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Research note

Anther culture and cold treatment of floral buds increased symmetrical and extra nuclei frequencies in soybean pollen grains

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Abstract

Androgenic response is characterized by a multinucleate or multicellular stage of pollen development. Histological sections stained with toluidine blue and squashes in propionic-carmine and in 4′-6-diamidino-2-phenylindole (DAPI) were used for serial observations (0, 14 and 28 days) in soybean pollen grains from cultured anthers and floral buds submitted to cold treatment at 4 °C. In a total of 62,536 pollen grains, it were observed general averages of 2.06‰ of pollen grains with two symmetrical nuclei and of 1.41‰ pollen grains with typical extra nuclei (i.e. additional nuclei with typical morphology). Symmetrical and extra nuclei frequencies increased in both treatments but only the number of pollen grains with typical extra nuclei increased significantly with time of exposure to treatments. In addition, 8.59‰ of multinucleate pollen grains were recorded with atypical nuclei, smaller than vegetative or generative-types and with a fragmented shape. The frequency of these grains increased significantly with time of exposure to treatments. Thus, soybean multinucleate grains occurrence was not an exclusive response to culture. These preliminary results point to the need of further studies to clarify the relationship between typical and fragmented extra nuclei with both androgenesis and programmed cell death.

Abbreviations: DAPI – 4′-6-diamidino-2-phenylindole; G – generative; V – vegetative

In plants androgenesis implies a deviation from gametophytic to sporophytic development, characterized by multinucleate and multicellular stages of pollen grains. Cold pretreatment is a stress factor predisposing microspores to androgenesis in some plant species but, despite the assumptions of Horner and Street (1978) and Heberle-Bors (1985), studies have shown no evidence of pollen embryogenesis prior to culture.

In soybean (*Glycine max* L. Merrill, $2n = 2x = 40$) anther culture, Yin et al. (1982) recorded that the content of the multinucleate pollen grains disintegrated after a 25-day incubation and only multicellular grains were associated with androgenic response. Kaltchuk-Santos et al. (1997) recorded no multinucleate grains before culture, but the frequencies of such pollen grains increased *in vitro* after a 4 °C pretreatment in cv. IAS 5 and RS 7. The authors suggested that some of these grains might be the precursors of embryos obtained from anther culture.

In our recent cytological studies using carmine staining we have found multinucleate pollen grains of the soybean cv. IAS 5 in anthers submitted to low temperatures without having been placed in culture. In a subsequent histological analysis, a multinucleate pollen grain of cv. MG/BR 46 Conquista was also found, on the 18th day of culture, but the toluidine blue staining pattern indicated that it had already become non-viable.

Since pollen viability is overestimated by carmine staining, these two observations have raised two hypotheses. First, the occurrence of soybean multinucleate grains could not be an exclusive response to *in vitro* culture, and second, extra nuclei formation is not always an androgenic response.

In order to obtain qualitative and quantitative information on the significance of multinucleate grains, we obtained immature inflorescences from field-grown plants of IAS 5 and Conquista in January 2002. Part of the material was submitted to anther culture and the other part to cold treatment. For cold treatment, floral buds placed in hermetically sealed plastic vials were subjected to 4 °C in the dark. At 0, 14 and 28 days of treatment, 20 floral buds per cultivar were sampled in each fixative, Farmer (100% ethanol:glacial acetic acid, 3:1) and McDowell and Trump (1976) (glutaraldehyde 1% and formaldehyde 4%). For *in vitro* culture, 1500 anthers were dissected from floral buds (length 3–3.5 mm) not cold treated and cultured according to Kaltchuk-Santos et al. (1997) in 60-mm plastic Petri dishes (50 anthers per dish). Each Petri dish contained 13 ml of B5 Long medium (Gamborg et al., 1968; see Hu et al., 1996 for the organic ingredients) supplemented with Yeung's amino acids (Yeung and Sussex, 1979), 0.5 mg BA, 2 mg 2,4-D, 90 g sucrose and 2.5 g Phytagel l^{-1}. The pH of the medium was adjusted to 6.4 before autoclaving. At 0, 14 and 28 days of culture, 10 anthers per cultivar per dish were sampled in each fixative. Material fixed in Farmer's solution was squashed in propionic-carmine according to Kaltchuk-Santos et al. (1997) and in DAPI according to Coleman and Goff (1985). Material fixed in McDowell and Trump (1976) was submitted to histological preparations according to Kaltchuk-Santos et al. (1997).

Table 1. Responses (per thousand) of soybean pollen grains at 0, 14 and 28 days under anther culture and cold treatment of floral buds at 4 °C

	Soybean microspores and pollen grains (per thousand)						
	With typical nuclei	P type	With two symmetrical nuclei	With typical extra nuclei	With fragmented extra nuclei	Degraded	Number total analysed
Anther culture							
Day 0	953.80	0.67	0.79	0.87	2.43	41.45	9,862
Day 14	317.39	0.00	0.07	4.39	11.72	666.43	8,415
Day 28	138.88	0.00	3.80	5.04	30.81	821.47	4,926
Cold treatment (4 °C) of buds without culture							
Day 0	929.60	0.14	0.37	0.00	2.18	67.71	10,950
Day 14	879.50	0.39	9.16	1.76	8.57	100.63	16,169
Day 28	783.20	0.00	0.13	0.59	13.85	202.23	12,214
General average	–	–	2.06	1.41	8.59	237.30	–
Sum	46,929	13	129	88	537	14,840	62,536
Pr > F							
A. Cv	–	–	0.2852	0.3294	0.2552	0.3804	–
B.Treat	–	–	0.8962	0.1052	0.2972	<0.0001	–
C.Day	–	–	0.6514	0.0021	<0.0001	<0.0001	–
AxB	–	–	0.5646	0.7279	0.0459	0.0251	–
AxC	–	–	0.5285	0.0838	0.5530	0.9449	–
BxC	–	–	0.0674	0.0882	0.3570	<0.0001	–
AxBxC	–	–	0.2682	0.8000	0.6633	0.8767	–
CV%	–	–	30.67	29.48	22.94	20.96	–
Transf	–	–	Root(x + 1)	Log(x + 0.001)	Log(x + 0.001)	Root(x + 1)	–

Anthers were fixed in Farmer's solution and squashed in propionic-carmine. The column 'Degraded' included non-stained and plasmolyzed pollen grains. The column 'P-type' was classified according to Kaltchuk-Santos et al. (1993) as a class of smaller grains which do not follow gametophytic route.

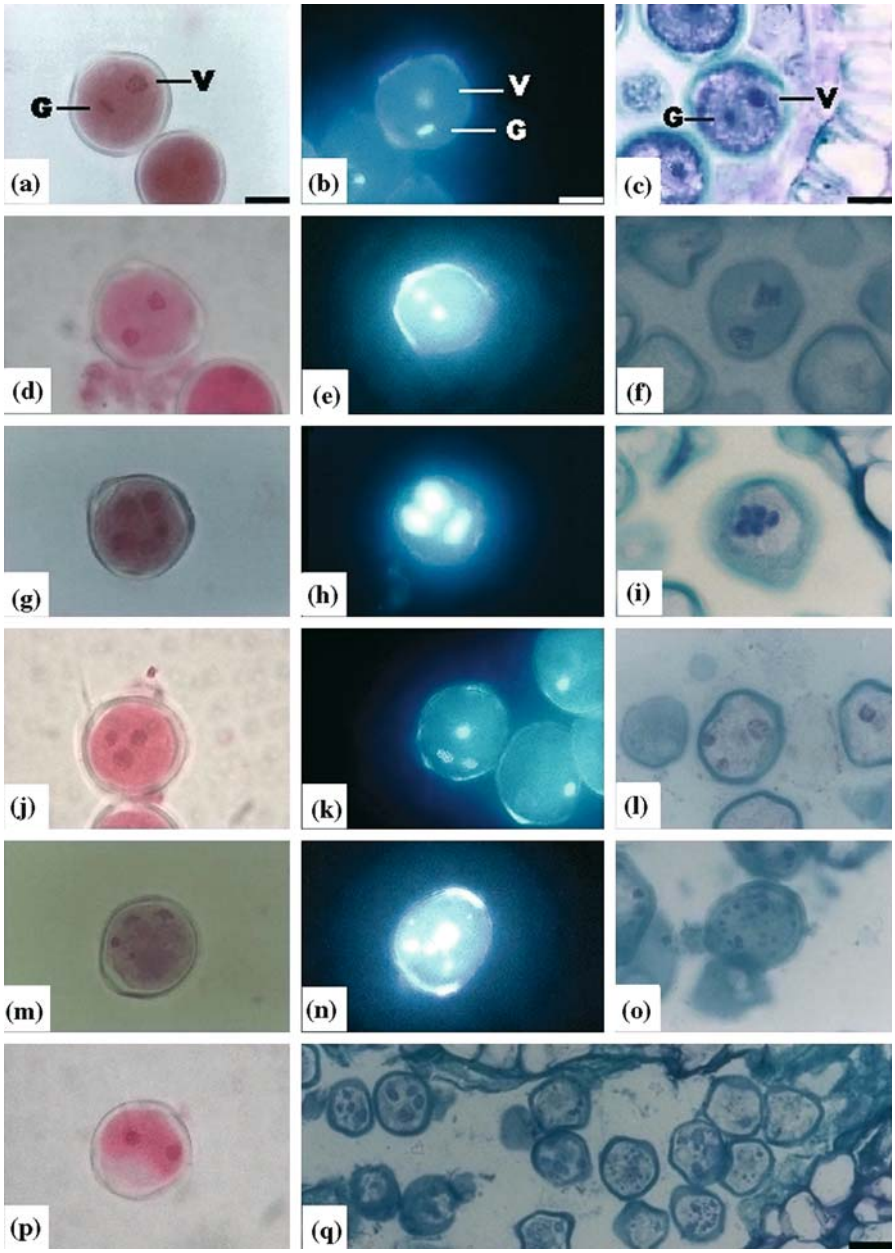

Figure 1. Soybean pollen grains recorded at 0, 14 and 28 days of anther culture and cold treatment of floral buds at 4°C. Figures *a*, *d*, *g*, *j*, *m* and *p* show propionic-carmine staining; *b*, *e*, *h*, *k* and *n* show fluorochromatic reaction to DAPI; *c*, *f*, *i*, *l*, *o* and *q* show 6–10 μm histological sections stained in toluidine blue. (*a–c*) Pollen grains with G (generative) and V (vegetative) typical nuclei at 0 day. (*d–f*) Pollen grains with symmetrical nuclei type V (*d*) and G (*e*) at 14 day of 4°C treatment and type V at 14 days of culture (*f*). (*g–i*) Multinucleate pollen grains with extra round nuclei at 28 days of 4°C treatment (*g*) and at 14 day of culture (*h,i*). (*j–l*) Multinucleate pollen grains with 3V nuclei at 14 days of 4°C treatment (*j*), with 2V1G nuclei at 14 day of culture (*k*) and with 1V2G nuclei at 28 day of 4°C treatment. (*m–o*) Multinucleate pollen grains with small extra nuclei whit a fragmented shape at 28 day of 4°C treatment (*m,n*) and at 14 day of culture (*o*). (*p*) Plasmolysis in pollen grain with two V nuclei at 14 day of 4°C treatment. (*q*) Anther locule filled with multinucleate pollen grains, with both typical and fragmented-type extra nuclei, at 14 day of culture (bars in (*a–c*) = 10 μm) and applies to (*d–p*) figures. (bar in $q = 20$ μm).

A total of 480, 320 and 600 anthers was processed in propionic-carmine (Figure 1a, d, g, j, m, p), DAPI (Figure 1b, e, h, k, n) and toluidine blue (to histological sections) (Figure 1c, f, i, l, o, q), respectively. ANOVA was performed on the carmine staining data.

There were no statistical differences between cultivars in all factors analyzed: pollen grains with two symmetrical nuclei, pollen grains with typical extra nuclei (i.e. additional nuclei with typical morphology), pollen grains with fragmented extra nuclei and degraded pollen grains (Table 1). General averages of 2.06‰ of pollen grains with two symmetrical nuclei (G and V type) (Figure 1d–f) and of 1.41‰ pollen grains with extra nuclei were observed, from day 0 until 28 of treatment, in the total of 62,536 pollen grains stained in propionic-carmine. The multinucleate pollen grains presented 3–6 round nuclei, frequently with different sizes (Figure 1g–i), or presented different nuclei types: 3V, 2V1G, 3V1G, 1V2G, 1V3G and 1V4G (Figure 1j–l). Symmetrical and extra nuclei frequencies increased in both culture and cold treatment but only the number of pollen grains with extra nuclei increased significantly with time of exposure to treatments, without difference between treatments (Table 1).

Besides the categories described above, 8.59‰ of multinucleate pollen grains were recorded with atypical nuclei with a fragmented shape and smaller than V or G nuclei. Observations in DAPI and in toluidine blue confirmed that these fragments contained DNA (Figure 1m–o). V and G nuclei of large size, with expanded shape, were also recorded in both treatments and associated to a DNA uncoil (not shown).

Pollen grain degradation, including the non-stained and plasmolyzed ones (Figure 1p), increased with time of exposure to treatments, and was significantly greater in anther culture. This result could be accounted to somatic proliferation that causes *in vitro* competition for space and nutrients (Rodrigues et al., 2004).

In histological sections, symmetrical and extra nuclei pollen grains frequently occurred assembled in the same anther locule (Figure 1q) but were absent in the other sporangia. This distribution could explain the high data variation and the lack of statistical significance of multinucleate grain frequencies in Kaltchuk-Santos et al. (1997) study.

Thus, the occurrence of soybean multinucleate grains was not an exclusive response to culture. As fragmented-type nuclei were recorded, not all nuclei had a typical morphology. These observations may cause confusion in the evaluation of microspores and pollen grain response to culture. These preliminary results indicate the need of further studies to understand the relationship between typical and fragmented extra nuclei with regard to both androgenesis and programmed cell death.

The hypothesis that, after extra nuclei formation, without appropriate environmental conditions to form a cell wall and take the sporophytic developmental route, soybean multinucleate grains degraded by plasmolysis or by fragmented-shape nuclei formation, cannot be ruled out.

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