African Journal of Biotechnology Vol. 10(70), pp. 15717-15724, 9 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2445
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Comparison of *in vivo* and *in vitro* Colchicine Application for Production of Dihaploid 'Kirkagac' and 'Yuva Hasanbey' Melons

İlknur Solmaz*, Nebahat Sarı, Irmak Gürsoy and Serkan Kasapoğlu

Department of Horticulture, Faculty of Agriculture, University of Cukurova, Adana, Turkey.

Accepted 9 September, 2011

This study was performed to compare the efficiency of different colchicine application methods for doubling of haploid melon plants in melon breeding programs. Doubled haploids assist breeding by reducing time for the recovery of homozygous breeding lines. Irradiated pollen technique provides successful results in melons and pure breeding lines can be produced in relatively shorter time. Despite the advantages, low frequency of haploid embryos, difficulties for detecting and excising the embryos and low percentage of duplication rate are the limiting factors of this technique. The objective of this study was to determine the effect of different colchicine treatments to double haploid melon plants. The same dose of colchicine (0.5 %) was applied either *in vitro* or *in vivo* and the resulting doubled haploids were compared. The results indicated that the most convenient method was found *in vivo* application of colhicine (0.5 %) by shoot tip immersing method for 2 hours. The mean duplication rate was 10.13 % after *in vitro* colchicine application and 46.03 % after *in vivo* colchicine application among the genotypes of 'Kirkagac' and 'Yuva Hasanbey melon groups.

Key words. Irradiation, Co^{60}, haploid, duplication, embryo rescue.

INTRODUCTION

Doubled haploids are very advantageous in the breeding of vegetable crops. Dihaploid lines are developed to obtain 100 % homozygosity and are used as parental lines of hybrid cultivars or directly as homozygous cultivars. The haploidization technique is beneficial in breeding programs as saving time to generate pure breeding lines and contributes to the selection efficiency. By this technique, 100% homozygosity can be achieved in a single generation. It has long been used in diverse crops like wheat, corn, rice, barley, colza, pepper, eggplant and gerbera (Caranta, 1992; Gürsoy et al., 2010; Szarejko and Forster, 2007).

Haploid embryos can be recovered by two methods as anther-microspore culture and ovary-ovule culture. Although these two have been prevalent in many economically important species, parthenogenetic embryos attained by irradiated pollen technique are more successful in *Cucurbitaceae* family. Several studies were reported on embryo inductions by using irradiated pollen in melon (Sauton, 1988; Sari et al., 1992; Ficcadenti et al., 1995; Gursoy et al., 2010; Sari et al., 2010), watermelon (Sari et al., 1994), cucumber (Niemirowicz-Szczytt and Dumas de Vaulx, 1989; Sauton, 1989; Caglar and Abak, 1999; Lotfi et al., 1999) and squash (Kurtar et al., 2002).

Although many advantages there are also drawbacks in the application of haploidy technique as detecting and excising haploid embryos (Lotfi et al., 2003) and doubling of the haploid chromosome to obtain fertile plants (Lim and Earle, 2008). The frequency of spontaneous chromosome doubling of haploid plants is very low so chromosome doubling is achieved by using colchicine or other antimitotic agents like oryzalin to obtain fertile plants (Lim and Earle, 2008, 2009; Yetisir and Sari, 2003). Various methods can be used to apply colchicine in *in vitro* and in *in vivo* growth conditions like adding colchicine to the growth media in *in vitro* culture,

*Corresponding author. E-mail: isolmaz@cu.edu.tr. Phone: +90 322 338 63 88; Fax: +90 322 338 63 88.

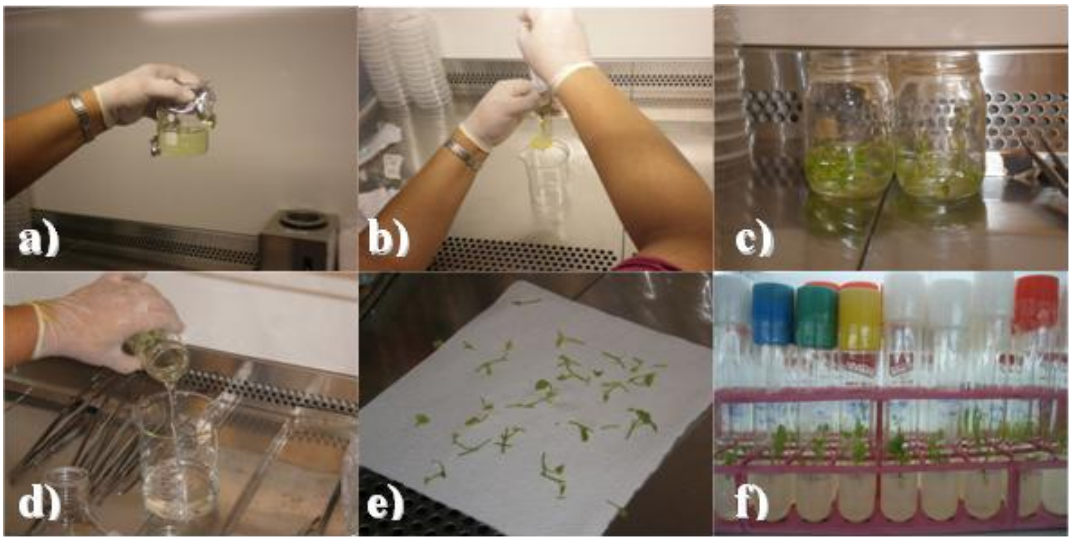

Figure 1. Steps of the *in vitro* colchicine application: a) Colchicine solution. b) Sterilization of colchicine solution by filtering. c) Immersion of plantlets into colchicine solution. d) Rinsing plantlets with sterile distilled water. e) Microcutting of plantlets. f) *In vitro* colchicine treated plantlets in test tubes.

immersing roots, plants and single node cuttings into colchicine solution, application of colchicine to lateral buds by medicine dropper and immersing shoot tips of *in vivo* grown plants (Yetisir and Sari, 2003). Besides the rate of *in vitro* chromosome duplication is low in haploid melons it was reported that shoot tip immersion into colchicine solution in cantaloupe melon was the most efficient method (Köksal et al., 2002; Yetisir and Sari, 2003).

Melon is an important vegetable crop in Turkey and the production ranks second in the world with 1.749 million tons (Anonymous, 2008). It is widely grown in all regions mostly by using winter types belong to *Cucumis melo* var. *inodorus.* Although there are many significant genotypes like 'Kirkagac', 'Hasanbey', 'Yuva', 'Sereflikochisar' and 'Kuscular', they are not hybrid cultivars and highly susceptible to fusarium wilt (*Fusarium oxysporum* f. sp. *melonis*) which highly constrains the production (Sari et al., 2010). A breeding program has been initiated in 2002 and a population was developed resistant to fusarium wilt in winter type melons ('Kirkagac' and 'Yuva Hasanbey') in Çukurova University. Haploid melon plants were obtained by irradiated pollen technique.

The objective of this study was to compare *in vitro* and *in vivo* colchicine application methods for the chromosome duplication of haploid 'Kırkagac' and 'Yuva Hasanbey' genotypes.

MATERIALS AND METHODS

This study was conducted at the research and application greenhouse and biotechnology laboratory at the Çukurova University, Faculty of Agriculture, Department of Horticulture.

In this study, eight 'Kirkagac' and six 'Yuva Hasanbey' melon genotypes derived from 53 backcrosses (BC1) were used as plant material and grown in glasshouse in the spring season. One day before anthesis male flowers were picked and induced with 300 Gray doses of Co^{60} gamma rays. Irradiated pollens were stored at room temperature overnight and in the following day emasculated hermaphrodite flowers were pollinated with at least three to four irradiated male flowers during one month period with two to three days of intervals.

Parthenogenetic immature fruits were harvested when they were three to four weeks old and taken to laboratory. Disinfection was done first washing with 1 % NaOCl solution. Later, fruits were surface sterilized with 96 % alcohol. Seeds were opened individually under a stereomicroscope in laminar flow cabin. The rescued haploid embryos were cultured in plastic Petri dishes containing E20A medium (Sauton and Dumas de Vaulx, 1987; Sari et al., 1994) and were incubated in growth chamber at 25-26^{o}C with a 16/8 h day/night photoperiod. After 7-10 days cultured embryos converted to plantlets and were transferred to glass test tubes containing the same medium till they had 6-8 nodes (Yetisir and Sari, 2003). *In vitro* micro-cutting was performed to propagate haploid plantlets.

In vitro Colchicine Application

Plants which had sufficient (10-12 plantlets) plantlets after *in vitro* micro-cutting were treated with colchicine (0.5 %) for doubling of chromosome number. For each use 0.5 grams of colchicine (Sigma) was dissolved in 100 ml of sterile distilled water and sterilized by filtering with sterile microfilter comprised of 0.20 µm pores.

Plantlets were kept into 0.5 % doses of colchicine solution for two h in culture jars. After rinsing 4 times with sterile distilled water, old leaves were striped of and plantlets were cut into pieces which have minimum one node. Later on they were dried for one to two minutes on sterile filter paper and were placed into test tubes containing MS medium. The treated micro-cuttings were placed in growth chamber which have 25-26^{o}C temperature and 16/8 h day/night photoperiod conditions. Steps of the *in vitro* colchicine application were shown in Figure 1.

Figure 2. *In vivo* colchicine application steps: a) Stripping of the leaves and tendrils of apex. b) Dipping apex into colchicine solution. c) Washing apex with water. d) Labeling application point with rope.

Acclimatization

In vitro colchicine treated plants and control haploid plants which will be *in vivo* colchicine treated were acclimatizated to greenhouse when they have roots and 6-8 nodes (about 10 cm). First, the lids of the tubes were opened in the growth chamber to avoid shock and one week later the plants were taken to greenhouse. Plantlets were transferred into trays carefully which have sterile peat : perlite mixture (2:1 v/v) after washing and dipping their roots into fungicide solution. The transferred plants were watered regularly and plastic tunnel was covered over the trays. Additionally, a shade coat was covered on the plastic tunnel. Cold steam machine was used for moisture controlling. The plastic covers were opened periodically to allow ventilation and after acclimatization they were totally removed over the plants. When the plants were grown and formed enough roots they were transferred from the trays to plastic pots (8 x 8 cm) and later on planted into the greenhouse.

***In vivo* Colchicine Application**

In vivo colchicine application was begun when main stem of the plants were reached about 30-40 cm. The application was done by shoot tip method (Yetisir and Sari, 2003). In this method, the apex of the shoot (5-10 cm) was stripped of leaves and dipped into colchicine solution (0.5 %) in test tubes for 2 h. Afterwards the plants were taken out from the tubes, washed with tap water and labeled with colored plastic rope from the application point. Side shoots were removed under the application point to prevent haploid shoot growing. The steps of the *in vivo* colchicine application were presented in Figure 2.

Haploid and dihaploid plants were identified by the presence or absence of the pollen. Dihaploid plants were selfed to produce seeds.

Results and Discussion

Results of *in vitro* Colchicine Application

The number of treated explants, number of survived explants, surviving rate in *in vitro* conditions (%), surviving rate after acclimatization (%), duplication rate (%) and number of fruit set were presented in Table 1. A total of 2742 haploid explants (1817 'Kirkagac' and 925 'Yuva Hasanbey') were treated with colchicine in *in vitro* conditions. The surviving rate of the explants after *in vitro* colchicine application varied between 22.58-52.70 % and the general mean was 38.37 % when two groups were considered together. 'Kirkagac' group (38.47 %) had a very slight difference than 'Yuva Hasanbey' group (.38.25 %). Both the highest (52.70 %, genotype no: 1) and the lowest (22.58 % genotype no: 8) surviving rates after *in vitro* colchicine application were obtained from 'Kirkagac'.

Table 1. Number of *in vitro* colchicine applied explants, number of survived explants, survivig rate after *in vitro* application (%), surviving rate after acclimatization (%), duplication rate (%) and number of fruit set in Kirkagac and Yuva-Hasanbey melons.

Genotype	Number of applied explants	Number of survived explants	Surviving rate after application (%)	Surviving rate after acclimatization (%)	Duplication rate (%)	Number of fruit set
1	220	116	52.70	23.30	0.00	0
2	337	153	45.40	8.69	12.50	1(1)*
3	265	111	41.88	33.33	0.00	0
4	25	7	28.00	0.00	0.00	0
5	160	70	43.75	89.47	11.11	1(1)
6	272	101	37.13	60.86	3.57	0
7	507	184	36.29	75.55	30.76	1(1)
8	31	7	22.58	0.00	0.00	0
Kirkagac Total/mean	1817/227.12	749/93.62	38.47	36.40	9.66	3 (3)
100	92	43	46.73	100	0.00	0
101	54	25	46.29	0.00	0.00	0
102	73	20	27.39	0.00	0.00	0
103	16	5	31.25	40.00	0.00	0
104	197	87	44.16	53.90	16.66	4(2)
106	493	166	33.67	59.30	26.69	22(6)
Yuva-Hasanbey Total/mean	925/154.16	346/57.67	38.25	42.20	10.84	26(8)
General Total/mean	**2742/195.86**	**1095/78.21**	**38.37**	**38.89**	**10.13**	**29(11)**

*: Numbers in the parentheses indicate the number of different pure lines derived from the same genotype.

The surviving rate after acclimatization was 38.89 % in general. 'Yuva Hasanbey' group melons showed better performance (42.20 %) than 'Kirkagac' (% 36.40) group. The highest surviving rate after acclimatization was belong to genotype 5 (89.47 %) which is a 'Kirkagac' genotype while there was no success in genotype 4 and 8 in 'Kirkagac' group and genotype 101 and 102 in 'Yuva Hasanbey' group.

Dihaploid and haploid melon plants were identified as controlling the pollen existence in male flower during flowering period in each day and doubled plants were labeled. Besides the pollen existence doubled plants were vegetatively strong, their leaves and flowers were normal in size and shape. It was easy to select haploid plants after colchicine application due to weak growing habit and having smaller leaves and flowers than diploids (Figure 3). The abnormal leaf (Figure 4) and flower shapes were also observed in these plants.

The mean duplication rate was 10.13 % after *in vitro* colchicine application. It was observed that 'Yuva Hasanbey' group melons revealed higher success (10.84 %) than 'Kirkagac' (9.66 %). The highest duplication rate was 30.76 % (no: 7) in 'Kirkagac' and 26.69 % (no: 6) in 'Yuva-Hasanbey'.

After selfing the doubled plants three fruits from 'Kirkagac' group and 26 fruits from 'Yuva Hasanbey' group were obtained which represent 11 different pure lines.

Results of *in vivo* Colchicine Application

The number of plants transferred for *in vivo* colchicine application was 1736 (886 'Kirkagac' and 850 'Yuva Hasanbey'). After acclimatization the mean surviving rate of plants was 23.69 % and 'Kirkagac' showed better performance (26.36 %) than 'Yuva-Hasanbey' (20.57 %) as seen in Table 2. For the *in vivo* colchicine application a total of 447 plants (240 'Kirkagac' and 207 'Yuva Hasanbey') were treated with colchicine solution (0.5 %) for 2 h. After controlling the pollens it was determined that the general mean of the duplication rate was 46.03

Figure 3. Male flower of dihaploid and haploid melon plant.

Figure 4. Abnormal leaf shape of haploid melon plant.

Table 2. Number of transferred explants for acclimatization, surviving rate after acclimatization, number of in vivo colchicine applied plants, duplication rate (%), number of fruit set in 'Kirkagac' and 'Yuva Hasanbey' melon genotypes.

Genotype	Number of transferred haploid explants for acclimatization	Survivig rate after acclimatization (%)	Number of *in vivo* colchicine applied plants	Duplication rate (%)	Number of fruit set
1	88	44.31	38	28.94	1(1) *
2	133	48.87	62	50.00	1(1)
3	131	32.06	41	34.14	1(1)
4	1	0.00	0	0.00	0
5	132	23.48	31	25.80	2(2)
6	198	16.60	31	32.35	0
7	203	19.21	37	37.83	3(2)
8	0	0.00	0	0.00	0
Kirkagac Total/mean	886/110.75	26.36	240/30.00	34.84	8 (7)
100	38	18.42	6	33.33	0
101	47	19.14	9	44.44	0
102	43	23.25	10	60.00	0
103	23	4.34	1	100	0
104	166	27.10	34	55.88	4(4)
106	533	31.14	147	49.65	6(5)
Yuva-Hasanbey Total/mean	850/141.66	20.57	207/34.50	57.22	10 (9)
General Total/mean	**1736/124**	**23.69**	**447/31.93**	**46.03**	**18 (16)**

*: Numbers in the parentheses indicate the number of different pure lines derived from the same genotype.

% among the genotypes of 'Kirkagac' and 'Yuva Hasanbey'. As well as in *in vitro* colchicine application the duplication rate in *in vivo* colchicine application 'Yuva Hasanbey' group had better performance (57.22 %) than 'Kirkagac' group (34.84 %). The highest duplication rate (100 %) was obtained from a 'Yuva-Hasanbey' genotype (no:103) while the lowest was belong to genotype no:5 (25.80 %) which is a 'Kirkagac' genotype.

Selfing was performed after the identification of doubled lines. In 'Kırkagac' group eight fruits from seven pure lines and ten fruits from nine pure lines were obtained in 'Yuva-Hasanbey' group. Totally 18 fruits representing 16 pure line were acquired.

The number of selfed lines were 27 from the doubled pure lines by both *in vitro* and *in vivo* colchicine application. The doubled plants were not grown normally as normal diploids. Leaf and flower shape disorders appeared in these plants. Despite they produce fertile pollen their efficiency were not as the pollen of diploid plants. Due to these reasons it was not easy to make selfing for generating the seeds of pure lines.

The results of this study revealed that doubling with *in vivo* colchicine application was more successful than doubling with *in vitro* colchicine application. This finding is in concordance with the previous studies carried out by different researchers. It was reported that in melons dihaploidization rate was 89 % in *in vivo* colchicine application and this rate was three times greater than *in vitro* colchicine application (Yetisir and Sari, 2003).

Köksal et al. (2002) compared different *in vivo* methods for chromosome duplication in muskmelon. The results showed that immersing shoot tips into colchicine solution (0.5 %) was determined the most successful technique. The dihaploidization efficiency was about 91.67 %.

Despite these studies and our results highlight *in vivo* colchicine treatment for obtaining higher frequency of dihaploidization rate Lim and Earle (2008) found the most effective procedure as *in vitro* exposure of shoot tip explants to 500 mg/l colchicine for three h. They obtained 83 % survival rate of explants and 26 % conversion to diploidy. In their study 132 plants were treated with colchicine *in vivo* dipping the tips and the first leaves of plants into colchicine solution (1000 mg/l for 12 h or 5000 mg/l for 2h or 4 h). However many *in vivo* colchicine treated plants showed abnormal growth. Researchers declared *in vivo* technique resulted in less surviving rate (78 %). This contrast may be caused by the higher dose of colchicine used or long exposure

period (1 week) into colchicine solution. Same authors reported in 2009 that regeneration of nodal explant treated with 500 mg/l colchicine for 12 h was increased from 40 to 88 % by transferring the treated explants to a medium supplemented with growth regulators. In another study carried out by Lotfi et al. (2003) treating colchicine (250-500 mg/l) *in vitro* to the shoot tips of greenhouse grown plants for 3 to 6 h resulted in 10 diploid plants among 156 plantlets. Most were mixoploid and the remaining plantlets were still haploid. It was also reported that in attempts doubling by *in vivo* colchicine application to the greenhouse grown plants were unsuccessful.

It can be concluded that the dose and the exposure period are the key factors of the high duplication rate; however, the content of transferring medium, stage of the explant and genotype effect are also important. Doubled haploidy has improved to obtain haploid plants but this technique has limitations as low induction of haploid embryos in plants like melon. Efficiency of doubling haploid plants also became one of the drawbacks of this technique. We recommend *in vivo* colchicine (0.5 %) application to shoot tips of greenhouse grown plants for 2 h in melons in a large scale dihaploid line production in melon.

ACKNOWLEDGEMENT

The authors are grateful to TUBITAK (Turkish Scientific and Technological Council) for the financial support of this project (106T760).

REFERENCES

Anonymous (2008). Turkish Statistical Database. http://www.tuik.gov.tr/bitkiselap/bitkisel.zul

Caglar G, Abak K (1999). Progress in the production of haploid embryos, plants and doubled haploids in cucumber (*Cucumis sativus* L.) by gamma irradiated pollen, in Turkey. Acta Hortic. 492:317-322.

Caranta, C (1992). Essai d'Induction de la Parthénogenése Haploide par du Pollen Irradié chez le Frasier Cultivé (*Fragaria xananassa* Duch.) et Etude du Gametophyte Femelle. Mémoire (D.E.A) Option Sciences Argonomiques: Amélioration des Plantes, Faculté des Sciences de Luminy, Marseille, p. 15.

Ficcadenti N, Veronese P, Sestili S, Crino P, Lucretti S, Schiavi M, Saccardo F (1995). Influence of genotype on the induction of haploidy in *Cucumis melo* L. by using irradiated pollen. J. Genet. Breed. 49:359-364.

Gursoy I, Solmaz I, Kasapoglu S, Sarı N, Szamosi C, Park KS (2010). The comparison of embryo rescue and direct seed sowing method for obtention of haploid Kirkagac and Yuva-Hasanbey melons. Cucurbitaceae 2010 Proceedings, 14-18 November 2010, South Carolina, USA, 181-184.

Köksal N, Yetisir H, Sari N, Abak K (2002). Comparison of different *in vivo* methods for chromosome dublication in muskmelon (*Cucumis melo*), Acta Hort. 588: 293-298.

Kurtar ES, Sari N, Abak K (2002). Obtention of haploid embryos and plants through irradiated pollen technique in squash (*Cucurbita pepo* L.). Euphytica 127: 335-344.

Lim W, Earle ED (2008). Effect of *in vitro* and *in vivo* colchicine treatments on pollen production and fruit set of melon plants obtained by pollination with irradiated pollen. Plant Cell Tissue Organ Cult, 95: 115-124.

Lim W, Earle ED (2009). Enhanced recovery of doubled haploid lines from parthenogenetic plants of melon. Plant Cell Tiss. Organ Cult, 98: 351-356.

Lotfi M, Kashi A, Onsinejad R (1999). Induction of parthenogenetic embryos by irradiated pollen in cucumber. Acta Hortic. 492: 323-326.

Lotfi M, Alan AR, Hening MJ, Jahn MM, Earle ED (2003). Production of doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. Plant Cell Rep. 21: 1121-1128.

Niemirowicz-Szczytt K, Dumas de Vaulx R (1989). Preliminary Data on Haploid Cucumber (*Cucumis sativus* L.) Induction. Cucurbit Genet. Coop. Rep., 12: 24-25.

Sari N, Abak K, Pitrat M, and Dumas de Vaulx R (1992). Induction of parthenogenetic haploid embryos and plant obtention in melon (*Cucumis melo* L. var. *inodorus* Naud. and C. *melo* L. var. *reticulatus* Naud.). Turk. J. Agric. For. 16: 302-314.

Sari N, Abak K, Pitrat M, Rode JC, and Dumas de Vaulx R (1994). Induction of parthenogenetic haploid embryos after pollination by irradiated pollen in watermelon. Hort. Sci. 29: 1189–1190.

Sari N, Solmaz I, Kasapoglu S, Gursoy I, Szamosi C, Unlu H, Park KS (2010) Effect of different pollination dates with irradiated pollens on fruit set, haploid embryo induction and plant obtention in Turkish (Kirkagac, Yuva and Hasanbey) melons Acta Hort., 871: 639-648.

Sauton A, Dumas de Vaulx R (1987). Obtention de plantes haploides chez le melon (*Cucumis melo* L.) par gynogénèse induite par du polen irradié. Agronomie 7: 141–148

Sauton A (1988). Effect of season and genotype on gynogenetic haploid production in muskmelon (*Cucumis melo* L). Sci. Hortic. 35: 71–75.

Sauton A (1989). Haploid gynogenesis in *Cucumis sativus* by irradiated pollen. Rep. Cucurbit Genet. Coop. Rep. 12: 22-23.

Szarejko L, and Forster BP (2007). Doubled haploidy and induced mutation. Euphytica, 158: 359-370.

Yetisir H, Sari N (2003). A new method for haploid muskmelon (*Cucumis melo* L.) dihaploidization. Sci. Hortic. 98: 277-283.