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Mass propagation of *Solanum surattense* Bum. using direct adventitious shoot organogenesis from internode

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ABSTRACT

An efficient and reproducible procedure was established for direct shoot regeneration of *Solanum surattense* Bum. using internodal explants. The shoot regeneration efficiency on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) was superior in compared to 6-furfurylaminopurine (Kn). The highest mean number of shoots per explant was recorded on MS medium containing 0.5 mg l⁻¹ BAP. BAP alone was more effective for shoot regeneration than combination of BAP and α -naphthaleneacetic acid (NAA). The number of shoot per explant was highest in the 4th subculture and then it was remained stable up to 6th subculture. The shoot number drastically reduced from 7th subculture when explants were repeated subculturing up to 8th subculture. Hundred percent adventitious shoots were rooted successively on half strength MS medium fortified with 0.05 mg l⁻¹ NAA. At about 90% of the *in vitro*-propagated plantlets survived when they were transferred to *ex vitro* conditions for acclimatization.

Key words: Direct shoot organogenesis, internodal explant, *in vitro*, medicinal plant, *Solanum surattense*

IZVLEČEK

RAZMNOŽEVANJE *Solanum surattense* Bum. Z UPORABO INTERNODIJSKIH EKSPLANTOV

Avtorji so razvili učinkovit in reproducibilen način neposredne organogeneze zdravilne rastline *Solanum surattense* Bum. z uporabo internodijskih eksplantov. Regeneracija na gojišču Murashige in Skoog (MS) je bila bolj uspešna z dodatkom 6-benzilaminopurina (BAP) v primerjavi z uporabo 6-furfurilaminopurina (Kn). Največje število poganjkov na eksplant je bilo na gojišču MS z 0,5 mg/l BAP. BAP sam je bil bolj učinkovit za regeneriranje poganjkov kot kombinacija BAP in α -naftalenocetne kisline (NAA). Število poganjkov na eksplant je bilo najvišje v četrti subkulturi in je ostalo stabilno vse do šeste, močno pa se je zmanjšalo v sedmi in osmi subkulturi. Adventivne korenine so se pojavile pri vseh poganjkih pri MS mediju polovične jakosti z dodatkom 0,05 mg/l NAA. Približno 90 % *in vitro* razmnoženih rastlinic je preživel prenos *ex vitro* in aklimatizacijo.

Ključne besede: neposredna organogeneza poganjkov, eksplanti internodijev, *in vitro*, zdravilna rastlina, *Solanum surattense*

INTRODUCTION

Solanum surattense Bum. (Solanaceae) is a perennial herb. It is usually found in India, Pakistan, Malaya, and Australia. In Bangladesh, it was found as wild herb in almost all northern parts and it was very common in the Barind region. Nowadays, this plant rarely gets in the Barind region only. The solasodine and glycosides are rich in this plant, are very common properties for anti-cancer (Cham, 2007). Besides, this plant is widely used as folk medicine for breathing trouble, heart diseases

and pain. Some drug companies (Unani, Handard Laboratories, Ayurvedic) are developed in Bangladesh based on plant extract and they are attracted by the people. These companies are using extract of *S. surattense* as to prepare remedy for breathing disease, as well this plant is widely planted in highland of Bangladesh. Since this herb becoming a potential medicinal plant in south Asia, more advance investigations are needed concerning modification of

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characteristics including rapid growth, increase essential chemicals content, disease resistant and stress tolerance in this plant.

Limited reports have been published on the *in vitro* propagation as well as genetic transformation systems of *S. surattense*. Pawar *et al.* (2002) developed a technique for direct shoot organogenesis from shoot tip and leaf segments. Using nodal and shoot tip segments, a micropropagation technique also established on this plant by Rama Swamy *et al.* (2004). Rama Swamy *et al.* (2005a) established a protocol on plantlet regeneration through somatic embryogenesis from cotyledon and leaf explants. Callus induction and shoot organogenesis system from floral bud were also reported earlier for this plant proliferation (Prasad *et al.*, 1998). Rama Swamy (2006) reported *Agrobacterium-mediated* genetic transformation systems using leaf explants of *S. surattense*. (Rama Swamy *et al.* 2005b) established streptomycin-resistant *S. surattense* plantlets using *in vitro* mutagenesis.

MATERIALS AND METHODS

Explant preparation

Shoot apices (20-25 cm) with leaves of *S. surattense* were collected from field growing plant in a Barind region, Rajshahi, Bangladesh. The shoot apices were defoliated and segmented at 4-5 cm contains 2-3 nodes after bringing to laboratory. Stem segments were washed thoroughly under running tap water for 30 min and finally disinfected with 0.1% HgCl₂ for 10 min under laminar airflow followed by washing thrice with autoclaved distilled water. The internodal segments (1 cm) were prepared from the disinfected plant materials.

Culture media and conditions

Three basal media were used in the present study: Murashige and Skoog (MS) medium, Gamborg (B5) medium and McCown Woody Plant medium (WPM). All variants of medium were supplemented with 3% (w/v) sucrose. The pH of medium was adjusted to 5.7 ± 0.2 before autoclaving at 121°C for 20 min under 1.1 kgcm⁻² pressure and medium was solidified with 0.8% (w/v) agar (Type M, Sigma). The MS medium was variously supplemented with 6-benzylaminopurine (BAP) at 0.2, 0.5 and 1 mg l⁻¹, 6-Furfurylaminopurine (Kn) at 0.5, 1 and 1.5 mg l⁻¹ alone or in combinations with α -Naphthaleneacetic acid (NAA) at 0.1 and 0.2 mg l⁻¹ for shoot regeneration. Half strength MS medium supplemented with various concentrations of NAA, Indole-3-butyric acid (IBA) or Indole-3-acetic acid (IAA) singly was used for rooting. Plant growth regulator (PGR)-free medium was considered as control for both shoot regeneration and rooting experiments. All cultures were maintained in the growth chamber under 25 ± 1 C with 16 h photoperiod (50 μ mol m⁻² s⁻¹) provided by cool white fluorescent light.

For genetic improvement of plant, we usually use selection method as well as *in vitro* molecular breeding technique. Plant breeders showing great interest on molecular breeding technique for plant modification genetically because conventional selection method takes long time, tedious and occurs large variation within clones. For molecular breeding based genetic transformation, we know, efficient regeneration systems are prime requirement. Stem segments are used as important explant for genetic transformation system, described in many plant species (e.g., Rastogi and Dwivedi, 2006). Adventitious shoot regeneration from internodal segments of *S. surattense* was not published in the previous reports so far.

In the present study an effort was made to establish a protocol for the *in vitro* propagation of *S. surattense* from the internodal explants of field grown plant through direct adventitious shoot organogenesis.

Culture and acclimatization procedures and data collection

After disinfection, internodal explants were cultured on the MS medium fortified with PGRs for shoot multiplication. Cultures were transferred to fresh medium routinely at 15-day-interval. Elongated shoots (at least 3 cm) were excised from shoot clusters prior to subculture. Large-sized shoot clusters were divided before transfer to fresh medium. Data were collected on percentages of explant forming shoot, total number and average length of shoots per explant 30 days after culture. Using 0.5 mg l⁻¹ BAP as standard PGR, shoot regeneration ability was evaluated among three basal media.

The effect of repeated subculturing on shoot regeneration efficiency was evaluated by another experiment. Ten internodal segments were passed (15-day-interval) continuously up to 8 subcultures on shoot-regenerating medium. Elongated shoots (at least 3 cm long) were excised from shoot clumps prior to transfer onto fresh medium. Data on total number and average length of elongated shoots per explant were recorded in each subculture (15-day-interval).

For rooting, leafy shoots (3 cm to above) were implanted onto half strength MS basal medium containing auxins and one shoot was cultured in one test tube. Data were collected on percentages of shoot forming root, total number and average length of roots per plantlet 21 days after culture.

For acclimatization of plantlets, rooted shoots were washed gently under running tap water, planted onto pot containing composed soil and maintained in the room conditions for 7 days. Plantlets were covered with polyethylene bag for avoiding over transpiration and watered regularly 2-day-interval. Seven days after hardening in room conditions, plantlets were transferred to out side conditions and survival rate was recorded 2 months after transfer.

Statistical analysis

All experiments were consisted of five replicates and repeated at least thrice. For shoot regeneration experiment, 6 internodal segments were used for each replicates, which were collected from one field grown plant. Five *in vitro* shoots were taken in a replication for rooting experiment, which were regenerated from one explant taken from one field grown plant. Therefore,

variation within replications indicates difference within genotypes. Data were arranged as a completely randomized design and significant difference was determined by analysis of variance. Differences among mean values were evaluated by least significance difference (LSD) at the 5% ($p < 0.05$) level.

RESULTS AND DISCUSSION**Direct adventitious shoot organogenesis**

The stem segments of field growing plant were very prickly, in which disinfection was problematic; however, surface-disinfection with 0.1% HgCl₂ solution for 10 min resulted in the highest contamination free of about 45% explants. The frequencies of shoot regeneration varied from 20 to 100% depending on the PGRs (Table 1). On MS medium supplemented with BAP (0.5 mg l⁻¹), the adventitious shoot induction at the cut side was first observed 7 days after inoculation without any callus intervention. Within 15 days, shoot induction obtained from not only from cut sides but also from the intact portion of internode, while some of first induced shoots were reached a height of above 3 cm (Fig. 1a). After separation of elongated shoots, shoot clusters were divided prior to subculture onto fresh same variant of medium, resulted in the highest production of adventitious shoots, on which 100% internodal segments produced 58.2 shoots per explant within 30 days after subculture (Fig. 1b). No regeneration of any shoot was observed on PGR-free

medium. The differences in the shoot regeneration efficiency were significant ($p < 0.05$) among the PGRs supplemented (Table 1). Within replications, the significant differences were not observed, in which differences in the shoot regeneration efficiency did not exhibit within genotypes. The shoot regeneration performances were low in MS medium supplemented with Kn in compared to medium containing BAP. The superior effect of BAP in compared to Kn was reported on direct adventitious shoot regeneration from leaf explant of *Santalum album* (Mujib, 2005). In contrast to present observations, medium containing BAP or Kn showed similar responses on *in vitro* shoot production of *Solanum nigrum* (Bhat *et al.*, 2010). In previous report on adventitious shoots regeneration of *S. surattense* (Pawar *et al.*, 2002), adventitious shoots were produced from shoot tip and leaf explants on MS basal medium fortified with BAP and kinetin individually and in their combination.

Table 1: The adventitious shoots proliferation from the internodal explants of *S. surattense* on MS medium fortified with PGRs 30 days after culture.

PGRs (mg l ⁻¹)			Percentages of explant forming adventitious shoot (Mean ± SE)	Number of total shoots per explant (Mean ± SE)	Average length (cm) of shoots per explant (Mean ± SE)
BAP	Kn	NAA			
0	0	0	0	0	0
0.2			43.3 ± 2.8f	12.4 ± 1.2d	4.0 ± 0.3c
0.5			100 ± 0.0a	58.2 ± 4.6a	6.7 ± 0.4a
1.0			90 ± 12.1b	25.5 ± 2.4b	5.5 ± 0.3b
2.0			30 ± 2.8g	7.5 ± 0.8d	3.4 ± 0.2d
	0.5		26.7 ± 3.5g	4.5 ± 0.5de	3.5 ± 0.4cd
	1.0		66.7 ± 5.4d	16.7 ± 1.3c	5.2 ± 0.2b
	1.5		80 ± 6.5c	30.6 ± 3.1b	6.5 ± 0.3a
	3.0		30 ± 2.8g	5.7 ± 0.4d	3.1 ± 0.4d
0.5		0.1	56.7 ± 4.5e	22.6 ± 3.5bc	4.8 ± 0.4bc
0.5		0.2	43.3 ± 2.6f	13.5 ± 3.8cd	4.0 ± 0.3c
1.0		0.1	30 ± 4.3g	6.2 ± 0.6d	3.2 ± 0.2d
1.0		0.2	23.3 ± 2.8g	5.6 ± 0.2de	3.0 ± 0.3d
	1.0	0.1	23.3 ± 2.4g	4.1 ± 0.6e	3.2 ± 0.3d
	1.0	0.2	20 ± 3.2gh	3.7 ± 0.3e	3.0 ± 0.4d
	1.5	0.1	40 ± 3.4f	12.4 ± 1.0d	4.5 ± 0.4bc
	1.5	0.2	20 ± 2.1gh	5.2 ± 0.5d	4.1 ± 0.3c

Means with the same letters are not significantly different by the LSD test at the 5% level ($p < 0.05$).

Table 2: Ions in three basal media used for shoot organogenesis of *S. surattense*.

Macro and microelement combinations (mM)			
Ions	MS	WPM	B5
NO ₃ ⁻	39.310	9.700	24.730
H ₂ PO ₄ ⁻	1.250	1.240	1.090
SO ₄ ²⁻	1.620	7.300	2.086
Cl ⁻	5.980	1.300	2.040
K ⁺	19.955	12.615	24.735
Ca ²⁺	2.999	3.000	1.020
Na ⁺	0.102	0.102	1.190
Mg ²⁺	1.500	1.500	1.010
NH ₄ ⁺	20.610	5.000	2.020
Total N	59.920	14.700	26.750
NH ₄ ⁺ : NO ₃ ⁻	1 : 0.524	1 : 0.515	1 : 0.082

Table 3: Root formation from the *in vitro* grown shoots of *S. surattense* on half strength MS medium fortified with auxins 21 days after culture.

Auxins (mg l ⁻¹)			Percentages of shoot forming root (Mean ± SE)	Number of roots per plantlet (Mean ± SE)	Length of roots per plantlet (Mean ± SE)
NAA	IBA	IAA			
0	0	0	65 ± 4.2c	2.7 ± 0.3de	2.5 ± 0.2b
0.01			100 ± 0.0a	9.2 ± 0.8b	3.0 ± 0.3a
0.05			100 ± 0.0a	12.5 ± 1.2a	3.2 ± 0.4a
0.1			80 ± 6.2b	5.8 ± 0.3c	2.8 ± 0.3ab
	0.01		0	0	0
	0.05		80 ± 5.6b	3.6 ± 0.3d	2.0 ± 0.3c
	0.1		0	0	0
		0.01	0	0	0
		0.05	60 ± 6.4cd	2.8 ± 0.2d	1.4 ± 0.2d
		0.1	0	0	0

Means with the same letters are not significantly different by the LSD test at the 5% level ($p < 0.05$).

The MS medium supplemented with the combination of NAA and BAP or Kn significantly ($p < 0.05$) reduced the shoot regeneration efficiencies. On medium supplemented with combinations of NAA and BAP, the abnormalities such as thick shoot and light green leaves were observed in the induced shoots, which were finally turned lethal browning at the 3rd subculture (45 days after culture). The results are in agreement with the finding of Teo *et al.* (1997) on the shoots multiplication of *Brassica rapa*, they observed that regenerated shoot shows abnormalities after addition of high concentration NAA to the medium. The contrary result was observed in the direct shoot regeneration of *Drymaria cordata* from leaf segment (Ghimire *et al.*, 2010) where they reported that the application of NAA and BAP in the medium was enhanced the shoot regeneration. In *S. surattense*, the combination of BAP and NAA resulted in the best somatic embryogenesis when cotyledon and leaf explant were used (Rama Swamy *et al.*, 2005a). Although the presence of NAA in combination with cytokinins enhancing morphogenesis and somatic embryogenesis reported in earlier studies, this combination inhibited direct shoot regeneration of *S. surattense* in the present investigation but reasons for this fact are unknown.

As shown in the Table 2, the content of ions in three basal media was presented. Total nitrogen content was higher in MS medium than in WPM and B5 media although ratio of NH₄⁺ and NO₃⁻ was similar in MS and WPM media. Of three basal media tested, the percentage of explant forming shoot, number and length of shoot per explant were the highest in MS medium. The percentage and length of shoot were lower nearly twice in WPM and B5 than MS medium, while the number of regenerated shoot was about trice higher in MS than those of WPM and B5 media (Fig. 2). The lack of responses in the shoot regeneration efficiency may be occurred in WPM and B5 media due to nitrogen (N) deficiency. This trend is in agreement with the finding of Pinto *et al.* (2008). An appropriate NO₃⁻ : NH₄⁺ ratio influenced morphogenesis and embryogenesis (Ramage and Williams, 2002) but the most favorable range depends on the species (e.g., Tazawa and Reinert, 1969). Also different NO₃⁻ : NH₄⁺ levels may have indirect effects to pH changes, which control cation absorption and eventually morphogenesis (Cousson and Tran Thanh Van, 1993). Other ions such as Ca²⁺, Mg²⁺, SO₄²⁻, H₂PO₄⁻, Cl⁻, and K⁺ were also correlated with morphogenesis (Benson, 2000; Pinto *et al.*, 2008). In the present study, shoot regeneration of *S. surattense* was

influenced on MS medium, may be reasons were high content of nitrogenous elements and even appropriate proportion of NO_3^- and NH_4^+ . Using MS medium, *in vitro* propagation of *S. surattense* was inspected by all previous reporters although they not did compare with other basal media. In the present study, we examined comparative study on three basal media and observed that MS medium was superior effective on *in vitro* shoot regeneration.

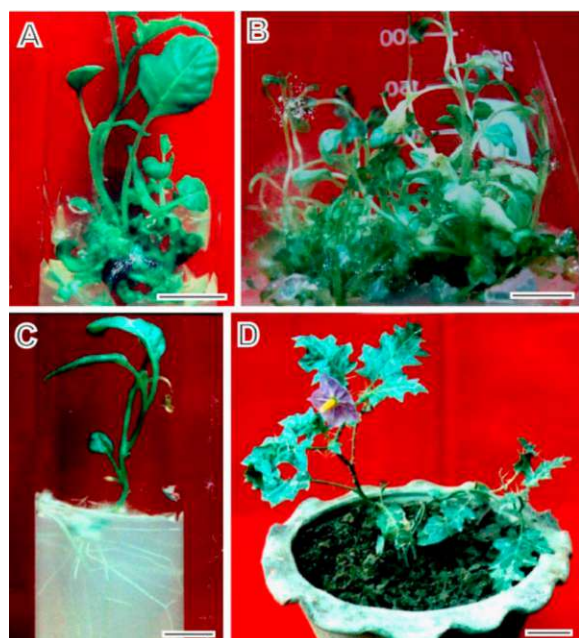


Fig. 1. *In vitro* multiplication of *Solanum surattense* through direct shoot organogenesis. A. Adventitious shoots induced from an internodal segment on MS medium supplemented with 0.5 mg l^{-1} BAP 15 days after inoculation (bar = 2 cm). B. A part of shoot cluster regenerated from an internodal segment on same variant of medium 30 days after culture (bar = 2 cm). C. Rooted shoot produced on half strength MS medium supplemented with 0.05 mg l^{-1} NAA 21 days after culture (bar = 2 cm). D. Acclimatized 2-month-old plantlet with flowers growing in the flowering season onto a pot containing compost soil in the ex vitro conditions (bar = 4 cm).

The internodal segments were subcultured repeatedly on MS medium supplemented with 0.5 mg l^{-1} BAP to achieve continuous production of healthy shoots. The number of shoots per explant rapidly increased from the 1st subculture up to the 4th subculture and more than 150 shoots were produced per explant at the 4th subculture. Similar shoot number was observed until the 6th subculture and then drastically decreased in the subsequent subcultures. No remarkable differences were

observed in the length of shoots throughout subcultures (Fig. 3). The stability of the shoot regeneration efficiency in the subculture depends on the plant species. The shoot number per leaf segment-derived calluses was reached highest level at the 5th subculture and stable up to the 7th subculture in *Elaeocarpus robustus* (Rahman *et al.*, 2009) and the similar investigation also carried out in *Lagerstroemia speciosa* (Rahman *et al.*, 2010) where stability of highest shoot production was continued from 6th to 8th subculture. Lethal browning in the tissue could be occurred by long-term subculture onto shoot-induction medium, which could lead to reduction of shoot regeneration efficiency in the subcultures. Horgan (1987) observed that the presence of cytokinin in the medium seems to enhance tissue browning for long-term culture, which inhibit shoot regeneration efficiency.

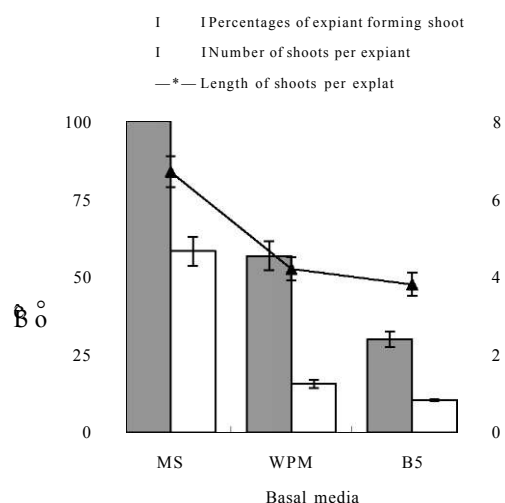


Fig. 2. Effect of three basic media on shoots regeneration from internodal segment 30 days after culture. All basal media were fortified with 0.5 mg l^{-1} BAP.

Rooting of shoot and acclimatization of plantlet

The MS medium containing 0.5 mg l^{-1} BAP-induced shoots were cultured on rooting-medium. Root initiation occurred within 7 days; an efficient root system was observed 21 days after culture (Fig. 1c). The addition of 0.01 and 0.05 mg/l NAA to the half strength MS medium was the most effective for root induction without basal callusing. On both variants of rooting medium, 100% shoot induced root but significant differences ($p < 0.05$) were observed in the root number (Table 3). The root formation was observed at the cut base of 65% shoots with a few number (2.7) on auxin-free medium; in contrast, the best rooting of *S. surattense* was observed on PGR-free MS medium (Pawar *et al.*, 2002). The differences in the root

induction efficiency were not observed within replications, in which variation in the root induction efficiency did not affected by genotypic variation of donor plants. The induction of root was also obtained on medium containing 0.05 mg/l of IBA and IAA but they showed stunt growth and thin, while callus was formed at shoot base. The other concentrations of IBA and IAA could not induce any root without forming callus. The best rooting responses from shoots on medium containing NAA were reported in many plant species (e.g., Teo *et al.*, 1997). The callus induction at the cut base was a vital reason for inhibition of root induction, reported in *Drymaria cordata* (Ghimire *et al.*, 2010).

About 90% of the plantlets survived onto compost soil 2 months after transfer and seasonal flowering observed in the *ex vitro* conditions. The detectable variation in the *in vitro* grown potted plants was not found when compared with the donor plants (Fig. 1d). The established shoot regeneration technique in the present study has a potential impact for clonal propagation of elite genotypes and improvement characteristics by molecular breeding technique of *S. surattense* for plantation and utilization in the drug industries.

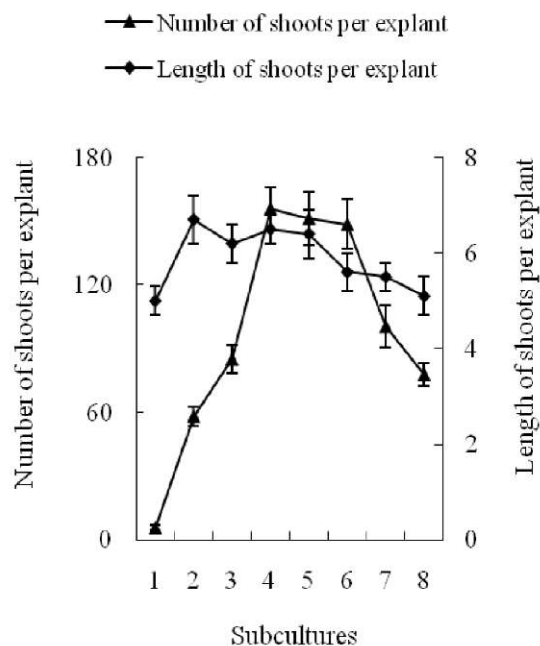


Fig. 3. The shoot regeneration ability per internodal segment throughout eight subcultures. The explants were repeated subculturing on MS medium supplemented with 0.5 mg^l⁻¹ BAP.

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