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The Effect of Growing Season and Culture Media on Anther Culture Response of Some Pepper Cultivars (*Capsicum annuum* L.) Grown in Tunisia

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ABSTRACT

The creation of pepper cultivars using traditional breeding is time consuming. In fact, 8 to 10 years are required to fix a determined character. In this way, anther culture is considered as an interesting tool in practical plant breeding and basic research to obtain haploid and diploid plants. However, the production and frequency of haploids is influenced by many factors, particularly growing season and composition of culture media. Therefore, the effects of different growing season (autumn or spring) and different culture medium on the anther culture response of three pepper cultivars ('Marconi', 'Baker' and 'J27') grown in Tunisia were examined. The suitable growing season for anther culture was spring (March-April). The addition of activated charcoal at 0.25% and $AgNO_3$ at 10 mg/L was found to increase the yield of embryo-like structures, particularly for cultivar 'J27'.

Keywords: activated charcoal, autumn, $CuSo_4$, silver nitrate, spring

INTRODUCTION

The pepper (*Capsicum annuum* L.) is one of the main vegetable crops grown and consumed in Tunisia and is therefore of economic importance. In fact, in 2006, 200,000 ha were dedicated to this crop and its production amounted 256, 000 t (DGPA 2007).

Conventional breeding to obtain and create new pepper cultivars and the fixation of interesting agronomic characters (disease resistance, precocity and nutritional quality) include a number of limiting factors and difficulties such as uncontrolled foreign pollination, the need for a large isolation space, cryptogamic diseases, biotic and virus disease (Liljiana *et al*. 2007).

To overcome these difficulties, it is possible to consider *in vitro* production of haploid regenerants from anther culture (Wang *et al*. 1973; Abak 1983; Gupta *et al*. 1998; Ercan *et al*. 2001; Liljiana *et al*. 2007; Kim *et al*. 2008). This method has several advantages, including the total expression of genetic potential, particularly different mutations, the controlled self pollination and breeding of separate individual plants and the possibility of rapidly obtaining stable and homozygous lines (Dumas De Valux *et al*. 1981).

The success of anther culture depends upon various factors such as genotypic differences, growing conditions of the donor plant, pre-treatment of buds or anthers, microspore development stage as well as incubation conditions (Bajaj 1990; Liljiana *et al*. 2007; Kim *et al*. 2008). Previously Kriastiansen and Andersen (1993) revealed that donor plant age and temperature are the most important factors to effectively obtain embryos derived from pepper anther culture. Ellialtıoğlu *et al*. (2001) studied the influence of silver nitrate $AgNO_3$ and $CuSO_4$ as elicitors in callus suspension cultures from different pepper genotypes and found that $AgNO_3$ at 0.2 mM was the best elicitor. Cömlekcioğlu *et al*. (2001) focused on the effect of $AgNO_3$ on haploid embryo induction by anther culture in pepper and found that $AgNO_3$ significantly improved the frequency of haploid embryogenesis during pepper anther culture. Boyaci (2001) examined the effect of activated charcoal (AC) on haploid plant production via anther culture of five pepper varieties (two hybrids namely 'Sirena F1' and 'Amazon F1' and three standards namely 'Demri Sivirsi', 'Bağci Carliston' and 'Yolva Carliston'). They found that the highest frequency of embryo formation was obtained in N medium (5 mg/L 2,4-D + 5 mg/L kinetin + 1% AC. but not all of the embryos obtained could survive. Supena *et al*. (2006) confirmed the importance of AC as embryo formation almost completely failed without its addition. In fact, they reported that the total embryo yield as well as the yield of normal-looking embryos increased with increasing concentrations of AC from 0 to 0.2 g/L. Recently, Lantos *et al*. (2009) studied the improvement of isolated microspore culture of pepper via co-culture with ovary tissue of pepper or wheat and found that anthers with microspores at the uninucleate stage were the preferred starting material for *in vitro* cultures. However, a limited number of studies focused on the effect of donor plant age and seasonal effect in pepper anther culture (Ercan *et al*. 2006). In addition, data regarding the effect of different media on pepper anther culture are often contradictory.

Therefore, and based on these facts, the aim of this study was to determine the effects of growing season (autumn and spring) and different culture media elicitors (AC and $AgNO_3$) on anther culture response of three pepper ('Marconi', 'Baker' and 'J27') cultivars grown in Tunisia.

MATERIALS AND METHODS

Field experiment

In this study, three pepper cultivars were used 'Marconi' (long, sweet type), 'Baker' (long hot type) and 'J27' (long medium-hot type). Seeds were sown in September (autumn) and seedlings were

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Research Note

moved to an unheated greenhouse in October. Flower buds were harvested and cultured at weekly intervals from December to January. On the other hand, for spring, seeds were sown in February and seedlings were moved to an unheated greenhouse in March. Plants were grown under insect-proof conditions from April to June. Flower buds were harvested when the corolla had the same length as the calyx or somewhat longer as recommended by Dumas de Valux *et al*. (1981). The collected flower buds were surface sterilized in 70% ethanol then transferred in 5% Ca(Clo) + 2-3 drops of Tween 20 for 10 min and rinsed 3 times in sterile distilled water and cultured as described by Murashige and Skoog (1962) with 8 g/L agar and 30 g/L sucrose. Cultures were incubated for 8 days at 35°C in the dark as reported by Mytiko *et al*. (1995), then transferred to 25°C with a 16-hr photoperiod H at 3000 lux. After 2 weeks culture elicitor treatments were prepared as reported by Boyaci (2001). Six different media were used with different elicitors added to the basal medium. M1: Dumas de Valux *et al*. (1981); M2: MS medium; M3: MS + 0.25% AC; M4: MS + 0.25% AC + 5 mg/L $AgNO_3$; M5: MS + 0.25% AC + 10 mg/L $AgNO_3$; M6: MS + 0.25% AC + 15 mg/L $AgNO_3$.

Flow cytometry

In this study, a Partec CII Chemunex flow cytometer was used. Small pieces approximately 1 cm² in size from the obtained plantlets were cut into small pieces using 1 mL of buffer prepared by Chemunex SA Co. The obtained suspension was then filtered into small tubes through 0.2 µm microfilters. For the coloration of these samples they were put into the flow cytometer after addition of one drop of DAPI and the DNA histograms were created.

Statistical analysis

All percentages of callused anthers and embryogenic anthers were subjected to analysis of variance (ANOVA) and mean values were evaluated by the LSD test at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of growing season and cultivar on pepper anther culture and percentage of embryo formation

The effect of growing season and pepper cultivar on the frequency of embryo formation is presented in **Table 1**. The data regarding embryo formation showed significant differences among the studied pepper cultivars ($P < 0.01$). In autumn, a total of 333 anthers from 'J27' were cultured and 12 embryos were obtained. However, only one converted into a plantlet. Therefore, the overall percentages for embryo formation and plant formation were 3.60 and 8.33, respectively. 'Marconi' had statistically similar percentages to 'J27' (3.03 and 6.25% for embryo formation and plant formation, respectively). However, 'Baker' had the lowest percentages of embryo and plant formations (1.68 and 0%, respectively). Flow cytometric analysis revealed that all the obtained plantlets during this experiment were haploids.

Embryo-like structures developed from the anthers of 'J27' during spring (**Fig. 1**). In spring, 'J27' had the highest embryo and plant formation percentages, 7.88 and 21.42%, respectively. In addition, in 'J27', plant formation was more than 2-fold higher than 'Marconi' and 'Baker' although 'Marconi' had percentages similar to 'J27' regarding the frequency of embryo formation, but a 1.83% higher frequency of embryo formation than 'Baker'. Pepper plants grown during spring were significantly more suitable for anther culture since the percentages of embryo formation were 2.28-, 2.24- and 2.18-fold higher than in autumn in 'Marconi', 'Baker' and 'J27', respectively. Our results confirmed that the response of pepper cultivars to seasonal effects was dependent on cultivar, as was reported by Ercan *et al*. (2006). In fact they found that pepper cv. 'Kekova' gave the highest embryogenic yield in summer while this was true in winter season for cv. 'Sera Demre 8'.

The result of this study also confirmed the significant

Table 1 Effect of growing seasons on embryo formation (%) and haploid plant number.

	№ of anthers cultured	№ of embryos obtained	№ of plantlets obtained	Embryo formation (%)	Plant formation (%)
Autumn					
Marconi	395	0	0	3.03 a	6.25 a
Baker	207	0	0	1.68 b	0
J27	333	12 a	1 a	3.60 a	8.33 a
Spring					
Marconi	335	28	0	6.92 a	10 b
Baker	265	10	0	3.77 b	10 b
J27	335	20	0	7.88 a	21.42 a

Significance: Values in the same column followed by the same letters do not differ significantly (LSD test, P<0.05).

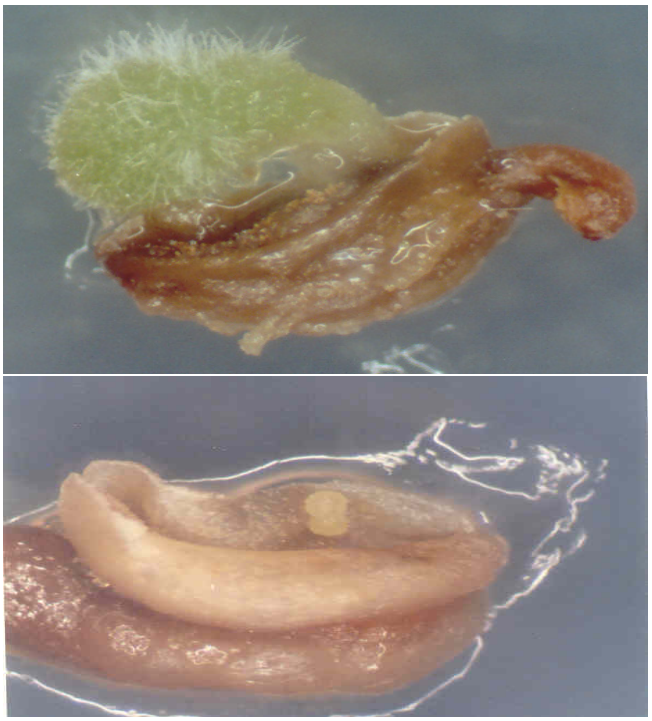

Fig. 1 Embryo-like structures development from an anther of 'J27' cultivar during spring.

importance of genotype, as reported by Liljiana *et al*. (2007), who found that the response of anthers from the nine pepper cultivars ('Feferona', 'Slatko Luta', 'Vezena Luta', 'Sivrija', 'Zlaten Medal', 'Kurtovska Kapija', 'California Wonder', 'Rotund' and 'Féherözön') depends highly on the genotype and growing conditions. In addition, Lantos *et al*. (2009) also found differences in the efficiency of establishment of isolated pepper microspore culture among three Hungarian ('Szegedi 80', 'Szegedi 178', and 'Reméný') and three Spanish ('Jeromin', 'Jariza', and 'Jaranda') pepper genotypes. Besides, the good results obtained in spring can be ascribed to different microspore conditions. In fact, the anthers containing microspores at the uninuclear stage and at first pollen mitosis are determined to be optimal for the induction of androgenesis for many plant species of *Solanaceae* (Vagera 1990). Moreover, the amino acid and growth regulator composition of anthers may result in a different androgenic response (Ellialtiöglu *et al*. 2001). Although Liljiana *et al*. (2007) reported that hot cultivars of the genus *Capsicum* were poor or non-responsive genotypes compared to sweet and bell pepper cultivars, in this study, the medium-hot cultivar 'J27' showed the highest percentage of embryo and plant formation independent of the growing season (autumn or spring).

Field-grown materials produced during the normal growing season are superior with respect to greenhouse-

Table 2 Effect of different culture media on embryo formation (%) and haploid plant number.

	№ of anther			№ of embryogenic anthers			Embryogenic anthers (%)			№ of haploid plants		
	J27	**Baker**	**Marconi**	**J27**	**Baker**	**Marconi**	**J27**	**Baker**	**Marconi**	**J27**	**Baker**	**Marconi**
M1	120	120	120	3 b	2 c	4 a	2.5 a	1.6 b	3.33 a	0	0	0
M2	120	120	120	3 a	5 a	4 a	2.5 b	4.1 a	3.33 b	0	0	0
M3	120	120	120	4 a	0 b	4 a	3.33 a	0 b	3.33 a	0	0	0
M4	120	120	120	9 b	14 a	10 b	7.5 c	11.6 a	8.33 b	1 b	0	2 a
M5	120	120	120	12 c	18 b	28 a	10 c	15 b	23.3 a	3 c	5 b	7 a
M6	120	120	120	0	0	0	0	0	0	0	0	0

M1: Dumas de Valux *et al*. (1981); M2: MS medium; M3: MS + 0.25% activated charcoal (AC); M4: MS + 0.25% AC + 5 mg/L $AgNO_3$; M5: MS + 0.25% AC + 10 mg/L $AgNO_3$; M6: MS + 0.25% AC +15 mg/L $AgNO_3$

Significance: Values in the same line followed by the same letters do not differ significantly (LSD test, $P < 0.05$).

grown material in most plant species. Furthermore, there exists a positive correlation between optimal temperature for plant growth and embryo formation from cultured anthers (Kristiansen and Andersen 1993). Therefore, during spring environmental conditions may influence not only pollen morphology but also physiological and biochemical processes in plant (Ercan *et al*. 2006). In addition, Lantos *et al*. (2009) found that anthers with microspores at the uninucleate stage were suitable starting material for *in vitro* cultures.

Effect of culture medium and cultivar on pepper anther culture and percentage of embryo formation

The effect of culture medium and cultivar on the percentage of pepper embryo formation is presented in **Table 2**. The number of haploid plants varied significantly among the studied pepper cultivars ($P < 0.01$). In M1, significantly higher percentages of embryo formation were obtained in 'J27' and 'Marconi' than in 'Baker'. The anthers of 'Baker' had the highest percentage of embryo formation on M2 medium (4.1%) although on M3, medium that was supplemented with 0.25% AC, both 'J27' and 'Marconi' had the highest percentage of embryo formation; 'Baker' was unresponsive. In contrast, on M4, M3 medium supplemented with 5 mg/L $AgNO_3$, the percentage of embryo formation in 'J27' and 'Marconi' was 2.25- and 2.50-fold higher than on M3 medium. Interestingly in 'Baker', the percentage of embryo formation increased from 0 to 11.6%, maximum among all three genotypes for this medium. In M5, where the dose of $AgNO_3$ was doubled (10 mg/L) compared to M4 the percentage of embryo formation increased for all three pepper cultivars: 10, 15 and 23.3%, respectively for 'J27', 'Baker' and 'Marconi' or 1.33-, 1.29- and 2.97-fold higher than the percentages for M4. Therefore, it seems that the improvement is mainly due to the dose of $AgNO_3$. This result is in agreement with that of Cömlekcioğlu *et al*. (2001) who reported that medium supplemented with 10 mg/L $AgNO_3$ significantly enhanced the frequency of haploid embryogenesis in two local pepper populations originated from 'Sanliurfa' and 'Kahramanmars'. However, in M6, where the [$AgNO_3$] was 3-fold higher than in M4, all pepper cultivars did not respond, most likely due to $AgNO_3$ toxicity. Lantos *et al*. (2009) reported that the microspore culture was improved when the ovaries of unrelated species (wheat) were co-cultured.

Regarding the number of haploid plants obtained, responses were only obtained for M4 and M5 media. In fact, on M4 medium 'J27' and 'Marconi' resulted in 1 and 2 haploid plants, respectively. However, on M5 medium, 'Baker' resulted in 5 haploid plants. Therefore, this study confirmed that haploid plant regeneration from anther culture is dependent on the *Capsicum* genotypes, as was reported by Nowaczyk and Kisiala (2006) who found that the effectiveness of androgenesis ranged from 4% for 'ATZ1' to 1.5% for the ('ATZ1 × PO') F1 in CP medium supplemented or not with AC. In addition, this study highlighted that the use of AC at the level (0.25%), when used with 10 mg/L $AgNO_3$ may ensure a better embryogenic response of anthers and consequently the number of haploid plants obtained might increase, in agreement with the results of Boyaci (2001). In fact, they reported that embryogenesis was recorded in all of the medium with AC and that the highest frequency of embryo formation was in N medium (5 mg/L 2,4-D + 5 mg/L Kinetin + 1% AC).

CONCLUDING REMARKS

This study confirmed the important role played by genetics in determining the response of pepper cultivars to different growing seasons and, of course, environmental conditions. The highest percentage of embryo and plant formation obtained by 'J27' was mainly due to the heterosis effect related to the background characters of its parents. Regarding the effect of different culture media and added elicitors, this study highlighted that the use of AC at 0.25% in association with 10 mg/L $AgNO_3$ ensured a higher percentage of embryo formation and subsequently more haploid plants were obtained. However, further studies on the effect of co-culture in the presence of other species' ovaries or the use of other elicitors are still needed.

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