



Overcoming dormancy, and establishment of a micropropagation protocol to conserve *Ziziphus spina-christi*

Mustapha Haruna¹ · Muhammad Nakhoda² · Shakira Shaik¹

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Abstract

Ziziphus spina-christi is a multipurpose wild tree that has been described as endangered in Nigeria and Saudi Arabia due to anthropogenic activities, seed dormancy, and ineffective vegetative propagation methods. The present study aimed to develop efficient seed germination and micropropagation protocols for conservation of *Z. spina-christi*. The effects of seed pre-treatments and environmental conditions on seed germination were investigated via conventional germination methods. Removal of the seed coat improved germination from 46.6 (control) to 86.6% after 28 days of sowing. Watering the soil every day resulted in significantly lower germination (53.3%) than watering on alternate days (100%) or every four days (86.6%). The optimal temperature for germination was between 21 and 35 °C (86.6%). In the micropropagation experiments, the highest number of shoots/explant (5) was achieved through node culture when the second node was treated with 2 mg L⁻¹ zeatin. Rhizogenesis was only achieved when the basal cut ends of shoots were dipped in 200 mg L⁻¹ indole-3-butyric acid for one hour, resulting in 80% rooting and 4 roots/shoot. Rooted shoots were acclimatised in a sterile potting mix: vermiculite (1:1) with 100% plantlet survival in the greenhouse. Plantlet regeneration via somatic embryogenesis was unsuccessful as embryo induction with 1 mg L⁻¹ each of 2,4-dichlorophenoxyacetic acid, picloram, indole-3-acetic acid, and naphthalene acetic acid inhibited embryo development on maturation media, attributable to the persistence of exogenous auxins in the tissue. This study provides cost-effective methods for conserving *Z. spina-christi* through seed propagation and also offers the alternate conservation approach of clonal propagation via node culture.

Key message

Efficient protocols for seed germination and in vitro propagation through node culture have been developed for *Ziziphus spina-christi*. The work opens new pathways for the propagation and conservation of *Z. spina-christi*.

Keywords Rhamnaceae · Seed germination · Clonal propagation · Plant growth regulators · Explant position

Abbreviations

AdS Adenine sulphate
 BAP Benzylaminopurine
 CH Casein hydrolysate

CIM Callus induction medium
 FGP Final germination percentage
 G Globular
 GH Globular-heart
 IAA Indole-3-acetic acid
 IBA Indole-3-butyric acid
 MS Murashige and Skoog (1962) medium
 NAA Naphthalene acetic acid
 NaOCl Sodium hypochlorite
 NH₄ N-Ammonium-nitrogen
 PGR Plant growth regulators
 PE Proportion of embryos
 SS Simple sugars (D-glucose, galactose, and arabinose)
 TDZ N-phenyl-N-1, 2, 3-thiadiazol-5-ylure

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✉ Shakira Shaik
shaiksh@ukzn.ac.za

¹ School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Private Bag X54001, Durban 4000, South Africa

² Department of Biotechnology and Consumer Sciences, Cape Peninsula University of Technology, Cape Town, South Africa

ZiP-6 γ - γ -dimethylallylaminopurine
 2,4 D-2,4 dichlorophenoxyacetic acid

Introduction

Ziziphus spina-christi (Rhamnaceae) is native to semi-arid regions of sub-Saharan Africa and the Middle East (Hutchinson and Dalziel 1972), and occurs as a shrub (*Z. spina-christi* var. *microphylla*) or a tree (*Z. spina-christi* var. *spina-christi*) (Hutchinson and Dalziel 1972). The primary distinguishing feature between the two forms is the fruit colour at the ripening stage, the shrub form has red fruit while the tree form has reddish-brown fruit (Saied et al. 2008a). Although natural populations of *Z. spina-christi* occur in the wild, cultivation of the plant in home gardens to derive economic benefit has been reported in Egypt and Sudan (Saied et al. 2008a), Nigeria (Hutchinson and Dalziel 1972), Iran and Ethiopia (Orwa et al. 2009), and Saudi Arabia (Natho 1991).

Ziziphus spina-christi is a multifunctional plant; its fruits are consumed raw or used to produce fine flour, the leaves are used as livestock feed for camels and goats, and the wood is used for fuel and furniture (Saied et al. 2008a). In traditional medicine, different parts of the plant are also used to treat various ailments, including pulmonary diseases, rheumatism, intestinal spasm, headaches, diarrhoea, and snake bites (Saied et al. 2008a). Despite its economic importance, natural populations of the species are reportedly declining in Nigeria (Danjuma and Abubakar 2017) and Saudi Arabia (Adgaba et al. 2013), the primary threats being overexploitation and land clearing for agricultural or construction purposes (Adgaba et al. 2013; Danjuma and Abubakar 2017; Wakawa et al. 2017). The lack of concerted efforts towards *in situ* and *ex situ* conservation programmes exacerbates the extinction risk of *Z. spina-christi*.

Although plants can be propagated via sexual (through seeds) or asexual (through cuttings) methods (Mazri et al. 2022), the objective of the propagation determines the method employed. The sexual method is the preferred route for conservation due to its capacity to maintain genetic diversity (Pence 2011). However, several factors, including seed dormancy, scarcity, and specific germination requirements, can limit propagation success by seeds (Maeda et al. 2021). Hence, for successful plant conservation, it is essential to understand the factors affecting seed germination and to establish strategies to overcome them. *Ziziphus spina-christi* possesses a hard seed coat (Saied et al. 2008a) that imposes physical dormancy, requiring scarification to facilitate imbibition and gas exchange that trigger germination. For example, Saied et al. (2008b) reported high seed germination of *Z. spina-christi* (91%) when seeds were

treated with 97% (v/v) sulphuric acid (H_2SO_4). However, scarification with concentrated acids poses health risks and is undesirable as a commercial propagation method. A safer and more cost-effective method to remove the physical dormancy should be sought by testing a range of pre-germination treatments (excluding acids). Other environmental factors that may affect germination, such as watering frequency, light conditions and temperature have not yet been investigated in *Z. spina-christi*, which grows naturally in dry (semi-arid or desert) areas with low rainfall and alternating low and high temperatures (Saied et al. 2008a).

The most popular asexual propagation technique is through stem or leaf cuttings, especially to mass-produce clones of elite, uniform genotypes, suitable for commercial propagation. However, this method can be limited by the difficulty to induce adventitious roots (Mazri et al. 2022). In *Z. spina-christi*, for example, Assareh and Sardabi (2005) achieved only 23% adventitious root formation from stem cuttings without rooting promoters. Hence, the role of plant growth regulators (PGR) that promote rooting, such as the auxins, remains to be investigated for this species. Maintaining cuttings is, however, resource-intensive, requiring significant land and water use (Pence et al. 2020). The alternative is to explore mass propagation systems for *Z. spina-christi*, such as through *in vitro* culture systems, as it affords a greater degree of control over propagation conditions, and allows for a better understanding of the physiological requirements of germination and propagation of the species. An added advantage of the *in vitro* route is that it is an important vehicle in preserving endangered plant species and conserving elite and commercially-important genotypes (Engelmann 2011; Pence et al. 2020).

Micropropagation can be achieved through two morphogenic routes viz. organogenesis or somatic embryogenesis, which can proceed directly from the explants or indirectly through an intervening callus phase (George and Debergh 2008). Direct organogenesis involves shoot initiation and multiplication from a pre-existing meristem using cytokinins, followed by root induction using auxins under the appropriate culture conditions (George 2008). In contrast, somatic embryogenesis is the process of inducing somatic cells to differentiate into embryogenic cells, which then undergo various morphological and biochemical changes to form somatic embryos that eventually develop into complete plants (Ammirato 1987). The micropropagation of *Z. spina-christi* via organogenesis using shoot tips and nodes as explants has been reported, but with low yields: 0.1–1 shoot per explant (Al-Sulaiman 2010; Al-Sulaiman and Barakat 2010; Sudharsan and Hussain 2003) and 4 shoots per explant (Hegazi et al. 2016). The improvement in shoot yield in the latter was attributed to using 80 mg L⁻¹ adenine sulphate in the multiplication medium instead of PGR

(Hegazi et al. 2016). However, this finding contradicts that of Al-Sulaiman (2010), who found 0.4 shoots per explant using the same medium composition for shoot multiplication. Therefore, further understanding of shoot multiplication in this species, is also required.

Given the challenges with established propagation methods for *Z. spina-christi*, the aims of the present study were two-fold. The first aim was to improve conventional seed germination through an understanding of the physical and physiological barriers to germination. The effects of pre-germination treatments, watering frequency, temperature, and light conditions on germination were investigated. The in vitro method for seed germination was also investigated as an alternative to the conventional method. The second aim was to understand the in vitro requirements for node culture and indirect somatic embryogenesis in *Z. spina-christi*, for commercial propagation and for the preservation of elite genotypes. As a result, the effects of different types and concentrations of PGR, and media components on shoot induction and multiplication, root induction, and somatic embryo induction, were investigated.

Materials and methods

Source of seeds

Ripe fruits of *Z. spina-christi* were purchased every January (2017–2022) from Yar’kutungu market, Katsina, Nigeria (13°0'15.85"N; 7°36'19.15"E). Each time, fruits were purchased from three different sellers and combined to form a genetically diverse composite sample of the natural population. The identity of the plant specimen was confirmed at the herbarium of the Botany Department, Ahmadu Bello University Zaria, Nigeria (ABU01622). Seeds were obtained by removing the pulp of ripe fruits, followed by washing with tap water, sun-drying for two days and maintaining in a room at ambient temperature (23–25 °C) until use. Permits were obtained from the Nigeria Quarantine Service, and the Department of Agriculture, Forestry and Fisheries, South Africa, to transport the seeds across international borders. The seeds were transported under ambient conditions by flight to the University of KwaZulu-Natal, Durban, South Africa, for laboratory work. Germination was done in a greenhouse and under in vitro conditions. A flowchart of the experimental design is presented in Fig. 1.

Seed germination

Effect of seed pre-treatments

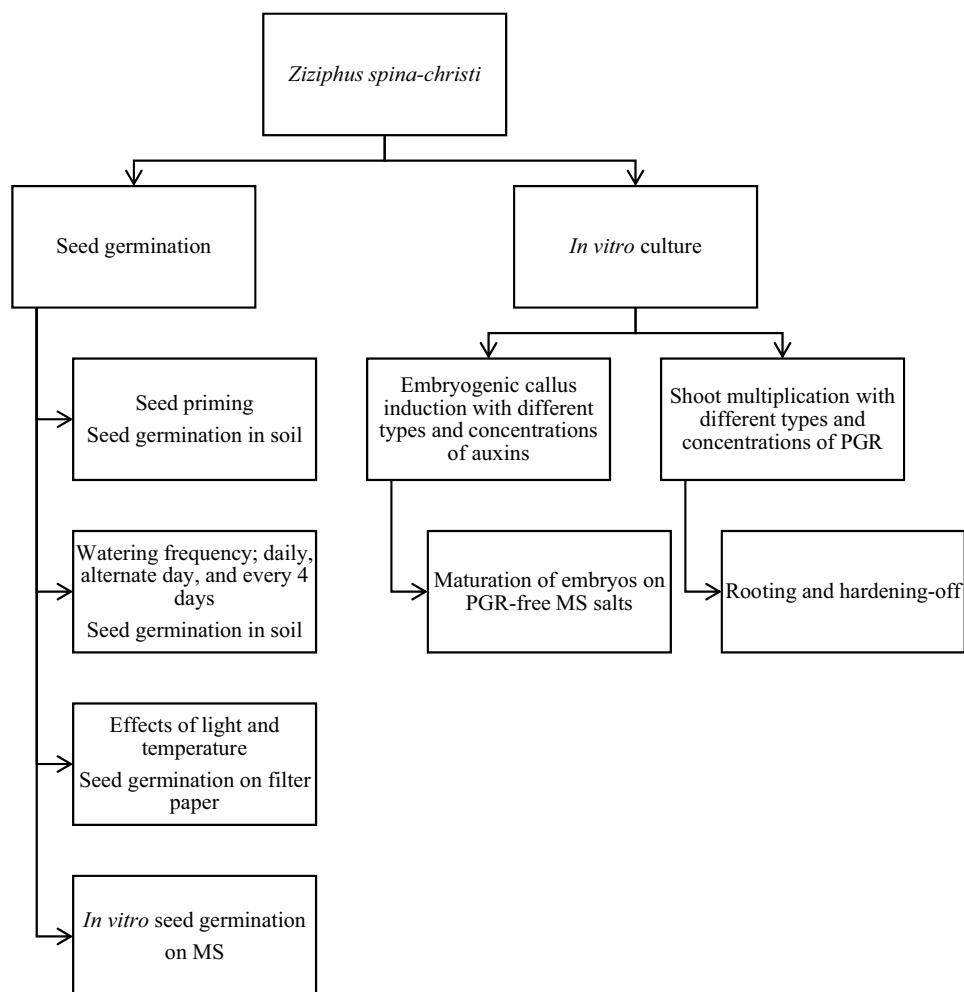
Seeds were subjected to different pre-germination treatments before planting out. The treatments included: (1) removal of the seed coat with a hammer (referred to as de-coated seeds in subsequent sections); (2) soaking seeds in tap water and shaking for 24 h on an orbital shaker (New Brunswick Scientific, USA) at a low speed (83 rpm) in 16-h light ($37 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8-h dark photoperiod in a growth room, at 25 and 21 °C, respectively; (3) soaking seeds in tap water and shaking for four days under the same conditions as in treatment 2; (4) incubating seeds in moist sand for three weeks at 10 °C in the dark to investigate physiological dormancy in the seeds; and (5) control (untreated seeds). At the end of the priming treatment, seeds were removed from the water or sand, rinsed under running tap water, and then surface dried with paper towel. Afterwards, ten seeds from each treatment were sown in 23 cm (length) x 15 cm (diameter) x 6 cm (depth) seedling trays (Plasgrow, South Africa) containing potting mix (Grovida, South Africa). Seedling trays were kept in a greenhouse (uncontrolled environment) in summer (February to March) at the University of KwaZulu-Natal Westville Campus (29°52'S; 30°59'E; 25 °C day/18°C night) and watered three times (120 ml per cycle) a day for three minutes each by an automatic water sprinkler using municipal water. A seed was considered to have germinated when a shoot emerged from the soil (≥ 1 cm). The number of germinating seeds was recorded every two days for 42 days, and there were three replicates per treatment.

Effect of watering frequency

De-coated seeds were sown in 23 cm (length) x 15 cm (diameter) x 6 cm (depth) seedling trays containing potting mix and placed in a greenhouse during summer (February to March). The soil in the seedling trays was watered to field capacity (well-watered) once every day, on alternate days or every four days (as indicated), using municipal water. A total of 15 seeds and four replicates were used for each treatment. A seed was considered to have germinated when a shoot emerged from the soil (≥ 1 cm.). Germination percentages were recorded following five weeks of initial sowing.

Effects of light and temperature

De-coated seeds were decontaminated in a laminar flow hood with 1% (v/v) sodium hypochlorite (NaOCl) (Reckitt Benckiser, South Africa) for 20 min, followed by three rinses in sterile distilled water, each for less than a minute. Seeds were then germinated on sterile filter paper (Filterking,

Fig. 1 A flowchart of the experimental design

India) in a 9 cm Petri dish. The filter paper was moistened with sterile distilled water (1–2 ml) and remoistened every four days. For the light experiment, three plates each containing 15 seeds were maintained in a growth room in 16-h light ($37 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8-h dark photoperiod, at 25 and 21 °C, respectively, or in the dark at ambient temperature (21 °C). In the temperature experiment, four Petri dishes with 15 seeds were used per treatment. The Petri dishes were incubated in plant growth chambers (GC-300TLH, JEIO TECH, Korea) which were set at different temperatures of 15, 25, 35, and 40 °C and in a growth room at ambient temperature (25 °C day/21 °C night) in 16-h light/8-h dark. A seed was considered germinated by the protrusion of a radicle ≥ 1 cm (Bewley 1997).

Chemicals and culture conditions

Unless otherwise stated, all chemicals and PGRs used in the present study for in vitro experiments were sourced from Sigma-Aldrich (USA). The pH of all in vitro culture media (for seed germination and in vitro stages) was adjusted to

5.6–5.8 before autoclaving at 121 °C and 1 kPa for 20 min. Additionally, cultures were kept in 16-h light ($37 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8-h dark photoperiod in a growth room, at 25 and 21 °C, respectively.

In vitro germination

Seeds were decontaminated as described previously. Seeds were cultured on a germination medium in 9 cm (height) x 2 cm (diameter) glass culture tubes (one seed per tube) containing 10 ml of half-strength Murashige and Skoog (1962) (MS) salts and vitamins, 10 g L⁻¹ sucrose, and 8 g L⁻¹ agar. After four weeks, seedlings were transferred onto 50 ml fresh medium in 9.6 cm (height) x 7.6 cm (diameter) Magenta™ (Bioworld, USA) culture vessels (one seedling per Magenta vessel) for a further four weeks. In this experiment, twenty seeds were used in each of four replicates.

Shoot induction and multiplication from in vitro nodal explants

Nodal segments from eight-week-old in vitro seedlings were used as explants for shoot induction and multiplication. All cultures were initiated in 9 cm (height) x 2 cm (diameter) glass culture tubes containing 10 ml of shoot induction and multiplication medium for four weeks, followed by subculture onto 30 ml fresh media in 8 cm (height) x 5 cm (diameter) glass culture bottles for another four weeks. Single nodes were excised from in vitro germinated shoots (approximately 1 cm long) and placed as one node per tube on MS salts and vitamins, 30 g L⁻¹ sucrose, and 8 g L⁻¹ agar for shoot induction and multiplication, along with the PGR listed below. Three experiments were conducted with different types, concentrations, and combinations of PGR designated E1, E2, and E3, as described below:

E1=PGRs were tested at 2, 3, and 5 mg L⁻¹ for 6-furfuryl-aminopurine (kinetin), 6-benzylaminopurine (BAP), and 6- γ -dimethylallylaminopurine (2iP), as well as 0.1, 0.5 and 1 mg L⁻¹ for N-phenyl-N-1, 2, 3-thiadiazol-5-ylurea (TDZ; Sigma-Aldrich, Switzerland); $n=25$.

E2=zeatin was tested at 0.2, 1 and 2 mg L⁻¹; $n=15$.

E3=2 mg L⁻¹ zeatin with or without 0.2 mg L⁻¹ indole-3-acetic acid (IAA) was tested on the first and second nodal explants from four-week-old in vitro seedlings; $n=25$. This experiment (E3) had four replicates.

Rooting and acclimatisation

Two methods of root induction (in vitro and *ex vitro*) were investigated using 25 and 45 shoots, respectively. In each method, eight-week-old in vitro shoots (about 3 cm long) from the shoot multiplication stage (E3, without IAA in the medium) were used. In vitro rooting media contained full-strength MS salts, 30 g L⁻¹ sucrose, 8 g L⁻¹ agar, and 0.1, 0.5, 1, or 2 mg L⁻¹ indole-3-butyric acid (IBA). The medium without auxin served as the control. Rooting protocols reported for *Ziziphus jujuba* using 0.1 mg L⁻¹ IAA (Melyan et al., 2013), and a combination of 2 mg L⁻¹ IBA, 0.5 mg L⁻¹ naphthalene acetic acid (NAA) and 3 g L⁻¹ activated charcoal (Soliman and Hegazi 2013), were also tested in the present study. For the *ex vitro* root induction, the basal cut ends of shoots were dipped in distilled water (control), 100 or 200 mg L⁻¹ IBA for one hour in the light. Thereafter, the shoots were transferred to 5.5 cm (diameter) x 5 cm (depth) plastic pots with sterile potting soil (nutrient-rich substrate): vermiculite (1:1) for *ex vitro* rooting and acclimatisation. The pots were covered in transparent plastic bags and kept in a plant growth room in 16-h light (37 μ mol m⁻² s⁻¹)/8-h dark photoperiod, at 25 and 21 °C, respectively. The shoots were occasionally sprayed with distilled water to maintain

humidity. After two weeks, the bags were punctured to gradually reduce the humidity, and at the end of the fourth week, the bags were removed. Afterwards, the plants were transferred into 10 cm (diameter) x 8.3 cm (depth) plastic pots containing potting soil and kept in a greenhouse, where they were watered three times a day for three minutes each by an automatic water sprinkler using municipal water, for four weeks.

Embryogenic callus initiation from in vitro leaf or cotyledon explants

For embryogenic callus induction and embryo maturation, 9 cm diameter Petri dishes and 26 ml of media were used throughout.

The embryogenic capacities of eight-week-old in vitro leaf and four-week-old in vitro cotyledon explants were investigated. Explants were excised across the midrib with a size 24 carbon steel surgical blade into 1 cm² sections and then cultured on callus induction medium (CIM) containing MS salts and vitamins, 30 g L⁻¹ sucrose or modified carbohydrate source as mentioned below, 10 g L⁻¹ agar, and PGR. Four explants were cultured per Petri dish and there were 10 Petri dishes per treatment. To establish the best medium for embryogenic callus induction, different types and concentrations of auxins and nutrients were tested in the CIM.

For embryogenic callus induction, three experiments were conducted. In the first experiment, leaf explants were cultured on: (1) 1 mg L⁻¹ 2, 4-D; (2) 3 mg L⁻¹ 2, 4-D; (3) 5 mg L⁻¹ 2, 4-D; (4) 1 mg L⁻¹ 4-amino-3, 5, 6-trichloropicolinic acid (picloram); (5) 5 mg L⁻¹ picloram; (6) 1 mg L⁻¹ IAA; and (7) 1 mg L⁻¹ NAA, for eight weeks without subculture. In the second experiment, leaf explants were cultured on MS modified with: (1) carbohydrate source (30 or 60 mg L⁻¹ sucrose, and 5 g/L each of D-glucose, galactose, and arabinose); (2) organic nitrogen source (0.5 mg L⁻¹ casein hydrolysate, and 20 or 60 mg L⁻¹ adenine sulphate); and (3) ammonium nitrogen (NH₄-N). All media contained 1 mg L⁻¹ 2, 4-D, and the culture period was reduced from eight weeks to six weeks with or without subculture at four weeks. The third experiment involved culturing cotyledon explants on 10 mg L⁻¹ 2, 4-D for four days followed by transfer onto MS medium without PGR, 0.5 mg L⁻¹ 2, 4-D or 1 mg L⁻¹ 2, 4-D for a further six weeks, or on 0.5 or 1 mg L⁻¹ 2, 4-D for six weeks without acute treatment on 10 mg L⁻¹ 2, 4-D. All cultures were kept in the dark in a growth room at ambient temperature (21 °C) for embryogenic callus induction.

Maturation of embryos

Following eight weeks (first experiment) or six weeks (second experiment) on CIM testing the effect of exposure duration to exogenous auxins, embryogenic calli were transferred to the maturation medium (MS salts with vitamins, 30 g L⁻¹ sucrose, and 10 g L⁻¹ agar) in Petri dishes (4 pieces/plate) for embryo development. Cultures were maintained for eight weeks in 16-h light (37 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/8-h dark photoperiod, at 25 and 21 °C, respectively.

Microscopy

Embryo morphology was observed using a stereomicroscope (NIKON AZ100; Tokyo, Japan) at the end of the six-and-eight-week callus induction periods. The stages (globular, and globular-heart) of embryo development were recorded based on the estimation of the microscopic field of view and presented as proportions in the results. Squash preparations were stained with 2.5% methylene blue and observed using NIKON ECLIPSE 80i microscope (Melville, USA).

Statistical analyses

Statistical analyses were performed with IBM Statistical Package for the Social Sciences (SPSS) software, version 25 (IBM Incorporation, USA). Percentage data were subjected to arcsine transformation before statistical analyses. A normality test was performed for all data types using a one-sample Kolmogorov-Smirnov (K-S) test ($p < 0.05$). Afterwards, data were subjected to the one-way analysis of variance (ANOVA) or paired samples t-test where appropriate. When the ANOVA indicated a significant difference, a Tukey's Honest Significant Difference (HSD) test was used

to compare the means at a 95% confidence interval. Significant differences among treatments were indicated by different letters assigned to the means.

Results

Seed germination

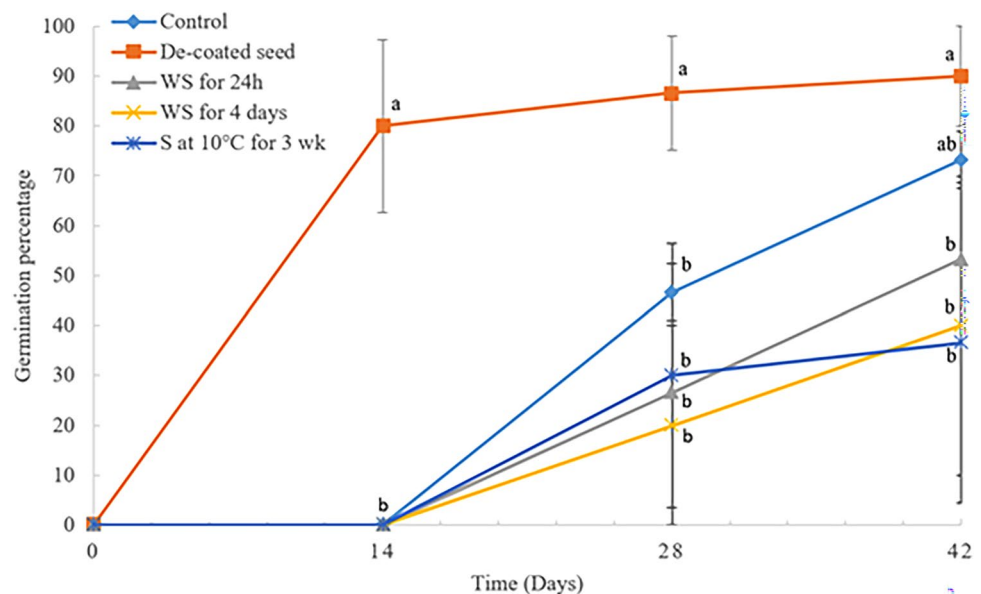
Effect of seed pre-treatments on germination

Removing the seed coat resulted in 80% germination within 14 days, whereas in other treatments and the control, seed germination started after 14 days resulting in 20–46.6% germination after 28 days. After 42 days, the final germination percentage (FGP) for de-coated seeds (90%) was not significantly different to the control (73.3%) but was different to those of the other priming treatments (36.6–53.3%) (Fig. 2). It was observed that, regardless of treatment, no further seed germination occurred after 42 days from initial sowing.

Effects of watering frequency, light and temperature on germination

The FGP was the lowest (53.3%) when soil was watered every day when compared with watering the soil on alternate days (100%) or every four days (86.6%) (Fig. 3). Germination in the light doubled the time to reach the FGP (91%) from seven days to 14 days. However, at 14 days, there were no significant differences in the FGPs between the light and dark treatments (Table 1). Seeds incubated at 25 and 35 °C reached maximum germination at day 7 (85 and 87%, respectively). During this period, germination was either inhibited at 15 °C (0%) and 40 °C (5%), or was

Fig. 2 Germination time-course of primed *Ziziphus spina-christi* seeds. Values describe mean $\pm$ standard deviation, means with different letters are significantly different (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, $n = 10$). WS = Water soaked, MS = Moist stratification, h = hour, wk = week



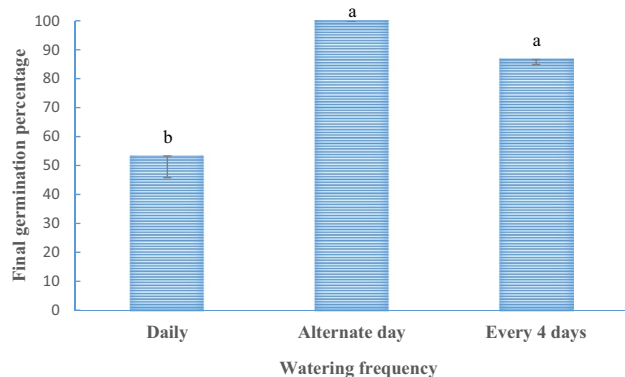


Fig. 3 Effect of watering regime on seed germination after five weeks of seed sowing. Mean $\pm$ standard deviation within columns followed by different letters denote significant differences amongst treatments (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, $n = 15$)

Table 1 Effect of light and temperature on germination of de-coated seeds on filter paper

Treatment	Germination (%)	
	Day 7	Day 14
<i>Light condition</i>		
16-h photoperiod	48.8 $\pm$ 3.8 ^b	91 $\pm$ 3.8 ^a
Dark	91 $\pm$ 3.8 ^a	91 $\pm$ 3.8 ^a
<i>Temperature (°C)</i>		
15	0 $\pm$ 0.0 ^c	65 $\pm$ 15.7 ^{ab}
25	85 $\pm$ 8.3 ^a	85 $\pm$ 8.3 ^a
35	87 $\pm$ 13.3 ^a	87 $\pm$ 13.3 ^a
40	5 $\pm$ 0.8 ^c	42 $\pm$ 23.3 ^b
25/21 (16-h light/8-h dark)	48.8 $\pm$ 3.8 ^b	91 $\pm$ 3.8 ^a

Mean $\pm$ standard deviation within columns followed by different superscript letters are significantly different ($p \leq 0.05$, $n = 15$); paired samples t-test for the light condition, and one-way ANOVA, post hoc Tukey test for the temperature treatment

moderate at 25/21°C (16-h light/8-h dark) (48.8%). At 14

days, seeds incubated at 40 °C had significantly lower FGP compared to those incubated at 25 and 35 °C and 25/21°C (16-h light/8-h dark) (Table 1).

In vitro germination

The FGP in vitro (96%) was achieved following two weeks of culture on half-strength MS salts (results not shown). Additionally, the cultures were devoid of contamination when seeds were decontaminated with 1% (v/v) NaOCl for 20 min. Thus, the protocol was adopted for subsequent in vitro seed germination to produce explants for in vitro organogenesis.

Shoot induction and multiplication from in vitro nodal explants

Among the cytokinins tested in the initial experiment, only 3 mg L⁻¹ kinetin significantly promoted shoot multiplication (2 shoots/explant), whereas high BAP concentrations (3 and 5 mg L⁻¹) and TDZ (0.1, 0.5 and 1 mg L⁻¹) promoted callus formation instead of shoots (Table 2). A less-stable cytokinin, zeatin, was tested at 0.5, 1 and 2 mg L⁻¹ to preclude callus formation and improve shoot multiplication. It was found that 2 mg L⁻¹ of zeatin significantly increased the number of shoots per explant from 1 to 4 (results not shown). Having established that, the effect of node position on the parent plant on shoot multiplication was investigated using 2 mg L⁻¹ zeatin with or without 0.2 mg L⁻¹ IAA. The first and second nodal explants were obtained from four-week-old in vitro seedlings having two nodes. The combination of 2 mg L⁻¹ zeatin with 0.2 mg L⁻¹ IAA significantly decreased the number of shoots per explant, irrespective of the node position on the parent plant, first node (3 to 2), and second node (5 to 3). The results also showed that the second node was the best explant for multiple shoot formation compared

Table 2 The effect of different types and concentrations of cytokinins on shoot induction and multiplication in in vitro nodal explants after eight weeks in culture

	Cytokinin	Concentration (mg L ⁻¹)	Survival (%)	Explant with callus (%)	Bud break (%)	Number of shoots/explant
Mean $\pm$ standard deviation within columns followed by different superscript letters indicate significant differences in the parameters investigated (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, $n = 25$)	Control	0	96 $\pm$ 8.9 ^a	16 $\pm$ 16.7 ^b	38 $\pm$ 38.9 ^a	1 $\pm$ 0.0 ^b
	Kinetin	2	40 $\pm$ 24.4 ^{bc}	8 $\pm$ 10.9 ^b	36.6 $\pm$ 25.4 ^a	1 $\pm$ 0.3 ^b
		3	80 $\pm$ 24.4 ^a	8 $\pm$ 10.9 ^b	58.6 $\pm$ 16.6 ^a	2 $\pm$ 0.5 ^a
		5	48 $\pm$ 30.3 ^{bc}	16 $\pm$ 18.9 ^b	55.5 $\pm$ 15.69 ^a	1 $\pm$ 0.1 ^b
	2iP	2	48 $\pm$ 26.8 ^{bc}	40 $\pm$ 28.2 ^b	24 $\pm$ 28.5 ^{ab}	0.2 $\pm$ 0.0 ^{cd}
		3	32 $\pm$ 22.8 ^c	16 $\pm$ 16.7 ^b	16.6 $\pm$ 23.5 ^{ab}	0.2 $\pm$ 0.1 ^{cd}
		5	80 $\pm$ 24.4 ^a	40 $\pm$ 24.4 ^b	26.6 $\pm$ 25.2 ^{ab}	1 $\pm$ 0.1 ^b
	BAP	2	40 $\pm$ 37.4 ^{bc}	36 $\pm$ 35.7 ^b	22.2 $\pm$ 19.2 ^{ab}	0.4 $\pm$ 0.5 ^{bc}
		3	12 $\pm$ 10.95 ^c	40 $\pm$ 24.4 ^b	0 $\pm$ 0.0 ^b	0 $\pm$ 0.0 ^d
		5	32 $\pm$ 30.3 ^c	24 $\pm$ 26.0 ^b	0 $\pm$ 0.0 ^b	0 $\pm$ 0.0 ^d
	TDZ	0.1	76 $\pm$ 26.0 ^{ab}	96 $\pm$ 8.9 ^a	0 $\pm$ 0.0 ^b	0 $\pm$ 0.0 ^d
		0.5	60 $\pm$ 14.1 ^{bc}	13.3 $\pm$ 16.3 ^b	0 $\pm$ 0.0 ^b	0 $\pm$ 0.0 ^d
		1	92 $\pm$ 10.9 ^a	88 $\pm$ 10.9 ^a	0 $\pm$ 0.0 ^b	0 $\pm$ 0.0 ^d

Table 3 The effect of cytokinin-auxin combination and node position on shoot multiplication after eight weeks in culture

Zeatin (mg L ⁻¹)	IAA (mg L ⁻¹)	Node position	Survival (%)	Explant with callus (%)	Bud break (%)	Number of shoots/explant
2	0	1st	100±0.0 ^a	100±0.0 ^a	100±0.0 ^a	3±1.7 ^b
2	0.2		80±24.4 ^a	100±0.0 ^a	100±0.0 ^a	2±0.1 ^c
2	0	2nd	80±24.4 ^a	100±0.0 ^a	100±0.0 ^a	5±0.7 ^a
2	0.2		100±0.0 ^a	100±0.0 ^a	100±0.0 ^a	3±1.3 ^b

Mean±standard deviation within columns followed by different superscript letters are significantly different amongst treatments (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, $n = 25$)

Table 4 Effect of IBA concentrations on *ex vitro* rooting of shoots after three weeks

IBA (mg L ⁻¹)	Rooting (%)	Number of roots/shoot
0	15±10.0 ^b	1±0.5 ^b
100	35±10.0 ^b	5±2.4 ^a
200	80±28.2 ^a	4±0.3 ^a

Mean±standard deviation within columns followed by different superscript letters indicate significant differences (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, $n = 20$)

to the first node (Table 3). The best treatment for shoot multiplication was with the second node treated with 2 mg L⁻¹ zeatin alone which resulted in a significantly higher number of shoots per explant (5) than other treatments (2–3).

Rooting and acclimatisation

The present study investigated root induction through in vitro and *ex vitro* methods on shoots induced and multiplied on 2 mg L⁻¹ zeatin. Shoots failed to form roots in vitro, regardless of the type, concentration, or combination of auxin tested (results not shown). In contrast, root induction was achieved with the *ex vitro* method by dipping shoots in IBA at various concentrations for one hour. The 200 mg L⁻¹ IBA treatment significantly increased shoot rooting response (80%) than the 100 mg L⁻¹ IBA treatment (35%) or the control (0 mg L⁻¹ IBA; 15%). There were fewer roots in the control than in the treatments with 100 and 200 mg L⁻¹ IBA (Table 4). 100% of the plantlets survived acclimatisation in the greenhouse. Different stages of the in vitro seed germination and protocol development for direct organogenesis are shown in Fig. 4A–F.

Embryogenic callus initiation from in vitro leaf explants

Embryogenic callus was successfully induced in the leaf explant using different auxins. However, 2, 4-D at 1 mg L⁻¹ was chosen for subsequent callus induction because 100% of explants exposed to it formed callus with a relatively high proportion of embryos (Table 5). Early stage embryos (globular and globular-heart) were observed in the calli at four and eight weeks in culture (Fig. 5A–D). Additionally, embryogenic and non-embryogenic cells were recognised in calli within this culture period (Fig. 6). After eight weeks

on CIM, calli were transferred to the embryo maturation medium (MS without PGR). At this stage, embryos were mostly at the globular and globular-heart stages (Fig. 5A–D). It was observed that some explants/calli became necrotic on CIM over the culture period, and the necrosis continued when calli were transferred to the maturation medium. In order to reduce explants/callus necrosis, different carbohydrate sources and concentrations were tested in the CIM in the second experiment. Additionally, the nitrogen content of the CIM was increased by adding organic nitrogen sources as additives or doubling the amount of NH₄-N. Callus necrosis significantly decreased by increasing the nitrogen content of the CIM or by subculturing explants/calli on fresh CIM with a high sucrose concentration (60 g L⁻¹) or a combination of simple sugars with sucrose. Additionally, increasing the nitrogen content (double strength of NH₄-N or addition of 20 mg L⁻¹ AdS) of the CIM prevented the decline of the proportion of embryos in calli after four weeks in culture (Table 6). Cotyledons (first leaves) were also used as explants to investigate their embryogenic capacity using acute and chronic auxin treatments, but these formed rhizogenic callus instead of embryogenic callus, irrespective of the PGR treatment (results not shown). Hence, no further experiments were conducted using the cotyledonary explants.

Maturation of embryos

When embryogenic calli from experiments 1 and 2 were transferred to the maturation medium (MS salts without PGR), embryos, which were mainly at the globular and globular-heart stages (Fig. 5A–D), did not mature (develop further) on this medium. Similarly, reducing the length of explant exposure to exogenous auxins on CIM from eight weeks to six weeks did not promote embryo maturation.

Discussion

Seed germination

Seed germination can be affected by various factors including dormancy (Bewley 1997; Baskin and Baskin 2004). The

Fig. 4 In vitro propagation of *Z. spina-christi* through seed germination and direct organogenesis; (A) Initiation of seed germination with de-coated seed on half-strength MS salts; (B) In vitro seedling after four weeks on half-strength MS salts; (C) shoot development on MS with 2 mg L^{-1} zeatin after four weeks; (D) shoot multiplication on MS with 2 mg L^{-1} zeatin after four weeks, following subculturing onto the same fresh medium; (E) *ex vitro* rooting response in eight-week-old in vitro shoots after three weeks, following a pulse treatment with 200 mg L^{-1} IBA for one hour; (F) two-month-old acclimatised plants

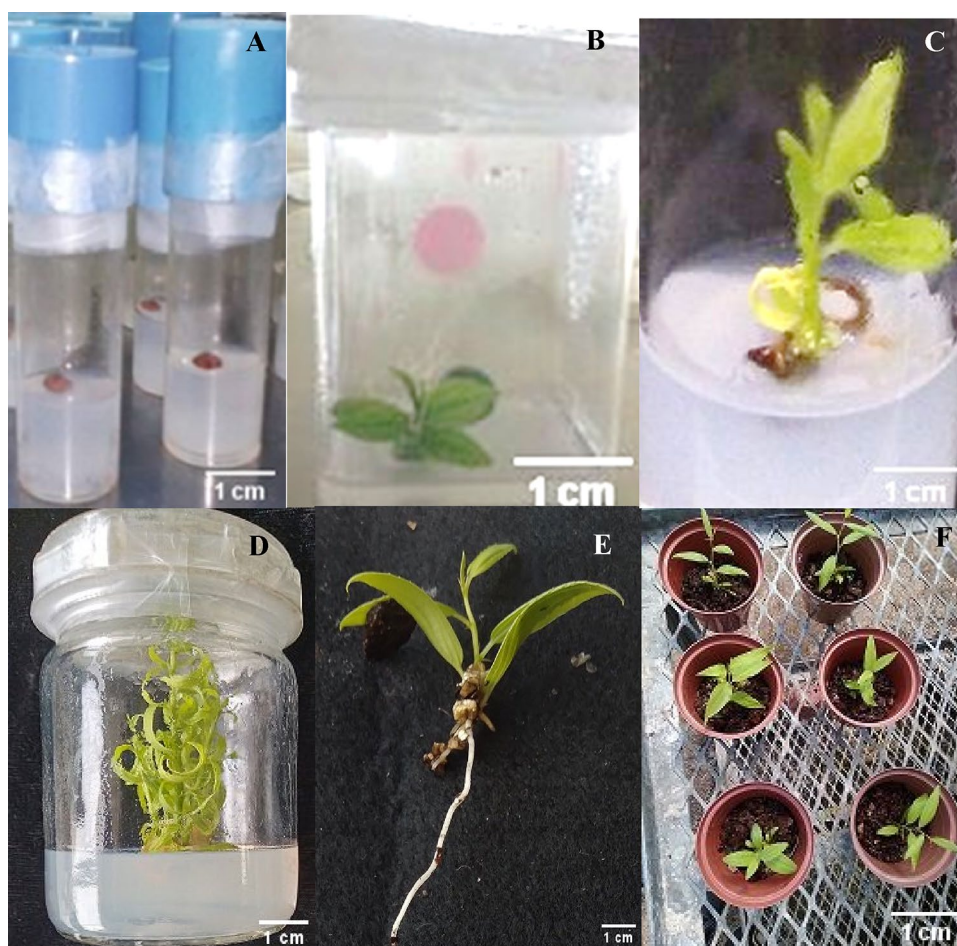


Table 5 The effect of auxin types and concentrations on embryogenic callus induction from in vitro leaf explants after eight weeks in culture

Auxin	Concentration (mg L^{-1})	Explants with callus (%)	Proportion of embryos per plate
2,4-D	1.0	100 ± 0.0^a	0.78
	3.0	2.5 ± 7.9^d	0
	5.0	25 ± 31.1^{cd}	0
Picloram	1.0	52.5 ± 47.7^{bc}	0.92
	5.0	35 ± 47.4^{cd}	0.99
IAA	1.0	60 ± 51.6^{abc}	0.46
NAA	1.0	85 ± 33.7^{ab}	0.38

Mean $\pm$ standard deviation within columns followed by different letters are significantly different (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, $n = 10$)

best priming treatment presently tested for *Ziziphus spina-christi* was removing the seed coat which resulted in the highest FGP. This finding indicates that seeds of *Z. spina-christi* are physically dormant and the seed coat appears to be the main barrier to germination. These are consistent with investigations by Saied et al. (2008b), who found significant differences in the FGP between the untreated control (42%) and acid (H_2SO_4)-treated seeds (70–91%) after

28 days of sowing. The deduced physical dormancy was also supported by intact seeds germinating after extended time in soil (after 42 days there was no difference in FGP between these seeds and the control); the longer the intact seed stayed in the soil, the more the coat weakened through the activity of soil microbes. It has been demonstrated that soil fungi and bacteria, can gradually break down the seed coat, facilitating water uptake and gas exchange for the germination process (Wu et al. 2023).

Ziziphus spina-christi occurs in semi-arid to desert regions (Naghmouchi and Alsubeie, 2021) implying that moisture level can have a significant impact on seed germination. When the effect of watering frequency on seed germination was investigated, it was found that seeds germinated better at low moisture levels than at a higher one. These are adaptive characteristics of the seed in plants from drier environments. It implies that high soil moisture levels can signal unfavourable conditions for seed germination, cause leaching of vital nutrients or promote seed rot due to bacterial or fungal infections. The gradual decline in soil moisture levels (watering every two or four days) investigated in the present study was designed to resemble conditions in arid regions, where the gradual decline in soil water

Fig. 5 Early-stage embryos (G=globular; GH=globular-heart) observed in calli induced on different auxins (mg/L), after 8 weeks; (A) globular-heart embryos in calli induced on 1 2, 4-D; (B) globular embryos in calli induced on 1 IAA; (C) globular and globular-heart embryos in calli induced on 1 NAA; (D) globular-heart embryo in calli induced on 1 picloram; bar = 1000 μ m

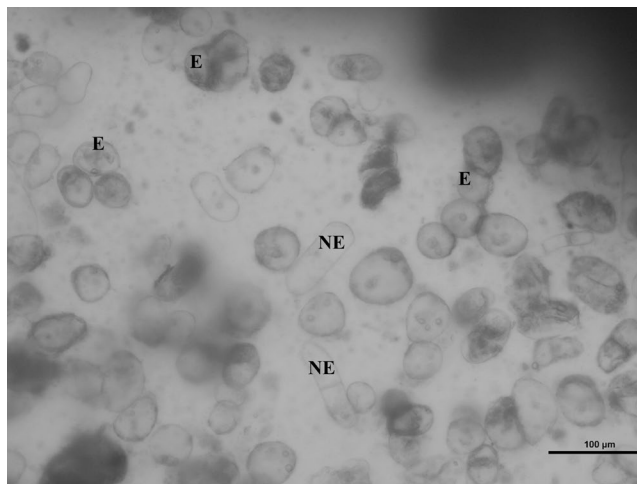
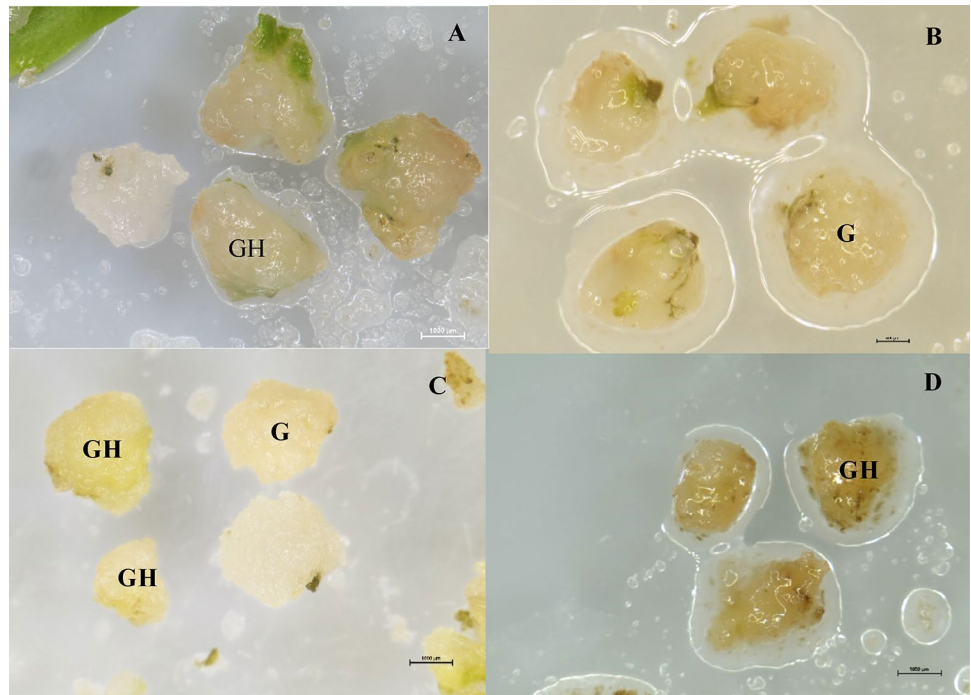


Fig. 6 Light micrograph of embryogenic (E) and non-embryogenic (NE) cells in calli induced with 1 mg L⁻¹ 2,4-D; bar = 100 μ m

potential is typical. To our knowledge, this is the first report on the effects of watering frequency on the germination of *Z. spina-christi* seeds.

Light and temperature are also important environmental factors that can affect seed germination. Given that the seed coat of *Z. spina-christi* was found to hinder water absorption and its removal promoted germination, de-coated seeds were used to test the effects of light and temperature on in vitro germination. Seeds kept in the dark on filter paper reached their maximum germination after 7 days while those maintained in a 16-h photoperiod reached the same after 14 days; *Z. spina-christi* seeds are therefore non-photoblastic. It appears that seeds of different *Ziziphus* species

differ in their light requirement for germination. While seeds of *Z. obtusifolia* var. *obtusifolia* are photoblastic (Speer and Wright 1981), those of *Z. mucronata* var. *mucronata* are non-photoblastic (Zietsman and Both 1987). The effect of light on seed germination has been widely reported to be species-dependent (Wartidiningsih and Geneve 1994; Wang et al. 2009; El-Keblawy et al. 2014; Bu et al. 2017; Zhang et al. 2021; Chen et al. 2022).

When the effects of varying temperature on germination were studied using filter paper, the FGP of seeds incubated at 40 °C was significantly lower than those of seeds incubated at 25 and 35 and 25/21°C (16-h light/8-h dark), while statistically similar to that of seeds incubated at 15 °C. High temperatures are known to cause protein structural changes and tissue damage, which negatively affect germination (Yan and Chen 2020). In northern Nigeria where *Z. spina-christi* occurs, fruiting starts from January to February, when it is cold (14 °C on average) at night and warm (30 °C on average) during the day (Amadi et al. 2014). This could explain why the FGP of seeds incubated at 40 °C was lower than seeds incubated between 21 and 35 °C, which is the optimum temperature range for *Ziziphus* species seed germination; this corresponds to those reported in the literature for *Z. obtusifolia* (Speer and Wright 1981), *Z. mucronata* (Zietsman and Botha 1987), *Z. mauritiana* (Danthu et al. 1993) and *Z. lotus* (Maraghni et al. 2010). Temperatures above 40 °C, and floods due to climate change, would negatively affect the regeneration of *Z. spina-christi* through seeds under natural environmental conditions. This may lead to further decline of the natural populations in the

Table 6 The effect of carbohydrate type and concentration, and nitrogen additive on the inhibition of explant and callus necrosis in the callus induction medium

Carbohydrate source (g/L)		Nitrogen source		Necrosis (%)		Explants with callus (%)		Proportion of embryos per plate	
First Media	Second Media	First Media	Second Media	Week 4	Week 6	Week 4	Week 6	Week 4	Week 6
30 sucrose	-	-	-	15±17.4 ^b	63±44.4 ^a	100±0.0 ^a	100±0.0 ^a	0.74	0.41
30 sucrose	60 sucrose	-	-	10±17.4 ^b	10±17.4 ^b	100±0.0 ^a	100±0.0 ^a	0.74	0.49
30 sucrose	15 sucrose + 5 each of SS	-	-	33±44.1 ^b	33±44.1 ^b	100±0.0 ^a	100±0.0 ^a	0.74	0.49
15 sucrose + 5 each of SS	-	-	-	73±39.8 ^a	73±39.8 ^a	32±27.8 ^c	100±0.0 ^a	0.58	0.25
30 sucrose	-	0.5 g/L CH	-	30±40.4 ^b	38±48.9 ^b	100±0.0 ^a	100±0.0 ^a	0.99	0.82
30 sucrose	-	20 mg/L AdS	-	33±40.9 ^b	33±40.9 ^b	100±0.0 ^a	100±0.0 ^a	0.66	0.83
30 sucrose	-	60 mg/L AdS	-	30±30.7 ^b	30±30.7 ^b	79±17.2 ^{ab}	79±17.2 ^{ab}	0.91	0.49
30 sucrose	-	Double strength NH ₄ -N	-	33±35.4 ^b	33±37.3 ^b	75±38.1 ^b	75±30.6 ^b	0.32	0.57

Mean±standard deviation within columns followed by different letters indicate significant differences (one-way ANOVA, post hoc Tukey test, $p\leq 0.05$, $n=10$)

future, which suggests that controlled conditions may be necessary to achieve high germination.

In vitro seed germination on half-strength MS resulted in FGPs of 95–100% within 14 days of culture, indicating the suitability of this basal salt in terms of exogenous nutrient supplementation for germination and seedling establishment. Hence, this in vitro approach was used to generate explants for the in vitro organogenesis studies.

Shoot induction and multiplication

Cytokinins exhibit variable physiological effects due to differences in their stability, receptor sensitivity, transport and metabolic pathways (Mok and Mok 2001; Kieber and Schaller 2014). In the present study, various cytokinin types and concentrations, including kinetin, 2iP, BAP, TDZ, and zeatin, were tested on nodal explants for shoot induction and multiplication. Among these, zeatin proved to be the most effective cytokinin in promoting shoot induction and multiplication. The superiority of zeatin over the other tested cytokinins was attributed to differences in cytokinin metabolism and sensitivity at the receptor level. The presence of a hydroxyl group in the side chain of zeatin enables the formation of an O-glucoside metabolite, which is a metabolically stable storage form that can be reversibly converted into active cytokinin bases when needed (Kieber and Schaller 2014). This reversible sequestration ensures sustained availability of bioactive cytokinin, thereby supporting prolonged and efficient shoot proliferation in vitro (Strnad 1997; Sakakibara 2006). Moreover, the hydroxyl group in zeatin contributes to a higher binding affinity for certain cytokinin receptors compared to cytokinins lacking it (Lomin et al. 2015; Jaworek et al. 2019). Such increased

receptor affinity often translates into greater biological activity (Mok and Mok 2001; Romanov et al. 2018). The effectiveness of zeatin on shoot induction and multiplication has been previously demonstrated in *Ziziphus jujuba* (Sherfed and Bensaad 2024) and other plant species, including *Labisia pumila* (Najhah et al. 2021), *Zingiber officinale* (Zahid et al. 2021), *Ficus carica* (Ling et al. 2022), and *Kazimierz odnowiciel* (Nowakowska et al. 2021). In agreement with the present findings, previous studies have reported low shoot yields (0.1–1 shoot per explant) when BAP was used for shoot induction in *Ziziphus spina-christi* (Sudharsan and Hussain 2003; Al-Sulaiman 2010; Al-Sulaiman and Barakat 2010), suggesting its unsuitability for shoot organogenesis in this species. Similarly, TDZ was found to be unsuitable for shoot induction in *Z. spina-christi*, as it promoted callus induction instead of shoot organogenesis. It is widely known that TDZ is a growth regulator that imitates the effect of both auxin and cytokinin (Phillips and Garda 2019), hence, can be antagonistic to shoot organogenesis in species that require a cytokinin bias.

Having established that zeatin at 2 mg L⁻¹ resulted in the highest number of shoots/explant, the capacity of the first and second (from the shoot apex) nodal explants to produce shoots was investigated using this concentration of zeatin (2 mg L⁻¹), with or without 0.1 mg L⁻¹ IAA. The results showed that combining zeatin with IAA significantly decreased shoot multiplication, indicating that *Z. spina-christi* does not require exogenous auxin to form shoots. Although auxin is known to suppress bud break (Machakova et al. 2008), in some cases, it promotes shoot multiplication (Rama and Pontikis 1990; Mathur et al. 1995; Anis et al. 2010; Watt 2014). This suggests that species requiring exogenous application of auxin for shoot organogenesis

require this to supplement endogenously-produced auxin, and to work synergistically with cytokinins to sufficiently induce shoot development, which does not appear to apply to *Z. spina-christi*. Additionally, more shoots were produced from the second node than from the first node of the shoot explant. This difference may have resulted from the differential accumulation and distribution of endogenous PGRs, with the first node being closer to the site of auxin synthesis (shoot apex) than the second node. Thus, the negative effect of exogenous auxin on shoot multiplication and the requirement of a relatively high concentration (2 mg L^{-1}) of the correct cytokinin analogue (zeatin) to multiply shoots indicates that *Z. spina-christi* contains elevated levels of endogenous auxins, which can be detrimental to shoot organogenesis through its potential antagonistic interaction with exogenously-applied cytokinin.

The rate of shoot multiplication achieved in the present study using the tested PGR was not obtained in previous studies for *Z. spina-christi* (Al-Sulaiman 2010; Al-Sulaiman and Barakat 2010; Sudharsan and Hussain 2003). The exception was the study by Hegazi et al. (2016), who obtained 4 shoots per explant using 80 mg L^{-1} AdS in the medium without PGRs. However, the attempt made in the present study to produce shoots using the same medium component and explant type (node) as that study, resulted in the formation of a large basal callus (result not shown), which is unsuitable for plant regeneration via direct organogenesis due to a potential risk of somaclonal variation. Given that genotypic effects could explain these differences in results among studies with the same species, the shoot multiplication protocol established in the present study can work for several genotypes as seeds were randomly sourced to represent a diversity of genotypes as opposed to sourcing explants from a single elite genotype (Hegazi et al. 2016). Additionally, it was observed that only shoots produced from zeatin treatments had the spiny texture observed in the natural populations, compared to shoots produced by other tested cytokinins. *Ziziphus spina-christi* has branches with spines that occur in pairs (one straight and the other curved) (Saied et al. 2008a).

The success of micropropagation can be limited by the adventitious rooting of shoots, which often represents the most challenging stage of in vitro protocols (Arya et al. 2009). Although the rooting process occurs in three physiological phases, including induction, initiation, and expression, each phase has specific requirements for root development (de Klerk et al. 1999). In the present study, different concentrations of IBA were tested to induce adventitious rooting of shoots in vitro, as this auxin is widely used to promote rhizogenesis in tree species (Mng'omba et al. 2007; Ilczuk and Jacygrad 2016; Lerin et al. 2021; Gautam et al. 2024), including *Ziziphus* spp. (Mathur et al. 1995;

Soliman and Hegazi 2013). In vitro shoots from the 2 mg L^{-1} zeatin treatment failed to root in vitro on MS salts with or without IBA. Additionally, the rooting protocols developed for *Z. jujuba* by Melyan et al. (2013) and Soliman and Hegazi (2013) did not presently work for *Z. spina-christi*. This variation could be due to differences in the levels of endogenous growth regulators between the two species, which can influence rhizogenesis by interacting with exogenous growth regulators (Yuan et al. 2025). In contrast, shoots rooted *ex vitro* when exposed to a pulse of 100 and 200 mg L^{-1} IBA for one hour (Table 4). Although cytokinin used in stages preceding rooting can persist in tissues and inhibit root induction (Boggetti et al. 1999; Nakhouda et al. 2012; Stevens and Pijut 2018), in the present study, zeatin, a highly metabolised cytokinin was used for shoot induction and multiplication. Therefore, the failure of shoots to form roots in vitro may have been caused by supra-optimal auxin levels as indicated in the shoot multiplication stage. Auxins are necessary for the initiation of root primordia but are known to become inhibitory to the development and emergence of roots (de Klerk et al. 1999). Some authors have reported that for selected *Eucalyptus* species, shoots rooted better when exposed to an IBA pulse treatment than when they were subjected to chronic auxin treatment (Blomstedt et al. 1991; Fett-Neto et al. 2001).

All rooted plantlets were successfully acclimatised. The advantage of *ex vitro* rooting is that the process occurs concurrently with acclimatisation, which can save time and require less work than in vitro rooting followed by acclimatisation. Additionally, in vitro roots can be susceptible to damage during transfer to an acclimatisation medium or they may not do well on the substrate to which they were transplanted, both of which might cause plant death (Debergh and Maene 1981).

Somatic embryogenesis

Auxins like 2, 4-D, picloram, NAA and IAA have been effective for the induction of somatic embryogenesis in a variety of tree species (Watt et al. 1991; Su et al. 1997; Hazubska-Przybył, and Bojarczuk, 2008), including *Ziziphus jujuba* (Kim et al. 2006) and *Z. mauritiana* (Ibrahim et al. 2011). In this study, these auxins were tested to determine their capacity on embryogenic callus induction in *Z. spina-christi* leaf explants. The results showed that all the tested auxins were able to induce embryogenic callus. The synthetic auxin 2, 4-D at 1 mg L^{-1} was, however, selected for subsequent callus induction because 100% of explants exposed to it formed callus with a relatively high proportion of early-stage embryos. Furthermore, it resulted in a higher proportion of embryogenic cells than non-embryogenic cells. Many researchers have thoroughly described

the characteristics of these cells: embryogenic cells are relatively small, isodiametric with prominent nuclei, and mostly appear in clusters; non-embryogenic cells have irregular shapes, and are large and highly vacuolated (Vasil et al. 1990; Fehér et al. 2003; Vilela et al. 2019; Kessel-Domini et al. 2022). It is well established that 2, 4-D is widely used for the induction of somatic embryos in plants (Machakova et al. 2008; Leljak-Levanić et al. 2015; Garcia et al. 2019). Some explants/calli became necrotic over time on CIM, and the necrosis persisted when calli were transferred to the maturation medium. In an effort to reduce explants/callus necrosis, the effects of carbohydrate sources, organic nitrogen sources and doubling the amount of $\text{NH}_4\text{-N}$ of the CIM were investigated. Explants were cultured for six weeks with or without subculture at week four. Callus necrosis significantly decreased when the nitrogen content of the CIM was increased. Similarly, subculturing explants/calli on fresh CIM with a high sucrose concentration (60 g L^{-1}) or simple sugar and sucrose combination also reduced callus necrosis. It suggests that necrosis was caused by insufficient nitrogen available to the cells, either by depletion or inadequate carbohydrate to convert the complex form of inorganic nitrogen to the simple form for cell uptake (George and de Klerk 2008; Abbas et al. 2020). The type and concentration of carbohydrates in the culture medium (Hew et al. 1988; Schofield et al. 2009; Abbas et al. 2020) or the ratio of nitrate-nitrogen ($\text{NO}_3\text{-N}$) and carbohydrates in the culture medium (Fletcher 1980; Gabryszewska and Sochacki 2012) can have a significant effect on the uptake of inorganic nitrogen under in vitro conditions.

Following eight weeks of callus induction on different auxin types, calli were transferred to MS medium without PGR for embryo development. Although a relatively high level of auxin is required for the induction of embryos, prolonged exposure inhibits their development (She et al. 2013; Fehér 2019). Although calli were transferred to the PGR-free medium, the embryos did not develop further, probably caused by auxin persistence in the callus tissue. When the culture duration was reduced from eight to six weeks during the callus induction stage, through medium modification with nitrogen and carbohydrate sources, embryos failed to develop further. This resulted in testing a different explant type (cotyledon) for callus induction as different explants can vary in their sensitivity to exogenous PGR (Machakova et al. 2008).

Cotyledons (first leaves) from four-week-old in vitro seedlings were given either a pulse treatment with 10 mg L^{-1} 2, 4-D for four days followed by transfer onto MS without PGR, or 0.5 or 1 mg L^{-1} 2, 4-D for a further six weeks, or they were cultured directly on 0.5 mg L^{-1} and 1 mg L^{-1} 2, 4-D for six weeks. Results showed that cotyledonary explants formed rhizogenic callus instead of embryogenic

callus, irrespective of the PGR treatment. Since the cotyledon explants were younger than the leaf explants, the former was likely to have contained higher levels of endogenous auxins (Machakova et al. 2008). Auxin is abundant in floral organs, young leaves and developing fruits and seeds, and the concentration decreases with age (Machakova et al. 2008). In contrast to the results of the present study, *Ziziphus jujuba* was regenerated via somatic embryogenesis when cotyledons from seedlings were treated with a combination of 0.9 mg L^{-1} 2, 4-D, 0.09 mg L^{-1} IBA and 0.09 mg L^{-1} BAP (Kim et al. 2006). Nevertheless, irrespective of the same genus, the species tested in the present study did not respond similarly.

Conclusion

The present study showed that the hard coat of *Z. spinachristi* seeds caused physical dormancy and removing it manually is the most effective approach to break the dormancy. Low moisture levels (watering the soil every two or four days rather than every day) were found to increase germination. Seeds were found to be non-photoblastic, but a temperature between 21 and 35°C was conducive to germination. According to the results of this study, *Z. spinachristi* contains high endogenous auxin to cytokinin ratio, which resulted in variable in vitro morphogenesis, depending on the type and concentration of the exogenous PGR applied or type of explant used in the culture. Zeatin at a high concentration (2 mg L^{-1}) promoted shoot multiplication more than BAP, 2iP, and kinetin. Shoots from the zeatin treatment, failed to form roots in vitro on MS supplemented with different types and concentrations of auxins, caused by supra-optimal endogenous auxin levels. This was confirmed by the successful *ex vitro* rooting of shoots following a pulse IBA treatment for one hour.

Although the somatic embryogenesis protocol was not completely successful, the seed germination protocols developed in this study provide cost-effective means for the conservation of genetically diverse *Z. spinachristi* trees through the conventional propagation approach. However, the protocol presently established through the nodal culture would be especially useful for conservation and propagation of elite genotypes. Future research should focus on understanding the PGR requirements for embryo development, germination and plantlet establishment.

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Author contributions Mustapha Haruna designed and performed experiments, recorded and analysed data, and wrote the manuscript; Muhammad Nakhoda and Shakira Shaik assisted with designing experiments, editing the manuscript and supervised the research.

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Data availability Data will be made available on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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