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CROP BREEDING AND
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Colchicine treatments in anther culture of soybean

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ABSTRACT - *The effect of colchicine on the symmetrical division and embryo induction in cultured anthers of soybean was investigated. The anthers were incubated in various concentrations of colchicine for 24 or 72 hours. The* in vitro-*incubated anthers were cytologically analyzed throughout the first 15 days of culture. The study showed that colchicine did not have a significant effect on the symmetrical division or multinucleated pollen formation. Embryo induction was also not affected by colchicine. Embryos showed histodifferentiation and were classified in various morphological classes that resembled somatic embryos obtained from immature cotyledons.*

Key words: androgenesis, anther culture, multinucleated pollen, symmetrical division.

INTRODUCTION

The phenomenon of pollen embryogenesis was first demonstrated in *Datura innoxia* by Guha and Maheshwari (1964). Since then the production of pollen-derived plants from anther culture has been documented in over 170 species of angiosperms (Reynolds 1997). Androgenesis has been studied intensively in model plants such as rapeseed, tobacco, and barley (Mardhorst et al. 1997). However, in important crops like soybean the induction of androgenetic embryos is still a troublesome process. The recalcitrant nature of this species makes the progress in haploid plant induction sluggish and limited published information is available (Hu et al. 1996).

The ability of cultured anthers to develop haploid embryos emerges from a change of the gametophytic program of microspores towards a sporophytic pattern. In sporophytic development the first embryogenic division may be asymmetric or symmetric (Sunderland and Dunwell 1974).

Studies on the initial segmentation of microspores in culture have shown that androgenetic embryos are derived from symmetrical mitosis in several species (Fan et al. 1988, Zaki and Dickinson 1990, 1991, 1995). These authors consider the symmetry of mitosis a key step to subsequent pollen embryogenesis. According to Telmer et al. (1993) the symmetrical division blocks further pollen development. The occurrence of symmetrical binucleated pollen has been observed in soybean and is a possible route for callus formation (Yin et al. 1980, Kaltchuk-Santos et al. 1993, Kaltchuk-Santos et al. 1997).

Different stress pretreatments of anthers or microspores, such as heat shock (Keller and Armstrong 1979, Telmer et al. 1993), cold treatment (Nitch and Norreel 1973), and starvation (Kyo and Harada 1986), have been reported to induce the androgenetic pathway. There are several chemical treatments as well, which enhance the embryogenic

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ability of microspores. The effect of the antimicrotubule drug colchicine on microspore embryogenesis has been investigated by several authors (Tanaka and Ito 1981, Wan et al. 1989, Barnabás et al. 1991, Zaki and Dickinson 1991, 1995, Iqbal et al. 1994, Szakács and Barnabás 1995, Zhao et al. 1996, Arzani and Darvey 2001, Obert and Barnabás 2004). These researchers found that colchicine promotes the androgenesis process by increasing symmetrical mitosis and induction of somatic embryos. Colchicine disrupts pollen development by depolymerizing microspore microtubules (Zhao et al. 1996).

The aim of the present work was to determine the effect of colchicine on the androgenetic pathway of soybean and to improve the efficiency of embryo induction and differentiation.

MATERIAL AND METHODS

Plant material

The cultivar IAS 5 of soybean (*Glycine max* (L.) Merr., 2n = 40) was used in this study. Young inflorescences of field-grown plants were harvested and pretreated for 5 days at 4 °C. Floral buds of 3.0-3.5 mm length, containing uninucleate microspores were selected and surface sterilized with 70% ethanol for 1 min, followed by 1% sodium hypochlorite solution with a trace of detergent for 20 min.

Colchicine treatments

Anthers were excised and exposed to various concentrations of colchicine added to the induction medium (Kaltchuk-Santos et al. 1997) by ultrafiltration. The tested concentrations were: 0, 5, 10 and 20 mg L^{-1} in experiment I; 0, 25, 50 and 100 mg L^{-1} in experiment II, and 0, 100, 200, and 400 mg L^{-1} in experiment III. One hundred anthers, dissected from 10 floral buds, were placed in each culture petri dish, with four replications per treatment. The treatments lasted either 24 or 72 hours. After these periods the anthers were transferred to fresh induction medium without colchicine. Petri dishes were incubated for 16 h at 25 °C under 22.5 mE m^{-2} s^{-1} photoperiod provided by fluorescent light.

Callus and embryo formation was determined after 60 culture days. The embryos formed after 60 days were transferred to a solid MSM6AC medium (Bailey et al. 1993) for histodifferentiation. After 35 days, the embryos were counted, separated and transferred to MSM6 medium (Finer and McMullen 1991) for maturation. Seven days later the histodifferentiated embryos were transferred to MSO medium (MS salts, B5 vitamins, 3% sucrose, 0.3% Phytagel™, pH 5.8) for germination. These androgenetic embryos were classified in morphological classes according to Buchheim et al. (1989).

Cytological analysis

Twenty-four anthers per treatment were fixed in ethanol: acetic acid (3:1) during 0, 5, 10, and 15 days after culture for the cytological analysis. After squashing in propionic-carmine, the microspores were staged and classified under a Zeiss Axioplan Universal microscope.

Statistical Analysis

A split-plot analysis of variance was conducted with a number of symmetrical binucleate and multinucleate pollen. A preliminary analysis of data indicated that transformation was necessary for both variables. A log (y + 0.001) proved to be the most adequate for experiment I data, log (y + 0.0001) for experiment II, and a log (y + 0.01) for experiment III (for symmetrical binucleate pollen). Means were compared by Tukey´s test at α=0.05. No transformation of data fitted the analysis of variance assumptions for multinucleate pollen in experiment III. Kruskal-Wallis's nonparametrical test was used and the calli frequencies analyzed in a two-factor factorial design.

RESULTS AND DISCUSSION

Colchicine Test

It has been proposed that microtubule reorganization is the key event in changing the microspore developmental pathway, where altered division symmetry and cell dynamics define the induced embryogenic structure (Zhao et al. 1996). Taking advantage of these events, efforts have been made to induce symmetric division. The microtubule inhibitor colchicine is considered an androgenesis promoting compound. According to Zhao et al. (1996), colchicine induces microspore embryogenesis through cytoskeleton reorganization, which leads to the loss of the original cell asymmetry that blocks the gametophytic development. In the present study, the effect of colchicine on the pattern of microspore division, multinucleate pollen formation, and embryo induction from anther culture of soybean was investigated in three experiments (Table 1).

The cytological analysis of anthers showed that symmetrical binucleated pollen can be found at the moment of incubation. The *in situ* occurrence of this type of soybean pollen was reported in a previous study (Kaltchuk-Santos et al. 1993). As a general tendency, the frequency of symmetrical pollen increased during the culture period.

No multinucleated pollen grains were found at inoculation but were detected after 10 days of culture in

experiment I, and after 5 days in experiments II and III (data not shown). In experiment I, multinucleated pollens were detected in only two treatments (10 mg L^{-1} colchicine, 24 h; and 20 mg L^{-1} colchicine, 72 h) after 15 culture days. In experiments II and III, the frequencies of such grains were higher. The statistical analysis of experiment II data confirmed a significant increase in frequencies of multinucleated grains after 10 days of culture (data not shown).

Our results did not provide any evidence of the ability of colchicine to induce symmetrical divisions and/or multinucleate pollen formation in cultured anthers of soybean. Differences among colchicine concentrations in percentages of these pollen grain types were not statistically significant in any experiment.

Embryos were formed from treated and non-treated anthers (Table 1). Although in all experiments some colchicine treatments had higher percentages of embryo formation, the differences among treatments were not statistically significant.

Table 1. Effect of colchicine concentration and treatment duration on the formation of symmetrical binucleated pollen grains (II_{sym}), multinucleated pollens and embryos in soybean anther culture

Colchicine treatment		at inoculation			after 15 day culture			after 60 day culture	
Concentration	Duration	N^1	II_{sym} [2](%)	Multinucleated[3]	N	II_{sym} (%)	multinucleated	N^4	anthers with embryos (%)
— mg L^{-1} —	— hours —			— % —			— % —		
Experiment I									
0	24	1193	0.08	0	1615	0.37	0	80	2.50
5	24	717	0	0	1044	0.29	0	69	1.45
10	24	1801	0.11	0	2276	0.79	0.09	58	0
20	24	828	0.12	0	1871	0.21	0	54	5.55
0	72	1198	0	0	660	0.76	0	79	3.80
5	72	956	0.31	0	328	0	0	52	5.77
10	72	2176	0.09	0	1019	0.10	0	58	0
20	72	1359	0	0	957	0.83	0.10	55	3.39
Experiment II									
0	24	1659	0.66	0	2428	0.53	0	88	7.95
25	24	2624	0.04	0	3014	0.43	0.23	103	2.91
50	24	3180	0	0	2538	0.16	0.08	85	8.23
100	24	2481	0.08	0	2277	0.31	0	118	3.38
0	72	2340	0.04	0	2700	0.59	0.59	69	4.34
25	72	2988	0.07	0	2411	0.29	0.08	97	2.06
50	72	2561	0.55	0	1988	0.85	0.40	69	2.90
100	72	2540	0.04	0	1742	0.75	0.57	76	0
Experiment III									
0	24	7395	0.3	0	9805	0.14	0.04	123	1.62
10	24	9570	0.7	0	7188	0.32	0.08	121	3.30
200	24	9329	0.45	0	8865	0.54	0.08	85	5.88
400	24	9940	0.13	0	8917	0.09	0.02	129	6.20
0	72	3341	0.06	0	7660	0.14	0.07	150	4.0
100	72	5574	0.02	0	7551	0.26	0.05	156	3.20
200	72	4659	0.82	0	8413	0.36	0.08	126	3.17
400	72	5572	0.02	0	5881	0.10	0	118	1.69

[1]total of microspores; [2]number of II_{sym}/total number of microspores; [3]number of multinucleated/total number of microspores; [4]total of anthers calli^{-1}

Data obtained in the present study did not show an androgenic promoter potential of colchicine as demonstrated in *Brassica* (Zaki and Dickinson 1991, 1995, Iqbal et al. 1994). In wheat, both positive (Szakács and Barnabás 1995) and negative (Ghaemi et al. 1994) results were obtained regarding the effects of colchicine on androgenesis. In diploid potato, the androgenic response did not differ significantly among five colchicine treatments (Teparkum and Veilleux 1998).

Zaki and Dickinson (1991) tested five colchicine levels and different periods of exposure in microspore cultures of *Brassica napus*. They verified that colchicine promoted both symmetrical mitosis and embryogenesis most actively when microspores were exposed to the levels above 25 mg L^{-1} for 12 h. According to the authors, the compound exerts a dramatic effect on the number of cells differentiating into embryos, and this effect is strikingly cultivar-specific. The same authors (Zaki and Dickinson 1995) investigated the effect of colchicine treatments on anther and isolated microspore culture using five cultivars of *Brassica*. In both, a promotional effect of the drug was observed, although the embryogenic response varied among cultivars. In poorly responding cultivars, only small increases were observed.

More recently, Zamani et al (2000) found significant differences in the induction frequency of microspore-derived structures among three wheat genotype. In the winter genotype 'Mv. Szigma' colchicine caused a significant reduction in such structures. By contrast, the colchicine treatment increased the frequency of the induced structure in the spring variety 'Acheloos'.

Only one cultivar, IAS 5, was tested in the present study, which might be a poor responding genotype. Assuming that colchicine action is cultivar-dependent, it is possible that clearer results could be obtained testing other cultivars.

Some results obtained by Iqbal et al (1994) in *Brassica* differ from those found by Zaki and Dickinson (1991). The last authors believe that the positive effect of colchicine might be associated with a particular developmental stage. Also in rapeseed, Zhao et al. (1996) verified that the microspore responsiveness to colchicine was development stage specific. They claim that low embryogenesis is usually the result of either culturing microspores at an unresponsive stage of development or a highly heterogeneous population of microspores with few responsive cells.

In our study, the bud size was used as an indicator of the microspore developmental stage. Buds that presented mostly uninucleate pollen were selected. However, we observed that anthers from a given bud differed in the developmental stage of microspores (data not shown). Thus, the low embryogenic response in cv IAS 5 might be partially attributed to the heterogeneity of the microspore population within a flower bud.

Most of the above reports used the microspore culture system to test the effect of colchicine on pollen embryogenesis induction. The use of the anther culture procedure in the present study might have hindered the evaluation of the colchicine effect on soybean androgenesis.

Androgenetic embryo production and histodifferentiation

After 30 days of culture, some of the anthers inoculated on induction medium began to form calli. Androgenetic embryos in globular stage were produced on the surface of anthers and on calli (Figure 1a) around 6 weeks after inoculation. After 60 culture days, the embryos were transferred to solid MSM6AC medium for histodifferentiation. The number of histodifferentiated embryos was low for all treatments, with a total of 25 embryo clusters (2 to 8 embryos per cluster).

Several morphological embryo types were found in this experiment (Figure1b-g): monocotyledonous, dicotyledonous, polycotyledonous, fused cotyledon, long hypocotyl-vestigial cotyledon, moderately fasciated, and grossly fasciated. Many embryos were partially or completely fused. Normal dicotyledonous embryos were rare (8%) and the most frequent type was a long hypocotyl-vestigial cotyledon (40%). These androgenetic soybean embryos resemble somatic embryos induced from immature cotyledons incubated on a high 2.4-D-containing medium (Buchheim et al. 1989, Santos et al. 1997). Previous study showed that androgenetic embryos were histologically similar to zygotic embryos (Kaltchuk-Santos et al. 1997).

The histodifferentiated embryos were transferred to a maturation and then to germination medium, but none of them converted to plants. An optimization of the conversion protocol is underway.
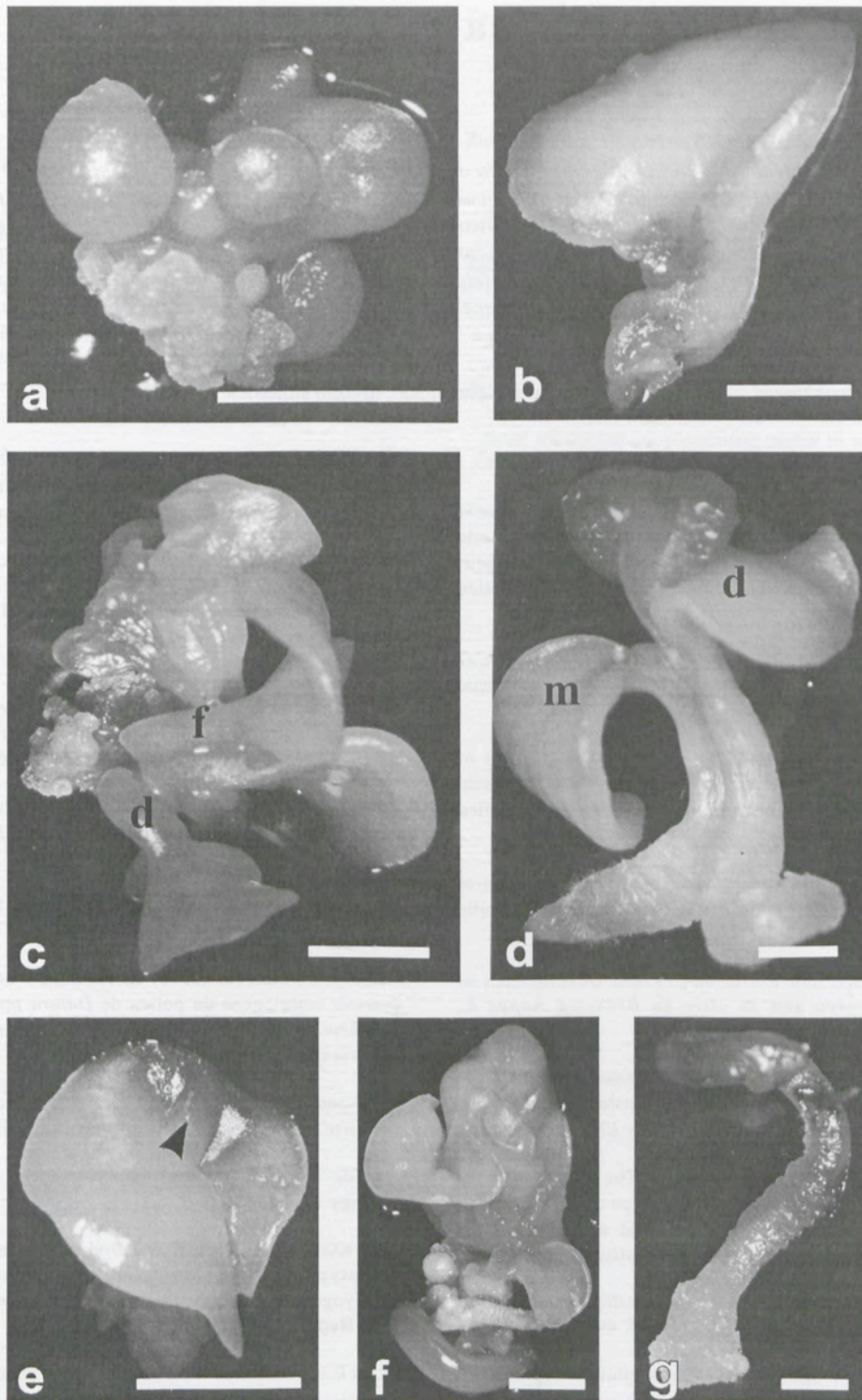
Figure 1. a. Globular embryos formed from soybean anther culture; b-j. Different morphologies of soybean androgenetic embryos: b. monocotyledonous embryo; c. dicotyledonous cluster (*d*) and fused embryos (*f*); d. fused embryo (*m*=monocotyledonous; *d*=dicotyledonous); e. embryo with fused cotyledons (arrow shows shoot apex); f. moderately fasciated embryo; g. embryo with long hypocotyl and vestigial cotyledon. Bars=2mm

Tratamentos com colchicina na cultura de antera em soja

RESUMO - *No presente trabalho foi investigado o efeito da colchicina na divisão simétrica e na indução de embriões em anteras de soja cultivadas* in vitro. *As anteras foram incubadas em diferentes concentrações de colchicina por 24 ou 72 horas. A análise citológica das anteras incubadas* in vitro *foi feita ao longo dos primeiros 15 dias de cultura. O estudo mostrou que a colchicina não exerce um efeito significativo sobre a divisão simétrica ou sobre a formação de pólen multinucleado. A indução de embriões também não foi afetada pela colchicina. Os embriões mostraram histodiferenciação e foram classificados em várias classes morfológicas assemelhando-se aos embriões somáticos obtidos a partir de cotilédones imaturos.*

Palavras-chave: androgêneses, cultura de antera, pólen multinucleado, divisão simétrica.

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