Indian Res. J. Genet. & Biotech. 9(4): 469 – 477 November (2017)

Gynogenic approach of haploid induction in eggplant (*Solanum melongena* L.)

Subhada Pattanayak[1] and R. Gnanam[2]

[1] Center for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, 641003
[2] Imayam Institute of Agriculture and Technology, Thuraiyur, Trichy, 621010

(Received : September, 2017 : Revised : October, 2017; Accepted : October, 2017)

Abstract

To determine the potential for haploid induction via *in vitro* gynogenesis in eggplant, the ovaries from five different eggplant cultivars were selected. Un-pollinated ovaries were cultured on six selected induction media to induce *in vitro* gynogenesis. During the culture, ovaries enlarged markedly, with callus having opaque white color appearance which after transfer to regeneration media showed subsequent greening. Continuous sub-culturing in the regeneration medium MS + Zeatin 1.0 mg L^{-1} + Kinetin 3.0 mg L^{-1} led to leaf primordia development and shoot regeneration in the cultivar HD-2. The results show that stage of flower bud and growth hormone combinations play an important role in callus induction as well as regeneration. Gynogenesis can be used as an alternative approach for haploidy induction in the recalcitrant Solanaceous crops.

Key words: *Solanum melongena* L, ovary, *in vitro* gynogenesis, haploid.

Introduction

Eggplant [*Solanum melongena* L.] is one of the most important vegetable crops in the world. Eggplant haploids have a particular significance in eggplant breeding, especially for the development of molecular markers, genetic map construction, gene mapping, and gene cloning. *In vitro* androgenesis and gynogenesis are the two approaches often used for haploid induction in plants. The androgenesis approach, using anther and microspore cultures, has been widely employed in barley [Kuhlmann and Foroughi, 1989], maize [Gaillard *et al*., 1991], rice [Genovesi and Clint, 1979], tobacco [Barinova *et al*., 2004], rapeseed [Lichter, 1982], cabbage [Arnison and Keller, 1990] and garlic [Suh and Park, 1986]. With respect to the ability of eggplant to be induced to androgenesis, the first report on plant regeneration from anther cultures with a gametophytic origin for the regenerants was demonstrated [Raina and Iyer, 1973]. After 44 years of the pioneering study on anther culture, several media compositions and inductive treatments have been used to produce doubled haploids through anther culture in different eggplant F_1 hybrids and cultivars under different experimental conditions [Isouard *et al.* 1979; Dumas de Vaulx and Chambonnet 1982; Borgel and Arnaud 1986; Rotino *et al*. 1987;

Corresponding author's e-mail : subhabiotech19@gmail.com
Published by Indian Society of Genetics, Biotechnology Research and Development,
5, E Biotech Bhawan, Nikhil Estate, Mugalia Road, Shastripuram, Sikandra, Agra 282007
Online management by www.isgbrd.co.in

Tuberosa *et al.*, 1987; Rotino *et al*. 1991; Matsubara *et al*. 1992; Alpsoy and Seniz, 2007; Segui-Simarro, 2007; Li *et al*., 2008; Salas *et al.*, 2011; Segui-Simarro *et al.* 2011]. But there has not been any significant success achieved through gynogenesis in eggplant.

The gynogenic approach is an alternative way to induce haploid plants [Chen *et al.,* 2011]. Gynogenesis is the development of sporophytes from an unfertilized element of the female gametophyte. Since the cells of the embryo sac are haploid, the plants derived from them are also haploid [Sant and Prem, 2013]. The gynogenesis approach includes the culture of non-fertilized ovaries, ovules, or female gametophytes. Gynogenesis has been examined in seed plants of at least 40 species, belonging to 20 families. Gynogenic haploids have been successfully obtained in 25 of these species, belonging to 10 families. It has been used successfully in wheat [Zhu *et al*., 1981], rice [Zhou and Yang, 1981], tobacco [Zhu and Wu, 1979], sugar beet [Hosemans and Bossoutrot, 1983], potato [Tao, 1985], *Allium* [Campion and Alloni, 1990; Keller, 1990], carrot [Agnieszka and Adela, 2010], and cotton [Stella and Demetrios, 2009].

Despite the success of haploid production through gynogenesis in other crops, it has been difficult to achieve in Solanaceous crops due to their recalcitrant nature. In tomato, San and Gelebart [1984] examined ovary culture, and their results indicated that the excess callus from the ovary wall hindered gynogenetic haploid development. Bal and Abak [2003a, b] pre-cultured ovaries on a starvation medium, and then subcultured on NLN medium. This approach confirmed the hindering effect of the ovary reported by San and Gelebart [1984]. However, they did not report further callus and shoot development. Earlier research on *in vitro* gynogenesis concentrated only on ovary culture but the results obtained were not satisfactory [San and Gelebart, 1984; Bal and Abak, 2003 a, b]. But this shows that gynogenesis can be attempted in other Solanaceae species.

Compared with androgenesis approach, the gynogenic approach has the advantage that it is an alternative way to induce haploid plant for the varieties with low frequency of haploid induction [Zhou and Yang, 1981]. The gynogenic approach increases the frequency of green plant regeneration and has low frequency of aneuploidy and character variation. As no literature data were available on the successful development of haploid eggplants via *in vitro* gynogenesis, this experiment was carried out with the aim to develop a protocol for developing gynogenically derived haploid plants. In this study, the possibility of haploid induction through gynogenesis has been explored.

Materials and Methods

Stages of bud development, and plant material

Flower buds of various sizes were excised from plants growing in greenhouse at the Center for Plant Molecular Biology and Biotechnology, TNAU. In this study five different cultivars of eggplant (HD-1, HD-2, PLR-1, PLR-2 and Anamalai-1) were used. Flower buds of different sizes were collected from plants and immediately taken to the laboratory. The flower bud length and diameter of ovaries were noted

for each sampled bud. Based on the length and ovary diameter, the flower buds were classified into four stages (Table 1). Ovaries were then excised from buds, and surface disinfected in 4% sodium hypochlorite solution for 10–15 min; they were then rinsed with sterile distilled water 3–4 times. The ovaries were then dried on sterile filter paper and transferred to callus induction media. All subsequent operations took place under sterile conditions.

Culture on induction media

For selecting the most suitable stage of flower bud development for further studies, ovaries of all the four stages from the five different cultivars were inoculated on MS basal medium (without any growth regulator). As per the survival and callus induction, the best performing stage was selected. Five combinations of growth regulators in the culture medium for induction based on Murashige and Scoog (MS) medium were used for callus induction studies. All media contained sucrose (30 g L^{-1}), and phytagel (2.5 g L^{-1}). In all cases, the pH of the medium was adjusted to 5.8–6.0 before autoclaving, heat labile compounds were filter sterilized and added after autoclaving. MS basal medium was used as control. The ovaries were cultured in the above mentioned induction media and kept in dark at 25°C. The survival rate, days to callusing and the callus induction was monitored. The percentage of callus induction was calculated.

Sub-culture on regeneration media

Once calli had developed on induction media, after 20 days, they were ready to be transferred to regeneration medium. Different growth regulator combinations were added to the basal media for evaluation and ten regeneration media were evaluated. All media contained sucrose (30 g L^{-1}), and phytagel (2.5 g L^{-1}). The pH of the media was adjusted to 5.8–6.0 before autoclaving. Heat labile compounds were filter sterilized and added after autoclaving. Calli, approximately 1.5-2 cm in diameter, were transferred to petriplates with regeneration media. Plates were then placed under conditions of 16-h light / 8-h dark cycles, at 28°C. Sub-culturing was done at 15 days interval. Shoot production was the desired endpoint. Once the shoots had developed to 2 to 3 leaves, they were placed on root induction medium (MS with the addition of 1.0µmol L^{-1} IBA, 3% (w/v) sucrose, and 0.3% (w/v) phytagel).

Data analysis: The experiment was conducted and arranged in Completely Randomized Design. All data on callused ovaries were subjected to analysis of variance (ANOVA), and mean values were evaluated at the $p < 0.05$ level of significance using Duncan's Multiple Range Test.

Results and Discussion

The suitable stage of flower buds for ovary culture

The initial attempts at ovary culture clearly indicated that the stage of ovary development was very important. Most of the ovaries obtained from the flower buds at stages I and II did not form calli in the test culture medium used, as they gradually turned brown and died. Only a limited number of ovaries obtained from flower buds at stage IV, expanded and subsequently

produced calli. However, ovaries obtained from flower buds at stage III began expansion after 8–12 h in culture, with the rate of callus induction reaching 50–60%. Therefore, the buds at stage 3 were found to be most suitable for callus induction (Table 2).

In this research, sampling criteria were developed to select the flower buds at the appropriate stage for ovule isolation and culture. Relationships between bud size, color, and ovary diameter differ among eggplant genotypes, which make it difficult to select ovaries at the appropriate stage. The use of the ovary diameter as an indicator of ovule development considerably enhanced the ability to harvest ovaries at the appropriate stage required for producing cell clumps in the embryo sacs. Additionally, the results confirmed the reports of Li *et al*. (2008), who successfully used a similar technique to identify ovule development stage in eggplant.

Induction culture of isolated ovaries

The ovaries enlarged markedly after 8–12 h culture on three induction media, with an opaque white color. After one week, they doubled in size and calli began to form. After 20 days calli grew to 1.5-2.0 cm in diameter. Usually, 6-7 calli were produced in each petriplate out of the 10 ovaries inoculated. Not much difference was noted in the performance of ovaries of the five eggplant varieties cultured on the evaluated media regarding the callus induction. The data analysed for the results of the cultivar HD-2 are mentioned in Table-3. In case of HD-2, best growth regulator medium for callus induction was found to be EG_7 [MS + NAA (2.0) + BAP (2.0)]. In EG_7 medium the number of days for callusing was the least (12 days) while the survival percentage (80.24) and callus induction frequency (76.20) was the highest.

Plant regeneration

Calli, derived from isolated ovary culture on induction media, were cultured on ten regeneration media separately. After one week in culture, the calli had enlarged. Subsequently the surface of the callus became pale white and then began to turn greenish after three weeks in regeneration medium. The performance of callus growth was similar on all of the ten media. After a period of two months, the calli became fully green.

Shoot regeneration occurred only in HD-2 variety when its calli were transferred to the regeneration medium MS + Zeatin 1.0 mg L^{-1} + Kinetin 3.0 mg L^{-1} after continuous sub-culturing at 15 days interval over a period of 2 months. Shoot primordia formation was first observed. For other cultivars, no shoot regeneration was observed. After 15 days, the developing shoots were transferred to rooting media MS + IBA 1.0 mg L^{-1} for rooting induction.

Conclusion

The development of a rapid, efficient and genotype-insensitive method for eggplant haploid production is a highly desired goal for eggplant breeders. Haploid production would also facilitate research on molecular markers, genetic map construction, gene mapping, gene cloning and numerous other studies in eggplant. This study can be considered as an attempt towards gynogenesis induction in a recalcitrant

Solanaceous crop like eggplant. The selection of appropriate flower bud stages, growth regulators for callus induction medium and regeneration medium was important as they are critical for enlargement of ovules and produce cell clumps in embryo sacs which further lead to better plantlet regeneration. Genotype of plant also played an important role as out of the five cultivars in only one cultivar shoot regeneration was there. In conclusion, the findings provided potential for further research in the gynogenic haploid induction in a recalcitrant crop like eggplant as well as other recalcitrant but economic important crops of the Solanaceae family.

Table 1: Stage of flower bud with respect to bud length and ovary diameter

Sl No	Bud Length(cm)	Ovary Diameter (cm)	Stage
1	0.8	0.2	I
2	1.2	0.3	II
3	1.8	0.6	III
4	>2.0	0.8	IV

Table 2: Effect of stage of flower bud on survival and callus induction from ovaries

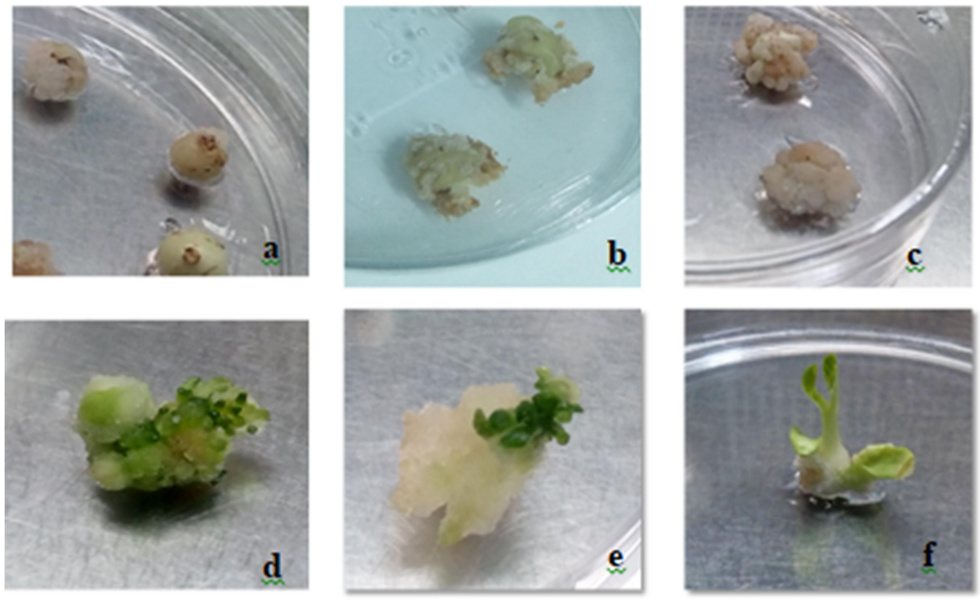
Cultivar	Size of flower bud			
	I	II	III	IV
HD-1	-	-	+	-
HD-2	-	-	+	+
PLR-1	-	-	+	-
PLR-2	-	-	+	-
Annamalai-1	-	-	+	+

Table 3: Effect of different growth regulator combinations on callus induction from the HD-2 ovaries

Code	Media and growth regulators (mg L^{-1})	Survival percentage (%) of ovaries	Days for callus initiation	Callus induction frequency
EG0	MS Basal	54.64^{g}	21.46^{a}	50.78^{g}
EG1	MS + BAP (1.0)	64.78^{e}	15.33^{e}	59.96^{e}
EG3	MS + BAP (2.0)	75.86^{b}	14.78^{c}	71.62^{b}
EG4	MS + NAA (1.0) + BAP (2.0)	70.46^{c}	18.58^{c}	65.66^{c}
EG5	MS + IAA (1.0) + BAP (2.0)	58.45^{f}	20.46^{b}	53.78^{f}
EG6	MS + 2,4-D (1.0) + BAP (2.0)	69.66^{d}	15.78^{d}	65.22^{d}
EG7	MS + NAA (2.0) + BAP (2.0)	80.24^{a}	12.66^{f}	76.20^{a}
	$LSD_{(0.05)}$	1.81	0.67	0.97
	CV (%)	10.46	6.45	9.08

Table 4 : Performance of callus derived from HD-2 ovaries in different regeneration medium

Code	Medium	Response
RG_1	MS + Zeatin (0.5) + Kinetin (0.5)	-
RG_2	MS + Zeatin (1.0) + Kinetin (1.0)	-
RG_3	MS + Zeatin (0.5) + Kinetin (2.0)	-
RG_4	MS + Zeatin (1.0) + Kinetin (2.0)	-
RG_5	MS + Zeatin (0.5) + Kinetin (3.0)	-
RG_6	MS + Zeatin (1.0) + Kinetin (3.0)	+
RG_7	MS + Zeatin (2.0) + Kinetin (3.0)	-
RG_8	MS + Thidiazuron (0.5) + Kinetin (1.0)	-
RG_9	MS + Thidiazuron (1.0) + Kinetin (2.0)	-
RG_{10}	MS + Thidiazuron (1.0) + Kinetin (3.0)	-



The photographs denote the different stages starting from inoculation to shoot development in HD-2:

a) Enlarged eggplant ovary 2 days after inoculation
b) Development of callus 7 days after inoculation
c) Proliferation of callus 15 days after inoculation
d) Initiation of leaf primordia from callus
e) Development of leaf primordia from callus
f) Development of shoots

References

1. **Agnieszka K. and Adela A.** 2010. In vitro culture of unfertilized ovules in carrot (*Daucus carota* L.). *Pl. Cell T. Org. Cult.*, **102**: 309–319.
2. **Alpsoy H.C. and Seniz V.** 2007. Researches on the *in vitro* androgenesis and obtaining haploid plants in some eggplant genotypes. *Acta Hortic.,* **729** : 137–141.
3. **Arnison P. and Keller W.** 1990. A survey of the anther culture response of *Brassica oleracea* L. cultivars grown under field conditions. *Pl. Breed.,* **104**: 125-133.
4. **Bal U. and Abak K.** 2003a. Attempts of haploidy induction in tomato (*Lycopersicon esculentum*Mill.) via gynogenesis I: pollination with *Solanum sisymbriifolium* L am. pollen. *Pak. J Biol. Sc.*, **6**: 745-749.
5. **Bal U. and Abak K.** 2003b. Attempts of haploidy induction in tomato (*Lycopersicon esculentum*Mill.) via gynogenesis II: In vitro non-fertilized ovary culture. *Pak. J Biol. Sc.*, 6: 750-755.
6. **Barinova I., Clement C., Martiny L., Baillieul F., Soukupova H., Heberle-Bors E. and Touraev A.** 2004. Regulation of developmental pathways in cultured microspores of tobacco and snapdragon by medium pH. *Planta*, **219**: 141-146.
7. **Borgel A. and Arnaud M.** 1986. Progress in eggplant breeding, use of haplomethod. *Capsicum Newsl.,* **5** : 65–66.
8. **Campion B. and Alloni C.** 1990. Induction of haploid plants in onion (*Allium cepa* L.) by in vitro culture of un-pollinated ovules. *Pl. Cell T. Org. Cult.*, **20**: 1-6.
9. **Chen J. F., Li C., Ahmed A. M., Kere G. M.** 2011. *In vitro* haploid and dihaploid production via unfertilized ovule culture. *Pl. Cell T. Org. Cult.*, **104**: 311–319.
10. **Dumas de Vaulx R. and Chambonnet D.** 1982. Culture *in vitro* d'antheres d'aubergine (*Solanum melongena* L.): stimulation de la production de plantes au moyen de traitements a` +35˚C associes a` de faibles teneurs en substances de croissance. *Agronomie.,* **2** : 983–988.
11. **Gaillard A., Vergne P. and Beckert M.** 1991. Optimization of maize microspore isolation and culture conditions for reliable plant regeneration. *Pl. Cell Rep.*, **10**: 55-58.
12. **Genovesi A. and Clint W.** 1979. Improved rate of callus and green plant production from rice anther culture following cold shock. *Crop Sci.*, **19**: 662-664.
13. **Gresshoff P. and Doy C.** 1972. Development and differentiation of haploid *Lycopersicon esculentum* (tomato). *Planta*, **107**, 161-170.

14. **Hosemans D. and Bossoutrot D.** 1983. Induction of haploid plants from in vitro culture of unpollinated beet ovules (*Beta vulgaris* L.). *Zeitschrift fur Pflanzenkrankheiten und Pflanzenaschutz*, **91**: 74-77.
15. **Isouard G., Raquin C. and Demarly Y.** 1979. Obtention de plantes haploides et diploides par culture in vitro d'anthe`res da'ubergine (*Solanum melongena* L.). C R Acad Sci Paris., **288**: 987–989.
16. **Keller J.** 1990. Culture of un-pollinated ovules, ovaries, and flower buds in some species of the genus *Allium* and haploid induction via gynogenesis in onion (*Allium cepa* L.). *Euphytica*, **47**: 241-247.
17. **Kuhlmann U. and Foroughi W. B.** 1989. Production of doubled haploid lines in frequencies sufficient for barley breeding programs. *Pl. Cell Rep.*, **8**, 78-81.
18. **Li H., Sun Z., Lian Y. and Xu X. H.** 2008. Cytological analysis of heat shock effects and developmental capacity of in vitro cultured microspore in eggplant (*Solanum melongena* L.). *Seeds*, **27**: 1-5.
19. **Lichter R.** 1982. Induction of haploid plants from isolated pollen of *Brassica napus*. *Zeitschrift fur Pflanzenkrankheiten and Pflanzenaschutz,* **105**: 427-434.
20. **Matsubara S., Hu K.L. and Murakami K.** 1992. Embryoid and callus formation from pollen grains of eggplant and pepper by anther culture. *J. Jpn. Soc. Hortic. Sci.,* **61**: 69–77.
21. **Murashige T. and Skoog F.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Pl. Physiol.*, **15**: 473-497.
22. **Raina S.K. and Iyer R.D.** 1973. Differentiation of diploid plants from pollen callus in anther cultures of *Solanum melongena* L. *Z Pflanzenzucht.,* **70**: 275–280.
23. **Rotino G.L , Restaino F., Gjomarkaj M., Massimo M., Falavigna A., Schiavi M. and Vicini E.** 1991. Evaluation of genetic variability in embryogenetic and androgenetic lines of eggplant. *Acta Hortic.,* **300**: 357–362.
24. **Rotino G.L., Falavigna A. and Restaino F.** 1987. Production of anther-derived plantlets of eggplant. *Capsicum Newsl.,* **6**: 89–90.
25. **Salas P., Prohens J., Segui-Simarro J.M.** 2011. Evaluation of androgenic competence through anther culture in common eggplant and related species. *Euphytica,* **182**: 261–274.
26. **San L. and Gelebart P.** 1984. Production of gynogenetic haploids. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants: *Plant regeneration and genetic variability*. Academic Press, Orlando: 305-322.
27. **Sant S. B. and Prem K D.** 2013. Gynogenesis. *Pl. Tissue Cult.* : 113-118.
28. **Segui-Simarro J. M.** 2007. Embryogenesis induction, callogenesis, and plant regeneration by in vitro culture

of tomato isolated microspores and whole anthers. *J Experi. Bot.*, **58**: 1119-1132.

29. **Segui-Simarro J.M., Corral-Martinez P., Parra-Vega V. and Gonzalez-Garcia B.** 2011. Androgenesis in recalcitrant solanaceous crops. *Plant Cell Rep.,* **30**: 765–778.
30. **Stella K. K. and Demetrios G. R.** 2009. *In vitro* gynogenesis in cotton (*Gossypium sp*.). *Pl. Cell T. Org. Cult.*, **96**: 53–57.
31. **Suh S. K. and Park H. G.** 1986. Studies on the anther culture of garlic (*Allium sativum L.*) I. Callus formation and plantlet regeneration. *J Kor. Soc. Hort. Sci.*, **27**: 89-95.
32. **Tao Z. R.** 1985. *In vitro* production of haploid plantlets from the un-pollinated ovaries of potato. *Hereditas*, **7**: 24.
33. **Tuberosa R., Sanguineti M.C. and Conti S.** 1987. Anther culture of eggplant *Solanum melongena* L. lines and hybrids. *Genet Agr.,* **41**: 267–274.
34. **Zhou C. and Yang H Y.** 1981. Induction of haploid rice plantlets by ovary culture. *Pl. Sci. Lett.*, **20**: 231-237.
35. **Zhu Z. C. and Wu H. S.** 1979. In vitro production of haploid plantlets from the un-pollinated ovaries of *Triticum aestivum* and *Nicotiana tabacum*. *Acta Genetica Sinica*, **8**: 63-65.
36. **Zhu Z. C., Wu H. S., An Q. K.and Liu Z. Y.** 1981. Induction of haploid plantlets from un-pollinated ovaries of *Triticum aestivum* cultured *in vitro*. *Acta Genetica Sinica*, **8**: 586-590.