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Haploid Plant Regeneration from Unpollinated Ovules of *Cucumis melo* L. Var. *Conomon* cv. Mudicode

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Authors' contributions

This work is a portion of the Ph. D. thesis of the first author SPK. Second author HNM gave the idea, supervised the research work and approved the final manuscript. All authors read and approved the final manuscript.

Short Communication

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ABSTRACT

Major objective of the present work was to induce haploid plants in *Cucumis melo* var. *conomon* cv. Mudicode (Common name: Kani vellari). Ovules of Kani vellari plants were collected one day before anthesis and were cultured *in vitro* on MS medium supplemented with IAA, IBA, NAA, 2, 4-D, KN, BAP, TDZ and 2-iP (1.0, 2.0 and 5.0 µM) and BAP/TDZ/KN (1.0, 2.0 and 5.0 µM) in combination with IAA, IBA, NAA, 2, 4-D (1.0, 2.0 and 5.0 µM). The cultured ovules involved in callus induction and an optimum quantity of 93.15 mg of callus from cultured ovules was obtained on the MS medium fortified with 1 µM BAP + 2 µM NAA. The callus induced on medium supplemented with 1 µM BAP+2 µM NAA was subcultured on MS medium supplemented with 5 µM BAP was involved in shoot organogenesis and developed a maximum of 5.55 shoots. The rooting of the regenerated shoots was achieved on MS medium supplemented with 1 µM IAA. Cytological analysis of the root tips of regenerated plants confirmed their haploid nature.

Keywords: Haploids; melon; ovule culture.

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ABBREVIATIONS

BAP = 6-benzylaminopurine; 2, 4-D = 2, 4-diclorophenoxy acetic acid; IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; KN = kinetin; MS = Murashige and Skoog medium; NAA = Naphthalene acetic acid; 2-iP = N6-2-isopentenyl adenine; TDZ = thidiazuron.

1. INTRODUCTION

Melons (*Cucumis melo* l.; 2n=24; Cucurbitaceae) are a diverse group of fresh, dessert fruits that includes the orange flesh cantaloupes, the green flesh honeydew and the mixed melons. *Cucumis melo* var. *conomon* is considered to be the most ancient form of melon domesticated in China [1,2], and is also held that it had originated from wild melon (var. *Agrestis*) in China [2]. It is cultivated in Asia particularly in India, China, Japan, Korea and Southeast Asia. *Cucumis melo* var. *conomon* is popularly known as 'kani vellari' in Kerala state, India. Both mature and immature fruits are eaten raw or cooked, pickled or made into sweets and juice.

Pure lines are considered to be a first step in the genetic improvement of vegetable crops. Pure lines can be obtained in a short time by *in vitro* anther or ovule culture. The morphogenesis occurring in gametophytic cells is remarkably efficient for the production of pure lines and detection of mutation. According to Guis et al. [3] and Nunez-Palenius *et al*. [4] the production of haploids through androgenesis has not been successful in melon plants. Studies have been conducted on gynogenic plant regeneration in *C. melo* via ovule or ovary culture by Ficcadenti et al. [5] and also haploid, diploid and mixoploids plant regeneration was reported. However, the induction of parthenogenetic development of the egg cell by pollination with irradiated pollen followed by haploid embryo rescue was first reported successfully in *Cucumis melo* var. *cantalupensis* by Sauton and Dumas de Vaulx [6]. Subsequently, haploid plant regeneration was reported in different cultivars of melon using the irradiated pollen [7-14]. Nonetheless, successful haploid plant regeneration via ovule or ovary culture or parthenogenetic embryo culture depends upon genotype, culture medium and supplementation of growth regulators to culture medium [15]. Haploid induction was attempted in Indian cultivars of melon (snapmelon and culinary melon) by Godbole and Murthy [8, 9], yet, frequency of haploid plant regeneration was very low. In the present study *in vitro* ovule culture in *Cucumis melo* var. *conomon* cv. Mudicode has been attempted and a successful haploid production is reported here.

2. MATERIALS AND METHODS

2.1 Plant Material

Germplasm of *Cucumis melo* var. *conomon* cv. Mudicode was collected from Kerala Agricultural University, Trissur, India and plants were raised in the experimental garden, Department of Botany, Karnatak University, Dharwad, India.

2.2 Ovule Culture and Plant Growth Regulator Treatment

Unpollinated ovaries were harvested one day before anthesis. The ovaries were surface sterilized using 70% ethanol for 3 mins, followed by 2 min soaking in 0.1% mercury chloride and thorough rinsing in sterile distilled water for three to four times. Individual ovules were isolated from the ovaries under ZEISS (Stemi 2000-C) strereozoom microscope and cultured

onto the MS semisolid medium supplemented with 3% sucrose and plant growth regulators like IAA, IBA, NAA, 2, 4-D, KN, BAP, TDZ and 2-iP (1.0, 2.0 and 5.0 µM) individually and BAP/TDZ/KN (1.0, 2.0 and 5.0 µM) in combination with 2, 4-D/NAA/IBA (1.0, 2.0 and 5.0 µM). The pH of the medium was adjusted to 5.8 before autoclaving at 121ºC for 20 min and solidified with 8.0 g l^{-1} agar (HiMedia, Mumbai). Six ovules were cultured per culture tube containing 20 ml of medium (150 × 25 mm; Borosil, Mumbai). The cultures were incubated at 25ºC ± 2ºC at 16 hours light (40 mmol m^{-2} s^{-1} PFD) provided by cool-white fluorescent lamps (Phillips, New Delhi) and 8 hours dark. For each treatment 72 ovules were cultured (6 ovules per culture tube, 12 replicates per treatment) and the experiments were repeated three times. The number of ovules involved in callus induction was recorded at the end of eight weeks. The callus derived from ovules on medium supplemented with 1 µM BAP+2 µM NAA was cultured on MS medium supplemented with 1.0, 2.0, 5.0 and 10 µM BAP/TDZ/KN/2-iP for shoot regeneration. Shoots regenerated from callus were cultured on MS medium supplemented with 0.5, 1.0 and 2.0 µM NAA/IAA/IBA for induction of roots. Three replicates were maintained for each treatment, shooting and rooting data were collected after eight weeks of culture. The data were subjected to analysis of variance according to Duncan's multiple range tests.

2.3 Cytological Studies

Haploid nature of the regenerated plants was confirmed by cytological analysis of the root tips. The root tips of regenerants were harvested and treated with an aqueous solution of 8-hydroxyquinoline (2.0 mM) at room temperature for 4 h to improve chromosome spreading and staining [16]. The pretreated root tips were fixed in Carnoy's solution (3 parts ethanol: 1 part glacial acetic acid) for 24 h. Subsequently, the root tips were washed in water, hydrolyzed in 1 N HCl at 60ºC for 10 min, stained with Feulgen reagent for 1 h and squashed in 45% acetic acid for microscopic observation.

3. RESULTS AND DISCUSSION

The ovules cultured on the MS medium supplemented with individual growth regulators did not show any response even after eight weeks of culture. Whereas, the ovules cultured on MS medium supplemented with combinations of BAP/TDZ/KN (1.0, 2.0 and 5.0 µM) with 2, 4-D/NAA/IBA (1.0, 2.0 and 5.0 µM) induced callus from the ovule at the end of six weeks. The combinations of BAP (1.0 and 5.0 µM) with NAA (1.0 and 2.0 µM), 2.0 µM KN+ 5.0 µM NAA and 2.0 µM TDZ +2.0 µM 2, 4-D yielded better results among the various combinations tested and the results are represented in Fig. 1. The MS medium supplemented with combination of 1 µM BAP+2 µM NAA was proved best for induction of callus from unpollinated ovules (90.73%; Fig. 1), which produced a maximum of 93.15 mg of white, friable non-embryogenic callus after eight weeks of culture. The non responding ovules dried within four weeks. The cultured ovules became bulged and turned green at the end of 2 weeks of culture (Fig. 2A). Callogenesis was initiated at the micropylar end of the ovules cultured on the MS medium supplemented with 1 µM BAP+2 µM NAA after six weeks and proliferated within eight weeks (Fig. 2B). *In vitro* cultured ovules respond either by inducing the embryogenic callus or non-embryogenic callus and subsequently leading to formation of either embryos (embryogenesis) or organs (organogenesis) depending upon the growth regulators supplemented to the culture medium [15]. Growth regulators, especially auxins or

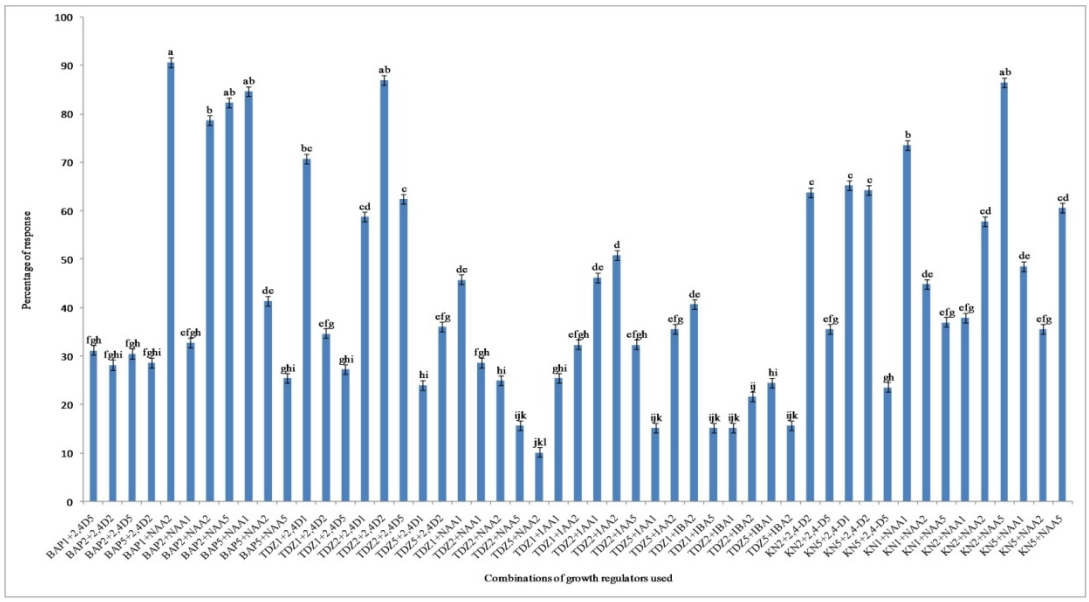

Fig. 1. Effect of combinations of cytokinins with auxins supplemented to MS medium on induction of callus from cultured ovules

cytokinins have been used for induction of gynogenesis and their optimum concentration/combination may vary considerably from species to species. Tang et al. [17] obtained gynogenic haploids in maize on 2, 4-D supplemented medium whereas, Bhat and Murthy [18] reported direct parthenogenetic embryo development in niger on medium supplemented with NAA, whereas Lakshmi Sita and Ravindran [19] reported the production of gynogenetic haploids in mulberry by using BAP or KN in the culture medium. In the present study, induction of non-embryogenic callus was observed from the cultured ovules of *Cucumis melo* var. *Conomon* cv. Mudicode only on the medium supplemented with combinations of auxins and cytokinins (Fig. 1). Optimum callus was induced on the medium supplemented with combination of 1 µM BAP+2 µM NAA. In concurrent with our results, a combination of auxin and cytokinin was useful for gynogenesis in *Allium* species [20]. Bhagyalakshmi [21] found that neither NAA nor BAP alone supported caulogenesis in saffron. Specific ratios of NAA and BAP supported, caulogenesis, whereas an increase in NAA (up to 26.9 µM) progressively increased the percentage response, and higher levels of BAP enhanced callus and subsequent shoot formation. Similarly, callus mediated plant regeneration from unfertilized ovule culture was reported in maize, egg plant and *Gerbera jamesonii* [22,23].

The non-embryogenic callus induced on medium supplemented with 1 µM BAP+2 µM NAA was used for regeneration of shoots and callus was cultured on medium supplemented with cytokinins (1.0, 2.0, 5.0 and 10.0 µM BAP/TDZ/KN/2-iP) and results are presented in Table 1. BAP was superior in induction of shoots from ovule derived callus and on medium supplemented with 5 µM BAP optimum 5.55 shoots were developed from the callus. The callus cultured on MS medium supplemented with 5 µM BAP initially turned green after two weeks of subculture. Further, the green callus produced a number of shoots at the end of 4 weeks (Fig. 2C), which later developed into shoots at the end of eight weeks. Similar to the present results 1 µM BAP in combination with 1µM 2-iP was proved responsive for shoot

regeneration from leaf derived callus of *C. melo* [24]. Shoots with two to four leaves were then subcultured onto the MS medium supplemented with IAA, IBA or NAA (0.5, 1.0 and 2.0 µM) for root induction. Optimum 3 roots were developed from shoots cultured on MS medium with 1 µM IAA (Table 2; Fig. 2D). Effectiveness of IAA in rooting of shoots has been reported in *Cucurbita pepo* [25].

Root tip analysis of regenerants showed haploid nmber of chromosomes (*n* = 12; Fig. 3A). The diploid number being 2n=24 (Fig. 3B). Whiteker and Bemis [26] indicated that there are ample of evidences that *Cucumis melo* have twelve pairs of chromosomes. However, small size of the chromosomes in this genus makes troublesome to count accurately.

Table 1. Effect of different cytokinins supplemented to MS medium on shoot induction from ovule derived callus of *Cucumis melo* var. *conomon* cv. Mudicode after eight weeks of culture

Growth regulators	Concentration (µM)	% of response*	Mean number of shoots*
BAP	1.0	0.00b	0.00b
	2.0	0.00b	0.00b
	5.0	94.44a	5.55a
	10.0	0.00b	0.00b
TDZ	1.0	0.00b	0.00b
	2.0	0.00b	0.00b
	5.0	0.00b	0.00b
	10.0	0.00b	0.00b
KN	1.0	0.00b	0.00b
	2.0	0.00b	0.00b
	5.0	0.00b	0.00b
	10.0	0.00b	0.00b
2-iP	1.0	0.00b	0.00b
	2.0	0.00b	0.00b
	5.0	0.00b	0.00b
	10.0	0.00b	0.00b

**Mean values followed by same letters are not significantly different according to DMRT at p = 0.05.*

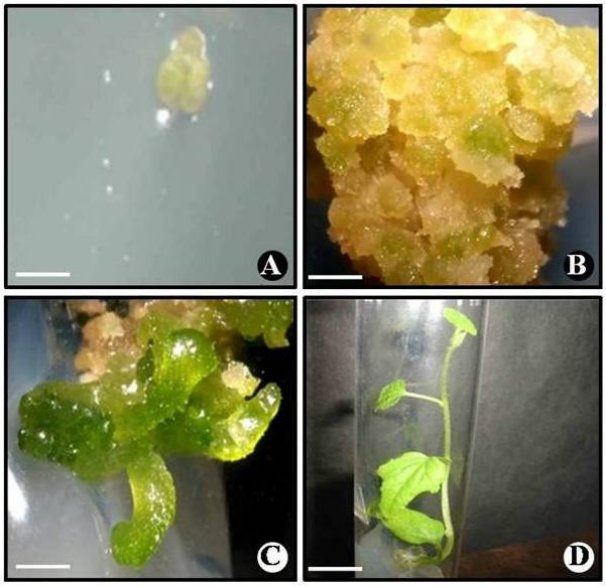

Fig. 2. *In vitro* ovule culture of *Cucumis melo* var. *conomon* cv. Mudicode: A. Bulged green ovule cultured on MS medium supplemented with 1 µM BAP + 2 µM NAA after 2 weeks of culture (Bar=0.2cm) B. Callus developed after six weeks of culture on the MS medium supplemented with 1 µM BAP + 2 µM NAA (Bar=0.1cm) C. Green shoots after 4 weeks after subculture on the MS medium supplemented with 5 µM BAP (Bar=0.23cm) D. Rooting of shoots after eight weeks of culture on the MS medium supplemented with 1 µM IAA (Bar=0.62cm)

Table 2. Effect of auxins supplemented to MS medium on *in vitro* rooting of shoots of *Cucumis melo* var. *conomon* cv. Mudicode after eight weeks of culture

Growth regulators	Concentration (µM)	No. of shoots cultured	Mean number of roots*
NAA	0.5	3	0.00b
	1.0	3	0.00b
	2.0	3	0.00b
IAA	0.5	3	0.00b
	1.0	3	2.22a
	2.0	3	0.00b
IBA	0.5	3	0.00b
	1.0	3	0.00b
	2.0	3	0.00b

**Mean values followed by same letters are not significantly different according to DMRT at p = 0.05*

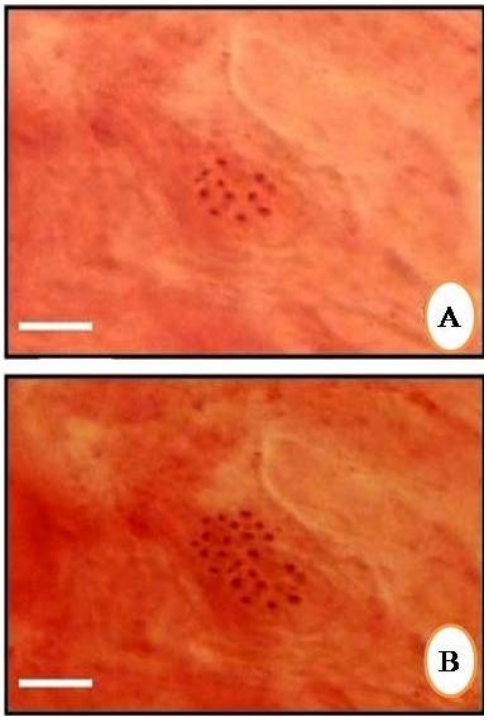

Fig. 3. Cytological analysis of *Cucumis melo* var. c*onomon* cv. Mudicode: A. Cytology of haploid plant showing 12 chromosomes (Bar=0.009cm) B. Cytology of controlled plant showing 24 chromosomes (Bar=0.009 cm)

4. CONCLUSION

In vitro haploid production reported in several species depended upon growth regulators, especially auxins and/or cytokinins and their optimum concentration in induction of haploid plants. In the present study, 90.73% of the ovules cultured on MS medium supplemented with 1 µM BAP and 2 µM NAA produced the callus, thus increasing the rate of haploid production when subcultured onto MS medium supplemented with 5 µM BAP by producing 5.55 shoots. In conclusion, the methodology developed here is simple and could be used for the production of haploids/double haploids for breeding programme of this cultivar.

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COMPETING INTERESTS

Authors declare that there are no competing interests between individual and organizations that can affect the publication of this work.

REFERENCES

1. Jeffrey C. A review of the Cucurbitaceae. Bot. J. Linn. Soc. 1980;81:233-47.
2. Walters TW. Historical overview on domesticated plants in China with special emphasis on the Cucurbitaceae. Econ. Bot. 1989;43:297-313.
3. Guis M, Roustan JP, Dogimont C, Pitrat M, Pech JC. Melon biotechnology. Biotechnol. Genet. Eng. 1998;15:289-311.
4. Nunez-Palenius HG, Hopkins D, Cantlie DJ. Powdery mildew of cucurbits in Florida. Hort Sci Depart, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Document HS1067. 2009;9.
5. Ficcadenti N, Sestili S, Annibali S. *In vitro* gynogenesis to induce haploid plants in melon (*Cucumis melo* L). Journal Genet. Breed. 1999;53:255–7.
6. Sauton A, Dumas de Vaulx R. Induction of gynogenetic haploid plants in muskmelon (*Cucumis melo* L.) by use of irradiated pollen. Agronomie 1987;7:141–8.
7. Lofti M, Alan AR, Henning MJ, Jahn MM, Earle ED. Production of haploids and doubled haploid plants in melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. Plant Cell Rep. 2003;21:1121-8.
8. Godbole M, Murthy HN. *In vitro* production of haploids via parthenogenesis in culinary melon (*Cucumis melo* var. *acidulous*). Ind. J. Biotechnol. 2012;11:495-7.
9. Godbole M, Murthy HN. Parthenogentic haploid plants using gamma irradiated pollen in snapmelon (*Cucumis melo* var. *momordica*). Plant Cell Tiss. Org. Cult. 2012;109:167-70.
10. Sauton A. Effect of season and genotype on gynogenetic haploid production in muskmelon, *Cucumis melo* L. Sci. Hort. 1998;35:71-5.
11. Cuny F, Grotte M, Dumas de Vaulx R, Rieu A. Effect of gamma irradiation of pollen on parthenogenetic haploid production in muskmelon (*Cucumis melo* L.). Environ. Exp. Bot. 1993;33:301-12.
12. Ficcandenti N, Veronese P, Sestili S, Crino P, Lucretti S, Schiavi M, Saccardo F. Influence of genotype on the induction of haploidy in *Cucumis melo* L. by using irradiated pollen. J. Genet. Breed. 1995;49:359-64.
13. Lim W, Earle ED. Enhanced recovery of doubled haploid lines from parthenogenetic plants of melon (*Cucumis melo* L.). Plant Cell Tiss. Org. Cult. 2009;98:351-6.
14. Yanmaz R, Ellialtoglu S, Taner KY. The effect of gamma irradiation on pollen viability and haploid plant formation in snake cucumber (*Cucumis m*elo L. var. *flexosus* Naud.) Acta Hort. 1999;492:307-10.
15. Chen JF, Cui L, Malik AA, Mbira KG. *In vitro* haploid and dihaploid production via unfertilized ovule culture. Plant Cell Tiss. Org. Cult. 2011;104:311-9.
16. Owen HR, Miller AR. A comparison of staining techniques for somatic chromosomes of strawberry. Hort Science. 1993;28:155-6.
17. Tang F, Tao Y, Zhao T, Wang G. *In vitro* production of haploid and double haploid plants from pollinated ovaries of maize (*Zea mays*). Plant Cell Tiss. Org. Cult. 2006;84:233-7.
18. Bhat JG, Murthy HN. Haploid plant regeneration from unpollinated ovule cultures of niger (*Guizotia abyssinica* (L. f.) Cass.). Russ. J. Plant Physiol. 2008;55:241-5.
19. Lakshmi Sita G, Ravindaran S. Gynogenic plants from ovary culture of mulberry (*Morus indica*). In: Prakash J, Pierik RLM, editors. Horticulture: New Technologies and Applications. Dordrecht: Kluwer; 1991.
20. Alan AR, Mutschler MA, Brants A, Cobb E, Earle ED. Production of gynogenic plants from hybrids of *Allium cepa* L. and *A. roylei* Stern. Plant Sci. 2003;165:1201-11.
21. Bhagyalakshmi M. Factors influencing direct shoot regeneration from ovary explants of saffron. Plant Cell Tiss. Org. Cult. 1999;58:205-11.

22. Uchimiya H, Kameya T, Takahashi N. *In vitro* culture of unfertilized ovules in *Solanum melongena* and ovaries in *Zea mays*. Jap. J. Breed. 1971;21:247-50.
23. Meynet J, Sibi M. Haploid plants from *in vitro* culture of unfertilized ovules in *Gerbera jamesonii*. Z. Pflanzenzuchtg. 1984;93:78-85.
24. Kathal R, Bhatnagar SP, Bhojwani SS. Regeneration of plants from leaf explants of *Cucumis melo* cv. Pusa Sharbati. Plant Cell Rep. 1988;7:449-51.
25. Kathiravan K, Vengedesan G, Singer S, Steinitz B, Paris H, Gaba V. Adventitious regeneration *in vitro* occurs across a wide spectrum of squash (*Cucurbita pepo*) genotypes. Plant Cell Tiss. Org. Cult. 2006;85:285-95.
26. Whitaker TW, Bemis WP. Cucurbits. In: Simmonds NW, editor. Evolution of Crop Plants. 3rd ed. Essex: Longman Scientific & Technical; 1976.

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