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In vitro shoot Proliferation from Nodal Explants of *Aegiceras corniculatum* L. (Blanco.)

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A B S T R A C T

The micropropagation protocol of tropical, small evergreen, true mangrove, and woody shrub or tree species, *Aegiceras corniculatum*, has been standardized. Axillary shoot proliferation was induced *in vitro* from nodal explants excised from 3-4 years field-grown old plant which was then used as explants for the establishment of *in vitro* cultures. Surface-sterilized explants were cultured on the Murashige and Skoog (MS) basal medium supplemented with different concentrations and a combination of growth regulators. Nodal explants, collected during January-March, exhibited 63% shoot proliferation and 1.799 ± 0.013 number of shoots per explants when cultured on Murashige and Skoog (MS) basal medium supplemented with 6-benzylaminopurine (BAP) alone at 2.0 mg/l. The maximum shoot proliferation (75%) and the number of shoots per explants (1.933 ± 0.011) were recorded on the medium containing 3.0 mg/l BAP, 0.5 mg/l Kinetin (Kn), and 150 mg/l Ascorbic acid (as an antioxidant) which helped to reduce browning of the explants and facilitated the induction of shoots. Harder shoot explants containing 1-2 nodes were found to be better explants as compared with soft apical and axillary shoots. This is the first report of shoot proliferation by *in vitro* propagation techniques using nodal segments of *A. corniculatum.*

Introduction

Aegiceras corniculatum L. (Blanco.) is a tropical, small evergreen, true mangrove, and woody shrub or tree species, that grow in the intertidal zone, belonging to the Myrsinaceae family, commonly known as river mangrove and Khalsi in Bengali. It is also known as a crypto viviparous species of mangrove and is one of the pioneer mangroves which can thrive in 3% salinity [(6)] by secreting salt though its leaf glands [(3)]. Moreover, it is an important honey-producing mangrove species in the Sundarban of West Bengal and

Bhitarkanika mangrove forests of Orissa. The species forms a potential source for high-quality honey and bee-wax from its flower and the wood is used as firewood, fencing materials, cores and mud wall, *etc*. Siddiqi [19] also stated that honey bees produce the best quality honey from the nectar of *A. corniculatum.* Its wood is used for fuel and charcoal production. Rahaman[14] recorded that the height of the plant in Sundarban varies from 2 m to 4 m and individual trees can reach up to 6-7 m, but usually much shorter. The bark is rough and dark grey or black. Mangrove and associated plants provide a wide domain for therapeutic application in recent years, most yet to be explored. The mangroves and their associated plants have various economic values and environmental functions [9]. Bandaranayake [4] informed that extracts from mangrove plant and associates has been used worldwide for medicinal purposes and have been recorded around 349 metabolites which turn out to be a rich source of steroids, diterpenes, and triterpenes, saponius, flavonoids, alkaloids, and tannins. Banerjee *et al.* [5] reported that the leaves of *A. corniculatum* are rich in flavonoids. Roome *et al.* [15,16] recorded that, *A. corniculatum* extract has analgesic, anti-arthritic and anti-inflammatory properties, whereas, leaf suspension of this species showed a moderate reduction in blood glucose [8].

This species has great medicinal, economical and ecological values for which it was being exploited indiscriminately for a very long time and became a threatened species according to ICUN red list 2010[7]. Tissue culture is a very important technique and widely used technologies in the tree improvement program. Sharp [18] reported that micropropagation may be useful for forest trees characterized by poor seed set, absence of uniform seed production, and seed prone to genetic damage or loss of viability during storage-features common in mangroves[17]. Many numbers of endangered and threatened species have been successfully regenerated using *in vitro* culture methods using nodal segments. Many researchers reported that mangrove species are recalcitrant to tissue culture studies [1,11]. During *in vitro* culture of mangrove plants frequently turn brown or black and eventually die shortly after inoculation [2, 10], as it excretes high tannin and phenolic compounds. Though, *in vitro* propagation of *A. corniculatum* has not yet been done and maybe the best alternative method for propagating this species. With this background, an experiment was conducted to explore the possibility of *in vitro* shoot proliferation from nodal explants of *Aegiceras corniculatum* L. (Blanco.) for the development of a protocol for future work.

Materials and Methods

Explant selection and surface sterilization

Seedlings of *A. corniculatum* were collected from Sundarbans Mangrove forest of India and grown at the experimental garden of the Institute of Forest Productivity campus, Ranchi during the monsoon season of the year (since May-June, 2014), Fresh axillary shoots produced during January-March and November-December which served as the source of explants. Young juvenile and healthy shoots were excised from field-grown plants of *A. corniculatum*. Removed all the leaves and cut into convenient sizes (3-4 cm long and 0.3-0.5 cm thick) each with 1-2 nodes was washed with running tap water for 30 minutes to remove adhering dust particles from the surface, then immersed in water containing 2-3 drops of surfactant solution (Tween-20) for 10 minutes followed by Cetrimide 0.1% for 5 minutes in gentle agitating condition and rinsed thoroughly. After that, treated with 0.1% (w/v) fungicide (Bavistin) solution for18-20 min and cleaned

4-5 times with sterilized double-distilled water. These were then surface sterilized with 0.1 % (w/v) $HgCl_2$ for 3-9 min and rinsed with autoclaved water 4-5 times and transfer to the culture medium.

Media preparation and cultural conditions

After surface sterilization, explants trimmed to (2-3 cm size) were immunized on Murashige and Skoog's (MS)[13] basal medium augmented with several concentrations of cytokinin BA, Kn, and TDZ (0.5-5.0 mg/l) alone and in combination with different concentrations of auxins NAA (0.5-2.0 mg/l) with additives (ascorbic acid 150 mg/l) together with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar were used for the shoot initiation. The final pH of the medium along with the Plant Growth Regulator (PGR) was adjusted to 5.6-5.8 using an electronic pH meter with the use of 0.1N NaOH or 0.1 N HCl solutions. The media was dispensed about 25 ml to each 25x150 mm culture tube, capped with non-absorbent cotton plug, and autoclaved for 15 min at 15 psi at 121°C. The culture was maintained at 25 ± 2°C under a 16-hour photoperiod.

Bud break and shoot induction

Aseptic transfer of explants was done in a laminar air-flow hood. The interior was swabbed with 70% ethanol before inoculation. After sterilization, explants were excised aseptically and inoculated on MS medium supplemented with various concentrations of cytokinin BA, Kn, and TDZ (0.5-5.0 mg/l) alone and in combination with different concentrations of auxins NAA (0.5-2.0 mg/l) with additives (ascorbic acid 150 mg/l). The addition of auxins and cytokinins to the medium had a positive effect on shoot formation. Explants were evaluated in terms of percentage of shoot induction, the number of shoots per explants, shoot length, and the number of leaves per explants after 6 weeks of culture. Each treatment was replicated 3 times using 20 explants for each treatment.

Statistical analysis

Data recorded, were analysed using Systat-12 software [20] for the computation of descriptive statistics (*i.e.*, mean, standard deviation, and critical difference). The Completely Randomized Design (CRD) was followed for statistical analysis.

Result and Discussion

Plant tissue culture is an important tool not only for plant propagation and conservation but also it is used to enhance the plant bioactive compounds through cell culture [19]. This is the study mainly focused on developed a protocol for shoot proliferation of *A. corniculatum* through nodal explants using different concentrations of cytokinin (BA, Kin, and TDZ) alone and in combination with different concentrations of auxins (NAA) with additives (ascorbic acid 150 mg/l). The results on the effect of different concentrations of cytokinin on *in vitro* establishment from nodal explants of *A. corniculatum* have been presented in (Table 1 & 2). Maximum shoot induction (63 %), number of shoots per explants (1.799 ± 0.013), shoot length (1.924 ± 0.043 cm), and number of leaves per explant (1.833 ± 0.050) were recorded in media supplemented with BAP 2.0 mg/l. In the case of media supplemented with Kinetin, 3.0 mg/l has induced maximum shoot induction (42%) with the number of shoots per explants (1.756 ± 0.126), shoot length (1.811 ± 0.041 cm), and the number of leaves per explants (1.776 ± 0.067 cm). The maximum per cent of shoot induction in TDZ treatment was observed in TDZ 3.0 mg/l (30%) with the number of shoots per explant (1.571 ± 0.038), shoot length (1.477 ± 0.030

cm), and the number of leaves per explants (1.586 ± 0.073cm). MS medium fortified with other additives had helped in the proliferation of the shoots, which might be due to the effect of cytokinin/auxins/antioxidants used either in alone or in combination. Similar *in vitro* propagation in other mangrove species have already been reported where shoot proliferation achieved on MS medium fortified with 0.13 µM of **α-** Naphthalene-acetic acid and 4.44 µM of 6-Benzylaminopurine from the mangrove species *Candelilla* [12]. Species like *Avicennia marine* produced shoot in MS medium containing 0.01 mg/l IBA, 2 mg/l kinetin, and 0.5 mg/l BA [1].

Table.1 Effect of Cytokinin (BAP, Kn and TDZ) on shoot proliferation from nodal explant of *Aegiceras corniculatum*

PGR	Conc. (mg/l)	Treatment code	% of cultures response	Mean No of shoots /explant	shoot length in (cm)	Mean No of leaves/explant
Control	0	BKN1	10	1.244 ± 0.244	1.139 ± 0.139	1.244 ± 0.244
BAP	0.5	BKN2	30	1.596 ± 0.032	1.474 ± 0.077	1.716 ± 0.056
	1.0	BKN3	60	1.763 ± 0.091	1.788 ± 0.023	1.767 ± 0.107
	2.0	**BKN4**	**63**	**1.799 ± 0.013**	**1.924 ± 0.043**	**1.833 ± 0.050**
	3.0	BKN5	52	1.724 ± 0.043	1.833 ± 0.060	1.733 ± 0.035
	4.0	BKN6	38	1.509 ± 0.057	1.474 ± 0.057	1.728 ± 0.030
	5.0	BKN7	28	1.509 ± 0.064	1.440 ± 0.110	1.617 ± 0.022
KN	0.5	BKN8	20	1.648 ± 0.044	1.526 ± 0.102	1.589 ± 0.038
	1.0	BKN9	25	1.551 ± 0.137	1.387 ± 0.030	1.610 ± 0.044
	2.0	BKN10	35	1.558 ± 0.093	1.605 ± 0.114	1.663 ± 0.059
	3.0	**BKN11**	**42**	**1.756 ± 0.126**	**1.811 ± 0.041**	**1.776 ± 0.067**
	4.0	BKN12	38	1.506 ± 0.046	1.650 ± 0.167	1.686 ± 0.079
	5.0	BKN13	15	1.489 ± 0.037	1.436 ± 0.127	1.524 ± 0.063
TDZ	0.5	BKN14	10	1.332 ± 0.173	1.293 ± 0.034	1.438 ± 0.223
	1.0	BKN15	20	1.414 ± 0.000	1.293 ± 0.034	1.525 ± 0.056
	2.0	BKN16	27	1.542 ± 0.066	1.353 ± 0.064	1.542 ± 0.066
	3.0	**BKN17**	**30**	**1.571 ± 0.038**	**1.477 ± 0.030**	**1.586 ± 0.073**
	4.0	BKN18	25	1.414 ± 0.000	1.259 ± 0.034	1.414 ± 0.000
	5.0	BKN19	10	1.382 ± 0.212	1.293 ± 0.034	1.276 ± 0.138
			S.E. (±m)	***0.105***	***0.081***	***0.098***
			C.D. 5%	***0.300***	***0.234***	***0.282***
			C.V. (%)	***11.734***	***9.426***	***10.652***

*The figures in parentheses are square root transformed values. Data are shown as mean values of variables ±SE.

Table.2 Effect of cytokinin and auxin on the induction of shoot development in *Aegiceras corniculatum* from nodal segment

Hormone con. (mg/l)	Treatment code	% of cultures response	No. of shoots /explant	Shoot length in (cm)	No. of leaves/explant
control	BKN1	10	1.138 ± 0.138	1.109 ± 0.109	1.000 ± 0.000
BAP 1.0 + KIN 0.5	BKN2	30	1.443 ± 0.029	1.293 ± 0.034	1.414 ± 0.000
BAP 2.0 + KIN 0.5	BKN3	50	1.709 ± 0.106	1.639 ± 0.069	1.549 ± 0.095
BAP 3.0 + KIN 0.5	BKN4	70	1.883 ± 0.037	1.905 ± 0.048	1.859 ± 0.024
BAP 4.0 + KIN 0.5	BKN5	42	1.452 ± 0.037	1.446 ± 0.078	1.524 ± 0.063
BAP 1.0 + KIN 3.0	BKN6	32	1.524 ± 0.063	1.592 ± 0.002	1.648 ± 0.018
BAP 2.0 + KIN 3.0	BKN7	35	1.596 ± 0.049	1.639 ± 0.069	1.569 ± 0.032
BAP 3.0 + KIN 3.0	BKN8	32	1.470 ± 0.056	1.470 ± 0.101	1.480 ± 0.034
BAP 4.0 + KIN 3.0	BKN9	20	1.138 ± 0.138	1.109 ± 0.109	1.470 ± 0.056
BAP 1.0 + KIN 4.0	BKN10	30	1.276 ± 0.138	1.248 ± 0.127	1.545 ± 0.018
BAP 2.0 + KIN 4.0	BKN11	20	1.138 ± 0.138	1.109 ± 0.109	1.452 ± 0.037
BAP 3.0 + KIN 4.0	BKN12	20	1.138 ± 0.138	1.075 ± 0.075	1.138 ± 0.138
BAP 4.0 + KIN 4.0	BKN13	10	1.138 ± 0.138	1.138 ± 0.138	1.138 ± 0.138
BAP 1.0 + NAA 0.5	BKN14	40	1.563 ± 0.018	1.666 ± 0.042	1.507 ± 0.049
BAP 2.0 + NAA 0.5	BKN15	50	1.661 ± 0.018	1.739 ± 0.071	1.569 ± 0.056
BAP 3.0 + NAA 0.5	BKN16	70	1.710 ± 0.059	1.880 ± 0.039	1.704 ± 0.032
BAP 4.0 + NAA 0.5	BKN17	41	1.540 ± 0.049	1.504 ± 0.049	1.515 ± 0.063
BAP 1.0 + NAA 1.0	BKN18	45	1.590 ± 0.032	1.477 ± 0.030	1.491 ± 0.040
BAP 2.0 + NAA 1.0	BKN19	65	1.736 ± 0.037	1.809 ± 0.072	1.823 ± 0.092
BAP 3.0 + NAA 1.0	BKN20	75	1.933 ± 0.011	2.036 ± 0.031	1.949 ± 0.034
BAP 4.0 + NAA 1.0	BKN21	52	1.580 ± 0.030	1.756 ± 0.102	1.604 ± 0.012
BAP 1.0 + NAA 2.0	BKN22	38	1.542 ± 0.066	1.764 ± 0.023	1.493 ± 0.040
BAP 2.0 + NAA 2.0	BKN23	45	1.570 ± 0.023	1.786 ± 0.064	1.512 ± 0.057
BAP 3.0 + NAA 2.0	BKN24	48	1.637 ± 0.053	1.417 ± 0.052	1.538 ± 0.007
BAP 4.0 + NAA 2.0	BKN25	30	1.470 ± 0.056	1.357 ± 0.030	1.623 ± 0.023
		S.E. (±m)	***0.080***	***0.075***	***0.059***
		C.D. 5%	***0.227***	***0.215***	***0.167***
		C.V. (%)	***9.201***	***8.605***	***6.670***

*The figures in parentheses are square root transformed values. Data are shown as mean values of variables ±SE.

Fig.1 Shoot induction from nodal segment of *A. corniculatum*

Fig.2 Shoot development from nodal *segment* of *A. corniculatum*

In another experiment, after surface sterilization, the nodal explants (2-3 cm size) were immunized on MS basal medium supplemented with various concentrations of cytokinin (BAP and Kn) in combination with different concentrations of auxin (*i.e.,* NAA) along with additives (*i.e.,* ascorbic acid 150 mg/l). The results on the effect of different concentrations and combinations of cytokinin and auxins on *in vitro* establishment (Fig. 1 & 2), from nodal explants of *A. corniculatum*, have been presented in Table 2. Maximum shoot induction (75%), number of shoots per explants (1.933 ± 0.011), shoot length (2.036 ± 0.031 cm) and number of leaves per explants (1.949 ± 0.034) were recorded on the medium of BKN20 (*i.e.,* BAP 3.0 mg/l + Kn 0.5 mg/l +Ascorbic acid 150 mg/l). Comparatively harder shoot explants containing 1-2 nodes were found to be better explants as compared with soft apical and axillary shoots. This is the first tissue culture report on this species *Aegiceras corniculatum.*

In conclusion, the present study was proposed to develop a standard protocol for shoot proliferation from nodal explants followed by standardization of media and concentration and combinations of plant growth regulator for *Aegiceras corniculatum.* The shoots were proliferated from nodal explants when inoculated on MS medium supplemented with a combination of growth regulators *i.e.,* 3.0 mg/l of BAP and 1.0 mg/l of NAA. It is recommended to select harder shoot explants of *A. corniculatum* containing 1-2 nodes for better results under *in vitro.*

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