Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology

Official Journal of the Societa Botanica Italiana

ISSN: 1126-3504 (Print) 1724-5575 (Online) Journal homepage: https://www.tandfonline.com/loi/tplb20

Efficient plant regeneration through callus in *Zanthoxylum armatum* DC: an endangered medicinal plant of the Indian Himalayan region

Sumit Purohit, Kuldeep Joshi, Vivek Rawat, Indra D. Bhatt & Shyamal K. Nandi

To cite this article: Sumit Purohit, Kuldeep Joshi, Vivek Rawat, Indra D. Bhatt & Shyamal K. Nandi (2019): Efficient plant regeneration through callus in *Zanthoxylum armatum* DC: an endangered medicinal plant of the Indian Himalayan region, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, DOI: 10.1080/11263504.2019.1610107

To link to this article: https://doi.org/10.1080/11263504.2019.1610107

PLANT BIOSYSTEMS - AN INTERNATIONAL JOURNAL DEALING WITH ALL ASPECTS OF PLANT BIOLOGY
https://doi.org/10.1080/11263504.2019.1610107

Efficient plant regeneration through callus in *Zanthoxylum armatum* DC: an endangered medicinal plant of the Indian Himalayan region

Sumit Purohit, Kuldeep Joshi, Vivek Rawat, Indra D. Bhatt and Shyamal K. Nandi

G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi Katarmal, Almora, Uttarakhand, India

ABSTRACT

An efficient *in vitro* propagation protocol for *Zanthoxylum armatum* DC has been developed via indirect organogenesis using aseptic leaf explants. The explants were soaked for different time duration (12, 24 or 36 h) in liquid woody plant medium (WPM) supplemented with various concentrations (15.0, 25.0 or 50.0 μM) of thidiazuron (TDZ). The pre-exposed explants transferred for callus induction onto WPM supplemented with different concentrations of TDZ (2.0, 4.0, 6.0, 8.0 and 10.0 μM) either alone or in combination with varied concentrations (0.5, 1 and 1.5 μM) of naphthaleneacetic acid (NAA). Of the tested concentrations and combinations, best response for pretreated (15 μM TDZ for 24 h) explants was achieved on WPM augmented with 6.0 μM TDZ and 0.5 μM NAA after 8 weeks of incubation. For shoot induction, the callus clumps were excised into small pieces (~0.5 g) and were transferred onto WPM fortified with different concentrations (2.0–9.0 μM) of benzylaminopurine (BA), indole-3-acetic acid (IAA, 1.0 μM) and gibberellic acid (GA_3, 0.5–3.0 μM). Maximum shoot number (10.4 ± 0.74) and average shoot length (4.75 ± 0.71 cm) were observed in WPM enriched with 2.0 μM BAP, 1.0 μM IAA and 1.5 μM GA_3 after 8 weeks of incubation. The developed shoots (4 cm) were excised, pulse-treated for 24 h in half-strength WPM containing indole-3-butyric acid (IBA, 50.0 μM) prior to their transfer on hormone-free MS medium, where 100% rooting was achieved. The regenerated plants were implanted in soil-filled poly bags, acclimatized properly and subsequently placed under sunlight with 80% survival rate after 60 days recorded. This is the first report for propagation of *Z. armatum* via callus phase with high rate of shoot proliferation and can be effectively utilized for generating sufficient planting material in promoting its re-cultivation and conservation programme.

ARTICLE HISTORY
Received 19 July 2018
Revised 6 April 2019
Accepted 12 April 2019

KEYWORDS
Endangered; de-differentiated tissue; *in vitro* propagation; thidiazuron; medicinal plants

Introduction

Zanthoxylum armatum DC, commonly known as winged prickly ash/Timur of the family Rutaceae, is an erect tree/shrub distributed in Himalayan range at an altitude of 1000–2100 m and lesser Himalayan ranges of northeastern parts of India, and in eastern ghats of Orissa and Andhra Pradesh at around 1200 m above sea level (Gaur 1999). The genus *Zanthoxylum*, including *Z. armatum*, are used for the treatment of stomach and tooth ache, intestinal worms, snake bites, rheumatism, scabies, fever and cholera (Anonymous 1976). The pericarp of the seed is extensively used as spice; further, on account of deodorant, disinfectant and antiseptic properties, it is also used in dental hygiene. The stem and bark are used in the preparation of toothpaste. Analysis of *Zanthoxylum* extracts has revealed antibacterial and antioxidant properties (Hisatomi et al. 2000; Tiwary et al. 2007; Joshi et al. 2009; Upadhyaya and Ashok 2010). High levels of linalool, an essential oil, are reportedly found in *Z. armatum* seeds (Jain et al. 2001). Gas chromatography-mass spectroscopy (GC-MS)-based studies on the extracts of *Z. armatum* have revealed the presence of over 100 volatile compounds (Kim et al. 1989). Recently, Tamblin (a flavonoid) isolated from the fruits of the species showed endothelium-independent relaxation in porcine coronary artery rings (Mushtaq et al. 2019). Similarly, anti-diabetic activity of aqueous leaf extract of the species is reported (Rynjah et al. 2018).

The species is generally propagated through seeds but natural regeneration through seed has been reported to be poor (Kala 2010). Moreover, propagation via stem cuttings takes several months for proper root formation. Studies on seed germination (Purohit et al. 2015a) and vegetative propagation through air layering (Purohit et al. 2015b) have, however, been reported but slower speed of seed germination and poor rooting response hamper mass and speedy production of planting material. Large-scale collection and unsustainable harvesting of this species from the wild due to its medicinal properties have caused severe threat to its natural populations and have placed *Z. armatum* in a list of "endangered" plants in the Indian Himalayan region (Samant et al. 2007). To meet the commercial demand and improve conservation status of this species, tissue and cell culture approaches offer a rapid method of multiplication (Bhatt and Dhar 2000, 2004, Giri et al. 2012a, 2012b; Nandi et al. 2002, 2016).

Tissue culture studies in some other species of *Zanthoxylum*, namely, *Z. piperitum* (Imaizumi et al. 1993;

CONTACT Indra D. Bhatt idbhatt@gbpihed.nic.in G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi Katarmal, Almora, Uttarakhand 263643, India

Published online 04 Jul 2019

Hwang and Hwang 2003), *Z. dissitum* (Ying et al. 2009) and *Z. bungesnum* (Wang and Jing 2006), have appeared. There are two previous reports on *in vitro* studies with *Z. armatum* (Bisht and Bhandari 2007; Purohit et al. 2016). Using nodal explants, Purohit et al. (2016) described a complete protocol with successful transfer of plants in soil and assessment of their fidelity. However, shoot generation was found to be rather low (only 4.76 shoots/explant). On the other hand, Bisht and Bhandari (2007) reported successful establishment of callus followed by shoot regeneration in *Z. armatum*; nevertheless, rooting of shoots and complete regeneration of plantlets could not be achieved. Further, clonal fidelity of micropropagated shoots was not checked. In view of these shortcomings, there is a need to develop an improved protocol which can enhance shoot multiplication rate not only to meet the demand of planting material for cultivation but also to enhance the conservation status of this species. The present study, therefore, focuses on developing a more efficient regeneration protocol for mass multiplication and commercial cultivation of *Z. armatum* using leaf explants via a callus phase.

Materials and methods

Plant material and surface disinfection

The nodal explants of *Z. armatum* were collected from Dummar village, Munsiyari region, District Pithoragarh, Uttarakhand, India (1663 m asl; 30° 06′ 16.0″ N, 80° 14′ 57.1″ E) during the month of November 2010 and used for establishment of *in vitro* cultures. Leaves were excised from *in vitro* raised shoots (Purohit et al. 2016) and used for the callus induction and further organogenesis of the multiple shoots in woody plant medium (WPM; McCown and Lloyd 1981) containing varying concentrations of plant growth regulators (PGRs).

Culture medium and conditions

The WPM (basal medium) was supplemented with sucrose (3.0%, w/v), phytagel (0.2%, w/v) and various concentrations of PGRs. All other chemicals were procured from Himedia, Mumbai (India), while PGRs used were obtained from Sigma-Aldrich (St. Louis, MO). The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl. Approximately 30 ml medium was dispensed into individual flasks (Borosil, India), plugged with non-absorbent cotton bungs wrapped in two layer of muslin cloth and covered with aluminum foil on the top. These were then sterilized by autoclaving at 121 °C for 20 min (1.05 kg cm^{-2}). The leaf explants from *in vitro* shoots were cultured in flasks containing full strength WPM medium variously supplemented with PGRs. All cultures were kept in a culture room at 25 ± 1 °C, with 16-h light and 8-h dark cycles and low irradiance (42 μEm^{-2} s^{-1}; inside culture tubes and flask) maintained by cool white fluorescent tubes (Philips; 40 W).

Callus induction, organogenesis, rooting and acclimatization of plantlets

The fully expanded green leaves were excised from the *in vitro* shoots (40 days old; Figure 1(A)) and cut into pieces (size ~1 cm^2, Figure 1(B)) using a surgical blade. The leaf pieces were first soaked in WPM liquid medium supplemented with three different concentrations of *N*-phenyl-*N*′-1,2,3-thiadiazol-5-urea (thidiazuron/TDZ; 15.0, 25.0 and 50.0 μM) and maintained on a shaker for three different time periods (12, 24 and 36 h; Table 1). Thereafter, these were removed from the flask and blotted with sterilized tissue paper. The excised leaves were then cultured on WPM semi-solid medium containing phytagel (0.1%, w/v) and sucrose (3%, w/v) supplemented with different concentrations of TDZ (0–10.0 μM) alone or in combination with 0.5–1.5 μM NAA. Calli with different shapes and colours were formed in these leaf pieces after 40 days of inoculation. For further proliferation, the callus was transferred on WPM medium containing 6.0 μM TDZ with 0.5 μM NAA where sufficient amount of callus generated. Calli pieces (0.5 g) were then subcultured onto WPM medium supplemented with different concentrations of BA (2.0–9.0 μM), IAA (1.0 μM) and GA_3 (0.50–3.0 μM) for shoot induction and multiplication. Healthy shoots (4 cm) were excised from multiplication medium after 8 weeks of subculture and kept for rooting. A two-step procedure for rooting was adopted. In the first step, microshoots were transferred to full-strength WPM medium containing sucrose (3% w/v), phytagel (0.2%, w/v) and different concentrations of IBA (25.0, 50.0, 75.0, 100.0 μM) for 12 or 24 h. In the second step, these shoots were transferred onto PGR-free half-strength MS medium. After 6 weeks in rooting medium, healthy plantlets with well-developed roots were then removed from the medium, thoroughly washed with tap water and transferred into plastic cups containing autoclaved garden soil. These were covered with polythene bags with small perforations, placed in a green house and irrigated with 1/4 WPM medium without sucrose, micronutrients and vitamins once every 6 days. After 6 weeks, the polythene covers were removed. Acclimatized plants were transferred to earthen pots (30 cm × 25 cm size) containing garden soil and placed under shade in a mist chamber for another 4 weeks. Subsequently, the plants were moved to open-air conditions under full sunlight.

Data analysis

Twenty explants in each treatment were used for data analysis and the experiments were repeated three times. The mean values of results obtained under various PGR treatments were subjected to analysis of variance using SPSS version 16. The level of significance was determined ($P < 0.05$), and the means were separated using Duncan's multiple range test (DMRT). Data are presented as mean values ± standard error (SE).

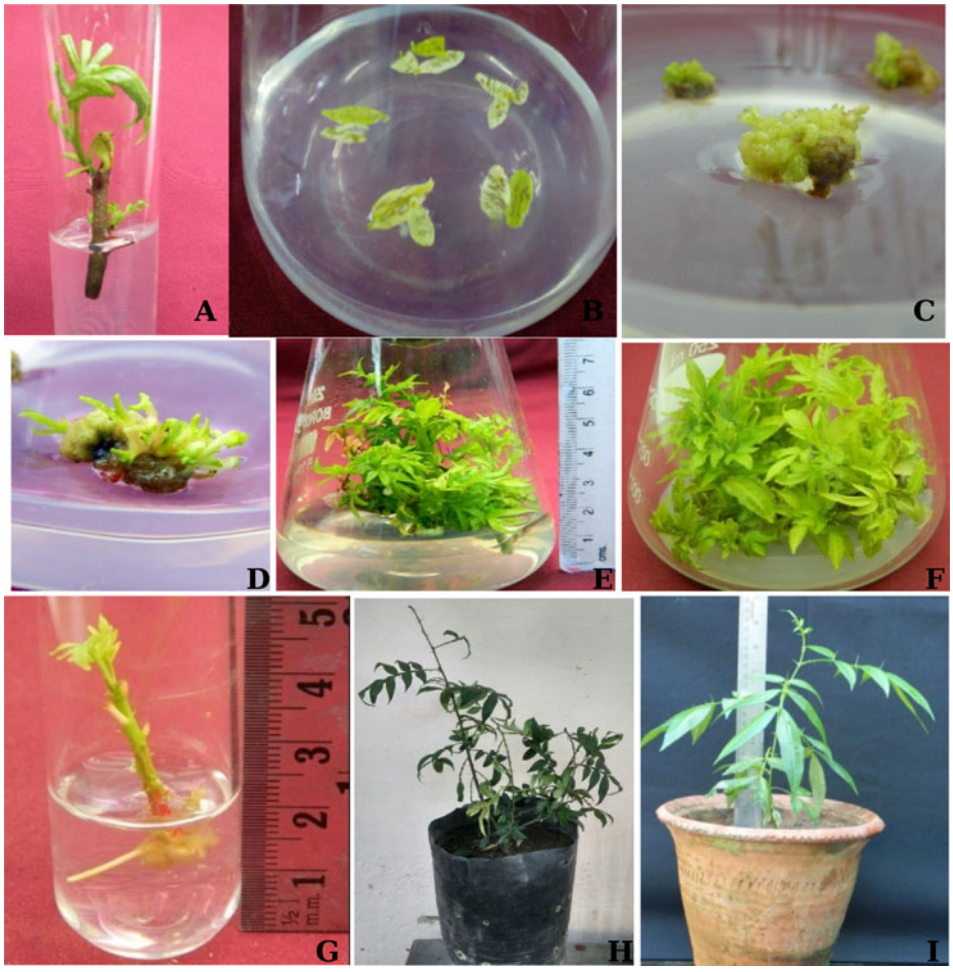

Figure 1. Direct shoot regeneration from leaf explants of *Z. armatum*. (A) *In vitro* sprouting observed in a nodal explant. (B) Leaf explants cultured on WPM. (C) Callus formation in leaf explants. (D–F) Shoot regeneration on WPM medium supplemented with 2.0 μM BA + 1.0 μM IAA + 1.5 μM GA_3 and multiple shoot development on WPM medium. (G) Root induction on *in vitro* raised shoots after initial (pulse) treatment with IBA (50.0 μM, 12 h) free half-strength MS medium. (H–I) *In vitro* raised plants after 60 days.

Table 1. Pretreatment of leaf explants with different concentrations of TDZ in different time periods.

S. No	Thidiazuron (μM)	Time (h)		
1	15	12	24	36
2	25	12	24	36
3	50	12	24	36

Results and discussion

Effect of PGRs on induction of shoot organogenesis from leaf explants

The present investigation describes high-frequency shoot induction from the callus induced on leaf explants. The pretreated leaves did not produce any shoots on PGR-free medium (control), and leaves became dry after few days of inoculation. Pretreatment of leaf explants with 15.0 μM TDZ for three different time periods (12, 24 and 36 h) showed callusing, however, maximum (90%) cultures induced callus following 24 h pretreatment (Table 2). Leaf explants when pretreated with TDZ at 25.0 μM for 12, 24 or 36 h alone and cultured on WPM medium supplemented with TDZ (6.0 μM) with NAA (0.5 and 1.0 μM), 70% of the explants showed callus induction but the calli formed appeared yellow (data not shown). When leaf explants were pretreated with 50 μM TDZ for three different time periods (12, 24 and 36 h), maximum callus formed appeared yellow color following 24 and 36 h treatments in TDZ and NAA combinations (Table 3). Compact and green-colored callus was formed following 24 h pretreatment in 15.0 μM TDZ and, therefore, selected for further experiments (Figure 1(C)). Instead of the different concentrations of TDZ tried, 6 μM TDZ along with 0.5 μM NAA gave maximum callus biomass along with green and compact calli. Compact calli, which when transferred to a secondary medium containing different concentrations of BA (2.0–9.0 μM), IAA (1 μM) and GA_3 (0.5–2.0 μM), maximum shoot formation (10.4 shoots/callus with 4.75 cm length) was obtained in 2.0 μM BA, 1.0 μM IAA and 1.50 μM GA_3 after 45 days of culture (Table 4; Figure 1(D–F)). The presence of BA with IAA and GA_3 in the medium improved the shoot induction both in terms of length and number of shoots per callus piece. However, at a higher concentration of BA (9.0 μM) with IAA (1.0 μM) and GA_3 (3.0 μM), the number of multiple shoots formed (2.6 ± 0.37) and average shoot length (3.96 ± 0.33 cm) were found to be comparatively low (Table 4). There are several reports on high-frequency shoot induction using BA and TDZ alone or in combination with NAA

Table 2. Effect of different concentrations of TDZ (15 μM) alone or in combination with lower concentration of TDZ and NAA on callus culture from leaf explants.

S. No	Plant growth regulators (TDZ, 15 μM)		Time (h)	Callus %	Time (h)	Callus %	Time (h)	Callus %
	TDZ	NAA	12		24		36	
1	0.0	0.0	Dry	0	Dry	0	Dry	0
2	2.0	0.0	Dry	0	Dry	0	Dry	0
3	2.0	0.5	Dry	0	Dry	0	Dry	0
4	2.0	1.0	Dry	0	Dry	0	Dry	0
5	2.0	1.5	Dry	0	Yellow	0	Yellow	20
6	4.0	0	Yellow	40	Yellow	60	Yellow	20
7	4.0	0.5	Green	40	Green	60	Yellow	10
8	4.0	1.0	Green	40	Green	70	Yellow	20
9	4.0	1.5	Green	40	Yellow	50	Yellow	20
10	6.0	0.0	Yellowish green	30	Yellowish green	60	Yellow	20
11	6.0	0.5	Green	50	Green	90	Yellowish green	30
12	6.0	1.0	Green	50	Green	70	Yellow	10
13	6.0	1.5	Green	30	Yellowish green	20	Yellow	10
14	8.0	0.0	Yellow	20	Yellow	20	Yellow	20
15	8.0	0.5	Yellow	20	Yellow	20	Yellow	20
16	8.0	1.0	Dry	0	Dry	0	Dry	0
17	8.0	1.5	Dry	0	Dry	0	Dry	0
18	10.0	0.0	Dry	0	Dry	0	Dry	0
19	10.0	0.5	Dry	0	Dry	0	Dry	0
20	10.0	1.0	Dry	0	Dry	0	Dry	0
21	10.0	1.5	Dry	0	Dry	0	Dry	0

Table 3. Effect of different concentrations of TDZ 50 μM and in combination with TDZ and NAA on callus culture from leaf explants of *Z. armatum*.

S. No	Plant growth regulators (TDZ, 50 μM)		Time (h)	Callus %	Time (h)	Callus %	Time (h)	Callus %
	TDZ	NAA	12		24		36	
1	0.0	0.0	Dry	0	Dry	0	Dry	0
2	2.0	0.0	Dry	0	Dry	0	Dry	0
3	2.0	0.5	Dry	0	Dry	0	Dry	0
4	2.0	1.0	Dry	0	Dry	0	Dry	0
5	2.0	1.5	Dry	0	Dry	0	Dry	0
6	4.0	0	Yellow	40	Yellow	30	Yellow	30
7	4.0	0.5	Green	40	Yellow	30	Yellow	40
8	4.0	1.0	Yellow	40	Yellow	30	Yellow	20
9	4.0	1.5	Yellow	20	Yellow	30	Yellow	20
10	6.0	0.0	Yellow	30	Yellow	30	Yellow	50
11	6.0	0.5	Yellowish green	60	Yellow	50	Yellow	50
12	6.0	1.0	Yellow	50	Yellow	50	Yellow	30
13	6.0	1.5	Yellow	70	Yellow	60	Yellow	20
14	8.0	0.0	Dry	0	Dry	0	Dry	0
15	8.0	0.5	Dry	0	Dry	0	Dry	0
16	8.0	1.0	Dry	0	Dry	0	Dry	0
17	8.0	1.5	Dry	0	Dry	0	Dry	0
18	10.0	0.0	Dry	0	Dry	0	Dry	0
19	10.0	0.5	Dry	0	Dry	0	Dry	0
20	10.0	1.0	Dry	0	Dry	0	Dry	0
21	10.0	1.5	Dry	0	Dry	0	Dry	0

using explants from different plant species (Herve et al. 2001; Guo et al. 2005; Espinosa et al. 2006; Raghu et al. 2006; Thomas 2007; Purohit et al. 2015c) but not in *Z. armatum*. The induction frequency as well as number of shoots per explant was low in some of the PGR combinations. Hence, pretreatment of callus induced from leaf explants was attempted to improve shoot induction. The control treatment, i.e. WPM medium without any PGR, did not induce any shoots. TDZ, known to mimic cytokinin-like activity (Wang et al. 1986) and initially used to promote breaking of dormant buds in apple, has been used regularly in tissue culture studies. Thus, pretreatment of explants using TDZ has been found to be more effective than many commonly used PGRs (Singh and Syamal 2001; Prathanturarug et al. 2003, 2005; Thomas 2007). In the present investigation too, explants pretreated with different concentrations of TDZ for varying periods had very significant effect on shoot induction. Generally, low TDZ concentration (1.0–10.0 μM) is used for shoot induction since higher concentrations result in shoots becoming hyperhydric and fasciated (Huetteman and Preece 1993). TDZ has been reported to promote the synthesis and accumulation of purine and alters cytokinin metabolism to increase the levels of endogenous cytokinins by inhibiting the action of cytokinin oxidase (Hare and Van

Table 4. Effect of different concentrations and combinations on shoot regeneration from callus culture of *Z. armatum*.

	Plant growth regulator concentrations (μM)				
S. No	BA	IAA	GA_3	Av. shoot length (cm)	Av. shoot number
1	2.00	1.00	0.50	$2.04 \pm 0.16^{g,h}$	$3.6 \pm 3.17^{e,f}$
2	2.00	1.00	1.00	$2.3 \pm 0.18^{g,h}$	$6.4 \pm 0.60^{b,c}$
3	2.00	1.00	1.50	$4.75 \pm 0.71^{a,b}$	10.4 ± 0.74^{a}
4	2.00	1.00	2.00	$4.29 \pm 0.06^{a,b}$	$5.4 \pm 0.83^{c,d}$
5	2.00	1.00	3.00	$3.25 \pm 0.28^{c-e}$	$4.5 \pm 0.76^{d,e}$
6	3.00	1.00	0.50	1.51 ± 0.10^{h}	$3.4 \pm 0.60^{e,f}$
7	3.00	1.00	1.00	$3.87 \pm 0.18^{b,c}$	$6.6 \pm 0.76^{b,c}$
8	3.00	1.00	1.50	$3.05 \pm 0.08^{d-f}$	$4.4 \pm 0.69^{d,e}$
9	3.00	1.00	2.00	$3.6 \pm 0.24^{b-d}$	$3.32 \pm 0.45^{e,f}$
10	3.00	1.00	3.00	$4.03 \pm 0.21^{a-c}$	$6.6 \pm 0.34^{b,c}$
11	6.00	1.00	0.50	$3.89 \pm 0.17^{b,c}$	7.7 ± 0.52^{b}
12	6.00	1.00	1.00	$4.12 \pm 0.24^{a,b}$	$6.8 \pm 0.59^{b,c}$
13	6.00	1.00	1.50	$4.37 \pm 0.19^{a,b}$	$5.7 \pm 0.67^{c,d}$
14	6.00	1.00	2.00	$2.48 \pm 0.20^{f,g}$	2.5 ± 0.32^{f}
15	6.00	1.00	3.00	$2.08 \pm 0.20^{g,h}$	2.5 ± 0.27^{f}
16	9.00	1.00	0.50	$2.54 \pm 0.20^{e-g}$	2.4 ± 0.37^{f}
17	9.00	1.00	1.00	$2.16 \pm 0.21^{g,h}$	2.3 ± 0.40^{f}
18	9.00	1.00	1.50	$2.19 \pm 0.19^{g,h}$	2.3 ± 0.40^{f}
19	9.00	1.00	2.00	$2.37 \pm 0.23^{g,h}$	2.6 ± 0.40^{f}
20	9.00	1.00	3.00	$3.96 \pm 0.33^{b,c}$	2.6 ± 0.37^{f}
21	0.00	0.00	0.00	0.00 ± 0.00^{i}	0.00 ± 0.00^{g}

Values are mean ± standard error, mean values followed by the same letter(s) in a column are not significantly different ($P < 0.05$).

Table 5. Effect of different concentrations of PGRs on rooting response.

S. No	IBA concentration (μM)	Time (h)	Rooting %	Root length (cm)	Root number	Callus formation
1	25.00	12	0	NA	NA	+
2	25.00	24	0	NA	NA	+
3	50.00	12	100	5.89 ± 0.39^{a}	8.40 ± 0.41^{a}	−
4	50.00	24	62	3.22 ± 0.22^{b}	5.62 ± 0.22^{b}	−
5	75.00	12	0	NA	NA	+
6	75.00	24	40	1.22 ± 0.52^{c}	2.89 ± 0.79^{b}	+
7	100.00	12	0	NA	NA	+
8	100.00	24	0	NA	NA	+
9	Control	0	0	NA	NA	−

Values are mean ± standard error, mean values followed by the same letter(s) in a column are not significantly different ($P < 0.05$). (+) Callus, (−) no callus observed at the basal portion of the shoot; NA: not applicable.

Staden 1994; Murthy et al. 1998). But for short and/or pulse treatment, higher concentrations of TDZ (i.e. up to 200 μM) can be employed (Singh and Syamal 2001). In the present study, three different concentrations (i.e. 15.0, 25.0 and 50.0 μM) of TDZ were employed for pretreatment, and of these, 15.0 μM TDZ for 24 h gave highest frequency of callus regenerating from leaves when cultured on 6.0 μM TDZ and 0.5 μM NAA. Following proliferation of multiple shoot buds, healthy shoots were excised for rooting. A pulse treatment with IBA (50 μM) for 12 h followed by culture on PGR-free MS medium (Murashige and Skoog 1962) resulted in quick root initiation within 35 days. An average number of 8.4 roots were formed with the longest root measuring 5.89 cm after 6 weeks (Table 5; Figure 1(G)). Most other treatments showed callus formation at the basal portion of the transferred shoots. All shoots with well-developed roots were taken out of the culture medium. To remove all media, their basal portion was washed under running tap water for 2 min. The plantlets were then treated with 0.5% Bavistin (w/v; BASF, Mumbai, India) solution to prevent fungal contamination and transferred into small plastic cups containing soil and farmyard manure (3:1, v/v). Subsequently, the plantlets were allowed to acclimatize by covering the top of plastic cups with polythene bags to maintain humidity, and plants were watered every 3 days. The plastic cups with tissue-culture-raised plants were kept in the green house. After 1 week, four to six holes were punctured in the polythene bags, and plants were irrigated every 6 days. After 6 weeks, polythene bags were removed and the hardened plants were transferred to polythene bag containing garden soil and kept under shade in a mist chamber for another 4 weeks before shifting the plantlets in open field under full sunlight. The growth under *ex vitro* conditions was initially slow during the first 15 days, but improved thereafter. The survival of plantlets was 80% after 60 days of transfer to polythene bags (Figure 1(H–I)).

This is the first report of an efficient *in vitro* propagation protocol of *Z. armatum* through leaf explants (via callus) with

high shoot multiplication (10.4 shoots/callus piece) and 100% rooting. This method can be effectively utilized for obtaining large number of planting material of the *Z. armatum* following field trials.

Conclusion

In conclusion, an efficient and reproducible protocol for high rate of adventitious shoot regeneration and excellent rooting was developed in *Z. armatum* through callus induced from leaf explants. The protocol established can be effectively used for mass scale production and cultivation following field trials. This will be useful not only for commercialization of the species but also help in reducing the pressure on its natural populations, thereby improving its threatened status.

Acknowledgements

The authors are thankful to the Director, G.B. Pant National Institute of Himalayan Environment & Sustainable Development, Kosi-Katarmal, Almora, MoEF & CC, Govt. of India, New Delhi, for facilities; Dr. V. K. Purohit (HAPRC, Srinagar) for technical help.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Uttarakhand State Council for Science and Technology, Dehradun, is thanked for financial assistance.

References

Anonymous. 1976. The wealth of India – a dictionary of Indian raw materials & industrial products raw materials. Vol. 11. New Delhi: Council of Scientific and Industrial Research; p. 17–25.

Bhatt ID, Dhar U. 2000. Combined effect of cytokinin on multiple shoot production from cotyledonary node explants of *Bauhinia vahlii*. Plant Cell Tissue Organ Cult. 62(1):79–83.

Bhatt ID, Dhar U. 2004. Factors controlling micropropagation of *Myrica esculenta* Buch-Ham ex D.Don: a high value wild edible of Kumaun Himalaya. Afr J Biotechnol. 3:534–540.

Bisht NS, Bhandari SL. 2007. Callusing response and direct *in vitro* regeneration of nodal explants of *Zanthoxylum armatum*. In: Trivedi PC, editor. Biotechnology in plant improvement. Jaipur, India: Pointer Publishers; p. 195–212.

Espinosa AC, Pijut PM, Michler CH. 2006. Adventitious shoot regeneration and rooting of *Prunus serotina in vitro* cultures. Hort Sci. 41:193–201.

Gaur RD. 1999. Flora of the district Garhwal North Himalaya. Srinagar (Garhwal), India: Transmedia Publishers; p. 811.

Giri L, Dhyani P, Rawat S, Bhatt ID, Nandi SK, Rawal RS, Pande V. 2012a. *In vitro* production of phenolic compounds and antioxidant activity in callus suspension cultures of *Habenaria edgeworthii*: a rare Himalayan medicinal orchid. Ind Crops Prod. 39:1–6.

Giri L, Jugran A, Dhyani P, Rawat S, Andola H, Rawal RS, Bhatt ID, Dhar U. 2012b. *In vitro* propagation, genetic and phytochemical assessment of *Habenaria edgeworthii*: an important Astavarga plant. Acta Physiol Plant. 34(3):869–875.

Guo DP, Zhu ZJ, Hu XX, Zheng SJ. 2005. Effect of cytokinins on shoot regeneration from cotyledon and leaf segment of stem mustard (*Brassica juncea* var. tsatsai). Plant Cell Tiss Organ Cult. 83(1):123–127.

Hare PD, Van Staden J. 1994. Inhibitory effect of thidiazuron on the activity of cytokinin oxidase isolated from soybean callus. Plant Cell Physiol. 35(8):1121–1125.

Herve P, Jauneau A, Paques M, Marien J, Boudet AM, Teulie'Res C. 2001. A procedure for shoot organogenesis *in vitro* from leaves and nodes of an elite *Eucalyptus gunnii* clone: comparative histology. Plant Sci. 161:645–653.

Hisatomi E, Matsui M, Kobayashi A, Kubota K. 2000. Antioxidative activity in the pericarp and seed of Japanese pepper (*Xanthoxylum piperatum*). J Agric Food Chem. 48(10):4924–4928.

Huetteman CA, Preece JE. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss Organ Cult. 33(2):105–119.

Hwang SJ, Hwang B. 2003. An efficient *in vitro* propagation of *Zanthoxylum piperitum* DC. Kor J Crop Sci. 11:316–320.

Imaizumi K, Totsuka Y, Oda S, Yamada Y. 1993. Production of shoot-tip culture lines of Asakura-sanshoo (*Zanthoxylum piperitum* var.f. *inerme* Makino) with high aroma emanation. Biosci Biotechnol Biochem. 57(4):591–593.

Jain N, Srivastava SK, Aggarwal KK, Ramesh S, Kumar S. 2001. Essential oil composition of *Zanthoxylum alatum* seeds from Northern India. Flavour Fragr J. 16(6):408–410.

Joshi B, Lekhak S, Sharma A. 2009. Antibacterial property of different medicinal plants: *Ocimum sanctum*, *Cinnamomum zeylanicum*, *Zanthoxylum armatum* and *Origanum majorana*. Kathmandu Univ J Sci Eng & Technol. 5:143–150.

Kala CP. 2010. Assessment of availability and patterns in collection of Timroo (*Zanthoxylum armatum* DC.): a case study of Uttarakhand Himalaya. Med Plants Int J Phytomed Relat Ind. 2:91–96.

Kim JH, Lee KS, Oh WT, Kim KR. 1989. Flavor components of the fruit peel and leaf oil from *Zanthoxylum piperitum* DC. Korean J Food Sci Technol. 21:562–568.

McCown BH, Lloyd G. 1981. Woody plant medium (WPM) – a mineral nutrient formulation for microculture of woody plant species. Hort Sci. 16:453–453.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 15(3):473–497.

Murthy BNS, Murch SJ, Saxena PK. 1998. Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. In Vitro Celldevbiol-Plant. 34(4): 267–275.

Mushtaq MN, Ghimire S, Alamgeer Akhtar MS, Adhikari A, Auger C, Schini-Kerth VB. 2019. Tambulin is a major active compound of a methanolic extract of fruits of *Zanthoxylum armatum* DC causing endothelium-independent relaxations in porcine coronary artery rings via the cyclic AMP and cyclic GMP relaxing pathways. Phytomedicine. 53:163–170.

Nandi SK, Palni LMS, Kumar A, editors. 2002. Role of plant tissue culture in biodiversity conservation and economic development. Gyanodaya Parkashan, Nainital: Hima Vikas Occasional Publication No-15. ISBN: 81-85097–55-0.

Nandi SK, Palni LMS, Pandey H, Chandra B, Nadeem M. 2016. Selection of elites and *in vitro* propagation of selected high-value Himalayan medicinal herbs for sustainable utilization and conservation. In: Anis M, Ahmad N, editors. Plant tissue culture: propagation, conservation and crop improvement. Singapore: Springer Science and Business Media; p. 15–44.

Prathanturarug S, Soonthornchareonnon N, Chuakul W, Phaidee Y, Saralamp P. 2003. High-frequency shoot multiplication in *Curcuma longa* L. using Thidiazuron. Plant Cell Rep. 21(11):1054–1059.

Prathanturarug S, Soonthornchareonnon N, Chuakul W, Phaidee Y, Saralamp P. 2005. Rapid micropropagation of *Curcuma longa* using bud explants pre cultured in thidiazuron supplemented liquid medium. Plant Cell Tiss Organ Cult. 80(3):347–351.

Purohit S, Jugran AK, Bhatt Palni LMS, Bhatt A, Nandi SK. 2016. *In vitro* approaches for conservation and reducing juvenility of *Zanthoxylum armatum* DC: an endangered medicinal plant of Himalayan region. Trees. 31(3):1101–1108.

Purohit S, Nandi SK, Palni LMS, Giri L, Bhatt A. 2015a. Effect of sulfuric acid treatment on breaking of seed dormancy and subsequent seedling establishment in *Zanthoxylum armatum* DC: an endangered medicinal plant of the Himalayan region. Natl Acad Sci Lett. 38:301–304.

Purohit S, Rawat V, Jugran AK, Singh RV, Bhatt ID, Nandi SK. 2015b. Micropropagation and genetic fidelity analysis in *Valeriana jatamansi* Jones. J Appl Res Med Arom Plants. 2:15–20.

Purohit S, Bhatt A, Bhatt ID, Nandi SK. 2015c. Propagation through air layering in *Zanthoxylum armatum* DC: an endangered medicinal plant in the Himalayan region. Proc Natl Acad Sci, India, Sect B Biol Sci. 86(3):607–610.

Raghu AV, Geetha SP, Martin G, Balachandran I, Ravindran PN. 2006. Direct shoot organogenesis from leaf explants of *Embelia ribes* Burm. F.: a vulnerable medicinal plant. J Res. 11(1):57–60.

Rynjah CV, Devi NN, Khongthaw N, Syiem D, Majaw S. 2018. Evaluation of the antidiabetic property of aqueous leaves extract of *Zanthoxylum armatum* DC. using *in vivo* and *in vitro* approaches. J Tradit Complement Med. 8(1):134–140.

Samant SS, Butola JS, Sharma A. 2007. Assessment of conservation diversity: status and preparation distribution of management plan for medicinal plants in the catchment area of parbati hydroelectric project stage—III in northwestern Himalaya. J Mount Sci. 4:34–56.

Singh SK, Syamal MM. 2001. A short pre culture in thidiazuron or for chlorfenuron improves axillary shoot proliferation in rose micropropagation. Sci Hort. 91(1-2):169–177.

Thomas TD. 2007. Pretreatment in thidiazuron improves the *in vitro* shoot induction from leaves in *Curculigo orchioides* Gaertn., an endangered medicinal plant. Acta Physiol Plant. 29(5):455–461.

Tiwary M, Naik SN, Tewary DK, Mittal PK, Yadav S. 2007. Chemical composition and larvicidal activities of the essential oil of *Zanthoxylum armatum* DC (Rutaceae) against three mosquito vectors. J Vector Borne Dis. 44(3):198–200.

Upadhyaya K, Ashok PK. 2010. Concentration dependent antioxidant activity of *Zanthoxylum armatum*. J Pharm Res. 37:1581–1582.

Wang LY, Jing RY. 2006. Culture *in vitro* of *Zanthoxvlum bungesnum* and rapid propagation of regenerated plantlet. Acta Agriculturae Boreali-Sin. 21:67–69.

Wang SY, Steffens GL, Faust M. 1986. Breaking bud dormancy in apple with a plant growth regulator, thidazuron. Phytochemistry. 25(2): 311–317.

Ying ZM, Wang P, Wang XM, Wang HX, Wang LP. 2009. *In vitro* culture and plant regeneration system of *Zanthoxylum dissitum* Hemsl. J Nat Sci Hunan Normal Uni. 32:106–111.