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Regeneration of *Solanum villosum* Mill., via Direct Organogenesis *in vitro*: A Novel Study

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Abstract: Hormonally controlled efficient regeneration system has been developed *in vitro* by inducing direct shoot bud organogenesis from *Solanum villosum* Mill. leaf explants using Murashige and Skoog's culture medium supplemented with various factorial combinations of benzyleadenine (2.8-11.2 μm) and α-naphthalene acetic acid (0.11-0.54 μm) within 20 days of incubation in dark and room temperature conditions. The objective of the present study is to introduce *Solanum villosum* Mill. to *in vitro* regeneration technology. The best ratio of organogenic explants (18/20) was obtained using (8.4 μm BA plus 0.54 μm NAA). The masses of shoot buds obtained were divided into smaller clusters and transferred to hormone free half strength MS medium to allow their elongation to suitable length (about 15-20 mm). Shoots were individually excised from the mother clusters and allowed to continue growth on a fresh hormone-free half strength MS medium where they rooted normally. The maximum number of shoots (38.7±1.2) per explants was observed in explants previously treated with 8.4 μm BA plus 0.54 μm NAA. Growth of regenerants obtained from that hormonal combination was compared to the growth of corresponding regenerants obtained by stem nodal segments culture on ½ strength, hormone-free Murashige and Skoog's culture medium over a period of 40 days. Shoot length of regenerants obtained by organogenesis significantly exceeded those obtained from stem nodal segments. Both types of shoots rooted normally on the same culture medium, successfully acclimatized and transferred to soil.

Key words: *Solanum villosum* Mill., micropropagation, shoot morphogenesis, growth regulators, leaf explants

INTRODUCTION

According to the various studies, *Solanum villosum* has never been regenerated *in vitro* although many of the other closely related medicinal species e.g., *S. aethiopicum* (Gisbert *et al*., 2006); *S. virginum* (Borgato *et al*., 2007); *S. sessiliflorum* (Medina-Rivas *et al*., 2008) ; *S. trilobatum* (Dhavala *et al*., 2009); *S. nigrum* (Bhat *et al*., 2010); *S. americanum* (O'Connor-Sanchez *et al*., 2010) and *S. surattense* (Yadav *et al*., 2010) were successfully regenerated. Leaves and ripe fruits of *Solanum villosum*, commonly known as red-fruit nightshade, are used by many Indian (Yesodharan and Sujana, 2007) and African (Masinde *et al*., 2009) tribes. Leaves usually eaten as boiled salad while ripe orange berries are considered fruits (Edmonds and Chweya, 1997). The leaves contain carbohydrates, proteins, high levels of vitamins (A, B and C), mineral fibres (iron, calcium and phosphorus) and a vast array of useful secondary metabolites (Yang *et al*., 2008). Phytochemical analysis of chloroform methanol extract of green berries carried by Chowdhury *et al*. (2008) has also proved the presence of alkaloids, essential oils, flavonoids, steroids, terpenoids, saponins and phenolics. The same author reported also that leaves and berries extracts exhibited larvicidal and antimicrobial activities. In traditional medicine of African tribes, the unripe fruits are used to soothe toothache, squeezed on baby gums to ease pain during teething; leaves are used to treat stomachache; extracts from leaves and fruits are used to treat tonsillitis; roots boiled in milk usually given to children as a tonic; fruit juice is used to calm sore eyes; leaves are added to the diet as a treatment of hypertension-associated fever (Grubben and Denton, 2004).

Shoot organogenesis is the process by which shoots can be derived from meristematic or non meristematic plant cells either directly or indirectly *in vitro* by a sequence of developmental changes. *In vitro* organogenesis depends on many factors like type and age of explants, specific media formulations, definite growth regulators, genotype, carbon source, gelling agent, light regime, temperature and humidity (Gantait and Mandal, 2010). Micropropagation of plants via organogenesis has recently been reported for *Artemisia scoparia*

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(Aslam *et al*., 2006); *Valerina officinalis* (Abdi and khosh-Khui, 2007), *Capsicum annum* (Otroshy *et al*., 2011) and *Stevia rebaudiana* (Das *et al*., 2011).

The demand for Plants with nutritive and aurovedic properties like *S. villosum* (Edmonds and Chweya, 1997) is increasing worldwide (Prohens *et al*. 2003).The main purpose of the present work is to regenerate *Solanum villosum* Mill. *In vitro* which a key step towards micropropagation, improvement and many other biotechnological manipulations of this species.

MATERIALS AND METHODS

The present study was carried out from April, 2010 to January, 2011.

Explant preparation: Ripe fruits of *Solanum villosum* Mill. were collected from identified wild plants growing in Ibb Mountains (Yemen Republic) and a voucher specimen is available in herbarium of Department of Biology, University of Ibb. Fruits were pressed gently and the seeds were harvested, washed, dried and kept in Petri-dish for 3 weeks. The seeds were sown in pots and allowed to grow in greenhouse where they needed 3-4 weeks to germinate. Plants of 3 month old were used as a source of explants (leaf and Stem nodal segments). Leaf segments were prepared from distal parts of fully expanded leaf blades after cutting off and discarding the proximal ends (Fig. 1).

Surface sterilization of explants: Explants were subjected to a triple sterilization process using 70% ethanol for 30 sec, 20% (v/v) commercial bleach (5.25% free chlorine) for 3 min and 0.1% solution of mercuric chloride for 3 min and received 6 rinses with sterile distilled water (one between ethanol and chlorine, one between chlorine and mercuric chloride and four rinses after mercuric chloride treatment). The explants were then dried between two layers of sterile filter papers in a Petri-dish.

Shoot organogenesis: Leaf explants were cultured on Murashige and Skoog's (1962) basal medium enriched with 58.5 mM sucrose and supplemented with 2.8, 5.6, 8.4 or 11.4 μm benzyladenine combined with 0.11, 0.32 or 0.54 μm α-naphthqalene acetic and incubated in dark for 20 days at room temperature for induction of direct shoot organogenesis. For elongation of the shoot buds obtained by direct organogenesis from leaf explants, ½ strength hormone-free Murashige and Skoog's culture media was used (incubation conditions: 25°C and photoperiod 16 h light/8 h dark). Stem nodal segments were cultured directly on ½ strength Murashige and Skoog (1962) basal medium enriched with 58.5 mm sucrose and incubation conditions (25°C a photoperiod of 16 h light/8 h dark). Shoots obtained in both cases developed roots normally on hormone free medium.

Acclimatization and greenhouse cultivation: After suitable shoot and root growth of regenerants obtained from both types of explants was achieved, the plantlets were taken out from culture tubes, washed under tap water to remove agar debris and planted individually in plastic cups containing equal amounts of garden soil. Regenerants were covered with transparent plastic bags, acclimatized for a week by gradual exposure to normal greenhouse conditions and then transferred to larger pots and allowed to continue their *ex-vitro* growth.

Experimental design and statistical analysis: All experiments were repeated three times following a completely randomized block system. Statistical analysis was performed using SPSS statistical package software version 15 (USA). The results presented as Mean±Standard errors and analyzed with students t-test. The data were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Shoot bud organogenesis: Results of the present study (Table 1) have shown that direct shoot bud organogenesis was observed on leaf explants cultured on hormone-containing MS culture media without a callus phase in all hormonal treatments tested but the No. of such organogenic explants varied considerably according to the combination of BA and NAA used. The minimum ratio of explants responded (5/20) was obtained in culture

Table 1: Effect of various hormonal treatments (in μm) on the number of organogenic explants and the No. of shoots obtained per single leaf explants

	BA (μm)							
	2.8		5.6		8.4		11.2	
NAA (μm)	Org.	Sh No.	Org.	Sh No.	Org.	Sh No.	Org.	Sh No.
0.11	5/20	15.3±1.5	14/20	12.7±2.3	11/20	13.7±2.0	8/20	20.7±2.2
0.32	11/20	23.3±1.2	13/20	31.7±1.9	16/20	26.3±3.4	12/20	17.7±2.2
0.54	10/20	22.0±2.7	10/20	18.3±2.3	18/20*	38.7±1.2*	9/20	15.0±2.3

Each value is a mean of 3 determinations±Standard error. *significant value at $p<0.05$.Org: Organogenic explants, Sh No.: Shoot number

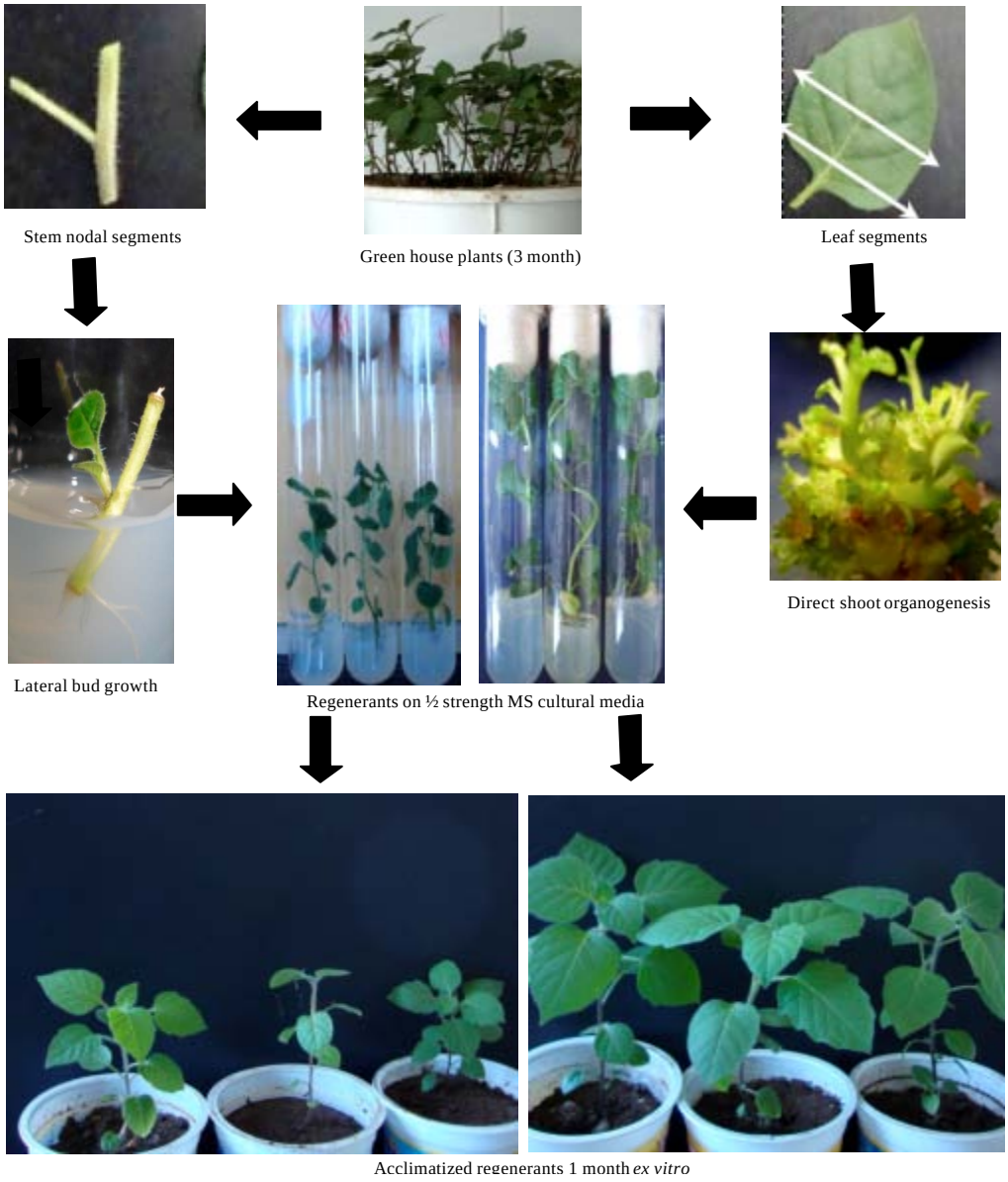

Fig. 1: Regeneration of *Solanum villosum in vitro*

medium which contained 2.8 μm BA combined with 0.11 μm NAA while the best response (18/20) was obtained with 8.4 μm BA and 0.54 μm NAA.

In agreement with this study, benzyladenine, alone or combined with auxins was also reported to regenerate other closely related herbaceous species like *S. nigrum* (Kolar *et al*., 2008; Xu *et al*., 2009); *S. trilobatum* (Arockiasamy *et al*., 2002; Jawahar *et al*., 2004) and *S. surattenese* (Yadav *et al*., 2010) but regeneration in these species differed from the regeneration obtained in this study in having a callus phase, producing-in most cases-a lower No. of regenerants per explant and needing auxin treatment for subsequent rooting of the obtained shoots. Quick and regeneration of plants via organogenesis without involving a callus phase is considered (Piccioni *et al*., 1997; Plader *et al*., 1998) reproducible and at the same time reduce the appearance of somaclonal variation.

Table 2: Shoot and root lengths (mm) and No. of leaves per regenerants obtained by stem nodal segments culture and shoot organogenesis

	Growth period in days							
Parameters	5	10	15	20	25	30	35	40
Shoot length								
S.E	7.2±1.1	14.4±2.2	22.3±2.4	30.3±2.9	34.4±2.9	38.5±2.9	44.9±3.0	48.9±33
L.E	12.2±1.3*	18.7±2.1*	27.9±2.2*	47.6±2.1*	56.8±2.3*	64.7±3.18*	70.7±2.9*	75.3±2.8*
Root length								
S.E	------	4.0±0.5	9.2±1.3	13.2±1.8	15.4±1.6	16.9±1.9	18.0±1.9	19.8±2.1
L.E	-------	4.1±0.6	9.1±1.7	14.1±2.2	15.7±2.1	17.0±1.9	18.0±2.1	18.8±2.3
Leaf number								
S.E	1.9±0.4	5.4±0.5	6.1±0.5	6.9±0.6	7.8±0.5	8.5±0.5	9.7±0.5	10.2±0.5
L.E	1.8±0.3	6.1±0.5	6.8±0.5	7.9±0.6	8.8±0.5	9.5±0.6	10.6±0.5	10.8±0.6

Each value is a Mean of 10 determinations±Standard error. *Significant value at $p<0.05$. S.E: Stem explants, L.E: Leaf explants

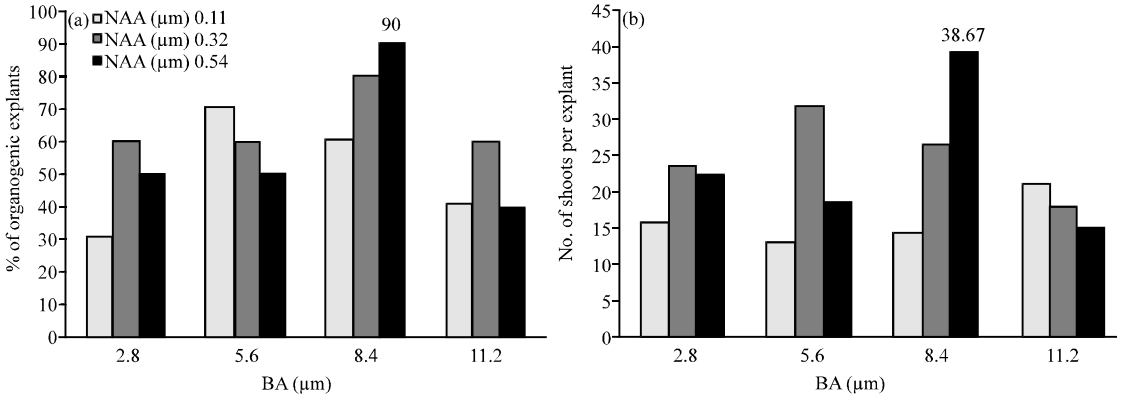

Fig. 2: (a) % of organogenic explants and (b) No. of shoots per explant under various hormonal combination treatments

Shoot elongation: In order to elongate the shoot buds obtained, the organogenic explants were transferred to half strength, hormone-free Murashige and Skoog's culture medium and allowed to grow under a photoperiod of 16 h illumination and 8 h dark per day and room temperature. The number of shoots which could be harvested from each organogenic explant differed according to the hormonal combination that was previously applied. After one week of growth of bud clusters on hormone free culture media it was possible to harvest a mean of 38.7±1.2 from each organogenic leaf explant from the treatment with 8.4 µm BA plus 0.54 µm NAA. The minimum number of shoots (11.7 per explant) was observed with the hormonal treatment (8.4 µm BA plus 0.11 µm NAA) so, considering the quality and quantity, it might be reported that the hormonal combination (8.4 µm BA plus 0.54 µm NAA) is the best. It seems also that the used growth substances for inducing organogenesis exhibited no adverse effects on subsequent shoot bud elongation and root morphogenesis that was reported in previous studies carried out by Marcotrigiano *et al.* (1996) and Fratini and Ruiz (2002) where direct organogenesis was obtained successfully in response to the use of the other synthetic cytokinins like thiadiazhuron (which is believed to be more effective than BA) but subsequent shoot elongation and rooting of the obtained regenerants got negatively affected.

With respect to stem nodal segments culture, the growth of lateral buds into shoots (one shoot per segment) could be observed few days only after cultivating the explants on hormone-free culture medium. Results presented in Table 2 and Fig. 1 and 2 show that no significant differences were observed between regenerants obtained from leaf or stem explants although significant differences were observed over the whole growth period in shoot length (this is probably due to the presence of residual amounts of the applied growth regulators that were used for induction of organogenesis). After 40 days of incubation on hormone-free culture media the regenerants obtained by stem nodal segments culture had a shoot length of 48.9 mm, a root length of 19.8 mm and a mean of 10.2 of leaves per regenerant while the corresponding regenerants obtained by direct organogenesis exhibited shoot length of 75.3 mm, root length of 18.8 mm and a mean number of leaves equal to 10.8. However, regeneration from nodal segments is also important, particularly, in avoiding somaclonal variation which was reported for example, in *S. aviculare* (Jasik *et al.*, 1997) regenerated via organogenesis *in vitro*.

Rooting and acclimatization: Regenerants rooted normally on MS culture media without the need of any exogenous application of auxins. Acclimatization and establishment of rooted plantlets from both stem nodal segments culture and organogenic explants in soil was carried out successfully (100%).

Regeneration of *S. villosum* Mill. which was carried out easily and successfully in this study can be used not only as a tool for quick and mass clonal micropropagation of selected heterozygous plants and for rootstock propagation (Gisbert *et al*., 2006) but also as a tool for plant improvement and production of pathogen-free plants. Efficient *in vitro* regeneration is a key step towards many successful genetic manipulations and transformation (Gisbert *et al*., 2006; Xu *et al*. 2009; O'Connor-Sanchez *et al*., 2010).

CONCLUSION

Solanum villosum Mill. was successfully regenerated *in vitro* via direct shoot organogenesis from leaf explants using various BA and NAA treatments. The best ratio of organogenic explants (18/20) and maximum number of shoots per explant (38.7±1.2) could be obtained using MS culture medium supplemented with 8.4 μm BA plus 0.54 μm NAA. Regenerants obtained from leaf segments or stem nodal segments culture rooted normally on half strength hormone-free culture media. Acclimatization and transfer to soil was carried out successfully (100%).

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