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Effects of Silver Nitrate and Donor Plant Growing Conditions on Production of Pepper (*Capsicum annuum* L.) Haploid Embryos via Anther Culture

S. Buyukalaca[1)], N. Comlekcioglu[2)], K. Abak[1)], E. Ekbic[1)] and N. Kilic[1)]
([1)]University of Cukurova, Faculty of Agriculture, Department of Horticulture, Adana, Turkey and [2)]University of Harran, Faculty of Agriculture, Department of Horticulture, Sanlıurfa, Turkey)

Summary

The responses of pepper anthers to different concentrations of silver nitrate and in dependence of the donor plant growing conditions were studied for haploid embryo production via anther culture. U-247 and U-238 genotypes, which were selected from a local paprika pepper population, were used as plant materials. Flower buds were collected from the plants grown either in greenhouse or in open field conditions. Four different silver nitrate concentrations (5, 10, 15 and 20 mg l^{-1}) were tested. Haploid embryos were obtained from all the concentrations tested, but with different production rates. The highest embryo formation (45.7 embryos per 100 anthers) was obtained from the genotype U-247 in the medium containing 15 mg l^{-1} silver nitrate. The anthers taken from the plants grown in the greenhouse produced more embryos than that of the open field conditions.

Zusammenfassung

Wirkung von Silbernitrat und Spenderpflanzen-Kulturbedingungen auf die Bildung haploider Embryonen aus Paprikaantheren. In dieser Arbeit wurden die Wirkungen unterschiedlicher Silbernitrat-Konzentrationen in Kombination mit den Kulturbedingungen der Spenderpflanze auf die Bildung haploider Embryonen aus Paprikaantheren *in vitro* untersucht. Als Pflanzmaterial dienten ausgewählte Genotypen (U-247 und U-238) einer lokalen Paprika-Population. Die Blütenknospen wurden sowohl von Gewächshaus- als auch von Freilandpflanzen genommen. Im Versuch wurden vier unterschiedliche Silbernitrat-Konzentrationen (5, 10, 15 und 20 mg l^{-1}) getestet. Haploide Embryonen wurden bei allen getesteten Konzentrationen allerdings in unterschiedlichen Mengen produziert. Die von Gewächshauspflanzen entnommenen Antheren bildeten mehr Embryonen als die aus dem Freiland. Die höchste Embryonenproduktion (45,7 Embryonen pro 100 Antheren) wurde von Antheren des Genotyps U-247 auf einem Medium mit 15 mg l^{-1} Silbernitrat erzielt.

Key words. pepper – androgenesis – pollen embryogenesis – haploid embryo

Introduction

Anther culture provides a rapid way for the production of haploid lines and these lines can be easily used for subsequent production of doubled haploids and F_1-hybrids. Doubled haploids have proved to be particularly valuable in the analyses of genetically complex basis of some resistances to pest and diseases (ABAK et al. 1982; PICKERSGIL 1997). Anther culture for the production of doubled haploid lines has been applied extensively to many plants species such as *Brassica* (DIAS and MARTINS 1999; WANG et al. 1999; ARNISSON and KELLER 1999; ANCHAR 2002), cucumber (KUMAR et al. 2003), and eggplant (KASHYAP et al. 2003). However the frequency of haploid embryo production with anther culture is low in many species (KRISTIANSEN and ANDERSEN 1993). Embryo production rate was affected by various factors such as genotype, culture conditions of donor plant, microspore development stage, pretreatment of anthers and culture medium. It has been reported that ethylene inhibits somatic embryogenesis and shoot regeneration (BIDDINGTON 1992), and callus growth (SONGSTAND et al. 1991). Ethylene is produced during plant tissue culture process as a result of the stress during explant excision (YANG and HOFFMAN 1984) and the presence of auxin in the culture medium (YU and YANG 1979). Silver nitrate ($AgNO_3$) is an inhibitor of ethylene production (BEYER 1976).

$AgNO_3$ application improved the regeneration in *Brassica campestris* (PALMER 1992) and *Nicotiana plumbaginifolia* (PURNHAUSER et al. 1987), the somatic embryogenesis in *Daucus carota* (ROUSTAN et al. 1990) and *Solanum tuberosum* (TIAINEN 1992), and the embryo production by anther culture in *Brassica oleracea*. It has been reported that no embryo was obtained in some *B. oleracea* morphotypes without the addition of $AgNO_3$ (DIAS and MARTINS 1999).

Adding $AgNO_3$ into the callus induction medium significantly enhanced embryogenic callus production of male immature inflorescence cultures of buffalograss (FEI et al. 2000). FERNANDEZ et al. 1999 reported that $AgNO_3$ affected both the percentage of em-

bryogenic explants and the number of somatic embryos per explant. This was explained with the possible involvement of $AgNO_3$ in the ethylene metabolism. $AgNO_3$ increased the induction of direct somatic embryogenesis (more than 22 folds) of durum wheat (*Triticum durum* Desf.). Root initiation and elongation of in vitro axillary bud cultures of *Decalepis hamiltonii* were improved when $AgNO_3$ was added. These beneficial effects of $AgNO_3$ on rooting may result from inhibition of ethylene action (BAIS et al. 2000).

Our previous work in anther culture of Sanlıurfa and Kahramanmaras pepper (*Capsicum annuum* L.) populations resulted in low frequencies of production of haploid embryos and plants (COMLEKCIOGLU et al. 1999). Later we studied (COMLEKCIOGLU et al. 2001) for the first time the effects of single $AgNO_3$ concentration on embryo yields in pepper. Results of this study showed that the number of embryos cultured increased significantly compared with the study without $AgNO_3$ (COMLEKCIOGLU et al. 1999).

Since the positive effect of $AgNO_3$ on embryo production became clear, we decided to study different concentrations of $AgNO_3$. Four different $AgNO_3$ concentrations and two different donor plant growing conditions were investigated in this study.

Materials and Methods

Plant materials

Two local genotypes named U-247 and U-238, which are mainly used for paprika and pepper paste production, were used in the study.

Donor plants were grown either in open field or in greenhouse using 5 liter plastic pots.

Flower buds were collected at the optimal stage, in which anthers have uninucleate microspores, for anther culture. At this stage the length of corolla should be equal to that of calyx or slightly longer, and almost half of the anthers have anthocyanin. The flower buds were surface sterilized by using 15 % calcium hypochlorite for 15 minutes and then rinsed 3–4 times with sterile distilled water. The anthers were removed without their filaments and placed in petri dishes under aseptic conditions.

Culture media and conditions

The MS (MURASHIGE and SKOOG 1962) medium supplemented with 4 mg l^{-1} Naphthalene acetic acid (NAA), 0.1 mg l^{-1} 6-Benzylaminopurine (BAP), 0.25 % activated charcoal, 30 g l^{-1} sucrose and four different concentrations of $AgNO_3$ (5, 10, 15 and 20 mg l^{-1}) was used. Since no embryo formation was obtained in the medium without $AgNO_3$ in our previous study (COMLEKCIOGLU et al. 2001), this medium was not used.

The anthers were incubated at 35 °C in dark for the first 2 days and then transferred to 28 °C with 16 hours light/8 hours dark photoperiod conditions. Illumination was provided by white fluorescent lamps (15 µmol m^{-2} s^{-1}).

The embryos in torpedo shape were transferred to the hormone-free MS medium with 30 g l^{-1} sucrose and cultured at 25 °C with the same photoperiod conditions for germination.

For both genotypes, different numbers of anthers (110–185) were cultured for each $AgNO_3$ concentration and donor plant growing condition. Five anthers were placed in a petri dish. The cultured anthers were observed periodically and morphological changes were recorded. The number of embryos per anther for each treatment were counted and recoded.

Statistical analysis

Experiments were arranged in a completely randomized design with 3 replications. The data that were calculated as a percentage were subjected to angle transformation before the statistical analysis. Data were subjected to analysis of variance (ANOVA) and means were separated by least significance difference (LSD) at 5 % probability level.

Results and Discussions

In a previous study (COMLEKCIOGLU et al. 2001) we observed some indications of a positive influence of $AgNO_3$ on pepper androgenesis. Therefore, we decided to investigate deeply this influence and conducted the studies reported here. Although our focus was on the determining the optimum $AgNO_3$ concentration, the effects of genotype and donor plant growing conditions were also tested. In the studies conducted through the spring of 2002, approximately half of 2505 cultured anthers were taken from the greenhouse-grown plants, and the others from the open field. Due to infections, a small amount of anthers were lost. The number of the remaining anthers was 2325. The first embryos were obtained in the 5th week of culturing (Fig. 1a), but intensive embryo production was observed in 6th and 7th weeks. Mature embryos (Fig. 1b) were collected and placed the hormone-free MS medium for germination. Plantlets (Fig. 1c) were obtained 4 weeks after transfer of embryos to the germination medium.

Table 1 shows the number of the cultured anthers, total embryos, and embryos per 100 anthers of different genotypes, donor plant growing conditions and $AgNO_3$ concentrations. As can be seen from Table 1, all the combinations of "genotype x donor plant growing condition x $AgNO_3$ concentration" produced embryos but in different numbers. Analysis of variance showed that differences between donor plant growing conditions, genotypes and $AgNO_3$ concentrations were found to be statistically important. The interaction between genotype, donor plant growing condition and $AgNO_3$ concentration was also found to be significant. Open field grown U-247 genotype produced the minimum number of embryos (0.9 embryo/100 anthers) when cultured in the medium containing 5 mg l^{-1} $AgNO_3$. The maximum number of embryos was obtained again from U-247 genotype (45.7 embryos/100 anthers). However, in this case the donor plants were from the greenhouse and $AgNO_3$ concentration was 15 mg l^{-1}.

When both donor plant growing conditions were considered, the results of the experiment showed that the optimum concentration of $AgNO_3$ for pepper anther culture is 15 mg l^{-1}. In one of our previous studies (COMLEKCIOGLU et al. 1999) in which similar genotypes were used, embryo yield of genotypes in a medium without $AgNO_3$ varied between 0.0 and 18.4 em-

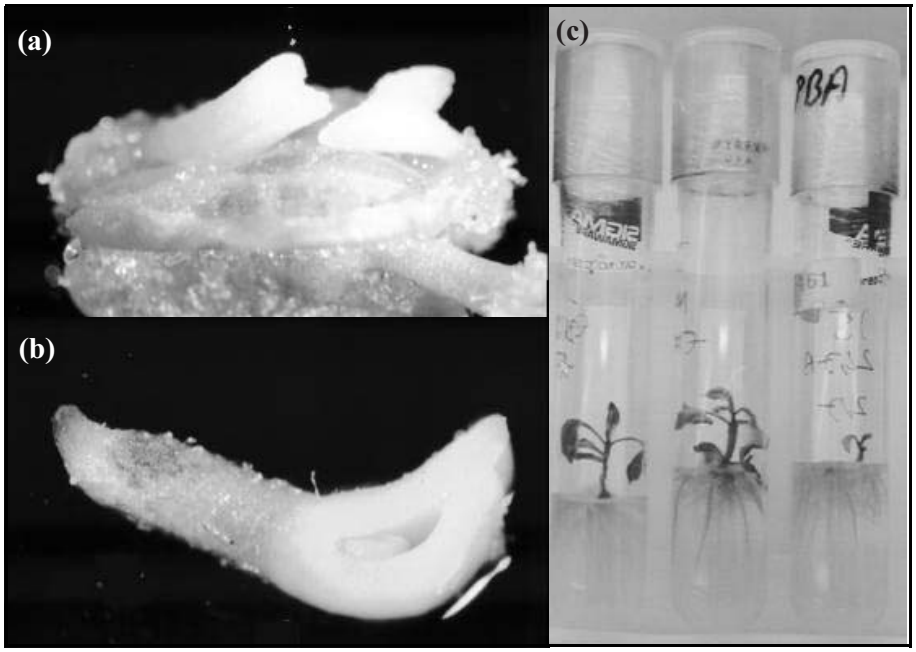


Fig. 1. Pepper anther culture: (a) haploid embryos coming from the anthers (b) the haploid embryo taken for germination (c) the plantlets from the haploid embryos 4 weeks after planting.

bryos/100 anthers with an average of 3.8 embryos/100 anthers. Embryo yield was increased slightly (1.2–19.3 embryos/100 anthers) when 10 mg l^{-1} $AgNO_3$ was added to the medium in our other study (COMLEKCIOGLU et al. 2001) that was the first study in which $AgNO_3$ was used for pepper anther culture. In this study, we obtained similar results for embryo yield (2.3–22.9 embryos/100 anthers) with 10 mg l^{-1} $AgNO_3$ concentration. When the concentration was increased from 10 to 15 mg l^{-1}, embryo yield was nearly doubled (1.3–45.7 embryos/100 anthers). Increasing concentration to 20 mg l^{-1} provided no increment in embryo yield.

Results of this study also clearly showed that growing conditions of the donor plants affect significantly the success of the anther culture. The plants grown in the greenhouse produced almost 4 times more embryos than the plants grown in open field for all $AgNO_3$ concentrations and genotypes. The average number of the embryos was 17.48 embryos/100 anthers for the first case and 4.87 embryos/100 anthers for the second case.

Influence of growing conditions of donor plants on androgenetic response was also observed for other species. For example, FOROUGHI-WEHR and WENZEL 1993 found that wheat variety 'Igri', which has a high haploid efficiency, produces only albino plants when grown under unsuitable conditions. In their study with tomato, SHTREVA et al. (1998) reported that the plants grown in greenhouse exhibited higher androgenetic

Table 1. Results of the pepper anther culture obtained from two different donor plant growing conditions and four different $AgNO_3$ concentrations.

Donor plants growing condition (GC)	Genotype (G)	No of the cultured anthers $AgNO_3$ (mg l^{-1})					No of the embryos obtained $AgNO_3$ (mg l^{-1})					Embryo yield (Embryo no /100 anthers) $AgNO_3$(mg l^{-1}) (Ag Con.)				
		5	10	15	20	Total	5	10	15	20	Total	5	10	15	20	Average GC
Greenhouse	U-247	160	175	175	165	675	30	40	80	29	179	18.8 bc	22.9 b	45.7 a	17.6 bc	
	U-238	115	150	110	115	490	13	10	10	9	42	11.3 de	6.7 fg	9.1 efg	7.8 efg	
Greenhouse Total		275	325	285	280	1165	43	50	90	38	221					*17.5 a*
Open field	U-247	110	145	180	185	620	1	8	19	12	40	0.9 h	5.5 g	15.6 cd	10.3 ef	
	U-238	125	130	150	135	540	2	3	2	2	9	1.6 h	2.3 h	1.3 h	1.5 h	
Open field Total		235	275	330	320	1160	3	11	21	14	49					*4.9 b*
General Total		510	600	615	600	1325	46	61	111	52	270					
Avarage Ag Con.												8.2 b	9.4 b	18.0 a	9.3 b	11.2

Means followed by the same letter do not differ according to least significance difference (LSD) test (P≤ 0.05).
LSD 5 %: Ag NO_3 : 2.24 *LSD 5 % GC: 1.59* LSD 5 %: Ag NO_3 x GC x G: 4.49

ability than the plants grown in open field. Callus formation and organogenesis were also better for the plants grown in greenhouse.

There are significant differences in terms of embryo production between two genotypes tested in this study (Table 1). U-247 produced 3 and 4.5 times more embryos than U-238 for greenhouse-grown plants and open field grown plants, respectively. The response of genotypes to $AgNO_3$ concentration was different. 15 mg l^{-1} yielded the maximum embryos for U-247 genotype, whilst no considerable difference was observed in the case of U238 genotype. ACHAR (2002) found similar results in cabbage.

Parallel to the results of other studies, it was found in this study that genotype has a clear effect on the androgenetic response. Donor plant growing conditions are also important for embryo formation from anthers. $AgNO_3$ should be added to the culture medium to increase embryo yield in pepper.

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Addresses of authors: S. Buyukalaca (corresponding author), K. Abak, E. Ekbic and N. Kilic, University of Cukurova, Faculty of Agriculture, Department of Horticulture, 01330 Adana, Turkey, and N. Comlekcioglu, University of Harran, Faculty of Agriculture, Department of Horticulture, Sanlıurfa, Turkey,
e-mail: sbircan@cukurova.edu.tr.