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Morphogenetic induction and organogenic differentiation from foliar explants of *Strobilanthes flaccidifolious* Nees: a natural dye yielding plant

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Abstract

Strobilanthes flaccidifolious is a shrub and its leaf is the source of blue color natural dye used for dying fibers and play important role in handloom industry. During the present investigation, an efficient *in vitro* regeneration protocol is developed from foliar segments of *in vitro* sourced plant materials. The first sign of response from the cultured foliar segments was recorded as swelling and curling of leaf segments within 7 days of culture followed by callusing and shoot buds formation within 3-4 wk. Amongst the different plant growth regulators tested, optimum response was recorded on MS medium enriched with 3% sucrose, 6 µM BA while other growth regulators and their concentrations did not support satisfactory morphogenetic response. Under optimum condition ~75% explants responded positively where as many as 9 shoots buds developed per leaf segment. The shoot buds converted into plantlets/micro shoots on MS medium when supplemented with 3 µM Kn where as many as 7.3 shoot buds developed per subculture of 3-4 wk duration. The micro shoots induced roots on MS medium enriched with 3 µM NAA where as many as 14 roots developed per plant within 4 wk times. The well rooted plantlets were transferred in potting mix and maintained in the poly-shed (50% light) for two months before transferring to the field.

Keywords: Dye yielding plant, Leaf explants, Organogenesis, Morphogenesis, Plant regeneration, *Strobilanthes flaccidifolious*

INTRODUCTION

Clonal propagation of high-value forest trees through organogenesis has the potential to rapidly capture the benefits of breeding or genetic engineering programme and to improve the quality and uniformity of stocks [1-4]. In forest plant species seed propagation does not assure genetic stability and a particular characteristic can be lost. Assays of vegetative propagation can be made by conventional vegetative propagation methods such as rooting of cuttings and by micropropagation methods [5,6]. However, the frequencies of rooting are quite low especially when mature cuttings are used [6-8]. Various *in vitro* techniques have been employed for *in vitro* propagation of forest plants and economically important plants such as somatic embryogenesis [1,9-12], organogenesis [13], axillary shoot proliferation [14-16].

Strobilanthes flaccidifolius Nees. (Acanthaceae) is a very important natural dye yielding plant and has very important role in handloom industry. The leaves are pounded and boiled with water, which produces a light to deep blue color dye and used for dying cloths and fiber by the handloom industry. The population of the species is down sized because of over collection of young twigs and leaves for dying fibers. The seed propagation of this species is very limited as seed germination rate is very poor. Besides seed propagation the species may also be propagated through stem cutting. Conventionally propagated population/progenies exhibits variability due to cross pollination [2,17]. Thus, the expanding plantation of economically important plants requires clonal propagation of *elite* clones through *in vitro* propagation. Hence, it is important to develop suitable tools for improving the genetic quality and uniformity of the planting stock. Furthermore, *in vitro* culture techniques are required to develop genetic transformation systems, and to establish cell suspension for production of secondary metabolites.

The present work is a report on the regeneration of *S. flaccidifolious* from leaf explants based on organogenic differentiation, together with a novel protocol for shoot regeneration from leaf explants. The morphogenic process on the basis of organogenic nodule differentiation has already been described in *Solanum nigrum* [18], *Sphaeranthus indicus* [19], *Stevia rebaudiana* [20], *Humulus lupulus* [21], *Charybdis numidica* [22], *Pinus radiate* [23], *Populus euphratica* [24], *Coelogyne suaveolens* and *Taenia latifolia* [25]. The present communiqué describes the morphogenetic potential and organogenesis of leaf explants of *Strobilanthes flaccidifolious* under *in vitro* condition.

MATERIALS AND METHODS

Plant materials, initiation of culture and culture conditions: Leaves of (~5.0 cm long) of *S. flaccidifolious* were isolated from *in vitro* stock plants (donor plants). The donor plants were raised from nodal segment culture on MS medium [26] containing sucrose (2%, w/v) and α-naphthalene acetic acid (NAA) (3 µM) and benzyl adenine (BA) (3 µM) in combination (Deb and Arenmongla, data under publication). The isolated leaves were soaked in sterilized distilled water till used to wash off the dye released from the petiole. The leaf explants were cut into segments horizontally. Each leaf was

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transversely cut in to ~1.0 cm pieces. Besides leaf segments, whole leaf was also cultured for comparative study. The leaf segments were cultured on MS medium containing sucrose (0-4%, w/v), polyvinyl pryolidone (200 mgl^{-1}) and supplemented with different concentrations of different plant growth regulators (PGRs) NAA, BA and kinetin (Kn) (0-9 µM) either singly or in combination. Agar (0.8%, w/v) (Hi-media, India) was used as gelling agent and *p*H of the medium was adjusted to 5.6 with NaOH and HCl (0.1 N). About 15 ml medium was dispensed in each borosilicate test tube (size: 15x150 mm), plugged with non-absorbent cotton and wrapped with muslin cloth before autoclaving at 1.05 Kg cm^{-2} pressure and 121°C for 20 min. Leaf explants/segments presenting callus and organogenic nodules were maintained for two more passages on the respective initiation medium for further proliferation and differentiation.

Shoot regeneration, micropropagation and rooting of micro shoots: The resulting organogenic loci and micro shoots were transferred on MS medium enriched with sucrose (3%, w/v) and different PGRs such as BA and Kn (0-9 µM) either singly or in combination for plant regeneration and culture proliferation. The micro shoots were separated at every subculture and transferred on fresh regeneration medium. The cultures were maintained for 2-3 passages for culture proliferation. In one set of culture activated charcoal (AC) (0-0.4%, w/v) was added in the optimum regeneration medium to study the effect of AC on plant regeneration, plant morphology and culture proliferation. About 5 cm long micro shoots with well expanded leaves from the regeneration medium were selected for inducing rooting. The micro shoots were maintained on MS medium containing sucrose (2%) and NAA (0-8 µM) under normal laboratory condition for 4-6 wk for inducing rooting. Another set of micro shoots were pulse treated with NAA (0-20 µM) for 3 hr followed by culturing on MS medium containing sucrose (3%) and maintained for 6-8 wk.

Acclimatization of regenerates and transplantation: The well rooted plantlets from rooting condition were transferred on ½MS medium containing sucrose (2%) without any PGRs and maintained in normal laboratory condition for ~6 wk for acclimatization. The hardened plantlets were taken out from above condition and traces of agar was washed off with luke warm water before transferring onto plastic pots containing a mixture of soil, sand, decayed wood powder at 1:1 ratio with a moss topping. The pots were initially covered with transparent plastics for two wk and maintained in the poly house (~50% light). The plants were fed with $1/10^{th}$ MS salt solution once in a week for ~3-4 wk. The potted plants were maintained in the poly house for two months or till sprouting of new leaves before transferring them in the natural habitat.

Experimental design: A completely randomized experimental design was performed. In all experiments, each treatment had at least five replicates and there were 20 explants per replicate. The cultures were maintained at 25±2°C under cool white fluorescent light at 40 µmol m^{-2} s^{-1} and 12/12 h (light/dark) photoperiod. All the cultures were sub-cultured at 4-5 wk interval unless mentioned otherwise. Morphogenetic efficiency of leaf segments was evaluated based on the callus mediated regeneration and number of buds formed directly per segment 8 wk of culture and data was expressed as the mean of replicates ± standard error.

RESULTS

Organogenic response

Soaking of leaf explants in water after excising from the *in vitro* raised cultures improved the morphogenetic response over non-soaked explants. It was observed that pre-soaked leaf explants leached dye in the water and promoted healthy culture initiation. While, the leaf explants which were not soaked released dye and phenolic compounds in the medium, became necrotic and degenerated subsequently.

Under the condition employed, the primed leaf explants were free from dye and phenolic exudates; the meristemetic loci were invoked followed by shoot bud formation. Swelling of leaf segments was observed within 7 days of culture from the cut ends followed by differentiated into shoot buds or formed callus within 3-4 wk (Table 1, Fig. 1 a, b). The highest percentage of cultivated leaf segments forming protuberances/shoot buds was 75% and highest mean number of protuberance regenerating buds per segment was 9 (Table 1). Incorporation of sucrose in the medium was pre-requisite for induction of morphogenetic response. On medium free of sucrose, leaf explants failed to respond and degenerated subsequently. Of the different concentration of sucrose tested, better morphogenetic response was achieved on medium fortified with 3% (w/v) sucrose (data not presented). Medium containing higher concentration of sucrose, explants turned brown while, at lower concentration fewer shoot buds formed. For morphogenetic induction, three different PGRs were incorporated at different concentrations. All the concentrations of NAA either singly or in combination with BA and Kn supported callus induction while both BA and Kn supported either shoot bud formation or callus and shoot bud formation (Table 1). At lower concentration of BA supported callus formation while at higher concentration of BA shoot bud induction was recorded. At a concentration of 6 µM BA, ~75% of explants responded positively where as many as 9 shoot buds/micro shoot formed (Table 1, Fig. 1 b) accompanied by callus formation. For morphogenetic response, leaf segments as well as whole leaf was tested. In the present study morphogenetic response was initiated from the cut ends of leaf segments while response was comparatively poorer in whole leaf.

Table 1: Effect of quality and quantity of PGRs on inducing morphogenetic response in foliar explants of *Strobilanthes flaccidifolious*.

PGRs Conc. (µM)* NAA	BA	KN	Time for initial response (days)	% response (±SE)**	Morphogenetic pathway***	No. of shoot buds formed /explants
0	0	0	-	-	-	-
3	-	-	15	25±0.50^g	Ca-Rt	1
6	-	-	7	66±1.00^c	Rt	1
9	-	-	7	66±0.75^c	Rt	1
-	3	-	7	70±1.50^e	Ca	3
-	6	-	10	75±0.75^a	Ca-Sb	9
-	9	-	7	70±2.50^b	Ca-Sb-Rt	6
-	-	3	15	60±2.00^d	Sb	4
-	-	6	20	50±1.50^f	Sb	3
-	-	9	20	50±1.75^f	-	-
3	3	-	12	25±1.00^g	Ca	2
3	6	-	10	75±1.50^a	Ca-Rt	2
3	9	-	8	65±2.50^c	Ca	-
6	3	-	10	60±3.00^d	Ca	-
6	6	-	7	60±2.50^d	Ca	-
6	9	-	10	50±1.00^f	Ca	1
9	6	-	10	63±2.50^d	Ca	-
9	9	-	20	48±1.00^f	Ca-Rt	1
3	-	3	25	45±2.00^f	Ca	2
3	-	6	30	55±1.50^e	Ca	2
6	-	6	30	20±1.00^g	Ca	1

* In MS medium containing sucrose (3%, w/v), ** Standard error, *** Ca: Callus, Sb: Shoot buds, Rt: Roots.
Only the significant treatments are computed.
Data represents the mean of five replicates, Data scored after 5 wk of culture initiation.
In the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

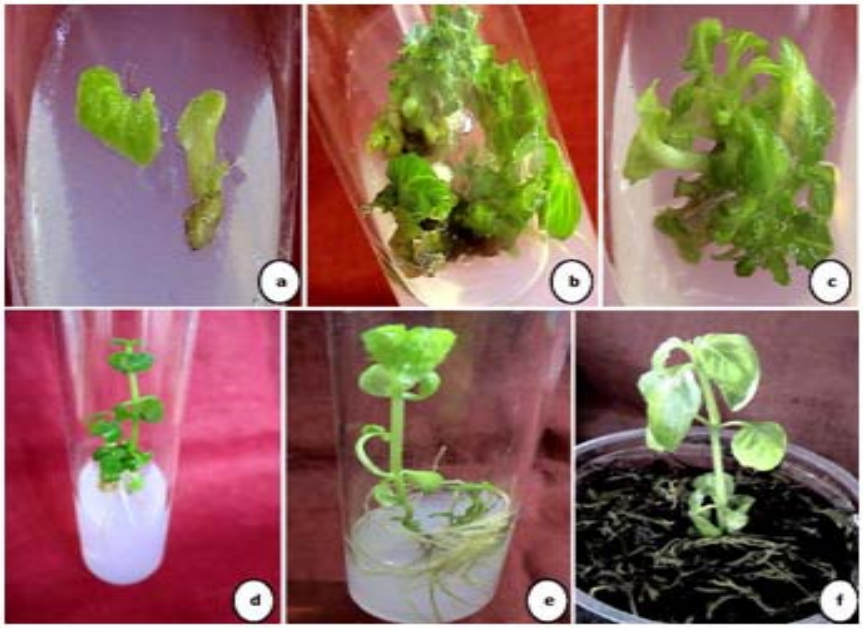

Fig. 1: Different stages morphogenetic response and plant regeneration from foliar segments of *Strobilanthes flaccidifolious*. a. Cultured leaf segments showing swelling from the cut ends, b. Micro shoots/shoot buds developed from the leaf segments, c. Multiple shoots formation on regeneration medium, d. Regenerated micro shoot in rooting medium, e. A well rooted plant under hardening condition, f. A potted plant.

Plant regeneration and *in vitro* multiplication

Leaf segments presenting organogenic loci and micro shoots/buds were maintained for two more passages on the respective medium for further proliferation and differentiation. The micro shoots were than maintained on MS medium containing 3% sucrose and different PGRs. For regeneration of plantlets, incorporation of one of the PGRs was obligatory. In medium freed of any PGRs, cultures exhibited stunted growth and degenerated. Presence of BA across the concentrations either singly or in combination did not promote healthy plantlet regeneration or culture proliferation and plantlets exhibited stunted growth. Singly treatment of BA (3 µM) supported mean 4.2 numbers of shoot bud formation after 7 wk of culture where mean height of plantlets was 3.2 cm. Alternatively when used in combination with Kn (3 µM each) formed 5.2 shoot buds. But incorporation of Kn singly proved to be optimum under the given conditions for shoot proliferation and plant growth. A

mean of 7.3 shoot/micro shoots of ~4.5 cm height were formed on medium 3 µM Kn (Table 2, Fig. 1C).

In one set of regeneration medium with optimum PGRs, AC was incorporated to study the effect on plant regeneration pant plant morphology. It was observed that incorporation of AC had very or no effect on culture proliferation and plant growth. It was observed that on AC rich medium plantlets developed roots which is absent in AC control medium. As many as 8 roots per plant formed on medium containing 0.1% (w/v) AC. While at higher concentrations of AC culture proliferation as well as culture growth inhibited (Fig. 2).

Table 2: Effects of different cytokinins on plantlets regeneration and culture proliferation of *Strobilanthes flaccidifolious*.

PGRs Conc. (µM)		No. of shoot buds formed/explants	Mean plantlet height (cm.)	Type of response*
BA	Kn			
0	0	0	0	Growth stunted and degenerated
3	0	4.2^{b}	3.2±0.1^{c}	Plantlets with dark green small leaves but plantlets etiolated
6	0	3.1^{c}	4.0±0.1^{b}	Plantlets slightly light green and not healthy
9	0	2.2^{e}	3.6±0.2^{b}	Small leaves and slight callusing at the base
0	***3***	***7.3***^{a}	***4.5***±0.2^{a}	***Healthy plantlets with dark green and broad leaves, plantlets with few roots***
0	6	3.2^{c}	3.0±0.3^{c}	Poor plant growth, yellowish-green leaves
0	9	2.1^{d}	2.9±0.2^{c}	Stunted plant growth
3	3	5.2^{b}	4.3±0.1^{a}	Dark green leaves, stunted growth with fewer roots
3	6	3.3^{c}	3.5±0.3^{b}	As above
3	9	3.2^{c}	3.6±0.2^{b}	As above
6	3	3.4^{c}	3.0±0.2^{c}	Stunted plant growth and leaves light green
6	6	2.3^{d}	2.7±0.2^{d}	As above
6	9	3.2^{c}	3.2±0.5^{c}	Many small leaves crowded at top, leaves light green
9	3	2.5^{d}	2.7±0.1^{d}	Broad green leaves, healthy plantlets
9	6	3.4^{c}	2.5±0.2^{d}	As above
9	9	2.3^{e}	2.9±0.2^{c}	Leaves light green and plantlets unhealthy

* On MS medium containing sucrose (3%, w/v).
Data represents the mean of five replicates.
Data scored after 7 wk of culture initiation.
In the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

Table 3: NAA stimulated *in vitro* rooting of micro shoots of *Strobilanthes flaccidifolious*

NAA Conc. (µM)	No. of roots invoked/shoot	No. of secondary shoot formed/shoot	Type of response*
0	3^{d}	-	Roots were very small and degenerated
1	5^{c}	2	Plantlets etiolated, roots short
2	8^{b}	3	Slightly etiolated plantlet, shoots branched but short
3	14^{a}	3	Healthy plants with profuse rooting with distinct root hairs
4	9^{b}	2	Healthy roots but poor root hairs
5	6^{c}	2	Swelling at the basal part of plants as well as roots
6	6^{c}	-	As above
7	5^{c}	-	Roots swelled and callusing at the base of the shoot
8	4^{c}	-	As above

* On MS medium containing sucrose (2%, w/v)
Data represents the mean of five replicates
Data scored after 4 wk of culture on the above medium.
In the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

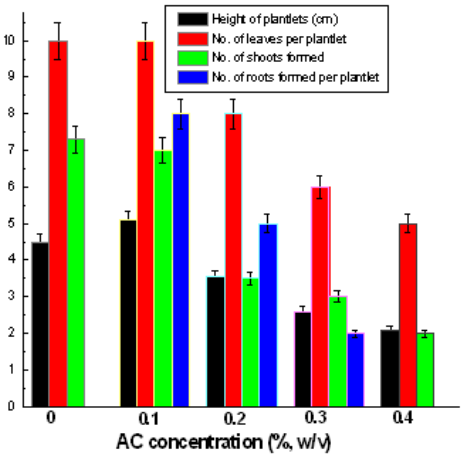

Fig. 2: Effect of activated charcoal on plant regeneration and culture proliferation of *Strobilanthes flaccidifolious*.

Rooting, hardening and transplantation to potting mix

On regeneration medium though there were some roots formation in some regenerated shoots, but roots were not fully developed. Regenerated shoots (~5 cm in length) from regeneration medium induced roots when transferred on rooting medium (Fig. 1d). The shoots were treated differentially for inducing roots. In general, pulse treatment with NAA was found to be inferior over incorporation of NAA in the medium. Of the different concentration of NAA used for inducing roots, a concentration of 3 µM supported maximum root growth where as many as 14 roots per plant developed after 4 wk of culture (Table 3, Fig. 1e). At lower concentrations roots were shorter and plantlets were etiolated while, at higher concentrations, roots formation was impaired accompanied by swelling of plants as well as roots.

In comparison to above, the pulse treatment of micro shoots with NAA, roots formation as well as shoot growth was poor. Under optimum condition only 8 roots were formed after ~4 wk of culture against 14 roots when NAA incorporated in the medium. Amongst the different pulse treatments given for inducing roots, pulse treatment with NAA concentrations of 10 and 15 µM were equally effective for inducing roots and vertical increase in plant height (data not presented).

The rooted plants were hardened on medium with ½MS salt solution containing sucrose (2%) and maintained for 6-7 wk under normal laboratory condition. The hardened plants were transferred to plastic pots as mentioned in the materials and methods (Fig. 1f). The plants were successfully transferred to a shade-house (50% shade) and then to field. About 65% survival was registered after two months of transfer.

DISCUSSION

In the present study, priming of explants by soaking the explants in water after excising from the *in vitro* raised cultures improved the morphogenetic response over non-soaked explants. During this treatment the leaf explants leached dye and other phenol compounds in water and promoted healthy culture initiation. While, the leaf explants which were not soaked released dye and phenolic compounds in the medium, became necrotic and degenerated subsequently. Earlier Lakshmanan *et al* [3] in sugar cane leaf culture observed that soaking of leaf explants after excision promoted morphogenetic response *in vitro*.

The primed leaf explants were free from dye and phenolic exudates before culture initiation. Within 7 days of culture initiation the explants started swelling and marked as the first sign of response and within 3-4 wk of culture differentiated into shoot buds and under optimum condition ~75% explants responded positively. Incorporation of sucrose in the medium was pre-requisite for induction of morphogenetic response. On medium free of sucrose, leaf explants failed to respond and degenerated subsequently. Of the different concentration of sucrose tested, better morphogenetic response was achieved on medium fortified with 3% (w/v) sucrose (data not presented). Medium containing higher concentration of sucrose, explants turned brown while, at lower concentration fewer shoot buds formed. Earlier the effect of organic carbon source on *in vitro* morphogenetic response was described in *Stevia rebaudiana* [27], in *Pogostemon cablin* [28].

In the present study for morphogenetic induction different PGRs were tested. NAA across the concentrations either singly or in combination with auxins supported callusing of explants while, BA at lower concentration supported callus formation and at higher concentration shoot bud induction. For morphogenetic response, leaf segments as well as whole leaf was tested. In the present study morphogenetic response was initiated from the cut ends of leaf segments while response was comparatively poorer in whole leaf. Many stimuli are communicated across the plant body by PGRs which consequently play an important role in diverse aspects of plant growth and development [29]. At a cellular level, auxin affects division, expansion and differentiation. Cytokinins are necessary in concerts with auxin in many cases for cell division at G1-S and G2-M transitions in a variety of cultured plant cells as well as in plants. Progression through the cell cycle is central to cell proliferation and fundamental to growth and development of higher plants [29,30].

In the present study our results show that uptake of BA by *Strobilanthes flaccidifolious* leaves cultured on MS medium promoted partial callus followed by shoot bud formation. It is important to highlight that mother plants which provided the leaves for the present study were cultured on MS medium containing BA and NAA. Thus, in the absence of NAA in the initiation medium, BA singly could promote shoot bud formation and this might be related to absorption of NAA from medium and accumulation in the mother plants. As a result when NAA used either singly or in combination with BA or Kn promoted callusing. Earlier Lakshmanan *et al* [31] noted that the cytokinin (BA) treatment is required during the induction period of six days for fully competent cells to enter into a caulogenically determined state in leaf explants of *Gracinia mangostana*.

Incorporation of one of the cytokinin in the medium to convert shoot buds into plantlets was obligatory. In medium freed of any PGRs, cultures exhibited stunted growth and degenerated. In

general BA in its all the concentrations tested in the present study did not promote healthy plantlet regeneration or culture proliferation and plantlets exhibited stunted growth. But when used in combination with Kn (3 µM each) formed 5.2 shoot buds. But incorporation of Kn singly proved to be optimum under the given conditions for shoot proliferation and plant growth. The promontory effect of cytokinin on culture proliferation is reported by many other workers [32], [33] while BA, Kn and NAA in combination was found to be superior over other treatment in *Acacia confuse* [4].

For rooting of micro shoots, two different techniques were followed. NAA was incorporated in differential concentrations directly in the medium and micros hoots were pulse treated with NAA followed by maintaining them on MS plain medium. Of the different concentration of NAA used for inducing roots, a concentration of 3 µM supported maximum root growth where as many as 14 roots per plant developed after 4 wk of culture. In comparison to above, the pulse treatment of micro shoots with NAA, roots formation as well as shoot growth was poor. Auxins have been shown to act as a local morphogenetic trigger on the formation of lateral roots in Arabidopsis, leading to the specification of founder cells of the new organ from previous differentiated cells [34]. The promotory effect of NAA on rooting is also described in rice [35], in *Populus euphratica* [24]. It was reported that NAA was better option followed by IAA and IBA. While in some other reports IBA was found to be superior over other PGRs for rooting [4, 33].

CONCLUSION

In the present study, an effective *in vitro* regeneration protocol from foliar segments for rapid propagation of *S. flaccidifolious* is developed. The procedure reported in this paper suggests that multiplication from foliar explants culture could be commercially feasible methods for *S. flaccidifolious*. Micropropagation is more rapid, continuous and efficient than propagation via conventional cutting because it can supply uniform and consistent plant materials for investigations of important secondary metabolites (dye) produced by this species.

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