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High frequency and reproducible plant regeneration from nodal explants of *Ammannia baccifera* L. a folkloric medicinal plant for the production of vital compounds

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Abstract
Ammannia baccifera L. is folkloric medicinal plant used in the treatment of female infertility. The present study demonstrates efficient protocol for large scale in vitro plant regeneration from nodal explants. The highest frequency (90.9±0.20) of multiple shoot bud regeneration with maximum number of shoots (81.01±0.58 shoots/explant) was noticed on MS medium with 1.0 mg/l BA. Large scale plant production, in vitro derived axillary buds were cultured on MS medium fortified with BA (1.0 mg/l) + TDZ (0.2 mg/l) + GA_3 (1.5 mg/l) + IAA (2.5 mg/l) combination; about 355.4 shoots/explant were obtained after five subcultures on the same media composition. Elongated shoots were excised from the in vitro proliferated clumps were cultured on MS medium containing different concentrations of IAA and IBA (0.5–2.5 mg/l) for root induction. Highest frequency of rooting (95±0.57) with length of root (8.60 cm±0.17) was achieved on full-strength MS medium fortified with 1.5 mg/l IBA + 2.5 mg/l IAA. The rooted plantlets were successfully acclimatized with the sand soil ratio of 1:2 and subsequently they were established in the greenhouse. The present protocol would facilitate an alternative method for fast and large scale propagation of this folkloric medicinal plant to explore vital compounds.

Keywords: direct organogenesis, large scale production, nodal explants, cytokinins, auxins

1. Introduction
Ammannia baccifera L. belongs to the family Lythraceae and is commonly called "blistering Ammannia," "monarch red stem," "acrid weed" and "Bhatjambol." In Hindi, it is called "agni-buti," "ban mirich" "jaungali mehndi" and in Tamil "Neer Mel Neruppu" [1]. In India it occurs in tropical and sub-tropical regions particularly in Tamil Nadu it occurs in wet cultivated lands (Mainly in paddy fields) and their surroundings. The plants are erect and branched, and are annual herbs, growing up to 60 cm in height. *A. baccifera* is widely used in traditional Chinese/Indian herbal formulations for treating human female infertility [2], gastroenteropathy [3], spinal disease [4], hemorrhoids [5], urethritis [6], common cold [7], abscess, sore, itching and other skin diseases [8, 9]. It has been reported to possess anticancer, antirheumatic, antidiuretic, antipyretic, antisteroidogenic, antimicrobial and rubefacient activities [10]. And also the ethanolic extract of *A. baccifera* have been proved antiurolithic activity in male albino rats, by reducing formation of stones, primarily of magnesium ammonium phosphate with traces of calcium oxalate, and also dissolving pre-formed stones [11]. The plant *A. baccifera* L. is reported to have hentriacontine, dotriacontanol, betulinic acid, lupeol, ellagic acid, quercetin, lawsone which are responsible for the medicinal and pharmacological activities [12, 14].
Plant tissue culture techniques can be effective method for mass production of such overexploited and important medicinal plants. For an efficient large-scale culture, perpetual explant source that is fast and stable growing is needed for securing the culture materials. Root has been regarded as a good explant source for the production of both plantlets [15, 16] and secondary metabolites [17, 18]. Efficient, repeatable and rapid in vitro regeneration system is prerequisite for using recent advances in biotechnology to improve the plant species. The present research work is based on twofold objectives, viz., to develop an efficient and rapid propagation protocol of *A. baccifera* for the fulfillment of market demand in terms of medicinal and requirement of pharmacological industry. This is a first attempt to study the effect of different growth regulators on in vitro organogenesis of *A. baccifera* using different explants sources. 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 plants produced in limited time and space through ex vitro rooting for the production of vital compounds.

2. Materials and Methods
2.1 Plant material and Culture media conditions
Shoot and multiple shoot induction of mature nodal explants of field-grown *A. baccifera* L. were collected from 3 month old plants and were washed in running tap water. Explant were washed with few drops of 10% (v/v) Tween-20 to remove the superficial dust particles including microbes. Then, they were surface sterilized with 0.1% (w/v) mercuric chloride solution for 2 minutes followed by rinsing them for five times with sterile distilled water. Sterilized nodal explants (10 mm) were inoculated onto MS [19] solid medium containing 3% (w/v) sucrose with different concentrations of growth regulators for shoot and multiple shoot induction. This

hormonal composition was selected on the basis of the earlier reports [20]. The pH of the medium was adjusted to 5.6 with 0.1 N NaOH (Sodium Hydroxide) or HCl (Hydrochloric acid) before adding 0.8% (w/v) agar. Media (15 ml) were poured into 25 mm x 150 mm culture tubes (Borosil, Mumbai) and autoclaving (20 min at 121°C; 1.4 × 104 kg m^{-2}). All the cultures were incubated at 25±2°C, with 16-h photoperiod (50 µmol m^{-2} s^{-1}).

2.2 Influence of plant growth regulators on shoot, multiple shoot proliferation and shoot elongation

The individual nodal explants were placed vertically in each culture tube. Each tube containing MS medium supplemented with different concentrations of BA (0.2–2.5 mg/l), KN (0.2–2.5 mg/l) and Zeatin (0.2 – 1.5 mg/l) and 2iP (6-(α, α - Dimethylallylaminopurine) (0.2 – 1.5 mg/l) TDZ (0.1–0.5 mg/l) ("Table 1"). After 4 weeks on culture initiation, the efficacy of cytokinins on shoot proliferation was observed by recording (1) the frequency of primary explants developing shoots and (2) the number of shoots per explant. Multiple shoot induction was observed from cut shoot apex after 12 days of culture initiation [21]. The explants with shoot clusters after 4 weeks of culture on PGR supplemented media were subcultured on full or half strength-MS basal medium without PGR or supplemented with varied concentrations of GA_3 (0.05–2.0 mg/l) ("Table 2") to allow for the elongation of shoot buds. The frequency of elongation, mean shoot length and mean number of nodes per shoot were recorded after four weeks of culture [21].

Table 1: Effect of different concentrations of cytokinins on field-grown nodal explants of *Ammannia baccifera* (L.) for shoot and multiple shoot proliferation.

Cytokinins (mg/l)	Frequency of responding explants (%)	Mean number of shoots per responsive explant
Control	29.8 ± 0.60	7.63 ± 0.015
BA		
0.2	40.9 ± 0.57	31.65 ± 0.11
0.4	50 ± 1.52	42.05 ± 0.11
0.6	63.63 ± 0.20	60.50 ± 0.25
0.8	72.7 ± 0.20	65.75 ± 0.11
1.0	90.9 ± 0.20	81.01 ± 0.58
1.5	81.8 ± 0.26	70.05 ± 0.11
2.0	72.7 ± 0.10	60.34 ± 0.10
2.5	59.0 ±1.73	49.55 ± 0.05
KN		
0.2	36.3 ± 0.26	15.10 ± 0.15
0.4	45.4 ± 0.26	30.50 ± 0.15
0.6	59.0 ± 2.00	49.75 ± 0.15
0.8	86.3 ± 0.26	70.01 ± 0.05
1.0	77.2 ± 0.26	62.55 ± 0.15
1.5	68.1 ± 0.26	50.55 ± 0.11
2.0	54.5 ± 0.26	38.01 ± 0.17
2.5	27.2 ± 0.26	19.55 ± 0.15
Zea		
0.2	81.8 ± 0.26	71.60 ± 0.15
0.4	72.7 ± 1.00	65.30 ± 0.05
0.6	59.0 ± 2.64	51.23 ± 0.05
0.8	54.5 ± 0.26	43.96 ± 0.15
1.0	50 ± 2.64	39.65 ± 0.15
1.5	40.9 ± 0.10	30.01 ± 0.17
2ip		
0.2	72.7 ± 2.00	31.10 ± 0.11
0.4	68.1 ± 0.26	22.65 ± 0.11
0.6	59.0 ± 3.60	20.55 ± 0.11
0.8	50 ± 3.60	18.93 ± 0.11
1.0	45.4 ± 0.30	16.55 ± 0.10
1.5	40.9 ± 0.87	15.16 ± 0.05
TDZ		
0.1	79.2 ± 0.20	31.32 ± 0.20
0.2	87.1 ± 0.20	35.10 ± 0.11
0.3	70.3 ± 0.26	27.55 ± 0.17
0.4	61.5 ± 0.30	18.31 ± 0.15
0.5	55.4 ± 0.20	13.15 ± 0.11

Each value represents the mean± SE of three replicates, each with 22 explants. $P \leq 0.05$ level, according to Duncan's multiple range test.

Table 2: Effect of MS strength and GA_3 concentration on shoot elongation developed from mature plant nodal in *Ammannia baccifera* (L.) after 4 weeks of culture initiation.

Medium	GA_3 (mg l^{-1})	Percent of response (Mean±SE)	Length of shoot (cm) (mean ±SE)	Number of node (mean±SE)
Control				
Full MS	0	5 ± 0.11	1.56 ± 0.15	1.05 ± 0.20
Full MS	0.05	20 ±1.52	1.85 ± 0.15	2.01 ± 0.57
Full MS	0.1	35 ±1.00	2.50 ± 0.15	2.80 ±0.20
Full MS	0.5	45 ±1.15	3.40 ± 0.15	3.60 ± 0.15
Full MS	1.0	80 ±1.52	4.00 ± 0.15	4.03 ± 0.15
Full MS	1.5	95 ±1.52	5.97 ± 0.15	4.90 ± 0.15
Full MS	2.0	70 ±1.52	4.10 ± 0.05	4.02 ± 0.15
Control				
½MS	0	5 ± 0.11	1.06 ± 0.30	1.08 ± 0.20
½MS	0.05	15 ±1.52	2.10 ± 0.15	1.95 ± 0.15
½MS	0.1	25 ±1.73	3.00 ± 0.20	2.10 ± 0.15
½MS	0.5	50 ± 0.57	3.50 ± 0.05	2.50 ± 0.10
½MS	1.0	65 ±1.15	4.00 ± 0.01	3.01 ± 0.05
½MS	1.5	85 ±1.15	4.93 ± 0.01	3.50 ± 0.05
1/2MS	2.0	70 ±1.52	3.50 ± 0.01	3.00 ± 0.05

Each value represents the mean± SE of three replicates, each with 20 explants. $P \leq 0.05$ level, according to Duncan's multiple range test.

2.3 Rooting and Acclimatization

The elongated shoots (4–5 cm) having 2 or 3 expanded leaves were excised and transferred to root induction medium ("Table 3"). Elongated shoots were cultured on full or half-strength MS medium without PGR or supplemented with IAA (0.5 – 2.5 mg/l) and IBA (0.5–2.5 mg/l) ("Table 3"). Plantlets with well-developed roots were removed from the culture vessels and after washing the roots gently under running tap water, the plantlets were placed into paper cups filled with sterilized soilrite. The cups were covered with transparent polythene bags and irrigated daily with 1– 2 ml of sterilized MS salt solution for six days followed by sterilized distilled water, the plants were maintained in culture room at 25±2°C under 16-h/day illumination of 50 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. After two weeks the polythene bags were gradually removed, the plants were kept in the culture room for another two weeks. Further plantlets were transplanted into earthen pot containing garden soil and kept under shade in a net house for another two weeks before transferring to field conditions.

Table 3: Effect of different concentrations of IBA and IAA media on rooting of micro shoots of *Ammannia baccifera* (L.) after 4 weeks of culture initiation.

Medium	IBA(mg/l)	IAA(mg/l)	Percent of response (Mean±SE)	Number of root/explant (mean±SE)	Length of root (cm) (mean±SE)
Control			10 ±1.52	1.01 ± 0.15	1.23 ± 0.15
Full MS	0.5		20 ±1.15	1.39 ± 0.15	1.50 ±0.15
Full MS	1.0		40 ±1.52	1.50 ± 0.11	2.00 ± 0.10
Full MS	1.5		75 ±1.52	2.00 ± 0.10	3.50 ±0.15
Full MS	2.0		65 ±1.52	4.50 ± 0.15	5.80 ±0.15
Full MS	2.5		45 ±1.52	3.50 ± 0.15	4.50 ±0.15
Full MS		0.5	30 ±1.52	1.30 ± 0.15	1.86 ± 0.05
Full MS		1.0	40 ±2.00	1.75 ± 0.15	2.66 ± 0.05
Full MS		1.5	50 ±2.08	2.10 ± 0.05	3.10 ± 0.15
Full MS		2.0	70 ±1.52	5.10 ± 0.15	4.89 ± 0.10
Full MS		2.5	85 ±1.52	3.96 ± 0.05	3.55 ± 0.11
Full MS	0.5	1.0	45 ±1.52	2.50 ± 0.15	2.96 ± 0.15
Full MS	1.0	0.5	65 ±1.00	3.80 ± 0.15	4.10 ± 0.15
Full MS	1.5	2.5	95 ± 0.57	7.95 ± 0.15	8.60 ± 0.17
Full MS	2.0	1.0	80 ± 0.57	5.89 ± 0.36	6.30 ± 0.05
Full MS	2.5	2.0	60 ±1.52	4.80 ± 0.15	5.20 ± 0.15
Control			5 ±1.52	0.93 ± 0.15	0.98 ± 0.15
1/2MS	0.5		15 ±1.52	1.00 ± 0.15	1.80 ± 0.15
1/2MS	1.0		30 ±1.52	2.00 ± 0.17	2.70 ± 0.05
1/2MS	1.5		60 ±1.52	2.60 ± 0.10	3.50 ± 0.10
1/2MS	2.0		45 ±1.52	4.00 ± 0.15	5.80 ± 0.15
1/2MS	2.5		35 ±1.15	3.10 ±0.05	4.00 ± 0.05
1/2MS		0.5	10 ± 0.57	1.20 ±0.05	1.80 ± 0.05
1/2MS		1.0	25 ±1.52	1.98 ±0.05	2.55 ± 0.05

1/2MS		1.5	40 ±1.52	2.50 ±0.05	3.20 ± 0.05
1/2MS		2.0	50 ±1.52	3.86 ±0.05	4.50 ± 0.05
1/2MS		2.5	70 ± 0.57	4.30 ±0.05	5.84 ± 0.05
1/2MS	0.5	1.0	35 ±1.15	2.01 ±0.05	2.60 ± 0.05
1/2MS	1.0	1.5	60 ±1.52	3.67 ±0.05	4.63 ± 0.05
1/2MS	1.5	2.5	75 ±1.52	5.89 ±0.05	6.89 ± 0.05
1/2MS	2.0	1.0	65 ±1.15	4.01 ±0.05	5.10 ± 0.05
1/2MS	2.5	2.0	40 ±1.52	3.96 ±0.05	4.38 ± 0.05

Each value represents the mean± SE of three replicates, each with 20 explants. $P \leq 0.05$ level, according to Duncan's multiple range test.

2.4 Large scale plant production of *Ammannia baccifera* L.

For large scale plant production, in vitro regenerated shoot buds were subcultured onto MS medium supplemented with 1.0 mg/l BA + 0.2 mg/l TDZ + 1.5 mg/l GA_3 + 2.5 mg/l IAA combination. The cultures were subcultured onto the fresh same media composition once in 2 weeks interval. This process was repeated for another five subcultures (each 15 days) to examine the effect of subculture on production of large scale shoot buds. After 75 days of culture multiple shoots were counted for analysis of total number of regenerated shoot buds ("Table 4").

Table 4: Large scale plant production in *Ammannia baccifera* (L.) using in vitro regenerated nodal as explant source.

Hormone concentration BAP (1.0 mg/l) + TDZ (0.2 mg/l) + GA_3 (1.5 mg/l) + IAA (2.5 mg/l)	No. of shoot clumps cultured for multiplication			Total number of shoots
	Clump 1	Clump 2	Clump 3	
1st Subculture (15 days interval)	20.98 ±0.25	19.10 ±0.15	23.60 ± 0.15	63.68
2nd Subculture (15 days interval)	41.36 ±0.15	36.32 ±0.20	45.36 ± 0.15	123.04
3rd Subculture (15 days interval)	69.01 ±0.11	59.55 ±0.15	75.92 ± 0.15	204.48
4th Subculture (15 days interval)	86.96 ±0.15	71.02 ±0.15	91.73 ± 0.11	249.71
5th Subculture (15 days interval)	112.17±0.11	106.37±0.15	136.86 ± 0.15	355.4
Total No. of days at 5th passage of culture –105 days				

Number of shoots/clump represents mean ± SE in three independent experiments.

2.5 Statistical analysis

Experiments were set up in a completely randomized block design and each experiment usually had three replicates. The number of culture tubes containing single explant per replicate was twenty culture tubes. The analysis of variance (ANOVA) was carried out to detect the significance of differences among the treatment means. Comparison between the mean values of treatments was made using Duncan's multiple range test at the 5% level of probability [22].

3 Results and Discussion

3.1 Plant material and multiple shoot induction

In this study, the nodal explants collected from wild grown plants results maximum amount of shoot and multiple shoot induction on MS (Murashige and Skoog's) solid medium supplemented with 1.0 mg/l of BA (6-Benzyl aminopurine) and 0.2 mg/l TDZ (Thidiazuron) separately. Different explants like shoot tip, single bud, leaf, axillary bud, node and cotyledon were utilized by many researchers using solid MS medium in variety of medicinal plants [23, 27]. Though, in shoot culture, the shoot proliferation has declined due to the nature of solid medium and hence, in the present study, full strength MS medium has been chosen for mass production of shoots and plantlets.

3.2 Influence of plant growth regulators on shoot and multiple shoot proliferation

To find out the accurate stage for the maximum amount of shoots and maximum plantlets are produced and to evaluate the production of in vitro plants was standardized and it showed maximum number of shoots, multiple shoots were given in ("Table 1; Figure 1"). Among the diverse cytokinins examined, MS medium fortified with 1.0 mg/l BA alone was most efficient for shoot production and multiplication with a mean number of (81.01 ± 0.58). Cytokinins applied exogenously amend the development of axillary meristems, augment proliferation of the meristematic cells in the axillary buds and enhance the number of bud primordia from the pre-existing meristems [28]. In the present study, elevated multiple shoot production was attained due to reliable contact and increased absorption of BA by the growing shoots in MS medium ("Table 1"). It was found that multiple shoot effectiveness was synergistically increased by manipulating the balance of cytokinins. Cytokinins played an essential role in shoot regeneration and multiplication due to formation of de novo meristems and pre-existing meristems from nodal explants of medicinal plants utilizing solid system [26].

In this study, inoculating the shoot cluster with elevated concentration of individual cytokinins has directed to modify morphology of multiple shoots like internode shortening or lengthening, differentiation of smaller or bigger leaves, suppression of shoots and reduced proliferation rate. MS medium fortified with TDZ at higher concentration (above 0.5 mg/l) exposed longer shoots and larger leaf area. Also 34 shoots and 16 shoots/explant on MS solid medium amended with TDZ and BA, respectively were observed by Kulkarni *et al.*,[29]. Likewise 145 shoots/shoot tip and 120 shoots/shoot tip on BA, IBA (Indole-3-butyric acid) and BA, 2,4-D (2,4-Dichlorophenoxy acetic acid) supplemented solid medium, respectively were recorded by Sen and Sharma [23]. 120 shoots from a single bud in the Nitsch and Nitsch solid medium [30] fortified with BA and IBA had obtained by Furmanowa [25]. Ray [31] obtained 37 microshoots/shoot tip explant in MS liquid medium supplemented with BA and coconut water.

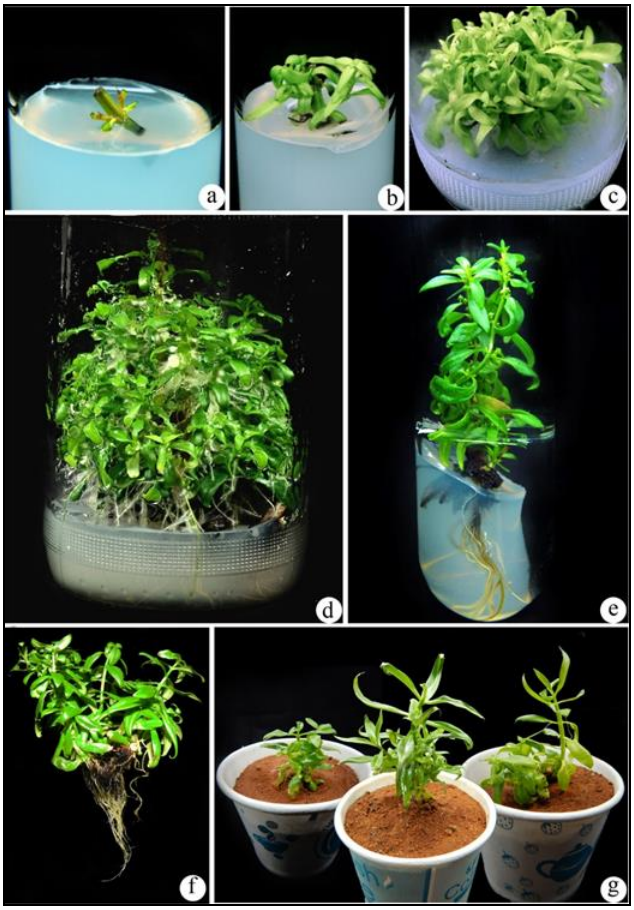

Fig 1: Large scale *in vitro* propagation of *Ammannia baccifera* L. using single nodal explants. (a) Nodal explant. (b) Shoot bud proliferation. (c) Multiple shoot bud proliferation. (d) Regeneration of multiple shoots with elongation. (e) Initiation of roots from the elongated shoot (f) Regenerated plantlets with well-developed roots. (g) Acclimatization of *in vitro* derived plantlets growing in paper cups.

3.3 Effect of MS strength and GA_3 (Gibberellic acid) concentration on Shoot elongation

Shoot elongation in regenerated plantlets is a crucial step, which requires alteration in media, composition [32, 33], plant growth regulator substitution [34] change in light conditions, etc. In the current study, multiple shoots developed on BA containing media abortive to elongate on the same medium. It is dissimilar to previous reports [35, 36], conceivably it is due to divergent explants. BA has frequently been reported to arouse shoot proliferation while inhibiting shoot elongation [37, 38]. Multiple shoots developed on BA fortified media further transferred to plant growth regulator free full-strength and half-strength MS media to examine its effect on shoot elongation of *A. baccifera*. Full-strength MS medium was found to be superior than half-strength MS medium for shoot elongation. Shoot elongation rate, length of shoot and number of nodes per shoot was not promising on PGR (Plant Growth Regulator) free medium. Thus, it was essential to develop a proper media for elongation of shoots. GA_3 at 0 – 2 mg/l ("Table 2") was utilized to augment shoot elongation. Addition of 1.5 mg/l GA_3 in full-strength MS medium found to be optimum for shoot elongation ("Figure 1"). ANOVA (Analysis of variance) exposed a considerable effect of treatments on shoot length and number of nodes per shoot. The best shoot growth in conditions of shoot length (5.97 ± 0.15 cm) and number (4.90 ± 0.15) of nodes per shoot was achieved on MS medium fortified with GA_3 1.5 mg/l. GA_3 alone or in combination with cytokinin found promontory for shoot elongation in several plants [39].

3.4 Large scale production of shoots

In order to produce large scale plants, the finest concentrations of growth regulators combination was selected depends on above results and the subculture was repeated as channel for five times as explained in. Shoot bud multiplication was commonly obtained by splitting of the shoots into 2–3 small clumps (4–5 shoots/clump). Shoot clusters were sub-cultured on the same hormonal combination for shoot bud multiplication at 15 days interval. At the end of the first subculture, the maximum number of shoots (23.60 ± 0.15 shoots/clump) was achieved. The number of shoots in a cluster was increased with consequent path of subcultures. The shoot bud multiplication was executed up to five passages and the shoot buds were also elongated on the same media composition. The elongated shoots were excised out separately and utilized for rooting. The present study is quite achievable to regenerate multiple shoots continuously without showing any decrease/reduction and a total number of shoots obtained was 355.4 shoots/explant at the end of the 5th subculture (105 days) ("Table 4"). On the other hand [40, 41], described that in vitro regenerated multiple shoots of medicinal plants (*Gymnema*) did not endure even after

subculturing on the similar media constituents while, Komalavalli and Rao [42] reported that it was feasible to regenerate multiple shoots 2–3 subcultures (3 months) and reduced thereafter. And similarly Sairam Reddy *et al.*, [43] explained that GA_3 and kinetin (KN) did not improve the number of multiple shoot bud proliferation in medicinal plants (*Gymnema).* The current results strongly recommended that the development of multiple shoots could be successful without showing any decrease in the quantity of shoots up to 5 subcultures. In addition, it is fascinating to note that the quantity of multiple shoots achieved was 355.4 from single nodal explant after 5 subcultures. Similarly the maximum number of shoots regeneration was 45 shoots/explant on MS medium supplemented with BA (1.0 mg/l) + IAA (Indole-3-acetic acid) (0.5 mg/l) + 100 mg/l B2 vitamin combinations after 30 days of culture were reported by Subathra [41]. Likewise Sairam Reddy *et al.*, [43] described that induction of multiple shoots achieved 7.4 shoots / explant on MS medium containing BA (5.0 mg/l) and NAA (α – Naphthalene acetic acid) (0.2 mg/l) combination after 5–6 weeks of culture.

3.5 Rooting

In vitro grown shoots were excised separately and transferred to MS medium fortified with different concentrations of IAA or IBA (0.5 – 2.5 mg/l) for root induction. Inoculated shoots were produced roots after two weeks of culture. The initial roots emerged directly from the basal part of the shoots after 21 days of culture. The maximum percent of root induction (95%) were achieved on MS medium supplemented with 1.5 mg/l IBA followed by 2.5 mg/l IAA and maximum number of roots achieved was 7.95 ± 0.15 roots/shoot (Fig. 1) (Table 3). The increasing concentrations of IBA and IAA were significantly increased the rooting percentage. Same observation was made by [44, 47]. Though, the rooting response was negatively correlated with NAA concentrations and no root initiation was noticed at higher concentration 2.0 mg/l NAA [48]. The present results were supported by the findings of earlier reports [49, 50]. Among the two auxins used, maximum percent of rooting was obtained on MS medium fortified with 1.5 mg/l IBA and 2.5 mg/l IAA was found to be finer auxin for rooting over other auxins. Generally, IBA was demonstrated a strong rooting response; in a wide range of plant species. Same kind of observation has been obtained in *Ceropegia candelabrum* [51], *Mucuna pruriens* [52], and *Jatropha curcas* [53].

3.6 Acclimatization

The rooted plantlets with matured leaves were successfully transferred into paper cups containing sand and soil in the ratio of 1:2 and covered with polythene bags to ensure high humidity. In the beginning the plantlets were kept in the fully closed and controlled conditions for two weeks and the polybags were gradually discarded in order to acclimatize the plantlets under greenhouse conditions. Consequently the plantlets were transferred to the field conditions and the survival rate noticed was 81% ("Figure 1"). The in vitro regenerated plants were grown well and phenotypically similar to the parental stock.

4. Conclusion

In conclusion, this study describes a two phase culture protocol for large scale multiple shoot bud regeneration from nodal explants of *A. baccifera* L. The maximum shoot bud induction (90.9 ± 0.20) as well as shoot number (81.01 ± 0.58) without attaining callus on MS medium supplemented with BA in the first phase, whereas, the combination of BA (1.0 mg/l) + GA_3 (1.5 mg/l) produced 355.4 shoots/explant after 5th subculture in the second phase. The in vitro regeneration protocol demonstrated here is different from that of medicinal plants which reported earlier. Therefore, the culture of shoot clumps in front of GA_3 containing medium increased the number of multiple shoots in *Ammannia*. The current study robustly recommended that the superiority of BA and other cytokinin in combination with GA_3 was found to be essential for rapid multiple shoot bud induction as well as improved rate of shoot proliferation in *Ammannia*. Full-strength MS medium supplemented with 1.5 mg/l IBA combined with 2.5 mg/l IAA were found to be best for maximum rooting response and regenerated plantlets were successfully established in the field condition with 81% survival rate. To the best of our knowledge, this in the first ever report on production of large scale in vitro plantlets from this important medicinal plant. The protocol standardized here can be used for commercial scale plant production of vital compounds like flavonoids (quercetin) as well as conservation of this important folkloric medicinal plant from the probable extinction in near future.

5. Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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