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Optimization of culture media for *in vitro* Zygotic embryo culture of *Senna alata* L. Roxb –An Ethnomedicinal plant

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Abstract: *Senna alata L. Roxb, is an ethnomedicinal plant belonging to Fabaceae family. The plant is known to possess potent medicinal properties include laxative, antitumor, antimutagenic, antioxidant and antifungal. Seeds of S. alata possess a hard seed coat which acts as a barrier for seed germination. Zygotic embryos were cultured on various media (half and full - strength Murashige and Skoog's medium, Gamborg's medium) with different concentrations of sucrose(10-30g/L) and plant growth regulators (PGRs). Maximum percentage of embryo germination and healthy plantlet formation were observed on MSO (Murashige and Skoog basal) medium containing 20 g/L sucrose without PGRs. Multiple shoot formation was observed on MS (Murashige and Skoog's) medium containing 20g/L sucrose supplemented with 2 mg/L BAP. The formed seedlings were acclimatized in sterile vermiculite: garden soil (1:1) and later shifted to field conditions. The newly formed plantlets resembled the donor plant phenotypically. This protocol can be used for multiplication and conservation of the species.*

Key Words: *Acclimatization, Culture medium, In vitro zygotic embryo culture, Multiple shoots, Plantlet formation, Seed dormancy, Senna alata.*

I. Introduction

The species *Senna alata* L. Roxb. has ethnomedicinal importance which belongs to family Fabaceae. The plant is known to possess laxative properties [1]. The tea prepared from the leaves of the plant is used for the treatment of constipation and for killing intestinal worms. The leaves of the plant are known to possess potent antifungal activities due to the presence of a fungicide chrysophanic acid [2] and can be directly rubbed on the skin to cure fungal infections like *Pityriasis versicolor* [3], ring worm [4], white spot fungal infections [5] and eczema. The plant also possess other important medicinal properties including antimicrobial [6, 7], analgesic [8], choleretic [9], anti hyperglycemic [10] and hepatoprotective [11] activities. It was also proved that the leaf extracts are effective in curing opportunistic infections in AIDS patients [12]. In cattle the ethanolic extracts of the plant are used to cure acute lesions of bovine dermatophilosis and prevented its reoccurrence [13].

As it is a non domestic species to India, seed germination depends on many factors like temperature, light, etc [14]. Seeds of *S. alata* posses a hard seed coat which hinders the absorption of oxygen and water which leads to seed dormancy in the species [15]. A number of studies demonstrated that seed dormancy of *S. alata* can be broken down by mechanical scarification or by chemical scarification using sulfuric acid for a period of 60 minutes [15]. However, subjecting seeds to acid scarification or mechanical scarification may reduce the percentage of seed germination as they may cause damage to the embryo.

Embryo culture is considered as a natural tool for overcoming seed dormancy and improving germination percentage [16]. We report in the present study the optimization of a reproducible protocol for *in vitro* propagation and rapid multiplication of the species *S. alata* through zygotic embryo culture.

II. Materials And Methods

2.1 Plant material

The pods of *S. alata* were collected from the medicinal garden, Department of Biotechnology, Kakatiya University, and dried under shade for 2-3 months. Later, the seeds were separated from the pods and stored at room temperature for further use.

The seeds were washed under running tap water for 30 minutes followed by soaking for 30 minutes in 2% (w/v) Bavistin and subsequently washed 5-6 times with sterile distilled water. These seeds were sterilized with 0.1% (w/v) $HgCl_2$ for 5-6 minutes followed by 3 rinses in sterile distilled water under aseptic conditions, blot dried on sterile tissue paper. These sterilized seeds were subjected to mechanical scarification using a sterile surgical blade to allow the passage of water. After scarification the seeds were soaked in sterile distilled water overnight. The embryos were carefully separated from the soaked seeds and dried on a sterile tissue paper and were inoculated on different types of culture media.

2.2 Culture Media and Culture Conditions

The zygotic embryos isolated from the seeds were carefully transferred onto (i) half and full strength MS [17] and B5 [18] media supplemented with varying concentrations of sucrose (10 -30 g/L) and (ii) half and full strength MS and B5 media supplemented with different concentrations of plant growth regulators (PGRs- BAP/ KIN/ 2,4 D). The medium was solidified using 0.8% (w/v) agar (Hi-Media, India). The P^H of the media was adjusted to 5.7±0.1 either with 0.1N HCl or 0.1N NaOH before autoclaving at 121^{o}C for 15-20 minutes.

The effect of the orientation of zygotic embryos was also studied by placing the embryos horizontally, vertically, half-dipped and embryos with a small portion of cotyledon. All the cultures were incubated at 25±2ºC under a 16 hr photoperiod with light intensity of 40-50 $\mu mol^{-2}s^{-1}$ maintained by using white fluorescent tubes.

2.3 Plantlet establishment

After embryo germination and maturation, the seedlings were taken out from the culture vessels and washed with sterile distilled water to remove traces of agar and transferred to plastic cups containing sterile vermiculite: garden soil (1:1) and were covered with polythene bags to maintain the relative humidity (85-90% RH) and kept in culture room for 3 weeks. Later the plantlets were transferred to plastic pots containing garden soil and kept in growth chamber at 25-27ºC. After hardening/acclimatization these plantlets were shifted to earthenware pots and maintained under shady conditions in the research field.

2.4 Data Analysis

Data of 20 replicates were recorded and each experiment was repeated thrice. Data on embryo germination and multiple shoots formation were recorded periodically and results were analyzed statistically.

III. Results

3.1 Effect of medium and concentration of sucrose

Zygotic embryos of *S. alata* were cultured on full and half strength MS and B5 basal media supplemented with different concentrations (10-30 gm/L) of sucrose (Table-1; Fig. 1). Germination of the zygotic embryos started within 2-3 days of inoculation. Colour change of the zygotic embryo from white to green, which is the first visible sign of embryo germination was observed within 2 days of inoculation followed by radical elongation. Shoot emergence was observed after 6-7 days of inoculation and well developed seedlings with healthy shoots and roots were formed by end of the 3rd week of culture.

Among the various concentrations of sucrose used, absolute percentage of embryo germination (100%) was observed on both full strength MS medium with 20, 25 and 30g/L sucrose and also on half strength MS medium containing 20, 25 and 30gm/L sucrose in comparison to B5 medium where a maximum of 82% germination was observed. Less percentage of zygotic embryo germination was observed on half strength MS medium with 10 g/L sucrose (Table-1). As the concentration of sucrose decreased, gradually the percentage of germination was decreased in both the types of (MS, B5) media used. The zygotic embryos were converted into callus on half strength MS and B5 media containing 10, 20g/L sucrose while at 10 g/L sucrose on B5 medium, embryos did not respond. Early germination of zygotic embryos was also observed on full strength MS medium containing 20-30g/L sucrose. High percentage of germination with healthy seedlings formation was observed on MS medium compared to B5 medium.

Of all the concentrations of sucrose and media used, healthy seedlings with well developed shoot and root systems was observed on full strength MS basal medium supplemented with 20 g/ L sucrose.

3.2 Effect of PGRs

The zygotic embryos were also cultured on MS medium containing 20 g/L sucrose supplemented with various concentrations (0.5-5mg/L) of 2, 4 D/ BAP/ KIN (Fig.1). They have shown varying response in germination percentage and multiple shoot induction. Absolute percentage of germination was observed in all the concentrations of 2, 4 D/ BAP/ KIN (0.5-5 mg/L) with a reduced time for germination of 3 days (Table.2). Although 100% of zygotic embryo germination was observed on all the concentrations of 2, 4 D, whole embryos were converted into callus after 6 weeks of incubation.

Highest number of multiple shoots was recorded on MS medium containing 20 g/L sucrose supplemented with 2.0mg/L BAP (3.1 ± 0.32) followed by 2.5 mg/L KIN (2.8±0.51) and 2.5mg/L BAP (2.7±0.82) with an average shoot length of 7.3±0.91, 3.0±0.17 and 6.3±0.42 cms respectively (Table.2). The seedlings developed on MS medium supplemented with KIN showed stunted growth with profuse rooting. The radical portion of the embryos elongated during germination in the medium with 2, 4 D turned into callus after 7-9 days of culture hindering the further development of roots (Fig.1d). More number of multiple shoots was observed on all the concentrations of BAP tested than KIN.

3.3 Effect of Embryo orientation

The zygotic embryos were cultured on MS medium with 20g/L sucrose in different orientations (Table.3). The zygotic embryos placed vertically showed 100% germination followed by the embryos with a portion of cotyledon attached to it (96%). The germination percentage was reduced when the embryos placed half dipped in the medium while the embryos which were placed horizontally elongated first and later turned into callus (Table.3).

3.4 Plantlet Establishment

Healthy and mature seedlings formed from zygotic embryos cultured on MS and B5 media were taken out from the culture vessels and washed with distilled water to remove traced of agar. These seedlings were then transferred to plastic cups containing sterile vermiculate: garden soil (1:1), covered with plastic bags to maintain the relative humidity of 85-90% RH and incubated in culture room for 3 weeks. After 3 weeks seedlings showing healthy growth are kept in growth chamber with a temperature of 25± 2°C. The plantlets were later shifted to earthenware pots and maintained under field conditions in the research field. The survival percentage of plantlets was recorded as 78%.

IV. Discussion

Zygotic embryo culture is a technique which involves isolating and culturing of immature or mature zygotic embryos on a nutrient medium under aseptic conditions. This technique also helps in understanding concepts related to nutrient requirements of the growing zygotic embryo, shortening the breeding cycle and overcoming seed dormancy [19].

In vitro propagation of *S. alata* using zygotic embryo explants was achieved by culturing the overnight soaked zygotic embryos on MS and B_5 media (Full and Half strength) with various concentrations of sucrose and on MS medium supplemented with different concentrations (0.5-5mg/L) of PGRs (2,4 D, BAP, KIN) as a sole growth regulator. The strength of the media, percentage of sucrose and orientation of the zygotic embryos on the culture medium played a vital role on the germination of the zygotic embryos and also on the development of healthy seedlings in *S. alata*.

Sucrose concentration is important in early stages of zygotic embryo germination as young embryos require a complex medium for growth and maturation [20]. It also helps in maintaining the osmolarity of the medium [21] and also prevents the embryos from entering into elongation state from division state [22,23]. MS basal medium with 20 g/L sucrose is the most efficient medium for maximum percentage of zygotic embryo germination and plantlet formation in the present investigations.

Nitrogen source is supplied in the form of ammonium nitrate and potassium nitrate. These are major constituents of MS and B5 media which play a major role in proper growth and differentiation of embryos [24,25]. Concentration of agar also plays a vital role in the development of zygotic embryos *in vitro*. Higher concentration of agar inhibits the growth by reducing the availability of water required for the proper development of the embryos [21].

Maximum number of multiple shoots was observed on MS medium containing 20 g/L sucrose supplemented with 2.0 mg/L BAP in contrast to the results reported by Veen (1963). He reported that cytokinins are ineffective and slightly promote the growth of young embryos when used as the sole PGR in the culture medium. Simultaneous rooting of the multiple shoots was also observed on the same medium without the addition of any auxins which agrees with the statement of Norstog (1979) that addition of exogenous auxins to the culture medium is not required for plant embryo growth in in vitro conditions.

The orientation of the zygotic embryos on the culture medium also affected the rate of germination. Embryos showed maximum percentage of germination when placed vertically on the medium as observed by Rambabu et al in *Givotia rottleriformis* [28]. The plantlets thus obtained were acclimatized by transferring them to plastic cups containing sterile vermiculite: garden soil (1:1), shifted to green house and transferred to the research field.

V. Conclusion

Seed dormancy is a major problem in woody legumes like *Senna alata* due to the presence of a hard, water proof seed coat that prevents its rapid multiplication. From our present investigation it can be concluded that such problems associated with seed dormancy can be overcome by culturing zygotic embryos *in vitro* under aseptic conditions. Our results also suggest that MS medium supplemented with 20 g/L sucrose supplemented with 2.0mg/L BAP (3.1 ± 0.32) was found to be the best medium for culturing zygotic embryos *in vitro*.

VI. Figures And Tables

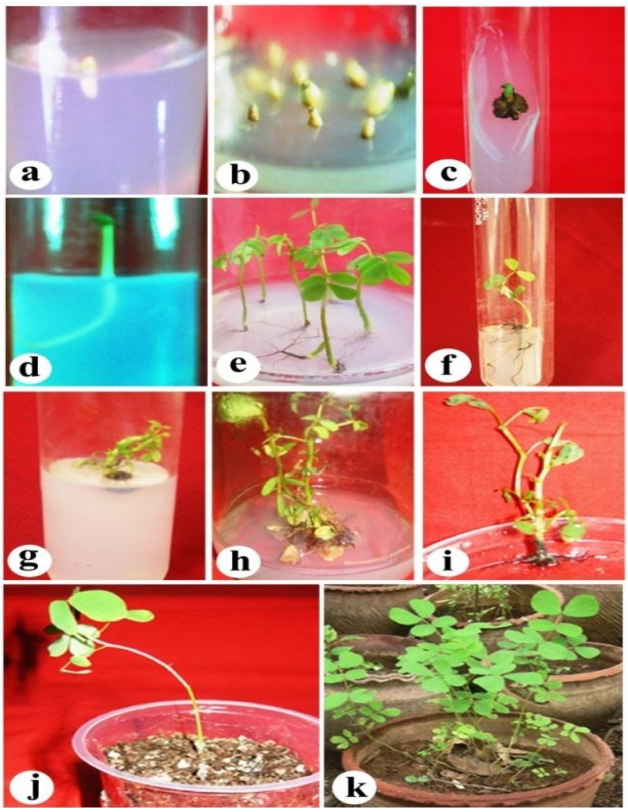

Fig.1 a-k: In vitro zygotic embryo culture of *Senna alata*.

a) zygotic embryo placed vertically on MSO medium **b)** Morphogenic response on MSO medium after 2 days **c)** Callus induction on MSO medium from embryos placed horizontally **d)** Zygotic embryos after 7 days of inoculation on MSO medium **e)** Healthy seedlings formed on MS medium containing 20g/L sucrose **f)** Profuse rooting of the plantlets developed from zygotic embryo on MS + 3mg/L KIN **g)** Multiple shoot induction on MS + 2 mg/L BAP **h)** Elongated multiple shoots on MS + 2 mg/L BAP after 1st subculture **i)** Elongated shoots with well developed roots **j**) Acclimatization in green house **k)** Plants growing in the research field.

Table-1: Effect of MS and B5 media containing various concentrations of sucrose on *in vitro* zygotic embryo culture of *S. alata*

Medium strength	Conc. of sucrose (gm/L)	No. of days for embryo germination	% of embryo germination	Average shoot length (cm) ± SE[a]	Average root length (cm) ± SE[a]
MS+Sucrose					
Full	10	9	64	callus	callus
Full	20	4	100	9.2 ±0.11	12.6± 0.37
Full	25	4	100	9.0 ±0.08	11.6 ±0.09
Full	30	4	100	8.6 ±0.22	11.2 ±0.16
Half	10	19	40	callus	callus
Half	20	13	84	callus	callus
Half	25	6	100	7.6±0.16	5.4±0.11
Half	30	19	40	7.9±0.62	6.2±0.09
B5+ Sucrose					
Full	10	17	82	7.2 ±0.43	4.6 ±0.46
Full	20	10	82	6.9 ±0.46	4.4 ±0.07
Full	25	6	79	4.8 ±0.54	3.7 ±0.05
Full	30	6	32	callus	callus
Half	10	19	NR	NR	NR
Half	20	19	48	callus	callus
Half	25	9	54	1.6±0.42	1.09±0.63
Half	30	9	73	3.2±0.05	2.1±0.09

[a] Mean ± Standard Error
NR= No response

Table-2: Effect of MS medium containing 20 g/L sucrose supplemented with 2, 4 D, BAP, KIN on *in vitro* zygotic embryo culture of *S. alata*

Conc. of PGR (mg/L)	% of germination	No. of days for germination	No. of shoots/ explant (±SE)[a]	No. of roots/ explant (±SE)[a]	Average shoot length (±SE)[a]	Average root length (±SE)[a]
2, 4 D						
0.5	100	5	1.1±0.61	--	2.1±0.65	Callus
1.0	100	5	1.0±0.01	--	2.6±0.82	Callus
2.0	100	5	1.6±0.41	--	2.9±0.23	Callus
2.5	100	5	1.3±0.31	--	3.1±0.11	Callus
3.0	100	5	1.0±0.21	--	1.8±0.57	Callus
4.0	100	5	1.1±0.18	--	1.7±0.19	Callus
5.0	100	5	1.0±0.31	--	1.7±0.09	Callus
BAP						
0.5	100	4	1.0±0.24	1.0±0.31	5.2±0.06	8.2±0.04
1.0	100	4	1.0±0.51	1.1±0.30	5.9±0.75	9.6±0.50
2.0	100	4	3.1±0.32	1.0±0.51	6.3±0.42	7.8±0.31
2.5	100	4	2.7±0.82	1.0±0.18	7.3±0.91	7.2±0.82
3.0	100	4	1.7±0.28	1.1±0.41	7.2±0.77	7.2±0.09
4.0	100	4	1.0±0.62	1.0±0.18	5.1±0.11	7.0±0.54
5.0	100	4	1.0±19	1.1±0.72	5.1±0.03	7.0±0.39
KIN						
0.5	100	3	1.1±0.42	1.0±0.16	3.6±0.06	9.5±0.72
1.0	100	3	1.1±0.17	5.6±0.17	3.7±0.57	10.6±0.41
2.0	100	3	2.1±0.28	5.9±0.58	3.8±0.82	12.8±0,05
2.5	100	3	2.8±0.51	9.6±0.31	3.0±0.17	9.5±0.61
3.0	100	3	2.1±0.31	4.7±0.19	2.9±0.09	9.4±0.71
4.0	100	3	1.0±0.61	2.9±0.73	3.9±0.11	9.2±0.54
5.0	100	3	1.0±0.18	2.9±0.11	3.4±0.16	8.6±0.67

[a] Mean ± Standard Error

Table-3: Effect of the orientation of zygotic embryos on germination percentage (MS medium containing 20g/L sucrose) in *S. alata*

Orientation of zygotic embryo	% of germination	No. of days for germination	Average length of seedling (cms) ± SE[a]
Vertical	100	3	7.2±0.54
Half dipped	82	7	5.4±0.20
Horizontal	62	5	callus
With a portion of cotyledon	96	3	6.3±0.82

[a] Mean ± Standard Error

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