

Original Research Article

<https://doi.org/10.20546/ijcmas.2021.1002.308>

Efficient Protocol for *in vitro* Regeneration and *Agrobacterium*-Mediated Transformation of Brinjal cv.CO₂

Priyanka Eraivan¹, S. Varanavasiappan¹, E. Kokiladevi¹, L. Arul¹,
B. Vinoth Kumar² and K. K. Kumar^{1*}

¹Department of Plant Biotechnology, Centre for Plant Molecular Biology & Biotechnology,
²Department of Agricultural Entomology, Centre of Plant Protection Studies,
Tamil Nadu Agricultural University, Coimbatore 641003, India

*Corresponding author

ABSTRACT

A reliable protocol for efficient shoot induction in brinjal cv. CO 2 was developed. The effects of different plant growth regulators and explant types in *in-vitro* shoot induction were studied. The 12-15 days old cotyledonary leaves showed the maximum shoot induction. The regeneration efficiency was significantly increased by using Thidiazuron (TDZ) in culture medium as a growth regulator. Following the standardized regeneration protocol, *Agrobacterium*-mediated transformation of brinjal cv. CO 2 was optimized. Cotyledonary leaf bits were used for co-cultivation with the *Agrobacterium* strain LBA4404 carrying pCAMBIA 2301 vector. A transformation efficiency of 33.5% was obtained by using the cotyledonary leaves as explants. The transformation efficiency was significantly enhanced when the explants were cocultivated on medium overlaid with Whatman No.1 filter paper.

Keywords

Brinjal
transformation,
Agrobacterium,
Shoot induction,
Cotyledonary leaf,
Thidiazuron

Article Info

Accepted:
20 January 2021
Available Online:
10 February 2021

Introduction

Brinjal (*Solanum melongena* L., 2n=2x=24), which is also known as eggplant is one of the most important solanaceous vegetables in the tropical and subtropical regions of the world including India (Daunay, 2008). Brinjal is rich in soluble fibre and mineral contents such as calcium, iron, potassium, phosphorus and

vitamins like vitamin C, vitamin B-6, vitamin K, folate and choline. Brinjal consumption is likely to contribute towards lowering the risk of cardiovascular diseases, diabetes and several other diseases due to the low calorie value and fat content (Bhat *et al.*, 2013).

It can be cultivated throughout the year but its quality and productivity is highly affected by

various biotic and abiotic factors. CO₂ is a brinjal variety released by Tamil Nadu Agricultural University, Tamil Nadu, India. It is a pure line selection from the cultivar varikatthiri and yields upto 35 tonnes/ha in 150 days.

However the crop productivity is hugely influenced by several pests and diseases, particularly fruit and shoot borer. The major bottleneck in the trait improvement in brinjal is genetic incompatibility, undesirable linkage, and the wide prevalence of progeny sterility (Bhat *et al.*, 2013).

To rectify the hindrance of conventional breeding and to impart new traits, various biotechnological methods like genetic transformation and somatic hybridization can be utilized as an alternative approaches for the improvement of this crop (Rao *et al.*, 1979).

Since the first report by Guri and Sink (1988) on *Agrobacterium*-mediated transformation of brinjal, several successful reports were made for the various characters like insect resistance, virus resistance, disease resistance and abiotic stress tolerance (Filippone and Lurquin, 1989; Rotino and Gleddie, 1990; Fari *et al.*, 1995; Billings *et al.*, 1997; Kumar *et al.*, 1998; Franklin and Lakshmi Sita, 2003). Stable *Agrobacterium*-mediated transformation has been achieved using leaf and cotyledonary explants from *in vitro* grown plants (Fari *et al.*, 1995).

However the efficiency of transformation and the *in-vitro* regeneration was relatively low in earlier reports.

Hence, the present investigation was carried out with a view to standardize the efficient regeneration for brinjal variety CO 2 by studying two different explants with different combinations of plant growth regulators. Further, high efficiency genetic transformation

of brinjal was successfully demonstrated using by *Agrobacterium* strain LBA4404 harbouring pCAMBIA2301 vector.

Materials and Methods

Plant materials

The brinjal (CO 2 variety) seeds (obtained from the Horticultural College and Research Institute, TNAU, Coimbatore) were surface sterilized in 0.1% HgCl₂ for 1.30 min, followed by sterile distilled water for three times and blot dried with sterile tissue paper.

Surface sterilized seeds were then transferred into ½ MS medium (Murashige and Skoog, 1962; Table 2) for germination and grown under cool white fluorescent light with photoperiod of 16 hrs at 25°C.

In-vitro shoot regeneration from explants

To analyse the regeneration efficiency of CO 2 brinjal, cotyledons and leaf from the 12- 15 days old seedlings (Fig.1) were transferred into different combinations of regeneration medium. From the seedlings, the cotyledons and leaf were excised and cut at both the ends.

The explants were transferred into regeneration medium containing growth regulators *viz.*, TDZ, BAP, Kinetin, Zeatin and IAA in different combinations (Table 1) and incubated at 25 °C with 16 hrs photoperiod.

The explants were sub-cultured every two weeks and the observation of shoots was recorded after six weeks of culture. The shoot induction efficiency is estimated by the following formula:

$$\text{Shoot induction efficiency} = \frac{\text{Number of shoots induced}}{\text{Number of explants inoculated}} \times 100$$

Agrobacterium-mediated transformation of brinjal

Preculture

The cotyledonary leaf from 12-15 days old seedlings were isolated and precultured on preculture medium (PCM, Table 2) for two days under 16 hrs photoperiod at 25°C.

Co-cultivation

Agrobacterium tumefaciens strain harbouring pCAMBIA2301 was grown to 1.0 O.D in LB medium (Chilton *et al.*, 1974) containing 10 mg/l rifampicin and 50mg/l kanamycin at 28°C in a rotary incubator shaker with 200 rpm agitation.

Bacterial suspension was centrifuged at 3400 g for 5 min and the pellet was resuspended in half the volume of AA medium (Toriyama *et al.*, 1985) containing 20 µM acetosyringone.

The explants were immersed in the bacterial suspension for 3 min and blot dried with sterile tissue paper. Then the explants were transferred to cocultivation medium (CCM, Table 2) overlaid with Whatman No.1 filter paper and wetted with 1mL of AA medium containing 20 µM Acetosyringone and incubated at 25°C in dark for 2 days.

Selection and *in-vitro* regeneration of transformed explants

To remove the *Agrobacterium* after infection, co cultivated explants were given a wash with ½ MS broth containing 250 mg/l of Cefotaxime and blot dried.

After that explants were transferred to the pre-selection medium (PSM, Table 2) and incubated under 16 h photoperiod at 25°C for 2 days.

After 2 days of pre selection, the explants were transferred into the selection medium containing kanamycin (SM, Table.2) to screen the transformed explants and subsequent subcultures were made every week.

Screening of transformed shoots from the selection medium

Stable GUS assay

Stable expression of *gusA* was performed as described by Jefferson (1987) with the calli that survived two rounds of kanamycin selection and with leaf tissue from shoots that formed in selection medium. A bit of callus or leaf tissue were incubated with X-Glu staining solution and incubated overnight at 37 °C to observe GUS expression.

Results and Discussion

Effect of explant type and growth regulators on regeneration of brinjal cv. CO 2

Several factors influences the regeneration and *Agrobacterium*-mediated transformation, among them most important factors are genotype, type of explants and growth regulators in medium. Earlier reports indicated that the cotyledonary leaves and leaf are the highly used explants for the genetic transformation of brinjal (Guri and Sink, 1998; Filippone and Lurquin, 1989; Magioli *et al.*, 1998; Kumar *et al.*, 1998; Rao *et al.*, 2010; Foo *et al.*, 2018). However the efficiency of transformation was relatively low. In this study, leaf and cotyledonary leaves were inoculated as explants in different combinations of growth regulators to analyse the shoot induction (Fig. 2 & 3). The cotyledonary leaves were found to be more effective and responsive than the leaves (Table 3). Cotyledonary leaves showed the maximum callus, shoot induction and regeneration after

six weeks of inoculation (Fig.3). Previous reports also shows that cotyledons were more responsive for regeneration and had a high efficiency of shoot induction (Sarker *et al.*, 2006; Zayova *et al.*, 2012). During the stage of 12- 15 days, the cells in the seedlings will be in dividing phase and it makes the cells to be more competent for *Agrobacterium*-mediated transformation (Fari *et al.*, 1995).

The combination and the concentration of plant growth regulators used can change the morphogenic responses effectively. The plant growth regulators *viz.* BAP, IAA, Kinetin, Zeatin and TDZ in different combinations were used to analyse the efficiency of *in vitro* shoot induction in CO 2.

Three different regeneration medium (Table 1) with different combinations of plant growth regulators were tested for shoot induction. Among the regeneration medium, Kinetin + BAP +IAA developed high amount of callus and few shoots. Zeatin in the regeneration medium developed abundant callus and healthy shoots. TDZ in medium produced more number of shoots in both cotyledonary leaf and leaf explants (Fig. 2 & 3).

Among all the combinations tested, regeneration medium with TDZ 0.05 mg/l shown the regeneration as high as frequency of 96% shoot induction in CO 2 brinjal (Table 3). Even though BAP & kinetin are most frequently used in earlier reports, the regeneration potential is relatively low.

Several studies reported that Zeatin riboside, TDZ are having the high regeneration potential more than other cytokinins (Sarker *et al.*, 2006; Magioli *et al.*, 1998). TDZ, a substituted phenylurea compound was used in many studies because of its prominent role on *in-vitro* regeneration of different plant species,

with the action of both auxin and cytokinin (Murthy *et al.*, 1998).

***Agrobacterium*-mediated transformation of brinjal cv. CO 2**

Transformation efficiency is highly depends on various factors like *Agrobacterium* concentration, infection duration, *Agrobacterium* overgrowth control and composition of selection medium. In this study, *Agrobacterium* culture of OD₆₀₀ = 1.0 with infection time of 3 minutes were found sufficient for CO 2. In contrast, earlier reported show that *Agrobacterium* at OD₆₀₀ = 0.2 with infection time of 3 minutes increased the transformation efficiency (Jadhav *et al.*, 2015). For efficient *Agrobacterium* infection, acetosyringone at 20 µM concentration was used in the cocultivation medium. To control the *Agrobacterium* overgrowth, co-cultivation was done only for two days.

The *Agrobacterium* overgrowth was greatly reduced by placing Whatman No.1 filter paper over the cocultivation medium. Transformation increased four fold from 8 % to 33.5 % on using the filter paper laid on co-cultivation medium (Fig.4). Use of 250 mg/l cefotaxime in pre-selection and selection medium effectively controlled the *Agrobacterium* overgrowth. Selection done using 50 mg/l concentration of kanamycin was found to be suitable for efficient selection of transformed tissue. Earlier studies also show that 50 mg/l concentration of kanamycin in selection medium was optimum to select the putative transgenic shoots(Pratap *et al.*, 2011; Akhter *et al.*, 2012; Jadhav *et al.*, 2015). Shoots regenerated after 7 weeks of kanamycin selection (Fig.4). Histochemical GUS assay confirm the genetic transformation in callus and regenerated shoots of brinjal (Fig. 5).

Table.1 Different regeneration medium compositions tested for regeneration in brinjal cv. CO 2

Chemical	RM 1	RM2	RM3
MS salts (%)	100	100	100
Vitamins (%)	100	100	100
Myo inositol (mg/l)	100	100	100
TDZ (mg/l)	-	-	0.05
BAP (mg/l)	0.4	-	-
IAA (mg/l)	0.1	-	-
Kinetin (mg/l)	0.1	-	-
Zeatin (mg/l)	-	0.1	-
Sucrose (g/l)	20	20	20
Phytigel (g/l)	3	3	3

Table.2 Media composition used in *Agrobacterium*-mediated transformation of brinjal cv. CO 2

Chemical	GM	PCM	CCM	PSM	SM
MS Salts (%)	100	100	100	100	100
Vitamins (%)	100	100	100	100	100
Myo inositol (mg/l)	100	100	100	100	100
TDZ (mg/l)	-	0.05	0.05	0.05	0.05
Acetosyringone (µM/l)	-	-	20	-	-
Kanamycin (mg/l)	-	-	-	-	50
Cefotaxime (mg/l)	-	-	-	250	250
Sucrose (g/l)	20	20	20	20	20
Phytigel (g/l)	3	3	3	3	3

Table.3 Regeneration efficiency of brinjal leaf and cotyledons in different media composition

Type of the explant		BAP+ Kinetin+ IAA	Zeatin	TDZ
Leaf	No of explants inoculated	50	50	50
	No of explants showed shoot generation	19	31	41
	No of shoots formed per explant	3	7	10
	Regeneration Efficiency	38	66	82
Cotyledonary leaf	No of explants inoculated	50	50	50
	No of explants showed shoots generation	23	37	48
	No of shoots formed per explant	4	8	11
	Regeneration Efficiency	46	74	96

Table.4 Effect of agrobacterium overgrowth control in influencing transformation efficiency

Explant type	Cocultivation method	No. of explants taken for cocultivation	No. of explants producing shoots	Transformation efficiency (%)
Cotyledonary leaf	-filter paper	100	8	8
	+ filter paper	128	43	33.5

Fig.1 *In-vitro* explant initiation of brinjal variety CO 2.

a. Inoculated surface sterilized seeds in the $\frac{1}{2}$ MS germination medium

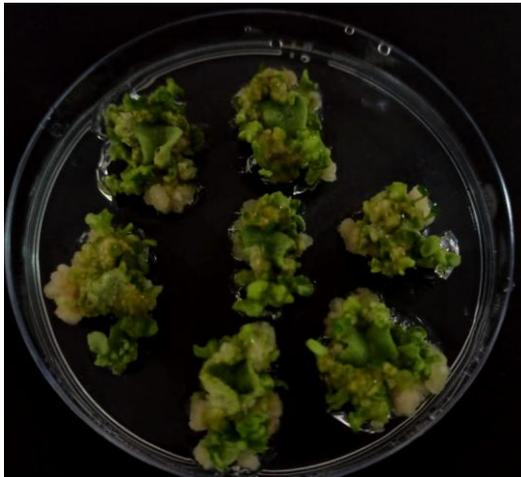


b. Explant initiation in $\frac{1}{2}$ MS – 15 days after germination



Fig.2 Standardization of regeneration in CO₂ brinjal using leaf as explant

a. Leaf explants in regeneration medium containing Kinetin+ BAP+IAA (6 weeks)



b. Leaf explants in regeneration medium containing Zeatin (6 weeks)



c. Leaf explants in regeneration medium containing TDZ (6 weeks)

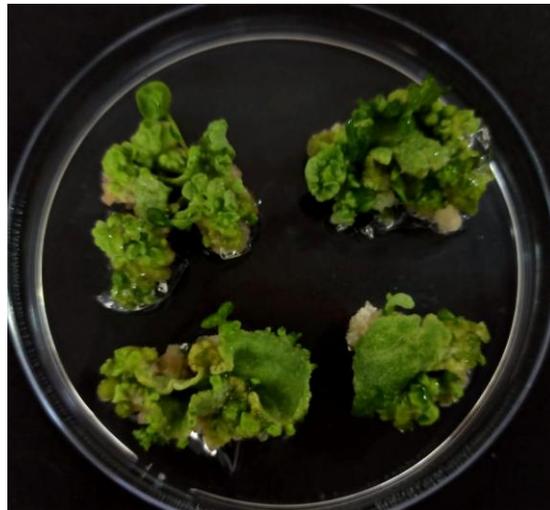
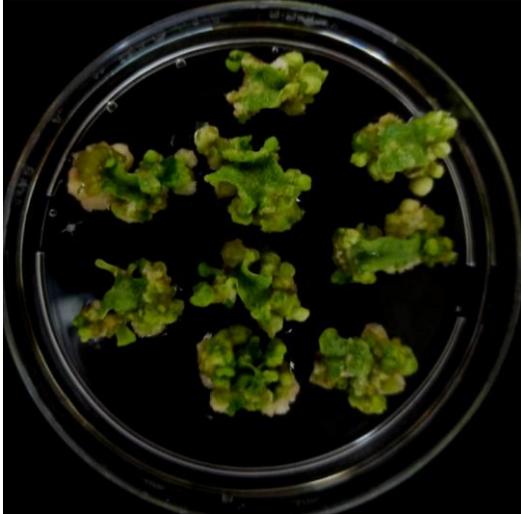


Fig.3 Standardization of regeneration in CO 2 brinjal using cotyledonary leaf as explants

a. Cotyledonary explants in regeneration medium containing Kinetin +BAP+IAA (6 weeks)



b. Cotyledonary explants in regeneration medium containing Zeatin (6weeks)

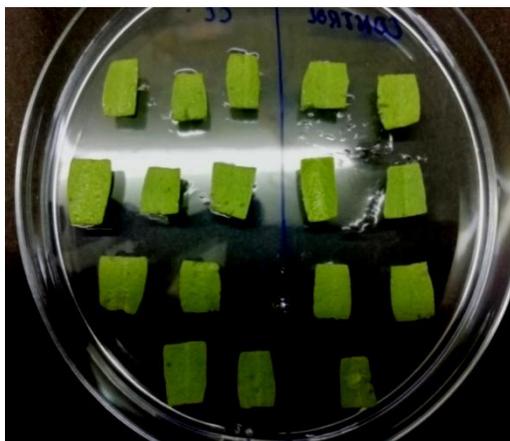


c. Cotyledonary explants in regeneration medium containing TDZ (6 weeks)



Fig.4 *Agrobacterium*-mediated transformation of brinjal cultivar CO 2

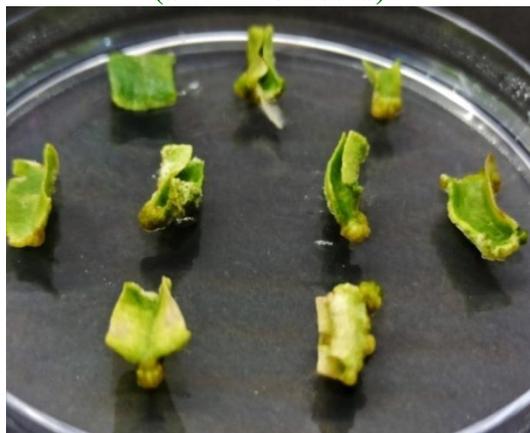
a. Precultured cotyledonary leaf explants



b. Explants in cocultivation medium overlaid with Whatman No.1 filter paper



c. Explants in the selection medium (after two weeks)



d. Shoot induction from the explants in selection medium (after five weeks)



e. Regenerated shoots from the transformed explants (after seven weeks)

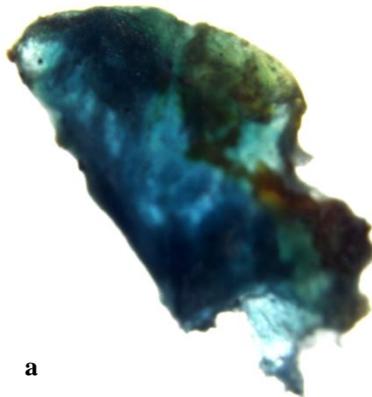


f. Developed shoots in the selection medium (after 8 weeks)



Fig.5 Histochemical GUS analysis of transformed callus and leaf of brinjal

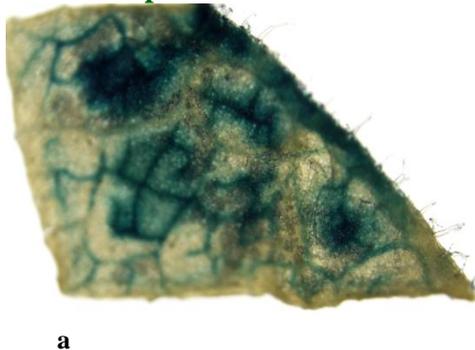
5A GUS expression in transformed callus of brinjal. a. Transformed callus



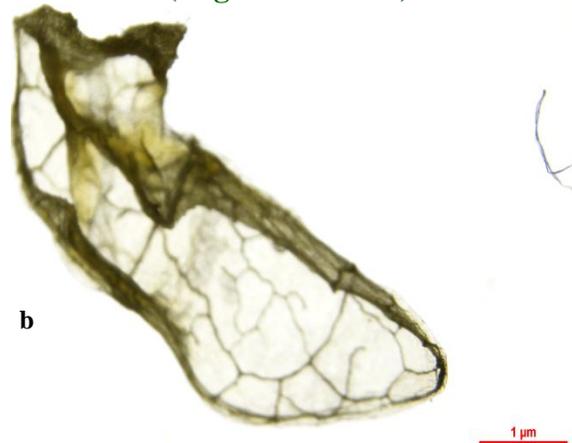
b. Untransformed callus (Control)



5B GUS expression in leaf of brinjal. a. Leaf from putative transformed shoot



b. Leaf from untransformed shoot (Negative control).



The efficiency of brinjal *in-vitro* shoot induction was enhanced significantly by using 0.05 mg/l TDZ in medium and the transformation efficiency was increased by using cotyledonary leaf as explants for cocultivation and reducing the *Agrobacterium* infection injury by growing the explants on medium overlaid with Whatman No.1 filter paper during cocultivation. This protocol could be extended for transforming various other brinjal cultivars to get improved transformation frequency.

Acknowledgement

The authors are grateful to the Department of Plant Biotechnology, Centre for Plant

Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, for allowed us to utilize the lab facilities throughout the work.

References

- Akhter, M.S., Ahamed, A. and Hossain, M.M., 2012. *Agrobacterium* Mediated Genetic Transformation of Eggplant (*Solanum melongena* L.). *International Journal*, 3(2), pp.275-280.
- Bhat, S.V., Jadhav, A.S., Pawar, B.D., Kale, A.A., Chimote, V.P. and Pawar, S.V., 2013. In vitro shoot organogenesis and plantlet regeneration in brinjal

- (*Solanum melongena* L.). *The Bioscan*, 8(3), pp.821-823.
- Billings, S., Jelenkovic, G., Chin, C.K. and Eberhardt, J., 1997. The effect of growth regulators and antibiotics on eggplant transformation. *Journal of the American Society for Horticultural Science*, 122(2), pp.158-162.
- Daunay, M.C., 2008. Eggplant. In *Vegetables II* (pp. 163-220). Springer, New York, NY.
- Fári, M., Nagy, I., Csányi, M., Mitykó, J. and Andrásfalvy, A., 1995. *Agrobacterium*-mediated genetic transformation and plant regeneration via organogenesis and somatic embryogenesis from cotyledon leaves in eggplant (*Solanum melongena* L. cv. 'Keckskemeti lila'). *Plant Cell Reports*, 15(1-2), pp.82-86.
- Filippone, E. and Lurquin, P.F., 1989. Stable transformation of eggplant (*Solanum melongena* L.) by cocultivation of tissues with *Agrobacterium tumefaciens* carrying a binary plasmid vector. *Plant Cell Reports*, 8(6), pp.370-373.
- Foo, P.C., Lee, Z.H., Chin, C.K., Subramaniam, S. and Chew, B.L., 2018. Shoot Induction in White Eggplant (*Solanum melongena* L. Cv. Bulat Putih) using 6-Benzylaminopurine and Kinetin. *Tropical life sciences research*, 29(2), p.119.
- Franklin, G. and Sita, G.L., 2003. *Agrobacterium tumefaciens*-mediated transformation of eggplant (*Solanum melongena* L.) using root explants. *Plant cell reports*, 21(6), pp.549-554.
- Ghosh, A., Igamberdiev, A.U. and Debnath, S.C., 2018. Thidiazuron-induced somatic embryogenesis and changes of antioxidant properties in tissue cultures of half-high blueberry plants. *Scientific reports*, 8(1), pp.1-11.
- Guri, A. and Sink, K.C., 1988. Organelle composition in somatic hybrids between an atrazine resistant biotype of *Solanum nigrum* and *Solanum melongena*. *Plant science*, 58(1), pp.51-58.
- Guri, A. and Sink, K.C., 1988. *Agrobacterium* transformation of eggplant. *Journal of plant physiology*, 133(1), pp.52-55.
- Habde, S., Jadhav, A., Kulwal, P. and Pawar, B., 2017. Development of fruit and shoot borer resistant transgenic brinjal with *CryIAabc* gene: An assessment of factors influencing transformation efficiency. *Journal of Pharmacognosy and Phytochemistry*, 6(6), pp.2009-2013.
- Jadhav, M., Jadhav, A., Pawar, B., Kale, A. and Kute, N., 2015. *Agrobacterium*-mediated genetic transformation of brinjal with *cryIF* gene for resistance against shoot and fruit borer. *Journal of Crop Improvement*, 29(5), pp.518-527.
- Kumar, P.A., Mandaokar, A., Sreenivasu, K., Chakrabarti, S.K., Bisaria, S., Sharma, S.R., Kaur, S. and Sharma, R.P., 1998. Insect-resistant transgenic brinjal plants. *Molecular Breeding*, 4(1), pp.33-37.
- Magioli, C. and Mansur, E., 2005. Eggplant (*Solanum melongena* L.): tissue culture, genetic transformation and use as an alternative model plant. *Acta Botanica Brasiliica*, 19(1), pp.139-148.
- Mir, K.A., Dhatt, A.S., Sandhu, J.S. and Sidhu, A.S., 2011. Effect of genotype, explant and culture medium on organogenesis in brinjal. *Indian Journal of Horticulture*, 68(3), pp.332-335.
- Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, 15(3), pp. 473-497.
- Murthy, B.N.S., Murch, S.J. and Saxena, P.K.,

1998. Thidiazuron: A potent regulator of *in-vitro* plant morphogenesis. *In Vitro Cellular & Developmental Biology-Plant*, 34(4), p.267.
- Naik, M.L. and Ravali, B., 2016. Plant regeneration in eggplant (*Solanum melongena* L.) A review.
- Narasimha Rao, N., 1979. Barriers to hybridization between *Solanum melongena* and some other species of *Solanum*. In *Linnean Society symposium series*.
- Prakash, D.P., Deepali, B.S., Asokan, R., Ramachandra, Y.L., Anand, L. and Hanur, V.S., 2007. Effects of growth regulators and explant-type on *Agrobacterium*-mediated transformation in brinjal (*Solanum melongena* L.) cv. Manjarigota. *Journal of Horticultural Sciences*, 2(2), pp.94-98.
- Rashid, H., Yokoi, S., Toriyama, K. and Hinata, K., 1996. Transgenic plant production mediated by *Agrobacterium* in indica rice. *Plant Cell Reports*, 15(10), pp.727-730.
- Rotino, G.L. and Gleddie, S., 1990. Transformation of eggplant (*Solanum melongena* L.) using a binary *Agrobacterium tumefaciens* vector. *Plant Cell Reports*, 9(1), pp.26-29.
- Saini, D.K. and Kaushik, P., 2019. Visiting eggplant from a biotechnological perspective: A review. *Scientia Horticulturae*, 253, pp.327-340.
- Sarker, R.H., Yesmin, S. and Hoque, M.I., 2006. Multiple shoot formation in eggplant (*Solanum melongena* L.). *Plant Tissue Culture and Biotechnology*, 16(1), pp.53-61.
- Sidhu, M.K., Dhatt, A.S. and Sidhu, G.S., 2014. Plant regeneration in eggplant (*Solanum melongena* L.): A review. *African Journal of Biotechnology*, 13(6), pp.714-722.
- Zayova, E., Vassilevska-Ivanova, R., Kraptchev, B. and Stoeva, D., 2012. Indirect shoot organogenesis of eggplant (*Solanum melongena* L.). *Journal of Central European Agriculture*, 13(3), pp.0-0.

How to cite this article:

Priyanka Eraivan, S. Varanavasiappan, E. Kokiladevi, L. Arul, B. Vinoth Kumar and Kumar, K. K. 2021. Efficient Protocol for *invitro* Regeneration and *Agrobacterium*-Mediated Transformation of Brinjal cv.CO₂. *Int.J.Curr.Microbiol.App.Sci*. 10(02): 2785-2796. doi: <https://doi.org/10.20546/ijcmas.2021.1002.308>