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Original article

Regeneration efficiency based on genotype, culture condition and growth regulators of eggplant (*Solanum melongena* L.)

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ABSTRACT

Several experiments were carried out to establish an efficient regenerating protocol for cultivated eggplant varieties. Among the five varieties cultured on Murashige and Skoog (MS) medium with free plant growth regulator (PGR), *Nayantara* performed better considering the number of shoots/explant (2.48). Considering explant types and culture conditions, better performance was observed (3.68 shoots/explant) when seed germination in the dark was proceeded by bottom hypocotyl segments cultured under dark conditions. A higher rate of shoot regeneration was observed in *Nayantara* when cultured in Zeatin Riboside (ZR) and Thidizuron (TDZ) supplemented MS medium. The highest number of shoots per explant was produced on MS medium supplemented with 2.0 mg/L ZR and 0.1 mg/L indole acetic acid (6.65 shoots/explant). Proliferation and elongation of the regenerated shoots were obtained in the MS medium with free PGR. The best rooting performance was observed in MS medium supplemented with 2.0 mg/L indole butyric acid. Plantlets with well developed roots and shoots were successfully transferred to soil.

Introduction

Eggplant (*Solanum melongena* L.) (2n = 24), is an important vegetable crop of tropical and temperate regions and is a popular and principal vegetable crop in Bangladesh where it is the second most important vegetable crop after potato in terms of area under cultivation (Yearbook of Agricultural Statistics of Bangladesh, 2007). The crop is susceptible to several diseases (*Phomopsis* blight, southern wilt, anthracnose) and pests especially brinjal shoot and fruit borer that causes serious yield losses, but efforts to overcome these problems, using hybridization with wild *Solanum* species are limited by sexual incompatibilities (Collonier et al., 2001; Kashyap et al., 2003). In addition, traditional improvement methods are hampered by the scarcity of natural resistance sources. Introgression of desired traits such as parthenocarpy, improved nutritional value and post harvest qualities into the cultivated varieties is difficult to achieve due to the lack of appropriate sexually compatible varieties or species (Collonier et al., 2001).

Eggplant tissues show a high morphogenetic potential under *in vitro* culture conditions that could be useful for developmental studies as well as for establishing biotechnological approaches to produce improved varieties. Organogenesis from various explants such as leaf, cotyledon, hypocotyl, epicotyl, anther and isolated microspores has been reported previously (Isouard et al., 1979; Gleddie et al., 1983; Sharma and Rajam, 1995; Magioli et al., 1998). Furthermore, culture conditions affecting regeneration via organogenesis (Mukherjee et al., 1991) have been well documented. In those protocols, the regeneration efficiency has been reported to be affected by different factors, such as the combination of plant growth regulators, explant type and genotype. Such variability in the tissue culture response necessitates the optimization of culture protocols for particular, elite varieties in different geographical regions. Bangladesh being one of the centre of origins of this crop, having available efficient protocols for *in vitro* regeneration and genetic transformation will offer an excellent model system to investigate plant physiology *in vitro* and pave the way for the development of pest resistance and stress tolerance in this crop.

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Materials and methods

Plant materials

Seeds of five eggplant varieties—*Nayantara, Kazla, Islampuri, ISD-006* and *Uttara*—were collected from the Horticulture Research Center, Bangladesh Agricultural Research Institute, Gazipur. Seeds were first dipped into 100% alcohol for 20–30s followed by washing four times with sterile distilled water. After that Tween 20 (3–5 drops) was added into 100 mL 40% Clorox (2% sodium hypochlorite) and shaken gently. Seeds were dipped in a 50 mL conical flask containing the prepared solution for 20 min with frequent shaking followed by washing with sterile distilled water four times. Finally, 25–30 seeds were inoculated in each baby jar containing 30 mL agar-solidified MS medium with 3% sucrose for supporting seedling development. Culture conditions were maintained by germinating the seeds both in the dark and in the light followed by incubation in the dark and light according to the experimental objectives. After seed germination, incubation was maintained for 2 wk for both cases. The explants from the dark culture treatment were transferred to light after 2 wk to allow proper growth of the regenerated shoots.

Effects of genotypes on organogenesis

The first experiment evaluated genotypic variation among genotypes. Five varieties of eggplants—*Nayantara*, Kazla, Islampuri, ISD-006 and Uttara—were tested for their innate ability to regenerate multiple shoots on growth-regulator-free, MS medium using hypocotyl explants on the basis of a previous observation that hypocotyl segments of eggplant can produce shoots on growth-regulator-free, MS medium (Akhond and Bhuiyan, 2001). This experiment had four replications with nine explants plated for each replication. According to the results, *Nayantara* was the best variety to produce maximum effective shoots. Therefore, *Nayantara* was used in the subsequent experiment.

Effects of dark pretreatment and type of explant

This experiment aimed to determine the dark pretreatment effect (both in seedling raising and culturing) and to study the morphogenetic response of different regions of the hypocotyl in MS medium. Seeds of *Nayantara* were germinated under dark and light conditions and cultured under both dark and light conditions. In the dark treatment, after 2 wk of dark, the germinants were transferred to light conditions. The samples under the light conditions received normal culture as described below. Each hypocotyl was cut into 6–8 mm long top, middle and bottom segments that were cultured on growth-regulator-free, MS medium to determine the suitable culture conditions as well as the suitable explant type. Responses of bottom hypocotyl segments were found to be better compared to the top and middle parts. Furthermore, seed germinated in the dark followed by culture under dark conditions was found to be best among the treatments. This experiment had four replications with nine explants plated for each replication.

Regeneration response against plant growth regulator

This experiment was carried out to determine the effects of growth regulators on adventitious shoot development. Treatments were composed of PGR-free, MS medium, naphtaleneacetic acid (NAA; 0.0, 0.5 and 1.0 mg/L), 6-benzylaminopurine (BAP 0.0, 2.5 and 5.0 mg/L), thidizuron (TDZ; 0.1 and 0.2 mg/L), zeatin riboside (ZR; 1.0 and 2.0 mg/L) and indole acetic acid (IAA; 0.1 mg/L) either singly or in combinations. The combinations of these five PGR are shown in Table 3. Each treatment contained nine explants with four replicates.

Table 1
Regeneration efficiency of different eggplant varieties on MS medium.

Cultivar	Responsive explant (%)	Number of shoots/ explant	Rooted shoots (%)
Kajla	80.67^{ab}	1.63^{bc}	79.17^{ab}
Nayantara	84.67^{a}	2.48^{a}	95.00^{a}
ISD-006	67.00^{c}	2.05^{ab}	100.00^{a}
Islampuri	70.33^{bc}	1.27^{c}	60.00^{b}
Uttara	83.00^{ab}	1.47^{bc}	77.67^{ab}

Means followed by different letters within a column are significantly different at $p < 0.05$ (Duncan, 1955).

Table 2
Effect of culture condition and explants type on shoot formation of eggplant variety Nayantara

Germination conditions	Culture conditions	Number of shoots/explant		
		Top hypocotyl	Mid hypocotyl	Bottom hypocotyl
Dark	Dark	1.30	1.25	3.68
	Light	1.66	1.00	1.95
Light	Dark	1.00	1.20	1.74
	Light	1.00	1.00	1.48

Table 3
Effect of various concentration and combination of NAA, BAP, ZR, TDZ and IAA on callus, shoot and root formation of eggplant var. Nayantara using hypcotyl.

MS supplemented with growth regulators (mg/L)	Explant producing root (%)	Explant producing shoot (%)	Explant producing callus (%)	Number of shoots/ explant
NAA + BAP				
0.0 + 0.0	100.00^{a}	76.00^{a}	27.63+	1.40^{f}
0.0 + 2.5	0.00^{c}	24.00^{d}	100.00++	1.58^{e}
0.0 + 5.0	0.00^{c}	52.33^{c}	100.00++	1.42^{f}
0.5 + 0.0	0.00^{c}	100.00^{a}	100.00++	2.53^{b}
0.5 + 2.5	0.00^{c}	0.00^{e}	100.00++++	0.00^{g}
0.5 + 5.0	0.00^{c}	0.00^{e}	100.00++++	0.00^{g}
1.0 + 0.0	0.00^{c}	57.33^{c}	100.00++	2.20^{d}
1.0 + 2.5	0.00^{c}	0.00^{e}	100.00++++	0.00^{g}
1.0 + 5.0	24.00^{b}	0.00^{e}	100.00++++	0.00^{g}
ZR + IAA				
1.0 + 0.1	100.00^{a}	75.33^{b}	100.00++	2.27^{c}
2.0 + 0.1	6.33^{c}	100.00^{a}	100.00+++	6.65^{a}
TDZ + IAA				
0.1 + 0.1	0.00^{c}	100.00^{a}	100.00+	2.55^{b}
0.2 + 0.1	0.00^{c}	95.33^{a}	100.00++	2.49^{b}

NAA = naphtaleneacetic acid; BAP = 6-benzylaminopurine; ZR = zeatin riboside; TDZ = thidizuron; IAA = indole acetic acid.
Means followed by different letters within a column are significantly different at $p < 0.05$ (Duncan, 1955).
+ = poor, ++ = moderate, +++ = good, ++++ = very good.

Root development and plant establishment

For root induction, half-strength MS medium supplemented with different concentrations of IBA (0.0, 0.5, 1.0 and 2.0 mg/L) were used. Regenerated shoots from 2.0 mg/L ZR in combination with 0.1 mg/L IAA-treated media were carefully removed from the baby jar/Petri dish and each shoot was cut from the bottom end and transferred into growth-regulator-free, MS medium. Shoots were kept in this medium for up to 4 wk to allow sufficient elongation and to reduce any possible adverse effects of the growth regulators during regeneration. Shoots of 2.5–4.0 cm length were excised and cultured in rooting medium. The regenerated rooted plantlets at the 5–6 leaf stage were transferred from the culture room and kept at room temperature (28–30 °C) for 5 d. The plantlets were then

transferred to a soil mixture in 10 cm plastic pots and covered with poly bags for acclimatization.

Culture conditions

The temperature was set at 25 °C with an illumination of 2000–3000 lux from fluorescent lamps and the cultures were maintained under a photoperiod of 16 h light and 8 h dark and at 60–70% relative humidity for all four experiments.

Statistical analysis

Experiments were laid out in a single factor completely randomized design with four replications. The results were analyzed by using the MSTAT-C statistical package (www.sas.com). Differences among the means were compared using following Duncan's new multiple range test at the 5% level of significance.

Results and discussion

It is now well established that in a number of plant systems, several intrinsic and extrinsic factors influence tissue culture response. Among these factors the genotype, type of explant, type of plant growth regulators and their concentration play a vital role. This study conducted a series of experiments to establish an efficient regeneration protocol for cultivated eggplants in Bangladesh.

Genotypic variation in organogenesis

Hypocotyl explants of five varieties of eggplants—*Nayantara*, *Kazla, Islampuri, ISD-006* and *Uttara*—were cultured on growth-regulator-free, MS medium for multiple shoot formation. It was observed that among the cultivars, *Nayantara* produced the highest number of shoots/explant and percentage of rooted shoots (Table 1). Considering its higher regeneration ability, this cultivar was used in the subsequent experiments. However, only shoots obtained from hypocotyl segments on MS medium could be rooted and regenerated into whole plants. This was in agreement with earlier observations on eggplant (Kamat and Rao, 1978; Matsuoka and Hinata, 1979; Sarker et al., 2006). Genotypic differences have been reported for organogenesis in eggplant by other researchers (Matsuoka and Hinata, 1979; Alicchio et al., 1982; Sharma and Rajam, 1995).

Dark pretreatment and type of explant

Seed germinated under the dark conditions of 2 wk culture in the dark produced more shoots compared to the other conditions (Table 2). Culturing in the dark is required for adventitious bud formation and light conditions were essential for the enlargement of the bud to form shoots in apple (Jun et al., 1996). The complete mechanism involved in the organogenesis enhancement by dark pretreatments has not yet been completely elucidated but researchers have commented that the incubation of plant tissues in darkness may preserve light-sensitive, endogenous plant growth regulators and other compounds (Evans et al., 1981; Yalcin et al., 2009, 2010) The current results from the dark pretreatment, where darkness improved the adventitious shoot regeneration, is also consistent with Punja et al. (1990), Leblay et al. (1991), Mohammed et al. (1992) and Compton (1999).

One of the important criteria that influences regeneration is the type of explant and its position. In this study, differences were observed in the morphogenetic potential of explants collected from different regions of the hypocotyl system. The maximum number of adventitious shoots was obtained from the basal segment, followed by the mid and top segments (Table 2). These significant differences may have occurred due to a gradient of phytohormones existing within the same explant which has been known to exist (Ulvskov et al., 1992). These results were consistent with results reported in eggplant (Sharma and Rajam, 1995).

Regeneration response against plant growth regulator

The type and concentration of a given growth regulator in association with specific genotypes can cause significant differences in the morphogenetic responses of eggplant. For example, Kamat and Rao (1978) using hypotocyl explants induced only the development of rhizogenic calli in the presence of NAA and a naphthoxyacetic acid, while regeneration through organogenesis was obtained in the presence of IAA. Both organogenesis and embryogenesis were also observed by Matsuoka and Hinata (1979) in response to different NAA concentrations using the same explant type.

The current study investigated different levels of NAA, BAP, TDZ, ZR and IAA for multiple shoot induction either singly or in combination. NAA and BAP were widely used in previously reported experiments but the observed regeneration efficiency was very low (0.75–2 shoots/explant) according to Sarker et al. (2006) and Rahman et al. (2006). ZR and TDZ are two cytokinins having high regeneration potentiality. Magioli et al. (1998) obtained as much as 20 shoots/explant by applying low concentrations (100–200 nM) of TDZ. In the present study, among the different growth regulators 2.0 mg/L ZR in combination with 0.1 mg/L IAA were found to be most effective for shoot regeneration (6.65 shoots/explant) as shown in Table 3. Sarker et al. (2006) also reported regeneration frequencies using 2.0 mg/L ZR in eggplant cv. *Kazla* and *Singnath* for the production of transgenic shoot using hypocotyl explants. A distinct variation was also observed among the treatments having different levels of NAA with respect to shoot initiation (Table 3). The highest number (2.53) of shoots/explant was found in the medium supplemented with 0.5 mg/L NAA only.

Simultaneous callus formation and shoot induction were observed in some treatments. On the other hand, only callus was produced by the treatment combinations of NAA and BAP. No shoot regeneration was observed in media supplemented with a combination of NAA and BAP (Table 3). Differences were also observed in the amount and color of the induced calli. Combinations of NAA and BAP produced friable, whitish calli while single concentrations of either NAA or BAP formed callus as well as embryo on the same hormonal concentration but at different time intervals, the former during the initial period (up to 3 wk) of culture and the later after 5 wk. Though the explant biochemical or molecular basis of such a differential response as a function of culture time could not be ascertained from the present study, it may be conjectured from the information available from earlier works that endogenous hormonal levels as well as those of polyamines might be changing with culture time and may influence the final response (Sharma and Rajam, 1995). The present accessions and explant types might provide a suitable experimental system to undertake detailed analysis of these biochemical factors.

Root development and plant establishment

Root formation varied with different IBA concentrations. In this sense, root formation increased with higher IBA concentrations. The highest percentages (93.33%) of roots were induced in the treatment having 2.0 mg/L IBA (Table 4). Several researchers observed rooting of regenerated eggplant on growth regulator-free, MS medium (Akhond and Bhuiyan, 2001; Sarker et al., 2006); however, in the current experiment, no rooted plantlets

Table 4
Effect of indole butyric acid (IBA) on root development and ex vitro survival on eggplant variety Nayantara

Treatment (IBA mg/L)	Days to root initiation	Shoots inducing roots (%)	*ex vitro* survival (%)
0.0	0.0^{c}	0.0^{d}	0.0^{d}
0.5	18.08^{a}	36.67^{c}	51.52^{c}
1.0	15.76^{ab}	53.30^{b}	64.45^{b}
2.0	14.72^{b}	93.33^{a}	100^{a}

Means followed by different letters within a column are significantly different at $p < 0.05$ (Duncan, 1955).

were found in the control treatment. The number of days required for root initiation was longer at lower concentrations of IBA treatments. Moreover, a lower concentration of IBA produced roots at a very low frequency. In the treatment having 0.5 mg/L IBA, globular structures formed at the cut ends of the shoots of some plants. Some produced root after 3 wk but the roots were few in number and in poor condition, while the others failed to initiate any roots (Fig. 1G). This may have been due to the residual effect of ZR. Previously it was reported that the application of ZR inhibits adventitious root formation on cucumber hypocotyls (Kuroha et al., 2002). To overcome this problem, shoots were first transferred to growth-regulator-free, MS medium for 4 wk before transferring to the rooting medium. Among the treatments, root initiation was earliest (14.72 d) in the medium supplemented with 2.0 mg/L IBA (Table 4). After sufficient development of roots, the plantlets were successfully transplanted into small plastic pots containing soil (Fig. 1I). Following proper acclimatization, the plantlets were transferred to the field.

In summary, the selection of genotypes, explants type and appropriate pretreatment supplemented with the correct growth regulator combinations improved the shoot formation and plant regeneration of eggplant. Multiple shoot induction was improved by selecting the variety *Nayatara*. Moreover, this induction rate was further enhanced by pretreatment and explant type (germination in the dark and culturing in the dark using the bottom hypocotyl). Subsequently, maximum shoot formation was obtained in the MS medium supplemented with 2.0 mg/L ZR in combination with 0.1 mg/L IAA. This regeneration protocol will pave the way for future transgenic research in eggplant.

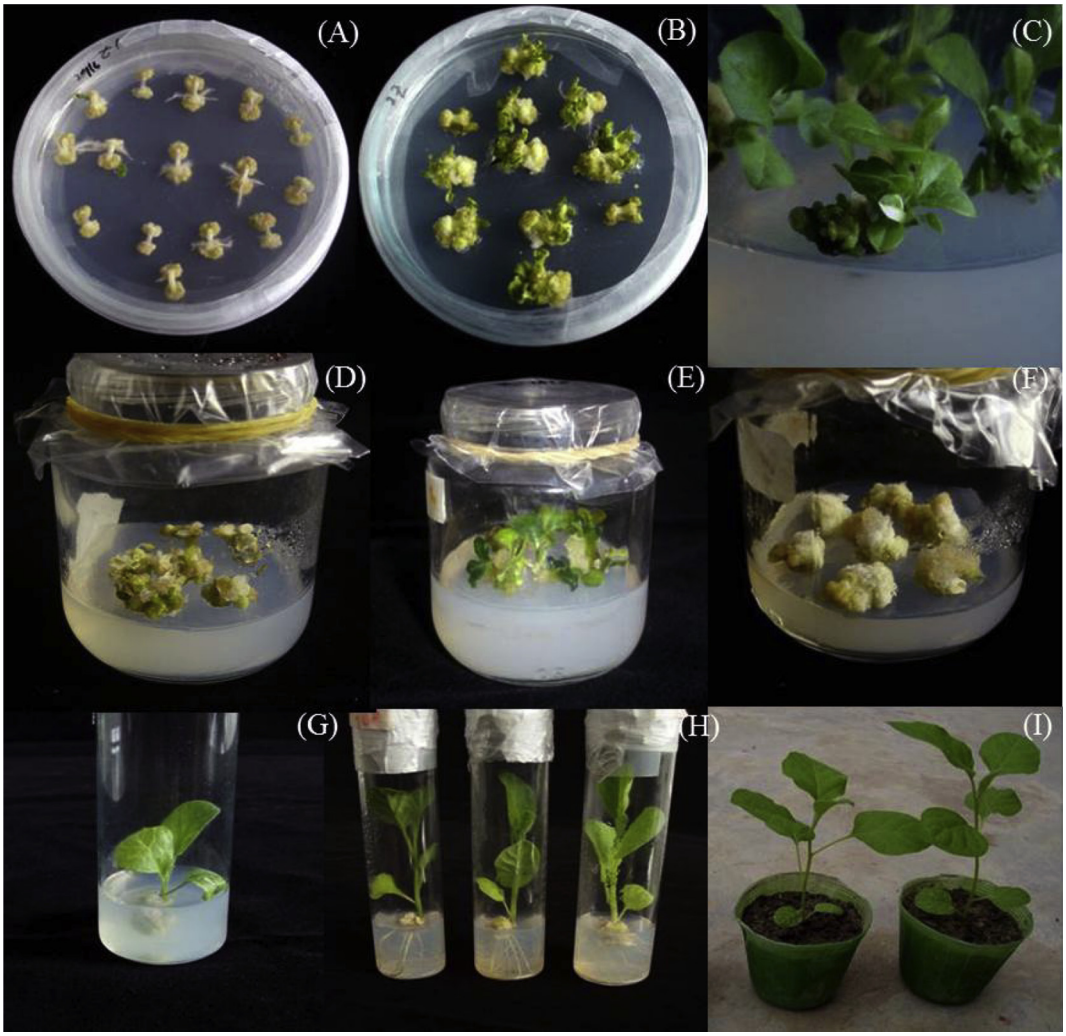

Fig. 1. (A) Shoot initiation observed from below of bottom hypocotyl supplemented with zeatin riboside (ZR); (B) Organogenesis in ZR treated medium; (C) Well developed shoots and chimera on thidizuron supplemented medium; (D) naphtaleneacetic acid (NAA) supplemented medium produce green callusing at 6 wk after culture; (E) NAA-supplemented medium shoots; (F) Callusing in treatment combination of NAA and 6-benzylaminopurine; (G) Lower concentrations of indole butyric acid (IBA) failed to produce roots; (H) Well developed root system on higher concentration, IBA-supplemented medium; (I) Plant establishment with well-developed shoot system (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Conflict of interest

The authors declare that they have no competing interests.

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