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Evaluation of Gelling Agents on Anther Culture: Response of Two Soybean Cultivars

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ABSTRACT

Anthers of two soybean cultivars were cultured in B5 long basal culture media gelled with agarose or Phytagel™. Cytological examinations of the anthers were carried out during the first 45 days of culture to assay the viability and developmental stage of microspores. Frequency of callus formation was recorded at 45 days of culture. The analysis of variance of the microspore viability assay showed significant Cultivar X Gelling Agent X Day of Culture interactions. The frequencies of viable microspores decreased significantly with time of culture, within each cultivar and gelling agent tested. The interaction Day X Cultivar was significant for the frequencies of binucleate symmetrical grains and multinucleate/multicellular structures. The effect of gelling agents on the frequency of binucleate symmetrical pollens grains and multinucleate/multicellular structures was not significant. About the frequencies of calli and embryogenic calli formed, a significant difference was detected between the cultivars (IAS5= 14.8% and BRS 133=6.6%). Gelling agents showed no effect over these frequencies.

Key words: Anther culture, *Glycine max*, culture media, binucleate pollen grains, multinucleate/multicellular structures, callus formation

INTRODUCTION

The unique phenomenon encompassed by *in vitro* pollen embryogenesis (androgenesis) is concerned with the redirection of normal gametophytic pollen development towards the embryo formation pathway. The symmetricity of the nuclear division upon stress has been implicated to be a major pathway of embryo formation when microspores are cultured prior to first pollen mitosis (Smýkal, 2000). Yin et al. (1980) reported that symmetrical binucleate pollen grains are one of the routes identified in soybean callus formation. Data obtained by Kaltchuk-Santos et al. (1997) showed that although symmetric division was an important pathway, it was not the exclusive route for the multinucleate structure formation in soybean.

It is accepted that plant genotype plays a predominant role in determining androgenic responses *in vitro*, with major differences between the genotypes in their reaction under given culture conditions. Not only do the species within a genus often show markedly dissimilar responses in culture, but also cultivars of the same species (for review, see Maheshwari et al., 1982; Góralski et al., 1999). Thus, culture procedure should be optimized for each genotype so that this genotypic effect can be decreased or even minimized (Atanassov et al., 1995).

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No single anther culture medium can be recommended for the general use. The concentration and composition of the sugars and the type of gelling agents have the greatest influence on medium efficiency (Foroughi-Wehr and Wenzel, 1993). According to Hadeler et al. (1995), the gellant strongly influences cellular differentiation and sensitivity of *in vitro* plant cell cultures to cytokinin. Gelling agents are believed to influence the availability of mineral salts and sometimes may result in the vitrification of cultured tissues (Nestáková et al., 2000).

Although agar has been for a long time the only gelling agent used to produce solid media, more reproducible results on anther culture have been obtained with Gelrite (gellan gum) and agarose (Atanassov et al., 1995). To avoid possible inhibitory agar impurities, agarose has been recommended to gel the medium for soybean anther culture (Hu et al., 1996). This gelling agent was used in previous studies (Kaltchuk-Santos et al., 1997).

Phytagel™ is a gellan gum PS-60 isolated from the bacterium *Pseudomonas elodea*. It is a linear polysaccharide composed of glucuronic acid, glucose, and rhamnose, requiring the presence of monovalent and divalent cations for gellation (Veramendi et al., 1997). Since Phytagel™ is purified from a single organism, it is highly purified and does not contain contaminants (Chevreau et al., 1997).

The aim of this work was to assess the effect of two gelling agents, agarose and Phytagel™, on microspore viability, androgenetic induction and callus formation from cultured anthers of two soybean cultivars.

MATERIAL AND METHODS

Soybean cultivars IAS-5 and BRS 133 were grown in the field conditions at the Universidade Federal do Rio Grande do Sul, Brazil. Harvested inflorescences were stored at low temperature (~4°C) for 1 or 2 days. Flower buds were sterilized in 70% ethanol for 15s, followed by immersion in a 2% sodium hypochlorite solution for 12 min and rinsed three times with sterilized distilled water.

Young inflorescences were harvested and anthers of 3.0 to 3.5 mm flower buds were dissected and cultured in B5 long culture medium. This medium consisted of B5 formulation (Gamborg et al., 1968), enriched with 16 organic compounds and supplemented with Yeung's amino acids (Yeung and Sussex, 1979), 2 mg. L^{-1} 2,4-D (dichlorophenoxy acetic acid), 0.5 mg. L^{-1} BA (benzyladenine), 9% sucrose. The medium was gelled with 0.3% Phytagel™ (Sigma Chemical Co.) or 0.8% Agarose (Type VII, Sigma). One thousand and six hundred anthers (100 per 90 mm ∅ Petri dish) were cultured per treatment per cultivar, with a total of 6,400 anthers. Cultures were incubated at 25°C under a 16-h photoperiod of 22.5 $\mu mol.m^{-2}.s^{-1}$ with fluorescent light.

Ten anthers per Petri dish were collected at 0, 15, 30, and 45 days after culture initiation for the cytological analysis. Anthers were fixed in ethanol:acetic acid 3:1. Five anthers per Petri dish were then squashed in propionic carmine to evaluate the viability and developmental stages of the microspores. A total of 8000 microspores were analyzed per treatment per cultivar at each collection date. The other five fixed anthers were stored in freezer to be analyzed using another cytological technique (data not presented). The remaining cultured anthers were transferred to smaller Petri dishes (∅ 60 mm), containing the same culture medium. The frequency of callus formation was recorded 45 days after the inoculation. Calli were classified into three morphological types: rough (Fig. 1a), rough/smooth (Fig. 1b) and smooth (Fig. 1c). Zhuang et al. (1991) and Hu et al. (1996) reported that smooth-surfaced calli demonstrated embryogenic potential.

Analysis of variance was performed on microspores viability, binucleate symmetrical microspores, multinucleate/multicellular structures and callus formation data. Arcsin transformation was used on viability data; on binucleate symmetrical grains and multinucleate/multicellular structures data repeated measures analysis was used weighed least-squared transformation method. Callus formation data were analyzed by a two-factor factorial Analysis of Variance, considering cultivar and gelling agent.

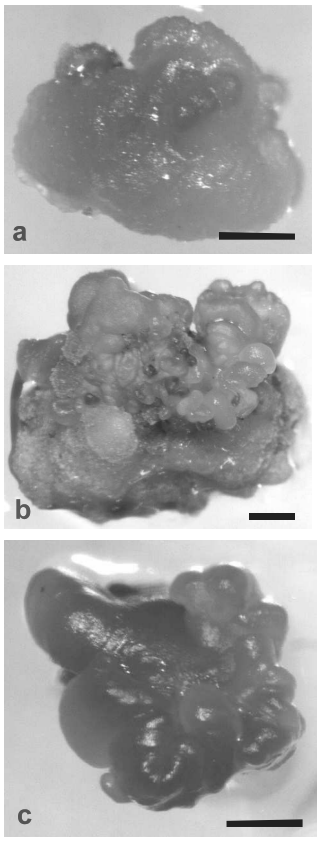

Figure 1 - Calli formed in soybean anther culture. a) rough; b) rough/smooth; c) smooth. Bars=1 mm

RESULTS AND DISCUSSION

Viability of microspores

The results of the microspores viability assay by propionic-carmine staining (Fig. 2a) during the first 45 days of culture are presented in Table 1. The analysis of variance showed significant Cultivar X Gelling Agent X Day of Culture interactions (Prob>0.0173). As expected, the frequencies of viable microspores decreased significantly with the time of culture, within each cultivar and gelling agent tested. Similar results were obtained by Rodrigues et al. (2005), which observed an increase of pollen degradation with time of culture. However, considering the frequency of viable microspores within day of culture, very similar means were obtained when comparing gelling agents in both cultivars.

Frequency of Binucleate Pollen Grains and Multinucleate Structures

Cytological analysis of the cultured anthers was carried out to determine the developmental stage at inoculation time and microspore segmentation throughout the culture period, using samples of anthers in the first 45 days of culture. In addition to the asymmetrical mitotic division of the microspores, pollen grains with symmetrical division were observed (Fig. 2b and 2c). In agreement with a previous study on soybean (Kaltchuk-Santos et al., 1997), the multinucleate structures (Fig. 2d) arose from either symmetrical or asymmetrical pathways, and both vegetative and generative nuclei might undergo division. The presence of symmetrical binucleate and multinucleate pollen grains were observed before the culture (0 day - Table 2). Considering that the flower buds were cold pretreated, these results indicated that the stress induced a gametophytic route deviation, even before the anthers inoculation in culture medium. Rodrigues et al. (2005) also showed that soybean multinucleate

grains occurrence was not an exclusive response to culture, since these pollen grains were recorded in anthers submitted only to cold treatment. In addition to multinucleate pollen grains, the presence of multicellular structures was noted from day 15 on (Fig. 2e). Finally, the mass of cells was released from pollen by exine rupture (Figs. 2f and 2g).

Considering the importance of symmetrical pollen grains and multinucleate/ multicellular structures for androgenetic callus and embryo formation, the statistical analysis only included this information. Results are summarized in Table 2.

The effect of gelling agents on the frequency of binucleate symmetrical and multinucleate/ multicellular pollen grains was not statistically significant (Prob>0.7823 and Prob>0.9384, respectively). The overall means for binucleate symmetrical pollen were 0.0272 for agarose and 0.0288 for Phytagel™. For multinucleate/multicellular structures, the means were 0.0125 for agarose and 0.0137 for Phytagel™. Similar frequencies were reported for cultivar IAS-5 in previous studies (Kaltchuk-Santos et al., 1997; Cardoso et al., 2004). Thus, results indicated that gelling agents had no influence on the segmentation pattern of microspores.

The interaction Day X Cultivar was significant for the frequencies of binucleate symmetrical grains (Prob>0.0002) and multinucleate/multicellular structures (Prob>0.0001). Comparing days of culture within each cultivar, it was observed that frequencies of binucleate symmetrical and multinucleate/multicelular pollen grains increased during the culture period. Comparing the cultivars within each day of culture, the means for binucleate symmetrical and multinucleate/ multicellular pollen grains in cultivar IAS–5 were higher than those obtained with cultivar BRS 133. However, significant difference was detected just for the multinucleate/multicellular structures at 15 days. The difficulty in detecting the significant differences could be accounted to extremely low frequencies of such kind of structures.

Androgenetic Structures

Data on the frequency of calli formed 45 days after culture initiation are summarized in Table 3. Gelling agents revealed to have no effect on the frequencies of calli and embryogenic calli formed. Similar results were reported by Jähne et al. (1994) who could not detect any difference in barley anther culture response to agarose-solidified or Gelrite-solidified media.

A statistically significant difference was detected between the cultivars (Prob>0.0005). The mean of embryogenic calli obtained with the cultivar IAS–5 (0.148) was higher than that of cultivar BRS 133 (0.066). Jian et al. (1986) reported that the frequency of callus and shoot formation in anther culture of soybean was quite different among the 85 varieties tested. Only one variety showed plantlet regeneration. These results reinforced the observation that the success in soybean anther culture was genotype-dependent.

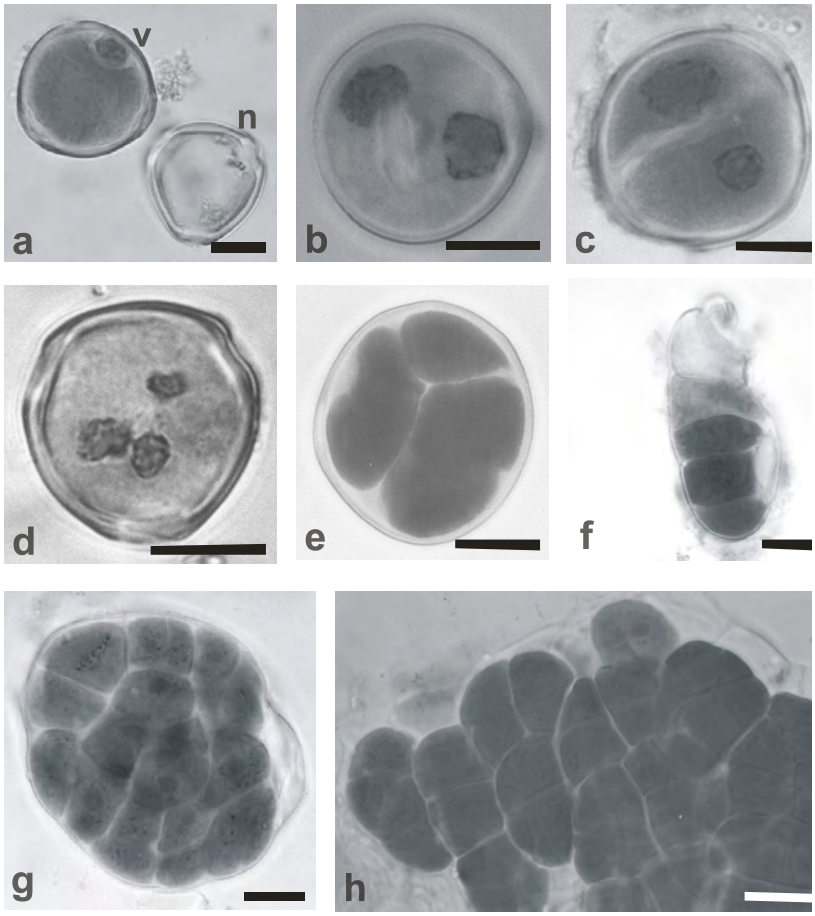

Figure 2 - Viability and microspore development through the culture period. a) v=viable and n=nonviable microspores; b) binucleate symmetrical without cell wall; c) binucleate symmetrical with cell wall; d) multinucleate; e) multicellular; f) mass of cells released by pollen exine rupture; g) and h) cell proliferations. Bars=10 μm

Table 1 - Effect of interaction between soybean cultivars, gelling agents and days of culture on viable pollen frequency.

Cultivar	**IAS-5**				**BRS 133**			
Days of culture	**0**	**15**	**30**	**45**	**0**	**15**	**30**	**45**
Agarose	a 1.31 A (0.96)	a 0.71 B (0.65)	a 0.73 B (0.66)	a 0.74 B (0.67)	a 1.28 A (0.95)	a 0.95 B (0.81)	a 0.75 C (0.68)	a 0.71 C (0.65)
Phytagel	a 1.30 A (0.96)	a 0.71 B (0.65)	a 0.69 B (0.64)	a 0.69 B (0.64)	a 1.26 A (0.95)	a 0.90 BC (0.78)	a 0.77 D (0.69)	a 0.79 CD (0.71)

( ) Original means

Capital letters on the right of transformed means indicate the comparison among days of culture within the same cultivar and gelling agent.

Small letters on the left of transformed means indicate the comparison between means of gelling agents within the same day of culture.

Means accompanied by the same letter (horizontally for upper class and vertically for lower class letters) are not significantly different (α= 0.05)

Table 2 - The effect of interaction between soybean cultivar and days of culture on binucleate symmetrical and multinucleate pollen grain frequencies

Days of culture	Number of microspores examined	Binucleate symmetrical *		Multinucleate / Multicellular *	
		IAS-5	BRS 133	IAS-5	BRS 133
0	60,674	a 0.0051 A (0.0052)	a 0.0018 A (0.0019)	a 0.0020 A (0.0020)	a 0.0009 A (0.0008)
15	26,915	a 0.0470 B (0.0521)	a 0.0181 AB (0.0207)	a 0.0179 B (0.0201)	b 0.0052 AB (0.0080)
30	21,905	a 0.0351 B (0.0420)	a 0.0242 B (0.0280)	a 0.0188 B (0.0208)	a 0.0112 B (0.0144)
45	27,591	a 0.0378 B (0.0426)	a 0.0292 B (0.0318)	a 0.0180 B (0.0202)	a 0.0169 B (0.0182)

* Transformed Mean
( ) Original Mean
Capital letters on the right of transformed means indicate the comparison among days of culture within the same cultivar.
Small letters on the left of transformed means indicate the comparison between cultivars within the same day of culture.
Means accompanied by the same letter are not significantly different (α= 0.01)

Table 3 - Callus formation from cultured anthers of two soybean cultivars.

Cultivar	Gelling Agent	Number of anthers	% of anthers with calli	% of anthers with embryogenic calli *
IAS–5	**Agarose**	884	42.7	15.7
	Phytagel	910	44.0	13.8
	Mean	1,794	43.4 A	14.8 A
BRS 133	**Agarose**	843	42.9	6.7
	Phytagel	872	40.1	6.5
	Mean	1,717	41.5 A	6.6 B

* Smooth + Smooth / Rough
Letters on the right of overall means indicate the comparison between cultivars. Means followed by the same letter are not significantly different (α= 0.01)

RESUMO

Anteras de duas cultivares de soja foram cultivadas em meio de cultura basal B5 longo gelificado com agarose ou Phytagel®. Análises citológicas das anteras foram conduzidas durante os primeiros 45 dias de cultura para avaliar a viabilidade e o estágio de desenvolvimento dos micrósporos. A freqüência de formação de calos foi analisada após 45 dias do início da cultura. A análise da variância da viabilidade do micrósporo mostrou interações significativas de Cultivar X Agente Gelificante X Dias de Cultura. As freqüências de grãos de pólen viáveis diminuíram significativamente com o tempo de cultura, dentro de cada cultivar e agente gelificante testado. A interação Dia X Cultivar foi significante para as freqüências de grãos de pólen binucleados simétricos e estruturas multinucleados/multicelulares. O efeito do agente gelificante na freqüência de grãos de pólen binucleados simétricos e estruturas multinucleados/multicelulares não foi significante. Com relação às freqüências de calos e estruturas embriogênicas formadas, houve diferença significativa entre cultivares *(IAS5= 14.8% e BRS 133=6.6%)*. O agente gelificante não mostrou efeito em tais freqüências.

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