

Micropropagation of an antidiabetic medicinal plant, *Artemisia pallens*

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Abstract: *Artemisia pallens* Wall. is an important aromatic medicinal plant used as a folk remedy for the treatment of diabetes mellitus. The present study was initiated to explore in vitro propagation of *Artemisia pallens* using different explants on Murashige and Skoog (MS) media supplemented with varying concentrations and combinations of growth regulators. Highest callogenic response (100%) was shown by shoot tip explants with 2 mg/L 2,4-dichlorophenoxyacetic acid. The highest number of shoots (14.25 ± 1.65) and highest shoot length (4.25 ± 0.47 cm) were observed on MS medium with 3 mg/L kinetin. After 40 days, shoots grown in vitro were transferred to rooting media. The highest number of roots (12 ± 0.08) and root length (8.15 ± 1.13 cm) were recorded on MS medium with 3 mg/L indole-3-butyric acid. The regenerated plantlets after 30 days were hardened in plastic cups containing sterile garden soil, farmyard soil, and sand (2:1:1) and were transferred to a greenhouse.

Key words: Acclimatization, *Artemisia*, callogenesis, MS medium, regeneration

1. Introduction

Artemisia pallens Wall. is a small aromatic herbaceous plant about 60 cm in height belonging to the family Asteraceae, native to the southern part of India, especially to the states of Karnataka, Tamil Nadu, Andhra Pradesh, and Maharashtra. It is commonly known as “davana”. An ethnomedical search conducted by Tropical Botanic Garden and Research Institute, Palode, Trivandrum (India), revealed that this plant is used in southern India as a folk remedy for the treatment of diabetes mellitus. Reports showed that oral administration of the methanol extract of the aerial parts of *Artemisia pallens* lowers the level of blood glucose in normal and diabetic rats (Subramoniam et al., 1996). Davanone, davan ether, davana furan, and linalool are the major constituents of davana oil. Methyl cinnamate, ethyl cinnamate, bicyclogermacrene, 2-hydroxyisodavanone, farnesol, geranyl acetate, sesquiterpene lactones, and germacranolides are also found (Pujar et al., 2000). Leaves and flowers are highly valued in the making of floral decorations and oils. Leaves are very small, bluish green with yellow flowers, and inconspicuous. It is utilized in traditional Ayurvedic medicinal formulations. Essential oil of *Artemisia pallens* is used as an antiseptic and disinfectant and is commercially important due to its fragrance (Misra et al., 1991). *Artemisia pallens* also

possesses antibacterial activity (Ruikar et al., 2009). Santonin, a sesquiterpene lactone, is widely distributed in the genus *Artemisia* within the family Asteraceae. It possesses antiinflammatory, antipyretic, and analgesic properties (Al-Harbi et al., 2000). The plant has also been screened for anthelmintic activity (Akhtar et al., 2000). Ruthless exploitation has resulted in severe reduction of this natural antidiabetic herb. Hence, it became extremely important to develop an in vitro protocol for rapid regeneration of *Artemisia pallens*. The aim of the present investigation was to establish an easy in vitro micropropagation protocol for *A. pallens* that would be an important parameter for the genetic transformation of *Artemisia pallens* to enhance the production of secondary metabolites. Plant tissue culture provides an efficient system for transgenic production (Hansen and Wright, 1999). We have reported an easy in vitro system to establish and maintain regeneration in *Artemisia pallens*. The present study reports the range of callus formation using different plant growth regulators for the first time.

2. Materials and methods

Experimental studies were conducted on *Artemisia pallens* Wall. in the Plant Tissue Culture Laboratory of the Department of Botany, Sant Gadge Baba Amravati University, Amravati, India.

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2.1. Plant material

Seeds of *Artemisia pallens* were collected from Chandur Bazar, Amravati (Maharashtra, India), in December 2010. The seeds were surface disinfected with 0.1% (W/V) aqueous mercuric chloride for 4 min, followed by rinsing 3 to 5 times in sterile distilled water. The seeds were then surface sterilized with 40% alcohol for 3 min and finally washed with autoclaved distilled water for 3–5 min to remove traces of surface sterilants. Surface disinfected seeds were inoculated into sterile test tubes containing 20 mL of plain Murashige and Skoog (MS) medium. Shoot tip (with 1 to 2 leaf primordia), leaf, and petiole explants excised from 40-day-old seedlings grown in vitro were used as the explant source for the present study. *Artemisia pallens* was identified by the Botanical Survey of India (BSI), Western Regional Centre, Koregaon Road Pune, India. A voucher specimen, as voucher specimen number GMYAP2, was deposited at the BSI. The plant specimens were also deposited in the Departmental Museum of the Department of Botany, Sant Gadge Baba Amravati University, Amravati, as voucher specimen number 0252.

2.2. Media preparation and explant inoculation

The basal medium used during the entire study was MS salt solution (Murashige and Skoog, 1962). Growth regulators used throughout the study were 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin. All culture media were fortified with 3% sucrose solidified with 0.8% agar. In all experiments, the chemicals used were of analytical grade (HiMedia, India; Sigma-Aldrich, USA; and E. Merck, Germany). All inoculations were carried out under aseptic conditions in a laminar air flow cabinet. Shoot tip, leaf, and petiole explants were inoculated by inserting their cut ends in MS medium supplemented with various concentrations and combinations of growth regulators. The cultures were maintained in a culture room incubated with a 16-h light cycle in every 24 h. The temperature was regulated at 20 ± 2 °C.

2.3. Callus induction

Shoot tip, leaf, and petiole explants excised from 40-day-old seedlings grown in vitro were inoculated on MS media supplemented with different concentrations of BAP, 2,4-D, IAA, kinetin, and NAA singly or in combination with each other (Table 1) for callus induction.

2.4. Multiple shoot induction

Calli formed after 40 days of culture were subcultured on MS media containing different concentrations/combinations of BAP, kinetin, NAA, and IAA (Table 2) to induce multiple shoots.

2.5. Multiple root induction

Multiple shoots formed were transferred to MS media with different concentrations/combinations of auxins (Table 3) to induce multiple rooting in them.

2.6. Acclimatization and transfer of plantlets to soil

Regenerated plantlets were isolated from the culture media and washed with sterile double distilled water to remove adhering medium. They were transferred to plastic cups containing sterile garden soil, farmyard soil, and sand (2:1:1). Each potted plantlet was irrigated initially with distilled water every 3 days for 3 weeks. The potted plantlets were initially maintained in a programmable environmental chamber for 5 weeks. The temperature and humidity were adjusted to 20 ± 2 °C and 50%, respectively. The temperature of the programmable environmental chamber was raised by 1 °C every 5 days. The plantlets were then transferred to normal laboratory conditions for 2 weeks. Finally, after the 57th day, the plantlets were transplanted to the Departmental Botanical Garden and placed under shade for further growth and development. The morphological characteristics, growth characteristics, and survival efficiency were observed.

3. Results and discussion

3.1. Seed germination

Surface sterilized seeds were germinated on MS media (Figure 1); 95% of the seeds had germinated after 5–7 days of dark exposure at 23 °C. Fully grown seedlings were observed within 40 days after transfer to photoperiodic conditions for 16/8 h of light/dark at 20 ± 1 °C. Similar results were reported in *A. vulgaris*, except that 10% filtered sterilized coconut water was added to media (Sujatha and Kumari, 2007). Shoot tip, leaf, and petiole segments of *A. pallens* were cultured on MS media supplemented with 2,4-D, kinetin, 2,4-D+kinetin, BAP+NAA, and kinetin+IAA in different concentrations to test the efficiency of sprouting of various explants. It was observed that after 9–12 days of inoculation, the explants showed callus initiation in all the concentrations.

3.2. Callus induction

The callogenic response showed variations among the hormonal combinations/concentrations and types of explants used. The callus color ranged from white to dark green, while texture ranged from soft to compact-hard and from embryogenic to nonembryogenic. The callus response varied among 70%–100%, 72%–90%, and 60%–80% in shoot tip, leaf, and petiole explants, respectively. Callus initiation was observed after the ninth day of inoculation. Among different types of explants used, shoot tips proved to be best for callus induction. Highest callogenic response, i.e. 100%, was noticed with shoot tip explants at 2 mg/L 2,4-D (Figure 1). The callus obtained

Table 1. Callus induction from shoot tip, leaf, and petiole explants at different concentrations and combinations of growth regulators.

PGR	Conc. (mg/L)	Shoot tip explants		Leaf explants		Petiole explants	
		RP (%)	Characteristics	RP (%)	Characteristics	RP (%)	Characteristics
2,4-D	1.0	85	Dark green, compact, hard, nonembryogenic	78	Light green, compact, embryogenic	77	Dark brown, compact, hard, nonembryogenic
	1.5	84	Light green, compact, embryogenic	88	Green, compact, hard, embryogenic	80	Dark green, compact, hard, nonembryogenic
	2.0	100	Green, compact, soft, embryogenic	82	Brown, compact, hard, nonembryogenic	70	Dark brown, compact, hard, nonembryogenic
	2.5	94	Light green, compact, embryonic	84	Brown, compact, hard, nonembryogenic	64	Dark green, compact, hard, nonembryogenic
	3.0	92	Light green, compact, soft, embryogenic	90	Dark brown, compact, hard, embryogenic	82	Light brown, compact, hard, nonembryogenic
Kin.	0.5	86	Whitish green, compact, soft, nonembryogenic	78	Light green, compact, embryogenic	60	Light brown, compact, hard, embryogenic
	1.0	78	Light brown, hard, embryogenic	88	Light green, compact, soft, embryogenic	82	Dark brown, compact, hard, nonembryogenic
	1.25	80	Green, compact, hard, nonembryogenic	78	Green, compact, hard, nonembryogenic	68	Light green, compact, soft, embryogenic
	1.50	75	Light green, compact, soft, embryogenic	80	Light green, compact, embryogenic	75	Dark green, compact, soft, embryogenic
	2.0	96	Green, compact, soft, embryogenic	92	Light green, compact, soft, embryogenic	86	Green, compact, soft, nonembryogenic
2,4-D + Kin.	1 + 0.5	77	Dark brown, hard, nonembryogenic	78	Brown, compact, hard, nonembryogenic	70	Whitish green, compact, soft, nonembryogenic
	1.5 + 1.5	78	Brown, compact, hard, nonembryogenic	72	Green, compact, hard, nonembryogenic	68	Light green, soft, embryogenic
	2.0 + 1.0	90	Light green, compact, soft, embryogenic	88	Brown, compact, hard, embryogenic	88	Dark brown, compact, hard, nonembryogenic
BAP + NAA	0.5 + 0.25	86	Whitish green, soft, nonembryogenic	72	Light green, compact, soft, nonembryogenic	68	Light green, compact, hard, embryogenic
	1.5 + 0.5	80	Light green, soft, embryogenic	60	Dark green, compact, hard, nonembryogenic	74	Green, compact, hard, embryogenic
	2.5 + 0.75	76	Green, compact, soft, embryogenic	90	Brown, compact, hard, nonembryogenic	60	Dark brown, compact, hard, embryogenic
Kin. + IAA	0.5 + 0.25	75	Whitish green, compact, hard, nonembryogenic	76	Green, compact, hard, nonembryogenic	66	Green, compact, hard, nonembryogenic
	1.0 + 0.5	70	Green, compact, hard, embryogenic	80	Light green, compact, soft, embryogenic	80	Dark brown, compact, soft, nonembryogenic
	1.5 + 0.75	90	Dark brown, compact, hard, nonembryogenic	90	Whitish green, compact, soft, embryogenic	70	Brown, compact, soft, nonembryogenic

Values are means of 3 replicates.

PGR = Plant growth regulator; Conc. = concentration, RP = response percentage.

Table 2. Multiple shoot induction from callus at different concentrations/combinations of growth regulators.

Plant growth regulator	Concentration (mg/L)	Response (%)	Average no. of shoots*	Average shoot length* (cm)
BAP	0.25	80	9 ± 1.29	2.75 ± 0.48
	0.5	78	5 ± 0.29	1.97 ± 0.36
	1.0	64	10.7 ± 1.10	2.02 ± 0.34
	1.5	78	7 ± 1.29	1.87 ± 0.42
	2.0	94	10 ± 1.82	2.32 ± 0.57
	3.0	80	9 ± 1.29	3.4 ± 0.40
Kin.	1.0	66	5.75 ± 0.85	2.42 ± 0.54
	1.5	75	6 ± 0.91	2.22 ± 0.66
	2.0	64	9 ± 1.29	2.3 ± 0.43
	3.0	92	14.25 ± 1.65	4.25 ± 0.47
Kin. + NAA	1.0 + 0.25	75	9.25 ± 1.49	1.87 ± 0.13
	1.5 + 0.50	88	6.5 ± 1.5	1.87 ± 0.21
	2.0 + 1.0	92	7 ± 1.29	2.05 ± 0.21
BAP + IAA	1.0 + 0.5	78	5.75 ± 0.85	1.37 ± 0.24
	2.0 + 0.75	64	11.2 ± 0.75	1.77 ± 0.33
	3.0 + 1.0	88	5.25 ± 0.94	2.37 ± 0.25

*Mean ± standard error of 3 replicates.

Table 3. Multiple root induction from shoots grown in vitro with different concentrations/combinations of growth regulators.

Plant growth regulator	Concentration (mg/L)	Response (%)	Average no. of roots*	Average root length* (cm)
IAA	1	80	2.5 ± 0.64	4.1 ± 0.98
	2	90	5.75 ± 0.85	3.52 ± 0.63
	3	88	6.75 ± 1.10	3.22 ± 1.09
IBA	1	75	4 ± 0.91	2.25 ± 0.33
	2	68	8 ± 0.70	3.62 ± 0.53
	3	94	12 ± 0.08	8.15 ± 1.13
NAA	0.5	72	4 ± 0.91	4.17 ± 1.08
	1.5	60	7 ± 1.29	2.87 ± 0.42
	2.0	78	8.25 ± 0.85	4.3 ± 0.50
	3.0	90	4 ± 1.08	3.85 ± 0.46
IAA + BAP	1.0 + 0.5	84	4.75 ± 0.75	3.95 ± 0.92
	1.0 + 0.75	74	3.75 ± 1.54	3.95 ± 0.73
	1.0 + 1.0	80	3 ± 1.08	5.1 ± 1.26

*Mean ± standard error of 3 replicates.

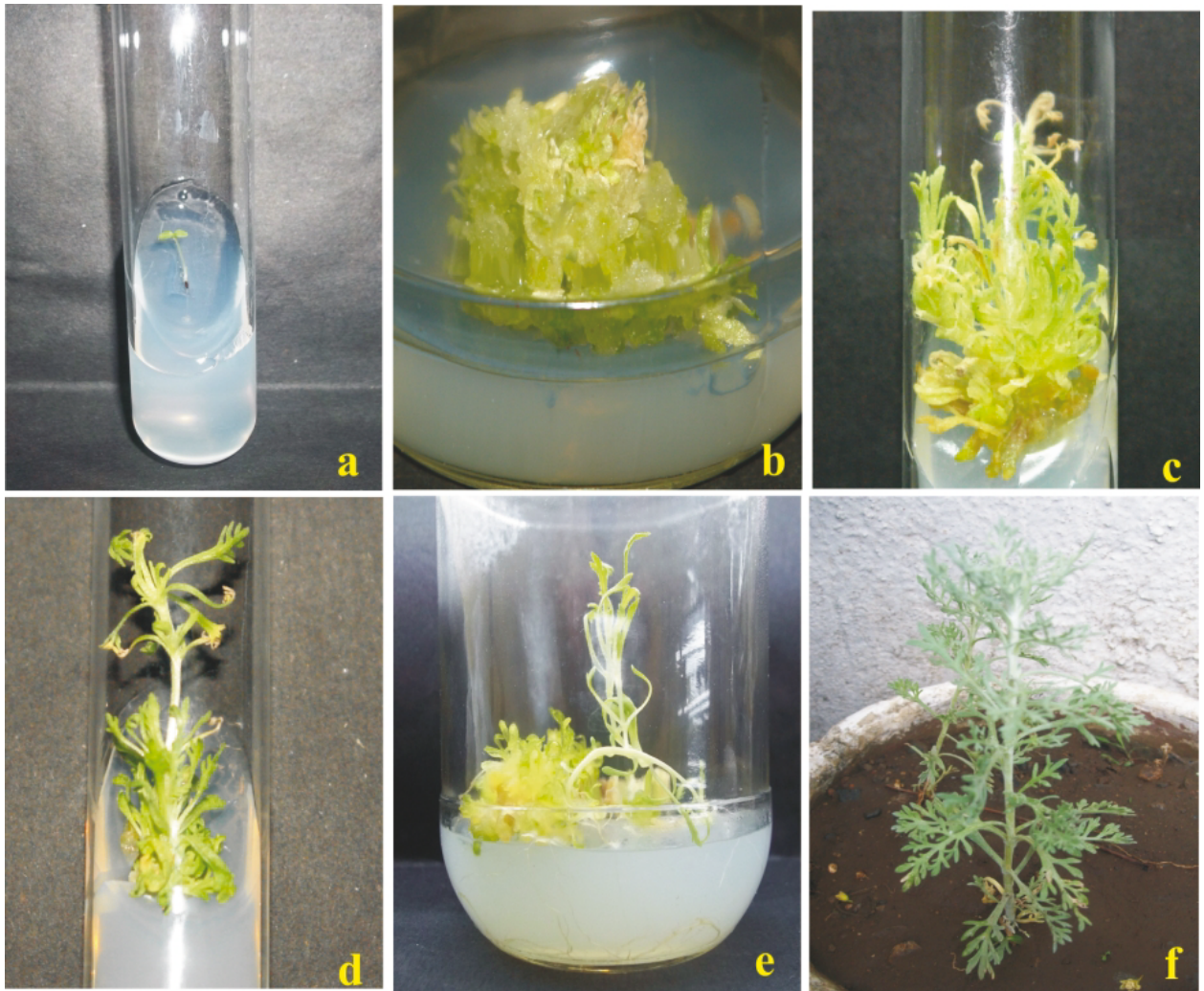


Figure 1. In vitro studies on *Artemisia pallens*. a- Seed germination on plain MS medium; b- callus formation from shoot tip explants with 2 mg/L 2,4-D; c and d- multiple shoot formation with 3 mg/L kinetin; e- multiple root formation with 3 mg/L IBA; f- acclimatized plant.

at this concentration was green, compact, soft, and embryogenic. Cytokinin (BAP) alone at low concentrations did not induce dedifferentiation, but at higher doses it was able to induce calli (Nin et al., 1996). Callus formation was also reported in *Artemisia scopario* with 2,4-D alone and in combination with kinetin (Aslam et al., 2006). Benjamin et al. (1990) reported 3 types of cultures in *A. pallens* on MS media supplemented with different concentrations/combinations of hormones. Unorganized calli were obtained on MS medium supplemented with BAP+2,4-D and semiorganized calli on BAP+IAA from shoot buds. The effect of plant growth regulators on callus formation showed that, in *Rosa gallica* L., 2 mg/L of 2,4-D and 1 mg/L of BAP were the optimum concentrations, increasing callogenesis more than 90% in vegetative (leaf, stem, and petiole) and petal explants and more than 80%

in flower explants (pistil and anther). Callus induction in *Artemisia absinthium* from leaf explant on MS medium supplemented with 2,4-D+NAA (1.0 + 0.5 mg/L) was also reported (Yatoo, 2010). The callus obtained after 40 days was subcultured on shooting media.

3.3. Multiple shooting

The calli that formed from shoot tip explants were used for multiple shoot induction. Multiple shoot initiation started from the callus after 10 days when subcultured onto fresh MS medium containing different cytokinins like BAP and kinetin and auxins like NAA and IAA. Kinetin among the various cytokinins proved best for multiple shoot induction. The highest number of shoots, i.e. 14.25 ± 1.65 , and highest shoot length, i.e. 4.25 ± 0.47 cm, were observed at 3 mg/L kinetin (Figure 1). The cytokinin BAP has been commonly used for the induction of organogenesis in

many plants (Zilis et al., 1979; Parrot et al., 1992; Mneny and Mantell, 2002). Effectiveness of different cytokinins to induce multiple shoot formation was revealed in the order of BAP > kinetin > zeatin > adenine (Prakash et al., 1994). BAP was essential for shoot production in both cultivars since no shoot development occurred on media without BAP (Gürel and Gülşen, 1998). Chalageri and Babu in 2012 showed that among different concentrations of growth regulators used in *Viola patrinii*, NAA (2.68 µM) with kinetin (23.25 µM) showed 88% shoot regeneration, which was achieved in 4 weeks. Multiple shooting was obtained on MS media with BAP+IAA+NAA (Benjamin et al., 1990). Several studies have demonstrated that an excess of cytokinins can promote the regeneration of shoots in tissue cultured plants (Kevers et al., 1984). BAP has been used in preference to other cytokinins to induce multiple shoots in *A. pallens*. Encapsulated shoot buds grown in vitro on MS media supplemented with NAA (5.3 µM) + BA (1.33 µM) + biotin (1 mg/L) + casein hydrolysate (3 mg/L) showed controlled multiplication (Sharief et al., 1997). Nair et al. (1979) stated that BAP is most effective for meristem, shoot tip, and bud culture. When BAP+kinetin in combination was tested, the frequency of response was still much better (Sujatha and Kumari, 2007). In *Macrotyloma uniflorum*, a large number of shoots developed from shoot tip explants on MS media supplemented with BAP alone or BAP+kinetin (Shamsudeen et al., 1999). Similar combinations produced greater numbers of shoots in *Arachis hypogaea*, *Carthamus tinctorius*, and *Eclipta alba* (Venkatachalam and Jayabalan, 1997; Baskaran and Jayabalan, 2005; Kumar and Kumari, 2005). In the present investigation, higher concentrations of BAP reduced shoot number and length, which is in agreement with the findings of Hu and Wang (1983) and Indhra and Dhar (2000).

3.4. Multiple rooting

To induce rooting, individual elongated shoots after 40 days were cultured on MS media augmented with different auxin concentrations. For root induction MS medium was augmented with different hormones like IAA (1–3 mg/L), IBA (1–3 mg/L), NAA (0.5–3 mg/L), and IAA+BAP (1 + 0.5–1.0 mg/L). Roots were visible within 8–12 days following the transfer of elongated shoots to the rooting media. The highest number of roots (12 ± 0.08) and highest root length (8.15 ± 1.13 cm) were encountered with 3 mg/L IBA (Figure 1). Gürel et al. in 2001 reported that over 90% of the regenerated shoots of *Beta vulgaris* L. could be readily rooted when cultured on medium containing 3.0 mg/L IBA.

IAA was reported as a potential auxin for rooting of *Arachis stenosperma* and *Arachis villosa* (Vijayalakshmi and Giri, 2003). Similar results were obtained in *Sesbania drummondii* (Cheepala et al., 2004). However, in the present

study, IBA was found to be most beneficial, followed by NAA. The present finding confirms the previous work on *Artemisia judaica* with IBA (Liu et al., 2003) and on *Morus alba* with NAA (Anuradha and Pullaiah, 1992).

3.5. Acclimatization

The hardened plantlets were initially maintained in a programmable environmental chamber for 5 weeks. Regenerated plantlets were isolated from the culture media and washed with sterile double distilled water to remove adhering medium. These plantlets were transferred to plastic cups that contained sterile garden soil, farmyard soil, and sand (2:1:1). The survival rate after hardening for the first 5 weeks was 100%. It decreased to 80% after 10 weeks of acclimatization. There was no detectable variation among the acclimatized plants with respect to morphological and growth characteristics. All of the micropropagated plants were free from external defects (Figure 1) and are maintained in the departmental garden.

4. Conclusions

The main objective of this study was to develop a system for mass propagation and aseptic growth of *Artemisia pallens*. The plant is used for different medicinal purposes. The literature reveals different regeneration systems for mass or in vitro propagation of many *Artemisia* species. Shoot tip explants supplemented with 2,4-D had the best callogenic response (Figure 2). Kinetin showed maximum shooting response both in terms of number of shoots and shoot length from subcultured calli (Figure 3). Maximum number of roots and maximum root length from shoots grown in vitro were achieved with NAA and IBA, respectively (Figure 4). In the present work, surprisingly, all the explants produced calli; this is the first report on in vitro callogenesis in *Artemisia pallens*. A wide range of

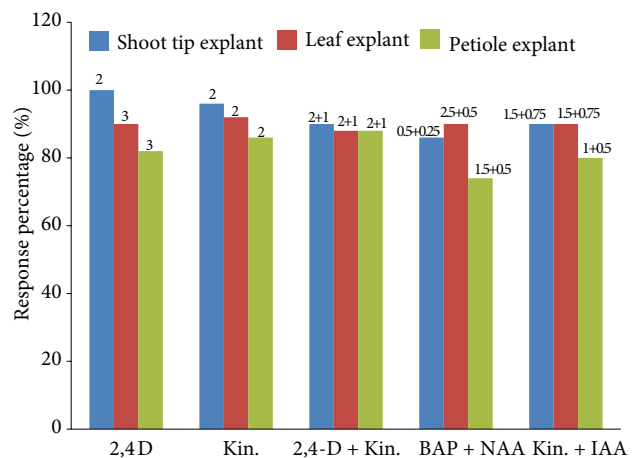


Figure 2. Maximum callogenic response from shoot tip, leaf, and petiole explants. The concentration of growth regulator is in mg/L.

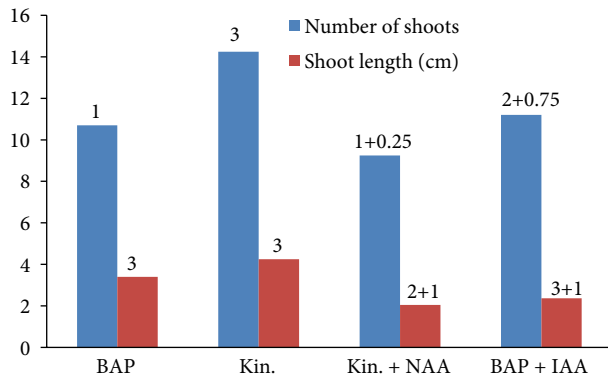


Figure 3. Multiple shoot induction from calli. The concentration of growth regulator is in mg/L.

calli were obtained, which make extraction of biologically active compounds easier. This will help in its conservation as well as in establishing a genetic transformation system.

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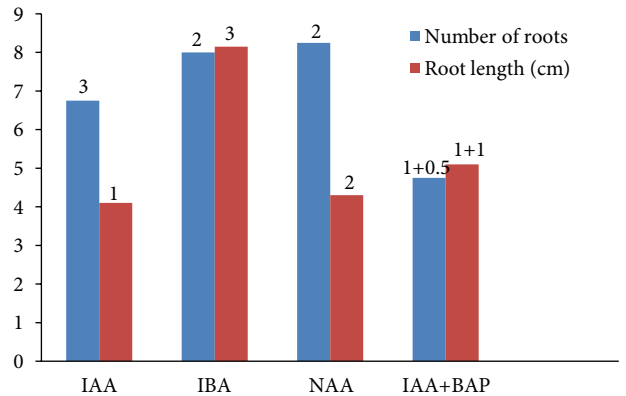


Figure 4. Multiple root induction from shoots grown in vitro. The concentration of growth regulators is in mg/L. Values are means \pm standard errors of 3 replicates.

The plants produced from excised tissues will be helpful for genetic engineering and cell culture techniques in the future.

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