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BRIEF COMMUNICATION

In vitro regeneration of *Solanum nigrum* with enhanced solasodine production

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Abstract

High frequency of shoot formation was achieved from *Solanum nigrum* L. leaves on Murashige and Skoog (MS) medium without any callusing stage. Shoot forming ability was more pronounced on leaves positioned dorsally. For shoot induction, 2.0 mg dm^{-3} benzylaminopurine and 1.5 mg dm^{-3} kinetin were observed to be the most effective plant growth regulators (PGRs). The present paper also describes first successful induction of *in vitro* flowering in *S. nigrum.* The leaf derived shoots were excised and treated with various root promoting PGRs and 0.25 mg dm^{-3} indole-3-butyric acid produced maximum number of roots (15.2 per plant). Plants were later transplanted in field with 100 % survival. Solasodine content was higher in *in vitro* raised shoots and leaf derived callus, compared to *ex vitro* grown shoots.

Additional key words: black nightshade, HPTLC, *in vitro* flowering, plant growth regulators.

The *Solanum nigrum* (commonly known as black nightshade) is an important medicinal plant of the family *Solanaceae.* This plant contains two important alkaloids solamargin and solasonine. Several *Solanum* species have been effectively propagated by plant tissue culture (Chandler and Dodds 1983) in which leaf, nodal section and other explants were used for callus induction, regeneration and other *in vitro* morphogenesis studies (Shahzad *et al.* 1999, Bhat *et al.* 2004). Recently, direct *in vitro* plant regeneration from leaf explants has been reported (Sreedhar *et al*. 2008, Kai *et al*. 2008, Saritha and Naidu 2008, Mingozzi and Morini 2009). Cultured tissues have often been considered as a useful raw source for the synthesis of alkaloids (Datta and Srivastava 1997) but the production of solasodine from differentiated tissues has not been attempted extensively. Beside the involvement of callus, other alternative and fast regeneration route is also necessary for obtaining *Solanum* in masses. Direct induction of plants on explants is one such type of regenerating method which offers several advantages including the possibility of reducing somaclonal variation, common in plants regenerated from callus or suspension (Misra and Datta 2001, Dayal *et al*. 2003, Mujib *et al*. 2005). Regenerated plants with *in vitro* flowering bear immense importance in selective hybridization that uses pollens from rare stocks. It also helps to understand the flowering physiology which is complex and largely influenced by interaction of applied phytohormones, sugars, minerals, phenolics and other compounds (Tanimoto and Harada 1981). *In vitro* cultivation conditions also affect flowering, thus can be manipulated to develop alternative system to study flower morphogenesis (Ziv and Naor 2006).

The present article describes a direct *in vitro* regeneration of *Solanum nigrum* using leaf discs as explant. The leaf size, positioning of explant and the requirement of PGRs were studied. It also reports *in vitro* flower induction. Lastly, we estimated the content of solasodine in various tissues by HPTLC.

The leaf explant, excised from *in vitro* grown *Solanum nigrum* plants, have been categorized into several groups according to their sizes (0.5, 1.0 and 1.5 cm^2; Bhat *et al.* 2008). The leaf section cut perpendi-cular to the mid vein were separately tested by placing them with the adaxial or abaxial surface on solid Murashige and Skoog (1962; MS) medium. Benzylaminopurine (BAP)

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Abbreviations: BAP - 6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; PGRs - plant growth regulators.

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and kinetin of the same concentrations (0.25, 0.50, 1.0, 1.5 and 2.0 mg dm^{-3}) were used individually; the medium also contained 3 % sucrose. The pH of the medium was pre-adjusted to 5.8 before sterilization (121 °C for 20 min). Inoculated cultures were kept at 25 ± 2 °C under 12-h photoperiod (100 µmol m^{-2} s^{-1} PFD). *In vitro* grown plantlets produced flowers when kept in medium amended with either BAP or kinetin for 16 weeks.

The shoots were excised and cultured on rooting MS medium amended with different (0.25 - 2.0 mg dm^{-3}) concentrations of indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA). Hardening of the rooted shoots was made by transferring plantlets from MS to half strength MS and finally to the medium without organics. The plantlets were maintained for 2 weeks on the above medium, later transferred to the pots containing *Soilrite*, covered with perforated transparent polythene bags, kept at culture room for 10 d. Later, the polythene bags were removed and the pots were kept at the same cultural condition, as mentioned earlier. After one week, the plants were transferred to the field.

The soluble protein content was estimated following Bradford's method (1976) where absorbance was measured at 595 nm by a spectrophotometer (*Blackman DU 640 B*, Fullerton, USA) and protein content was calculated using the standard curve of bovine serum albumin. The soluble sugar was estimated by the method of Dey (1990). Lee and Takahashi (1966) method was used for the estimation of soluble amino acids. Chlorophyll *a*, chlorophyll *b* and carotenoid contents were estimated according to the method of Hiscox and Israelstam (1979). The absorbance was recorded at 480, 510, 645 and 663 nm, respectively, with a *Blackman DU 640 B* spectrophotometer (Fullerton, USA).

The content of solasodine in field grown plants and in *in vitro* cultures was estimated using solasodine (*MP Biomedicals*, Eschwege, Germany) as standard. A modified Bradley's method was used for extraction in which dried and powdered field grown plant (leaf) and lyophilized *in vitro* cultures (500 mg each) were refluxed separately in 15 cm^3 of 1 M HCl on water bath for 2 h. It was filtered and washed with the same solvent. The pH of filtrate was adjusted to 10 using a dilute ammonia solution (10 % v/v); basic solution was extracted with $CHCl_3$ (20 cm^3 each) thrice to ensure complete extraction. The $CHCl_3$ extracts were pooled and evaporated on water bath (below 40 °C). Dried extracts were further dissolved in methanol for application to TLC plate for quantification. The samples were spotted in the form of bands (width 3 mm) with a *Camag* (Muttenz, Switzerland) syringe on precoated silica gel aluminium plate *60F-254* (E. Merck, Darmstadt, Germany). The mobile phase consisted of chloroform and methanol (5:1, v/v). TLC plates were then dried and densitometric scanning was performed on *Camag TLC* scanner *IV* (absorbance at 510 nm) after spraying with anisaldehyde sulphuric acid and dried at 100 °C. Deuterium and tungsten lamps were used as radiation sources.

Statistical analyses were performed using analysis of variance following Duncan's multiple range test in which each of the experiment was conducted at least twice with 5 replicates per treatment.

After week incubation the wounded regions of the excised leaf started to produce small protuberances which developed into microshoots. The number of shoots, increased with time. It is clear that the shoot forming ability was noticed in both dorsal and ventral surfaces but leaf length influenced shoot production (Table 1). Maximum number of shoots (19.6 from a leaf disc of 1.5 cm^2 placed dorsally) was observed on medium with 1.5 mg dm^{-3} kinetin. Shoot forming response was poor on low concentration of kinetin (0.25 mg dm^{-3}). BAP at 0.5 mg dm^{-3} showed also good response. Kinetin was observed to be more suitable compared to BAP, also in leaves placed ventrally. Maximum number of shoots (13 shoots per leaf disc) was obtained on 1.0 mg dm^{-3} kinetin. Shoots originated from dorsal side grew faster compared to shoots derived from ventral surface. Shoot length increased with enhanced concentration of kinetin.

Table 1. Effects of BAP and kinetin on number of shoots per explant. Explants of different sizes (0.5, 1.0 and 1.5 cm^2) were cultured either dorsally or ventrally on MS medium. Data were scored after 4 weeks. Values are the means of three replicates. Means in each column with the same letters are not significantly different at $P \leq 0.05$ according to DMRT.

PGR	[mg dm^{-3}]	Dorsally			Ventrally		
		0.5 cm^2	1.0 cm^2	1.5 cm^2	0.5 cm^2	1.0 cm^2	1.5 cm^2
BAP	0.25	2.6e	3.6e	9.0c	1.3d	2.6d	8.0d
	0.50	6.3c	5.3d	5.3de	2.6c	4.6c	4.3e
	1.00	3.3e	4.3e	4.3e	1.6d	3.0d	4.6e
	1.50	3.0e	3.6e	6.0d	2.6d	4.3c	4.0ef
	2.00	4.6d	6.6c	11.3b	4.6c	7.3b	10.0c
KIN	0.25	1.6f	1.3f	2.3f	1.0d	0.0e	3.0f
	0.50	7.3c	5.3d	8.0c	5.0b	4.3c	4.3d
	1.00	8.6b	9.0b	12.6b	7.0a	9.3a	13.0a
	1.50	9.6a	12.0a	19.6a	4.7b	7.0b	11.6bc
	2.00	8.0c	9.3b	12.3b	5.3b	5.3c	12.6b

The shoots from dorsally positioned leaves cultivated in kinetin added medium showed maximum soluble protein, soluble amino acid, sugar and pigment contents. Microshoots grown at MS + 0.5 mg dm^{-3} BAP or 0.5 mg dm^{-3} kinetin produced healthy *in vitro* flowers. The plants retained the flowers for over 2 weeks or more, but showed premature flower-senescence without setting any fruit.

Table 2. Root induction in different PGRs treated MS medium. Data were scored after 4 weeks. Means of three replicates. Means in each column with the same letters are not significantly different at $P \leq 0.05$ according to DMRT.

PGR	Concentration [mg dm^{-3}]	Root number [$plant^{-1}$]	Root length [cm]
IBA	0.25	15.2a	4.5a
	0.50	14.3a	4.5a
	1.00	6.5d	4.1a
	2.00	3.2g	3.5ab
IAA	0.25	15.2a	2.6b
	0.50	9.0c	2.9b
	1.00	7.5d	2.2b
	2.00	6.4de	2.1bc
NAA	0.25	12.0b	3.4ab
	0.50	5.0ef	2.5bc
	1.00	4.4f	2.1bc
	2.00	3.2g	1.1c

The *in vitro* raised shoots were transferred individually to MS added with various auxins at 0.25 - 2.0 mg dm^{-3}. Although rooting was observed in all the cultures (100 %), maximum number of roots (15.2 per plant) was observed at 0.25 mg dm^{-3} IBA (Table 2) with average root length of 4.5 cm after 4 weeks of incubation. The response was poor at higher auxin concentrations, *e.g.*, 2.0 mg dm^{-3} NAA produced only 3.2 roots with length 1.1 cm. The rooted plantlets were successfully transplanted to pots with *Soilrite*, kept at 30 ± 2 °C in plant growth chamber for hardening. The plantlets grew well and attained 15 cm height within 2 weeks. The morphological characteristics of *in vitro* raised plants like leaf size, shape and plant height were very similar to field grown plants.

The maximum solasodine content [2.34 mg g^{-1}(d.m.)] was observed in *in vitro* raised shoots derived from leaves, followed by non regenerative callus [0.76 mg g^{-1}(d.m.)] and the lowest [0.51 mg g^{-1}(d.m.)] was in field grown shoots.

In this study we observed direct shoot formation on leaves in *S. nigrum*. Green juvenile leaves of 1.5 cm^2 were efficient to induce higher number of shoots compared to leaves of smaller sizes. Shoot bud formation ability proceeded mostly on leaf margin compared to other regions; this study further demonstrated that dorsal surface was more responsive compared to ventral one. The different gradients of endogenous PGRs, existing in different parts of the leaf, are probably involved in such response (Wernicke and Milkovits 1986, Mujib *et al.* 1996). Similarly, the orientation of explant on nutrient medium seemed important as it affects auxin transport and nutrient uptake in cultured tissues (Mujib 2005). We found that kinetin was very active PGR, which is in contrast to several earlier observations where BAP's role on shoot production has been pointed out (Mujib 2005). These results are in agreement with previous study (Hassanien 1999) where *S. nigrum* regeneration was high and fast in comparison to many other plant species (Binding 1985, Binding *et al*. 1988). The proteins, amino acids, sugars and other biochemical reserves were noted to be high during shoot morphogenesis. In this study we also observed *in vitro* flowering but the shoots exhibited premature flower senescence without setting any fruit or seed. BAP has been found to promote flower buds in plans like tobacco (Smulders *et al.* 1990), maize (Mandal *et al.* 2000). There are a few reports of plant species, which developed *in vitro* flowers, for example cauliflower (Kumar *et al.* 1995), coriander (Stephan and Jayabalan 1998), bamboo (Nadgauda *et al*. 1990, Singh *et al*. 2000), maize (Mandal *et al.* 2000), *etc*.

Quantitative analysis of solasodine in cultivated tissues (callus, shoot) showed that its content was significantly higher in shoots as compared to callus and field grown plant. This result corroborated with earlier study where a significant increase in alkaloid content was reported in *Narcissus confusus* cultures (Selles 1999). The mechanism by which *in vitro* raised tissues overproduce alkaloid is not known. The enriched medium with added carbon source, optimized photoperiod or

Table 3. Contents of chlorophyll, sugars, amino acids and proteins [mg g^{-1}(d.m.)] in shoots regenerated from dorsally and ventrally positioned leaf, cultured on MS + 2.0 mg dm^{-3} BAP for 4, 8 or 12 weeks. Means of five individual readings. Means with the same letters in each row are not significantly different at $P \leq 0.05$ according DMRT.

Parameter	4 weeks		8 weeks		12 weeks	
	ventral	dorsal	ventral	dorsal	ventral	dorsal
Chlorophyll	1.58d	1.47e	1.73b	1.58d	1.82a	1.69c
Sugars	4.20f	9.64e	12.3c	11.9d	14.8a	13.1b
Amino acids	2.24e	1.97f	4.36c	3.57d	6.20a	6.12b
Proteins	9.67b	9.20d	9.83a	9.46c	9.84a	9.45c

the stresses may enhance synthesis of alkaloids in culture (Rocha *et al.* 2005, Bhat *et al.* 2008). We suggest that certain level of differentiation may be necessary for enhanced content of secondary metabolites (see also Pande 2002, Ma *et al*. 2006).

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