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Plant Regeneration via Somatic Embryogenesis in an Ethnomedicinal Plant *Senna alata* (L.) Roxb.

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Authors' contributions

This work was carried out in collaboration between all authors. Author RSN designed the study and corrected the manuscript. Author AP performed the experiments and wrote the manuscript and Author MB managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: To study the effect of various plant growth regulators (PGRs) for induction of somatic embryogenesis and plantlet regeneration from cotyledon and leaflet explants in *Senna alata* (L.) Roxb. (Syn. *Cassia alata*) an important ethnomedicinal plant used in the treatment of fungal skin infections.
Place and Duration of Study: Department of Biotechnology, Kakatiya University, Warangal, Telangana, India, between June 2013 to September 2014.
Methodology: Cotyledon and leaflet segments (1-3 cm, 20 day old) were cultured on MS medium supplemented with 0.5 mg/L N6-Benzylaminopurine (BAP) /Thidiazuron (TDZ) in combination with various concentrations of plant growth regulators (PGRs) viz., Indole acetic acid (IAA)/ α-Naphthaleneacetic acid (NAA)/2,4-Dichlorophenoxy acetic acid (2,4-D). The percentage of somatic embryo induction, maturation and plantlet formation is calculated.
Results: Maximum percentage of somatic embryogenesis (91%) was observed in cotyledon explants on MS medium augmented with 0.5 mg/L TDZ in combination with 3.0 mg/L NAA while the highest number of somatic embryos per explant (66.9±0.10) was formed in leaflet explants on MS medium supplemented with 0.5 mg/L BAP+3.0 mg/LNAA followed by 0.5 mg/L TDZ +3.0 mg/LNAA.
Conclusion: Among the explants tested, cotyledon explants were proved to be efficient for

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induction of somatic embryogenesis and plantlet development compared to leaflet explants and MS medium fortified with TDZ is more effective compared to BAP in both the cotyledon as well as leaf explants. The developed plantlets were acclimatized and transferred to the research field. The regenerated plants were found to be similar to the donor plant phenotypically.

Keywords: Senna alata; somatic embryogenesis; acclimatization; plantlet establishment.

ABBREVIATIONS

PGRs: Plant Growth Regulators; BAP: 6-Benzylamino purine; 2, 4-D: 2,4-Dichlorophenoxy acetic acid; NAA: α-Naphthalene acetic acid; IAA:Indole-3-acetic acid; TDZ: Thidiazuron; GA_3:Gibberelic acid; mg/L: Milligram/Liter

1. INTRODUCTION

Somatic embryogenesis is the formation of somatic embryos from a somatic cell lacking vascular connection with the pilot tissue by undergoing a series of morphological and biochemical changes on appropriate induction. It offers an alternative and efficient means of plant propagation [1]. The process of somatic embryogenesis is an example of developmental plasticity or totipotency of plant cells. The process begins with the transformation of somatic cells to embryonic state which can be induced by certain *in vitro* conditions [2]. Somatic embryos are different from zygotic embryos as they are induced from somatic cells without involvement of fertilization. The plants regenerated via somatic embryogenesis are of single cell origin with *true-to-type* and are produced in large numbers within a short period [3].

Induction of somatic embryogenesis is of two types: direct somatic embryogenesis and indirect somatic embryogenesis. Direct somatic embryogenesis is used for those conditions where embryos initiate directly from pre-embryogenic determined cells while indirect refers to those which are developed from *defferentiated* tissues *i.e*. from induced embryogenic determined cells. Of the two types, direct somatic embryogenesis is preferred because it allows production of plants without somaclonal variation and its efficiency for cloning and genetic transformation.

Somatic embryogenesis has been achieved in a number of tree species. However, very few reports are available in woody species [4] as woody species particularly woody legumes are recalcitrant to regenerate *in vitro* [5,6] through somatic embryogenesis. Somatic embryogenesis *in vitro* depends on genotype and physiological stage of the explant, composition of culture medium, PGRs and also culture conditions.

A few reports are available on the plantlet regeneration via somatic embryogenesis in *Cassia* species [7,8]. So far no report has been published on somatic embryogenesis in *Senna alata* (syn. *C. alata*). In the present investigation we report high frequency plant regeneration through direct somatic embryogenesis in *S. alata* an ethnomedicinally important plant by using cotyledon and leaflet explants.

2. MATERIALS AND METHODS

2.1 Plant Material

For present investigation**,** *in vitro* grown 2-3 weeks old healthy seedlings were selected. Cotyledon and leaflet explants were excised carefully with the help of sterilized scalpel and cut into small pieces of size 1-3 cm^2 and were used for induction of somatic embryogenesis.

2.2 Culture Media and Culture Conditions

The explants were inoculated on MS [9] medium containing 30 g/L sucrose supplemented with different concentrations of PGRs (IBA/NAA/2, 4-D) (0.5-3.0 mg/L) in combination with 0.5 mg/L BAP/ TDZ.

The pH of the medium was adjusted to 5.8 with either 0.1N NaOH or 0.1N HCl and solidified with 0.8% (w/v) Difco-bactoagar (Hi-media, India) and autoclaved at 121ºC under 15ψ for 15-20 min. All the cultures were maintained in a culture room at 25±2ºC under a photoperiod of 16/8hr with light intensity of 40 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent tubes. For subculturing, the cultures were transferred on to fresh medium containing the same concentration and combination of PGRs.

For germination of somatic embryos, the bipolar stage embryos were cultured on MSO and MS medium fortified with different concentrations of GA_3 in combination with 3.0 mg/L 2,4-D/NAA.

2.3 Plantlet Establishment

The regenerated plantlets were taken out from the culture vessels and washed with sterile distilled water under aseptic conditions to remove remains of agar medium. Later these were transferred to plastic cups containing sterile vermiculite, covered with polythene bags to maintain the RH (90%) and kept in culture room for 4 weeks. Later they were shifted to earthenware pots containing sterile garden soil: compost (1:1) and maintained in the research field under shady conditions.

2.4 Data Analysis

Data on somatic embryogenesis were recorded for every 6 weeks of culture. 30 replicates were maintained for each experiment and each experiment was repeated atleast thrice.

3. RESULTS

Cotyledon and leaflet explants of *S.alata* were excised from 2-3 weeks old *in vitro* grown seedlings and were cultured on MS medium supplemented with 0.5 mg/L BAP/TDZ in combination with various concentrations of IBA/NAA/2, 4-D (0.5-3.0 mg/L) respectively (Tables 1-2). Somatic embryos were induced directly from both the explants at 0.5 mg/L BAP/TDZ in combination withall the concentrations of IBA/ NAA /2,4-D used. Somatic embryogenesis was initiated from the cut ends of the cotyledon and leaflet explants within 2 weeks of culture in all the concentrations of PGRs tested. The number of somatic embryos increased with an increase in the concentration of all the PGRs used in both the explants studied. Maximum percentage of somatic embryogenesis with highest number of somatic embryos per explant was observed on MS medium fortified with NAA in combination with TDZ in comparison to 2,4-D and IBA in both the explants tested (Table 1).

A gradual initiation of globular somatic embryos has been noticed after 2nd week of culture which later turned into heart-shaped and torpedo-shaped (bipolar) somatic embryos within 4 weeks of 1st subculture (Figs. 1-2). For further maturation, these somatic embryoids were subcultured on the fresh medium containing the

Table 1. Effect of different concentrations of 2,4-D/IBA/NAA in combination with 0.5 mg/L BAP/TDZ on somatic embryogenesis from cotyledon explants in *S. alata*

Conc. of PGRs (mg/L)	Cotyledon explants			
	% of Somatic embryogenesis	Average number of somatic embryoids/ explant (±SE[a*])	% of Somatic embryogenesis	Average number of somatic embryoids/ explant (±SE[a*])
	0.5 mg/L BAP		0.5 mg/L TDZ	
2,4-D				
0.5	35	10.12±0.36	47	12.4±0.19
1.0	42	24±0.57	51	29.4±0.19
2.0	56	26.6±0.13	68	44.0±0.44
2.5	68	39.4±0.12	70	54.1±0.73
3.0	72	47.9±0.57	75	59.7±0.66
IBA				
0.5	32	10.8±0.24	45	13.8±0.43
1.0	39	21.1±0.31	52	27.3±0.19
2.0	50	24.5±0.07	63	43.4±0.51
2.5	62	36.0±0.12	68	51.3±0.19
3.0	64	47.0±0.18	71	58.4±0.82
NAA				
0.5	45	19.6±0.36	49	19.4±0.20
1.0	59	27.3±0.52	61	30.24±0.72
2.0	65	42.4±0.74	72	44.8±0.31
2.5	77	48.9±0.41	82	56.5±0.29
3.0	88	58.6±0.15	91	59.9±0.14

*[a]Mean ± Standard Error, *n=10; P<0.05*

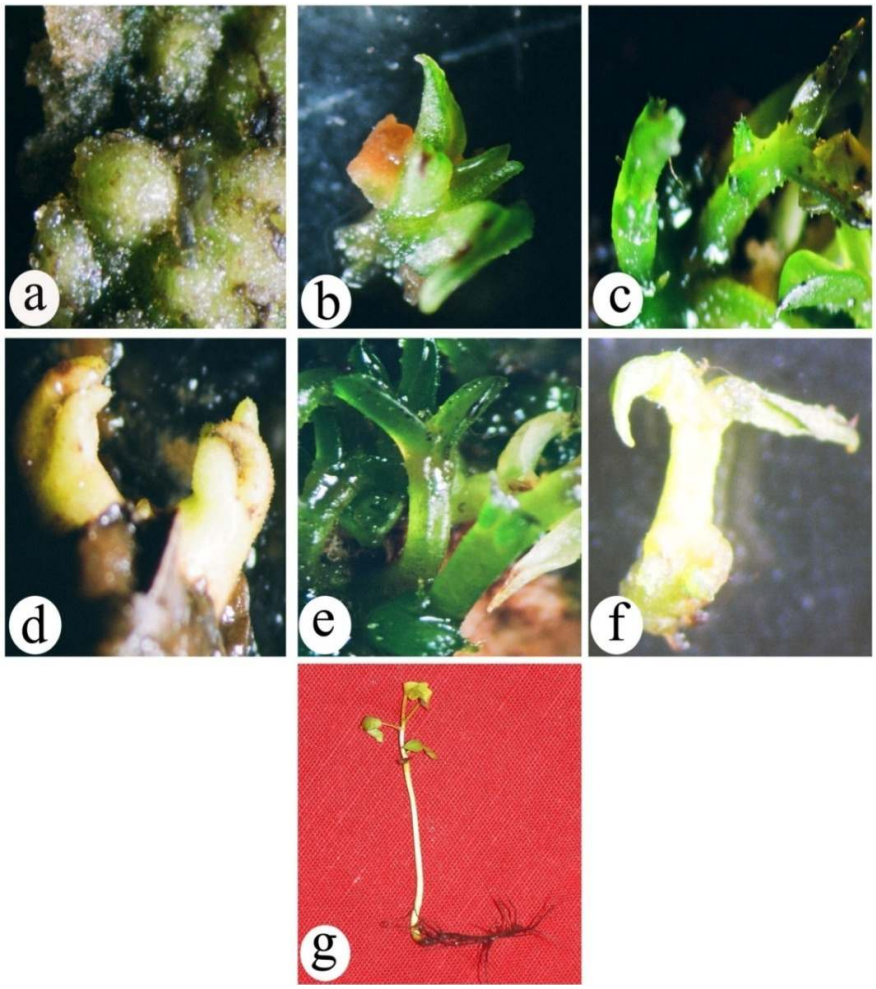

Fig. 1(a-g). Somatic embryogenesis and plantlet establishment from cotyledon explants in *S. alata*

a) Globular shaped somatic embryos formed on MS+ 0.5 mg/L TDZ+3.0 mg/L NAA; b) Different types of somatic embryoids including heart-shaped on the same;c) Developing torpedo- shaped embryos on the same; d) A group of germinating cotyledonary stage embryos on MS+3.0 mg/L 2,4-D+ 3.0 mg/L GA_3; e) Formation of a group of cotyledonary stage embryos on the same; f) Afully developed cotyledonary stage embryo of the same; g) Completely developed plantlet through somatic embryogenesis.

same concentrations of PGRs. Even after two cycles of subculture, we didn't find further morphogenesis in torpedo-shaped somatic embryoids of *S. alata*. The torpedo-shaped embryoids failed to undergo any further maturation upon subculturing on MSO medium too.

3.1 Somatic Embryogenesis in Cotyledon Explants

Cotyledon explants cultured on MS medium supplemented with 0.5 mg/L BAP/TDZ in combination with different concentrations of 2,4-D/IBA/NAA (0.5-3.0 mg/L) showed an increase in the percentage of somatic embryogenesis with an increase in the number of somatic embryos per explant in all the concentrations tested. As the concentration of auxins increased, an increase in the percentage of somatic embryogenesis and the number of somatic embryos per explant was observed (Table 1).

Maximum percentage (91%) of somatic embryogenesis with highest number (59.9±0.14) of somatic embryos development per explant was observed at 0.5 mg/L TDZ in combination with 3.0 mg/L NAA in comparison to all other

concentrations of auxins used (Fig. 1). Whereas maximum number of somatic embryoids per explant (59.7±0.66) was also recorded at 0.5 mg/L TDZ+3.0 mg/L 2,4-D followed by 0.5 mg/L BAP+3.0 mg/L NAA and 0.5 mg/L TDZ+3.0 mg/L IBA.

3.2 Somatic Embryogenesis in Leaflet Explants

The leaflet explants excised from auxenic cultures of *S. alata* were cultured on MS medium augmented with 0.5 mg/L BAP/TDZ in combination with different concentrations (0.5-3.0 mg/L) of auxins (2,4-D/IBA/NAA) to study the induction and proliferation of somatic embryogenesis (Table 2).

Maximum percentage (77%) of somatic embryogenesis was observed at 0.5 mg/L TDZ in combination with 3.0 mg/L NAA followed by 0.5 mg/L TDZ+3.0 mg/L 2,4-D (Table 2). Whereas maximum number of somatic embryos (66.9±0.10) at 0.5 mg/L BAP+3.0 mg/L NAA followed by 0.5 mg/L BAP+3.0 mg/L 2,4-D.

In both the cotyledon and leaflet explants, MS medium fortified with TDZ is more effective for somatic embryogenesis compared to BAP. NAA showed superiority in both the percentage of somatic embryogenesis and the maximum number of somatic embryos per explant in comparison to 2,4-D and IBA (Table 2).

Development of somatic embryos appeared to be asynchronous with a wide range of varied sizes and structures. As a result various stages of somatic embryos development was recorded in the same cluster of embryos originally from the explant.

3.3 Embryo Germination and Plantlet Establishment

The torpedo-shaped embryos were tested by transferring them onto medium containing the same concentrations and combination of PGRs(2,4-D/IBA/NAA+0.5 mg/L BAP/TDZ) and also on MS medium devoid of PGRs (MSO) for further maturation and germination. But these embroids failed to germinate even after second subculture too. However, germination of torpedo-shaped somatic embryos was observed on MS medium augmented with differerent concentrations of GA_3 (0.5-3.0 mg/L) in combination with 3.0 mg/L 2,4-D/NAA (Table 3).

Table 2. Effect of different concentrations of 2,4-D/IBA/NAA in combination with 0.5 mg/L TDZ on somatic embryogenesis from leaf explants in *S. alata*

Conc. of PGRs (mg/L)	Leaflet explants			
	% of Somatic embryogenesis	Average number of somatic embryoids/ explant (±SE[a])	% of Somatic embryogenesis	Average number of somatic embryoids/ explant (±SE[a])
	0.5 mg/L BAP		0.5 mg/L TDZ	
2,4-D				
0.5	31	12.0±0.53	42	10.14±0.32
1.0	37	26.1±0.24	47	23.6±0.69
2.0	43	49.6±0.73	56	36.9±0.57
2.5	51	60.2±0.52	63	47.9±0.93
3.0	56	64.1±0.28	68	53.3±0.64
IBA				
0.5	28	15.0±0.31	39	13.0±0.12
1.0	31	26.3±0.19	42	29.4±0.19
2.0	36	32.3±0.50	47	46.8±0.24
2.5	42	59.5±0.64	51	56.7±0.38
3.0	49	63.8±0.49	58	61.4±0.18
NAA				
0.5	38	23.6±0.51	48	24.6±0.86
1.0	42	30.6±0.20	52	34.8±0.72
2.0	53	49.24±0.93	59	47.2±0.04
2.5	60	61.49±0.20	67	58.6±0.92
3.0	65	66.9±0.10	77	62.3±0.85

*[a]Mean ± Standard Error, *n=10; P<0.05*

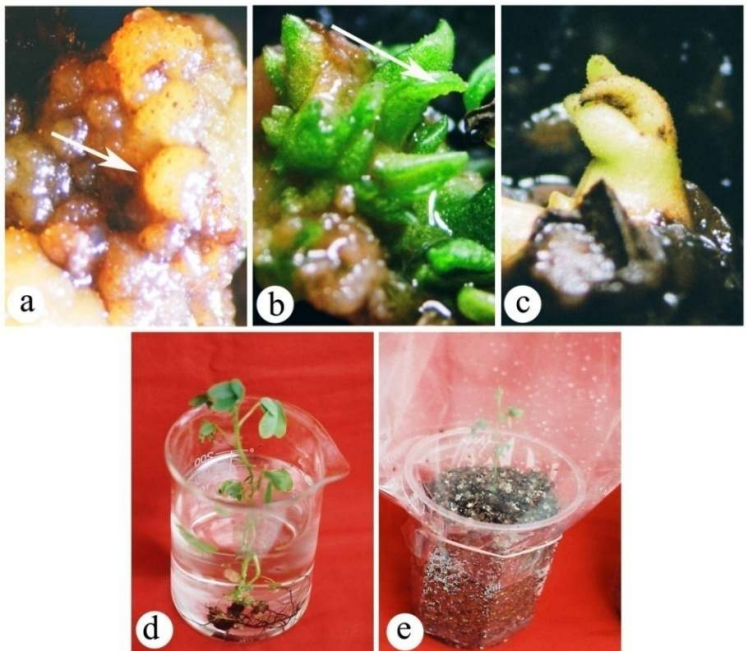

Fig. 2(a-e). Somatic embryogenesis and plantlet establishment from leaflet explants in *S. alata*
a) Globular somatic embryos developed on MS+ 0.5 mg/L TDZ+3.0 mg/L NAA; b) Somatic embryos in various stages on the same; c) Initiation of somatic embryo germination on MS +3.0 mg/L GA_3+3.0 mg/L 2,4-D; d) Completely developed plantlet with healthy root system; e) Acclimatization of plantlet developed through somatic embryogenesis

Table 3. Effect of GA_3+ 2,4-D/NAA on germination of torpedo shaped embryos in *S. alata*

Conc. of PGRs (mg/L)	Number of embryos cultured	Number of embryos germinated (%)*	% of germination
GA_3+3.0 mg/L 2,4-D			
0.5	50	34	45
1.0	50	42	47
2.0	50	46	59
3.0	50	56	63
GA_3+3.0 mg/LNAA			
0.5	60	33	38
1.0	60	38	43
2.0	60	45	56
3.0	60	51	58

**n=10; P<0.05*

Within 3-4 weeks of culture, complete plantlets with well developed shoot and root systems were obtained (Fig. 1 d-g; Fig. 2 c-d). As the concentration of GA_3 increased, there is gradual increase in the percentage of somatic embryo germination was observed in both the combinations of auxins (3.0 mg/L 2,4-D/NAA) used. However, maximum percentage of somatic embryo germination was observed on MS medium augmented with 3.0 mg/L GA_3+ 3.0 mg/L 2,4-D (Table 3).

The plantlets developed through somatic embryogenesis were hardened in culture room for 4 weeks (Fig. 2 d-e). Afterwards these were shifted to plastic pots containing garden soil: compost (1:1) and maintained in the research field under shady place. The plantlets survival

percentage was found to be 63%. The plants developed through somatic embryogenesis were similar to donor plants in morphology.

4. DISCUSSION

Somatic embryogenesis and plantlet establishment have been successfully achieved from cotyledon and leaflet explants in *S. alata* using various concentrations and combinations of different PGRs. Of both the explants tested, cotyledon explants were found to be highly responsive with maximum percentage (91%) of somatic embryogenesis at 0.5 mg/L TDZ+ 3.0 mg/L NAA (Table 1). The induction, development and germination was controlled by PGRs. Among the PGRs tested, TDZ in combination with NAA was found to be highly effective for the induction and proliferation of somatic embryos in *S. alata*. Somatic embryogenesis had also been reported in a number of other legumes [8,10]. Direct somatic embryogenesis proceeds under *in vitro* conditions from pre embryonic determined cells which require appropriate triggering phenomenon such as favorable conditions and usage of plant growth regulators (PGRs) for undergoing division and progression of embryogenesis. In the present investigation, highest percentage of somatic embryogenesis has been recorded in cotyledon explants compared to leaflet explants of *S. alata* which is in agreement with the reports of the previous investigators in other legume plants [11].

Selection of type of explant, concentration and combination of PGRs influence the induction of somatic embryogenesis and plantlet recovery in a number of species [12]. All the auxins tested in the present investigation, resulted in the formation of direct somatic embryos from both the explants, which suggests that the exogenous supply of auxins in *in vitro* enhanced the somatic embryogenesis in *S. alata*.

In addition to this, presence of cytokinins in the culture medium is also essential for the induction of high frequency somatic embryogenesis in *S. alata*. Induction of somatic embryogenesis by cytokinins alone is rare among legumes [13]. The results of our investigation are also in accordance with the above findings [13]. Whereas, cytokinins alone favored the induction of somatic embryos in woody legumes *viz*. *Calliandra tweedii* [14] and *Cassia angustifolia* [15]. Cytokinins in combination with auxins resulted in the formation of high frequency somatic embryogenesis in *S. alata* (Tables 1-2). Similar reports on the requirement of auxin-cytokinin combination for somatic embryo induction were observed by Rama Swamy et al. in *Solanum surattense* [16] and Agarwal and Sardar in *Cassia angustifolia* [15].

The transition of globular to heart-shaped to bipolar is critical step in somatic embryogenesis and also the maturation of somatic embryos, particularly, the ability of somatic embryos to germinate and form plantlets. Maturation of somatic embryos can be achieved by manipulating the PGRs that are required specifically for each stage from globular to torpedo-shaped somatic embryos and also for germination [3].

The torpedo-shaped somatic embryos germinated well on medium supplemented with GA_3. The addition of GA_3 appeared to promote further maturation and plantlet development. In the present investigation, GA_3 appeared to promote germination of somatic embryos and elongation; in its absence only torpedo-shaped embryos that usually didn't develop further. The plantlets formed from somatic embryos were shifted to field conditions and the survival percentage was recorded as 63% (Fig.1g). The *in vitro* regenerated plantlets were found similar to the parent plant phenotypically with healthy root and shoot system. Thus, we have established the protocol for regeneration through direct somatic embryogenesis in *S. alata* for its conservation and multiplication.

5. CONCLUSION

In conclusion, this is the first report of a successful procedure to regenerate *S. alata* via somatic embryogenesis. The cotyledon explants were proved to be efficient for *in vitro* somatic embryogenesis compared to leaflet explants in *S. alata*. MS medium supplemented with higher amounts of auxins in combination with lower concentrations of cytokinins favor the induction and proliferation of somatic embryogenesis.MS medium fortified with TDZ is more effective compared to BAP in *S. alata*.

Thus, the present reproducible regeneration protocol can be used for mass multiplication, genetic transformation, artificial seed production and cryopreservation of the important ethnomedicinal plant *S. alata*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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