OPTIMIZATION OF PARAMETERS AFFECTING *IN VITRO* REGENERATION FROM *Agrobacterium* ASSISTED TRANSFORMATION COMPATIBLE SHOOT APEX EXPLANTS OF *Sesbania aculeata*

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Abstract. Daincha (*Sesbania aculeata*) is an multipurpose significant leguminous crop that possesses many medicinal properties, used industrially and as food in some parts of the world. There are many attributes of the crop that needs to be improved via genetic engineering. The present work involves standardisation of various factors (type of medium and concentration of cytokinin, auxins and gibberellic acid, presence of thiol compounds, wounding stress and exposure to vacuum) affecting shoot organogenesis from shoot apex explants and optimization of the selective concentration of kanamycin for the first time. The 2-day-old shoot apex explants were cultured on medium containing Murashige and Skoog (MS) medium salts and Gamborg (B5) medium vitamins accompanied with 1 µM 6-benzylaminopurine and 656.8 µM thiourea and formed multiple shoots (6-7) with frequency of regeneration (100 %) under a period of 4 weeks. The selective concentration of kanamycin was found to be 171.6 µM for shoot organogenesis and 17.2 µM for root organogenesis. Compatibility of the regeneration system towards genetic transformation facilitated by *Agrobacterium* was assessed via β-glucuronidase assay in shoot apices and regenerated transformed plants, respectively. Transgene insertion in plants was also checked by polymerase chain reaction analysis and obtained 15% efficiency of transformation.

Key words: *Agrobacterium*; kanamycin; regeneration; shoot apex; thiol compounds; wounding stress.

INTRODUCTION

Sesbania aculeata (Pers.) is a leguminous crop mainly grown in India and several countries of Asia but found throughout the world [3]. It is a multipurpose crop species which is grown for fibre, gums (galactomannan), fuel wood, fodder, paper, dye industry, to increase soil fertility, flowers as food and decorative purpose [3, 19, 22, 24]. Plant possesses various medicinal properties such as alexiteric, anthelmintic, diuretic, antibacterial and anticancer properties and is used in treatment of dermal ailments, ophthalmic disorders and snake bites [3, 12, 13]. Being a legume crop, its seeds are rich in digestible protein (33%) along with carbohydrates, essential fatty acids, vitamins, minerals and fibre (11%) [6]. The fat level is just 6% and contains relatively high proportion of healthy, unsaturated fatty acids. But the use of seeds as food is restricted due to the existence of high level of anti-nutritive contents like phytic acid, phenolics, tannin and protease inhibitor. This problem could not be overcome by using traditional breeding approaches due to presence of limited gene pool. So to overcome such problems there is requirement of biotechnological approaches.

The most pre-requisite for genetic amendment is an competent and repeatable *in vitro* regeneration protocol. Like other grain legume crops, *S. aculeata* is also recalcitrant towards regeneration. At present, there is no report of regeneration using shoot apex explants in *Sesbania* species. Further, shoot apex explants have its own advantages like rapid and efficient multiplication, and broad meristem which can be made accessible to gene transfer methods. Shoot apices have been used previously in different legumes for *in vitro* regeneration and transformation studies [15, 21]. Moreover, previously using shoot apices no selection system was optimized which is required for efficient selection of transformants as well as the compatibility of the regeneration system towards *Agrobacterium* mediated gene transfer method was also not assessed. In this perception, the present work reports a rapid, single step and high potential shoot regeneration system compatible to *Agrobacterium tumefaciens* facilitated transformation using shoot apex explants and optimized the kanamycin selection system for efficient selection of putative transformants of *S. aculeata*.

MATERIALS AND METHODS

Plant material and explant preparation

Daincha (*S. aculeata*) seeds were procured from the district propagule centre, Thanesar, India. Sterilization of the healthy seeds from the exterior was done by immersing in ethanol (70%) for 1-3 minutes followed by using mercuric chloride solution (0.2%) (*w/v*) up to 4 – 6 minutes under laminar air flow cabinet. Sterilized seeds were rinsed several times with sterile distilled water to wash out any remnants of mercuric chloride. The seeds were germinated either by soaking in sterilized distilled water in a 250 mL flask or on semisolid Murashige-Skoog (1962) (MS) basal media [16] and incubation at 26 ± 2ºC temperature under light (16-h period per day) of intensity 80 µE m^{-2} s^{-1} provided by cool white fluorescent lamps. Shoot apex explants were excised from different age-old germinated seeds (16-hour, 2, 4, 6 and 8-day).

Optimization of *in vitro* multiplication of shoots

Variable factors affecting *in vitro* muliplication of shoots have optimized (Table 1). To investigate optimum media for efficient regeneration, excised shoot apex explants were cultured in plant tissue

culture boxes having different media viz. MS media [16], B_5 media [5] and MSB_5V media (containing salts of MS medium and vitamins of B_5 medium), supplemented with 1 µM benzyl-aminopurine (BAP). To determine the effective concentration of BAP, explants were cultured on MSB_5V medium supplemented with different concentrations of BAP (0.0–15.0 µM). The influence of kinetin and thidiazuron (TDZ) individually at equivalent concentration to BAP was tested and the best concentration of cytokinin was further checked in combination with different concentrations (0.5 and 1.0 µM) of gibberellic acid (GA_3), indole-3-acetic acid (IAA), and naphthalene-1-acetic acid (NAA).

The effect of sucrose concentration in the medium was tested by using its variable concentrations (10-50 g/L) in the optimized media. The explant's age affecting multiplication of shoots was evaluated by cultivating different age (16 hours - 8 day) old shoot apices on regeneration medium (MSB_5V + 1 µM BAP).

Effect of different thiol containing compounds (dithiothreitol, thiourea, L-cysteine, sodium thiosulphate) on regeneration potential of *S. aculeata* was also evaluated by culturing the explants on above used shoot organogenesis medium supplemented with thiol containing compounds in different concentrations (Table 1).

The effect of wounding stress on explants regeneration efficiency was also assessed. The injuries were made on the explants by observing under the stereo-zoom microscope (Olympus India) in different ways either by stabbing the apical dome with a fine needle (30 G), or by longitudinal partial slicing of meristematic part of shoot apices. The injured apices then cultured in vertically upright position on the optimized shoot regeneration medium (SRM - MSB_5V + 1 µM BAP + 656.8 µM thiourea).

The influence of vacuum infiltration on regeneration potential was checked by immersing the wound induced stress explants in liquid regeneration medium (MSB_5V + 1 µM BAP + 656.8 µM thiourea) and subjecting them to a time period of 2, 5, 7, 10 and 15 minute under vacuum (600 mm of Hg) in a sterilized desiccator. The vacuum infiltrated explants were then cultured on semi-solid shoot regeneration medium (SRM) and the data of regeneration potential was compared with non-infiltrated (control) explants.

Rooting and transplantation

Excised the healthy regenerated shoots of length 3-4 cm long and cultivated on the root induction medium (RIM) compose of salts (half composition) and vitamins (full composition) of MS medium with 3% sucrose, 0.375% Phytagel augmented with indole-3-butyric acid (IBA) in varying concentrations (0.0–5.0 µM). Shoots with healthy and branched roots were selected after 2 weeks and transplanted to soil in pots. High level of humidity was maintained initially which was gradually decreased and ultimately plants were planted to soil, where they grow to set the seeds.

Kanamycin sensitivity of organogenesis

Sensitivity of the non-transformed shoot apex explants of *S. aculeata* towards kanamycin on organogenesis was assessed by cultivating the shoot apices on optimized shoot regeneration medium (SRM) supplemented with different concentrations (0.0-171.6 µM) of kanamycin. After two weeks, cultures were transferred and maintained on to the same fresh medium. The data of kanamycin sensitivity was scored after 4 weeks. Inhibitory concentration of kanamycin on root organogenesis was also assessed as second round of selection by culturing the well-developed non-transformed shoots on optimized root induction medium (RIM) containing 2.5 µM IBA and supplemented with varying concentrations (0.0-25.7 µM) of kanamycin.

***Agrobacterium* assisted transformation**

To check the compatibility of the regeneration system, *Agrobacterium tumefaciens* (EHA105 strain, having plasmid vector pCAMBIA2301) [14] was cultured in liquid SRM (MSB_5V + 1 µM BAP + 656.8 µM thiourea) according to Chopra [4]. The stabbed shoot apices (25 in number) were then inoculated in this suspension and subjected to vacuum infiltration as optimized above. Likewise, intact shoot apices (without wounding) were also inoculated with agrobacterial suspension. Such inoculated explants

Table 1. Factors optimized for the *in vitro* multiplication of shoots along with variables used

Factor optimized	Variable (concentration in µM)
Media Composition	MS media, B_5 media, MSB_5V media
Age of explants excised	16-hour, 2, 4, 6 and 8-day
Growth regulators	BAP (0.0, 1.0, 2.5, 5.0, 10.0, 15.0)
	TDZ (1.0)
	Kinetin (1.0)
	BAP (1.0) + IAA (0.5 and 1.0)
	BAP (1.0) + NAA (0.5 and 1.0)
	BAP (1.0) + GA_3 (0.5 and 1.0)
Sucrose concentration	10, 20, 30, 40, 50 g/L
Thiol compound concentration	Thiourea (0.0 - 1642.0)
	L-Cystine (0.0 - 1031.7)
	Sodium thiosulphate (0.0 - 790.6)
	Dithiothreitol (0.0 - 810.3)
Wounding stress	None. Stabbing, Longitudinal slicing
Vacuum infiltration time (600 mm of Hg)	0, 2, 5, 7, 10 and 15 minutes

were co-cultured for 2 days in vertically upright position on semisolid SRM under the same physical culture conditions as used for regeneration. It was followed by washing of the co-cultured explants using autoclaved distilled water to remove the agrobacterial cells and final rinsing was made using cefotaxime (1047.1 µM). Some of the washed shoot apices were used to check the β-glucuronidase (GUS) gene expression (transient) while rest others (20 in number) were cultured on SRM containing kanamycin (171.6 µM) and cefotaxime (1047.1 µM) to regenerate the putative transformed shoots. The regenerated shoots were shifted to above optimized root induction medium (RIM) supplemented with kanamycin (17.2 µM) for rooting and followed by transfer of plantlets to pots containing soil, to collect the seeds. The histochemical GUS activity (stable) was assessed in leaves of primary putative transformants (T0) and germinated seedlings (T1) to confers the presence and expression of transgenes. Further, the presence of transgene neomycin phosphotransferase II (*npt II*) was checked by PCR analysis. The PCR and histochemical GUS analysis for transient and stable GUS activity was performed following the protocol used by Chopra [4].

Data analysis

The experiments of regeneration were set in replicates of 20 for each treatment and repeated thrice. Assessment of every experiment was based on the percentage of regenerated explants, percentage of rooting and the mean number and length of regenerated shoots. Multiple range test of Newman-Keuls [2] was used for the statistical analysis of the data.

RESULTS

***In vitro* multiple shoot regeneration**

Shoot apices cultured on basal medium gave rise to single shoot from apex along with rooting at the base (Figure 1a, b). The addition of growth regulators resulted in multiple shoot formation. The most effective medium for shoot induction was MSB_5V followed by MS, and B_5 medium supplemented with 1 µM BAP where 5.5, 4.6, and 4.2 shoots per explant were obtained. Addition of BAP induced multiple shoots within 5-6 days from the apex and the basal part of the explants converted into small callus. Maximum shoot regeneration potential was observed at BAP concentration of 1 µM, where multiple shoots (5.5 per explant) were obtained with 100% frequency of regeneration and mean height of shoots 5.4 cm (Table 2). Increasing the concentration of BAP was followed by decreasing the regeneration potential of the explants. Substituting the BAP with TDZ or kinetin in MSB_5V media resulted in multiple shoot formation but the morphogenetic response obtained was low and produced 4.6 and 3.8 shoots per explant, respectively as compared to BAP (5.5 shoots per explant) at 1 µM concentration (Table 2). Addition of auxins (IAA, NAA) and gibberellic acid (GA_3) in the medium containing 1 µM BAP resulted in decrease in the regeneration potential rather than increase. Although an increase in the shoot length was obtained by supplementation of GA_3 but formation of shoots and regeneration potential was decreased significantly (Table 2). Varying the concentration of sucrose significantly affects the regeneration potential, we obtained best response at sucrose concentration of 30 g/L with 100% regeneration and production of 5.5 shoots per explant. Further enhancing the concentration of it beyond 30 g/L resulted decrease in the regeneration potential. At 50 g/L sucrose concentration, 3.2 shoots per explant were obtained in 80% of cultures whereas at lower concentration of 10 g/L, average 3.7 shoots per explant were obtained in 90% of cultures.

Shoot apices excised from different age-old seedlings (16-hour, 2, 4, 6 and 8-day) were tested for efficient *in vitro* regeneration by culturing on MSB_5V medium supplemented with 1 µM BAP and out of

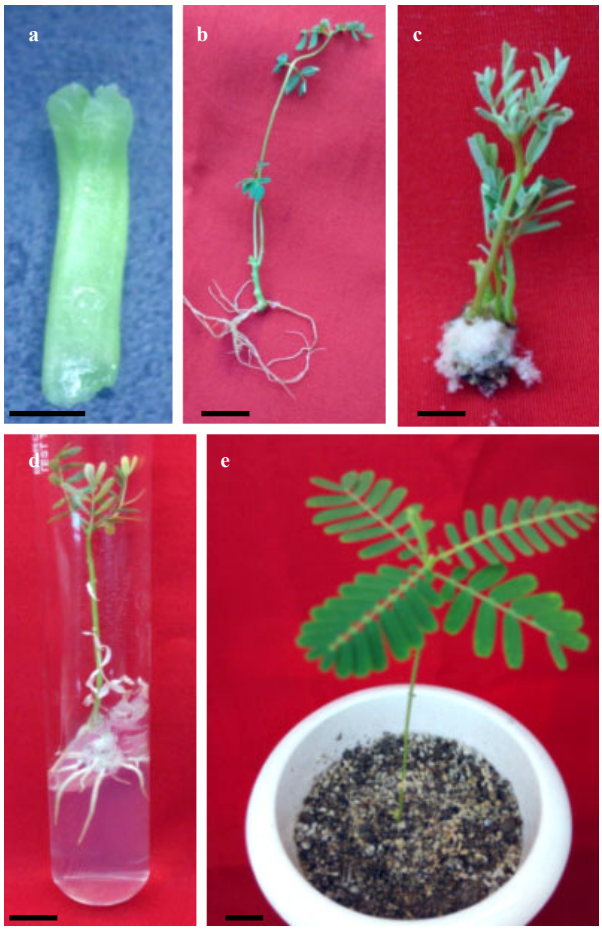

Figure 1. *In vitro* plant regeneration from shoot apex explants of *Sesbania aculeata*: (a) 2-day old explant; (b) Shoot and root formation from the explants on basal MSB_5V medium (control); (c) Multiple shoot regeneration from the shoot apices on MSB_5V supplemented with 1 µM BAP and 656.8 µM thiourea and callus induction at base. (d) Rooting of shoots on root induction medium (RIM) containing 2.5 µM IBA; (e) Growing plant transplanted to soil in pot. *Bar* 1 mm (**a**), 2 cm (**b**), 1.5 cm (**c**), 2 cm (**d**), 2.5 cm (**e**).

them 2-day old explant was found most effective towards multiple shoot regeneration with non-significantly different from 16-h-old explants (Table 3). With increasing the age of explants, there was significant decline in regeneration frequency up to 80% with 2.6 shoots per explant.

Influence of different thiol containing compounds such as dithiothreitol (DTT), thiourea, L-cysteine, sodium thiosulphate (STS) was checked. All the thiol containing compounds tested lead to an upsurge in regeneration efficiency at lower concentrations with thiourea at concentration of 656.8 µM giving best response with 6.8 shoots per explants in 100% of cultures (Figure 1c) whereas concentration of 324.1 µM DTT (5.9 shoots per explant), 474.3 µM STS (6.1 shoots per explant) and 825.3 µM L-cysteine (6.2 shoots per explant) produced similar results with non-significant differences (Table 4). Increasing the concentration of these thiol compounds beyond above mentioned concentrations resulted in decrease in regeneration potential.

Table 2. Effect of different growth regulators on multiple shoot regeneration from 2-d-old shoot apex explants of *Sesbania aculeata*[1]

Growth regulators	(µM)	(%) Regeneration	Mean average number of shoots/explants*	Mean average length of shoots* (cm)
BAP	**0.0**	100	1.0±00^{a}	7.1±0.35^{a}
	1.0	100	5.5±0.28^{b}	5.4±0.20^{bc}
	2.5	95	4.7±0.42^{bc}	3.9±0.25^{d}
	5.0	95	4.4±0.50^{cd}	2.6±0.30^{e}
	10.0	90	3.9±0.36^{cd}	2.1±0.22^{e}
	15.0	70	1.7±0.18^{ag}	0.9±0.10^{f}
KIN	**1.0**	95	4.6±0.25^{bc}	5.5±0.34^{bc}
TDZ	**1.0**	90	3.8±0.22^{cd}	1.9±0.18^{d}
BAP + IAA	**1.0 + 0.5**	70	4.3±0.51^{cd}	5.3±0.69^{bc}
BAP + IAA	**1.0 + 1.0**	60	4.1±0.46^{cd}	5.2±0.47^{c}
BAP + NAA	**1.0 + 0.5**	65	3.6±0.19^{de}	5.6±0.64^{bc}
BAP + NAA	**1.0 + 1.0**	60	3.2±0.31^{ef}	5.5±0.87^{bc}
BAP + GA_3	**1.0 + 0.5**	70	2.9±0.38^{ef}	5.9±0.55^{bc}
BAP + GA_3	**1.0 + 1.0**	65	2.6±0.17^{fg}	6.2±0.26^{ab}

[1]Data recorded after 28 days of cultivation on MSB_5V medium supplemented with or without growth regulators.
*Values are expressed in mean±standard deviation, and within the columns having same alphabet shows non-significant difference at *P*=0.05 (Newman-Keul's multiple range test).

Table 3. Effect of age of shoot apex explants of *Sesbania aculeata* on *in vitro* regeneration[1]

Age of explant	% Regeneration	Mean average number of shoots/explants*	Mean average length of shoots* (cm)
16-h	100	5.3±0.50^{a}	5.1±0.20^{a}
2-d	100	5.5±0.28^{a}	5.4±0.20^{a}
4-d	90	4.3±0.45^{ab}	3.7±0.28^{a}
6-d	80	3.3±0.40^{bc}	2.7±0.20^{ab}
8-d	80	2.6±0.27^{c}	1.9±0.15^{b}

[1]Data recorded after 28 days of cultivation on MSB_5V medium supplemented with 1 µM BAP.
*Values are expressed in mean±standard deviation, and within the columns having same alphabet shows non-significant difference at *P*=0.05 (Newman-Keul's multiple range test).

Table 4. Effect of thiol containing compounds on shoot organogenesis from shoot apices of *Sesbania aculeata*[1]

Thiol compounds	(µM)	(%) Regeneration	Mean average number of shoots/explants*	Mean average length of shoots* (cm)
None (control)	0.0	100	5.5±0.28^{ad}	5.4±0.20^{a}
L-cysteine	206.3	100	5.6±0.42^{ad}	5.4±0.18^{a}
	412.7	100	5.7±0.21^{ad}	5.3±0.54^{a}
	619.0	100	5.9±0.50^{ac}	5.3±0.43^{a}
	825.3	100	6.2±0.36^{ac}	5.3±0.31^{a}
	1031.7	70	3.7±0.18^{b}	4.9±0.19^{a}
Thiourea	328.4	100	5.8±0.62^{a}	5.4±0.20^{a}
	656.8	100	6.8±0.22^{c}	5.3±0.20^{a}
	985.2	80	4.8±0.12^{d}	4.9±0.18^{a}
	1313.6	65	3.6±0.28^{be}	4.5±0.34^{ab}
	1642.0	50	2.7±0.76^{e}	3.9±0.18^{bc}
DTT	162.1	100	5.7±0.08^{ad}	5.4±0.53^{a}
	324.1	100	5.9±0.50^{ac}	5.3±0.46^{a}
	486.2	80	4.6±0.65^{bd}	5.1±0.64^{a}
	648.2	70	3.8±0.14^{b}	3.8±0.26^{bc}
	810.3	55	3.2±0.85^{be}	3.4±0.19^{c}
STS	158.1	100	5.6±0.68^{ad}	5.4±0.33^{a}
	316.2	100	5.8±0.29^{a}	5.3±0.26^{a}
	474.3	100	6.1±0.38^{ac}	5.3±0.47^{a}
	632.4	75	4.5±0.54^{bd}	4.9±0.49^{a}
	790.6	60	3.2±0.44^{be}	4.5±0.34^{ab}

[1]Data recorded after 28 days of cultivation on MSB_5V medium supplemented with 1 µM BAP and various concentrations of thiol compounds.
* Values are expressed in mean±standard deviation, and within the columns having same alphabet shows non-significant difference at *P*=0.05 (Newman-Keul's multiple range test).

Wound induced stress did not change the percent of regeneration though average shoots regenerated declined when corelated to the control intact explants (Table 5). Though there was non-significant difference between the intact and stabbed explants but the effect of slicing on regeneration was more than stabbing with the fine needle and significantly different from results of intact explants.

The effect of vacuum infiltration on regeneration potential was assessed and it was found that vacuum infiltrating (600 mm of Hg) explants up to 7 minutes did not significantly affect the frequency and number of shoots formed. By further increase in time period of vacuum infiltration up to 15 minutes, regeneration potential was decreased and resulted in 75% regeneration frequency forming an average 4 shoots per explant.

Rooting and transplantation

Healthy, thick, and branched roots were induced in all the healthy shoots transferred to the RIM containing 2.5 µM IBA (Figure 1d). Hormone-free rooting medium also resulted in root formation in 70% of culture but the roots were thin and less branched. Addition of 1.0 µM IBA concentration increased rooting to 80% of culture but the thickness and branching of the roots was not changed significantly. Higher concentration of IBA (5.0 µM) resulted in more thickened, shorter in length and reduced branching of roots with callus induction in 60% of cultures at the root initiation point. On transplantation, 70% of the plants survived in soil and grew to produce seeds (Figure 1e).

Kanamycin sensitivity of organogenesis

The percentage explants survival, regeneration, and average number of shoots developed per explant decreased with increase in concentration of kanamycin (Table 6). Kanamycin concentration of 171.6 µM drastically reduced the regeneration potential resulting in formation of few bleached out shoots buds thus this concentration was considered as selective kanamycin concentration for shoot apices of *S. aculeata*. Likewise, root induction from shoots was inhibited completely at kanamycin concentration of 17.2 µM showing that roots induction is more sensitive than shoot induction.

***Agrobacterium* assisted transformation compatibility**

The regeneration system was found compatible to *Agrobacterium* assisted transformation. After inoculation with *Agrobacterium*, in case of the intact shoot apices (without any wounding stress) no transformed shoot was obtained while wound induced stress and vacuum infiltrated explants produced transformed shoots (5 shoots out of 20 explants). The GUS activity was observed, transient in co-cultured shoot apices and stable in leaves of primary transformants (T0) and sprouts (T1), while no GUS expression was observed among the non-transformed (controls) (Figure 2 a-f). No GUS activity in controls shows that no endogenous GUS activity is present in the non-transformed plants and confirms the existence and expression of transgene (*uid*A) in the transformed plants raised through shoot apices. Further the presence of transgene (*npt*II) was confirmed by the PCR analysis of the transformants which survived in soil (3 out of 5 shoots) (Figure 2 g). GUS expression and PCR analysis of the putative transformed plants of *S. aculeata* validate the compatibility of the developed shoot apices mediated regeneration towards *Agrobacterium* assisted transformation and obtained 15% efficiency of transformation.

Table 5. Effect of wound induced stresses on shoot organogenesis from shoot apices of *Sesbania aculeata*[1]

Wounding stress	(%) Regeneration	Mean average number of shoots/explants*	Mean average length of shoots* (cm)
None	100	6.8±0.22[a]	5.3±0.20[a]
Stabbing	100	5.9±0.21[ab]	5.4±0.37[a]
Longitudinal slicing	100	5.1±0.38[b]	5.3±0.32[a]

[1]Data recorded after 28 days of cultivation on shoot regeneration medium (MSB_5V+1 µM BAP+656.8 µM thiourea).
*Values are expressed in mean±standard deviation, and within the columns having same alphabet shows non-significant difference at *P*=0.05 (Newman-Keul's multiple range test).

Table 6. Effect of kanamycin on shoot organogenesis from shoot apices of *Sesbania aculeata*[1]

Kanamycin (µM)	(%) Survival of explant	(%) Regeneration	Mean average number of shoots/explants*	Remarks (if any)
0.0	100	100	6.8±0.22[a]	Healthy shoots
34.3	80	65	5.2±0.20[b]	Decreased regeneration and length of shoots
68.6	60	45	2.7±0.30[c]	Some explants bleached
102.9	45	34	1.9±0.24[c]	Bleaching of explants after regeneration
137.3	40	20	0.9±0.18[d]	Few shoots appeared, then bleaching occurs
171.6	30	15	0.7±0.15[d]	Shoots appeared initially, then all bleached out

[1]Data recorded after 28 days of cultivation on shoot regeneration medium (MSB_5V+1 µM BAP+656.8 µM thiourea) supplemented with or without kanamycin.
* Values are expressed in mean±standard deviation, and within the columns having same alphabet shows non-significant difference at *P*=0.05 (Newman-Keul's multiple range test).

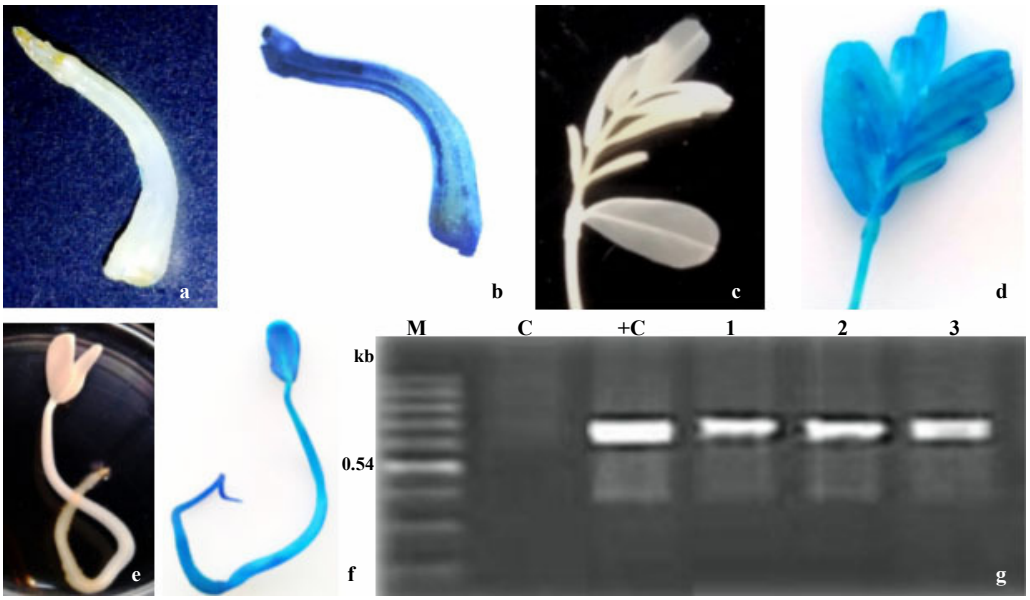

Figure 2. GUS gene expression and PCR analysis of transformants raised using *A. tumefaciens* and un-transformed plants of *Sesbania aculeata*: Transient GUS expression in shoot apices (a) control showing no activity, (b) transformed showing GUS activity. Stable GUS expression (c-f): control shoot (c), transformed shoot showing GUS gene expression (blue stain) (d), un-transformed germinated seeds (e), T_1 progeny germinated transformed seeds (f); (g) PCR analysis of kanamycin tolerant transformed plants using primars targetting *npt*II genes: Lane M - marker DNA, lane C - DNA from untransformed (control) plant, lane +C- plasmid DNA, lanes 1 to 3 – transformed plants.

DISCUSSION

Various factors such as growth medium components, mechanical injury, addition of thiol compounds and vacuum infiltration that affect the regeneration potential has been assessed and optimized. There was no earlier report of regeneration from shoot apex in *Sesbania aculeata*. The most responsive media for shoot regeneration has been found to be MSB_5V medium and the results are in compliance with the earlier findings in legume species [15, 20, 21] where MSB_5V has been found best for shoot organogenesis. Addition of BAP increased the regeneration potential and number of shoots with best response at 1 µM BAP. The results are similar with the earlier findings in other legume species such as *Vigna mungo* [21] and *Vigna unguiculata* [11] where BAP at low concentrations was found effective on shoot multiplication from shoot apices. However, in some other legume species such as *Vigna radiata* [15] and *Delonix regia* [18], a high concentration of BAP was required for efficient multiple shoot induction. Among different cytokinins tested, BAP was found better in shoot regeneration than TDZ and kinetin. Our findings are in agreement with the prior finding in other *Sesbania* species [7, 8] where BAP was found more responsive than its counterpart cytokinin. Addition of auxins and gibberellic acids to the medium did not increase the regeneration potential rather decreased it. Results are similar to the earlier findings in *Vigna mungo* [21] and *Annona squuamos* [17] where auxins addition didn't increase the regeneration efficiency. Sucrose concentration in the medium also affected the regeneration potential where 30 g/L concentration was found most effective. Earlier, it has been reported that increase in sucrose concentration is related with increase in the phenolic content of the culture resulting in the necrosis of tissues at high sucrose concentration [26]. So, our findings are in compliance with the previous reports where concentration of 30 g/L sucrose has been found best for regeneration [1] and this concentration is used for routine regeneration experiments in legumes. Among effect of age, it was found that younger age explants were more responsive than the older ones. Similar results have reported in other legume species such as *V. mungo* [20] and *V. radiata* [15] where younger aged shoot apex explants have been found more responsive towards multiple shoot regeneration. The results are contrasting with the other reports where 12-15-day old explants in *Arachis hypogaea* [10] and *Delonix regia* [18] was reported more responsive towards *in vitro* regeneration.

Most thiol containing compounds are natural anti-oxidants that have been reported to protect the plants against stress by removing the free radicals from the tissues [25]. Addition of the thiol compounds reduces the effect of phenols released in the medium by tissue thus preventing the necrosis of the explants. In the study, addition of thiol compounds increased the regeneration potential with best results at 656.8 µM thiourea. The outcomes are similar to the earlier report where low concentrations of thiol compounds resulted in increased regeneration potential whereas at high concentrations, tissue necrosis occurred [23]. Decreased regeneration efficiency at higher concentrations must be related to the formation of thiyl radicals that reacts with metals such as iron in the media and form transition metals ions which are toxic to the plant tissues [25]. Wound induced stresses were found to affect the regeneration negatively. Our findings are similar to the previous observations in *V. mungo* [21] where wound induced stresses to the shoot

apices reduced the number of shoots produced per explant. Similarly, vacuum infiltration was affecting regeneration non-significantly. The results are similar to the earlier findings [4, 9] where vacuum infiltrating explants at 600 mm of Hg for 5 minutes did not affect the regeneration significantly but plays an important role in enhancing efficiency or compatibility of explants to *Agrobacterium* assisted transformation.

Kanamycin based selection system has not been previously optimized using shoot apex explants of *Sesbania* species. Selection system uses the optimum concentration of the kanamycin that inhibits the growth of the explants and is useful in transformation procedures to select out the transformants. Complete inhibition of regeneration was reported at 171.6 µM kanamycin concentration. Similar results were reported using shoot apices in *V. radiata* [15] and *V. mungo* [21], where 171.6 µM and 128.7 µM kanamycin, respectively were found as optimal selective concentrations.

In conclusion, we have developed a promising regeneration system compatible to *Agrobacterium* assisted transformation and optimized kanamycin selection system using shoot apex explants of *Sesbania aculeata*. Shoot apices excised from 2-day old seedlings gave rise to multiple shoots (average 6.8 shoots per explant) on MSB_5V medium containing 1 µM BAP and 656.8 µM thiourea, in span of only 20 days. The developed system is simple and fast, giving rises to plantlets in just 6-7 weeks. Optimization of selection system gave the selective concentration of kanamycin for shoot organogenesis (171.6 µM) and root organogenesis (17.2 µM). Wound induced stressed explants showed compatibility towards *Agrobacterium*-mediated transformation, which was evident from the GUS gene expression (transient and stable) obtained in the transformed shoot apices, shoots, and germinated seedlings. Transfer of genes was also established by PCR analysis of the putative transformed plantlets and obtained 15% efficiency of transformation. In future, the developed regeneration protocol could be used for routine transformation experiments to genetically improve the crop for desirable agronomically important traits.

Acknowledgement: Authors are grateful to the CAMBIA Institute, Australia, for plasmid CAMBIA 2301. Nikhil Mehta and Priyanka Rao are thankful to UGC, New Delhi for the award of Senior Research Fellowship.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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Received: July 3, 2021
Accepted: August 23, 2021
Published Online: August 25, 2021
Analele Universităţii din Oradea, Fascicula Biologie
https://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House