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Research Article

The Effects of Cold Treatment and Charcoal on the In Vitro Androgenesis of Pepper (*Capsicum annuum* L.)

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Abstract: The effects of cold-shock treatment of *Capsicum annuum* L. buds at 4°C for 48 or 96 h, and the addition of 0.25% activated charcoal on embryo formation were examined. Murashige and Skoog (MS) basal nutrient medium was supplemented with combinations of 4 mg l^{-1} naphthalene acetic acid (NAA) and 1 mg l^{-1} benzyladenine (BA) or 1 mg l^{-1} NAA and 4 mg l^{-1} BA, together with 0.8% (w/v) agar and 3% (w/v) sucrose. The highest number of embryos was obtained from the control anthers which were not subject to cold treatments and were cultured on MS medium containing 4 mg l^{-1} NAA and 1 mg l^{-1} BA and activated charcoal. The mean androgenetic embryo production was found to be about 12.5%. The addition of growth regulators and activated charcoal had a greater effect than a cold pretreatment on embryo formation in pepper anther culture. On the other hand, the developmental stages of the microspores of this pepper genotype were investigated. Cytological studies were performed using the acetocarmine squash and paraffin methods. The bud size and morphological characteristics of the buds and anthers were defined and the microspore stages were determined. The buds 5 mm in diameter and 7 mm in length were determined to contain microspores at the uninucleate and 1st pollen mitosis stages. At this stage, the length of corolla was about the same as or slightly greater than that of the calyx.

Key Words: *Capsicum annuum* L., pepper, androgenesis, anther culture, cold shock treatment, activated charcoal.

Biberin (*Capsicum annuum* L.) In Vitro Androgenezisi Üzerine Aktif Kömür ve Soğuk Uygulamasının Etkileri

Özet: *Capsicum annuum* L. tomurcuklarına 4ºC' de 48 ve 96 saat sürelerde bekletilerek yapılan soğuk şoku uygulamalarının, besin ortamına %0,25 oranında aktif kömür katılmasının embryo oluşumuna etkileri incelenmiştir. Murashige Skoog (MS) temel besin ortamına 4 mg l^{-1} naftalen asetik asit (NAA) ve 1 mg l^{-1} benzil adenin (BA) veya 1 mg l^{-1} NAA ve 4 mg l^{-1} BA, %0,8 agar ve %3 sukroz katılmıştır. En yüksek embriyo oluşumu 4 mg l^{-1} NAA ve 1 mg l^{-1} BA ve aktif kömür içeren MS besin ortamında kültüre alınan hiçbir ön uygulama yapılmayan kontrol grubu anterlerinden elde edilmiştir. Ortalama androgenik embriyo oluşumu %12,5 civarında bulunmuştur. Besin ortamına katılan büyüme düzenleyicileri ve aktif kömür biber anter kültüründe embryo oluşturma frekansı üzerinde, soğuk şoku uygulamlarına göre daha etkindir. Diğer yandan bu biber genotipinin mikrospor gelişim aşamaları araştırılmıştır. Sitolojik incelemeler asetokarmin ezme yöntemi ve parafin metodu kullanılarak yapılmıştır. Tomurcuk büyüklükleri, tomurcuk ve anter morfolojileri tanımlanmış ve mikrospor aşamaları belirlenmiştir. Çapı 5 mm, uzunluğu 7 mm olan, korolla seviyesinin kaliks ile aynı veya biraz daha uzun olduğu gelişme dönemindeki tomurcukların uninucleat ve 1. polen mitozu aşamasındaki mikrosporları içerdiği saptanmıştır.

Anahtar Sözcükler: *Capsicum annuum* L., biber, androgenezis, soğuk şoku, anter kültürü, aktif kömür.

Introduction

Anther culture has been used in recent years as a tool for producing haploid plants in a variety of higher plants (Bajaj, 1983), but the low frequencies of microspore-derived plants restrict the use of the technique in plant breeding. There are several factors affecting androgenesis in many species, such as genotypes, growth of donor plants, pretreatments of anthers, composition of medium and culture conditions. Also the developmental stage of microspores within anthers is an important factor for the success in anther cultures. The anthers containing microspores at the uninuclear stage and at 1st pollen mitosis are determined to be optimal for the induction of androgenesis for many plant species of *Solanaceae* (Karakullukçu & Abak, 1992; Morrison et al., 1986; Vagera, 1990; Kristiansen & Andersen, 1993; Qin

& Rotino, 1993). Novak (1974) determined that anthers containing microspores at the uninuclear stage, taken from buds at a size between 2.6 and 5.0 mm, gave good results in pepper. It was also reported that flower buds, removed together with the corolla of the same size as the calyx or somewhat longer and containing anthers with microspores at the stage of 1st pollen mitosis, yielded more haploid plants in pepper (Sibi et al., 1979). Abak (1983) determined that the morphological characteristics of buds are different among pepper species and showed that the most suitable stage for the initiation of embryogenesis in pepper was the stage when the size of the corolla was the same as that of the calyx.

Some treatments can be applied before or during culture in order to increase the success obtained from anther culture. It has been shown that, for instance, the addition of activated charcoal (Anagnostakis, 1974; Vagera & Havranek, 1985; Tıpırdamaz & Ellialtıoğlu, 1998), cold pretreatment of whole buds or anthers (Morrisson et al., 1986) or different combinations and concentrations of NAA and BA (Rotino et al., 1987 a,b; Qin & Rotino, 1993; Ellialtıoğlu & Tıpırdamaz, 1997) could increase embryo production. The objective of the present study was to examine the effect of the cold pretreatment of buds, combinations of NAA and BA, and activated charcoal on the androgenetic capacity of pepper and also on the formation of haploid embryos or plants. Another aim of this investigation was to determine the developmental stages of microspores in relation to the morphological characteristics of buds and anthers.

Materials and Methods

Donor plants, *Capsicum annuum* L. (a local Turkish pepper genotype, Malatya), grown under field conditions, was used in the experiments.

1. Determining the developmental stages of microspores: During flowering, from late June to early August, flower buds were removed and classified into five groups according to their size and morphological characteristics.

The developmental stages of the microspores were determined in using microscopic slides prepared according to the acetocarmine squash (Elçi, 1994) and paraffin methods (Algan, 1981; Johansen, 1940). For the acetocarmine squash method, carmine (1g) was dissolved in glacial acetic acid (50 ml) by slight heating. The solution was then diluted to obtain 1% acetocarmine in 45% acetic acid (50 ml). An anther was placed into a drop of acetocarmine on the slide, and then a cover glass was placed on it and it was squashed. The paraffin method was carried out according to Algan (1981) and Johansen (1940). The anthers were dehydrated and then saturated with paraffin, by first using paraffin melted at 45ºC and then paraffin melted at 60ºC. Sections 10 µm in thickness were taken from the anthers, which had been embedded in paraffin and mounted on wood blocks. These sections were placed on the slides with glycerin albumin and stained by using the technique of Heidenhain Iron Hematoxylin (Johansen, 1940). Permanent slides were prepared using Entellan. These slides were examined under a light microscope and the microspores were photographed.

2. Pretreatments and anther cultures: Flower buds 5-7 mm in size were harvested from plants when the corolla was slightly longer than the calyx. The flower buds were placed in a flask and stored in a refrigerator at 4ºC for 48 or 96 hours. The control buds were not subjected to cold pretreatments. Before isolating the anthers, the buds were surface sterilized with 20% sodium hypochlorite for 15 minutes followed by 3 rinses in sterile distilled water. The anthers were then cultured on MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.8% (w/v) agar, and 4 mg l^{-1} NAA and 1 mg l^{-1} BA or 1 mg l^{-1} NAA and 4 mg l^{-1} BA with or without 0.25% activated charcoal. The cultures were incubated at 29ºC in continuous light conditions (2000 lux). Experiments were performed with 20 replications per treatment (each replication consisted of one petri dish, with 10 anthers). Data were collected after 3 months' culture. Data were analysed by multiple-way analysis of variance using ANOVA and the differences in the means were tested by the least significant difference (LSD) test (Winner, 1971). Before analysis, percentage data were angular transformed for homogeneity of variances.

Results

1. Determining the developmental stages of microspores

The appearance and size of the buds and the morphological characteristics of the buds, anthers and microspores are shown in Table 1. The microscopical

Table 1. The appearance and size of buds classified into five groups and the morphological characteristics of anthers buds (the values were the means of five replicants ± standard error).

Developmental stage of buds	Appearance of buds	Length of buds (mm)	Diameter of buds (mm)	Characteristics of buds, anthers and microspores
1		5 ± 0.5	4 ± 0.2	The buds are small, the colour of anthers is light green, and they contain microspore mother cells (Fig. 1).
2		6 ± 0.7	4.5 ± 0.5	The buds are unopened, the colour of anthers is green, and they contain microspores at tetrad and late tetrad stages (Fig. 2).
3		7 ± 0.3	5 ± 0.2	The length of the corolla of buds was about the same as or slightly greater than that of the calyx, and the colour of anthers is green with a slightly violet tinge on the anther sac ends, and they contain microspores at uninucleate and 1st pollen mitosis stages (Fig. 3).
4		8 ± 0.4	7 ± 0.3	The petal level of buds was longer than sepals, anthers are greenish violet, and they contain microspores undergoing mitosis (Fig. 4).
5		10 ± 0.9	7 ± 0.6	The buds are open and the anthers are dark violet, and they contain pollen grains (Fig. 5).

analyses showed the microspore stages, obtained from different sizes of buds. Anthers belonging to the buds at developmental stage 1 were found to contain microspore mother cells (Figures 1 a and b). Microspores taken from the buds at developmental stage 2 were at the tetrad and late tetrad stage (Figures 2 a and b). At developmental stage 3, microspores at the uninucleate stage and 1st pollen mitosis were found (Figures 3 a and b). Microspores of the buds at developmental stage 4 were found to undergo mitosis (Figures 4 a and b). Finally, at developmental stage 5, early binucleate pollen grains and late mature pollen grains were found (Figures 5 a and b). Our results show that buds at developmental stage 3 contained microspores at the uninucleate stage and 1st pollen mitosis, which was the suitable development stage for androgenesis of pepper.

2. Pretreatments and anther cultures

Microspore-derived embryoids were produced from control anthers of *C. annuum* cultured in MS medium containing 4 mg l^{-1} NAA and 1 mg l^{-1} BA and activated charcoal. The androgenetic embryo production was 12.5% (Table 2). Pretreating flower buds with cold at 4ºC for 48 or 96 hours resulted in a lower number of responding anthers and reduced embryo production, mainly by causing callus induction from the anthers.

The results of the analysis of variance are given in Table 3. It was found that the effects of all the factors

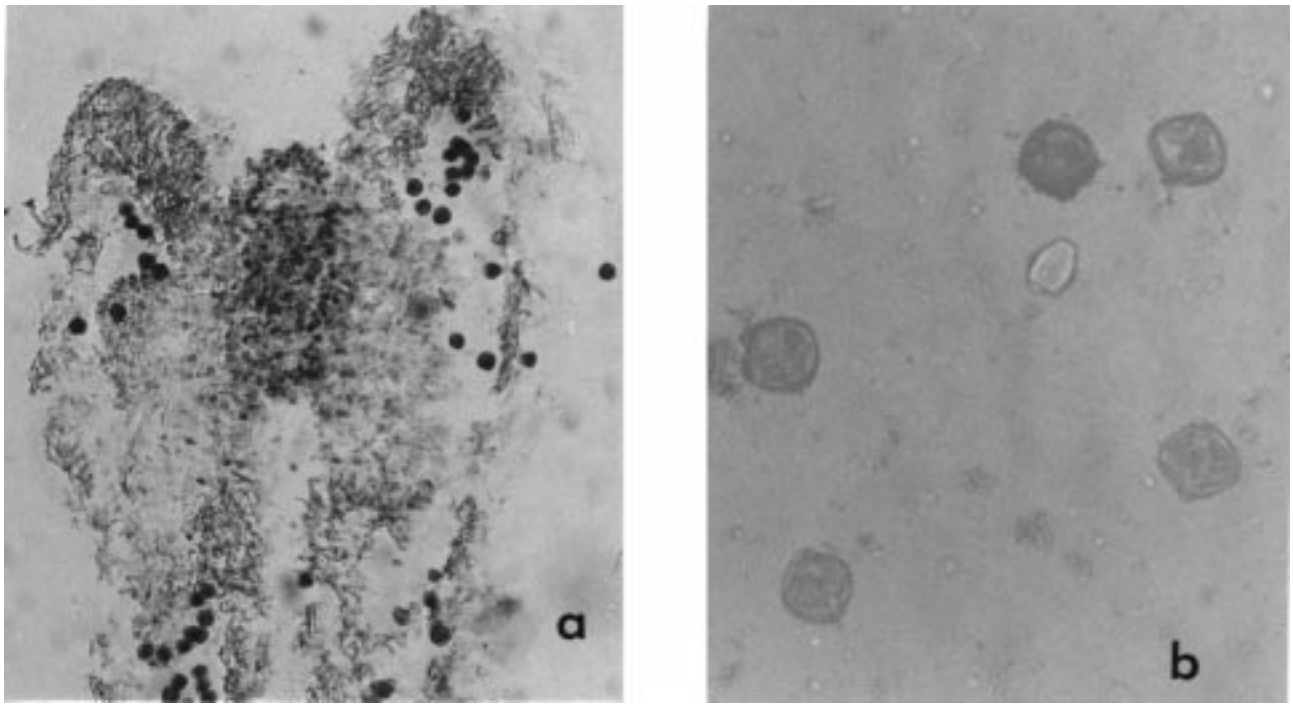

Figure 1. Microspore mother cells in anthers of buds at developmental stage 1 following a) paraffin (X100) and b) acetocarmine squash method (X400).

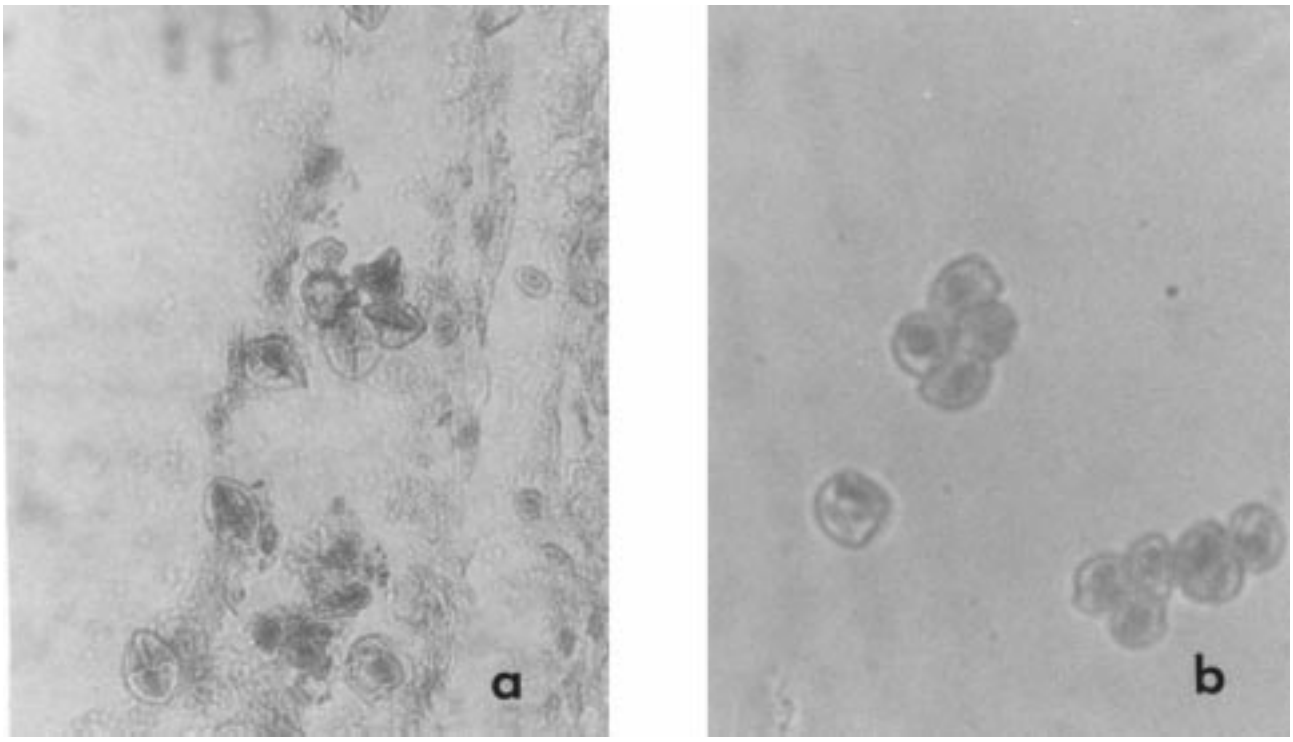

Figure 2. Microspores at tetrad and late tetrad in anthers of buds at developmental stage 2 following a) paraffin (X400) and b) acetocarmine squash method (X400).

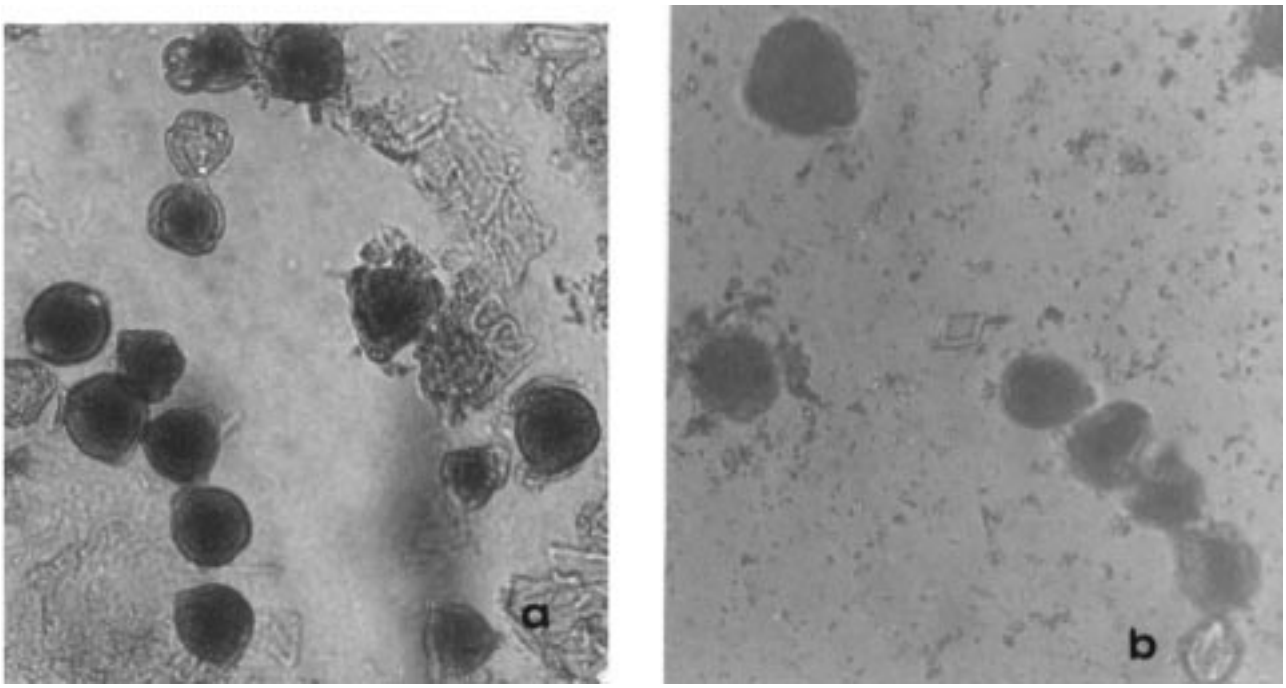

Figure 3. Microspores at uninucleate and 1st pollen mitosis in anthers of buds at developmental stage 3 following a) paraffin (X400) and b) acetocarmine squash method (X400).

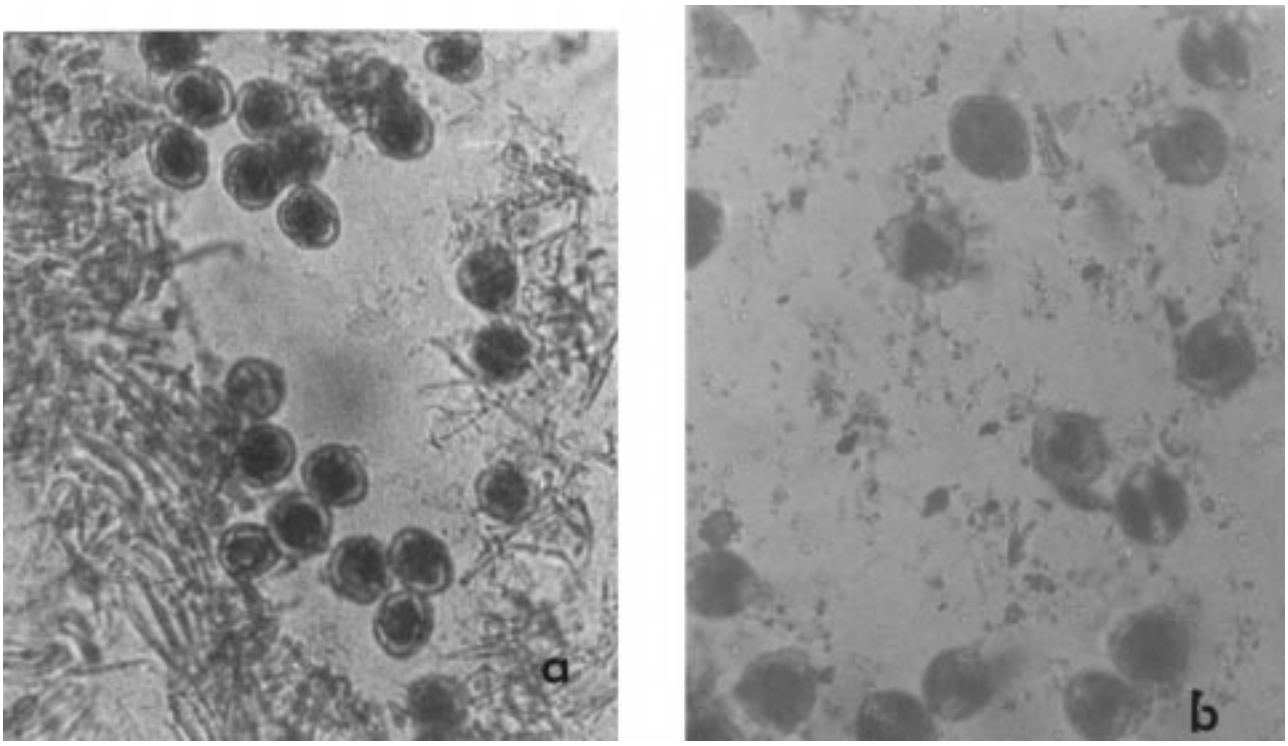

Figure 4. Microspores undergoing mitosis in anthers of buds at developmental stage 4 following a) paraffin (X400) and b) acetocarmine squash method (X400).

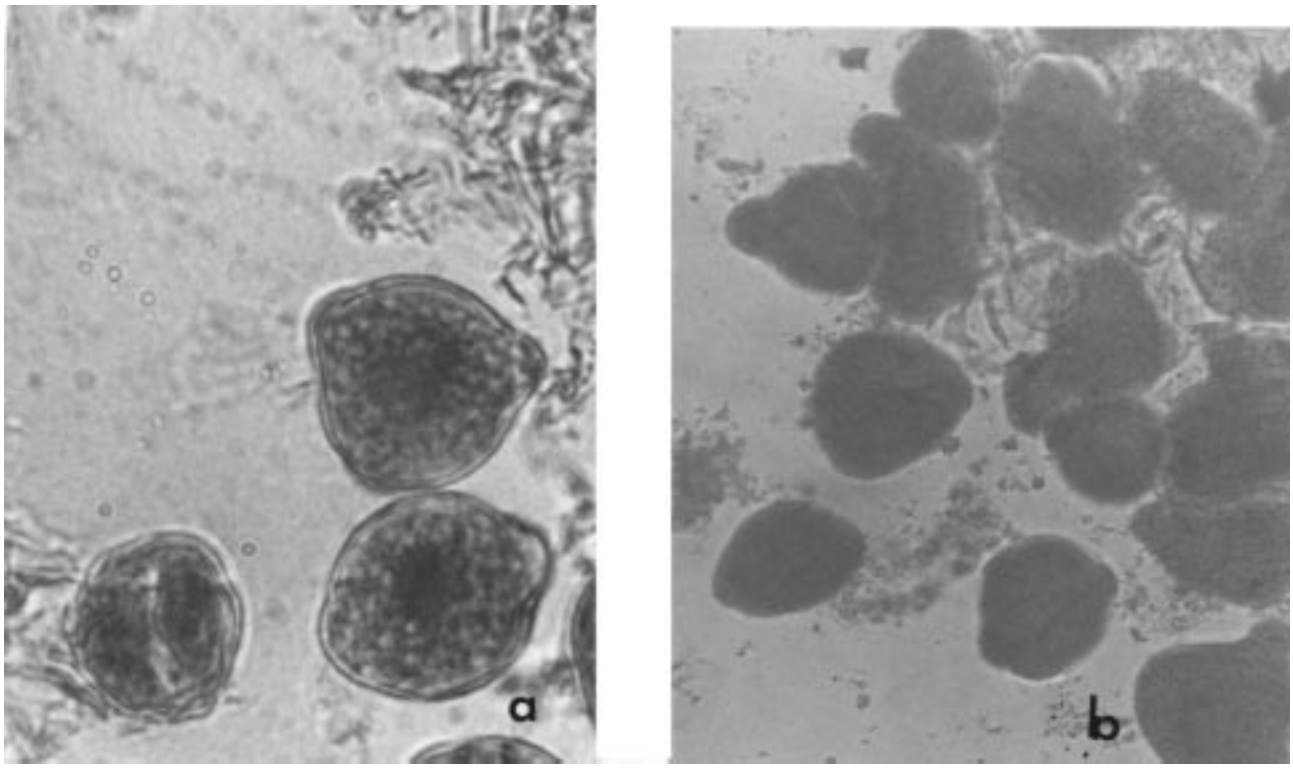

Figure 5. Early binucleate pollen grains and late mature pollen grains in anthers of buds at developmental stage 5 following a) paraffin (X1000) and b) acetocarmine squash method (X1000).

(i.e., cold pretreatment of buds, combinations of growth regulators and activated charcoal) on the androgenetic capacity of pepper, as well as interactions between the factors, were significant.

To compare the cold pretreatment durations in terms of androgenesis, the LSD test was performed, and the results are given in Table 4. It was shown that the control explants (without pretreatment) were significantly different when compared with cold pretreated explants (4ºC for 48 or 96 hours) with regard to the capacity for embryo formation. However, the differences between 48 and 96 hours were not significant.

Discussion

The studies on bud size and the stage of microspore development were undertaken in order to determine whether any external indicators like bud size or the morphological characteristics of anthers could be used in androgenesis to assess the stage of development of microspores. Such knowledge is essential because it is laborious and time consuming to examine the contents of every anther cytologically (Sopory & Maheswari, 1976).

In this study, the bud size and morphological characteristics of anthers, buds and microspores as well as the developmental stages of microspores were determined. The anthers, of buds 5 mm in diameter and 7 mm in length, were green with a slightly violet tinge on the anther sac ends and contained microspores at the uninucleate stage and 1st pollen mitosis, which was the most suitable stage for the induction of androgenesis in pepper (Karakullukçu & Abak, 1992; Morrison et al., 1986; Vagera, 1990; Kristiansen & Andersen, 1993). However, the anthers of buds smaller than those described above contained microspore mother cells and tetrad or late tetrad cells, which were not suitable for the androgenesis of pepper. Similarly, the anthers of the buds that were longer than this size contained microspores still undergoing mitosis or at early binucleate pollen grains and late mature pollen grains were not the correct stages of microspores for androgenesis (Abak, 1983; Karakullukçu & Abak, 1993).

Table 2. Frequency of *in vitro* androgenesis in anthers of Malatya pepper genotype on MS medium after cold pretreatment (4ºC) of excised buds (200 anthers were cultured for each combination).

Pretreatment time (h)	Hormone combination	Activated charcoal	% of callus in anthers	% of embryo in anthers	% of complete plants in anthers
Without pretreatment (control)	4 mg/l NAA 1 mg/l BA	+	84.5	12.5	0.5
	4 mg/l NAA 1 mg/l BA	–	100	0	0
	1 mg/l NAA 4 mg/l BA	+	88	6.5	0
	1 mg/l NAA 4 mg/l BA	–	99	0.5	0
48 h	4 mg/l NAA 1 mg/l BA	+	97	2.5	0
	4 mg/l NAA 1 mg/l BA	–	100	0	0
	1 mg/l NAA 4 mg/l BA	+	97	1	0
	1 mg/l NAA 4 mg/l BA	–	99.5	0	0
96 h	4 mg/l NAA 1 mg/l BA	+	98	2	0
	4 mg/l NAA 1 mg/l BA	–	99	0.5	0
	1 mg/l NAA 4 mg/l BA	+	100	0	0
	1 mg/l NAA 4 mg/l BA	–	100	0	0

Table 3. The analysis of variance of the data in relation to androgenetic embryo production.

Source	df	Mean Squares	F	Sig.
Pretreatment (A)	2	2.41	8.649	0.000
Hormone combination (B)	1	3.267	11.711	0.001
Activated charcoal (C)	1	6.017	21.569	0.000
A x B	2	1.079	3.869	0.022
A x C	2	2.254	8.081	0.000
B x C	1	3.267	11.711	0.001
A x B x C	2	1.504	5.392	0.005

Table 4. The LSD test among the pretreatments.

Pretreatment level (I)	Pretreatment level (II)	Mean difference (I-II)	Sig.
Control (without pretreatment)	+4ºC 48 hours pretreatment	1.230*	0.001
	+4ºC 96 hours pretreatment	1.480*	0.000
+4ºC 48 hours pretreatment	Control (without pretreatment)	-2.8750*	0.001
	+4ºC 96 hours pretreatment	2.500	0.590
+4ºC 96 hours pretreatment	Control (without pretreatment)	-3.1250*	0.000
	+4ºC 48 hours pretreatment	-2.500	0.590

The mean difference is significant at p= 0.05

It is essential to know the developmental stages of microspores because the morphological characteristics of buds and anthers and suitable developmental stages of microspores for androgenesis may be different for many plant species or even the different cultivar species (Abak, 1983).

The pretreatment of flower buds before excising the anthers for culture has been described as a means of increasing the androgenetic response (Maheswari et al., 1982; Morrisson et al., 1986). However, Vagera and Havranek (1985), working with pepper anther culture, reported no significant effect of a pretreatment of flower buds. The results obtained here showed that cold treatments did not increase the embryo formation and most of the embryos were from the control groups. The pretreatments resulted in reduced embryo formation, as a result of induction of callus on the explants.

The promotive effect of activated charcoal in anther culture medium has been described previously (Johansson, 1983; Anagnostagis, 1974; Tıpırdamaz & Ellialtıoğlu, 1998). Generally, the effect has been attributed to the absorption of inhibitory substances (phenolics, abscisic acid) from the medium. In the present study, the highest number of embryos was obtained from the control anthers on MS medium with 4 mg l^{-1} NAA and 1 mg l^{-1} BA and activated charcoal. It has been suggested (Rotino et al., 1987 a,b) that the additions of NAA/BA hormone combinations to MS medium can increase androgenetic embryo formation.

In conclusion, the use of growth regulators and activated charcoal was found to have a greater effect than cold pretreatments on embryo formation frequency in pepper anther culture.

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