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In vitro regeneration, flowering and seed formation from leaf explants of *Celsia coromandeliana* (Scrophulariaceae)

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Abstract
This study describes an efficient protocol for the induction of *in vitro* flowering and fruiting of *Celsia coromandeliana* family Scrophulariaceac), a multipurpose folk medicinal plant. Murashige and Skoog medium supplemented with kinetin (KIN; 13.93 µm) and indole-3-acetic acid (IAA; 1.14 µm) is optimal for the formation of multiple shoots (20.00±1.68), which induced loral buds (13±0.45). The regenerated plantlets developed lowers and fruits within 40-45 and 50-55 days, respectively. *In vitro* floral development was asynchronous. The morphology of the *in vitro* developed floral organs was almost similar to that of the parent plant The flowers produced *in vitro* developed ripe fruits with viable seeds. Our finding has important implications in understanding the influence of physiological factors and the molecular basis of floral organ formation in this valuable medicinal plant.

Keywords: Floral organ development, IAA, *in vitro* fruiting, kinetin, multiple shoots, ornamental potential

1. Introduction
The production of herbal medicines is one of the most important plant species remarkable contributions of plant biodiversity. This plant is used in traditional medicine. The widely accepted medicinal properties have featured this plant as a subject of intense research (Riel *et al.* 2002; Ahsan *et al.* 2003; Latha *et al.* 2006; Babincova *et al.* 2008; Latha *et al.* 2009; Mahendar *et al.* 2009) [1-6].
Previous studies with this plant revealed the presence of numerous phytochemicals such as coumarins, phenols, saponins, tannins, amino acids and flavanoids (Latha *et al.* 2006) [3], various terpenoids like scoparic acid A (Riel *et al.* 2002) [1], B and D (Latha *et al.* 2009) [5], scopadulciol and Scopa- dulin, useful for their therapeutic properties (Hayashi *et al.* 1990) [7], including antihyperalgesic, analgesic, anti-HIV (Mahendar *et al.* 2009) [6], neurological disorders, antimalarial (Riel *et al.* 2002) [1], and antiulcer (Babincova *et al.* 2008) [4], as well as cytotoxicity activity against cancer cells (Ahsan *et al.* 2003) [2]. Natural *Celsia coromandeliana* populations are dwindling at an alarming rate due to its overexploitation for medicinal use. The natural means of propagation of this plant suffers from low seed germination ability. The conservation of this plant can be achieved by *in vitro* clonal propagation which can also fulfill its increasing demand in the pharmaceutical sector.
In vitro flowering has been reported for many plant species such coriander (Stephen and Jayabalan, 1998) [8], tomato cultivar Microtom (Rao *et al.* 2005) [9]. However, reports on *in vitro* fruiting and seed set are limited Stephen and Jayabalan (1998) [8] and Rao *et al.* (2005) [9]. Induction of flowering under controlled *in vitro* conditions offers a unique possibility to study the hormonal regulation of floral organ development in this medicinally important plant species.
Our preveious finding describes an efficient protocol for *in vitro* propagation of *S. dulcis* (Mahender *et al.* 2008) [10]. No reports are available on the induction of *in vitro* flowering and fruiting for this plant species. In this paper, we present a suitable protocol for the rapid induction of invitro flowering and seed formation in S. dulcis. This protocol (from establishment of leaf cultures to fruit ripening) can be accomplished in a short period of 50-55 days under *in vitro* conditions. The present study has also led to the development of a regeneration protocol from mature leaf explants of *S. dulcis* which complements very well our earlier studies on clonal propagation of this valuable medicinal plant (Mahender *et al.* 2008) [10].

Materials and Methods

All chemicals and reagents used in the experiments were of laboratory reagent grade purchased from Himedia, Mumbai, India.

Plant material

The plant material was collected from a medicinal arboretum maintained at the Jayantikunj, Rewa (M.P.)

Surface sterilization

Leaves excised from field-grown mature plants were washed thoroughly under running tap water and placed in 5% Tween-20 for 5 min followed by 3-4 washes in sterile distilled water. The explants were surface sterilized with an aqueous solution of 0.1% $HgCl_2$ for 4-5 min and then washed 4-5 times with sterile distilled water.

Regeneration medium and sub culturing

Sterilized leaves were cut into explants 1.0 cm^2 each and cultured individually on Murashinge and Skoog (MS; Murashige and Skoog 1962) [11] regeneration medium supplemented with lkinetin (KIN, 4.64, 9.29, 13.93 and 18.58 µM) in combination with either indole-3-acetic acid (IAA, 1.14 and 2.85 µM) or α-naphthalene acetic acid (NAA, 1.07 and 2.68 µM). MS medium without plant growth regulators (PGRs) served as the control. MS media with 2% (w/v) sucrose and 0.8% (w/v) agar were used for regeneration and rooting studies. The pH of all media was adjusted to 5.8 with 0.1N NaOH before autoclaving at 121 °C for 15 min. Each culture tube (150 x 25 mm) containing 20 ml of media was inoculated with a single explant and plugged with non-absorbent cotton wrapped in two layers of cheese cloth.

After 35 days of culture on regeneration medium, all regenerating leaf cultures with or without flower buds were transferred onto fresh MS basal medium (without PGRs) for further proliferation and growth. Following a 2-week culture on fresh MS basal medium, the elongated micro shoots (4-5 cm) were excised and rooted onto MS medium supplemented with auxin, indole-3-butyric acid (IBA, 4.92 µM), or without IBA, which served as the control.

Culture conditions and analysis

All cultures were incubated under a 16-h photoperiod under cool white fluorescent lights (Phillips, India) providing a quantum flux density of 65 µE $m^{-2}s^{-1}$ at 25±2 °C. In order to estimate pollen viability, the petals were removed, and the anthers were squashed in distilled water and centrifuged at 5000 rpm for 2 min. After centrifugation, pollen grains were pelleted, collected, mixed in iodine stain solution, I_2-KI (2.5g KI, 250mg I_2 and 125ml H_2O) and allowed to incubate for 10-15 min (Pedersen *et al.* 2004). The stained suspension was placed on a cavity microscopic slide and examined at 40X magnification using an inverted phase contrast microscope (Nikon CO., Tokyo, Japan). The pollen grains that stained dark-red were considered to be fertile while those that stained light-red or remained unstained were treated as sterile (Pedersen *et al.* 2004). Pollen fertility was calculated in terms of percent viability using the following formula:

$$\text{Pollen viability (\%)} = \frac{\text{Number of pollen stained}}{\text{Total number of pollen counted}} \times 100$$

In order to study the internal morphology of the *in vitro* developed fruit and the process of seed formation, histological cross sections of *in vitro* derived fruits were made using a razor blade. Sections were incubated for 5 min in 2% acetocarmine (w/v). The stained images were photographed with Nikon camera (Nikon Co., Tokyo, Japan) mounted on a Nikon inverted phase contrast microscope. The data pertaining to explants exhibiting multiple shoot formation and the number of floral buds per explant, flowers per plantlet, and fruits per plantlet were analyzed on days 35, 45 and 55, respectively from the beginning of leaf culture.

The data recorded was initially used to calculate standard deviation and by standard error using Microsoft Excel. The data thus obtained was analyzed statistically using analysis of variance (ANOVA). The p values (p<0.05) obtained from the 'r' table were used to categorize significant differences between means. For each concentration, 10 explants were used with 2 replicates each; the experiment was repeated twice.

Results and Discussion

Multiple shoot formation and floral bud initiation

The efficiency of multiple shoot formation with or without the initiation of *In vitro* flowering differed with the concentrations of PGRs used in this study. The culture of leaf explants on MS medium with different combinations of KIN (4.64, 9.29, 13.93 and 18.58µM) with IAA (1.14 and 2.85µM) or NAA (1.07 and 2.68µM) induced direct or indirect (callus-mediated) plant regeneration from these explants, respectively. Leaf explants that responded on different regeneration media developed shoot buds within 20-25 days of culture. The explants cultured on KIN (4.64, 9.29, 13.93 and 18.58 µM) with IAA (1.14 and 2.85µM) induced more multiple shoots ranging from 9.00±1.35 to 20.00±1.68 shoots/explant. A combination of KIN (13.93µM) and IAA (1.14µM) induced more multiple shoots (20.00±1.68 µM) that also resulted in the development of maximum number of floral buds (13±0.45) (Table 1).

Table 1: Effect of Kn in combination with either IAA or NAA on the plant regeneration ability. *In vitro* floral bud induction, flowering and fruiting from leaf explants of *Celsia coromandeliana*

Hormone conc. (µM)	Shoot regeneration response (%)	No. of shoots/explant (±S.E.)	Flowering response	No. of floral buds/explant (±S.E.)	No. of flowers/plantlet (±S.E.)	No. of fruits/ plantlet (±S.E.)
Kn + IAA						
4.64+1.14	70	10.00±1.47 g a	NF	-	-	-
9.29+1.14	85	14.00±1.72 h b	F	8±0.36 c a	7±0.42 f a	4±0.44 f a
13.93+1.14	90	20.00±1.68 i c	F	13±0.45 g b	12±0.56 g b	10±0.45 g b
18.58+1.14	80	15.00±1.86 i b	F	9±0.68 f c	6±0.66 c c	4±0.32 f a
4.46+2.85	65	9.00±1.35 e a	NF	-	-	-
9.29+2.28	70	13.00±1.91 b d	F	6±0.63 d d	4±0.30 a d	2±0.22 a c
13.93+2.28	85	17.00±1.58 i e	F	10±0.52 f c	7±0.29 f a	4±0.24 c a
18.58+2.28	70	12.00±1.62 h d	F	8±0.58 c a	6±0.45 c c	4±0.32 c a
Kn + NAA						

4.46+1.07	55	6.00±0.96 d f	NF	-	-	-
9.29+1.07	65	8.00±1.19 e g	F	5±0.30 c d	5±0.26 c c	3±0.26 d d
13.93+1.07	75	10.00±1.35 g a	F	6±0.50 d d	6±0.36 d c	4±0.36 f a
18.58+1.07	80	9.00±1.08 f a	NF	-	-	-
4.46+2.68	55	5.00±1.80 a f	NF	-	-	-
9.29+2.68	60	6.00±1.14 c f	F	4±0.53 b c	5±0.26 c c	2±0.26 b c
13.93+2.68	60	6.00±1.20 c f	F	2±0.27 a f	4±0.36 b d	2±0.36 c c

The treatments of KIN (4.64 µM) with IAA (1.14 and 2.85µM) did not induce floral buds on the regenerated plants (Table 1). In the first set of experiments, MS medium containing KIN (13.93 µM), when combined with IAA (1.14µM), induced high shoot regeneration along with floral bud initiation followed by the combination of KIN (13.93 µM) with IAA (2.85 µM). Fewest multiple shoots without floral buds formed on MS medium with KIN (4.64 µM) and NAA (2.68 µM).

In another set of experiments, leaf explants were cultured on MS medium with KIN (4.64, 2.29, 13.93 and 18.58 µM) and NAA (1.07 and 2.68 µM). This PGR combination initiated the development of callus from cut ends of the explants followed by plant regeneration. Thus, an indirect mode (callus-mediated) of *in vitro* plant regeneration was observed in the presence of KIN and NAA. The formation of multiple shoots was observed at all concentrations of KIN and NAA tested (Table 1). However, in these experiments, medium containing KIN (13.93 µM) in combination with NAA (1.07 µM) efficiently regenerated plants (10.00±1.35) as well as floral buds (6.00±0.50). A combination of KIN (4.64 or 18.58 µM) with NAA (either 1.07 or 2.68 µM) failed to produce *in vitro* flowering. However, the presence of these PGR combinations lead to the development of multiple shoopts *in vitro*. In the presence of KIN (4.64-18.58 µM), an increase in the concenration of NAA (from 1.07 to 2.68 µM) resulted in an increase in the amount of callus from explants causing a decrease in the number of multiple shoots. The leaf culture did not regenerate in the control (MS medium devoid of PGRs) treatments.

In vitro flowering and fruiting

Floral bud induction occurred with 30-35 dyas of leaf culture on regeneration medium. These floral buds attained full bloom with 40-45 days of culture resulting in the development of normal (with all floral organs) bisexual flowers. The progression of *in vitro* flowering is asynchronous *i.e.* all flowering stages (From young floral buds to mature flowers) were observed on the regenerating cultures at any given time. The floral buds continued to bloom when explants were cultured on MS basal medium. Nevertheless, no new floral buds developed on MS basal medium. The flowers self-fertilized *in vitro* and mature fruits developed 50-55 d after the onset of primacy culture. The *in vitro* developed pollen grains were viable when staine with I_2-KI solution: viable pollen grains stained dark red and showed approximately 8-85% viability. Histological cross sections of the ripened fruits (Stained with 2% acetocarmine solution) showed normal seed development pattern. The seeds developed *in vitro* resumed normal growth and development under ex vitro conditions.

For root initiation, the elongated micro shoots (4-5 cm, 42-47 days-old) were transferred to MS medium containing 4.92 µM IBA. Well-developed roots (7-8 roots/micros hoot) were observed to emerge from the cut ends of the microe shoot with 10-12 days of culture.

This study aimed to develop an efficient protocol for direct leaf-based regeneration as well as for *in vitro* flowering and fruiting in *Celsia coromandeliana*. In all our experiments, the cytokinin KIN was used in combination with either IAA or NAA. Our previous *in vitro* studies have shown that the combination of BAP (22.2µM) and IAA (0.5µM) resulted in efficient induction of multiple shoots from mature leaf explants of *Celsia coromandeliana* although this PGR combination did not support *in vitro* flowering in *Celsia dulcis* (Mahender *et al*. 2008) [10]. The responses resulting in direct plant regeneration as well as the development of floral buds observed on medium supplemented with KIN and IAA or NAA were reported earlier (Thakur *et al.* 1998; Kintzios and Michaelakis, 1999) [12-13].

The protocol presented here demonstrates that an efficient plant regeneration can be obtained from leaf explants; thus, the protocol has important implications for the genetic transformation of this medicinal plant. Moreover, the entire life cycle of *Celsia coromandeliana* can be completed under controlled *in vitro* conditions within a short duration of 50-55 days unlike ex vitro conditions, which might need 5-6 months. The response to flowering is a complex process and its induction under *in vitro* conditions is rare. Under *in vitro* conditions, the supply and balance of the type of cytokinin and/or auxin can favor floral development and maturation (Rao *et al*. 2005) [9].

In conclusion, the protocol presented here is simple, efficient, and reproducible. Using this protocol, *in vitro* regeneration, flowering and fruiting can be achieved for the medicinal plant *Celsia coromandeliana,* This protocol facilitates the preparation of whole plant-based extracts for medicinal purposes, and also for the characterization of useful secondary metabolites. This protocol can be applied for understanding the physiological and chemical factors involved in the transition of vegetative to floral meristem in a medicinally important genus.

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References

1. Riel MA, Kyle DE, Milhous WK. Efficacy of scopadulcic acid a against *Plasmodium falciparum in vitro*. Journal of Natural Products. 2002;65(4):614-615.
2. Ahsan M, Islam SK, Grey AI, Stimson WH. Cytotoxic diterpenes from *Scoparia dulcis*, Journal of Natural Products. 2003;66(7):958-961.
3. Latha M, Ramkumar M, Puri K, Damodaran PN, Rajeshkannan V, Suresh T. Phytochemical and Antimicrobial study of an anti-diabetic plant: *Scoparia dulcis* L. Journal of Medicinal Food. 2006;9(3):391-394.
4. Babincova M, Schronerova K, Sourivong P. Antiulcer activity of water extract of *Scoparia dulcis*, Fitoterapia. 2008;79(7):587-588.
5. Latha M, Puri K, Ramkumar M, Rajaguru P, Suresh T, Dhanabal T, *et al*. Anti-diabetic effects of scoparic acid D isolated from *Scoparia dulcis* in rats with streptozotocin-induced diabetes. Natural Product Research. 2009;14(16):1-13.

6. Mahendar P, Mahendar A, Rao KV, Kranthi G, Pavan U, Rao AV, *et al*. *In vitro* HIV type I reserve transcriptase inhibitory activity of leaf extract of *Scoparia dulcis* L. Journal of Herbs, Spices and Medicinal plants. 2009;15(3):241-247.
7. Hayashi T, Kawasaki M, Miwa Y, Taga T, Morita N. Antiviral agents of plant origin III. Scopadulin, a novel tetracyclic diterpene from *Scoparia dulcis* L. Chemical and Pharmaceutical Bulletin. 1990;38(4):945-947.
8. Stephen R, Jayabalan N. *In vitro* flowering and seed setting formation of coriander (*Coriandrum sativum*). Current Science. 1998;74(3):195-197.
9. Rao KV, Kiranmayee K, Pavan U, Jayashree T, Rao AV, Sadanandam A. Induction of multiple shoot from leaf segments, *in vitro* flowering and fruiting of a dwarf tomato (*Lycopersicon esculentum*). Journal of Plant Physiology. 2005;162(8):959-962.
10. Mahendar A, Rao KV, Kota SR, Pavan U, Sadanandam A. Efficient *In vitro* regeneration from mature leaf explant of *Scoparia dulcis* L., an ethno medicinal plant. Journal of Herbs, Spices and Medicinal plants. 2008;14:200-207.
11. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiologia Plantarum. 1962;15:473-497.
12. Thakur R, Rao PS, Bapat VA. *In vitro* plant regeneration in *Melia azedarach* L. Plant Cell Reports. 1998;18:127-131.
13. Kintzios S, Michaelakis, A. Induction of somatic embryogenesis and *in vitro* flowering from inflorescences of chamomile (*Chamomilla recutita* L.). Plant Cell Reports. 1999;18:684-690.