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In vitro Propagation of *Stemona tuberosa* Lour. - A Rare Medicinal Plant through High Frequency Shoot Multiplication using Nodal Explants

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Abstract

Nodal segments of *Stemona tuberosa* Lour. were found to proliferated without any differentiation on to MS supplemented with 3.0 mg/l BAP and 0.5 mg/l NAA under continuous dark condition. After 20 days of inoculation under dark condition the cultures were transferred to a daily cycle of 16/8 hrs light/dark photoperiod and there the proliferated nodal segments underwent direct organogenesis producing huge number of shoot buds (25.87/culture). The shoot buds underwent rapid elongation on a range of BAP (0.1 - 1.5 mg/l) and IBA (0.1 - 1.0 mg/l) supplemented MS. Rooting of elongated shoot buds was successfully achieved (90%) in half strength MS with 1.0 mg/l NAA. The plantlets were finally established in outside environment through a successive phase of acclimatization.

Introduction

Stemona tuberosa Lour. is a perennial woody climber with tuberous roots. It belongs to Stemonaceae and prefers a rather dry climate. It is found in the hilly areas of North-east India, Bangladesh, Myanmar, Thailand, Vietnam, Laos, Combodia and Philippines (Tsi and Duyfjes 2000). In Bangladesh it has been reported from Chittagong, Rangamat and Sylhet (Huq and Mirza 1989). The family Stemonaceae is now the only source of medicinally important stemona alkaloids. Pilli and de Oliveira (2000) listed 14 alkaloids available in *Stemona tuberosa.* Extracts of *S. tuberosa* tubers have been used for many years in China for treating a variety of ailments including bronchitis, pertusis and tuberculosis

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(Jacobi and Lee 1997). In Bangladesh, the tribal people of Chittagong Hill Tracts (CHT) use tuberous roots and leaves of this plant for treatment of intestinal worm, cough and mental disorders. Naturally *Stemona tuberosa* propagates by seeds and rootstocks. But due to indiscriminate destruction of forest vegetation and its large-scale use for medicinal purpose this important plant species is becoming rare in natural habitat. Because of the essential medicinal use, attention has been paid to formulate rapid propagation of this important medicinal plant species. Propagation of a plant of such medicinal value can be achieved by adoption of tissue culture technique. In this perspective it was considered important to develop protocol for rapid propagation of *S. tuberosa*. In recent years much attention has been given both to cost-effective micropropagation and to medicinal plants as sources of curable compounds for several ailments. The development of rapid large-scale propagation systems for rare medicinal plants is a necessity in order to meet pharmaceutical needs and also to prevent the plants from becoming endangered and extinct (Martin 2003). Tissue culture technique has been used for conservation and multiplication of medicinal plants (Bhojwani 1980, Wawrosch et al. 2001, Martin 2002, 2003, Joshi and Dhar 2003). For this reason the development of an *in vitro* protocol will attach a great importance for large-scale production and conservation of this species. Direct regeneration from explants without an intervening callus phase has several advantages, including production of true type progenies (Manickavasagam et al. 2004). So far no report is available on the development of protocol for *in vitro* propagation of this species. The purpose of the investigation reported here was to develop an efficient *in vitro* protocol that would maintain and propagate this important species.

Materials and Methods

Tuberous rootstocks of *Stemona tuberosa* Lour. were collected from natural habitat of Chittagong Hill Tracts and planted in earthen pots in the experimental field of the Institute of Biological Sciences, Rajshahi University. Nodal, internodal and leaf segments of two months old twigs were used as explants. Those were washed first under running tap water for 20 min. After washing, the explants were dipped into 5% (v/v) Savlon solution with a few drops of Tween 80, shaked gently for 10 min and washed with sterile distilled water following 1 min treatment with 70% ethanol. The explants were then surface sterilized with an aqueous solution of 0.1% $HgCl_2$ for 6 - 8 min and finally washed in sterile distilled water for five times. Explants were then cut into small segments (0.5 - 0.1 cm) and aseptically cultured on 0.8% (w/v) agar solidified MS with 3% sucrose and fortified with different plant growth regulators (PGRs) *viz.* BAP (1.0

- 5.0 mg/l), Kn (1.0 - 5.0 mg/l), NAA, IAA and IBA (0.1 - 1.5 mg/l) at different concentrations and combination. pH of the medium was adjusted to 5.7 ± 0.1 before dispensing in culture tubes and autoclaving at 121ºC and 1.1 kg/cm^2 for 20 min. In order to see the effect of light and dark condition on multiple shoot induction, two sets of experiments were carried out: (i) for 16/8 hrs light dark photoperiod and (ii) for 15 - 20 days continuous dark followed by a 16/18 hrs light/dark photoperiod. All cultures were incubated at 25 ± 2ºC and regular subculturing was done at three weeks interval. For induction of roots the elongated shoots were individually grown on full and half strength MS with different growth regulators (NAA, IAA and IBA) at different concentrations. The experiments were set up in a completely randomized design. Each experiment was repeated thrice using 10 replicates. The data were analyzed statistically following DMRT. These tests were conducted using statistical software package by MSTAT-C.

After one week of acclimatization, rooted plantlets were transferred to small pots filled with sterile garden soil and kept in the growth chamber for hardening and then transferred to natural environment.

Results and Discussion

Among the three kinds of explants only the nodal segments gave positive response. It proliferated and then underwent direct organogenesis producing multiple shoot buds in different PGR supplemented media (Table 1). Internodes and leaves did not produce any adventitious shoots. So further experiments were conducted using only nodal explants. Induction of multiple shoot buds directly from nodal explants has been reported in a wide range of plant species (Murashige 1974, Selvakumar et al. 2001, Das and Handique 2002, Hassan and Roy 2004, Sultana and Handique 2004). No shoot induction took place when nodal explants were cultured in basal medium without any PGR supplement. However, this process was dependent on light/dark condition under which the cultures were maintained. Under continuous dark condition the nodal segments underwent swelling without any differentiation (Fig. 1). But after 15 - 20 days of culture in dark when the cultures were transferred to a daily cycle of 16/8 hrs light/dark the swollen nodal segments underwent direct organogenesis producing huge number (av. 12.7 shoots/segment) of shoot buds. On the other hand, under continuous light condition the nodal segments produced only one or two shoot buds without any proliferation even after two successive subcultures at 21 days interval. This finding indicates the influence of light and dark condition on tissue differentiation. From this experimental result it was

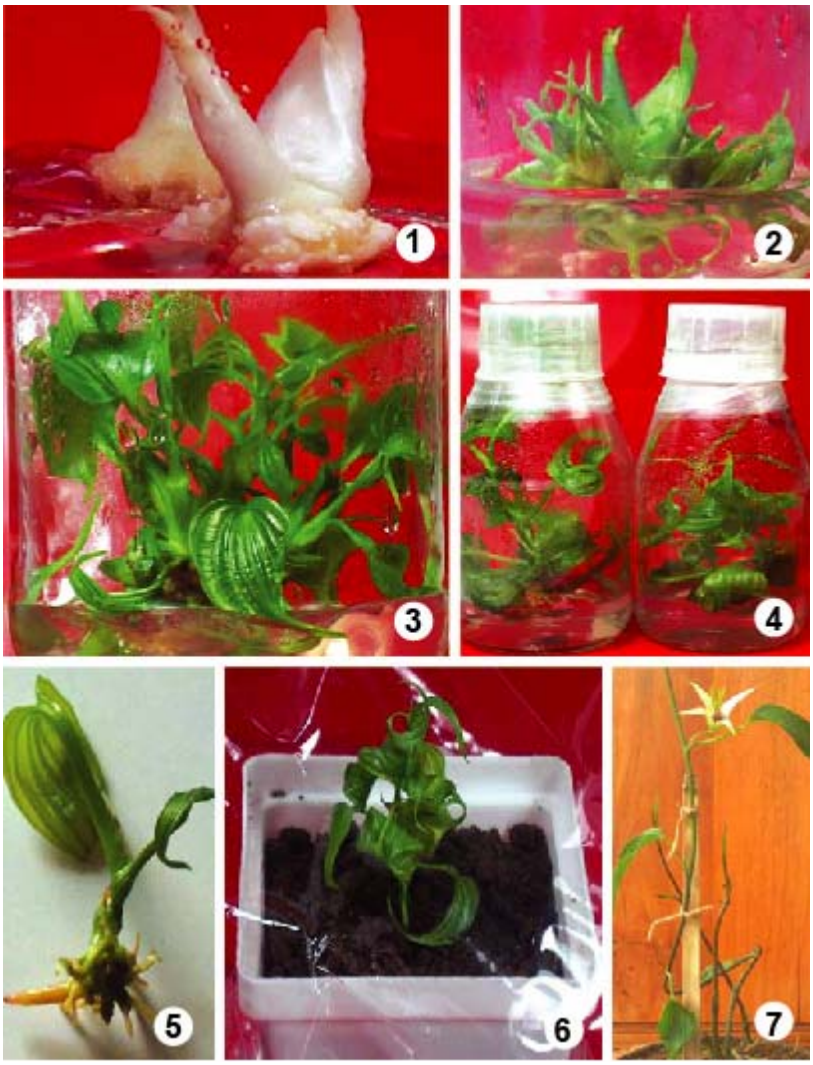
Table 1. Response of nodal segments of *S. tuberosa* in different PGR supplemented MS media after 60 days of culture.

Growth regulators (mg/l)					Percentage response	Number of shoots per culture	Length of shoots per culture (cm)
BAP	Kn	NAA	IBA	IAA	Mean	Mean ± SE	Mean ± SE
Control					0	0	0
1.0	-	-	-	-	23fg	0.57 ± 0.30gh	0.43 ± 0.02efg
2.0	-	-	-	-	33def	1.00 ± 0.00fg	0.72 ± 0.05ef
3.0	-	-	-	-	53c	2.17 ± 0.05de	1.56 ± 0.06c
4.0	-	-	-	-	37df	1.20 ± 0.03fg	0.93 ± 0.04de
5.0	-	-	-	-	30ef	0.77 ± 0.10gh	0.72 ± 0.07ef
-	1.0	-	-	-	-	-	-
-	2.0	-	-	-	-	-	-
-	3.0	-	-	-	20fg	0.77 ± 0.10gh	0.55 ± 0.10fegh
-	4.0	-	-	-	13fg	0.50 ± 0.05gh	0.32 ± 0.02fg
	5.0	-	-	-	-	-	-
3.0	-	0.1	-	-	47cd	4.27 ± 0.35c	1.82 ± 0.15c
3.0	-	0.5	-	-	97a	12.70 ± 0.25a	5.48 ± 0.31a
3.0	-	1.0	-	-	87b	8.97 ± 0.22b	4.22 ± 0.08b
3.0	-	1.5	-	-	33df	2.56 ± 0.16d	1.40 ± 0.15cd
3.0	-	-	0.1		-	-	-
3.0	-	-	0.5		-	-	-
3.0	-	-	1.0		-	-	-
3.0	-	-	1.5		-	-	-
3.0	-	-	-	0.1	27fg	1.20 ± 0.09fg	0.75 ± 0.04ef
3.0	-	-	-	0.5	30ef	1.70 ± 0.25ef	0.75 ± 0.07ef
3.0	-	-	-	1.0	10h	0.50 ± 0.16gh	0.43 ± 0.14efg
3.0	-	-	-	1.5	-	-	-

Values are the mean of three replicates with 10 explants. Mean values followed by the same letters are not significantly different at the 5% level.

clearly indicated that for induction of multiple shoots of *S. tuberosa* dark phase played an important role for stimulating the differentiated cells of explants to express its totipotency.

BAP is considered to be one of the effective cytokinins for induction of organogenesis and thereby effective micropropagation of plants (Sharma et al. 1993, Chandramu et al. 2003) and a combination of BAP and NAA is most often



Figs 1-7. *In vitro* propagation of *Stemona tuberosa*: 1. Initial culture of nodal segment on MS in dark. 2. Proliferating multiple shoots. 3-4. Elongation of shoots. 5. Roots developed in half-strength MS. 6. *In vitro* developed plant in pot after acclimatization. 7. Flowering *in vitro* cultured plant.

Table 2. Effect of auxins on *in vitro* rooting of *S. tuberosa* in half-strength and MS after 30 days of culture. Values are the mean of three replicates with 10 explants.

PGR (mg/l)		Half strength			Full strength		
		Rooting %	No. of roots per shoot	Root length (cm)	Rooting %	No. of roots per shoot	Root length (cm)
Control	0	0	0	0	0	0	0
NAA	0.5	53 b	1.73 ± 0.11b	0.95 ± 0.02b	23 b	0.63 ± 0.05 b	0.43 ± 0.04 b
	1.0	90 a	4.70 ± 0.23a	1.85 ± 0.07a	47 a	1.23 ± 0.08 a	0.77 ± 0.05 a
	1.5	37 bcd	1.03 ± 0.02cd	0.60 ± 0.03cd	-	-	-
IBA	0.5	13d	0.63 ± 0.05cde	0.43 ± 0.04de	-	-	-
	1.0	43 bc	1.17 ± 0.10 c	0.73 ± 0.05 c	-	-	-
	1.5	30cd	0.73 ± 0.05cd	0.48 ± 0.04de	-	-	-
IAA	0.5	17d	0.47 ± 0.03de	0.33 ± 0.06e	-	-	-
	1.0	17d	0.77 ± 0.04cd	0.42 ± 0.02 de	23 b	0.70 ± 0.11 b	0.43 ± 0.08 b
	1.5	-	-	-	7c	0.17 ± 0.10 c	0.08 ± 0.05 c

Mean values followed by the same letters are not significantly different at the 5% level.

used for shoot organogenesis (Tripepi 1997). Lower concentrations of auxins in combination with higher concentrations of cytokinin enhance the rate of shoot proliferation has been reported by many authors (Sharma et al. 1993, Rout and Das 1997, Sharma and Singh 1997). Media supplemented with 3.0 mg/l BAP + 0.5 mg/l NAA proved very effective in terms of organogenesis, shoot bud formation and its growth. Here 97% of the cultured explants became organogenic and maximum number of shoots (12.70 ± 0.25) and elongation (5.48 ± 0.31) were recorded after two subcultures in the same medium (Table 1, Figs 2 - 3). This finding is supported by Roy et al. (1994), who noted the suitability of 1.5 mg/l BAP with 0.5 mg/l NAA supplemented MS for shoot induction and multiplication of *Rauvolfia serpentina*. Almost similar observation was also reported in other medicinal plant species (Sudha et al. 1998, Lara et al. 2003, Hassan and Roy 2004, 2005). Other auxins IBA (0.1 - 1.5 mg/l) and IAA (0.1 - 1.5 mg/l) were also tested for shoot multiplication with 3 mg/l BAP but it was observed that IBA had no effect in combination with BAP. On the other hand, IAA had a little effect on production of multiple shoots. Kn alone also induced small percentage of multiple shoots. The maximum 33% cultures produced multiple shoots in 3 mg/l Kn.

In order to induce rapid elongation, multiple shoot at a stage of 2 - 4 cm long were individually subcultured on MS supplemented with reduced concentrations of BAP + IBA. Media fortified with 1.5 mg/l BAP + 0.5 mg/l IBA was proved effective for induction of rapid elongation (Fig. 4). Lui and Li (2001) noted that low BAP concentration appeared to be the key to successful shoot elongation. On elongation media shoot buds did not produce any roots. Thus the induction of strong and stout root systems the elongated shoot buds were individually cultured on full and half-strength MS with or without IAA, NAA and IBA (0.1-1.5 mg/l). Of the media combinations half strength MS with 0.5 mg/l NAA gave the best response and here 90% of the shoots produced on an average of 4.70 roots per shoot (Table 2, Fig. 5) within two weeks. Martin (2003) reported that half-strength MS fortified with NAA was superior to IAA and IBA supplemented with respect to the induction of roots in *Rotula aquatica*. Full-strength MS having same concentrations of NAA proved less effective with respect to *in vitro* root induction (Table 2). The effectiveness of NAA at lower concentrations for *in vitro* rooting has been reported in various medicinal plants (Mandal and Gupta 2001, Casado et al. 2002, Anand and Jeyachandran 2004). In PGRs free media no root formation occurred.

Shoots with well-developed roots were planted in small pots containing sterile garden soil. Those were acclimatized for another one week in growth chamber. To prevent evaporation and facilitated light transportation, pots were

covered by transparent polythene. Eighty per cent of the plantlets survived in out side natural conditions without any abnormal morphological changes (Figs 6 - 7).

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