

Optimization of Cytokinin and Auxin Applications for Effective In Vitro Micropropagation of *Ziziphus spina-Christi* (L.) Desf.

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Abstract

Ziziphus spina-christi (L.) Desf., a woody plant of considerable economic and ecological importance, continues to lack robust, standardized protocols for efficient in vitro multiplication. The present investigation was undertaken to optimize the hormonal regimen governing shoot proliferation and rhizogenesis in this species. Explants consisting of nodal segments were established on Murashige and Skoog (MS) basal medium fortified with graded levels of cytokinins and auxins, and cultured under standardized environmental parameters within a completely randomized experimental design comprising five replicates per treatment. Statistically significant variation ($p \leq 0.05$) emerged across the tested regimens. Superior performance in terms of bud initiation, shoot elongation, and overall biomass yield was recorded on medium supplemented with 1.0 mg L^{-1} zeatin, whereas root proliferation, root elongation, and subsequent survival following acclimatization were maximized using 1.0 mg L^{-1} indole-3-butyric acid (IBA). Collectively, these findings establish a reliable and reproducible micropropagation protocol with practical value for clonal mass propagation, ex situ germplasm conservation, and downstream biotechnological applications involving *Z. spina-christi*.

Keywords: *Ziziphus spina-christi*, in vitro, cytokinins, auxins, shoot multiplication, root induction

Introduction

Belonging to the family Rhamnaceae, *Ziziphus spina-christi* (L.) Desf. is an evergreen, perennial tree with a large number of ecological and economic values. It is naturally found throughout the Middle East, North and East Africa, and South Asia where it provides edible fruit, medicinal ingredients, animal fodder, fuelwood and shade cover to local communities. The tree is especially important for reforestation and rehabilitation of degraded land and for increasing climate-resilience in dry and semi-dry areas [1,2,3], due to its high resistance to drought, salinity, and high temperatures.

Despite these characteristics, there is still little information on ways to propagate this species on a large scale, either through traditional horticulture or tissue culture. Long dormancy periods, low seed germination and high genetic variability restrict propagation by seed and restrict rooting ability by vegetative propagation (cutting). Tissue culture thus becomes a useful alternative, providing genetically uniform, good quality planting material and conserving the genetic resources of the species [4,5,6].

One of the most critical factors in successful in vitro regeneration is plant growth regulators (PGRs). While auxins control cellular differentiation, vascular patterning and initiation of rooting,

cytokinins regulate cell division, axillary bud release, and shoot organogenesis. This balance between the two classes of hormones is an important factor in successful regeneration in woody species like *Z. spina-christi*. The different individual cytokinins, including 6-benzylaminopurine (BAP), kinetin (Kin), thidiazuron (TDZ) and zeatin, vary widely in their natural occurrence, biochemical activity, and shoot stimulating activity. Similarly, the other types of auxins, such as IBA, α -naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) exhibit varying degrees of effectiveness that are dependent on their nature, the source of the explants and culture parameters. The importance of correctly adjusting the ratio between cytokinin and auxin has been highlighted in contemporary literature, as it is essential for achieving strong shoot development, robust rooting, and successful acclimatization [1,7, 8].

[9] were the first to report successful regeneration of *Z. spina-christi* using shoot tips cultured in BAP-supplemented MS medium, while roots were obtained by growing shoot tips in half-strength MS medium containing NAA. This was followed by further studies which extended the practice of micropropagation with the species to an even greater extent.

Nevertheless, the PGR requirements of *Z. spina-christi* are not fully known. Comparative studies of the relative effectiveness of natural and synthetic cytokinins and auxins on shoot and root formation are limited and few studies have considered shoot regeneration, rooting and acclimatization in combination in a single experimental design.

Thus, the present study was aimed at assessing the effect of four cytokinins

[BAP, Kin, TDZ, Zeatin] and three auxins [IBA, NAA, IAA] on shoot multiplication, root induction and acclimatization performance of *Z. spina-christi*.

Material and Methods

Explant material was collected from 1.5–2.0 cm segments of vigorous and disease-free shoots from mature *Ziziphus spina-christi* trees in Kirkuk Province, Iraq and the segment with one axillary bud was used for initiation of culture. The excised material was washed under running tap water for 30 minutes before sterilization, with the aim of removing surface debris and contaminants, using a mild detergent solution. Disinfection of explants was then carried out aseptically in a laminar flow cabinet, which consisted of sequential immersion in 70% (v/v) ethanol for 30 seconds and 2% sodium hypochlorite solution with 0.1% Tween-20 for 15 minutes, followed by three successive rinses in sterile distilled water to remove any disinfectant residue before being placed on the culture medium [4,5].

The culture medium used was MS basal salts supplemented with 30 g L⁻¹ sucrose and 7 g L⁻¹ agar and the pH of which was adjusted to 5.8 ± 0.1 before autoclaving at 121°C and 1.1 kg cm⁻² for 20 min.

The cultures were incubated in a controlled environment growth chamber with a 16:8 light-dark photoperiod, using cool-white LEDs providing 40–50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 60–70% relative humidity (RH) and a temperature of 25 ± 2°C. The cultures were sub cultured onto fresh medium every 4 weeks as is common for woody plant species [1,4] to maintain adequate levels of

nutrients and hormones.

During the shoot multiplication stage, the MS basal medium was supplemented with four different cytokinins, namely 6-benzylaminopurine (BAP), kinetin (Kin), zeatin, and thidiazuron (TDZ), at different concentrations. The treatments consisted of a cytokinin-free control medium (0.0 mg L^{-1}) and three concentrations of BAP (0.5 , 1.0 , and 2.0 mg L^{-1}), kinetin (0.5 , 1.0 , and 2.0 mg L^{-1}), and zeatin (0.5 , 1.0 , and 2.0 mg L^{-1}). TDZ was incorporated at 0.1 , 0.5 , and 1.0 mg L^{-1} . Thus, a total of 13 cytokinin treatments were evaluated to determine their effects on shoot initiation, multiplication, elongation, leaf and node formation, and shoot biomass accumulation of *Ziziphus spina-christi* under in vitro conditions.

The different concentration ranges used for TDZ were intentional and were based on the substantially higher biological activity of TDZ compared with conventional adenine-type cytokinins such as BAP, kinetin, and Zeatin.

TDZ is a highly potent cytokinin-like regulator and is commonly evaluated at substantially lower concentrations because increasing its concentration excessively may result in undesirable physiological responses, including excessive shoot compactness, reduced shoot elongation, abnormal morphology, and other growth-related disorders.

Therefore, TDZ was evaluated at **0.1 , 0.5 , and 1.0 mg L^{-1}** , whereas BAP, kinetin, and Zeatin were evaluated at **0.5 , 1.0 , and 2.0 mg L^{-1}** . The purpose was not to compare identical numerical concentrations among all cytokinins, but rather to evaluate biologically relevant concentration ranges for each regulator.

Shoot regeneration was assessed after eight weeks by percentage of explants with shoot emergence, time to bud break, number of shoots per explant, shoot length,

number of leaves, number of nodes, and fresh and dry shoot weights.

During the rooting stage, the regenerated shoots were transferred to MS medium supplemented with three different auxins, namely indole-3-butyric acid (IBA), α -naphthaleneacetic acid (NAA), and indole-3-acetic acid (IAA), at three concentrations. A hormone-free medium (0.0 mg L^{-1}) was used as the control treatment. For each auxin, concentrations of 0.5 , 1.0 , and 2.0 mg L^{-1} were tested, resulting in a total of 10 rooting treatments. These treatments were used to evaluate the effects of auxin type and concentration on rooting percentage, time required for root initiation, number of roots per shoot, root length, fresh and dry root biomass, and subsequent plantlet survival during acclimatization.

The rooting frequency, the number of days to root emergence after plantlets were placed in the rooting medium, the number of roots per shoot, the mean and maximum root length, and fresh and dry root biomass were recorded six weeks after placing plantlets in the rooting medium.

Plantlets with roots were transplanted to plastic pots with peat-perlite substrate (1:1, v/v) during the acclimatization period and watered with sterile distilled water to remove any traces of agar. The pots were placed in clear polyethylene covers and, in a greenhouse, held at $25 \pm 2^\circ\text{C}$ and 70–80% relative humidity; the polyethylene covers were gradually removed over four weeks so the plants had time to harden off; the number of plants surviving eight weeks after the pots were removed was recorded.

The design was completely randomized with five replicate units and each replicate included five explants. Data were statistically analyzed by one-way analysis of variance (ANOVA) and mean separation was done by Duncan's Multiple

Range Test (DMRT) at 95% level of significance [10, 11].

The Results

The effect of the different cytokinin treatments on shoot regeneration is given in Table 1. All the cytokinin formulations increased shoot induction, vegetative expansion, multiplication potential and biomass accumulation compared to the untreated control but the level of response varied greatly among the treatments. The 1.0 mg L⁻¹ zeatin was the most effective among all the treatments examined, with the highest shoot induction rate (94.6%), number of shoots per explant (6.84), shoot elongation (5.82 cm), number of leaves per culture (15.74), number of nodes per culture (10.33), fresh shoot weight (2.82 g) and dry shoot weight (0.351 g). This treatment also advanced bud break as compared to the other treatments.

Higher zeatin concentration (2.0 mg L⁻¹) had only slight additional effects suggesting that the lower concentration was at the near-optimum level; however, zeatin at both concentrations did outperform all other cytokinins tested in this study. Similarly, the use of BAP at 1.0 mg L⁻¹ positively affected shoot proliferation while higher concentrations (2.0 mg L⁻¹) resulted in a reduction in shoot length, foliar production, and biomass accumulation, indicating an inhibitory effect at the high concentration. Kinetin not only promoted shoot induction but also biomass production, but its effectiveness was not as high as zeatin or BAP.

The control treatment always showed the lowest performance for all parameters measured. These results together demonstrate that the 1.0 mg L⁻¹ zeatin is the best cytokinin treatment for the in vitro micropropagation of *Z. spina-christi*.

Table 1. Effects of different cytokinins on shoot multiplication of *Ziziphus spina-christi* cultured in vitro.

Treatment	Shoot induction (%)	Days to bud break	Shoots explant ⁻¹	Shoot length (cm)	Leaves shoot ⁻¹	Node's shoot ⁻¹	Fresh weight (g)	Dry weight (g)
Control	38.50 f	18.40 a	1.20 f	1.45 e	3.10 f	2.12 f	0.42 f	0.051 f
BAP 0.5	72.30 d	13.20 c	3.65 d	3.42 c	8.25 d	5.14 d	1.24 d	0.152 d
BAP 1.0	88.40 ab	9.30 e	5.82 ab	4.65 ab	12.40 ab	8.41 ab	2.18 ab	0.281 ab
BAP 2.0	84.20 bc	10.10 de	5.31 b	4.21 b	11.62 b	7.82 b	2.02 b	0.264 b
Kin 0.5	63.70 e	14.10 bc	2.95 e	3.05 cd	7.45 e	4.63 e	1.03 e	0.130 e
Kin 1.0	74.60 d	12.4 cd	3.84 d	3.62 c	8.70 d	5.55 d	1.36 d	0.171 d
Kin 2.0	71.80 d	12.8 cd	3.46 de	3.55 c	8.12 de	5.30 d	1.29 d	0.164 d
Zeatin 0.5	82.70 c	10.20 de	5.12 bc	4.86 ab	12.60 ab	8.24 ab	2.10 b	0.273 b
Zeatin 1.0	94.60 a	7.4 f	6.84 a	5.82 a	15.74 a	10.33 a	2.82 a	0.351 a
Zeatin 2.0	90.80 ab	8.20 ef	6.35 a	5.41 a	14.92 a	9.86 a	2.63 a	0.331 a
TDZ 0.1	81.60 c	9.80 de	5.41 b	3.62 c	10.54 c	7.31 c	1.95 bc	0.245 bc
TDZ 0.5	86.30 b	9.50 e	5.92 ab	3.35 cd	10.11 c	7.05 c	1.90 bc	0.241 bc

TDZ 1.0	80.40 c	10.80 d	4.82 c	2.85 d	8.82 d	6.32 cd	1.71 c	0.216 c
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Means sharing no common letter within a given column differ significantly, as determined by Duncan's Multiple Range Test at the $P \leq 0.05$ probability level.

Rooting outcomes under the different auxin treatments are compiled in Table 2. Every auxin-supplemented medium exceeded the control in terms of root formation, biomass accumulation, and post-transfer survival. The 1.0 mg L⁻¹ IBA treatment delivered the strongest overall performance, producing the highest rooting percentage, a mean of 6.94 roots per shoot, a root length of 6.81 cm, fresh and dry root weights of 1.26 g and 0.162 g respectively, and the shortest interval to root initiation; plantlets subjected to this treatment achieved an acclimatization survival rate of 96.5%.

There was only a small difference between the 2.0 mg L⁻¹ IBA concentration and the effects of using even higher concentrations, showing a diminishing returns effect. NAA was more effective

than the control in promoting rooting but was not as effective as IBA overall, with lesser effects at concentrations greater than 1.0 mg L⁻¹. The control medium was not capable of adequate root development or survival, and IAA was the least effective of the three auxins tested, having the lowest values on each parameter measured.

Overall, the 1.0 mg L⁻¹ IBA treatment proved to be the best auxin for root induction and the establishment of successful plantlets of *Z. spina-christi*.

Table 2. Effects of different auxins on in vitro rooting of *Ziziphus spina-christi*.

Treatment	Rooting (%)	Days to root initiation	Roots shoot ⁻¹	Root length (cm)	Fresh root weight (g)	Dry root weight (g)	Survival (%)
Control	22.4 e	24.8 a	1.10 e	1.20 e	0.18 e	0.026 e	40.3 e
IBA 0.5	76.8 b	15.2 c	4.82 b	4.52 b	0.82 b	0.103 b	83.6 b
IBA 1.0	95.2 a	11.4 e	6.94 a	6.81 a	1.26 a	0.162 a	96.5 a
IBA 2.0	90.6 a	12.3 de	6.12 a	6.10 a	1.14 a	0.148 a	91.2 a
NAA 0.5	70.3 c	17.1 bc	3.91 c	3.85 c	0.70 c	0.091 c	77.4 c
NAA 1.0	78.8 b	15.9 c	4.43 bc	4.05 bc	0.76 bc	0.097 bc	80.6 bc
NAA 2.0	65.7 cd	18.4 b	3.34 cd	3.20 cd	0.62 cd	0.081 cd	72.5 cd
IAA 0.5	63.4 d	19.6 b	3.28 cd	3.52 c	0.58 d	0.076 d	70.4 d
IAA 1.0	69.5 c	17.8 bc	3.82 c	3.96 bc	0.67 c	0.087 c	76.8 c
IAA 2.0	58.6 d	20.5 b	2.95 d	3.18 cd	0.55 d	0.071 d	68.2 d

Means sharing no common letter within a given column differ significantly, as determined by Duncan's Multiple Range Test at the $P \leq 0.05$ probability level.

Discussion

The current study showed that the regeneration capacity of *Ziziphus spina-christi* was significantly improved by the addition of different cytokinin treatments

to the culture medium. Among the tested treatments, zeatin (1.0 mg L⁻¹) was the most effective cytokinin for shoot induction, multiplication, and biomass production. The group exposed to 1.0 mg L⁻¹ zeatin had the highest frequency of

induction (94.6%) , shoot elongation (5.82 cm), number of leaves (15.74), number of nodes (10.33) and output of biomass (2.82 g fresh weight, 0.351 g dry weight) and shortened

the bud break period, indicating a faster initiation of the shoot. The shoot induction, multiplication and biomass also increased with increasing zeatin concentration at 2.0 mg L⁻¹, although this was not significant compared to the 1.0 mg L⁻¹ concentration. On this basis, zeatin can be considered as the most effective cytokinin for the in vitro propagation of *Z. spina-christi* at 1.0 mg L⁻¹.

This superior response could be attributed to the unique biochemical properties of zeatin and its good binding affinity to the cytokinin receptors. Zeatin is a natural cytokinin and tends to be more effective in shoot organogenesis than synthetic cytokinins because it promotes balanced cell division without inducing the physiological problems that are often observed at high levels of synthetic PGRs. Additionally, zeatin has been linked to better chloroplast development, higher levels of photosynthesis, and more efficient nutrient assimilation, all of which lead to increased shoot biomass accumulation [12, 13].

BAP treatments also stimulated shoot proliferation, but not to the same extent as zeatin. An increase of BAP to 2.0 mg L⁻¹ reduced shoot length, leaf number and biomass production, suggesting that the 1.0 mg L⁻¹ level was the superior treatment for shoot proliferation, which aligns with previous studies that show that higher levels of BAP can negatively affect shoot quality (George et al., 2008; Phillips & Garda, 2019).

Kinetin significantly improved shoot induction, number of shoots per explant and biomass production though not to the

same extent as zeatin and BAP. TDZ also enhanced shoot induction and multiplication but generated shorter shoots as compared to zeatin and BAP. All the other treatments tested were superior to the control treatment for all the parameters measured.

In terms of rooting, the current study shows that 1.0 mg L⁻¹ IBA was the most effective auxin to induce roots, accumulate biomass and support acclimatization survival in *Z. spina-christi* plantlets. This treatment resulted in an increase in root number per shoot (6.94), root length (6.81 cm), fresh root weight (1.26 g) and dry root weight (0.162 g) and also reduced the time for the initiation of root formation. Moreover, IBA (1.0 mg L⁻¹) improved shoot acclimatization survival to 96.5%. Increasing the IBA concentration to 2.0 mg L⁻¹ also improved root induction and biomass production; however, the increases were marginal compared with those recorded for IBA (1.0 mg L⁻¹). Thus, it can be concluded that IBA (1.0 mg L⁻¹) was the most effective auxin for the in vitro propagation of *Z. spina-christi*.

IBA is a potent auxin with high physiological stability and has been reported to improve root induction, biomass production, and acclimatization survival of various woody plant species. It promotes root development by improving cell dedifferentiation, vascularization, and root primordia formation while reducing the risk of tissue necrosis and callus formation [5,14].

Conclusions

The current study proved that the success of in vitro regeneration of *Ziziphus spina-christi* was dependent on the type and concentration of plant growth regulators. Out of the tested cytokinins, Zeatin 1.0 mgL⁻¹, was found to be the most efficient regulator for shoot induction, proliferation,

shoots elongation, leaves production, biomass, and multiplication of the tested plant species. For the rooting process, IBA 1.0 mgL^{-1} resulted in the highest percentage of roots, root architecture, root biomass, and the highest acclimatization survival, which suggests that IBA 1.0 mgL^{-1} can be considered the best choice for growing strong plantlets with high chances of survival after acclimatization in the greenhouse.

The current study proved that it is important to optimize cytokinin and auxin treatments to achieve the highest rates of regeneration in this particular species. This technique can be applied for the rapid propagation of *Ziziphus spina-christi* and for the conservation of this economically important species.

The current research has implications in commercial production of *Ziziphus spina-christi*, as well as in other biotech industries involving plant tissue culture, including genetic transformation, gene editing, conservation of endangered plant species, and large-scale production of planting material suitable for agroforestry and ecological restoration in the semi-arid region.

Study Limitations

The current study aimed to optimize the effect of different cytokinin and auxin treatments on the in vitro regeneration of *Ziziphus spina-christi*. Therefore, the effect of combined growth regulator treatments was beyond the scope of this study. Further research should focus on exploring the synergistic effects of various plant growth regulators, testing the method on other genotypes of *Ziziphus spina-christi*, as well as evaluate genetic stability and field performance of the regenerated plants.

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