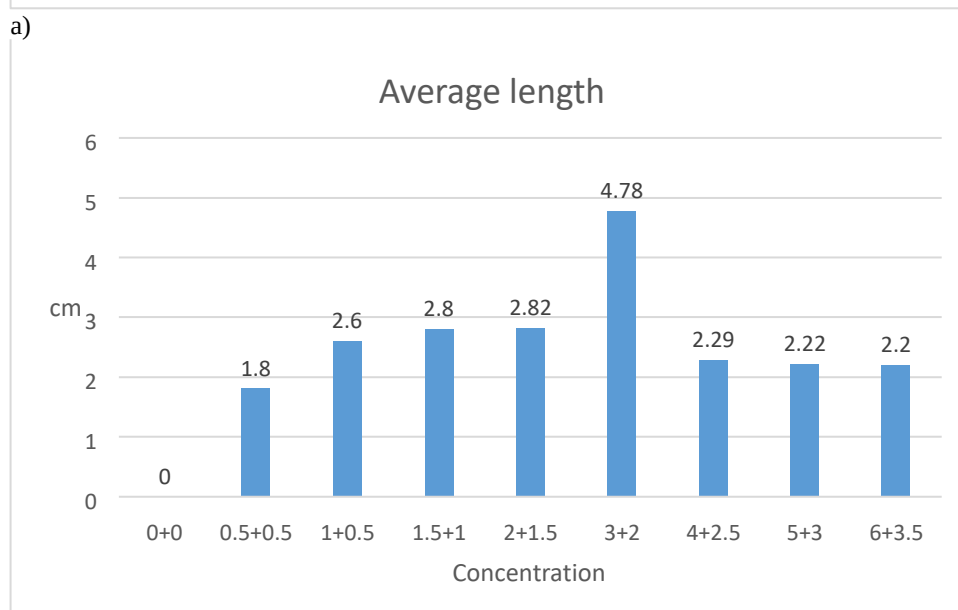
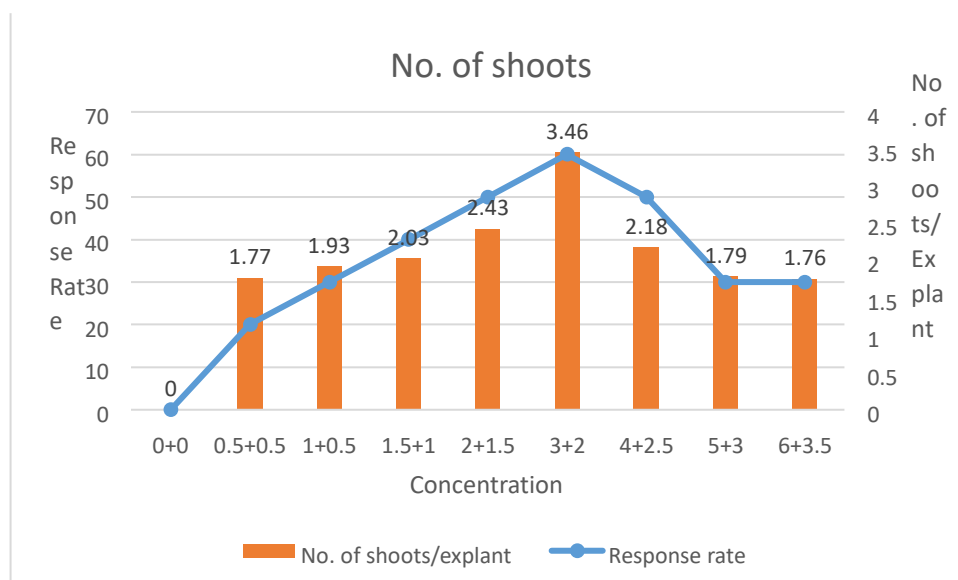
S.No	BAP (mg/L)	IBA (mg/L)	Response rate	No. of shoots/explant	Average length
1	0	0	0	0	0
2	0.5	0.5	20	1.77 ± 0.02 ^e	1.80 ± 0.23 ^f
3	1	0.5	30	1.93 ± 0.04 ^d	2.60 ± 0.13 ^c
4	1.5	1	40	2.03 ± 0.03 ^c	2.80 ± 0.28 ^b
5	2	1.5	50	2.43 ± 0.04 ^b	2.82 ± 0.13 ^b
6	3	2	60	3.46 ± 0.05 ^a	4.78 ± 0.18 ^a

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7	4	2.5	50	2.18 ± 0.03^c	2.29 ± 0.10^d
8	5	3	30	1.79 ± 0.03^e	2.22 ± 0.24^e
9	6	3.5	30	1.76 ± 0.01^e	2.20 ± 0.41^e

The response rate was 20% at the lowest BAP and IBA concentrations (0.5 mg/L each), with an average shoot length of 1.80 mm and an average of 1.77 shoots per explant. With an average shoot length of 2.60 mm and an average of 1.93 shoots per explant, the response rate increased to 30% as the concentrations of BAP and IBA were increased to 1 mg/L and 0.5 mg/L, respectively. A further increase in BAP and IBA concentrations to 1.5 mg/L and 1 mg/L, respectively, produced an average of 2.03 shoots per explant and 2.80 mm of average shoot length, yielding a 40% response rate. With an average of 2.43 shoots per explant and an average shoot length of 2.82 mm, the response rate increased to 50% at BAP and IBA concentrations of 2 mg/L and 1.5 mg/L, respectively.

At BAP and IBA concentrations of 3 mg/L and 2 mg/L, respectively, the maximum response rate of 60% was observed along with an average of 3.46 shoots per explant and an average shoot length of 4.78 mm. However, as BAP and IBA concentrations increased further, the response rate, average shoot length, and number of shoots per explant all decreased. Based on the response rate, average shoot length, and number of shoots per explant, the data indicates that 3 mg/L BAP and 2 mg/L IBA is the best concentration for inducing shoots in *Zanthoxylum armatum*. Statistically, a significant difference was observed in the number of shoots per explant and average length of the plant at various concentration ($P < 0.05$).



b)

Figure 2. Effect of plant hormone in various concentrations on *Zanthoxylum armatum* shoots bud, a) Number of shoots, b) average shoot length

4.2. Proliferation and multiplication of shoots

The effects of different concentrations of 6-furfuryl amino purine (Kinetin) and 6-benzyl amino purine (BAP) on the growth and culture of *Zanthoxylum*

armatum shoots in Murashige and Skoog (MS) medium are shown in the table. The average length of the shoots and the quantity of shoots per explant are two of the characteristics that are measured.

Table 3. Effects of various concentrations on *Zanthoxylum armatum* shoot proliferation and cultivation in MS media with a 6-benzyl amino purine (BAP) and 6-furfuryl amino purine (Kinetin).

S.No	BAP (mg/L)	Kinetin (mg/L)	No. of shoots/explant	Average length
1	0.0+0.0	0	0.0	0.0
2	1	0.5	3.91±0.49 ^f	2.09±0.16 ^g
3	1	1	4.24±0.38 ^e	3.04±0.13 ^f
4	2	0.5	6.36±0.48 ^d	4.07±0.38 ^c
5	2	1	8.25±0.61 ^b	4.54±0.34 ^b
6	4	0.5	9.42±0.29 ^a	5.06±0.16 ^a
7	4	1	8.78±0.35 ^b	4.57±0.44 ^b
8	6	0.5	7.57±0.46 ^c	3.34±0.21 ^d
9	6	1	6.28±0.29 ^d	3.15±0.20 ^e

After 28 days

At the lowest BAP and Kinetin concentrations of 1 mg/L and 0.5 mg/L, respectively, there were 3.91 on average shoots per explant and 2.09 mm on average shoot length. The average number of shoots per explant grew to 4.24, and the average shoot length increased to 3.04 mm, as the concentrations of BAP and kinetin climbed to 1 mg/L and 1 mg/L, respectively. An average of 6.36 shoots per explant and an average shoot length of 4.07 mm were obtained with a further increase in BAP and kinetin concentrations to 2 mg/L and 0.5 mg/L, respectively. The average shoot length increased to 4.54 mm and the average number of shoots per explant increased to 8.25 at BAP and kinetin concentrations of 2 mg/L and 1 mg/L, respectively. At BAP and kinetin concentrations of 4 mg/L and 0.5

mg/L, respectively, the longest average shoot length (5.06 mm) and the largest average number of shoots per explant (9.42) were noted. The overall number of shoots per explant and the average shoot length, however, decreased as BAP and kinetin concentrations increased higher. Statistically, a significant difference was observed in the number of shoots per explant and average length of the plant at various concentration ($P < 0.05$).

Based on average number of shoots per explant and length of shoot, the results indicates that 4 mg/L BAP and 0.5 mg/L Kinetin is the most effective concentration for shoot proliferation and culture in *Zanthoxylum armatum*. However, these characteristics decreased when BAP and kinetin concentrations increased further.

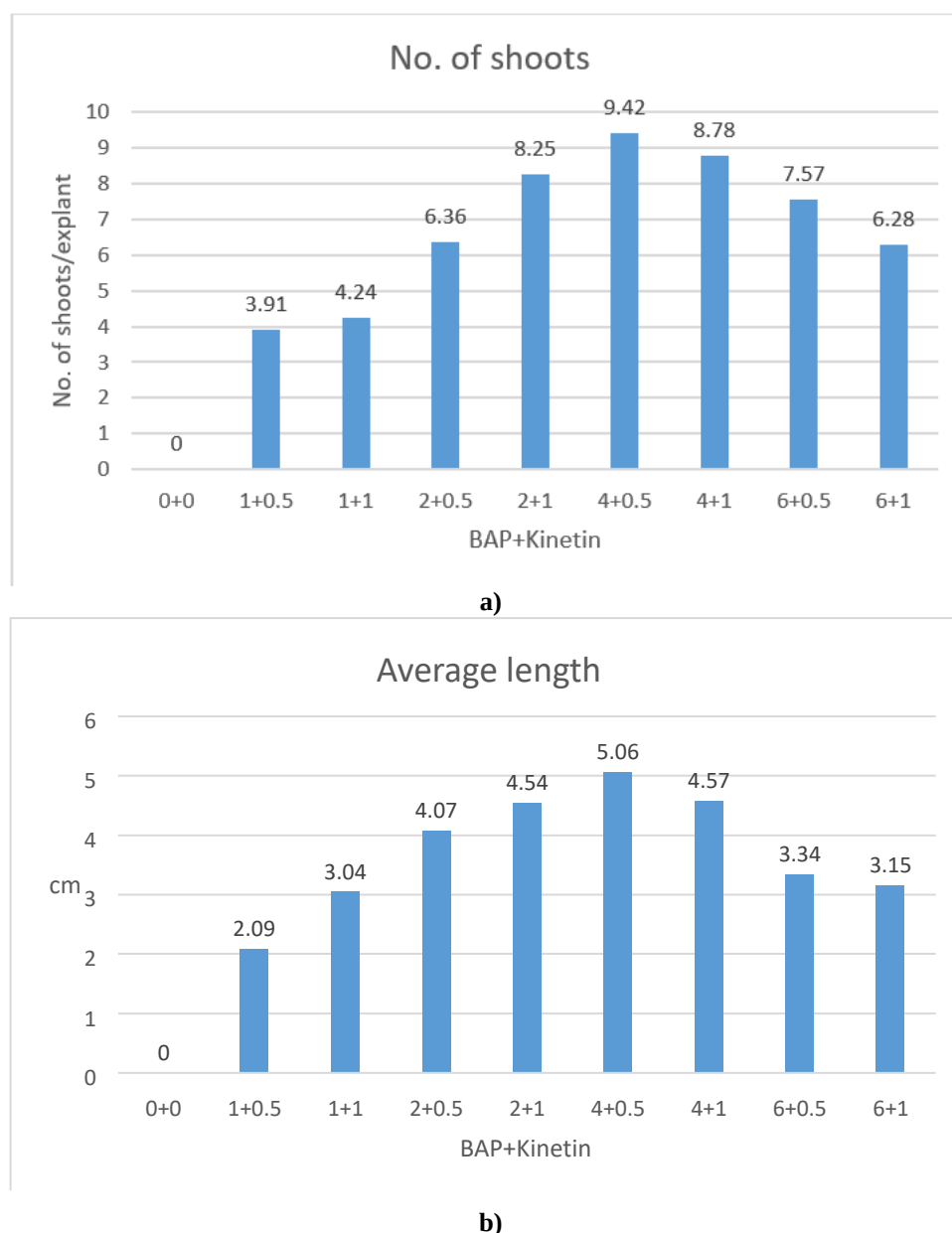


Figure 3. Effect of PGRs on shoot multiplication of *Zanthoxylum armatum* in varied concentrations of BAP and kinetin, a) Number of shoots, b) average shoot length

5. Discussion

The *in vitro* propagation of *Zanthoxylum armatum* has emerged as a crucial approach to address the challenges associated with its natural propagation. This study demonstrated that the application of plant growth regulators (PGRs) in varying concentrations significantly influences the regeneration, proliferation, and multiplication of *Z. armatum* shoots. These findings are consistent with previous studies that emphasize the role of PGRs in tissue culture systems for inducing desired morphogenetic responses.

5.1. Regeneration of Shoots

The data reveal that shoot regeneration is maximized with a combination of 3 mg/L benzylaminopurine (BAP) and 2 mg/L indole-3-butyric acid (IBA), resulting in a response rate of 60%, an average shoot length of 4.78 mm, and an average of 3.46 shoots per explant. These results align with prior research by

Singh et al. (2018), who reported that BAP, in combination with auxins like IBA, enhances shoot induction and elongation in *Zanthoxylum* species. The synergistic effect of BAP and IBA can be attributed to their roles in cell division and elongation, with BAP promoting cytokinin-mediated shoot induction and IBA facilitating root primordia formation (Sharma et al., 2020).

5.2. Proliferation and Multiplication

Shoot proliferation and multiplication were significantly influenced by the concentration of BAP and kinetin. The combination of 4 mg/L BAP and 0.5 mg/L kinetin yielded the highest proliferation rates, with an average of 9.42 shoots per explant and an average shoot length of 5.06 mm. However, higher concentrations of these PGRs resulted in reduced proliferation, possibly due to the inhibitory effects of supra-optimal cytokinin levels (Gupta et al., 2016).

These findings are in agreement with previous studies by Murashige and Skoog (1962), who highlighted the importance of balancing cytokinin and auxin concentrations for optimal shoot culture outcomes.

5.3. Statistical Significance and Optimization

The statistical analysis revealed significant differences in shoot regeneration and proliferation across various concentrations of PGRs ($p < 0.05$). This underscores the critical role of optimizing growth regulator combinations to achieve the desired results. Similar trends were observed in studies on other medicinal plants, where the precise concentration of PGRs determined the efficiency of in vitro propagation (Gupta et al., 2016).

5.4. Applications and Conservation Implications

The results of this study have significant implications for the conservation and commercialization of *Z. armatum*. The optimized protocol can be used for large-scale propagation, ensuring a sustainable supply of plant material for pharmaceutical, agricultural, and ethnobotanical applications. Moreover, *in vitro* propagation offers a viable solution for conserving this endangered species by reducing pressure on natural populations (Sharma et al., 2020).

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