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In-vitro Callus Induction and Regeneration of Brinjal (*Solanum melongena* L.) through Cotyledon

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ABSTRACT

Brinjal is one of the most popular, nutritional and vegetable crops in the world. It plays a vital role in the national economy as a cash crop. Tissue culture techniques used for *in-vitro* plant regeneration through cotyledon explants of eggplant (*Solanum melongena* L.) with different combinations of plant growth hormones BAP (4.44, 6.66, 8.88, 11.10 and 13.32µM) and IAA (0.57, 1.14 and 1.71µM) used for *in-vitro* regeneration of brinjal. The cotyledon explants used in this study, the highest callus induction found on BAP 8.88 µM and IAA 1.14 µM. The callus induction occurred after 15days from initiation, shoot induction occurred after 30 days from initiation and shoot elongation was carried out on the same medium, shoot elongation occurred after 45 days from initiation. MS hormone-free medium found best for root regeneration, the elongated shoots were selected and transferred to a test tube containing MS hormone-free rooting medium and the elongated shoots produce roots after 15 days. Then the rooted plantlets were transferred to poly-cup with a pre-sterilized mixture of coco peat for primary hardening under poly-tunnel for 10days. Subsequently, there generated plantlets acclimatized under the greenhouse. Then, hardened plants transferred to the open field for further development. This plant regeneration method can be useful for the production of the disease-free plant.

Key words: *Solanum melongena* L, Callus induction, Shoot induction, Shoot elongation.

INTRODUCTION

The brinjal (*Solanum melongena* L.), commonly known as eggplant under the family Solanaceae, is an economic importance vegetable crop of tropical and subtropical regions of world and is mostly grown in Asian subtropical regions (94% of world production). All over the world, there are about 25 refined species of a genus *Solanum,* which includes the potato, tomato and various eggplant species (Samuels, 2009). Brinjal is considered as King of Vegetables. It is a commercially important vegetable, as well as cash crop. In India nearly 40 eggplant varieties cultivated with an estimated area of 730.40 thousand hector and the total production is 12800.8 thousand Metric tons in 2017-18 (Anonymous, 2018). The major producing states of brinjal are West Bengal, Odisha, Gujarat, Madhya Pradesh, Bihar, Maharashtra, Karnataka, Uttar Pradesh and Andhra Pradesh. According to the Food and Agriculture Organisation of the United Nations (FAO, 2015), the world's largest eggplant producers are China and India with production of 28 Mt and 13 Mt per year respectively. The eggplant mainly cultivated in Turkey (827,000 tons), Italy (220,000 tons), Spain (206,000 tons) and Romania (123,000 tons/year) in Europe. The FAO (2015) has reported that both Ukraine (96,000 tons in 2013) and Lithuania (2,000 tons in 2013) now also grow this crop.

As compared to other crop plants like tomato, it is rich in vitamins and minerals that increase its total nutritional value (Kalloo, 1993). The eggplant consist of high soluble fiber and mineral contents as calcium, iron, potassium and phosphorus. Vitamins such as vitamin B-6, vitamin K, vitamin C, folate and choline are also considerably high in the fruits, making it beneficial to human health (Bhatti *et al.,* 2013).

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Brinjal has rich in antioxidant compounds, which leads to hepatoprotective properties (Concellon *et al.,* 2012). The crop is susceptible to several diseases like Damping-off, Phomopsis blight, fruit rot, Little leaf of brinjal, Bacterial wilt, Leaf spot, Late blight, Collar rot, *etc*., (Bhupendra Kumar Singh *et al*., 2014) and pests, causing massive yield losses. Biotic stress has become a significant risk of infections for the cultivation of brinjal (Krishnaiah, 1980).

The regeneration ability of brinjal has allowed the application of somaclonal variation, haploidy, hybridization and genetic transformation (Collonier *et al*., 2001). It is also a sound system for *in vitro* studies because plant regeneration can be achieved via the organogenic pathway from different explants. Organogenesis from hypocotyl (Magioli *et al*., 1998; Dobariya and Kachhadiya, 2004), root (Franklin *et al*., 2004) explants have been reported. Eggplant has been regenerated via somatic embryogenesis from leaf and cotyledon (Rao and Singh, 1991) and hypocotyl

(Matsuoka and Hinata, 1979) explants. The main objectives of this study are mass production of brinjal cultivar Arka Shirish and standardization of *in vitro* protocol for the Agrobacterium-mediated transformation with the hevein gene against fungal disease.

MATERIALS AND METHODS

Plant material

The seeds of Brinjal cultivar Arka Shirish obtained from the Indian Institute of Horticulture Research (IIHR- ICAR) Bangalore. Seeds sowed on 96-well nursery pro-tray and sprinkled the water once in a day up to 12 days. The seeds started germination from the fifth day on wards. Cotyledon excised from the 10-12 days old seedlings.

Culture media preparation

The MS medium (the composition of micronutrient, macronutrient, vitamins, amino acids and hormones (Himedia) adjusted to the pH 5.8 and 0.8% agar was added (Murashige and Skoog, 1962). The medium-boiled up to milky appearance for dissolving agar in the medium. Nearly 50 ml of medium dispersed in each culture bottle, 12 ml of the medium was dispersed in each culture tube and sealed with a clean wrap cover before autoclaving at 121°C for 15 minutes under 15 Psi. The medium in culture bottles left to cool in the culture room until use. Under laminar airflow cabinet, the surface-sterilized explants were inoculated aseptically in the MS medium supplemented with various concentrations of phytohormones combinations 4.44, 6.66, 8.88, 11.10, 13.32 µM of 6-Benzylaminopurine (BAP) and 0.57, 1.14, 1.71 µM of Indole-3-acetic acid (IAA) which is used for callus and shoot development, likewise induction of root in hormone-free medium.

Sterilization of explants and inoculation

The cotyledon excised from germinated seedlings and clean thoroughly under tap water for 10 min, then washed in an agitated solution of 2% Tween 20 for 10 minutes and washed with tap water. Then the explants were treated with 0.5% of bavistin for 30 min. Finally, the explants cleaned with double distilled water for clearing the fungicide on the explant. The treated explants transferred into the horizontal laminar airflow chamber (Sunrise enterprises, Bangalore) for further sterilization. Then the explants were submerged in sodium hypochlorite (2.0%) for 10min then 70% ethanol for 30 seconds, followed by thorough shaking in $HgCl_2$ (0.1%) for 1.5 minutes and washed thrice with sterile distilled water. After surface sterilization, both ends of the explant were cut and trimmed to 1 cm size.

Callus, shoot induction and regeneration

The 20 cotyledon explants inoculated into the MS media supplemented with different concentrations of BAP (4.44, 6.66, 8.88, 11.10 and 13.32 µM) and IAA (0.57, 1.14, 1.71 µM) on culture bottle. The cultures were incubated under the fluorescent lights with 1500-2000 lux for 10 hours per day. The culture room maintained with a temperature of 25 ± 1°C and 60±10 relative humidity during callus induction. The well-developed callus induces shoot and shoots elongation. The data recorded on the 15th, 30th and 45th days of incubation. Each of the experiments repeated thrice; the data recorded for callus formation, shoot initiation and elongation.

Rooting and hardening

For root induction, a 2-3 cm size shoot selected and transferred to test tubes containing rooting media without exogenous hormones (Pratap *et al*., 2011; Chen *et al*., 1995). The root induction data recorded on the 15th, 30th and 45th days of incubation. After root induction, rooted plantlets were taken out from the culture tubes and washed to remove adhered agar and traces of medium then washed with 20 ppm of bavistin solution for 10min to avoid microbial infection. Plantlet transferred to an 8.0 cm diameter poly cup containing sterilized Coco peat, 1% neem cake and sand mixture. These plants were maintained inside the growth chamber set at 26±1°C and 75-80% relative humidity were maintained by covering the plant with polythene cover for two weeks and irrigated gently every alternate days for acclimatization. After that, the plants were transferred to pots containing organic manure, garden soil and sand (1:1:1) and maintained in a greenhouse. Then, the plants transplanted to the field.

Statistical analysis

Tissue culture data were subjected to analysis of variance by One-Way ANOVA to detect the significant differences among the treatment means using Duncan's Multiple Range Test at $P<0.05$.

RESULTS AND DISCUSSION

Seed germination

Seeds of Brinjal Arka Shirish were sown on nursery pro-tray and sprinkled with water once a day, it germinated well. 73% of seed germination observed (Table 1; Fig 1A).

The sterilized cotyledonary explants (Fig 1B) inoculated in the various concentration of BAP (4.44-13.32 µM) combine with IAA (0.57-1.71) supplemented MS medium for callus initiation, shoot induction and also multiple shoot formation. The data observed on the 15th for callus, 30th for the shoot, 45th day for multiple shoots.

Callus induction

The combination of BAP and IAA initially induce the callus. The variation of callus induction observed on the 15th day of

Table 1: Seed germination percent of brinjal cultivar Arka Shirish.

Cultivar name	No of seeds	No of days	Germinated seeds	Germination percentage
Arka Shirish	100	10-12	73±0.00	73

observation. First, the formation of a compact callus later is formed friable callus (Fig 1C). The highest callus induction 18.00±0.00 observed on the MS medium fortified with 8.88 µM of BAP and 1.14 µM of IAA followed by the 8.88 µM of BAP and 0.57 µM of IAA fortified medium (Table 2; Fig 1C). The lowest callus induction 11.33±0.57 observed on the medium fortified with 4.44 µM of BAP and 0.57 µM of IAA. In the previous study, 100% callus induction observed on the MS media fortified with 8.88µM of BAP and 0.27µM of NAA from cotyledon explants of eggplant (Huda *et al.,* 2007) and callus induction in cotyledon (90.0%) and hypocotyls (63.3%) observed in BAP (2.22 µM) and Kinetin (9.29 µM) combination (Zayova *et al.,* 2008). Similar observation also observed on cotyledon and hypocotyl explants with BAP and IAA fortified MS medium but low frequency of callus induction found on root explants (Mir *et al.,* 2011).

Shoot initiation and multiple shoot induction

In the 30th day of observation, 12.00±0.00 compact callus are loosely formed friable callus. Later the friable callus initiate shoots out of 14.00±0.00 callus in the MS medium supplemented with 8.88 µM of BAP and 1.14 µM of IAA (Fig 1D). 60.00% (12.00±0.00) of multiple shoots observed on the 45th days of observation. A maximum of 3 multiple shoots are observed in the explant (Table 3; Fig 1E). Pei Ching Foo (2018) reported 2.0 mg/l kinetin + 8.88 µM BAP combinations induce an average number of 0.80 ± 0.25 multiple shoots per explant. But in our study, the low concentration of IAA (1.14 µM) combine with BAP (8.88 µM) induced a better number of shoots. Jamil *et al*. (2013) reported that kinetin is necessary to be included together with other plant growth regulators in inducing shoots for eggplant, but in this study, both BAP and IAA hormonal combination induced callus and shoots. The cotyledon explants showed a high frequency of shoot regeneration as compared to hypocotyl explants in eggplant (Bardhan *et al.,* 2012) and in *Vigna mungo* (Anandan *et al*., 2019). Ani Rani Borah *et al*. (2019) reported the maximum five shoots was achieved on 4.44 µM of BAP and 2.32 µM of kinetin supplemented MS medium in *Coccinia indica.*

Table 2: Induction of callus rate from different combination of media.

The concentration of Hormones (µM)		BAP				
		4.44	6.66	8.88	11.10	13.32
IAA	0.57	+	+	++	++	++
	1.14	+	+	+++	++	++
	1.71	+	+	++	++	++

Note: Diameter of callus mass: + (0.0-0.5 cm) - slight; ++ (0.5-1.0 cm) - moderate; +++ (1.0-2.0) – massive.

Table 3: Callus induction after 15 days, shoot induction after 30 days, both multiple shoot induction and shoot elongation after 45 days inoculation of cultivar Arka Shirish.

			Day of observation							
BAP (µM)	IAA (µM)	No of explants inoculated	No of explants induced callus - 15th day	Percen -tage	No of explants induced shoots - 30th day	Percen -tage	No of explants induced multiple shoots – 45th day	Percen -tage	No of shoots elongated (2-3 cm height) – 45th day	Percen -tage
4.44	0.57	20	11.33±0.57	56.66	07.33±0.57	36.66	05.33±0.57	26.66	03.33±0.57	16.66
4.44	1.14	20	12.66±0.57	63.33	08.66±0.57	43.33	06.66±0.57	33.33	04.66±0.57	23.33
4.44	1.71	20	11.66±0.57	58.33	07.66±0.57	38.33	05.66±0.57	28.33	03.66±0.57	18.33
6.66	0.57	20	13.33±0.57	66.66	09.33±0.57	46.66	07.33±0.57	36.66	05.33±0.57	26.66
6.66	1.14	20	14.66±0.57	73.33	10.66±0.57	53.33	08.66±0.57	43.33	06.66±0.57	33.33
6.66	1.71	20	13.66±0.57	68.33	09.66±0.57	48.33	07.66±0.57	38.33	05.66±0.57	28.33
8.88	0.57	20	15.66±0. 57	78.33	11.66±0.57	58.33	09.66±0.57	48.33	07.66±0.57	38.33
8.88	1.14	20	18.00±0. 00	90.00	14.00±0.00	70.00	12.00±0.00	60.00	10.00±0.00	50.00
8.88	1.71	20	16.00±0. 00	80.00	12.00±0.00	60.00	10.00±0.00	50.00	08.00±0.00	40.00
11.10	0.57	20	14.00±0.00	70.00	10.00±0.00	50.00	08.00±0.00	40.00	06.00±0.00	30.00
11.10	1.14	20	15.00±0.00	75.00	11.00±0.00	55.00	09.00±0.00	45.00	07.00±0.00	35.00
11.10	1.71	20	14.33±0.57	71.66	10.33±0.57	51.66	08.33±0.57	41.66	06.33±0.57	31.66
13.32	0.57	20	12.00±0.00	60.00	08.00±0.00	40.00	06.00±0.00	30.00	04.00±0.00	20.00
13.32	1.14	20	13.00±0.00	65.00	09.00±0.00	45.00	07.00±0.00	35.00	05.00±0.00	25.00
13.32	1.71	20	12.33±0.57	61.66	08.33±0.57	41.66	06.33±0.57	31.66	04.33±0.57	21.66

Note: Each experiment repeated thrice (3-replication), per experiment 15-combinations adopted (15-treatment) and per combination 20 explants inoculated.

Shoot elongation

In the 45th day of observation, above 3 cm height of the plant is considered as elongated shoot. In this study, 10.00±0.00 shoots lengths are more than 3 cm height grow on MS medium supplemented with 8.88 µM of BAP and 1.14 µM of IAA (Table 3). The 03.33±0.57 shoots lengths are more than 3 cm height on the 1 µM of BAP and 0.57 µM of IAA supplemented with MS medium (Fig 1E). The high concentration of BAP (8.88 µM) with a low concentration of IAA (1.14 µM) responsible for callus induction initially on the explant. The shoot initiation and multiple shoot formation on the subsequent days. These concentrations of the hormones are suitable for shoot elongation to compare among the tested BAP and IAA combination. In the present study, the culturing of cotyledon with an inverted position (a ventral portion of the cotyledon) touches the medium responds to the callus induction. It may occur due to the rapid meristematic activity that occurs in the ventral region, which is a direct contract with the nutrient medium under the influence of hormones reported by Padma Mallaya and Ravishankar (2013) in hypocotyl region of *S. melongena* and Kumar *et al*. (2005) in *Capsicum annuum*. The continuous incubation of explants in the same culture medium, which is responsible for inducing the multiple shoots and shoot elongation. The few numbers of elongated shoots observed on BAP and IAA combination medium in eggplant. A similar observation was observed in *Stevia rebaudiana* (Tajo Abraham and Smrithi, 2016).

This study is controversy to the earlier report in *S. melongina* require multiple shoot initiation culture in TDZ media (Padma Mallaya and Ravishankar, 2013), combination of GA3 and TIBA for elongation of shoot (Cambecedes *et al*., 1991) in *Lonicera nitida*.

Root induction and hardening

The elongated shoots excised and transferred in MS hormone-free medium for root induction (Fig 1F). The 17.66±0.57 shoots are response for roots induction out of 20 inoculated shoots. The average of 05.44±0.52 roots and 07.44±0.52 leaves per shoot on the 15th day of observation (Table 4; Fig 1G). Padma Mallaya and Ravishankar, (2013) reported that IBA (4.92 µM/l) need for root induction from the elongated shoots, which produce 4±0.7 roots per explants in eggplant. A similar type of results for the formation of the root in plant growth hormone-free medium has been described in *S. melongena* (Sarker *et al*., 2006) and *Elaeis guineensis* (Sparjanbabu *et al*., 2019). Magioli *et al*. (1998)

Table 4: Shoots response, leaves/plant and roots/plant from the ex-agar plants.

No of Shoots inoculated	No of shoots responded	No of leaves/plant	No of roots/plant
20	17.66±0.57	07.44±0.52	05.44±0.52

Note: Each experiment repeated thrice (3-replication), per experiment 20 shoots inoculated.

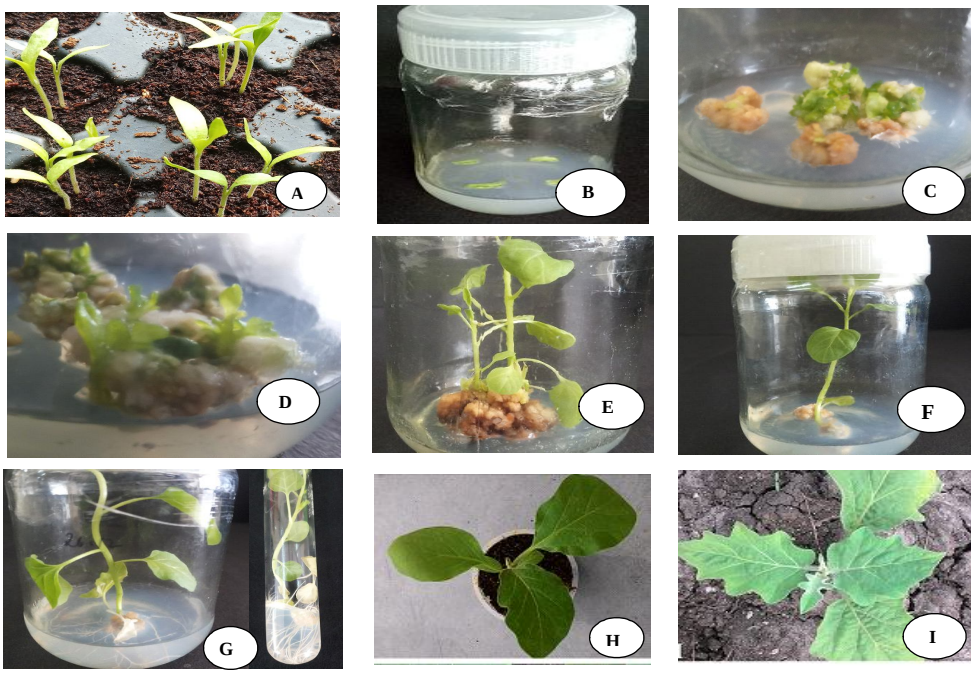

Fig 1: Brinjal cultivar Arka Shirish shows different regeneration stages: (A). Germination of seeds on 10th day from sowing, (B) Cotyledon explants inoculated on regeneration medium, (C) Callus induction on 15th day of inoculation, (D) Shoot induction on 30th day of inoculation, (E) Shoot elongation and multiple shoot induction on 45th day of inoculation, (F) Elongated shoot inoculated on root induction medium (G) Root induction on 15th day of transfer to rooting medium (H) Hardened plant in poly-cup on 30th day of hardening, (I) Field plant on 15th day of planting on-field.

reported the induction of roots using half strength of MS supplemented with 0.6µM IAA. The rooted plantlets was transferred to 8.0 cm diameter poly cup containing sterilized Coco peat and 1% neem cake sand mixture (Fig 1H). After that, the plants were transferred to pots containing organic manure, garden soil and sand (1:1:1) and maintained in a greenhouse. Then, the plants were transplanted to the field (Fig 1I).

CONCLUSION

The 10-12 days old cotyledon explants used in this study, the best callusing and shoot regeneration found regeneration medium supplemented with BAP 8.88µM and IAA 1.14µM. This study reveals that the higher performance of callus induction 90.00%, 70.00% of shoot induction, 60.00% of multiple shoot induction and 50.00% of shoot elongation observed on medium supplemented within BAP 8.88µM and IAA 1.14µM. In the present study concluded that the combination of BAP at high concentration with a low concentration of IAA supplemented MS medium responsible for callus induction on the cotyledonary explants initially, latter these hormones induced shoot initiation and elongation of shoots. The root induction occurred from elongated shoots on the MS hormone-free rooting medium. This protocol has efficient in being used for the development of desired types of disease-resistant eggplant plants following future genetic transformation.

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