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An efficient protocol for *in vitro* propagation of *Solanum nigrum* L. from nodal explants

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An efficient method for *in vitro* propagation of *Solanum nigrum* L has been developed. The nodal explants were cultured on MS medium supplemented with different concentrations of BAP and KIN. The two cytokinins tested, KIN was found to respond well in shoot multiplication and number of shoots from the nodal explants when compared to BAP. Large number of shoots was produced from all the concentrations of both the cytokinins. The highest frequency of 100% shoot induction was observed on MS medium supplemented with 10-15 µM KIN and BAP. The number of shoots produced on the basal medium supplemented with BAP ranged between 40 and 46 and 43 and 49 with KIN 30 days after inoculation. Excised shoots were transferred to rooting medium containing different concentrations of IBA and 2,4-D for root induction. The roots were initiated and well developed on a medium fortified with 10-15 µM of both the auxins. Of the two auxins tested, more number of roots were produced on the medium containing IBA. Maximum number of 47 roots per shoot were produced in 30 days on MS medium supplemented with 10 µM IBA. The well rooted plantlets were transplanted to the paper cup for hardening and the well established plants were transferred to the field for acclimatization.

Key words: *In vitro* regeneration, shoot induction, rooting, hardening

Introduction

Solanum nigrum L. (Black nightshade) a member of Solanaceae is a common herbaceous plant distributed everywhere. It is cultivated as a food crop, both for its leaves and fruits. The stem may be glabrous or hairy. The flowers usually white with five regular parts. The leaves alternate and some what ovate with irregularly toothed wavy margin. The fruit is a round fleshy berry and black when ripe. The seeds are brown and numerous (Gamble, 1921; Edmonds and Chewya, 1997). The leaves and fruits contain toxic alkaloid solanine. It is a glyco-alkaloid with the highest concentrations in the unripened

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berries (Cooper and Johnson, 1984). When ripe, the berries are the least toxic part of the plant and are sometimes eaten without ill effects (Watt and Breyer-Brandwijk, 1962). Solanine may be separated by chromatography into six components (Merck, 1989). Solanidine is obtained after hydrolysis of solanine, and is less toxic. The leaves and fruits are popularly used as vegetable in the preparation of soup. The leaves blend well with other greens and pulses to make porridges in southern parts of India. Besides being used for human consumption, the leaves serve as fodder and browse for domestic herbivorous animals (Akubugwo, *et al,* 2007). The whole plant is antiperiodic, antiphlogistic, diaphoretic, diuretic, emollient, febrifuge, narcotic, purgative and sedative (Singh and Kachroo, 1976; Emboden, 1979; Lust, 1983; Duke and Ayensu, 1985). It is harvested in the autumn when both flowers and fruit are upon the plant, and is dried for later use (Grieve, 1984). The leaves, stems and roots are used externally as a poultice, wash, etc. in the treatment of cancerous sores, boils, leucoderma and wounds (Duke and Ayensu, 1985; Moerman, 1998). Extracts of the plant are analgesic, antispasmodic, anti-inflammatory and vasodilator (Duke and Ayensu, 1985; Ravi *et al*, 2009). The plant has been used in the manufacture of locally analgesic ointments and the juice of the fruit has been used as an analgesic for toothaches (Chiej, 1984). The plant has also been used as insecticide (Merck, 1989) and larvicide (Singh, *et al*., 2001).

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. It is a method of vegetative propagation conducted in the laboratory condition and it has a significant impact on plant breeding, horticulture and medicine. It is the ever-ready tool for specialization in hybridization either by sexual or asexual means. It is a suitable method for obtaining a large quantity of genetically homogenous and healthy plant material which can be used for planting (Pierik, 1987). This technique is an alternative method of propagation as there is an increase in the propagation rate of plants, availability of plants throughout the year, protection of plants against pests and pathogens under controlled conditions and the availability of uniform clones and uniform production of secondary metabolites (Bajaj, *et al.*,1988). Some micropropagation works have been conducted from the various explants of *S. nigrum*. Akhterjahan and Hadiuzzaman (1996) obtained plant regeneration from shoot tip, stem, leaf and root segments of *S. nigrum* through callus culture. They obtained callus on MS medium with NAA, regeneration of shoots on BAP and rooting on IBA. Hassanein and Soltan (2000) cultured shoots from shoot cutting of germinated seeds of *S. nigrum* on different media (B5, MS or SH) and observed the best culture condition for shoot formation was the culture of stem internode segments on B5 medium supplemented with 0.5 mg dm^{-3}

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BAP at 16-h photoperiod (irradiance of 100 µmol m^{-2} s^{-1}). Direct organogenesis and *in vitro* flowering was obtained in *S. nigrum* by Venugopal, *et al.* (2005). The highest frequency and number of multiple shoots were obtained from leaf and nodal explants on MS medium supplemented with benzyladenine and IAA. Regenerated plants rooted and flowered on rooting medium supplemented with IBA or IAA. Basha, *et al.* (2008) made a successful induction of callus from *S. nigrum* L. on MS basal medium supplemented with IAA and BAP. Regeneration shoots from callus and *in vitro* flowering were obtained on MS medium fortified with BAP and IAA or NAA or 2,4-D. The best rooting was obtained on MS containing 0.5 mg/l IBA. Kannan *et al*. (2005) reported the *in vitro* regeneration of *S. nigrum* using different plant growth regulators and concluded that BAP 0.5 mg/l, 2, 4-D 1.0 mg/l and IBA gave the highest frequency of the well growing shoot. Yogananth, *et al* (2009) showed the accumulation of the alkaloid solasodine in the callus of *S. nigrum*. Hanan *et al.* (2010) produced *S. nigrum* with a high power of alkaloid accumulation through *in vitro* regeneration trials followed by *in vivo* plant acclimatization. MS-basal medium containing BA and NAA (0.5 mg/ml each) was the best for both plants. A series of *in vitro* and *in vivo* plants were successfully produced and chemical analysis revealed contents of glycoalkaloids higher than those reported for intact field plants. Bhat *et al.* (2010) obtained high frequency of shoots directly from the leaf explant of *S. nigrum* on MS medium supplemented with BAP and KIN without any callusing stage. Though some micropropagation studies have been conducted so far, this paper deals with the efficient plant regeneration system with large number of shoots within a short period.

Materials and Methods

Sterilization, Media and Explants

All the glassware was washed thoroughly with chromic acid (potassium dichromate and sulphuric acid, 2:1 w/v), rinsed in tap water and then with distilled water. Sterilization of glassware, forceps and scalpels for micropropagation was done in an autoclave at 121°C for 20 minutes at 1.06 kg cm^{-2}. MS basal medium (Musarhige and Skoog, 1962) was used along with different concentrations of Plant Growth Regulators for shoot multiplication (BAP and KIN) and rooting (IBA, NAA and 2,4-D). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 1 N HCl prior to autoclaving (121°C at

1.06 kg cm^{-2} for 20 min). The explants were collected from the field grown plants of *Solanum nigrum* L. in and around the college campus. The plant materials, nodal explants with axillary bud, were rinsed with running tap water with few drops of liquid soap (Teepol). The explants were then washed with distilled water 3 or 4 times and further sterilization was carried out in Laminar Air Flow chamber under aseptic condition prior to inoculation. The explants were sterilized with 70% alcohol for 30-45 sec and 0.1% (w/v) $HgCl_2$ for 5 min. The explants were then washed 4-5 times with sterile distilled water.

Inoculation Procedure

For multiple shoot induction, the nodal explants with axillary bud were placed on MS medium supplemented with different concentrations of benzylaminopurine (BAP: 5-25 µM) or kinetin (KIN: 5-25 µM). *In vitro* raised shoots of 2 cm and above were excised from the culture tube and subcultured into MS medium supplemented with various concentrations (2-10 µM) of indole butyric acid (IBA: 5-25 µM) or 2,4-dichlorophenoxy acetic acid (2,4-D: 5-25 µM). The root number and length were measured in each culture medium.

Culture maintenance and conditions

All cultures were maintained at 25±2°C in a culture room under cool white fluorescent lamps (Phillips, India) at intensity of 50 µmol m^{-2} s^{-1} with 16 hrs photoperiod.

Hardening and Acclimatization

Plantlets with well-developed roots were dislodged from the culture medium and roots were washed gently under running tap water to remove the adhering medium. Plantlets were transferred to plastic cups (10 cm diameter) containing autoclaved garden soil, farmyard manure and sand (2:1:1). Each plantlet was irrigated with distilled water every 2 days for 2 weeks followed by tap water for one week. The potted plantlets were initially maintained under culture room conditions in 3 weeks and later transferred to normal laboratory conditions in 2 weeks. The potted plantlets were initially covered with porous polyethylene sheets to maintain high humidity and were maintained inside the culture room. The relative humidity was reduced gradually. After 30 days the plantlets were transplanted to the field under shade for 3 weeks and then transplanted to the soil for further growth and development.

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Experimental Design, Data Collection and Statistical Analysis

All experiments were preformed using a randomized comletely block design and each experiment consisted of five explants per flask and five replicate culture flasks per plant growth regulator treatment. The parameters recorded were frequency (number of cultures responding in terms of multiple shoot proliferation and root development), number of shoots per explant, shoot length, number of roots per shoot, root length and survival rate (%). All of the experiments were repeated five times. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% level of significance (Gomez and Gomez, 1976).

Results and Discussion

Shoot Multiplication

Shoot multiplication was observed on MS medium supplemented with all the concentrations of BAP and KIN within 10 days of inoculation (Fig. 1a). Shoot multiplication frequency was observed on both the hormones (Fig. 1b,c). The percentage of shoot induction frequency ranged from 80-100% and 90-100% on MS medium supplemented with BA and KIN respectively (Table 1). Large number of shoots was produced in 30 days of culture. The highest frequency of 100% shoot induction was observed on MS medium supplemented with 10-15 µM KIN and BAP.

However, variations among the two hormones were observed in number of shoots and shoot length. The number of shoots produced on the basal medium supplemented with BAP ranged between 40 and 46 with the highest in 15 µM BAP 30 days after inoculation. The number of shoots ranged between 43 and 49 on MS medium supplemented with KIN with the highest in 10-15 µM KIN (Table 1) in the same period of culture. Of the two cytokinins tested, KIN was found to respond well in shoot multiplication and number of shoots from the nodal explants when compared to BAP. A large number of shoots was formed from the nodal explants of *S. nigrum* within a short period of 45-60 days.

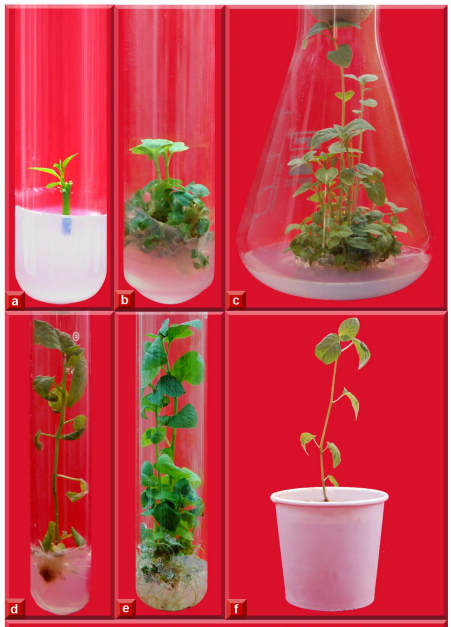

Fig.1. *In vitro* propagation of *Solanum nigrum* L. from nodal explant. a. nodal explant growing on MS basal medium supplemented with cytokinins; **b.** shoot proliferation 20 days after inoculation; **c.** shoot proliferation and elongation after 30 days; **d.** induction of root from the isolated shoot on MS medium supplemented with auxins; **e.** establishment of root; **f.** Hardening.

The shoot length varied from 7-9 cm in both the cytokinins. Shoots growing on MS medium supplemented with 15 µM BAP reached 9.24 cm height in 30 days of culture. Shoots growing on MS medium with 15 µM KIN reached 9.46 cm height in the same period (Table 1).These results showed both the cytokinins tested were found to initiate and proliferate shoots from the nodal explants of *S. nigrum*. However, KIN was found to be more suitable for shoot multiplication when compared to BAP.

The shoot induction and proliferation depend on plant growth regulators and types of explants (Patnaik and Chand, 1996; Mohamed *et al*., 1999). In many plants, multiple shoots were obtained from the shoot tips or axillary buds by administering BAP or KIN (Kumar *et al*., 1998; Sahoo and Chand, 1998; Velayutham, 2003; Baskaran and Jayabalan, 2005; Bhat *et al*., 2010). In the present study, a large number of shoots was produced from the nodal explant of *S. nigrum* on MS medium supplemented with both BAP and KIN separately within a short period of 30-45 days.

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Table 1. Effect of different concentrations of cytokinins on shoot induction from the nodal explant of *Solanum nigrum* L.

BAP	KIN	Percentage of response	Number of shoots	Shoots lenght
5 µM	-	95[b]	41 ± 0.96 [c]	7.26 ± 1.86 [de]
10 µM	-	95[b]	43 ± 1.24 [b]	8.68 ± 2.00 [b]
15 µM	-	100[a]	47 ± 1.86 [b]	9.24 ± 1.96 [a]
20 µM	-	95[b]	46 ± 1.56 [a]	7.58 ± 1.96 [c]
25µM	-	85[c]	40 ± 1.96 [cd]	7.48 ± 1.86 [d]
-	5 µM	90[c]	40 ± 1.33 [cd]	8.65 ± 1.32 [de]
-	10 µM	100[a]	45 ± 0.96 [c]	9.42 ± 1.96 [ab]
-	15 µM	100[a]	49 ± 1.32 [a]	9.46 ± 1.86 [a]
-	20 µM	95[b]	43 ± 0.96 [d]	9.12 ± 1.95 [c]
-	25 µM	95[b]	43 ± 0.96 [d]	8.84 ± 1.96 [d]

Values in the last two columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at $P<0.05$ by Duncan's Multiple Range Test (same letter within the column of the respective growth regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference)

Though several growth regulators are available for shoot multiplication, BAP and KIN are widely used. Of the two cytokinins tested, KIN was more effective in shoot induction and proliferation than BAP. In several studies BAP was more effective in inducing bud break resulting in the sprouting of a large number of shoots (Sahoo and Chand, 1998; Kadota and Niimi, 2003; Velayutham and Ranjithakumari, 2003; Martinussen *et al.*, 2004; Vasudevan *et al.*, 2004). However, in the present investigation, KIN was found to be more efficient in shoot multiplication.

Rooting

Excised shoots were transferred to rooting medium containing different concentrations of IBA and 2,4-D (5-25 µM) for root induction. Root initiation was observed on MS medium supplemented with all concentrations of IBA and 2,4-D in 10 days of culture (Fig.1d,e). The root induction frequency was more

or less similar in all the concentrations of both the auxins (Table 2). However, the roots were initiated and well developed on a medium fortified with 10-15 µM of both the auxins (Fig.1d,e; Table 2). Of the two auxins tested, more number of roots were produced on the medium containing IBA. Maximum number of 47 roots per shoot with the mean length of 5.5 cm were produced in 30 days on MS medium supplemented with 10 µM IBA (Table 2).

Table 2. Effect of different concentrations of auxins on root induction from the isolated shoots of *Solanum nigrum* L. (after 30 days)

BAP	KIN	Percentage of response	Number of shoots	Shoots lenght
5 µM	-	95	42± 1.96 ^{bc}	4.73± 2.01 ^{bc}
10 µM	-	95	47± 0.95 ^{a}	5.50± 0.75 ^{a}
15 µM	-	100	45± 1.20 ^{ab}	5.00± 1.96 ^{b}
20 µM	-	95	35± 2.66 ^{d}	4.65± 2.93 ^{cd}
25µM	-	85	30± 2.98 ^{e}	3.90± 2.26 ^{e}
-	5 µM	85	27± 2.91 ^{d}	3.80± 2.72 ^{cd}
-	10 µM	95	33± 2.36 ^{b}	4.45± 1.82 ^{b}
-	15 µM	100	36± 1.20 ^{a}	4.95± 0.96 ^{a}
-	20 µM	90	30± 2.52 ^{c}	3.90± 2.12 ^{c}
-	25 µM	80	25± 2.88 ^{de}	3.65± 2.72 ^{de}

Values in the last two columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at $P<0.05$ by Duncan's Multiple Range Test (same letter within the column of the respective growth regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference

In most of the studies IAA, IBA and NAA were used for root induction. High frequency of rooting was achieved by IAA in *Syzygium cuminii* (Jain and Babbar, 2003), *Gossypium arboreum* and *G. hirsutum* (Bajaj and Gill, 1986) and IBA in *Aristolochia indica* (Manjula *et al.*, 1997), *Gymnema sylvestris* (Komalavalli and Rao, 2000), *Avicennia marina* (Al-Bahrany and Al-Khayri, 2003) and *Eclipta alba* (Baskaran and Jayabalan, 2005). Higher frequency of roots was observed in *Cichorium intybus* at 5 µM NAA (Velayutham and Ranjithakumari, 2003), *Rubus chamoemorus* (Martinussen *et al*., 2004), *Viburnum odoratissimum* (Schoene and Yeager, 2005), *Plumbago zeylanica* at

3 µM NAA (Velayutham *et al.*, 2005) and *Aerva lanata* at 6 µM NAA (Suganya *et al.*, 2005). Jabeen *et al.* (2005) also showed that NAA was found to induce more number of roots when compared to IAA and IBA in *Solanum nigrum*. In the present study also IBA was found to induce more number of roots when compared to 2, 4-D.

Hardening and acclimatization

The well rooted plantlets were transplanted to the paper cup (Fig. 1f) containing a mixture of autoclaved red soil, farm yard manure and sand in the ratio of 2:1:1. The survival rate of these plants was 80%. The established plants were transferred to the field for acclimatization.

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