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IN VITRO MICROPROPAGATION AND CALLUS INDUCTION IN *Scutellaria discolor* COLEBR. — A MEDICINALLY IMPORTANT PLANT OF NEPAL

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A medicinally important plant *Scutellaria discolor* (family Lamiaceae) was studied for *in vitro* rapid multiplication and callus induction. Cotyledons with shoot tip were found to be the most responsive explant in Murashige and Skoog medium based experiments. Maximum multiple shoot production was observed in the treatment containing 1mg/1 BAP and 0.1 mg/1 NAA. From one explant, 15-20 shoots can be obtained within a period of ten weeks. These shoots (six to eight leaves) produce roots in two to three weeks when placed in non-sterile sand. Plants were hardened by gradually exposing them to the external atmosphere and then transferred to earthen pots. In the next 12 to 15 weeks of time these plants flowered and seed set was observed. Three auxins (NAA, 2, 4-D and IAA) at various concentrations were used individually for callus induction and 2,4-D was found to be most suitable.

Key words : *Scutellaria*, tissue culture, micropropagation, medicinal, germplasm

Nepal is very rich in plant diversity and more than 7000 flowering plants are already recorded. About 700 plants are known to have some sort of medicinal property. *Scutellaria discolor* Colebr. is an annual herb of the family Lamiaceae, distributed in Nepal, India and China. Leaves of this plant in Nepal are used as a folk remedy for cold, cut and insect stings. The dried parts of this plant are also a crude drug in China and has been used as antipyretic, antidotic and anti-inflammatory agent for the treatment of cold, gastroenteritis, tympanitis and other diseases (Tomimori *et al.*, 1985). Extract of *S. rivularis* has shown anti-inflammatory and hepatoprotective activity in test animal (Ching and Den, 1996).

In *S. discolor* at the time of flowering, plant is up to 30 cm tall, often with small paired leaves. Flowers are blue, in long slender leafy spikes (Fig. 9). *S. discolor* is a small plant growing at an altitudinal range of 1000 to 2400 m. with a view to domesticate wild plants of medicinal and ornamental importance, *S. discolor* was identified as having a good prospect as an indoor plant also. In the commercial application of tissue culture for floriculture, market requires new products at regular interval and wild plants are the best source to fulfill this demand. It is evident that it is a valuable non-timber forest product with a medicinal and commercial prospect. Keeping all these points in mind, work on *S. discolor* was initiated. Already some chemical work on this species has been done (Tomimori *et al.*, 1985, 1986). Selected plant flavonoids possess anti-allergic, anti-inflammatory, antiviral and antioxidant activities (Elliott Middletoon, 1996). Seventeen flavonoids from roots and ten

from aerial parts have been reported from *S. discolor*. There is already a patent for a flavone as sialidase inhibitor of influenza virus for therapeutic use from the leaves of *S. baicalensis* (Akishiro *et al.*, 1992). It has been suggested that the baicalin from *S. baicalensis* may be significant in the lipid metabolism, lipogenic and lipolytic pathways of adipose cells (Eun et al., 1994; Chung *et al.*, 1995). A variety of chemical compounds extracted from *Scutellaria* are flavones, flavonoides, chrysin, baicalin, iridoids, neo-clerodane derivatives, scutapins, isoscutellarein, wogonin etc. Baicalin has been produced in the callus cultures of *S. baicalensis* (Shin and Lee, 1995). One of the isolated diterpenoids, scutalpin C, has shown very high insect antifeedant activity against the larvae of *Spodoptera littoralis* (Munoz *et al.*, 1997).

MATERIALS AND METHODS

I. *In vitro* seed germination and culture

Mature seeds from the wild population of *S. discolor* plants growing in the Kathmandu University premises were collected during 1996. Seeds were washed in running tap water for thirty minutes prior to sterilization by 0.1% mercuric chloride (Qualigen) for 8-10 minutes. Seeds were washed four times by sterilized distilled water. Seeds were sown on agar-sucrose slants (1% agar + 1% sucrose). Seeds were germinated in dark at 24-26°C. In two to three weeks, uniform germination was observed. For shoot multiplication, cotyledon and hypocotyl were used as explants. Murashige and Skoog's (1962) medium (MS) was supplemented with various concentrations of a benzyl aminopurine (BAP, Sigma) and 1-naphthalene acetic acid (NAA, Sigma) individually or in combination for shoot induction. For the study of callus induction, three auxins at four concentrations (Table 1) were used individually. Medium was solidified with 0.8 per cent agar agar (Hi-Media) and pH was adjusted between 5.6-5.8. All experiments were repeated twice with five replicates per treatment. Hormonal combinations used in the experiment are given in Table 1. After inoculation cultures were

Table 1. Hormonal treatments used in the study of organogenesis and callus induction

S.N.	Treatment	Shoot/Explant (Average)	Callus induction	Callus Response
1	MS Basal (Control)	1 ± 0	MS Basal (Control)	No
2	MS 0.5 BAP	2 ± 0.35	MS + 0.1 NAA	No
3	MS 1.0 BAP	1.5 ± 0.25	MS + 0.5 NAA	No
4	MS 0.1 NAA	1 ± 0	MS + 1.0 NAA	No
5	MS 0.5 NAA	3 ± 0.46	MS + 2.0 NAA	No
6	MS 0.5 BAP + 0.1 NAA	3.5 ± 0.55	MS + 0.12, 4-D	Yes^{+}
7	MS 0.5 BAP + 0.5 NAA	3 ± 0.94	MS + 0.5 2, 4-D	Yes^{+}
8	MS 1.0 BAP + 0.1 NAA	6 ± 1.27	MS + 1.0 2, 4-D	Yes^{+++}
9	MS 1.0 BAP + 0.5 NAA	1 ± 0	MS + 2.0 2, 4-D	Yes^{++}
10	MS 2.0 BAP + 0.1 NAA	2.5 ± 0.25	MS + 0.1 IAA	No
11	MS 2.0 BAP + 0.5 NAA	1 ± 0	MS + 0.5 IAA	No
12	MS 2.0 BAP + 1.0 NAA	1 ± 0	MS + 1.0 IAA	No
13			MS + 2.0 IAA	No

+ small amount of callus; ++ medium amount of callus; +++ large amount of callus

maintained at 24-28°C. For each treatment, five replicates were maintained and regular examination was carried out visually and with the help of a dissecting microscope. Cultures were provided a 12h photoperiod (≈2,500 lux) by cool white fluorescent tubes.

Table 2. Rooting response in MS media

S.N.	Treatments	Rooting Response
1	MS Basal (Control)	Yes
2	MS 0.5 BAP	Yes
3	MS 1.0 BAP	No
4	MS 0.1 NAA	Yes
5	MS 0.5 nAA	Yes
6	MS 0.5 BAP + 0.1 NAA	Yes
7	MS 0.5 BAP + 0.5 NAA	Yes
8	MS 1.0 BAP + 0.1 NAA	Yes
9	MS 1.0 BAP + 0.5 NAA	No
10	MS 2.0 BAP + 0.1 NAA	No
11	MS 2.0 BAP + 0.5 NAA	No
12	MS 2.0 BAP + 1.0 NAA	No

II. Rooting and plantlet establishment

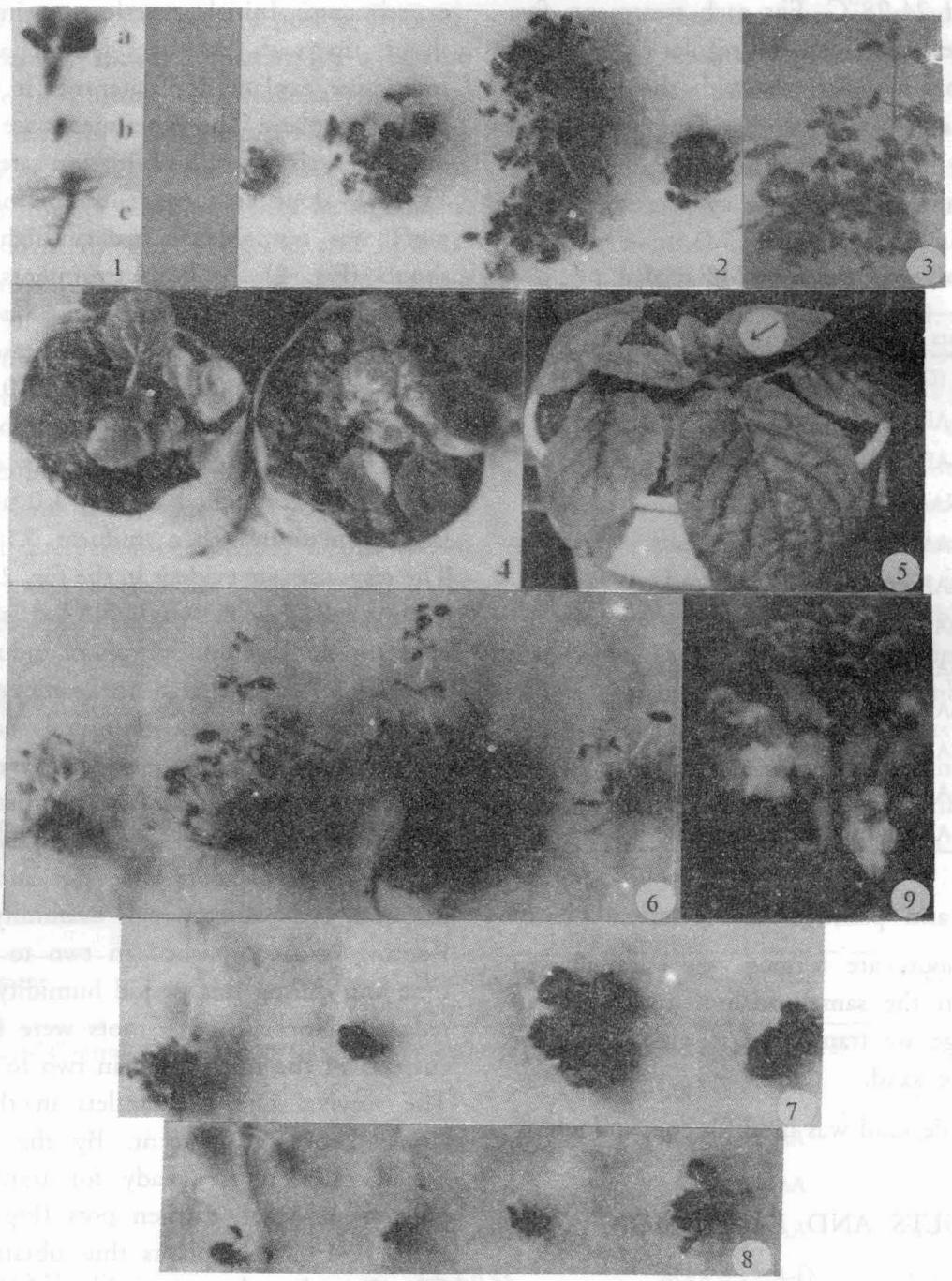
Once shoots are formed, they are allowed to elongate in the same medium and at six to eight leaf stage are transferred to glass house for rooting in the sand.

Non-sterile sand was used for root induction.

RESULTS AND DISCUSSION

I. Seed germination and multiple shoot initiation

Seeds were germinated on agar-sucrose slants and cultures were incubated at 25 ± 2°C and 12h photoperiod. At the end of third week 75-80 per cent seeds germinated. After two more weeks, explants (shoot tip with two cotyledons, hypocotyl and root; Fig. 1) were excised and used for experiments. Initial experiments indicated that shoot tip with two cotyledons as the most responsive explant and later on it was used as the sole explant. The experiments are divided into organogenesis and callus initiation categories (Table 1). BAP alone at a concentration of 0.5 or 1.0 mg/l was not able to induce high number of shoots (Fig. 2). In both treatments, 3-5 shoots were observed after three weeks but the height of shoots and rooting *in vitro* decreased drastically at 1.0 mg/l BAP in comparison to 0.5 mg/l BAP (Fig. 2). To achieve higher number of shoot buds, BAP and NAA were tried in combination. BAP was incorporated at 0.5, 1.0 and 2.0 mg/l in combination with a uniform 0.1 mg/l NAA. The responses are evident in the Fig. 2, establishing 1.0 mg/l BAP + 0.1 mg/l NAA as the optimum concentration for multiple shoot induction. Shoot elongation continued in the same medium and after four to six weeks-elongated shoots reached 4-8 leaf stage (Fig. 3). Few roots are seen in the culture during this phase and plantlets transferred with roots to non-sterile sand establish well. At this stage microshoots were cut and introduced to sand beds under high humidity condition. Rooting is accomplished in two to three weeks time and during this period humidity is gradually reduced. Normally, 3-5 roots were formed from cut end of the microshoot in two to three weeks. The survival rate of plantlets in the sand was always above 90 percent. By the end of this period plantlets are ready for transferring into polypots or small earthen pots (Figs. 4 and 5). After 12-15 weeks plants thus obtained flowered (Fig. 5) and seed set was observed. High rooting percentage of microshoots and very low mortality of plantlets is reported in other plants too (Kannan and Jasrai, 1996). *In vitro* techniques are being increasingly used for multiplication and conservation of germplasm of medicinal importance threatened with extinction (Bhojwani *et al.*, 1989; Sharma *et al.*, 1995).



In vitro studies in *Scutellaria discolor* (Fig. 1-9) : 1. Explants used (a- shoot tip with cotyledons, b- hypocotyl, c-root); 2. Response to 0.5B, 1.0B, 0.5B + 0.1N, 1.0B+ 0.1N, and 2.0B + 0.1N; 3. Shoot elongation in MS+1.0B+0.1N; 4. Plantlets in the soil; 5. Plantlets in pots and initiation of flowering (arrow); 6. Response to 0.1, 0.5, 1.0 and 2.0 N; 7. Response to 0.1, 0.5, 1.0, and 2.0 D; 8. Response to 0.1, 0.5, 1.0 and 2.0 I; 9. A flowering twig
Hormone concentrations are in mg/1 and are written in one letter symbol (B = BAp, N = NAA, D = 2, 4-D, and I = IAA)

II. Callus induction

A total of thirteen hormonal treatments were used in this study (Table 1). For this experiment explants used were shoot tip and leaf with petiole. Three auxins; NAA, 2, 4-D, and IAA were used at four different concentrations (0.1, 0.5, 1.0, and 2.0 mg/1) individually. Our initial results reveal that at the hormonal concentrations applied

here only 2, 4-D could induce callusing. NAA induced feathery roots in both the explants (shoot tip and leaf with petiole) and these roots mostly remain on the surface of the medium. A comparison of the efficacy of various auxins at different concentrations is evident in the Fig. 6, 7 and 8. Except one treatment (0.1 mg/l), 2, 4-D were very effective in inducing callus and covered the whole explant in a period of three weeks. Colour of callus whenever produced was light yellow. Callus is being worked out for regeneration.

At present role of hormones in combination (auxin/cytokinin) is being tried to study callusing with different media base. The main idea is to develop a suspension culture system for the study of secondary metabolite biosynthesis.

In this study protocols for *in vitro* multiplication and callus induction of *Scutellaria discolor* were standardized. *Ex vitro* method for the rooting of microshoots was tried and found to be very successful. No prior hormonal (long term or pulse treatment) was required for sand rooting. Using this protocol of multiple shoot production, about 300 plantlets were obtained from a single shoot tip explant within 4-5 months. Future experiments are being directed to obtain multiple shoots from explants of *in vivo* grown plants. Callus induction was easily achieved with the help of 2, 4-D but more experiments are required to obtain embryogenic callus.

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