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REGULAR ARTICLE

DIRECT SHOOT REGENERATION FROM MATURE LEAF EXPLANTS OF *SPHAERANTHUS INDICUS* L., A MULTIPURPOSE MEDICINAL PLANT

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SUMMARY

A rapid and reproducible protocol for *in vitro* regeneration of *Sphaeranthus indicus* (Asteraceae), a medicinal herb has been established. Leaf segments isolated from mature plants were cultured on MS medium with different concentrations of 6-benzyladenine (2.2, 4.4, 6.6 and 8.8 µM) or kinetin (1.3, 2.3, 4.6 and 6.9 µM). Inclusion of IAA into BA supplemented medium triggered a high frequency of regeneration response from leaf explants. Maximum number of shoots (12 ±1.15) with highest shoot length (3.0 ±0.73) were obtained directly (without intervening callus phase) from the leaf explants using combination of BA (4.4 µM) and IAA (1.71 µM) within 3-4 week of culture. The elongated shoots were rooted on MS medium fortified with IBA (2.46 µM).The regenerated plantlets were successfully hardened on earthen pots after proper acclimatization under greenhouse conditions.

Key words: Auxins, Cytokinins, Multiple shoots, Root induction, Greenhouse transfer
Abbreviations: BA, 6-benzyladenine; IAA, Indole 3- acetic acid; IBA, Indole-3-butyric acid; Kn, Kinetin; MS, Murashige and Skoog; PGR, plant growth regulators

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1. Introduction

Sphaeranthus indicus Linn. is a medicinal herb with round purple flowers, commonly known as gorakhmund and east Indian globe thistle. This plant belongs to the family *Asteraceae*, which includes several medicinal plants such as *Stevia rebaudiana, Vernonia anthelmintica, Taraxacum officinale* etc. The plant is distributed throughout India, Srilanka, Australia, Malaysia, China and Africa. The whole plant parts are used in traditional medicine for curing various disorders. In folk medicine the plant is reported to be used in treating hemicrania, epilepsy and mental disorders (1). It is used as a tonic, laxative, digestive, anthelmintic, alexipaharmic and the treatment of insanity, tuberculosis and disease of spleen, anaemia, bronchititis, elephantiasis, leucoderma. Leaves of the plant have anxiolytic, macro-filaricidal and antimicrobial activities (2,3,4). The paste prepared from the herb is beneficial in treating pruritus, edema, arthritis, gout, and cervical adenopathy.It also treats piles and hepatitis (5). Earlier work on the aerial parts of this plant revealed it to be rich in essential oil components, alkaloid sphaeranthine (6,7). Glucosides and eudesminoids, phenolic glycosides and sesquiterpene lactones, sphaerantholide, flavone and isoflavone glycosides were also identifed in this plant species (8,9,10,11,12). Several medicinal properties have been attributed to the extracts, fractions and isolated constituents of *Sphaeranthus indicus* flowers which includes hypotensive, peripheral vasodilatory and cathartic activity, antidiabetic, and immunomodulatory activity (13,14,15).

The natural means of propagation of *S. indicus* is *via* seeds. However, it encounters the problem of low seed set, viability and

germination. Further, the population of this plant is dwindling at an alarming rate due to its over exploitation for medicinal use. Therefore the immediate need is to propagate the plant for its conservation. Efforts are also needed to propagate this species on commercial scale to meet increasing demands of pharmaceutical firms for its medicinal importance. *In vitro* propagation can be used as an effective strategy for germplasm conservation and multiplication of this plant species that will also enable further investigation of its medicinally active constituents. Till date no reports are available describing direct organogenesis in *S. indicus*. This paper for the first time reports an efficient method for *in vitro* regeneration from leaf explants of *S. indicus*.

2. Material and Methods

Plant material was collected from field conditions at Warangal, India. Leaf segments were isolated and washed thoroughly under running tap water for 30 min then treated with 5% tween - 20 for 5 min followed by 3-4 rinses in sterile distilled water. These explants were surface sterilized with 0.1% (w/v) $HgCl_2$ for 4-5 min followed by 4-5 rinses with sterile distilled water.

A single leaf was aseptically cut in to 3-5 pieces of size 1x1 cm. These explants were inoculated on MS medium with various concentrations of BA (2.2, 4.4, 6.6 and 8.8 µM) or Kn (1.3, 2.3, 4.6 and 6.9 µM), and hormone combinations of BA (4.4µM) plus indole-3-acetic acid (IAA) (0.57, 1.71 and 2.85 µM) were tested. The regenerating shoots (3-4 week old) were transferred to MS basal medium for further elongation and cultured for 1 week on to this medium.

Rooting and greenhouse transfer

Microshoots regenerated from leaf explants (5-8 cm and 2-3 cm respectively) were excised and rooted on MS medium supplemented with indole-3-butryic acid (IBA) (2.46 µM). *In vitro* regenerated plantlets obtained after 10-12 days of culture on rooting medium were carefully removed from the culture tubes and washed under running tap water until agar was removed completely. These plantlets were transferred to pots containing autoclaved mixture of sand: soil (1:1) followed by acclimation in the greenhouse (28°C day, 24°C night, and 80 - 90 % RH).

Plant growth regulators (PGRs) used in the study were added prior to autoclaving the medium. The explants (leaf) were inoculated in culture tubes (150 x 25 mm) containing 20 ml of medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (Himedia, India). All media pH were adjusted to 5.8 with 0.1N NaOH before adding agar and sterilized at 121°C for 15 min. All cultures were maintained at 25 $\pm$ 2°C under white fluorescent light (65 µE/m2/s) with 16 h photoperiod.

For culture establishment and multiplication from leaf explants, 10 explants were used in each of two replicates for each treatment and the experiment was repeated twice. Data pertaining to number of shoots per culture, shoot regeneration percentage, and mean shoot length were recorded after 3 – 4 weeks. The data were analyzed statistically using Duncan's multiple range test.

3. Results

The leaf segments excised from field grown plants were cultured on MS medium fortified with BA, or Kn, or BA plus IAA. The efficiency of direct leaf regeneration differed with PGR concentrations used in the study. Green nodular buds developed from the cut ends of leaf cultures that further modified into shoot initials (Fig: a and b). This was a common morphogenic response observed for all regenerating leaf cultures. BA at concentrations of 2.2 and 4.4 µM induced significantly higher number of multiple shoots (7.0 shoots/explant). However, the per cent of such responding cultures were more at 4.4 µM BA (50%) as compared to 2.2 µM BA (30%). The regenerated shoots attained a mean length of 1.5 $\pm$ 0.25 and 2.5 $\pm$ 0.39 at concentrations 2.2 and 4.4 µM of BA respectively. Kn at 2.3 µM initiated multiple shoot induction from leaf explants with 45% of cultures showing such response. At this concentration, the mean number of shoots

per explant were 6.2 ± 0.97 that attained an average length of 2.6 ± 0.37. A lower level of Kn (1.3 µM) caused a decrease in percent of responding cultures (40%) (Table1). Higher level of BA (6.6 µM) or Kn (4.6 µM) induced callusing from cut ends of leaf cultures (Fig.: c). Under these conditions, the morphogenic response was callus initiation accompanied with shoot formation. Further increase in concentration of BA (8.8 µM) or Kn (6.9 µM) resulted in callus formation without shoot induction (Table).

Fig. 1: (a) Shoot bud initiation from cut ends of leaf culture (b) shoot development from shoot bud (c) multiple shoot induction from intermediate callus phase on MS medium supplemented with BA (6.6 µM) (d) direct leaf regeneration on MS medium containing BA (4.4 µM) plus IAA (1.71 µM) (e) development of roots from cut ends of microshoots on IBA (2.46 µM) (f) *in vitro* raised shoots under greenhouse condition.

Table 1: Effects of BA, or Kn, or BA + IAA on multiple shoot induction from leaf expalnts of *S. indicus* L.

Hormone (µM)	% of response	Morphogenic response	Mean number of shoots/explant ± S.E.	Mean length of shoot (cm ± S.E.)
BA				
2.2	30	S	7.0 ± 0.79a	1.5 ± 0.25a
4.4	50	S	7.0 ± 0.82a	2.5 ± 0.39b
6.6	30	C+S	4.0 ± 0.44b	2.2 ± 0.34b
8.8	45	C	-	-
Kn				
1.3	40	S	6.0 ± 0.70c	2.2 ± 0.35b
2.3	45	S	6.2 ± 0.97c	2.6 ± 0.37b
4.6	35	C+S	3.0 ± 0.35d	1.5 ± 0.34a
6.9	20	C	-	-
BA + IAA				
4.4 + 0.57	70	S	8.0 ± 0.93e	2.2 ± 0.54b
4.4 + 1.71	85	S	12.0 ± 1.15f	3.0 ± 0.73c
4.4 + 2.85	60	S	9.0 ± 1.05g	2.8 ± 0.31b

S-shoot; C-callus
Values are mean of 40 explant ± S.E
In each column mean followed by same letter were not significantly different ($p \leq 0.05$) according to DMRT

BA (4.4 µM) in combination with IAA (1.71 µM) showed optimal regeneration response with maximum number of shoots per leaf explant (12 ± 1.15) as compared to other BA plus IAA combinations that were tested (Fig: d, Table). Eighty five per cent of cultures responded to this particular BA and IAA combination. Addition of lower concentration of IAA (0.57 µM) to the medium supplemented with BA (4.4 µM) also resulted in multiple shoot induction from leaf cultures with 8.0 ±0.93 shoots per explant. The average shoot length at this concentration was 2.2±0.54. Further, a mean number of 9.1±1.05 shoots per explant was obtained with higher IAA (2.85 µM) concentration in combination with BA (4.4 µM) BA. The per cent of responding cultures

were 70% and 60% with IAA at concentrations of 0.57 µM and 2.85 µM respectively in combination with BA (4.4 µM) (Table). All combinations of BA plus IAA resulted in direct leaf regeneration without callusing phase.

Individual microshoots developed healthy roots within 10-12 days of culture on MS medium supplemented with IBA (2.46 µM) (Fig: e). All regenerated shoots showed 80% rooting efficiency on IBA medium. Rooted plantlets were hardened in sand : soil (1:1) followed by transfer to greenhouse for further growth. New leaves emerged in a week days and healthy plants were obtained within one month from the transfer to the greenhouse (Fig: e). The survival rate under greenhouse condition was 70%.

4. Discussion

The study described here was under taken to examine tissue culture response of mature leaf culture for *in vitro* shoot multiplication with the aim to establish an efficient and reproducible regeneration protocol for *S. indicus*. There are no reports available demonstrating *in vitro* propagation and regeneration methodologies for this plant species.

A high number of multiple shoots were produced from leaf explants in the presence of cytokinins, BA or Kn. Initially, we varied the concentration of BA in the range from 2.2 to 8.8 µM to evaluate the per cent explants forming shoots. The regenerating cultures forming shoots increased with increase in BA concentration from 2.2 to 4.4 µM. The number of shoots produced per explant were maximum at 4.4 µM BA. On the other hand, Kn at a concentration of 2.3 µM was found to be efficient for multiple shoot induction from leaf explants of *S. indicus*. It has been implicated that the regeneration potential of an explant is influenced by the type and/or concentration of cytokinins, BA or Kn in the medium, and that the number of shoots per explant could be increased by manipulating the balance of PGRs in the medium (17).

In the next set of experiments, effect of BA in combination with IAA on *in vitro* organogenesis in *S. indicus* was studied. Addition of IAA to medium containing BA (4.4 µM) resulted in an increase in mean number as well as mean length of the shoot. It implies that cytokinin in combination with auxin plays vital role in organogenesis from leaf explants of *S. indicus*. In at least three species (mulberry, tomato cv MicroTom and *Scoparia dulcis*) under different treatments we have established corelation between cytokinins and auxins concentrations leading to an efficient *in vitro* organogenesis (18,19,20). This suggests that a balance between cytokinin and auxin concentration can govern *in vitro* developmental pattern for a wide range of species. In the study, it was found that the percent response of explants forming shoot bud initials increased with increase in concentrations of IAA in medium with BA (4.4 µM), the optimum being 1.71 µM IAA. The number of shoots formed per explant was also maximum at these concentrations of BA plus IAA. An approximate two fold increase in shoot number following the inclusion of IAA (1.71 µM) in to BA supplemented media strongly suggests an interactive effect between the cytokinin and auxin on shoot proliferation in *S. indicus*. The percent of responding cultures decreased at higher level of IAA (2.85 µM) in combination with BA. This might be due to high endogenous auxin levels which lead to a decrease in per cent of explants forming shoots. Thus it is concluded that IAA (1.71 µM) when combined with BA (4.4 µM) was overall the most effective auxin-cytokinin combination in terms of multiple shoot induction from leaf explants of *S. indicus*. These results are in accordance with *in vitro* organogenesis in other Asteraceae members (21,22).

In conclusion, this is the first report in *S. indicus* with protocol for direct organogenesis from mature leaf explants. A rapid multiplication rate could be obtained from leaf explants by combining the hormones, BA and IAA. This protocol has great potential for rapid multiplication and conservation of *S. indicus*.

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