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Rapid *in vitro* multiplication and plant regeneration from Shoot tip explants of *Ammannia baccifera* L.: An important medicinal plant

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Abstract
Tissue culture technique was developed for mass production of multiple shoots from valuable medicinal plant of *Ammannia baccifera* Linn. High frequency of direct shoot proliferation was induced in shoot tip explants cultured on Murashige and Skoog's medium supplemented with 6-benzylaminopurine (BAP). Among the various cytokinins tested (BAP, kinetin and Zeatin), BAP proved to be the most effective. The most suitable medium for shoot regeneration was MS medium fortified with 2.0 mg/l 6-benzylaminopurine (BAP), on which highest percentage of shoot response (80±1.90), the maximum number (7.8±1.40) of shoots per explant, having a shoot length of (2.8±1.01cm) were produced. The highest number of shoots multiplication response (85±1.68), the maximum number shoot per explants (28.6±1.63) and shoot length (3.0±1.12 cm) was documented on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA. Further shoot elongation was achieved on MS medium containing 0.5 mg/l GA_3. The elongated micro shoots were rooted on MS medium fortified with 2.0 mg/l IAA and 0.8 mg/l IBA on highest percentage of rooting response (80±2.16), the maximum number of root per explants (18.4±1.50) and root length (4.2±1.34 cm) was recorded. The well developed shoot and rooted plantlets were successfully transferred into paper cups containing vermiculite, sand and soil in the ratio of 1:2:1 and subsequently they were established in the greenhouse. The results of this study provide the first successful report on *in vitro* direct plant regeneration from shoot tip explants of *A. baccifera* L.

Keywords: Shoot multiplication, growth regulators, direct organogenesis, *Ammannia baccifera*

1. Introduction
Medicinal plants are of great interest as pharmaceutical industries depend in part on plants for the production of secondary metabolites. Conventional methods like plant tissue culture techniques allow to produce enormous amount of plants within short period of time. *Ammannia baccifera* Linn. belongs to the family Lythraceae which is a glabrous, erect branching herb, found as weed in rice fields and moist or marshy places throughout India. It is commonly known as Blistering Ammannia, in Tamil Nirumelneruppu. The herb is reported to possess anti-typhoid, anti-tubercular and anti-tumor properties [1, 2]. *A. baccifera* is widely used in traditional Chinese herbal formulations for treating human female infertility, gastroenteropathy, spinal disease, hamorrhoids, urethritis, common cold, abscess, sore, itching, and other skin diseases [3, 4]. It has been also reported to possess anticancer, antirheumatic, antidiuretic, antipyretic, antisteroidogenic, antimicrobial, antiurolithic, and rubefacient activities [4]. This plant was found to possess hypothermic, hypertensive, antibacterial and CNS depressant activities [5, 6]. The methanolic extracts of *A. baccifera* showed low toxicity against mouse fibroblasts cell subselective cytotoxicity against different human gastric, colon and breast cancer cell lines [7].
In the present investigation, we describes the first report on rapid and efficient plant regeneration of *A. baccifera* using shoot tip explants excised from healthy mature plant. The improved method that we have established for plant regeneration in *A. baccifera* could be applied for large scale propagation and also ensure a continuous supply of secondary metabolites of plants produced in limited time. To conserve its germplasm and therapeutically uses.

2. Materials and Methods
2.1 Plant material and surface sterilization of explants
Mature shoot tip explants taken from the field grown healthy plant of *A. baccifera*. The explants were first washed under running tap water for 30 minutes and followed by washing with 5% (v/v) Teepol detergent for 10 minutes by continuous shaking. After repeated wash with sterile double distilled water (DDW) for 3-4 times. Further washing was done under

laminar air flow, where explants were treated with 0.1% (w/v) freshly prepared mercuric chloride ($HgCl_2$) for 2 minutes and then finally rinsed with sterile DDW 3-4 times. After sterilized, the shoot tip explants inoculated on sterile media.

2.2 Nutrient media and culture conditions
The culture medium contained Murashige and Skoog [8] medium with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Himedia, India) were used in all investigates. The p^H of the medium was adjusted to 5.6 with 0.1N NaoH or 0.1 N HCl after addition of the growth regulators. The culture vials containing the media were autoclaved at 121ºC, for 20 minutes and 1.4 × 104 kg m^{-2} pressure. All the cultures was maintained in a sterilized culture room at 25±2^{0}C, under 16 hours photoperiods provided by cool white fluorescent light 50 µmol m^{-2} s^{-1} (Philips, India) and with 55-60% relative humidity.

2.3 Shoot induction
MS medium lacking any plant growth regulators was kept as control with each experiment. Surface sterilized mature shoot tip explants were cultured on MS medium supplemented with different concentrations of Cytokinins like BAP (6-benzylaminopurine), KIN (kinetin) and Zea (Zeatin) 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l for shoot bud induction. After 12 days of culture, direct shoot origination from the mature shoot tip explants were observed. The induction rate and average number of regeneration shoots/explant were documented after 3 weeks of culture notice.

2.4 Shoot multiplication and shoot elongation
For shoot initiation derived from *in vitro* regenerated shoots were cultured on MS medium fortified with various concentration of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) in combination with NAA (α – Naphthalene acetic acid) (0.2, 0.3, 0.4, 0.5, 0.6 and 0.7) for multiple shoot development. To achieve shoot multiplication *in vitro* derive shoot were cultured on MS medium supplemented with various concentration of GA_3 (Gibberellic acid) (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8) alone for shoot elongation.

2.5 *In vitro* rooting
For root induction, individual shoots were separated from the multiple shoot bunches and inoculated on MS mediums augment with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l IAA (Indole-3-acetic acid) and combine with IBA 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 IBA (Indole-3-butyric acid). The percentage of rooting, root number and length of root per shoot were documented after 3 weeks of culture.

2.6 Acclimatization
The rooted plants were removed from culture vessels, washed carefully with rinse sterilized double distilled water to eliminate adhering medium and transferred to paper cups containing vermiculite, sand and soil in the ratio of 1:2:1. The plantlets were covered with transparent polythene bags to ensure high humidity, and watered every 2 days for 2 weeks. The polythene bags were removed after 3 weeks in order to acclimatize plants to natural conditions. After 5 weeks, these plantlets were transferred to paper cups containing normal garden soil and maintained in the greenhouse under natural conditions.

2.7 Statistical analysis
All the regeneration experiments were conducted in three replicates 20 explants per repetition were employed for each treatment. The effect of different treatments was quantified and data was analyzed statistically using software SPSS version 16.0 (SPSS inc., Chicago, USA). The significant differences between means were assessed by Duncan's multiple range test at $P<0.05$. The results are expressed as means±SE of three experiments.

3. Results and Discussion
3.1 Shoot bud induction
Mature shoot tip explants excised from *A. baccifera* plants were inoculated on MS medium fortified with different concentrations of BAP, KIN and Zea (0.5-3.0 mg/l) alone for shoot bud initiation. Among different cytokinins tested, BAP alone was performed highest frequency of shoot bud induction from shoot tip. After 2 weeks, the shoot buds developed directly from the mature shoot tip explants. The highest percentage of shoot induction (80±1.90), the maximum number of shoots per explants (7.8±1.40) and shoot length (2.8±1.01 cm) induction was observed on MS medium containing 2.0 mg/l BAP (Table 1 & Fig. 1B). Shoot bud regeneration was gradually decreased when the cytokinin concentration was increased beyond optimum level. Ananthakrishnan *et al.*, described that BAP enhanced highest number of shoots in *Cucurbita pepo* [9]. Vasudevan *et al.*, stated that BAP induced maximum number of shoot buds from shoot tip explants of *Cucumis sativus* [10]. Ganasan and Huyop reported that BAP was found to be the best cytokinin for induction of shoots from explants of *Citrullus lanatus* [11]. Agarwal and Kamal obtained shoot induction on MS medium fortified with BAP alone in *Momordica charantia* [12]. Lee *et al.*, affirmed that adventitious shoot bud regeneration was effectively achieved on MS medium augmented with BAP alone for *Cucurbita maxima* [13].

Table 1: Effect of different concentrations of Cytokinins (BAP, Kin and Zea) on shoot regeneration from shoot tip explants of *Ammannia baccifera* L.

Plant Growth Regulators (mg/l)			Shoot regeneration response (%) (Mean±SE)	Number of Shoots/explant (Mean±SE)	Shoot length (cm) (Mean±SE)
BAP	KIN	Zea			
00	00	00	00±00^{h}	00±00^{e}	00±00^{d}
0.5			30±1.84^{d}	1.6±0.75^{bcde}	0.7±0.20^{bcd}
1.0			45±1.80^{c}	2.3±1.30^{bcde}	1.6±0.65^{abc}
1.5			60±1.98^{b}	4.1±1.67^{b}	2.1±0.86^{ab}
2.0			80±1.90^{a}	7.8±1.40^{a}	2.8±1.01^{a}
2.5			55±1.61^{b}	3.4±1.24^{bcd}	1.6±0.56^{ac}
3.0			40±1.87^{c}	2.5±1.16^{bcde}	0.4±0.15^{cd}
	0.5		15±1.67^{fg}	1.1±0.60^{bcde}	0.3±0.15^{cd}
	1.0		30±2.28^{d}	1.7±0.58^{bcde}	0.9±0.36^{bcd}
	1.5		45±2.37^{c}	2.3±1.16^{bcde}	1.2±0.36^{bc}
	2.0		60±2.19^{b}	3.8±1.46^{bc}	1.9±0.47^{ab}
	2.5		25±2.06^{de}	2.1±0.81^{bcde}	0.8±0.30^{bcd}

	3.0		10±1.83^{g}	1.0±0.32^{bce}	0.3±0.15^{cd}
		0.5	15±1.77^{fg}	0.3±0.15^{de}	0.1±0.05^{d}
		1.0	25±1.85^{de}	0.7±0.25^{cde}	0.3±0.15^{cd}
		1.5	40±1.76^{c}	1.4±0.75^{bcde}	0.9±0.36^{bcd}
		2.0	20±1.83^{ef}	0.7±0.20^{cde}	0.4±0.25^{cd}
		2.5	10±1.76^{g}	0.3±0.20^{de}	0.1±0.05^{d}
		3.0	00±00^{h}	00±00^{e}	00±00^{d}

Each value represents the mean ± SE. Each mean value followed by the same letter does not differ significantly according to Duncan's Multiple Range Test (P ≤0.05).

3.2 Multiple shoot bud regeneration and elongation

Regeneration of multiple shoot buds from shoot tip explants were induced on MS medium augmented with various concentrations of BAP (0.5-3.0 mg/l) and combination with NAA (0.2-0.7 mg/l) tested. The highest frequency of shoot bud multiplication was observed on MS medium supplemented 2.0 mg/l BAP and combination with 0.5 mg/l NAA. The maximum percentage of shoot multiplication (85±1.68), highest number of shoots per explants (28.6±1.63) and shoot length (3.0±1.12 cm) was observed on 6 weeks of culture period (Table 2 & Fig. 1C). Similar observations with auxin and cytokinin combinations have been recorded in other species, e.g. *Petasites hybridus* [14] and *Hypericum perforatum* [15]. Nunes *et al.,* also described that BAP in combination with low concentration of auxin was found to be most effective for multiple shoot bud induction [16]. Similarly, synergistic effect of BAP and NAA has been also reported in *chrysanthemum* [17]. *In vitro* regenerated multiple shoot buds were cultured on MS medium containing different concentrations of gibbrelic acid (GA_3) (0.1–0.8 mg/l) for shoot bud elongation. After 2 weeks of culture, the shoot bud elongation was recorded. The shoot elongation response (75±2.15) and maximum shoot length (8.3±1.51cm) of shoot bud elongation was noticed on MS medium fortified with 0.5 mg/l GA_3 (Table 3 & Fig. 1D). When GA_3 concentration was increased above (0.5 mg/l) the length of the shoot bud elongation was reduced inversely. GA_3 had important role in shoot bud elongation as reported earlier for other plants such as *Eclipta alba* [18] and *Andrographis paniculata* [19]. GA_3 played an important role in shoot elongation as reported in *Canavalia virosa* [20] and *Helianthus annuus* [21].

Table 2: Effect of Cytokinin (BAP) along with Auxin (NAA) on Multiplication of Shoots from shoot tip explants of *Ammannia baccifera* L.

Plant Growth Regulators (mg/l)		Shoot multiplication response (%) (Mean±SE)	Number of Shoots/explant (Mean±SE)	Shoot length (cm) (Mean±SE)
BAP	NAA			
00	00	00±00^{g}	00±00^{f}	00±00^{b}
0.5	0.2	35±1.65^{e}	7.9±1.53^{de}	1.3±0.56^{ab}
1.0	0.3	55±1.36^{c}	13.4±1.58^{c}	1.8±0.91^{ab}
1.5	0.4	70±2.23^{b}	21.2±1.68^{b}	2.4±0.86^{ab}
2.0	0.5	85±1.68^{a}	28.6±1.63^{a}	3.0±1.12^{a}
2.5	0.6	45±1.87^{d}	11.8±1.93^{cd}	1.9±0.88^{ab}
3.0	0.7	20±2.27^{f}	6.8±1.57^{e}	1.0±0.60^{ab}

Each value represents the mean ± SE. Each mean value followed by the same letter does not differ significantly according to Duncan's Multiple Range Test (P ≤0.05).

Table 3: Effect of GA_3 on elongation of *in vitro* regenerated shoots from shoot tip explants of *Ammannia baccifera* L.

Plant Growth Regulators (mg/l)	Shoot elongation response (%) (Mean±SE)	Shoot length (cm) (Mean±SE)
GA_3		
00	00±00^{f}	00±00^{d}
0.1	20±1.95^{e}	1.7±0.85^{cd}
0.2	35±1.87^{d}	2.8±1.01^{bcd}
0.3	55±1.15^{bc}	4.7±1.33^{abc}
0.4	60±2.02^{b}	6.3±1.68^{ab}
0.5	75±2.15^{a}	8.3±1.51^{a}
0.6	50±2.27^{c}	4.6±1.41^{abc}
0.7	30±1.93^{d}	3.2±1.11^{bcd}
0.8	15±1.76^{e}	1.8±0.86^{cd}

Each value represents the mean ± SE. Each mean value followed by the same letter does not differ significantly according to Duncan's Multiple Range Test (P ≤0.05).

3.3 *In vitro* root induction

In vitro regenerated shoots were excised individually and transferred to MS medium augmented with different concentrations of IAA (0.5 – 3.0 mg/l) and combination with IBA (0.2 – 1.2 mg/l) for root induction was tested. The first *in vitro* roots emerged directly from the basal part of the shoots after 3 weeks of culture with no intervening callus. The highest percent of root induction (80±2.16), maximum number of roots (18.4±1.50) and root length (4.2±1.34 cm) was detected on MS medium fortified with 2.0 mg/l IAA and combined with 0.8 mg/l IBA (Table 4 & Fig. 1E). Similar results were also reported in *Decalepis hamiltonii* [22], *Stevia rebaudiana* [23].

Table 4: Effect of various concentrations of auxins for rooting in microshoots from shoot tip explants of *Ammannia baccifera* L.

Plant Growth Regulators (mg/l)		Rooting response (%) (Mean±SE)	Number of Roots/explant (Mean±SE)	Root length (cm) (Mean±SE)
IAA	IBA			
00	00	00±00^{e}	00±00^{d}	00±00^{c}
0.5	0.2	30±1.52^{d}	4.3±1.46^{cd}	1.0±0.26^{bc}
1.0	0.4	50±2.32^{c}	7.1±1.63^{c}	1.8±0.90^{abc}
1.5	0.6	65±1.41^{b}	14.8±2.56^{ab}	2.6±1.04^{abc}
2.0	0.8	80±2.16^{a}	18.4±1.50^{a}	4.2±1.34^{a}
2.5	1.0	60±1.63^{b}	13.1±1.84^{b}	3.2±1.32^{ab}
3.0	1.2	35±1.95^{d}	6.4±1.25^{c}	1.3±0.61^{abc}

Each value represents the mean ± SE. Each mean value followed by the same letter does not differ significantly according to Duncan's Multiple Range Test (P ≤0.05).

3.4 Acclimatization

The *in vitro* well developed rooted plantlets with expanded leaves were effectively transferred into paper cups containing vermiculite, sand and soil in the ratio of 1:2:1 and covered with polythene bags to safeguard high humidity. Primarily the plantlets were kept in the controlled environment for 3 weeks and the polybags were gradually removed in order to acclimatize the plantlets under greenhouse conditions. Consequently they were transferred to the field conditions and the survival rate observed was 84% (Fig. 1F). The acclimatized plants appeared morphologically uniform with normal leaves, shape, and growth patterns to the parental stock.

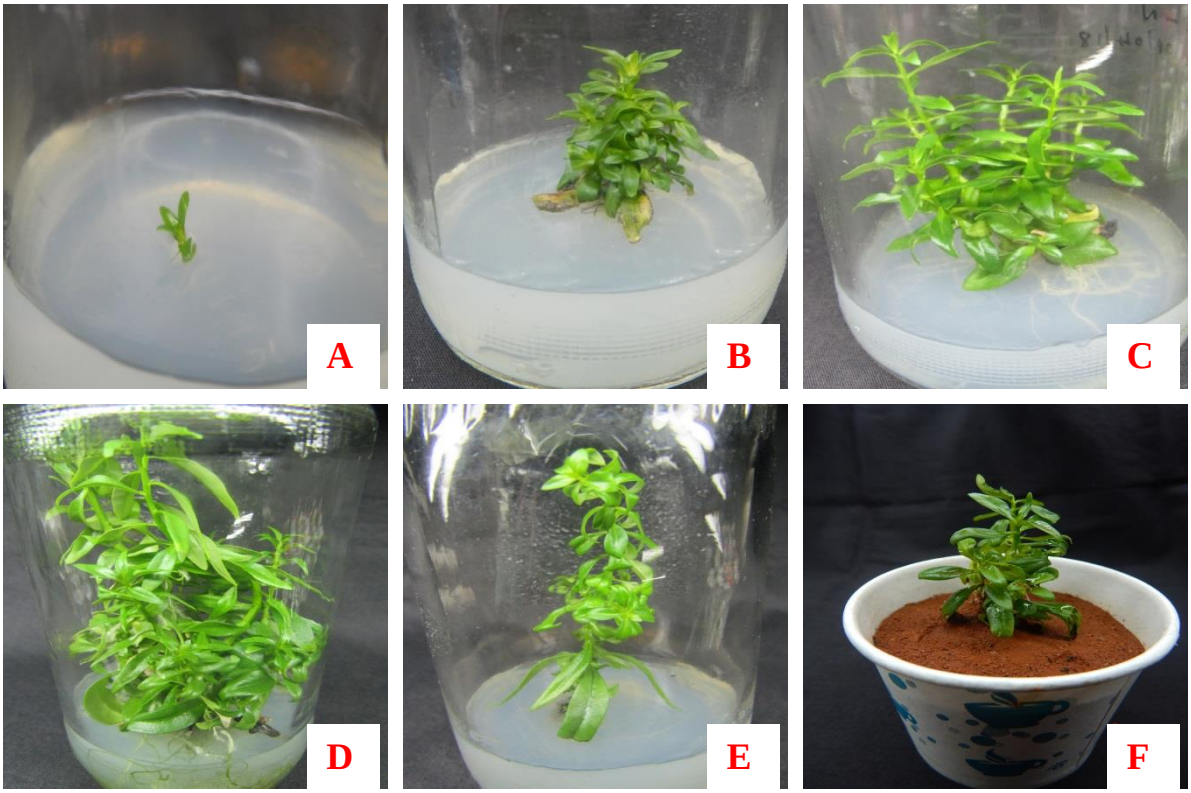

Fig 1: Micropropagation of *Ammannia baccifera* L. via direct regeneration using shoot tip explant.
A) Shoot tip explant of *Ammannia baccifera* L.
B) Shoot induction on MS medium containing BAP 2.0 mg/l
C) Multiple shoots proliferation on MS medium containing BAP 2.0 mg/l combined with NAA 0.5 mg/l.
D) Multiple shoots elongation on MS medium containing GA_3 0.5 mg/l.
E) Proliferation of shoot and rooted plantlet.
F) Acclimatized plant in soil.

4. Conclusion

The present investigation describes a simple and effective *in vitro* protocol for high frequency direct shoot regeneration from shoot tip explants of *A. baccifera* L. BAP at 2.0 mg/l was found to be best concentration for shoot induction. BAP 2.0 mg/l in combined with 0.5 mg/l NAA produced multiple shoots. GA_3 0.5 mg/l induced maximum shoot elongation. The highest frequency of rooted shoots was noted on 2.0 mg/l IAA and combination with 0.8 mg/l IBA. To be the best of our knowledge, this is the first report on high reproducible plantlet from shoot tip explant of *A. baccifera* L. Hence, current method can be used for large scale propagation of this important plant at commercial scale.

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