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EVENTOS EMBRIOGÊNICOS EM TECIDOS ESTAMINAIS DE *Glycine max* (L.) Merrill

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RESUMO

O cultivo de anteras de soja [*Glycine max* (L.) Merrill, 2n=40] iniciou na década de 1970 visando à obtenção de plantas androgenéticas haplóides e duplo-haplóides. Contudo, foram raras as ocasiões em que as condições de cultivo favoreceram o desenvolvimento embriogênico até a regeneração de plantas. No presente trabalho, pelo cultivo de anteras heterozigotas para um marcador molecular codominante, foi registrado que alguns genótipos segregantes originaram estruturas embriogênicas tanto a partir dos micrósporos quanto a partir dos tecidos diplóides da antera. Subseqüentemente, por meio de análises histológicas, foram registradas calogênese e embriogênese a partir do tecido conectivo, das camadas médias e da epiderme em anteras de quatro cultivares, sob as condições de cultivo até então recomendadas para desencadear androgênese. Estes eventos foram favorecidos pela presença do ácido 2,4-diclorofenoxiacético (2,4-D) e pela indução na luz. A constatação do potencial embriogênico do conectivo levantou a possibilidade de usar tecidos estaminais como uma nova fonte de explantes, a partir de indivíduos que já alcançaram a fase reprodutiva, quando a resposta morfogênica é considerada sem sucesso em soja. A formação de grãos de pólen multinucleados não foi uma resposta exclusiva ao cultivo: tanto o cultivo de anteras quanto o tratamento a 4ºC aumentaram as freqüências de núcleos simétricos e extranumerários em grãos de pólen, incluindo núcleos com formato fragmentado. De forma geral, os resultados comprovaram que, nas condições recomendadas, o cultivo de anteras é um sistema limitado para desencadear androgênese em soja. Por isso, uma seqüência de testes foi conduzida para o estabelecimento *in vitro* de micrósporos isolados. Foi desenvolvida uma técnica de isolamento que permitiu a obtenção de suspensões de micrósporos viáveis com densidade adequada e, em seguida, o estabelecimento de cultivos. Nestes cultivos, foram testados os efeitos de meios nutritivos e de genótipos. A técnica desenvolvida será útil para futuros testes visando identificar fatores que desencadeiam androgênese em soja. Neste trabalho, foram identificadas limitações e potencialidades morfogênicas do cultivo de anteras de soja. Em conseqüência, é proposta uma nova abordagem ao estudo da androgênese, pelo cultivo de micrósporos e grãos de pólen isolados.

ABSTRACT

Soybean [*Glycine max* (L.) Merrill, 2n=40] anther culture began in the 1970's in order to obtain haploids and double-haploids androgenic plants. However, only in rare occasions, culture conditions allowed embryogenic development to proceed as far as plant regeneration. In the present study, culturing heterozygous anthers to a codominant molecular marker, it was recorded that some segregating genotypes originated embryo-like structures from either microspores or anther diploid tissues. Subsequently, through histological analysis, it was recorded callogenesis and somatic embryogenesis from the connective, middle layer and epidermis in cultured anthers of four soybean cultivars, under culture conditions known to trigger an androgenic response. Morphogenic responses from anther walls and connective tissue was favoured by 2,4-dichlorophenoxyacetic acid (2,4-D) and induction under light. Embryogenic potential of the connective tissue provided the possibility of using staminal tissue as a new source of explants, from individuals that have reached the reproductive phase, when the morphogenic response has been considered unsuccessful in soybean. Multinucleate pollen grains formation was not an exclusive response to culture: both anther culture and 4ºC treatment increased symmetrical and extra nuclei frequencies in pollen grains, including atypical extra nuclei with a fragmented shape. On the whole, results indicated that, under recommended conditions, anther culture is a limited system to trigger androgenesis in soybean. Thus, a sequence of tests was carried out aiming to establish isolated microspores *in vitro*. It was developed a technique that allowed to obtain viable microspores suspensions at appropriate density and, subsequently, to establish cultures. The effects of medium constitution and genotypes were tested. This technique may be useful for further studies aiming to identify factors that are important to stimulate soybean androgenesis. In this study, morphogenic limitations and potentialities of soybean anther culture were identified. In consequence, it is proposed a new approach to study androgenesis, through the culture of isolated microspores and pollen grains.

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Introdução

INTRODUÇÃO

1.1. A SOJA

A soja é uma espécie anual e diplóide (2n=40) pertencente ao gênero *Glycine* da família Fabaceae (Hymowitz, 1976). Esta espécie é nativa do Oriente, principalmente da China, havendo divergências entre os autores quanto à região de origem e à época de domesticação (Hymowitz, 1976; Bonetti, 1988; Gomes, 1990).

Há evidências de que o nordeste da China seja a região na qual iniciou a domesticação da soja há, aproximadamente, treze séculos, de onde se expandiu para outras regiões chinesas, Coréia, Japão, sudeste da Ásia e, posteriormente, para a Europa e, finalmente, para os Estados Unidos da América (EUA), a partir de 1804. O registro mais antigo da introdução da soja no Brasil data de 1882 (Hymowitz, 1976). Os primeiros imigrantes japoneses também trouxeram soja para o Brasil em 1908, mas sua introdução oficial no Rio Grande do Sul ocorreu em 1914. A expansão da lavoura de soja no Brasil iniciou na década de 1970, com o interesse crescente da indústria de óleo e a demanda do mercado internacional (Bonetti, 1981).

As sementes da soja são ricas em óleo e em proteínas, principalmente caseína, albumina, lecitina, colesterina e dextrina (Gomes, 1990), por isso, esta planta tornou-se o quarto grão mais produzido no planeta e a oleaginosa mais cultivada, constituindo-se na maior fonte de óleos e de proteínas vegetais, tanto para a alimentação humana como animal (Reunião de Pesquisa de Soja da Região Sul, 1999).

De acordo com o Ministério da Agricultura (www.agricultura.gov.br acessado em 1º de janeiro de 2004), a participação do Brasil na produção mundial de soja tem aumentado progressivamente, sendo o segundo maior produtor do planeta. Durante a execução deste trabalho de doutorado, a produção brasileira aumentou de 32.345.000 toneladas, na safra 1999/2000, para 52.067.000 toneladas, na safra 2002/2003. Do total produzido na safra 2002/2003, 40% (21.019.000 toneladas) corresponderam à produção na Região Sul e 18% (9.437.000 toneladas) à safra recorde no Rio Grande do Sul.

Além da grande participação na alimentação humana e animal no país, a soja tem a perspectiva de contribuir ainda mais com a balança comercial brasileira. As perspectivas da lavoura brasileira de soja são excelentes devido ao progressivo aumento da demanda

mundial.

No Rio Grande do Sul, a soja participa da economia de pequenos, médios e grandes estabelecimentos rurais, estando presente em 33,14% das propriedades. Mais de 94% das propriedades que cultivam soja possuem áreas menores que 50 hectares (Reunião de Pesquisa de Soja da Região Sul, 1999).

Um dos grandes problemas para o lançamento de cultivares que venham a contribuir para a sustentabilidade do sistema produtivo é a falta de variabilidade genética disponível para o melhoramento: a soja atualmente cultivada passou por um longo processo de domesticação, que resultou em um desvantajoso estreitamento das bases genéticas (Abdelnoor *et al*., 1995). Por isso, o emprego de ferramentas biotecnológicas pode contribuir significativamente para a geração e manipulação da variabilidade genética nos programas de melhoramento. Dentre as inúmeras ferramentas, está o emprego de plantas haplóides e de linhagens duplo-haplóides, tecnologia já disponível para inúmeras outras espécies cultivadas.

É possível obter haplóides e duplo-haplóides *in vitro* por meio de eventos como a partenogênese, a eliminação somática, a ginogênese e a androgênese (Atanassov *et al.*, 1995; Mohan *et al*., 1996).

1.2. A ANDROGÊNESE

Para o desenvolvimento normal do grão de pólen, são necessários dois eventos seqüenciais que integram a ontogênese da antera: a microsporogênese (ou androsporogênese) e a microgametogênese (ou androgametogênese). A microsporogênese transcorre desde a meiose até a liberação do micrósporo (ou andrósporo) haplóide da tétrade. A microgametogênese é sinalizada pela formação de um grande vacúolo central na célula do micrósporo, resultante da coalescência de inúmeros pequenos vacúolos, o que força a polarização do núcleo haplóide: o citoplasma e o núcleo são comprimidos contra a parede do micrósporo. A célula do micrósporo sofre uma mitose assimétrica e origina duas células diferentes, separadas por uma parede pectocelulósica delgada: a célula vegetativa (sifonogênica) e a célula generativa (gametogênica) periférica (Mariath *et al*., 2003). O padrão assimétrico desta primeira divisão mitótica é essencial para a funcionalidade do gametófito (Tanaka, 1993).

O desenvolvimento do grão de pólen (ou gametófito masculino) pode ser

interrompido por inúmeros eventos degradativos. Também pode ser interrompido por condições de cultivo *in vitro* que acionem divisões celulares atípicas, as quais podem originar um novo esporófito, pela ocorrência da androgênese.

A androgênese é um processo reprodutivo uniparental, no qual a progênie possui o material genético nuclear apenas do genitor masculino. Tal evento pode ocorrer *in vivo* em espécies do gênero *Corbicula* (Corbuculidae) e do gênero *Bacillus* (Bacillidae), do reino animal, e na espécie vegetal *Cupressus dupreziana* (Cupressaceae) (McKone e Halpern, 2003).

A androgênese vegetal *in vitro* foi descoberta casualmente em *Datura innoxia* na década de 1960, durante estudos da fisiologia da meiose, nos quais anteras eram cultivadas *in vitro*. Os grãos de pólen apresentaram um desvio da rota gametofítica e originaram plantas haplóides (Guha e Maheshwari, 1964, 1966).

A partir da descoberta de Guha e Maheshwari (1964, 1966), foram conduzidos cultivos experimentais para obtenção de plantas androgênicas de inúmeras espécies vegetais (Maheshwari *et al*., 1982; Moraes-Fernandes *et al*., 1999). Nas condições de cultivo propostas, algumas espécies mostraram-se mais responsivas e seu estudo permitiu esclarecimentos quanto aos fatores que alteram a rota de desenvolvimento gametofítico, ao estádio de desenvolvimento responsivo do micrósporo ou grão de pólen, aos padrões iniciais da divisão celular, as etapas que envolvem a transição gametófito-esporófito e as condições de cultivo que aceleram a regeneração de plantas androgênicas.

A pesquisa em dimorfismo do pólen foi associada ao estudo da androgênese e vários trabalhos registraram que micrósporos atípicos (de menor tamanho, com fina camada de exina e contendo citoplasma pobre em ribossomos e com mitocôndrias condensadas), possivelmente resultantes de alterações meióticas, teriam maior predisposição para tomar a rota de desenvolvimento esporofítico quando estabelecidos *in vitro* (Horner e Street, 1978; Heberle-Bors, 1985; Kaltchuk-Santos *et al*., 1993).

Porém, foi demonstrado que tanto micrósporos quanto grãos de pólen típicos originam esporófitos androgênicos *in vitro* (Binarova *et al*., 1997). No micrósporo, o núcleo pode sofrer sucessivas divisões mitóticas simétricas, precedidas ou não pela autoduplicação cromossômica, originando um grão de pólen multinucleado ou multicelular. No grão de pólen, a célula vegetativa, a célula generativa ou ambas podem sofrer divisões mitóticas simétricas e originar um grão de pólen multinucleado ou multicelular (Sunderland e Dunwell, 1974; Pretová *et al*., 1993; Góralski *et al*., 1999).

Devido à inexistência de um termo mais adequado, a estrutura de transição entre um micrósporo ou um grão de pólen e um novo esporófito androgênico, é chamada neste trabalho de "grão de pólen multinucleado" ou "grão de pólen multicelular". Um grão de pólen pode ser chamado de multicelular apenas quando a técnica de análise permite a discriminação visual de mais de duas células dentro da esporoderme (parede do grão de pólen).

Preferiu-se o uso do termo grão de pólen, mesmo que a estrutura de transição tenha origem no micrósporo, porque, por definição, um micrósporo possui apenas um núcleo haplóide não-polarizado e constitui o resultado da fase anterior, a microsporogênese.

Pré-tratamentos e condições de cultivo desencadeadores de estresse podem acionar a rota androgênica (Kyo e Harada, 1986; Binarova *et al*., 1997; Touraev *et al*., 1997; Ríhova e Tupi, 1999; Smýkal, 2000) ou aumentar a proporção de micrósporos e grãos de pólen responsivos (Kiviharju e Pehu, 1998; Dias, 1999; Dias, 2001; Gu *et al*., 2004). Contudo, não foram encontrados registros de ocorrência de divisões celulares características da androgênese, anteriormente ao cultivo.

A continuidade do desenvolvimento androgênico requer a síntese das paredes das novas células do grão de pólen multinucleado (Idzikowska e Mlodzianowski, 1979). À medida que o número de células aumenta, ocorre o rompimento da esporoderme (Rao e Deepesh, 1987; Hu e Kasha, 1999) e a liberação de uma estrutura multicelular com variados graus de organização entre o padrão de desenvolvimento zigótico e calogênico (Maheshwari *et al*., 1982; Góralski *et al.*, 1999; Wang *et al.*, 2000). Normalmente, este conjunto de células apresenta o padrão embriogênico de desenvolvimento, sem uma fase intermediária de calos. Nos casos em que não apresenta a organização de um embrião, é chamado de microcalo (Van Geyt *et al*., 1985; Pretová *et al*., 1993) ou proembrião (Guo e Pulli, 2000; Silva *et al*., 2000).

Quando as condições de cultivo são favoráveis, há regeneração de plantas completas haplóides ou duplo-haplóides. A formação de plantas duplo-haplóides requer uma duplicação do material genético celular, que pode ser espontânea e anterior às primeiras divisões celulares (Henry, 1998) ou induzida por agentes antimitóticos, como a colchicina, ao final do processo (Moraes-Fernandes *et al*., 1999). Entretanto, o estudo de mais de duzentas espécies de plantas mostrou que variações no número cromossômico, incluindo diploidia, poliploidia e aneuploidia, são comuns entre plantas androgênicas obtidas de um mesmo cultivo (Henry ,1998).

Apesar dos termos “embriogênese haplóide” (Góralski *et al*., 1999), “embriogênese do micrósporo” (Touraev *et al*., 1997) e “embriogênese do pólen” (Sunderland e Dunwel, 1974; Raghavan, 1987; Reynolds, 1997) serem usados como sinônimo de androgênese, há registros de regeneração de plantas androgênicas pela via organogênica (Hughes *et al*., 1975). Além disso, há autores que consideram a embriogênese do micrósporo como um evento de embriogênese somática (Yeung, 1995). Entretanto, o termo “embriogênese somática” é referido neste trabalho apenas para eventos que partem de células somáticas diplóides.

Eventos androgênicos são desencadeados *in vitro* tanto por meio do cultivo de anteras quanto do cultivo de micrósporos e/ou de grãos de pólen isolados. Ambos os sistemas prestam-se a estudos básicos, em que a embriogênese do micrósporo ou do grão de pólen serve como modelo para estudo dos diferentes aspectos (molecular, fisiológico, histológico, etc) da embriogênese e da embriologia. A produção de plantas haplóides e duplo-haplóides também tem amplo emprego como ferramenta biotecnológica em trabalhos que visam à manipulação de variabilidade no melhoramento genético, permitindo o mapeamento de genes de interesse agronômico, a descoberta de mutações e a geração de linhagens duplo-haplóides. O cultivo de micrósporos isolados também pode ser um sistema alvo para indução de mutações, para fusão de protoplastos e para transformação genética pela biobalística .

1.3. A ANDROGÊNESE EM SOJA

A androgênese em soja é pesquisada desde o início da década de 1970, inicialmente por pesquisadores chineses (Tang *et al*., 1973) e americanos (Ivers *et al*., 1974) por meio do cultivo de anteras *in vitro*. A maioria dos trabalhos sobre o assunto integrou publicações não indexadas em chinês, sem repercussão no Ocidente. Neste período, o cultivo de micrósporos isolados foi alvo de apenas uma tentativa (Liu e Zhao, 1986). A última publicação chinesa com cultivo de anteras foi a de Zhao *et al*. (1998).

A pesquisa em androgênese da soja no Brasil iniciou em 1992, no Departamento de Genética do Instituto de Biociências da Universidade Federal do Rio Grande do Sul (UFRGS), pelo cultivo de anteras, com a colaboração do professor sino-americano Ching Yeh Hu, do Willian Patterson College (estado de New Jersey, EUA). Este doutor resgatou os resultados inacessíveis da pesquisa chinesa e contribuiu com a linha de pesquisa em soja

em andamento no Departamento de Genética da UFRGS (Hu *et al*. 1996).

Os resultados obtidos nesta linha de pesquisa foram promissores. Estudos citológicos foram executados em anteras *in vivo* (Kaltchuk-Santos *et al*., 1993) e *in vitro* (Kaltchuk-Santos *et al*., 1997), a partir dos quais foram registrados eventos celulares associados à androgênese, como divisão mitótica simétrica do micrósporo e a formação de grãos de pólen multinucleados. Kaltchuk-Santos *et al*. (1997) propuzeram que alguns destes grãos de pólen multinucleados eram precursores dos embriões formados *in vitro*.

Por meio do cultivo de anteras, Zhao *et al*. (1998) afirmaram ter obtido um número não definido de plantas as quais ainda estariam em estudo. No Departamento de Genética, alguns cultivos também geraram plantas isoladas que morreram por falhas no manuseio (Kaltchuk-Santos, 1999; Moraes, 2002). Os resultados destes trabalhos não apresentaram repetibilidade e a maioria dos embriões formados nos cultivos de anteras de soja não ultrapassou o estádio globular. Apesar da importância da regeneração de plantas, as causas destas falhas de desenvolvimento não foram pesquisadas.

Mesmo após três décadas de pesquisa, ainda não foi possível a obtenção de duplo-haplóides de soja. Apesar da destacada importância da soja na economia mundial e das numerosas empresas públicas e privadas atualmente envolvidas em gerar ferramentas biotecnológicas para o melhoramento genético, não há registro de um programa de melhoramento que faça uso da haplodiploidização pela via androgênica.

Reunindo os resultados de pesquisa publicados até 2000, quando este trabalho teve início, os seguintes passos básicos foram recomendados para a indução à androgênese através do cultivo de anteras de soja:

a. Coleta de racemos contendo botões florais no tamanho em que as anteras apresentam a maior proporção de micrósporos, o qual corresponde ao comprimento entre 2,5 e 3,5 mm, desde a base até a ponta da bráctea (Hu *et al*., 1996). Kaltchuk-Santos *et al.* (1997) e Cardoso (2002) consideraram vantajoso o uso de botões de comprimento entre 3 e 3,5 mm.

b. Tratamento dos botões florais ou das anteras *in vitro* com baixas temperaturas, variando de 4 a 8ºC por 3 a 10 dias (Hu *et al*., 1996; Kaltchuk-Santos *et al*., 1997) para aumentar a freqüência de grãos de pólen multinucleados. O pré-tratamento a 4ºC teve efeito genótipo-dependente, mas a freqüência de grãos de pólen multinucleados não aumentou significativamente (Kaltchuk-Santos *et al*., 1997).

c. Seleção dos botões florais ao estereomicroscópio; desinfestação dos botões;

abertura e excisão cuidadosa das anteras, separadamente dos filetes, em câmara de fluxo estéril (Hu *et al*., 1996).

d. Estabelecimento das anteras em placas de petri contendo meio de cultivo B5 (Gamborg *et al.*, 1968) enriquecido com componentes orgânicos (Hu *et al*., 1996) e acrescido de: aminoácidos de Yeung (Yeung e Sussex, 1979); 2 mg de ácido 2,4-diclorofenoxiacético (2,4-D); 0,5 mg de 6-benzilaminopurina (BA); 90 g de sacarose L^{-1} e gelificado com 8 g agarose ou 2,5 g Phytagel L^{-1}. Este meio de cultivo foi composto a partir de uma série de ajustes entre meios pesquisados por Yin *et al*. (1982), Jian *et al*. (1986), Kaltchuk-Santos *et al*. (1997) e Oliveira *et al*. (1998).

e. Incubação em sala de cultivo a 25±2ºC sob fotoperíodo de 10 (Jian *et al*., 1986), 12 (Ivers *et al*., 1972) ou 16 h (Kaltchuk-Santos *et al*., 1997; Oliveira *et al*., 1998) a 22,5 µmol m^{-2} s^{-1} (Kaltchuk-Santos *et al.*, 1997).

f. Excisão e descarte dos calos amarelo-claros, rugosos e de rápida proliferação que se formam a partir dos tecidos diplóides da antera nos primeiros 20 a 30 dias de cultivo (Ivers *et al.*, 1974; Yin *et al.*, 1982; Ye *et al.*, 1994).

g. Transferência dos explantes para meio de cultivo fresco, de igual composição, ao 30º dia de cultivo, para a produção de calos (Hu *et al*., 1996).

h. Transferência das estruturas embriogênicas para meios de histodiferenciação (Bailey *et al*., 1993), maturação (Finer e McMullen, 1991) e germinação de embriões, conforme procederam Kaltchuk-Santos *et al.* (1997).

i. Avaliação dos cultivos quanto à formação de calos e de estruturas embriogênicas ao estereomicroscópio, em torno do 60º dia de cultivo.

j. Avaliação da resposta dos micrósporos ao cultivo. No Departamento de Genética da UFRGS, esta avaliação foi feita principalmente através de amostragens de explantes ao longo do cultivo, submetidas a: fixação em Farmer (3 partes de etanol 100% e 1 parte de ácido acético glacial, em proporções volumétricas) por 12 a 24 h a temperatura ambiente; armazenamento em congelador, imersas no próprio fixador ou em etanol 70%; esmagamento em carmim propiônico 0,6% sobre lâmina, recoberto com lamínula; armazenamento no refrigerador para a observação após 24 h, quando a coloração torna-se suficientemente intensa.

Os resultados alcançados até 2000, não foram conclusivos sobre as condições mais adequadas para o sucesso do cultivo de anteras da soja. A pequena proporção de micrósporos responsivos, explantes embriogênicos, embriões histodiferenciados e plantas

regeneradas apontava a necessidade de otimização do protocolo. Havia a necessidade de aumentar a freqüência de embriões androgênicos e de promover a continuidade do seu desenvolvimento *in vitro*, para viabilizar a regeneração de plantas.

1.4. O DESAFIO DESTE TRABALHO

A execução deste trabalho de doutorado iniciou em janeiro de 2000, a partir de um projeto inicial fundamentado em Hu *et al*. (1996) e Kaltchuk-Santos (1999), reunindo todas as indicações publicadas até então sobre o cultivo de anteras de soja. O projeto inicial previa uma seqüência de experimentos visando reunir as condições de cultivo que aumentassem a freqüência das estruturas embriogênicas a partir do cultivo de anteras. Alguns fatores apontados na bibliografia como determinantes de desvios da rota gametofítica foram testados ao longo de sete experimentos. A combinação dos melhores resultados foi reunida em um teste final com quatro cultivares. Deste cultivo, foram feitas amostragens seriais para análises citológicas e histológicas. A expectativa era analisar e descrever os eventos celulares associados à rota androgênica da soja através destes estudos citológicos e histológicos.

A proposição inicial deste trabalho amparava-se nas afirmações de que os embriões gerados no cultivo de anteras tinham origem exclusivamente androgênica. A origem das estruturas embriogênicas, dos embriões e das raras plantas regeneradas *in vitro* foi atribuída à androgênese devido à baixa resposta morfogênica (ou recalcitrância) dos tecidos somáticos da planta em fase reprodutiva (Hu *et al*., 1996). Ainda que fosse possível obter calos a partir de tecidos nos diferentes estádios do desenvolvimento da planta, considerava-se que o potencial organogênico era perdido logo após a germinação e o potencial embriogênico restringia-se aos tecidos zigóticos (Saka *et al*., 1980; Ranch *et al*; 1985; Wright *et al*., 1987).

Entretanto, em agosto de 2000, em experimentos paralelos aos que integravam este projeto, foi observada a formação de estruturas embriogênicas a partir de calos conectivais. Esta observação desencadeou o progressivo abandono da proposição que fundamentava o projeto e a execução paralela de outros experimentos, os quais vieram a integrar esta tese.

A estrutura do projeto original foi comprometida porque a resposta androgênica não poderia ser avaliada separadamente da resposta dos tecidos diplóides. Os resultados dos experimentos deste projeto inicial não serão publicados pois se fundamentam em uma

premissa que já não tem mais validade científica. A mudança também levou a um redirecionamento dos objetivos do trabalho e esta tese constitui-se, em grande parte, de experimentos adicionais, executados paralelamente ou posteriormente ao projeto inicial. O redirecionamento dos esforços de pesquisa teve como objetivos:

- Comprovar a origem das estruturas embriogênicas obtidas no cultivo de anteras de soja;

- Por meio de procedimentos histológicos, descrever as respostas dos tecidos estaminais de quatro cultivares de soja ao cultivo em meio e condições recomendados para desencadear androgênese;

- Estabelecer uma metodologia para o cultivo de micrósporos e grãos de pólen isolados, a fim de viabilizar uma nova abordagem à androgênese em soja.

CAPÍTULO II

Origin of embryo-like structures in soybean anther culture investigated using SSR marker

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Research note

Origin of embryo-like structures in soybean anther culture investigated using SSR marker

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Key-words: androgenesis, anther culture, *Glycine max*, microsatellite, somatic embryogenesis

Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - 6-benzylaminopurine; ELS – embryo-like structure; SSR - simple sequence repeat

Abstract

The Satt418 microsatellite locus was examined in order to investigate the origin of embryo-like structures (ELS) obtained from soybean anther culture. Four heterozygous plants were used as anther donors. A total of 7000 anthers were placed on the induction medium under culture conditions known to trigger androgenic response. After 60 days of culture, the upper portion of 216 ELS were carefully removed and transferred to a proliferation medium, in order to obtain sufficient tissue for DNA extraction. Callogenic masses originated from 114 ELS were screened for the Satt418 microsatellite locus. Heterozygous and homozygous ELS were identified, suggesting the occurrence of somatic embryogenesis and androgenesis in the same system. This unexpected morphogenic response seems to be genotype-dependent.

Soybean [*Glycine max* (L.) Merrill] anther culture began in the 1970's in order to obtain androgenic haploids and double-haploids (Hu et al. 1996). However, only in rare occasions, culture conditions allowed embryogenic development proceed as far as plant regeneration (Kaltchuk-Santos et al. 1997; Zhao et al. 1998).

The diploid origin of early calluses in soybean anther culture was recorded by some authors (Yin et al. 1982; Ye et al. 1994), but it was assumed that, since the somatic tissues of mature soybean are known to be highly recalcitrant, it was unlikely that ELS were derived from anther diploid tissues (Hu et al. 1996). However, in the course of our studies, ELS originated from connective tissue derived calluses, similar to those obtained from anthers were observed indicating the embryogenic ability of the connective tissue.

Aiming to clarify the origin of ELS in soybean anther culture, a codominant molecular marker was used. Parental genotypes BRQ96-3065 and BRSMG-Liderança were previously tested for many SSR loci, at Centro Nacional de Pesquisa da Soja – Embrapa, Londrina, PR, Brazil, and revealed polymorphism at Satt418 locus. Individual F_2 seeds derived from the cross BRQ96-3065 x BRSMG-Liderança were grown under controlled conditions and tested for the homozygous or heterozygous condition. Four heterozygous plants were selected as anther donors.

Seven thousand heterozygous anthers were dissected from immature floral buds (length 3 to 3.5 mm) and cultured in conditions known to trigger androgenic response (Hu et al. 1996; Kaltchuk-Santos et al. 1997). Cultures were incubated under controlled conditions according to Kaltchuk-Santos et al. (1997) and all explants were transferred to fresh medium after 30 days of culture. After 60 days of culture, the upper portion of the more developed ELS from each of 216 embryogenic explants were carefully removed. Only tissues from individual globular ELS were detached, without visible callus portion.

These 216 ELS samples were subcultured in B5 medium with 30 g l^{-1} sucrose and pH 6, with an alternation of 3 mg l^{-1} BA and 1 mg l^{-1} 2,4-D + 2 mg l^{-1} BA growth regulators. This medium constitution was previously tested to trigger callogenic proliferation in order to obtain enough tissue for DNA isolation. Calluses were incubated at 25±1°C under 16h photoperiod of 25 μmol m^{-2} s^{-1}.

Calluses with minimal mass of 32 mg were submitted to DNA isolation according to Doyle and Doyle (1987) with modifications. DNA was extracted from 114 ELS and quantified on a 1% agarose gel. Microsatellite amplifications were performed according to Liu et al. (1996). The amplified products were separated on a 1.5% agarose gel.

Amplified fragments indicated presence of both heterozygous and homozygous ELS (Figure 1). It may be assumed that homozygous condition results from microspore embryogenesis, while heterozygous ELS could be originated from anther diploid tissue, since the occurrence of unreduced gametes has not been recorded in soybean.

The percentage of heterozygous ELS were different among genotypes (Table 1). ELS originated from plants number 1 and 2 were exclusively heterozygous, while 88% and 3% ELS formed from plant number 3 and 4, respectively, were homozygous. In plant 3, the occurrence of homozygous ELS only of type 2 (Table 1) may be due to sample size. It is possible that androgenic response is genotype-dependent, since anther donors were F_2 segregating plants.

Distinct morphogenic events have already been recorded in soybean anther culture, as for instance, callogenesis, indirect organogenesis and microspore embryogenesis (Hu et al. 1996; Kaltchuk-Santos et al. 1997; Zhao et al. 1998). However, somatic and microspore embryogenesis in a same culture is an unexpected and unrecorded response. It was not possible to establish if these two morphogenic events can occur concurrently in the same anther, because only one ELS was removed from each embryogenic explant.

Somatic embryogenesis from anther culture has been recorded in few species, but not simultaneously with androgenesis. In soybean, this response increases the difficulty in haploid and double-haploid plant production. Although SSR had been an efficient way to determining the origin of ELS, its usefulness is restricted to the use of heterozygous anther donors plants. Thus, anther culture is a limited system to trigger androgenesis in soybean, since ELS can also be originated from diploid tissue.

The present study included plants from only one cross and just one SSR locus was analysed. Nevertheless, the results achieved stimulate challenging questions that must be solved by our

complementary studies.

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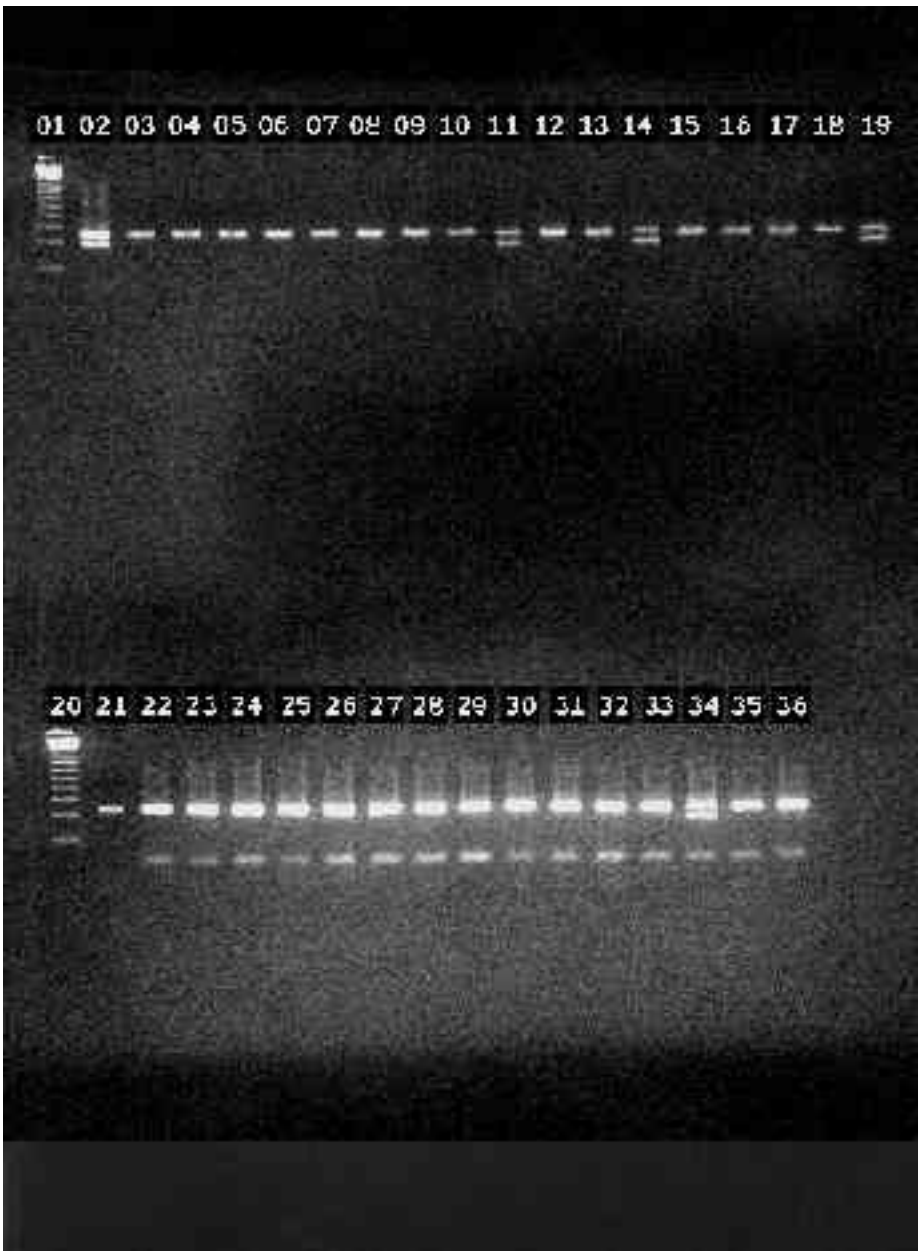
Table 1. Percentage of heterozygous and homozygous ELS obtained from anther culture of four heterozygous individual F_2 plants from cross BRQ96-3065 x BRSMG-Liderança.

Anther Donor	Number of ELS analysed	Homozygous 1		Heterozygous 1/2		Homozygous 2	
		n	%	n	%	n	%
1	30	0	0	30	100	0	0
2	18	0	0	18	100	0	0
3	33	0	0	4	12	29	88
4	33	1	3	32	97	0	0

Figure 1. Microsatellite pattern obtained at locus Satt418 in ELS originated from anther culture of soybean plant number 3. The 1.5% agarose gel was stained with ethidium bromide. Lane 1 and 20: 10 bp DNA Ladder. Lane 2: Heterozygous anther donor (control). Lanes 3-19 and 21-36: different ELS.


01 02 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 18 19
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

COMENTÁRIO AO CAPÍTULO II

Os resultados expostos no capítulo II fundamentam um questionamento quanto ao conceito até agora vigente sobre embriões androgênicos. No cultivo de anteras de soja, a formação de estruturas embriogênicas não pode mais ser usada como um indicativo de androgênese.

Não foram registradas diferenças morfológicas entre estruturas embriogênicas obtidas a partir das plantas 1 e 2 (que só originaram estruturas embriogênicas de origem somática) e da planta 3 (que originou uma maioria de estruturas embriogênicas de origem androgênica). No cultivo da planta 3, chamou a atenção a ocorrência de explantes embriogênicos em que apenas o embrião permanecia verde e em franco desenvolvimento, isolado das demais regiões do calo, que se tornaram oxidadas. Contudo, por não fazer parte do objetivo do trabalho, esta característica não foi mensurada.

Considerando que resultados inesperados foram obtidos a partir de uma pequena amostragem, restrita a apenas uma combinação de genótipos, tornaram-se necessárias observações histológicas seriais, para descrever os eventos embriogênicos no cultivo de anteras de soja.

CAPÍTULO III

Histology of embryogenic responses in soybean anther culture

Artigo científico aceito para publicação na revista Plant Cell, Tissue and Organ Culture

Original research papers

Histology of embryogenic responses in soybean anther culture

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Key words: anther anatomy, anther culture, *Glycine max*, histology, somatic embryogenesis

Abbreviations: ELS - embryo-like structures; ANOVA - analysis of variance; 2,4-D - 2,4-dichlorophenoxyacetic acid; BA- 6-benzyladenine

Abstract

In order to clarify the embryogenic responses in soybean anther culture, anthers of four cultivars were cultured under known conditions to trigger androgenic response. A histological study was performed with anthers *in vivo* and with approximately 100 explants sampled after 9, 12, 15, 18, 21, 30 and 45 days of culture. *In vitro* culture triggered the frequent accumulation of phenolic compounds on the locular and anther surfaces, and also caused the destruction of cells and tissues in complex structure such as the tapetum, microspores and early bicellular pollen. Somatic embryogenesis of unicellular origin was observed from the epidermis and the middle layer, and of multicellular origin from connective calluses. No androgenic response could be observed in the anthers of these four soybean genotypes, in the medium and conditions indicated. We point out to

the need of changing the approach to the study of androgenesis in soybean, either by using culture conditions unfavourable to the proliferation of diploid tissues, or by culturing isolated microspores.

Introduction

Anther culture is an established technique for the production of androgenic plants of numerous species. Androgenesis implies a deviation from gametophytic development to a sporophytic one, leading to the production of a whole haploid or double-haploid plant via microspore embryogenesis or organogenesis (Nitsch and Nitsch 1970; Maheshwari et al. 1982; Góralski et al. 1999; Pechan and Smykal 2001).

Induction of haploid tissue leading to plant formation from soybean (*Glycine max* L. Merrill) anthers has been rather unsuccessful so far. Numerous attempts have only led to the induction of callus, occasionally ending in shoot organogenesis (Jian et al. 1986), ELS (Hu et al. 1996; Kaltchuk-Santos et al. 1997) and rare plants that are weak and grow slowly (Zhao et al. 1998). Thus, double-haploid lines of soybean are not yet available.

Detailed cytological studies of soybean anthers were carried out *in vivo* (Kaltchuk-Santos et al. 1993) and *in vitro* (Yin et al. 1982; Kaltchuk-Santos et al. 1997) describing cellular events related to the androgenic pathway, such as the symmetrical mitotic division of the microspores and formation of multinucleate and multicellular pollen grains. It was assumed that these grains might be the precursors of the ELS obtained under culture conditions (Kaltchuk-Santos et al. 1997). However, culturing heterozygous anthers to a codominant molecular marker, we recorded that some segregating soybean genotypes originated ELS either via androgenesis or via somatic embryogenesis (Rodrigues et al. 2004). Thus, this study was undertaken in order to clarify the origin of the resultant ELS.

Material and methods

Four soybean cultivars (Bragg, IAS 5, MG/BR-46-Conquista and BRS 133) were used in the present study. Seeds were obtained from Centro Nacional de Pesquisa da Soja - Embrapa, Londrina, PR, Brazil.

Young inflorescences of field-grown plants were harvested in January, 2001. Floral buds (length 3-3.5 mm) were disinfected and anthers dissected according to Kaltchuk-Santos et al. (1997). Fifty anthers were placed in each 6 mm plastic petri dish containing B5 medium (Gamborg et al. 1968) enriched with organic compounds (Hu et al. 1996) and supplemented with 10 ml Yeung's amino acids (Yeung and Sussex 1979), 0.5 mg BA, 2 mg 2,4-D, 2.5 g Phytagel and 90 g l^{-1} sucrose at pH 6.4. One thousand anthers were cultured for each cultivar.

Five anthers per petri dish were sampled to verify the microspore development stage at the time of inoculation. For this cytological observation, anthers were fixed in Farmer´s fixative (100% ethanol: glacial acetic acid, 3:1) and squashed in propionic-carmine stain 0.6%.

Cultures were kept under controlled conditions (at 25±1ºC under 16h photoperiod of ~25 µmol m^{-2} s^{-1}) according to Kaltchuk-Santos et al. (1997). After 30, 60 and 90 days of culture, callus and ELS yields were scored and all explants were transferred to fresh medium. Normality test and ANOVA were performed on the culture data.

For histological analysis, floral buds 3-3.5 mm were processed to illustrate the developmental pattern of anthers *in vivo*. Explants were collected from five petri dishes of each cultivar after 9, 12, 15, 18 and 21 days of culture, totaling 100 samples. Explants of the IAS 5 cultivar were also collected after 30 and 45 days of culture to complement the study. Samples were fixed and treated for histological procedure according to Kaltchuk-Santos et al. (1997).

Results

The explants were analysed for their ontogeny and stages of microsporogenesis and

microgametogenesis. Anthers sampled at dissection and squashed in propionic-carmine presented 82, 38, 37 and 36% of unicellular microspores in BRS 133, Bragg, Conquista and IAS 5, respectively. The tetrad frequencies were 10, 4, 35 and 0.07% for the same cultivars. The other observations corresponded to pollen grains.

In the histological analysis of *in vivo* anthers, sporangia exhibits epidermis, endothecium, two-three middle layers and tapetum with one cell layer. The tapetum lines the whole locule, surrounding young pollen grains (Figure 1). Connective and middle layers have a parenchymatous structure.

The anthers responded to culture in very different ways: 49% did not respond to the stimulus of the environment and senesced. Approximately 3% of the senescent anthers completely lost their pigmentation. The others presented intense phenolic oxidation from inside the sporangia, advancing towards the whole explant.

As a rule, *in vitro* culture triggered the destruction of the tapetum and the male cells (for comparison, see anther locules in Figure 1 and Figure 2A). The endothecial cells did not give rise to callus, and in most cases underwent marked radial lengthening, sometimes accompanied by cells from the middle layers (Figure 2A,B). The accumulation of phenolic compounds occurred often at the locular and anther surfaces (Figure 2A,B). The male cells began to lose viability during the first days of culture, even before the histological observation. Although the histological sections do not favour the identification of gametophytic path deviations, rarely microspores were found with a symmetrical division. A multinucleate pollen grain of the cultivar Conquista that had already become unviable on the 18th day was also found (not shown).

ELS were observed growing from calluses after 30-90 days of culture. On the 90th day, 5.8% of the explants presented ELS. According to the non-parametric ANOVA (Kruskal-Wallis test), the proportion of embryogenic explants after 90 days of culture was significantly different among cultivars ($Pr>F = 0.009$), corresponding to 11% in Bragg, 6% in Conquista, 4% in IAS 5 and 2% in BRS 133.

Sometimes the calluses also presented organogenesis with the formation of roots after 90 days

of culture and phylloids formation in two cases. In the first case, a small trifoliated differentiated structure developed, which oxidized and regressed with the whole callus. In the second, a phylloid occurred concurrently with ELS.

Embryogenic proliferations occurred simultaneously from the epidermal, sporangial and connective sites.

Epidermal site

The epidermis underwent total or partial reabsorption, with the frequent accumulation of phenolic compounds directly on the endothecium (Figure 2A). The epidermal cells that were not resorbed dedifferentiated into meristematic cells (Figure 2A-C). The reddish purple toluidine blue staining pattern (O´Brien and McCully 1981) indicated a greater deposition of pectins on the wall of these responsive cells (Figure 2C), very different from the mature epidermal cells of *in vivo* anthers (Figure 1).

After 15 days of culture, some meristematic cells from the epidermis produced lines with few cells based on one or two periclinal divisions followed by anticlinal divisions, giving rise to spherical or lobulate to clavate ELS (Figure 2A,C), whose details of later development could not be followed.

Sporangial site

In some explants the middle layers and cells of the connective tissue adjacent to the locule gave rise to cell proliferations that invaded the sporangium. Some of these proliferations formed ELS of unicellular origin, when they came from the middle layer (Figure 3A-E) and calluses of multicellular origin when they came from the connective tissue.

The middle layer proliferation came from dedifferentiated primordial cell (Figure 3A) which divided in an organized way (Figure 3B) and gave rise to ELS with radial and bilateral symmetry (Figure 3C-E). Each initial cell presented an increased cytoplasmic density, reduced vacuole size

and increased parietal pectins concentration. It is possible that the embryogenic-type development has been interrupted. The later development of these ELS could not be followed. The potential of originating intralocular ELS from a single cell was limited to anthers that were established *in vitro* before the first mitosis of pollen, since after this stage the middle layers cells collapse.

Invasive proliferations from the connective layers occurred with the formation of small meristematic cell groups adjacent to the inner margin of the locule (Figure 4A). These cell proliferations originated calluses as they developed, taking up the intralocular space (Figure 4B,C) and breaking the sporangium. Filamentous embryogenic proliferations of unicellular origin may occur simultaneously with proliferations of multicellular origin, within a same locule (Figure 3E).

Connective site

Connective cells that are more distant from the locule produced cell rows in different directions, originating calluses. The accumulation of phenolic compounds between these calluses and the sporangia frequently caused a separation of these tissues. The connective cells exposed by separation from filament presented a vigorous callogenic potential.

After 21 days, typical cells of provascular tissue differentiated in the parenchymatous portion of the connective calluses (Figure 5A). These cells gave rise to tracheoidal elements (Figure 5B,C) that were densely distributed throughout the callus. These ordered tracheoidal elements were detected along the embryonic axis of ELS after 30 days of culture (Figure 6A). The position of the tracheoidal elements determined the central and cortical zones of ELS.

Thus, between 30 and 90 days of culture, the parenchymatous zone of the connective calluses gave rise to a large ELS of multicellular origin (Figure 6A).

The pattern and the position of cell divisions may indicate the beginning of cotyledons occurred after the radicle-like structure was complete (Figure 6A,C). It is possible that the cotyledons were formed from two or three subdermal cell layers that initially underwent periclinal divisions (Figure 6B). The size of these cotyledons depended on the extension of the area in which these divisions occurred. Then, usually, cellular divisions occurred forming a dome-like

protuberance in the central region between the cotyledons (Figure 6D,E). Thus, these ELS did not develop characteristic apical meristems. Exceptional cases were also observed in which the formation of cotyledons and dome-like protuberances occurred without the formation of radicle-like structure.

Unfortunately, the vast majority of ELS did not go beyond the globular stage. Even the more developed ones did not respond to transfers to regeneration media in order to obtain complete plants. Such structures suffered interruption in their development and perished as a consequence of progressive phenolic oxidation.

Discussion

This study made it possible to describe the embryogenic responses to *in vitro* anther culture of four cultivars, and thus explain a few morphogenic events previously observed (Yin et al. 1982; Hu et al. 1996; Kaltchuk-Santos et al. 1997; Zhao et al. 1998). Contrary to the statement that anthers at the tetrad stage did not respond to the culture conditions and would remain translucently white, without any obvious changes (Yin et al. 1982), it was observed that the anthers at the tetrad stage may form calluses and ELS.

The three embryogenic sites were characterized according to the ELS pattern formation, based on the proliferative behavior of their cells. As shown, the epidermal site is characterized only by epidermal cells that can form ELS. The sporangial site includes cells from the middle layers and connective cells adjacent to the inner tapetum. The proliferation of cells from this site leads to the formation of ELS invading the locular space. At the connective site, connective cells, including cells from the provascular tissue, form calluses that proliferate in the opposite direction of the sporangia, from which ELS are organized.

The anthers of the four cultivars sampled before culture presented a general structure similar to that described by Carlson (1973) and Albertsen and Palmer (1979). However, heterochrony and

different cultivar cycles allow anthers to be cultivated at different stages of microspore development, since there is a selection of floral buds by length. Floral buds 3-3.5 mm presented anthers at stages from the end of meiosis until mature pollen. Since the physiological differences between young and mature anthers may account for different responses to culture, we propose that the significant variation found in the responses among cultivars included the effect of the physiological conditions of the explant at the beginning of culture.

Cells that gave rise to ELS presented a parietal accumulation of pectins, possibly related to the acquisition of embryogenic competence (Verdeil et al. 2001). It was assumed that wall composition influences cell fate (Knox 1992). Alterations in the parietal structure were demonstrated in embryogenic plant cells (Filippi et al. 2001), including the thickness of small portions of the competent cells walls and callose deposit (Verdeil et al. 2001).

The ELS of unicellular origin described in this study are similar to the embryogenic masses recorded for other species such as *Foeniculum vulgare* (Anzidei et al. 2000), *Musa* spp (Filippi et al. 2001) and *Cocos nucifera* (Verdeil et al. 2001) but did not present further development and always gave rise to calluses, possibly due to the lack of appropriate culture conditions.

The connective tissue formed calluses whose outer layers were constituted by meristem cells from ordered cell divisions and inner parenchyma cells, similar to that recorded for *C. nucifera* (Verdeil et al. 2001). It is possible that the tracheoidal elements organized in the parenchymatous portions of the callus have produced the polarity needed for the initial differentiation of the ELS, as described for the initial formation of *Brassica juncea* embryos (Liu et al. 1993).

The polarized auxin transport is essential for the somatic embryos (Liu et al. 1993) and occurs through the parenchymatous cells that accompany the xylem (Taiz and Zeiger 1998). Since the tracheoidal elements have established polarity for the ELS, it is possible that the parenchyma associated with these tracheoidal elements have induced the polar transport of auxin. The very early differentiation of tracheoidal elements in the calluses and the differentiation of well-defined histological zones around them, suggest that these ELS have a non-meristematic origin, as proposed for some somatic embryos of *Musa* spp. (Filippi et al. 2001).

In cotyledonary explants obtained from immature zygotic embryos of soybean, ELS of multicellular origin came from subdermal cell divisions in the cotyledon mesophyll and the protuberance was covered by the epidermis. Tracheary differentiation was observed among the meristematic cells of these protuberances (Fernando et al. 2002). Different from the somatic embryos formed in cotyledonary explants, the ELS from connective calluses did not develop characteristic apical meristems, possibly due to the lack of an appropriate medium for histodifferentiation, as already mentioned for many species, such as *Daucus carota* (Nickle and Yeung 1993) and *Musa* spp. (Filippi et al. 2001). The root portion of these embryos presented an initial cell organization similar to that of zygotic soybean embryos at 20 days (Chamberlin et al. 1994), but the root meristem did not form completely.

In soybean, only parts of the immature zygotic embryo are used as explants for somatic embryogenesis (Ranch et al. 1985). In order to use embryogenesis of anthers as a biotechnological tool in plant breeding, it will be necessary to test appropriate culture conditions for histodifferentiation of ELS and to secondary embryogenesis of unicellular origin based on connective ELS, as occurs in cotyledonary explants (Finer 1988; Sato et al. 1993).

Although androgenesis was recorded simultaneously at somatic embryogenesis in anthers from two segregating plants (Rodrigues et al. 2004), it was not possible to confirm androgenic response in the anthers of these four genotypes, in the medium and in the conditions indicated. The lack of viability of the multinucleate pollen grain confirmed previous observations that multinucleate soybean pollen grains disintegrate (Yin et al. 1982) and, as in *Vitis spp*., nuclear divisions are sometimes not followed by cell segmentation and result in abortion (Cersosimo 1996).

Thus, proliferation from diploid cells may be detrimental to the microspore development process and hamper the production of androgenic soybean plants via anther culture. This may led to difficulties in isolating haploid tissues. Therefore, culture conditions may be adjusted to avoid the effects of diploid cells, by changing the approach to the study of androgenesis. In order to make the androgenic response feasible by anther culture, it should be necessary to develop selective culture

conditions, which could not favour the proliferation of diploid tissues. Otherwise, it is possible to change of system, by culturing isolated microspores, to test the androgenic response in the absence of influence from these tissues.

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Figure 1. Cross-section of sporangium of soybean cultivar BRS 133 *in vivo* with the degradation of the tapetum (stars), immediately after the first microspore mitosis. Young pollen grain (asterisk); ML = middle layer; EP = epidermis; ED = endothecium; CN = connective tissue. Bar = 50 μm.

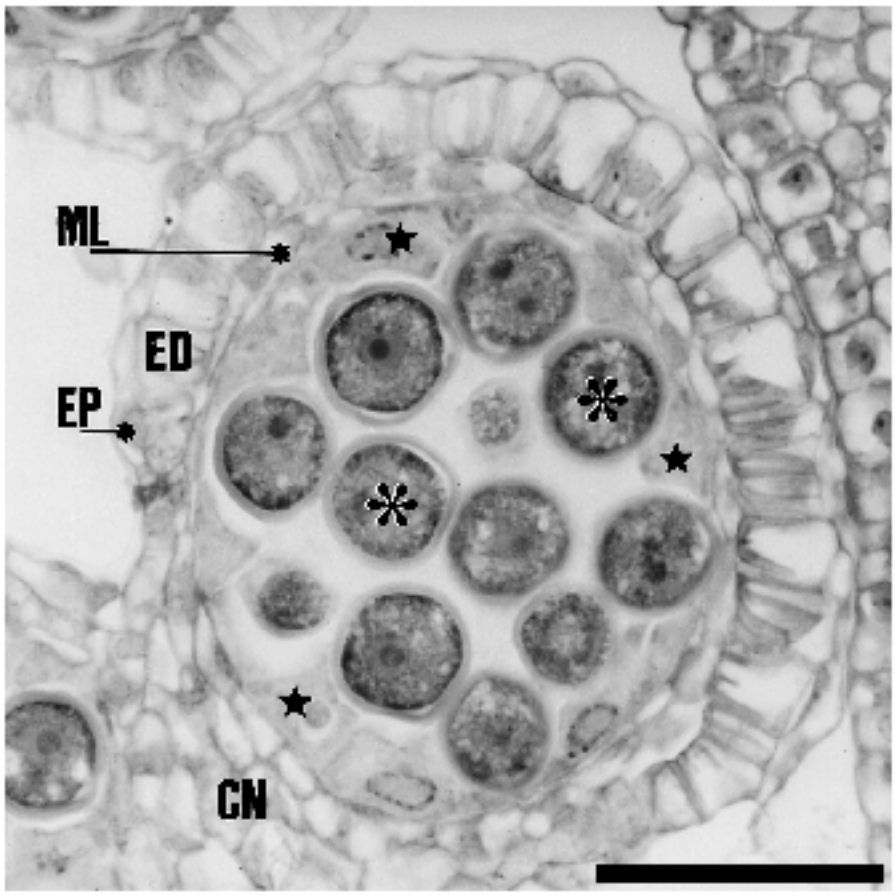
ML
ED
EP
CN

Figure 2. Unicellular origin of ELS in soybean anther culture. **A**: Cross-section of anther of the cultivar Bragg at 9 days *in vitro*, with the accumulation of phenolic compounds (arrowheads), radial lengthening of the endothecium cells (asterisks), cell proliferation at the connective site (star) and ELS formation (arrows). The intense degradation of the male cells is observed. LC = locular space; CN = connective tissue. Bar = 100 μm. **B**: Detail of a sporangium of the cultivar Conquista, in a cross-section, with the accumulation of phenolic compounds (arrowheads), radially lengthened endothecium and middle layer cells (asterisks) and dedifferentiated epidermal cells (arrows). Bar = 100 μm. **C**: Embryogenesis from non-resorbed epidermal cells of cultivar IAS 5. Dedifferentiated cells with parietal accumulation of pectins (arrows) give rise to ELS (star). Bar = 50 μm.

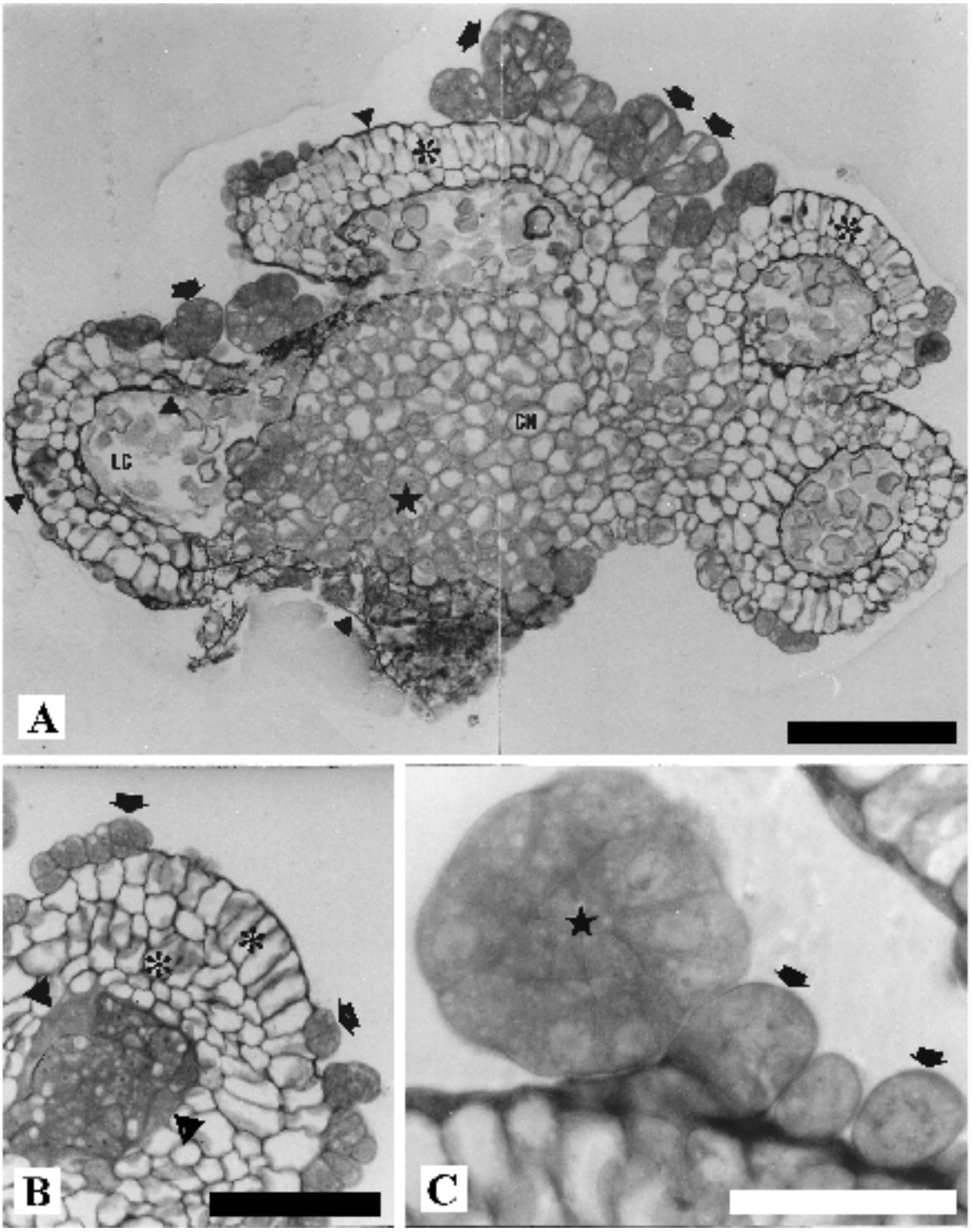
LC
CM
A
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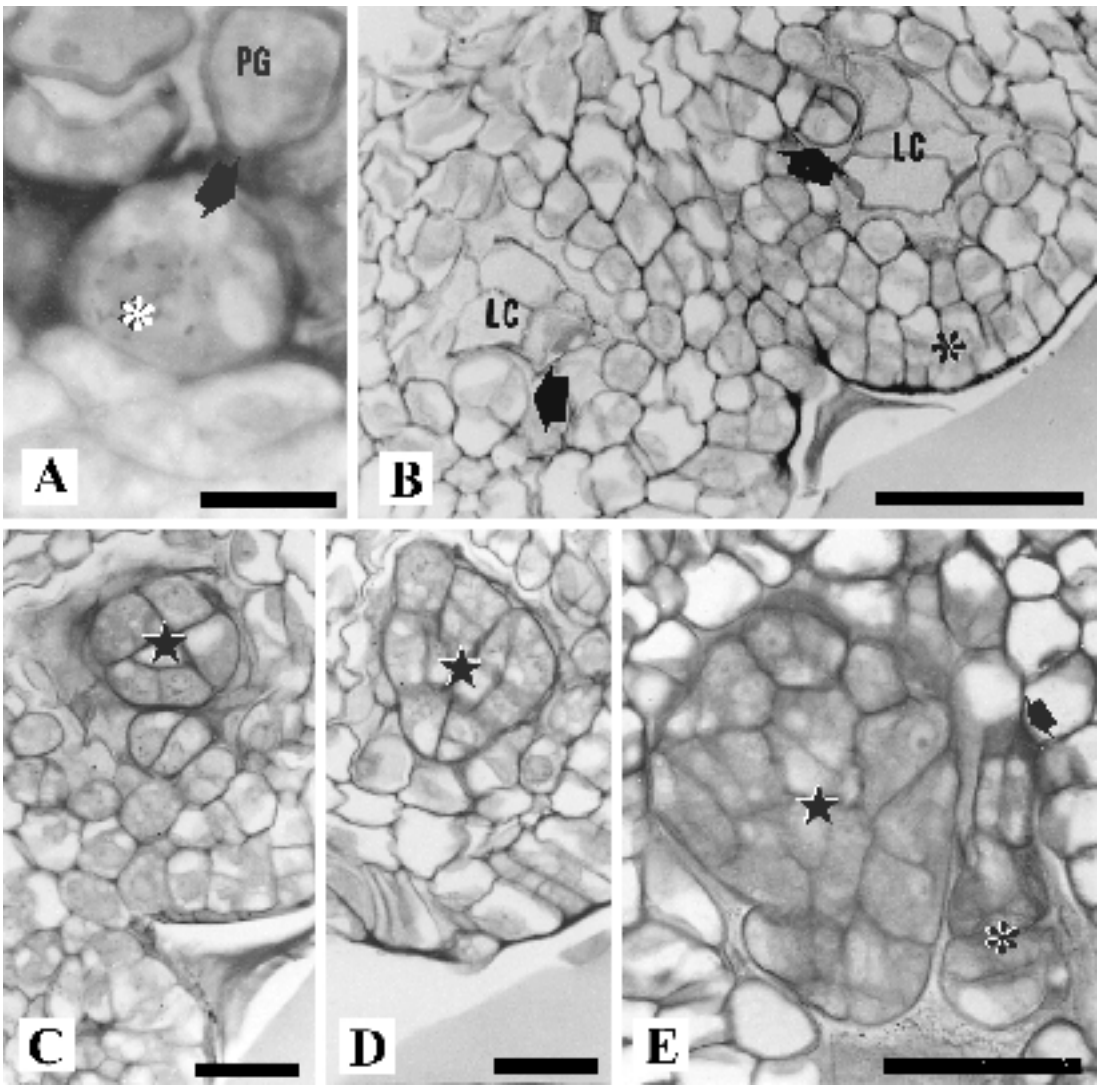
Figure 3. Unicellular origin of ELS in soybean anther culture. **A**: Initial embryogenic cells from the middle layer of the cultivar BRS 133 (white asterisk), with a greater accumulation of pectins in the primary wall (arrow). PG = aborted pollen grain. Bar = 12.5 μm. **B**: Three-celled ELS in an initial phase of formation (arrows). LC = locules in which male cells were already totally degraded. Radial lengthening of the endothecium cells is observed (asterisk). Bar = 100 μm. **C**: ELS with radial symmetry in the cultivar Bragg (star). Bar = 100 μm. **D**: ELS with bilateral symmetry (star) in the cultivar Conquista. Bar = 100 μm. **E**: ELS derived from the middle layer in cultivar IAS 5 (asterisk) occurring concurrently with ELS of multicellular origin derived from the connective tissue (star). The connection of the ELS of unicellular origin to the middle layer is observed (arrow). Bar = 50 μm.


PG
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LC
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D
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Figure 4. Connective proliferation taking up the intralocular space. **A**: Formation of initial cell rows (stars) in the anther locule of the cultivar IAS 5, in the presence of aborted pollen grains (asterisks). Bar = 100 μm. **B**: Calluses (black circle) invading the anther locule of the cultivar Conquista, in the presence of aborted tetrads (arrows). Bar = 100 μm. **C**: Anther locule of the cultivar Bragg filled by callus. The connection with the connective is observed (arrow). Bar = 50 μm.

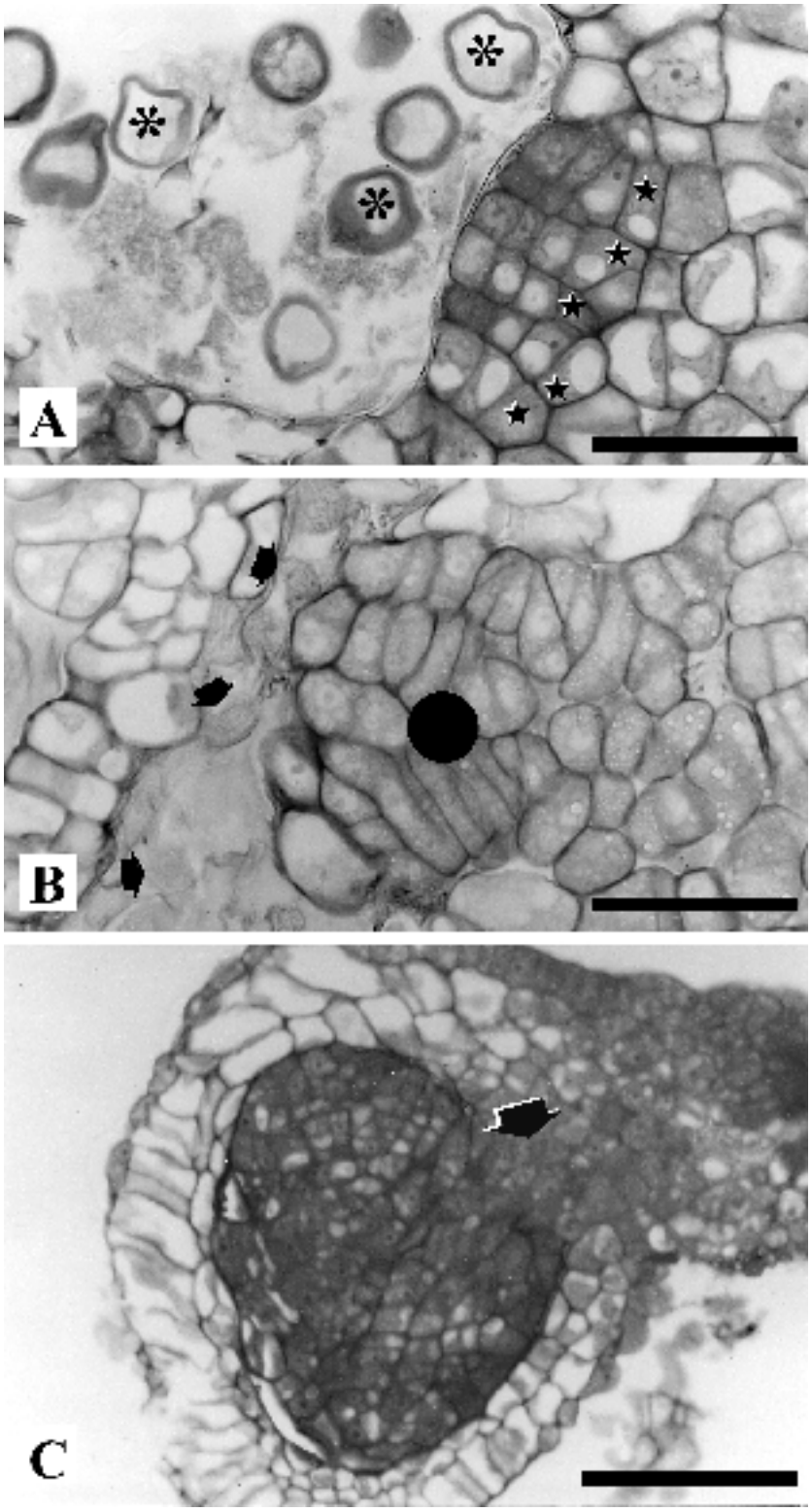
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Figure 5. Formation of tracheoidal elements in callus derived from connective tissue of soybean. **A**: Connective callus of the cultivar Bragg after 21 days of culture, with formation of provascular type tissue (arrow). **B**: General aspect of a callus of the cultivar BRS 133 after 30 days of culture, presenting tracheoidal elements (arrows). **C**: Details of tracheoidal elements of the cultivar Conquista (arrows). Bars = 100 μm.

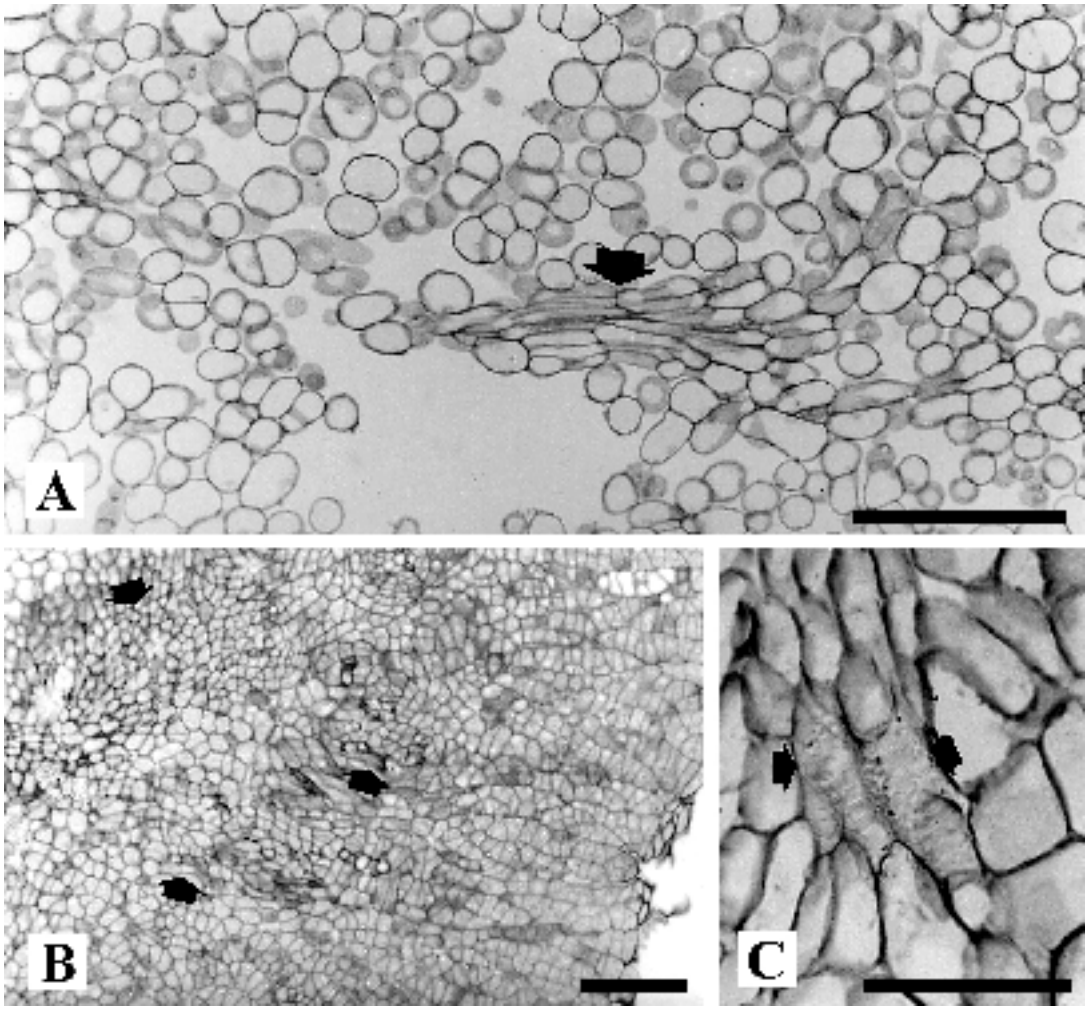
A
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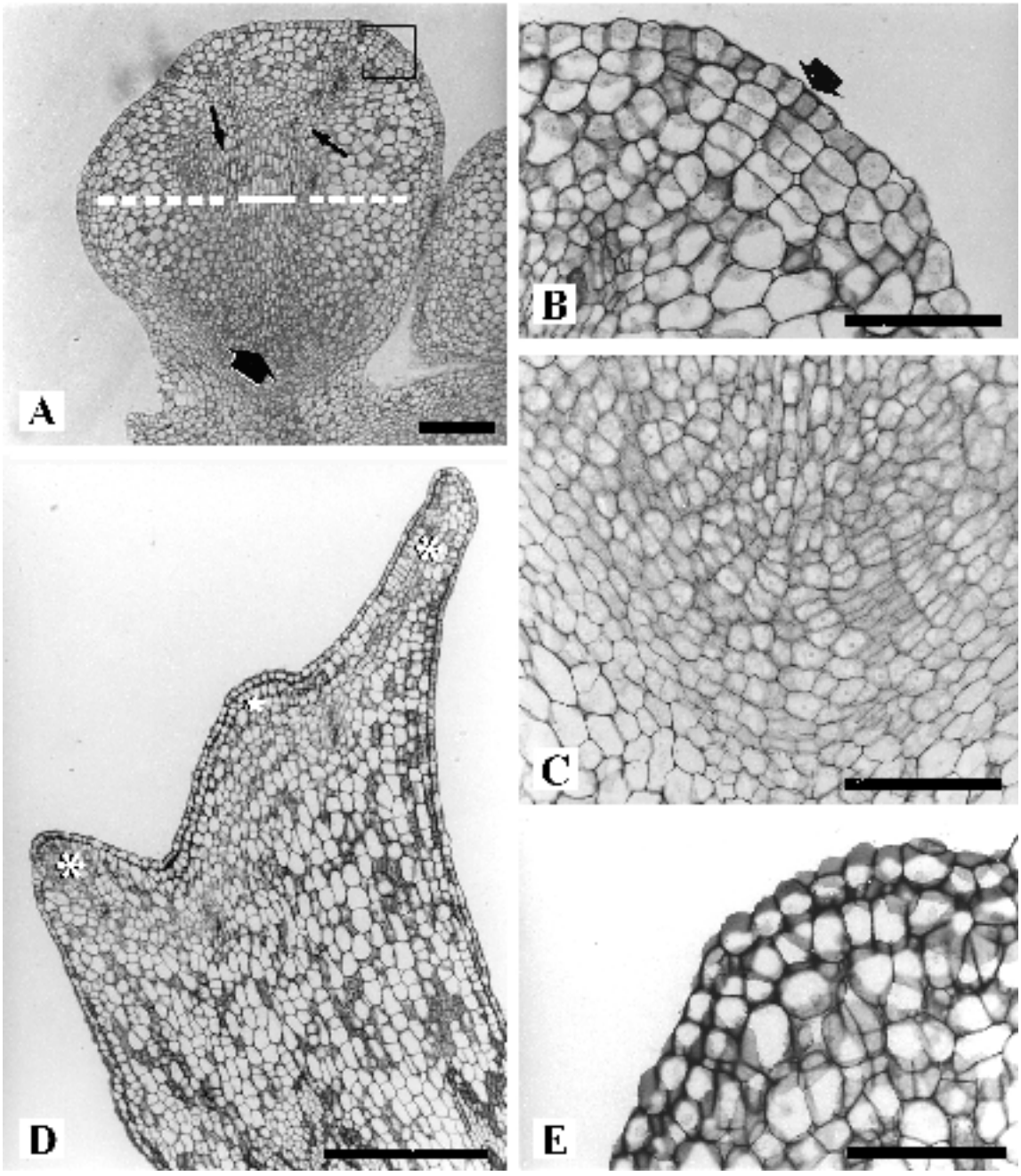
Figure 6. ELS of multicellular origin in cultured soybean anthers. **A**: ELS of connective origin of the cultivar Conquista, with central zone (white simple bar), cortical zone (white dashed bar), tracheoidal elements (small arrows), root apex (large arrow) and cell divisions that likely originate cotyledon-type structure (boxed area). Bar = 100 μm. **B**: Detail of the probable formation zone of the cotyledon-type structure (arrow) of the boxed area shown in Figure 6A. Bar = 50 μm. **C**: Detail of the root apex shown in Figure 6A. Bar = 50 μm. **D**: General aspect of the stem apex of incomplete ELS of the cultivar Bragg with a dome-like protuberance (white star) and cotyledon-type structures (white asterisks). Bar = 100 μm. **E**: Detail of the non-meristematic zone presented in Figure 6D. Bar = 50 μm.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COMENTÁRIO AO CAPÍTULO III

Inusitados eventos morfogênicos foram descritos neste capítulo, destacadamente a proliferação invasiva ao espaço intralocular. Entretanto, não foram observados embriões androgênicos em Bragg, MG/BR 46 Conquista, IAS 5 e BRS 133, como registrado no cruzamento BRQ96-3065 x BRSMG-Liderança. Análises histológicas e citológicas complementares confirmaram os resultados deste estudo histológico.

Desta forma, para o estudo da androgênese no cultivo de anteras, tornou-se necessário testar condições de cultivo seletivas, desfavoráveis à indesejável proliferação dos estratos parietais e do conectivo.

CAPÍTULO IV

Effects of light conditions and 2,4-D concentration in soybean anther culture

Manuscrito em preparação para submissão à revista Plant Growth Regulation

General papers

Effects of light conditions and 2,4-D concentration in soybean anther culture

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Key-words: androgenesis, culture conditions, 2,4-D, *Glycine max*, light, somatic embryogenesis

Abstract

The morphogenic response of anther walls and connective tissue is the greatest obstacle to androgenesis in soybean anther culture. Whereas androgenesis induction occurs in the dark in almost all plant species, soybean anthers have been cultured under light. In an attempt to establish culture conditions that simultaneously stimulate pollen embryogenesis and inhibit epidermal and connective cell proliferation, the effect of light and two 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations (2 and 10 mg l^{-1}) on the induction process was investigated. Higher 2,4-D concentration speeded up microspore plasmolysis and did not improve androgenesis. Callogenesis and embryogenesis induction from diploid cells were significantly lower in the dark, and some microspores showed major alterations in the sporoderm. Auxin 2,4-D and induction under light contributed to the morphogenic response of the anther walls and connective tissue under the conditions previously recommended to trigger androgenesis.

Introduction

After over 30 years of research, no protocol has yet been established for haploid and double-haploid plant regeneration in soybean (*Glycine max* L. Merrill, 2n=40) anther culture.

Cytological studies reported deviations from the gametophytic pathway in soybean (Yin et al. 1982; Jian et al. 1986; Kaltchuk-Santos et al. 1993; Kaltchuk-Santos et al. 1997), but most microspores and pollen grains degrade in culture (Rodrigues et al. *in press*). Our recent histological studies recorded callogenesis and somatic embryogenesis from the connective, middle layer and epidermis in cultured anthers of four soybean cultivars, under culture conditions known to trigger an androgenic response (Rodrigues et al. *in press*). Connective callus induction in soybean anther culture occurred predominantly in the first 30 days, overlapping the key-period of androgenesis induction in the majority of plant species. These calluses were frequently formed from connective cells exposed at the point of anther detachment from the filament and gave rise to embryo-like structures (ELS) of multicellular origin.

Culturing anthers heterozygous to a codominant molecular marker, we showed that some segregating soybean genotypes originated ELS either via androgenesis or via somatic embryogenesis (Rodrigues et al. 2004). A similar response had already been recorded in *Malus* spp anther culture, where green calluses were originated from connective and anther walls, and white calluses were initiated from microspores. The proliferation of both types of calluses depended on the genotype and on the culture media employed, especially with regard to the auxin (Ochatt and Zhang 1996). Therefore, in order to study a soybean androgenic response through anther culture, it is necessary to test selective culture conditions that will not favour the somatic proliferation that causes *in vitro* competition for space and nutrients (Rodrigues et al. *in press*).

Induction under darkness may be an important selective condition, since the anthers of most plant species are usually cultured in the dark and regenerated plantlets are transferred to the light (Maheshwari et al. 1982; Atanassov et al. 1995). Despite this, soybean anthers have been submitted to light in the incubation room with a 10 (Jian et al. 1986), 12 (Ivers et al. 1972) and 16 h

photoperiod (Kaltchuk-Santos et al. 1997; Oliveira et al. 1998) at the induction stage.

Auxin 2,4-dichlorophenoxyacetic acid (2,4-D) was shown to be essential for androgenesis induction in the soybean anther culture. The best response occurred in 2 mg 2,4-D combined with 0.5 mg 6-benzyladenine (BA) l^{-1} (Jian et al. 1986; Kaltchuk-Santos et al. 1997; Oliveira et al. 1998). However, Oliveira et al. (1998) suggested tests with higher 2,4-D concentrations.

Thus, this study was undertaken to test the effects of induction in darkness and higher 2,4-D concentration, aiming to inhibit connective and anther wall cell proliferation and simultaneously stimulate androgenesis in soybean anther culture.

Material and methods

Field-grown plants of the cultivar IAS 5 were used as anther donor, because this genotype showed gametophytic pathway deviation *in vivo* (Kaltchuk-Santos et al. 1993) and *in vitro* (Kaltchuk-Santos et al. 1997). Young inflorescences were harvested and 1000 anthers from 3-3.5 mm floral buds were dissected and cultured according to Kaltchuk-Santos et al. (1997) in 60 mm plastic petri dishes (50 anthers per dish) containing B5 medium (Gamborg et al. 1968) enriched with 16 organic compounds (Hu et al. 1996), 10 ml Yeung's amino acids (Yeung and Sussex, 1979), 0.5 mg BA, 90 g sucrose and 8 g l^{-1} agarose, at pH 6.4.

The factorial design included the sources of variation: 2,4-D concentration in the induction medium (2 and 10 mg l^{-1}) and presence/absence of light in the first 30 days. After this period, culture dishes were kept under a 16 h photoperiod at ±2000 lux (~25µmol m^{-2} s^{-1}) provided by fluorescent 20 W Sylvania® lamps ('Luz do Dia Plus' and 'Gro-Lux' alternately) at 26±2ºC.

At 30, 60 and 90 days of culture, all explants were scored and transferred to fresh medium, with 2 mg 2,4-D l^{-1}. The experiment was repeated twice and similar trends were obtained. Only results of the January 2001 culture are presented here.

For the histological study, 90 anthers were sampled at 15, 30 at 45 days of culture and

processed according to Kaltchuk-Santos et al. (1997). For the cytological study of microspore responses, 5 anthers per petri dish were fixed in Farmer solution (100% ethanol: glacial acetic acid, 3:1) at 0, 15, 30 and 45 days of culture and squashed in propionic-carmine 0.6% stain according to Kaltchuk-Santos et al. (1997). Responses of 27,774 microspores and pollen grains were scored. Normality test and analysis of variance (ANOVA) were performed on the transformed data.

Results and discussion

Connective and anther walls response

Anthers cultured in the light showed morphogenic responses similar to those described by Rodrigues et al. (*in press*). The presence/absence of light showed significant effects on the morphogenic response (Table 1, Figure 1 and 2a,b). Until the 30th day under darkness, few, small, non-chlorophyllated calluses were formed and embryogenic sites were not observed. Even after transferring to light, the number of explants originating calluses and ELS (Figure 2c) from anthers induced in the dark was significantly lower in all evaluations.

In histological sections, similar events were observed in material submitted to both light and dark: radial lengthening of endothecium cells; reabsorption of the majority of epidermal cells; accumulation of phenolic compounds; and microspore degradation (Figure 3a,b). However, anthers submitted to dark treatment presented important differences when compared to those submitted to induction under light: the cell division cycle was rarely activated in the non-resorbed epidermal cells, cells from the middle layer and the connective one. On the few occasions when undifferentiation of epidermal cells occurred, no proliferation was subsequently observed. After transfer to light, only connective cells exposed at the point where the anther is detached from the filament acquired the capacity to divide again in a few explants. Only a single connective proliferation was observed invading the locule.

Although the accumulate evidence of importance of light on morphogenic response, light conditions have been neglected in the study of androgenesis. Rare published information on this

subject is available. Light conditions appeared to be more important than pretreatments and culture media composition in the anther culture of *Lilium longiflorum*, where no androgenic callus formation was observed for anthers cultured under light (Arzate-Fernandez et al. 1997). Anther culture in darkness until the development of a callus (10 days) seems to be a crucial factor favouring androgenesis in *Helianthus annuus* (Saji and Sujatha 1998). In a *Lycopersicum esculentum* anther culture, the number of explants producing calluses increased proportionally to the duration of the dark period (Jaramillo and Summers 1991). In the present study, results indicated that induction in the dark inhibited somatic proliferation.

Induction at a higher 2,4-D concentration (10 mg l^{-1}) significantly increased the callogenic response (Table 1, Figure 1). However a 2,4-D concentration had no effect on embryogenesis. A high concentration (40 mg l^{-1}) of this auxin is employed to induce somatic embryogenesis from immature soybean cotyledons (Bailey et al. 1993; Droste et al. 2001).

Histological observations confirmed the somatic origin of ELS. As in our previous studies, ELS did not progress due to the lack of appropriate condition for histological differentiation and maturation. Media known to allow histodifferentiation and maturation of embryos obtained from cotyledonary explants (Bailey et al. 1993; Finer and McMullen 1991) were not efficient for plant regeneration in ELS from anthers (data not shown). When kept in induction medium, ELS always regressed to become calluses.

Microspores and pollen grains response

In histological sections, it was observed that microspores inside the anthers submitted to darkness suffered less compression due to the proliferation of sporangia tissues (Figure 3a,b).

After 30 days in culture under dark, some microspores showed major morphological differences in the pollen wall that had never been observed under light. The pollen wall presented a greater development of the outer exine layer due to pectin accumulation (Figure 3c). Pectin accumulation is one of the cell wall modifications associated to acquiring embryogenic competence (Verdeil et al. 2001) and also occurred in dedifferentiated cells from anther epidermis and middle

layer after the organized cell divisions that originated ELS (Rodrigues et al. *in press*). Pollen wall alterations were limited to a few explants, possibly because they depended on physiological and developmental conditions of the anther at the beginning of the culture.

In propionic-carmine analysis (Table 2), the difference in frequencies of stained and plasmolyzed microspores was not significant under light or dark treatment. Throughout the course of the culture, there was significant decrease in stained microspores (Table 2), mainly in 10 mg l^{-1} 2,4-D (Figure 4).

Auxin 2,4-D determined microspore embryogenesis in *Camellia japonica* (Pedroso and Pais, 1994), and is employed in callus-mediated systems, when direct embryogenesis is rare and the plants must be differentiated based on callus (Maheshwari et al. 1982). In the soybean anther culture, induction in 10 mg l^{-1} 2,4-D had no effect on androgenic response. On the contrary, it speeded up microspore collapse.

Microspores and pollen grains presented progressive plasmolysis (Figure 3d) that was scored in 2% of the microspores before culture, in 39% of the microspores at 15 days of culture, in 56% of the microspores at 30 days and in 32% of the microspores at 45 days. At 45 days, the more numerous category was non-stained microspores. Apparently microspores collapsed when the membrane integrity was reduced to a critical level, with critical loss of cell contents, without bursting the plasmatic membrane and loss of pollen wall integrity.

A total of twenty (0.07%) pollen grains were recorded with symmetrical and extra nuclei, deviations from the gametophytic pathway associated to androgenesis (not shown). The highest percentage of these pollen grains (0.13%) was recorded under darkness in 2 mg l^{-1} 2,4-D. In the other combinations of treatment, this percentage varied from 0 to 0.08%.

Conclusion

Morphogenic response from anther walls and connective was favoured by 2,4-D and by induction under light. It is advisable to submit soybean anthers to induction in the dark for later tests of culture conditions that will trigger the androgenic pathway, including other auxin types and concentrations.

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Table 1. ANOVA of number of anthers that gave rise to calluses and embryo-like structures (ELS). Averages were expressed in percentages.

Factors:		% Anthers					
		With calluses			With ELS		
		Averages		Pr>F	Averages		Pr>F
A) Auxin concentration	2 mg l^{-1} 2,4-D	21	b	**<0.0001**	3.5		0.5328
	10 mg l^{-1} 2,4-D	35	a		2.8		
B) Light condition	~25 µmol m^{-2} s^{-1}	40	a	**<0.0001**	4.6	a	**0.0001**
	Dark	14	b		1.5	b	
C) Days of culture	30	14	b	**<0.0001**	0.0	b	**<0.0001**
	60	38	a		4.1	a	
	90	33	a		5.6	a	
Interactions	A x B			**0.0076**			0.5528
	A x C			**0.0089**			0.7316
	B x C			0.5428			**0.0013**
	AxBxC			0.9771			0.9069
Transformations		Raiz (x+1)			Log (x+1)		
General average		28			3.2		
CV%		18			7		

Table 2. ANOVA of number of microspores and pollen grains stained and plasmolysed, scored by propionic-carmine staining. Averages were expressed in percentage.

Factors		Microspores					
		Stained			Plasmolyzed		
		Averages		Pr>F	Averages		Pr>F
A) Auxin concentration	2 mg 2,4-D l^{-1}	82	a	**0.0001**	13	b	**0.0001**
	10 mg 2,4-D l^{-1}	49	b		40	a	
B) Light condition	~25 µmol m^{-2} s^{-1}	62		0.8846	33		0.4389
	Dark	63			27		
C) Days of culture	0	98	a	**<0.0001**	2	c	**<0.0001**
	15	61	b		39	b	
	30	40	c		57	a	
	45	28	c		32	b	
Interactions	A x B			0.6424			0.3010
	A x C			**0.0090**			**0.0002**
	B x C			0.2845			0.3739
	AxBxC			0.7753			0.4638
Transformation			-			Raiz(x+1)	
General average			62			29	
CV%			37			29	

Figure 1. Percentage of soybean anthers that give rise to calluses (above) and ELS (below) throughout the course of the culture.

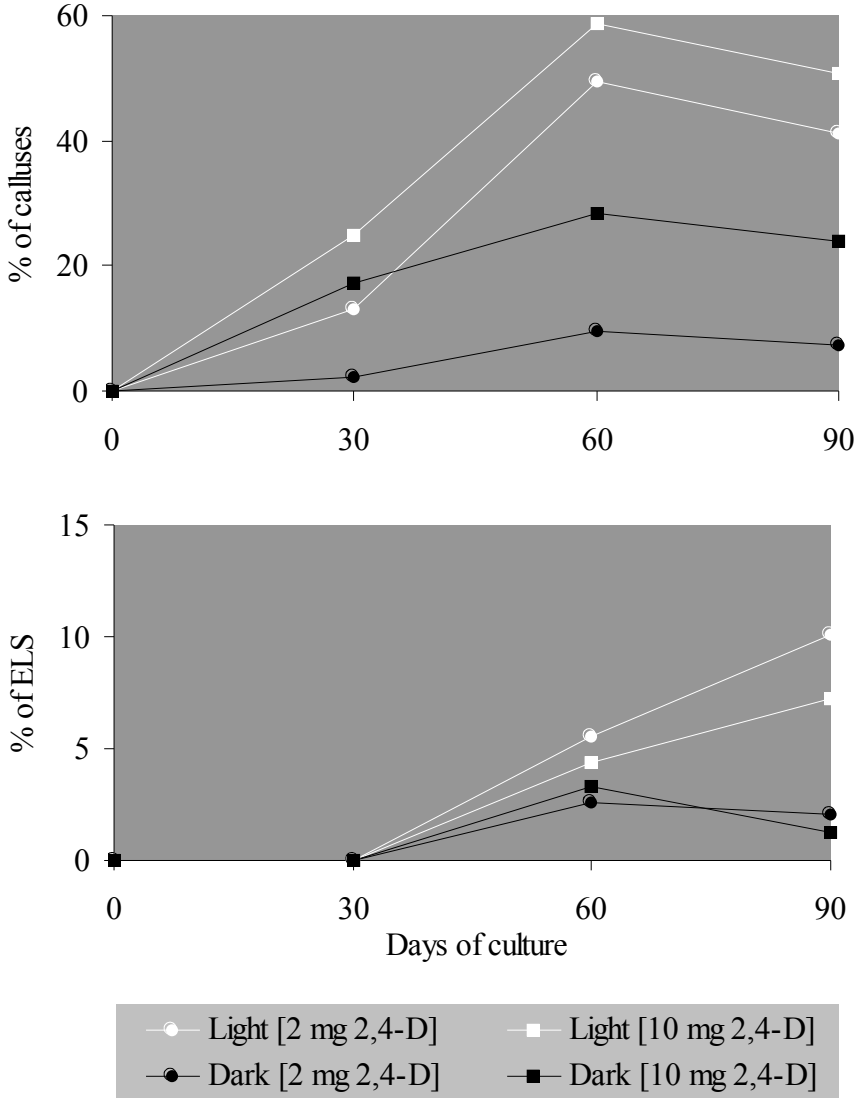

Figure 2. Culture observations. **a**: General view of a culture dish after 30 days under darkness in 2 mg l^{-1} 2,4-D, exhibiting explants without any embryogenic sites and few small non-chlorophyllated calluses (bar = 1 cm). **b**: General view of a culture dish after 60 days of culture under light in 2 mg l^{-1} 2,4-D (bars in a and b = 1 cm). **c**: ELS of somatic origin (arrow) at 60 days of culture under light (bar = 1 mm).

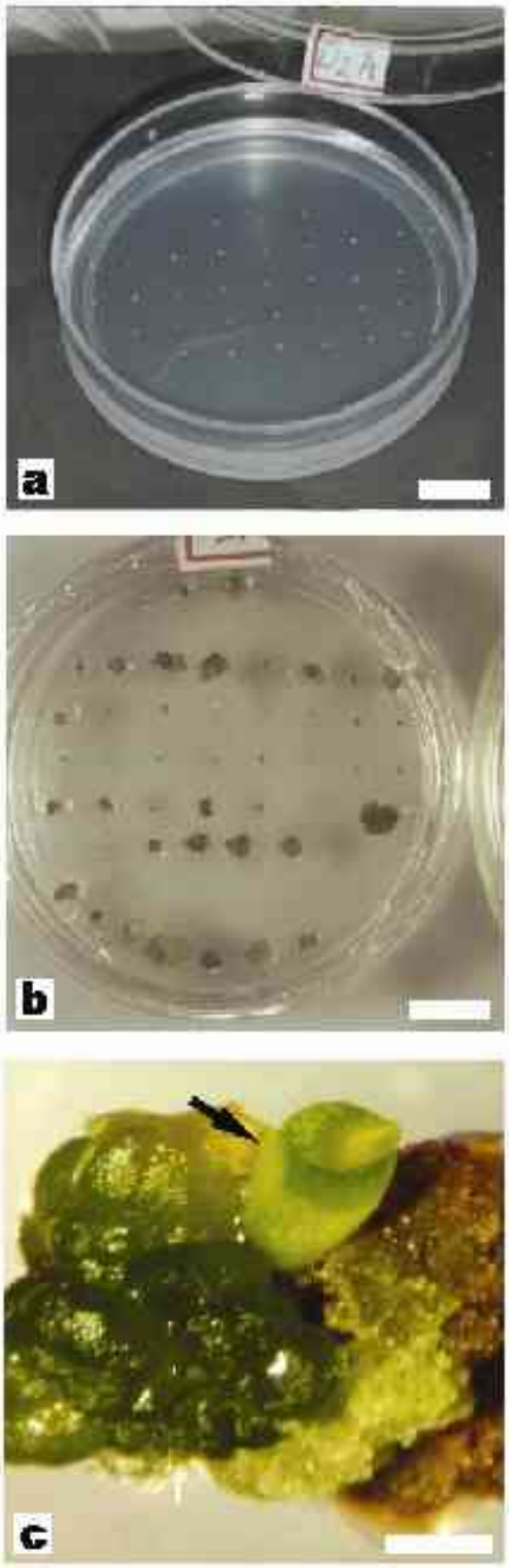
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Figure 3. Details of histological and cytological analysis. **a**: Anther locule after 15 days of culture under light showing microspore compression (arrow) by endothecium lengthening (asterisk) and intralocular proliferations from the anther walls (arrowhead). **b**: Anther locule at 30 days of induction in the dark with initial events that commonly occur under light: radial lengthening in endothecium cells (black asterisk); reabsortion of most of the epidermal cells; accumulation of phenolic compounds (arrowheads) and microspore degradation, except for one (white asterisk). Dedifferentiated epidermal cell (white arrow) that did not reactivate its cell division cycle (bars in a and b = 50 μm). **c**: Microspore at 30 days of culture in the dark presenting sporoderm with higher development of the external exine layer due to accumulation of pectins (arrows). **d**: Plasmolysis in microspore, after 30 days of culture under light (bars in c and d = 10 μm).

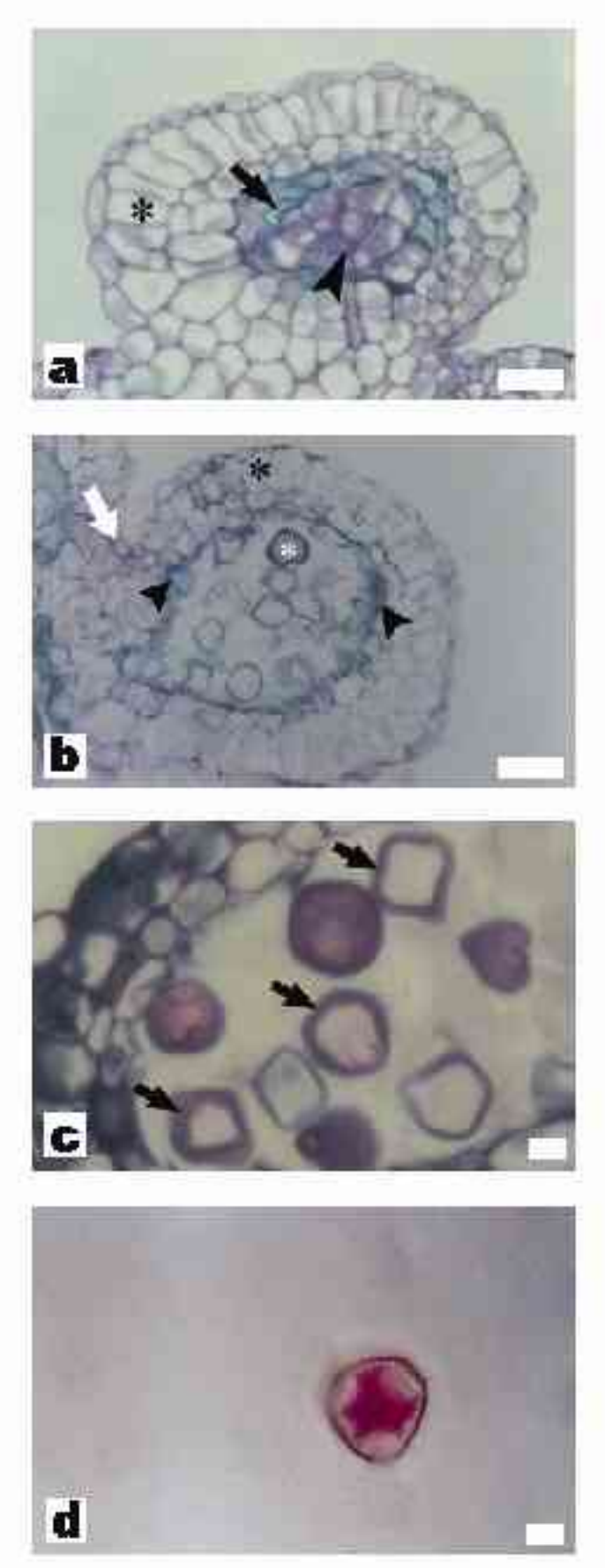
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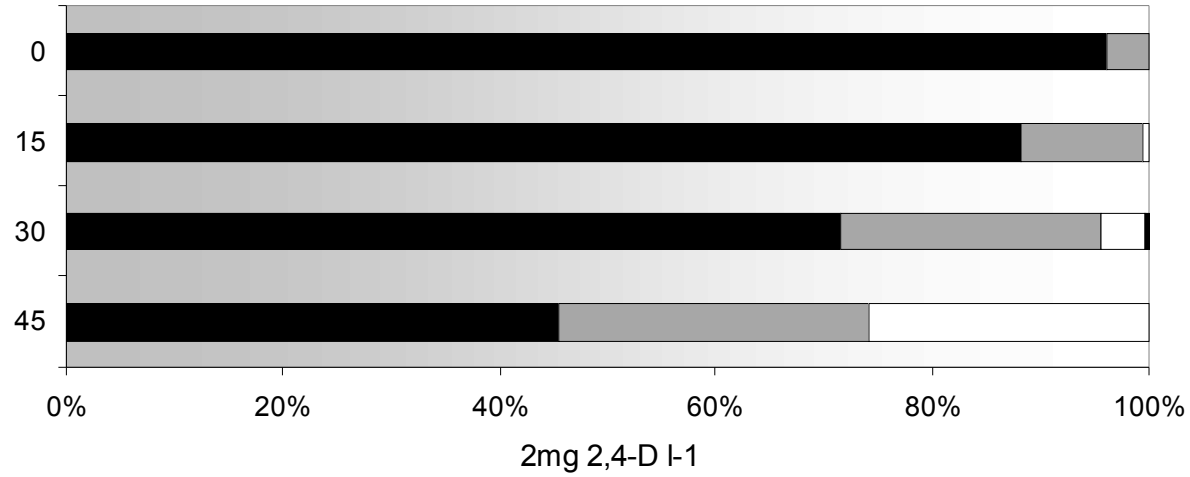
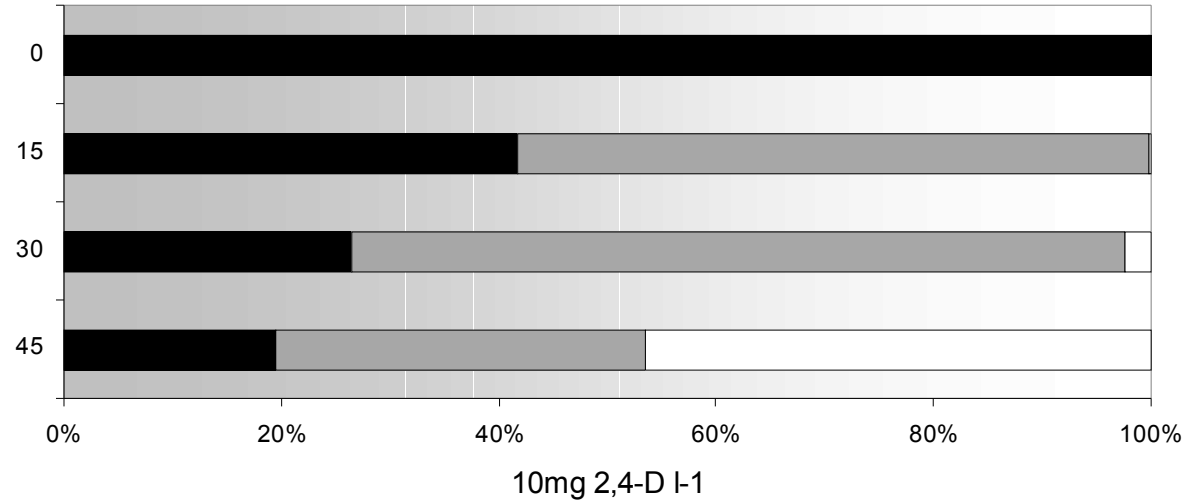
Figura 4. Cytological analysis in anthers sampled in Farmer fixative and squashed in propionic-carmine 0.6%. Percentage of microspores and pollen grains stained (black bar), plasmolyzed (striped bar) and non-stained (white bar) at 0, 15, 30 and 45 days of culture, in 2 mg l^{-1} 2,4-D (above) and in 10 mg l^{-1} 2,4-D (below). ANOVA detected a significant difference between 2,4-D concentrations.





COMENTÁRIO AO CAPÍTULO IV

Este capítulo inclui observações que contribuirão à Discussão. Entretanto, apesar de estar organizado em formato de artigo científico, sua submissão a um periódico dependeria de complementação por estudos adicionais.

Neste capítulo, mesmo sendo possível inibir a proliferação dos tecidos diplóides da antera e limitar a ocorrência de embriogênese somática, não foram observados embriões androgênicos. Assim, surgiu a dúvida quanto ao destino dos grãos de pólen com núcleos simétricos e extranumerários anteriormente registrados por alguns autores.

CAPÍTULO V

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

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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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Key-words: androgenesis, anther culture, Glycine max, multinucleate grains, cold treatment

Abbreviations: ANOVA- analysis of variance; DNA- deoxyribonucleic acid; DAPI- 4´-6-diamidino-2-phenylindole; G- generative; V- vegetative

Abstract

Androgenic response is characterized by a multinucleate or multicellular stage of pollen grains. Histological sections stained with toluidine blue and squashes in propionic carmine and in DAPI were used for serial observations in soybean pollen grains from cultured anthers and floral buds submitted to cold treatment at 4°C. Both treatments increased symmetrical and extra nuclei frequencies, including atypical extra nuclei with a fragmented shape. 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.

Plant androgenesis implies a deviation from gametophytic development to a sporophytic one, characterized by a multinucleate and multicellular stage of pollen grains. Cold pretreatment is a stress factor predisposing to androgenesis in microspores of 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=40) anther culture, Yin et al. (1982) recorded that the contents of the soybean multinucleate pollen grains disintegrated after a 25-day incubation and only multicellular grains were associated to 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 cultivars IAS 5 and RS 7. The authors signalized that some of these grains might be the precursors of the embryos obtained from anther culture.

In our recent cytological studies using carmine staining we have found multinucleate pollen grains of the soybean cultivar IAS 5 in non-cultured anthers submitted to low temperatures. In a subsequent histological analysis, a multinucleate pollen grain of the cultivar 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 unviable. Since the 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. Second, extra nuclei formation is not always an androgenic response.

In order to obtain qualitative and quantitative information on the multinucleate grains, immature inflorescences from field-grown plants of IAS 5 and Conquista were harvested in January 2002. Part of the material was submitted to anther culture and the other part to cold treatment. Cold treatment was performed by subjecting floral buds 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%). To perform *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 petry dishes (50 anthers per dish). At 0, 14 and 28 days of culture, 10 anthers per cultivar per dish were sampled in each fixative, Farmer and McDowell and Trump (1976). Material fixed in Farmer 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)

A total of 480, 320 and 600 anthers was processed in propionic-carmine (Figure1 a, d, g, j, m, p), DAPI (Figure 1 b, e, h, k, n) and toluidine blue (to histological sections) (Figure 1 c, 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. General averages of 2.06‰ of pollen grains with two symmetrical nuclei (G and V type) (Figure 1 d, e, f) and of 1.41‰ pollen grains with extra nuclei (Table 1) were observed, since day 0 until 28 of treatment, in the total of 62,536 pollen grains stained in propionic carmine.

The multinucleate pollen grains presented 3 to 6 round nuclei, frequently with different sizes (Figure 1 g, h, i), or presented different nuclei types: 3V, 2V1G, 3V1G, 1V2G, 1V3G and 1V4G (Figure 1 j, k, l).

Symmetrical and extra nuclei frequencies increased in both treatments 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 were constituted by DNA (Figure 1 m ,n, 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 1 p), increased with time of exposure to treatments, and was significantly greater in anther culture.

In histological sections, symmetrical and extra nuclei pollen grains frequently occurred assembled in the same anther locule (Figure 1 q) 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.

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

These preliminary results point to the need of further studies to clear the relationship between typical and fragmented extra nuclei with both androgenesis and programmed cell death. It is necessary to revise some assumptions based on carmine staining in soybean androgenic cultures, applying new techniques that enable more effective inferences.

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analysis of early microspore divisions and embryo formation in cultured soybean anthers. Plant Cell Tiss. Org. Cult. 49: 107-115

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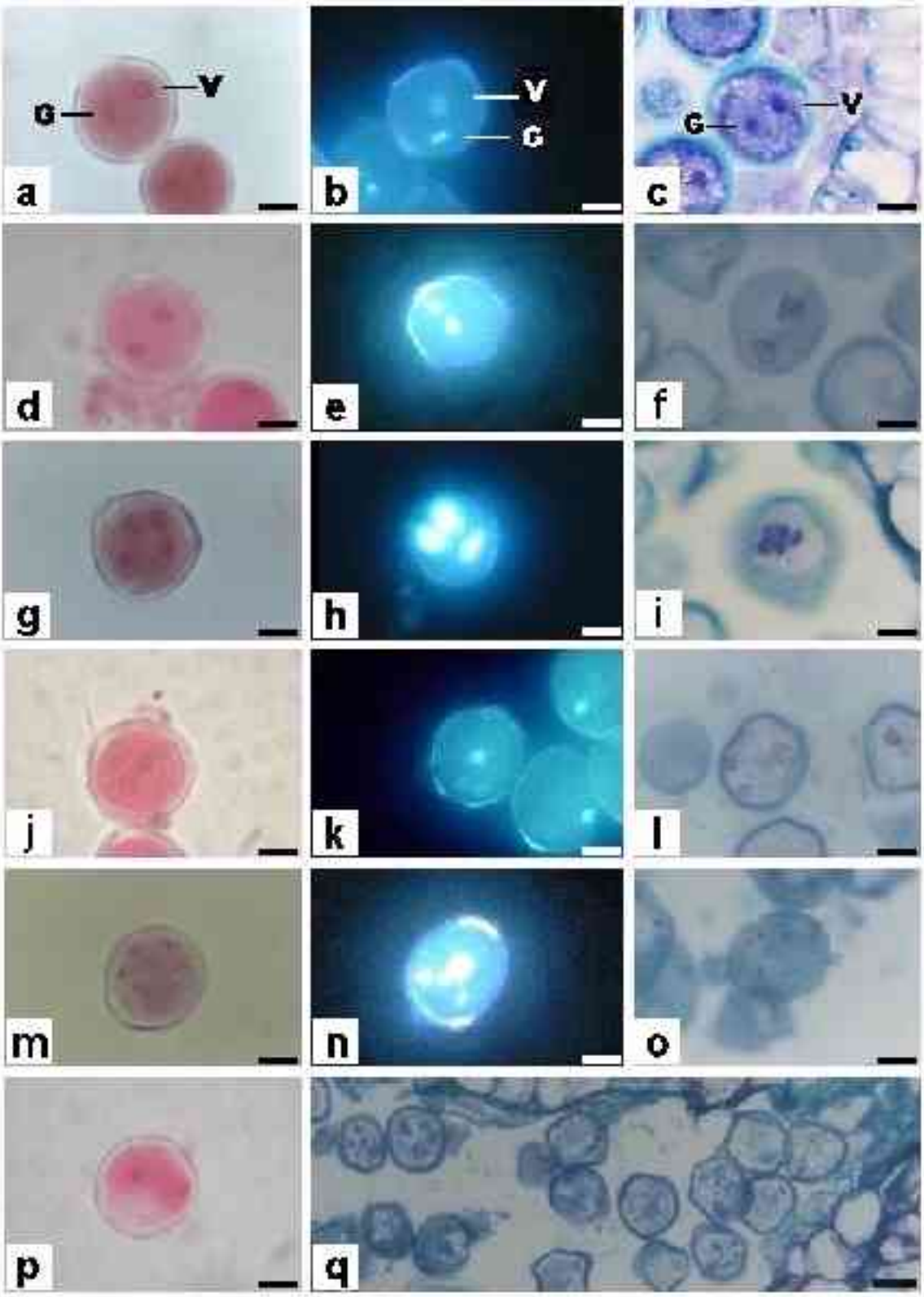
Table 1. Responses (per thousand) of soybean pollen grains at 0, 14 and 28 days under anther culture and cold treatment of floral buds at 4ºC. Anthers were fixed in Farmer and squashed in propionic carmine. The column 'Degraded' included non-stained and plasmolyzed pollen grains. The column 'P-type' was classified according to Kaltchuk-Santos et al. (1993).

	Soybean microspores and pollen grains (per thousand)						
	With typical nuclei	P type	With two symmetrical nuclei	With typical extra nuclei	With fragmented extra nuclei	Degra-ded	Number total analysed
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
4ºC							
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	**<.0001**	-
C)Day	-	-	0.6514	**0.0021**	**<.0001**	**<.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	**<.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)	-

Figure 1. Soybean pollen grains recorded at 0, 14 and 28 days of anther culture and cold treatment of floral buds at 4ºC. 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 6-10μm 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 4ºC treatment and type V at 14 days of culture (f). **(g-i)** Multinucleate pollen grains with round nuclei at 28 days of 4ºC treatment (g) and at 14 day of culture (h,i). **(j-l)** Multinucleate pollen grains with 3V nuclei at 14 days of 4ºC treatment (j), with 2V1G nuclei at 14 day of culture (k) and with 1V2G nuclei at 28 day of 4ºC treatment. **(m-o)** Multinucleate pollen grains with small extra nuclei whit a fragmented shape at 28 day of 4ºC treatment (m,n) and at 14 day of culture (o). **(p)** Plasmolysis in pollen grain with two V nuclei at 14 day of 4ºC 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-p = 10 μm, bar in q = 20 μm).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COMENTÁRIO AO CAPÍTULO V

Neste capítulo, o emprego das três técnicas para estudo do mesmo material vegetal submetido a condições distintas permitiu a observação de eventos androgênicos fora do ambiente *in vitro*. Contudo, serão necessários testes para confirmar a ocorrência de fragmentação de DNA.

Conforme os resultados apresentados neste capítulo, é possível propor uma hipótese quanto à origem e ao destino dos grãos de pólen multinucleados. O cultivo e o tratamento térmico são fatores de estresse que podem desencadear eventos similares em parte dos grãos de pólen, cuja resposta envolve divisões atípicas associáveis à androgênese. As condições ambientais são inadequadas à continuidade da rota esporofítica e não há formação das paredes celulares, de modo que os grãos de pólen multinucleados degradam junto com os demais. Uma das rotas de degradação assemelha-se bastante à fragmentação do DNA, evento relacionado à morte celular programada. Entretanto, a rota de degradação mais freqüente é a plasmólise. No cultivo de anteras, a degradação é mais intensa porque a proliferação dos tecidos diplóides compete com os micrósporos por espaço e nutrientes.

A partir das observações reunidas neste e nos capítulos anteriores, consolidaram-se as perspectivas deste trabalho: o cultivo de micrósporos isolados ofereceria condições mais adequadas para o estudo da androgênese em soja, enquanto o cultivo de anteras surgia como um novo sistema para a embriogênese somática a partir de tecidos da planta que já ingressou em fase reprodutiva.

CAPÍTULO VI

Observations on the embryogenic potential of soybean staminal tissues

Trabalho apresentado no V Latin American and Caribbean Meeting on Agricultural Biotechnology

Observations on the Embryogenic Potential of Soybean Staminal Tissues

L.R. Rodrigues, J.M.S. Oliveira, J.E.A. Mariath and M.H. Bodanese-Zanettini[1]

ABSTRACT

Our previous studies reported somatic embryogenesis from sporophytic tissues of cultured anthers providing the possibility to use staminal tissue as a new explant source in soybean. This approach would allow obtaining clonal plants from individuals that has achieved reproductive phase, when morphogenic response has been considered unsuccessful. The purpose of the present study, therefore, was to further analyze the embryogenic response of soybean staminal tissues. Anthers, filaments and whole stamen were established *in vitro* and compared as explants. The in *vitro* development was analyzed through serial histological analysis. The three explants originated embryo-like structures (ELS), but just ELS from connective calluses presented histodifferentiation beyond globular stage. To use staminal tissue culture as an *in vitro* system it will be necessary to overcome the low percentage of embryogenic explants, the lack of secondary embryogenesis and the low rate of histodifferentiation.

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It was assumed that soybean (*Glycine max* L. Merrill, 2n=40) is a recalcitrant specie for *in vitro* regeneration. Although calluses were induced readily from tissues at various developmental stages, the totypotency for organogenesis decreased soon after germination (Saka et al., 1980) and somatic embryogenesis was confined to early zygotic development (Ranch et al., 1985).

To shoot morphogenesis, explant sources were the cotyledonary node (Barwale at al. 1986; Wright et al., 1987) or primary leaf (Wright et al., 1987). To somatic embryogenesis, only parts of the immature zygotic embryo presented embryogenic potential as explant source (Ranch et al., 1985).

Although soybean has also been classified as a non-responsive species to anther culture (Matsubayashi and Kuranuki, 1975), several morphogenic events were recorded in cultured anthers, for instance, the formation of calluses (Tang et al., 1973; Ivers et al., 1974), roots (Ye et al., 1994), shoot (Yin et al., 1982; Jian et al., 1986) and androgenic embryo-like structures (ELS) (Zhuang et al., 1991; Hu et al., 1996; Kaltchuk-Santos et al., 1997; Zhao et al., 1998).

In our anther cultures, vigorous calluses have been obtained from connective cells exposed at detachment of anthers from filaments. These calluses originated ELS up to 90 days of culture. Moreover, culturing anthers heterozygous to a codominant molecular marker, we recorded that some segregating soybean genotypes originated ELS from both microspores and diploid tissues (Rodrigues et al., 2004). Subsequently, a histological study elucidated embryogenic responses: middle layer cells and epidermal cells originated ELS of unicellular origin, while ELS from connective calluses had a multicellular origin (Rodrigues et al., *in press*).

These observations offered the possibility of using staminal tissue culture as an *in vitro* system to regenerate genotypes for which the vegetative performance is already known, including segregant individuals from a F_1. Stamen should be an explant source during reproductive phase, when morphogenic response has been unsuccessful.

The purpose of the present study was further investigation of somatic embryogenesis from soybean staminal tissues.

MATERIAL AND METHODS

Test 1

Floral buds (length 3-3.5 mm) from field-grown plants of the cultivar Bragg were disinfected and dissected according to Kaltchuk-Santos et al., (1997). Anthers (A), filaments (F) and whole stamens (i.e., anther plus filament, S) were separately placed in 6 mm plastic petri dish (50 explants per dish) containing B5 medium (Gamborg *et al.,* 1968) supplemented with organic compounds (Hu et al., 1996), 10 ml Yeung's amino acids (Yeung and Sussex, 1979), 0.5 mg 6-benzyladenine (BA), 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D), 2.5 g Phytagel® and 90 g sucrose l^{-1} at pH 6.4. Cultures totaling 900 explants were kept under controlled conditions, at 25±1°C under 16 h photoperiod of ~25 µmol m^{-2} s^{-1}. After 30, 60 and 90 days of culture, callus and ELS yields were scored and all explants were transferred to fresh medium. Normality test and analysis of variance (ANOVA) were performed on the culture data. For serial histological observation, 15 explants of each type were sampled from culture dishes after 15 and 30 days of culture. Fresh floral buds 3-3.5mm (to illustrate the *in vivo* pattern) and a total of 90 explants were fixed and treated for histological analysis.

Test 2

Considering a better embryogenic response of A, a total of 2800 immature anthers from 3 mm floral buds of cultivars IAS 5 and MG/BR-46 Conquista were established in the same medium cited above but the initial 2,4-D concentration was 10 mg l^{-1}. Culture conditions were as described previously. For serial histological observation, one explant was sampled per culture dish after 15, 30 and 60 days of culture, totaling 168 samples.

Histological analysis

Samples were fixed in Mc Dowel & Trump solution (glutaraldehyde 1% and formaldehyde 4%) (McDowell and Trump, 1976) in phosphate buffer, pH 7.2, dehydrated in ethanolic series (Johansen, 1940) and embedded in hydroxyethylmetacrilate. The 4-8 µm histological sections were stained in toluidine blue 0.05%.

RESULTS AND DISCUSSION

At first, anther culture was used to trigger androgenic response from microspores and pollen grains (Guha and Maheshwari, 1964, 1966). However, morphogenic potential of the anther wall and connective has been recorded for some species, such as *Ranunculus sceletarus* (Konar and Nataraja, 1965), *Medicago sativa* (Saunders and Bingham, 1972), *Hevea brasiliensis* (Wang et al., 1980), *Vitis rupestris* (Altamura et al., 1992), *Manihot esculenta* (Mukerjee, 1995), *Oenothera hookeri* (Martinez and deHalac, 2000) and *Pometia pinnata* (Sudarmonovati et al., 2000). Our previous observations confirm that, also in soybean, anther culture is not an exclusively androgenic system.

Throughout the course of culture, the three explant types (A, F and S) originated typical calluses with a peripheral meristematic zone and an inner parenchymatous zone, both with intense cell division activity (Figure 1a).

Cytological abnormalities such as cytokinesis failures (data not shown) and nuclei with reduced cromossomal number were sometimes observed. A chromossome count confirm 20 chromosomes in one of these nuclei (Figure 1b), corresponding to the haploid level of soybean. Although it was impossible to determine the precise number by histological sections, the other nuclei probably also had 20 chromosomes.

Haploidy was recorded in embryogenic calluses and roots derived from diploid tissues of cultured anthers of *M. esculenta* (Canas and Roca, 1982) and *V. vinifera* x *V. rupestris* (Rajasekaran and Mullins, 1979). In these reports, due to the lack of histological studies on the course of callogenic, organogenic and embryogenic processes in anther culture, the origin of haploid cells was associated to androgenic response. Subsequently, it was recorded that haploid cells were present in embryogenic tissue of diploid origin (Altamura et al., 1992; Woodward and Puonti-Kaerlas, 2001). Yihua et al., (2001) recorded somatic meiosis-like reduction in cells of embryogenic callus of *Arabidopsis thaliana*. The latter authors supposed that somatic reduction has a relationship with cell dedifferentiation.

In the peripheral meristematic zone of the callus, cell divisions formed embryogenic cells clusters (Figure 1c) sometimes surrounded by a thickened outer wall. Since the physical and

physiological isolation of cells have been considered a prerequisite for further morphogenesis (Yeung, 1995), these cell clusters may have embryogenic potential. In *Cocus nucifera* similar structures give rise to ELS of unicellular origin (Verdeil et al., 2001).

In the inner parenchymatous zone of the callus, large ELS of multicellular origin differentiated according to Rodrigues et al (*in press*) (Figures 1d-f).

The S explant presented polarized response depending on the site of initial proliferation. Filament necrosis was observed when cell proliferation began in the anther. On the other hand, anther necrosis occurred when filament cells proliferated, forming smooth and light green callus (Figure 1d).

Explants that included filaments (S and E) showed significant higher callogenic proliferation but proportion of explants that originated ELS did not differ significantly among A, F and S (Table 1). Despite lower callus production from A, higher proportion of these calluses formed ELS. This result indicates that calluses formed from anthers, especially from connective tissue, present higher number of competent cells to respond to inductive signals for somatic embryogenesis. The anther is a complex organ, composed of cells with intense physiological and developmental dynamics, such as tapetum and microspores (Vasil, 1967) while filaments are constituted of parenchymatous tissue (Schmid, 1976). In additional tests, the comparison between A and F was repeated with four cultivars (Bragg, IAS 5, MG/BR 46 Conquista and BRS 133) and similar trends were obtained (data not shown).

ELS formed from filament tissue (both in S and F) did not develop beyond globular stage. Further stages of embryo development and histodifferentiation were observed in ELS from connective tissue. Similar results were obtained in subsequent tests, in which several culture media were tested (data not shown). Induction medium used to soybean cotyledonary explants (Bailey et al., 1993) and combinations of 2,4-D, BA, abscisic acid and gibberellic acid in B5 medium affected significantly the number of explants with ELS, but not the histodifferentiation rate. Secondary embryogenesis was not observed. Although organogenesis has been previously reported in calluses derived from anthers (Yin et al., 1982; Jian et al., 1986; Rodrigues et al., *in press*), it was not

observed in F cultures.

Culture conditions inducted soybean staminal tissues to somatic embryogenesis (Figure 1g-i). But, in order to use staminal tissue culture as an *in vitro* system, it will be necessary to overcome the low percentage of embryogenic explants, the lack of secondary embryogenesis and the low rate of histodifferentiation. Since distinct morphogenic events may occur in soybean cultured anthers (Rodrigues et al., 2004), cultures must undergo a serial histological analysis for a precise description of embryogenic response.

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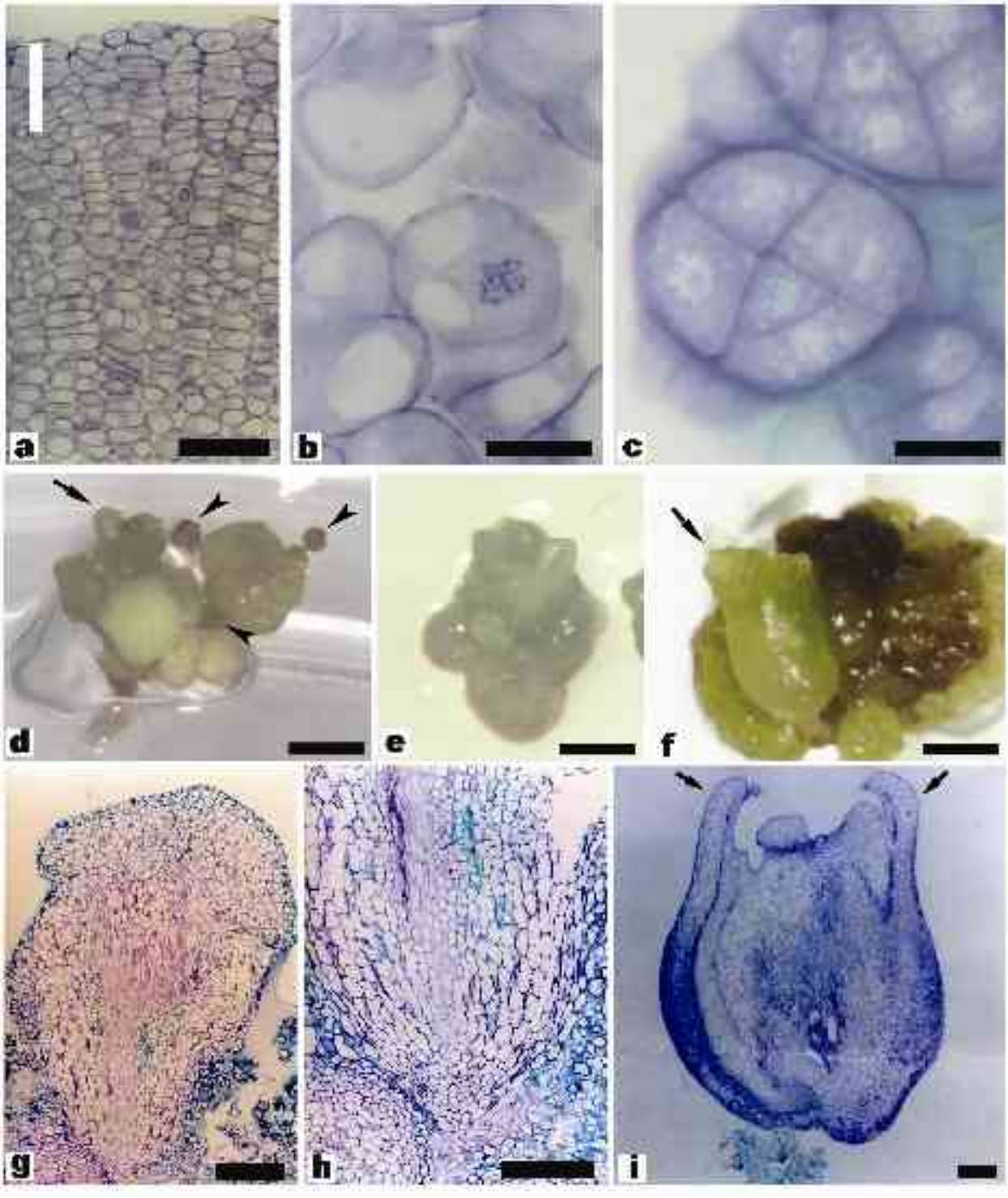
Table 1. Analysis of variance of the percentage of responsive explants after 60 days of culture.

Explant type	Explants with calluses		Explants with ELS	Embryogenic Calluses	
Anther	67	b	5	7	a
Stamen	85	a	5	6	ab
Filament	81	a	4	5	b
Pr>F	0.0224		0.2319	0.0448	
CV%	12		22	25	

Figure 1. Histological sections (a-c, g-i) and observations under stereomicroscope (d-f) on the embryogenesis from soybean staminal tissues. a) Peripheral meristematic zone (white bar) and inner parenchymatous zone in callus of the cultivar IAS 5; b) Haploid cell in connective callus of the cultivar MG/BR-46 Conquista; c) Embryogenic cells in the meristematic region of callus derived from connective tissue of the cultivar Bragg (bars in a,b end c = 10 µm); d) Polarized response from stamens of the cultivar Bragg after 30 days of culture. Due to the preferential proliferation of filaments, anthers presented phenolic oxidation (arrowheads). Callus derived from filament formed a globular embryo (arrow); e) ELS with cotyledon formation in callus derived from connective tissue of the cultivar IAS 5 after 60 days of culture; f) Trumpet-shaped ELS after 60 days of culture of the cultivar Bragg (bars in d, e end f = 1 mm); g) Longitudinal section of ELS of the cultivar MG/BR-46 Conquista without meristematic apexes; h) Detail of the root apex shown in g; i) Longitudinal section of ELS with initial cotyledon formation (arrows)of the Bragg cultivar (bars in g, h and i = 100 µm).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COMENTÁRIO AO CAPÍTULO VI

No total, foram executados seis cultivos experimentais testando comparativamente o potencial embriogênico dos tecidos estaminais de soja. As observações de apenas um destes cultivos foram suficientemente representativas do conjunto de resultados e, para uma abordagem objetiva, foram apresentadas neste capítulo. Também foram omitidas observações histológicas cuja discussão não contribuiria ao objetivo do trabalho.

Este capítulo foi acrescentado à tese por conter observações complementares à Discussão e, apesar de estar organizado em formato de nota de pesquisa, requer a execução de estudos adicionais para a publicação.

CAPÍTULO VII

Isolation and culture of soybean microspores and pollen grains

Manuscrito em preparação para submissão à revista Plant Cell, Tissue and Organ Culture

Original research papers

Isolation and culture of soybean microspores and pollen grains

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Key-words: androgenesis, Glycine max, microspore isolation, microspore culture.

Abbreviations: ANOVA - analysis of variance; FDA – fluorescein diacetate

Abstract

In the last three decades, research on soybean androgenesis was limited to anther culture, which presents several limitations such as the small number of microspores with androgenic response and the morphogenic potential of connective tissue, middle layers and epidermal cells. Therefore, a sequence of studies was performed to establish appropriate conditions for the isolation and culture of soybean microspore and pollen grains as an alternative to anther culture. First of all an isolation technique was developed using floral buds from four soybean cultivars (Bragg, IAS 5, MG/BR-46 Conquista and BRSMT Uirapuru). This technique made it possible to establish cultures with satisfactory density and characteristics to study androgenesis. Subsequently microspore viability was tested in different culture media. Despite the fact that B5 and MS media are recommended for androgenesis in soybean anther culture, the best result was obtained in PTA-15 modified medium, with the formation of enlarged microspores and 0.4% of multicellular pollen grains in the cultivar BRSMT Uirapuru.

Introduction

Soybean (*Glycine max* L. Merrill, 2n=40) androgenesis has been studied since the early 1970s (Tang et al. 1973; Ivers et al. 1974), mainly through anther culture (Hu et al. 1996). However, a protocol for haploid and double-haploid plant production is not yet available. Two main factors account for the lack of success in soybean anther culture. First, a small proportion of gametophytic pathway deviation recorded in microspores and pollen grains (Yin et al. 1982; Kaltchuk-Santos et al. 1997). Second, using SSR marker (Rodrigues et al. 2004) and histological analysis (Rodrigues et al. *in press*), it was proved that conditions known to trigger androgenesis also favoured callogenesis and the occurrence of somatic embryogenesis.

Isolated microspore culture could provide better conditions for androgenic response in soybean, however morphological features of floral buds, such as small size, presence of trichomes and low yield of microspores per floral bud, make it difficult to isolate microspores and to establish cultures.

As far as we know, no consistent information is available concerning the soybean microspore culture. In an extensive review, Hu et al. (1996) referred to a single Chinese publication (Liu and Zhao 1986), in which the authors claimed to have obtained haploid callus from isolated soybean microspores.

In an attempt to establish an alternative method to trigger androgenesis in soybean, a sequence of tests was carried out to develop a technique to isolate microspores at appropriate density and, subsequently to establish microspore cultures. The effects of medium constitution and genotypes were tested.

Material and methods

Plant material

Plants of four cultivars (Bragg, MG/BR-46 Conquista, IAS 5 and BRSMT Uirapuru) were grown in

a growth chamber with a 14h photoperiod, light intensity of 13,500 lux and temperature 26±3ºC. Plant material used in the test of PTA-15 modified medium was obtained from field-grown plants.

Microspore isolation test

Androeciums were dissected from 3-3.5 mm immature floral buds, gently macerated with a glass rod and suspended in 4.5 ml liquid medium + 0.5 ml propionic-carmine 0.6% in a 50 ml Erlenmeyer flask. The crude extract containing ~80 anthers ml^{-1} was stirred with a magnetic stirrer for 4 min. The medium was B5 (Gamborg et al. 1968) with 60 g l^{-1} sucrose at pH 6.4. Aliquots of 1 ml of crude extract were filtered through a 37 μm nylon mesh screen and washed with the same medium by: centrifugation at 2000 rpm for 3 min (previously tested); discarding 700 μl medium; and resuspension in the same volume of liquid medium. The number of washes (0, 1, 2, 3 and 4) was tested. Each combination (cultivar x number of washes) was repeated twice. The quality of the suspensions was evaluated in 60 μL samples of each aliquot. Microspores were counted, staged and classified on glass slides under a Zeiss Axioplan Universal microscope. An average of 300 observations per slide was performed. Constituent elements of the suspension were classified as: tetrads; whole microspores and pollen grains; non-viable or plasmolyzed microspores; burst microspores; whole diploid anther cells; and burst diploid anther cells (=debris). The normality test and ANOVA were performed on the data.

Culture density test

Floral buds from 4 cultivars were submitted to the isolating technique developed in the previous test. Suspensions were obtained using 4, 8 and 12 floral buds per ml of medium, with 5 repetitions. The density of the whole microspore and pollen grains was scored using a hemacytometer. The normality test, ANOVA and analysis of regression were performed on the data.

Microspore viability test

Floral buds 3-3.5 mm from cultivar BRSMT Uirapuru were disinfected according to Kaltchuk-Santos et al (1997). Microspores were isolated in sterile conditions, according to the previous isolation and density tests, in order to obtain a suspension with 5 x 10^4 cells ml^{-1}. Factors tested were: two culture media [B5 and MS (Murashige and Skoog 1962) including salts and vitamins]; sucrose concentration (60 and 120 g l^{-1}) and pH (6.0 and 6.4) in a triple factorial design. The suspension was divided into 1ml aliquots, placed in Corning™ plates (6 wells) and incubated at 26±1ºC in darkness. Each combination of treatments was repeated 5 times (wells). Microspore viability was determined by fluorochromatic reaction to FDA (Heslop-Harrison and Heslop-Harrison 1970) in samples at 0 and 28 days of incubation. Microspore responses during culture were analysed in fresh material sampled at 0, 14, 28 and 42 days, mixed with a drop of propionic-carmine on a glass slide, covered under a cover slip, and analysed under the microscope. The normality test, ANOVA and correlation test were performed on the data.

Culture in PTA-15 modified medium

Microspores of cv MG/BR-46 Conquista, IAS 5 e BRSMT Uirapuru were isolated in PTA-15 (Skinner and Liang, 1996) modified medium according to previous tests. Aliquots of 500 μL (density 5x10^4 cells ml^{-1}) were placed in Corning™ plates (24 wells) under two conditions: liquid medium and double phase medium (liquid spread on 8 g l^{-1} agarose gelled medium). IAS 5 had 4 repetitions per treatment and MG/BR-46 Conquista and BRSMT Uirapuru had 8 repetitions. Cultures were incubated at 26±1ºC in darkness. Microspore viability was determined by fluorochromatic reaction to FDA in samples at 0 and 28 days of incubation. Microspore responses during culture were analysed under the microscope at 0, 14, 28 and 42 days of incubation in samples fixed in Farmer solution (100% ethanol: glacial acetic acid, 3:1) and stained with propionic-carmine. Fifty four samples were collected from BRSMT Uirapuru suspension before culture and analyzed simultaneously by fluorochromatic reaction to mithramicin according to Coleman and Goff (1985) and propionic carmine staining. The normality test and ANOVA and correlation test were performed on the data.

Results and discussion

Microspore isolation test

Immature soybean anthers do not respond to *in vitro* stimulus to dehiscence (data not shown). Isolation soybean microspores demand individualized and selective removal of the androecium, so that no bract will be part of the crude extract. Trichomes make disinfestation difficult due to air bubbles trapping. Furthermore, trichomes obstruct mesh pores, hindering the passage of microspores and decreasing microspore density in the culture. After removal of the androecium, maceration with a glass rod and magnetic stirrer was essential for the fragmentation of staminal tissues and microspore release from the sporangia. Due to the lack of intercellular adhesion, few microspores bursted during this step. Magnetic stirring was also suitable to isolate *Oryza sativa* (Cho and Zapata 1990) and *Malus domestica* (Höfer et al. 1999) microspores.

According to our previous observations, the microspores of the soybean cultivars tested in the present study presented an average diameter of 25 μm. Thus, a 37 μm pore was appropriate for filtering, although this pore size allows the passage of tetrads.

As a rule, when the bud length is 3 to 3.5 mm, meiosis has already ended and only a small number of tetrads and mostly microspores and pollen grains remain. However, floral buds having the same size present differences in the microsporogenesis and microgametogenesis stages when comparing different soybean cultivars (Lauxen et al. 2003).

Floral buds of IAS 5 presented anthers in more advanced microsporogenesis stages while the other cultivars had anthers containing more than 94% of microspores (Figure 1a). When microspores were isolated, the percentage of degraded microspores (whole but non-stained or plasmolyzed) was significantly higher for IAS 5 (Table 1). ANOVA did not detect a significant effect of the number of washes on the percentage of degraded microspores.

The constituent elements of the suspension were: a small proportion of burst microspores and diploid cells and a majority of whole microspores and pollen grains (Figure 1b). In figure 1b, the category of whole microspores included either stained or non-stained microspores,.

The percentage of burst microspores was not significantly different among cultivars and decreased significantly with the number of washes (Table 1). This observation indicates that bursting preceded washes, resulting from the maceration procedure.

Concerning the proportion of debris, a difference was detected among the different genotypes: cultivars Bragg and IAS 5 presented a significantly higher proportion of debris when compared to MG/BR-46 Conquista and BRSMT Uirapuru. After the first wash, the proportion of debris decreased significantly.

The occurrence of whole diploid cells in culture was rare, totaling 5 out of the 12,272 observations. This occurrence could be accounted for mesh pore widening. In further experiments, the careful examination of the mesh under stereomicroscope avoided whole diploid cells in culture.

After three washes, the suspension presented enough quality to establish cultures. The same number of washes is used to isolate microspores in *Zea mays* (Huang and Keller, 1989; Pretová et al. 1993), *Brassica* species (Huang and Keller 1989; Barro and Martín 1999; Dias 1999) and *Malus domestica* (Höfer et al. 1999).

The technique described here made it possible to obtain mixed suspensions containing microspores and pollen grains. In order to separate homogeneous populations, exclusively with microspores or pollen grains, it will be necessary to complement the technique, for instance, by means of Percoll density gradient centrifugation (Kyo and Harada 1986).

Culture density test

Culture density has an important role in the proportion of microspores with androgenic response. Usually, microspore density in culture varies from 10^4 to 10^5 cells ml^{-1}. Very low embryo yields were found at microspores densities lower than 2 x 10^4 in *B. oleracea* (Duijs et al. 1992).

Both number of floral buds ml^{-1} ($Pr>F=<0.0001$) and genotype ($Pr>F=0.0004$) showed highly

significant effects on density, without interaction between sources of variation (Pr>F=0.3821).

The cultivar Bragg stood out from the other cultivars, given the greater number of microspores (Figure 2). The overall average was 5.7 x 10^5 cells ml^{-1} in Bragg, 4.1 x 10^5 in BRSMT Uirapuru, 3.8 x 10^5 in MG/BR-46 Conquista and 3.1 x 10^5 in IAS 5. These differences determined a significant linear regression equation for each cultivar. The correlation coefficient (R^2) between bud number and density was significant for 3 cultivars, except for IAS 5, which presented an advanced stage of microgametogenesis (Figure 1a). Microspore release and passage through mesh screen become progressively less efficient with bud development due to tissue rigidity and volume.

Despite the significant R^2, the final density varies as a consequence of differences among anthers, buds and cultivars. This observation indicates that culture density should be adjusted for each isolation procedure.

Obtaining an appropriate culture density is a very laborious task in soybean. To obtain 10 ml of suspension with 5 x 10^4 cells ml^{-1}, it was necessary to dissect androeciums of 100 to 170 floral buds. For comparison, 15 to 20 *Petunia* buds (Sangwan and Norrel 1975) and 10 *B. napus* buds (Huang and Keller 1989) were enough to produce 10 ml of liquid culture with a density of 1x10^4 and 2 to 3x10^4, respectively.

Microspore viability test

Table 2 shows the results of assessment of microspore stainability by propionic-carmine and fluorochromatic reaction to FDA (Figure 3d). The percentage of fluorochromatic reaction (35% in average) is lower than that observed previously in fresh anthers (approximately 50%; data not shown).

The percentage of carmine-stained microspores was higher than the percentage of microspores that reacted to FDA. The R^2 between carmine and FDA results was not significant either at the beginning of the culture (R^2=-0.296) or after 28 days (R^2=0.054). This result confirms previous observations (data not shown) that had indicated that the two methods provide different inferences

on the evaluation of microspore response to culture. ANOVA detected significant effects of media ($Pr>F=0.0301$) and pH ($Pr>F=0.0108$) on decreasing microspore viability after 28 days of culture. Although the effect of sucrose concentration was not significant ($Pr>F=0.3333$), there was significant interaction between medium and sucrose concentration ($Pr>F<0.0001$). Reduction of viability was smaller when 60 g l^{-1} sucrose was combined with B5 medium. On the other hand, higher reduction of viability was observed when 60g l^{-1} sucrose was combined with MS medium (Table 2).

Viability of microspores cultured in B5 medium with 60g l^{-1} sucrose and pH 6.4 was higher after 28 days than at the beginning of the culture (Table 2). This result can be accounted for experimental error, such as: defective sampling and variation on the optimum moment of fluorescein retention by plasmalema (Heslop-Harrison and Heslop-Harrison 1970).

Carmine squashes have been used to determine the nature of the embryogenic microspores ever since the first description of plant androgenesis *in vitro* (Guha and Maheshwari 1966). Carmine allows a distinction between vegetative and generative nuclei and is useful to follow cell division and events causing degradation. The disadvantage is that carmine indiscriminately stains both live and dead cells.

Only two multicellular structures were found out of the 12,272 observations: a four-celled structure in MS medium (not shown) and an eight-celled one in B5 medium (Figure 3a). The latter had a translucent covering similar to callose. Although such structures were formed *in vitro*, there is no proof of their androgenic origin. Anyway callose deposition is thought to be a prerequisite for somatic embryogenesis (Verdeil et al. 2001).

The higher survival of microspores in B5 indicates that this medium provides chemical and physical conditions for cell maintenance, but not necessarily to trigger androgenesis.

Culture in PTA-15 modified medium

In the *Medicago sativa* microspore culture, the PTA-15 medium has been used to trigger androgenesis. In the isolation procedure, the pelleted microspores were resuspended in PTA-15

liquid and spread on PTA-15 gelled with agar or Gelrite (Skinner and Liang 1996). Therefore, PTA-15 modified medium was tested in both liquid and double phase in soybean microspore culture.

A total of 24,218 carmine-staining observations was scored from samples at 0, 7 and 14 days of culture. Degraded (non-stained and plasmolysed) microspores represented around 1.5% of the total. This value was lower than those obtained in B5 and MS media. Multicellular structures similar to those already recorded in B5 and MS media were also observed (Figure 3b).

Typical androgenic events were observed in the PTA-15 modified medium: enlargement of the microspore size (Sangwan and Norrel 1975; Höfer et al. 1999) and multicellular pollen grain formation (Figure 3c). Multicellular pollen grains presented 3 to 5 cells, sometimes varying in size but with similar nuclei, without vegetative and generative features.

The multicellular pollen grains occurred indiscriminately in double phase and liquid medium, but at very low frequencies: only 7 out of 3,688 (0.2%) microspores in IAS 5 and 29 out of 7,173 (0.4%) in BRSMT Uirapuru. In the latter cultivar, the structures presented a higher number of cells.

Microspore viability evaluated by FDA decreased to 21%, 28% and 1% in MG/BR-46 Conquista, IAS 5 and BRSMT Uirapuru, respectively, after 28 days of culture, independently of the occurrence of embryogenic events.

Comparing fluorochromatic reaction to mithramycin and stainability to propionic-carmine, a total of 26,236 microspores and pollen grains were analyzed. The fluorochrome allowed significant better characterization of the morphology of typical and atypical nuclei. ANOVA detected significant differences between observations obtained by two techniques from the same samples ($Pr>F<0.001$). Only by means of fluorochromatic reaction to mithramycin, it was identified symmetrical nuclei in pollen grains before culture (Figure 3e).

Procedures developed in this extensive sequence of assays allowed to isolate, to culture and to analyze *in vitro* responses of soybean microspores in the absence of sporophytic tissues. Next challenge will be to establish a protocol to control developmental fate of soybean microspores.

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Table 1. ANOVA of the percentage of undesirable elements of the microspore suspension isolated from four cultivars, with an increasing number of washes. The column "Degraded microspores" included non-stained and plasmolyzed microspores

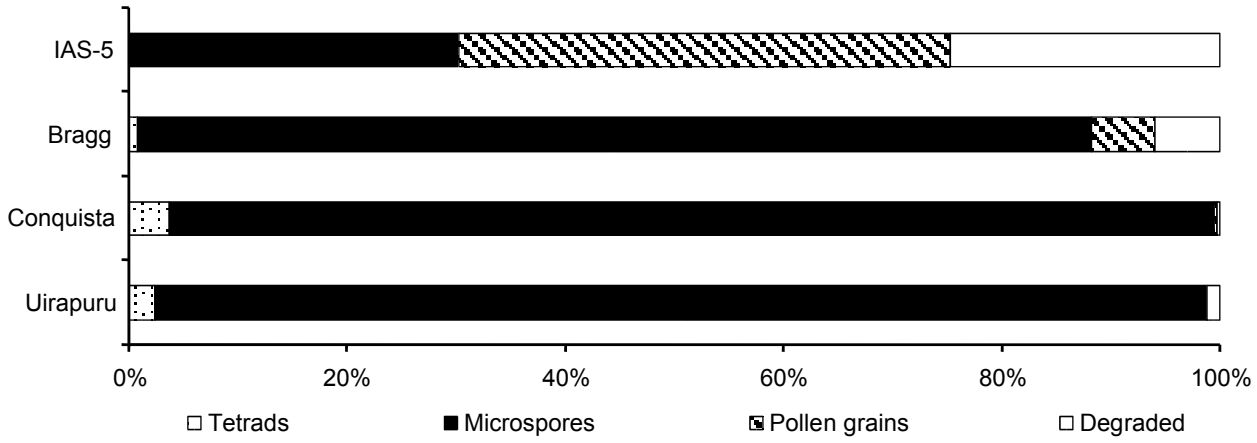
Sources of variation		Degraded Microspores			Burst Microspores			Debris		
		Average		Pr>F	Average		Pr>F	Average		Pr>F
Cultivars	Bragg	5.9	b	**0.0001**	2.5		0.1019	17.9	a	**0.0001**
	IAS 5	24.7	a		2.4			16.8	a	
	Conquista	0.3	c		1.1			8.4	b	
	Uirapuru	1.2	bc		1.5			8.8	b	
Washing number	0	8.4		0.8825	3.2	a	**0.0086**	19.0	a	**0.0001**
	1	9.4			2.5	ab		13.5	b	
	2	9.0			1.8	ab		13.4	b	
	3	7.4			1.0	b		10.0	b	
	4	6.0			0.8	b		9.0	b	
Interaction				0.9984			0.7542			0.2067
Transformation		Root (x+2)			Log (x+2)			-		
CV%		30			24			26		

Table 2. Fluorochromatic reaction to FDA and stainability to propionic-carmine in microspores and pollen grains of cultivar BRSMT Uirapuru at 0 and 28 days of culture in different constitutions of culture medium. The columns ≠ present the difference between day 0 and 28.

Treatments			Fluorochromatic reaction to FDA			Propionic-carmine staining		
Medium	Sucrose	pH	Day 0	Day 28	≠	Day 0	Day 28	≠
B5	60	6.0	29	22	7	99	99	<1
B5	60	6.4	31	39	-8	99	97	2
B5	120	6.0	37	19	18	99	96	3
B5	120	6.4	33	16	17	99	99	<1
MS	60	6.0	45	23	22	96	91	5
MS	60	6.4	40	20	20	97	85	12
MS	120	6.0	36	22	14	94	85	9
MS	120	6.4	28	25	3	97	82	15
General average			35	23	12	98	92	6

Figure 1. Microspore isolation test. **a**: Percentage of microspores in the suspension obtained from 3-3.5 mm floral buds according to developmental stage of four soybean cultivars (IAS 5, Bragg, MG/BR-46 Conquista and BRSMT Uirapuru). **b**: Percentage of constituent elements of suspension according to number of washes. Cultivars IAS 5 and Bragg (above) and cultivars MG/BR-46 Conquista e BRSMT Uirapuru (below) were separated as a consequence of statistical differences.

a



b

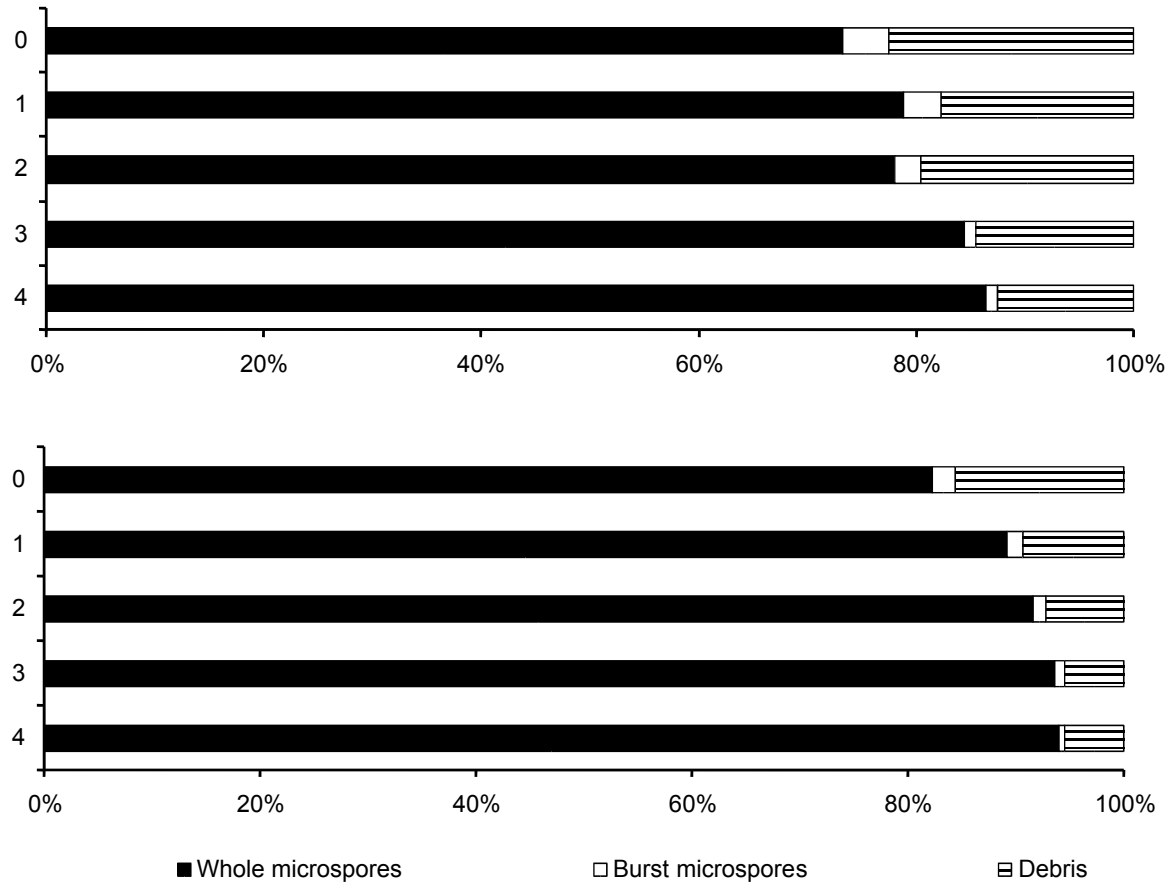


Figure 2. Culture density test. Trend lines relating the number of floral buds of four cultivars and the density (microspores per ml of culture) in the final suspension. Linear regression equation: Bragg: y= 6250x + 7000 (R^2=0,5921*); BRSMT Uirapuru: y= 5562x + 3333,3 (R^2=0,8558*); MG/BR-46 Conquista: y= 6125x -10833,3 (R^2=0,7852*); and IAS 5: y= 3062,5x + 6833,3 (R^2=0,4422, non-significant).

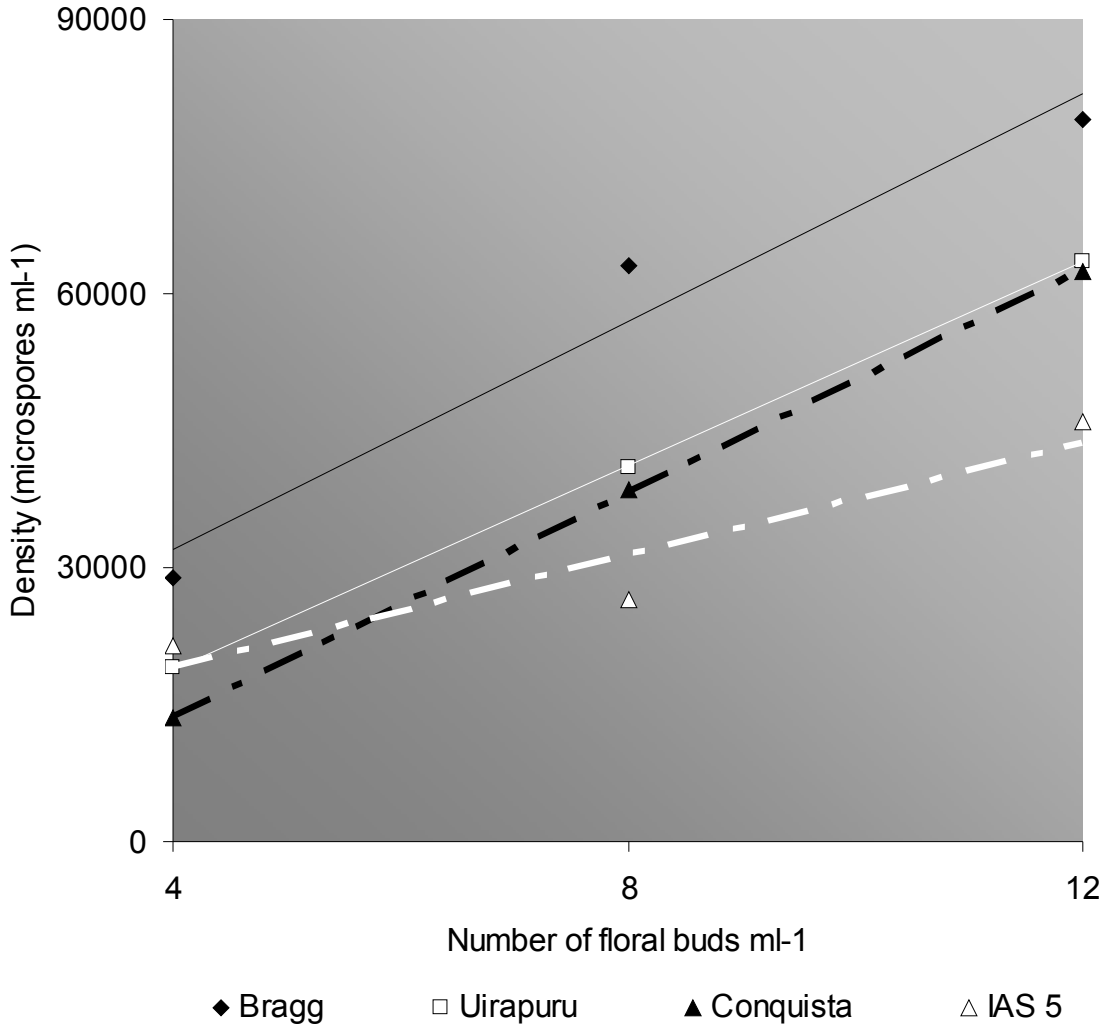
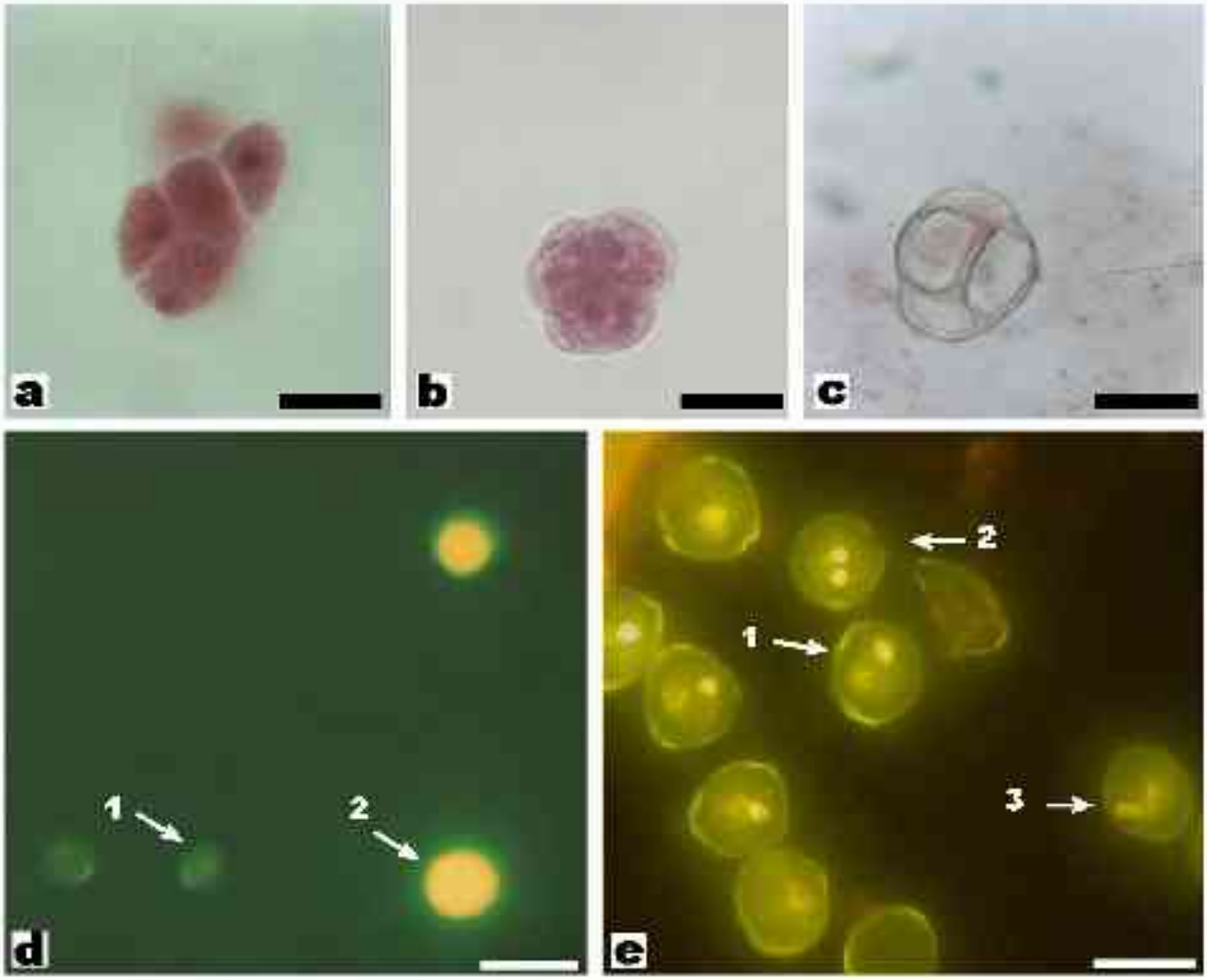


Figure 3. Culture of isolated microspores of the cultivar BRSMT Uirapuru. **a**) Eight-celled multicellular structure after 14 days of culture in B5 medium. **b**) Four-celled multicellular structure after 7 days of culture in PTA-15 modified medium. **c**) Multicellular pollen grain after 7 days of culture in PTA-15 modified medium. **d**) Fluorochromatic reaction to FDA was the criteria employed to discriminate non-viable (arrow 1) and viable microspores (arrow 2) at 0 and 28 days of culture. **e**) Fluorochromatic reaction to mithramycin, showing pollen grains with typical nuclei (arrow 1) and with symmetrical nuclei generative-like (arrow 2) and vegetative-like (arrow 3) before culture (Bars = 25 μm).


a
b
c
1
2
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2
1
3
e

CAPÍTULO VIII

Discussão Geral

DISCUSSÃO GERAL

No período compreendido entre janeiro de 2000 e janeiro de 2004, foram estabelecidos *in vitro* 41.576 explantes estaminais, principalmente anteras, e 20.225.000 micrósporos e grãos de pólen isolados; 480 explantes foram analisados em secções histológicas; e um número incontável de esmagamentos foi executado com carmim propiônico, FDA, laranja de acridina, DAPI e mitramicina. Apenas parte destes cultivos e destas observações integra esta tese.

Apesar de terem sido executados e escritos em uma ordem cronológica diferente, os capítulos desta tese foram organizados para, em um primeiro momento, esclarecer os eventos morfogênicos que ocorrem no cultivo de anteras de soja e, em um segundo momento, apresentar o cultivo de micrósporos e grãos de pólen isolados como uma alternativa para o estudo da androgênese. Não há uma premissa inicial e outra final. As constatações resultantes de um capítulo fundamentam ou levantam questões para o capítulo seguinte. Os resultados oferecem acréscimos e retificações às proposições de Hu *et al*. (1996), Zhao *et al*. (1998) e Kaltchuk-Santos (1999) quanto à androgênese em soja, a seguir apresentados:

1. Nem todos os tecidos da planta de soja que já ingressou em fase reprodutiva são recalcitrantes

Alguns autores constataram que os tecidos da planta madura de soja são recalcitrantes para a regeneração *in vitro* (Saka *et al*., 1980; Ranch *et al*., 1985). Inclusive, os protocolos que permitem regeneração de plantas, tanto via organogênese quanto via embriogênese somática, empregam partes de embriões zigóticos imaturos ou de embriões recém-germinados como fontes de explante (Barwale *et al*., 1986; Wright *et al*., 1987; Ranch *et al*., 1985).

Por esse princípio, ainda que a origem diplóide dos calos primários de anteras de soja já tivesse sido registrada pelos pesquisadores chineses (Yin *et al*., 1982; Ye *et al*., 1994), os embriões não poderiam ter origem nos estratos parietais e no conectivo (Hu *et al.,* 1996).

Estas observações não se confirmaram neste trabalho, sugerindo que o emprego da palavra recalcitrante deve ser mais cuidadoso. Uma vez que é difícil testar todas as

condições de cultivo em todos os explantes de todos os genótipos, é inadequado afirmar que determinada espécie, tecido ou célula não tem potencial morfogênico.

2. O cultivo de anteras não é um sistema exclusivamente androgênico

Na literatura e em eventos científicos, é freqüente a citação do cultivo de anteras como sinônimo de androgênese. Entretanto, em 1963, antes mesmo do primeiro registro da androgênese *in vitro* (Guha e Maheshwari, 1964), a calogênese a partir do conectivo já tinha sido observada (Vasil, 1967). Desde então, o potencial morfogênico de tecidos estaminais de outras espécies tem sido registrado (Tabela 1).

Tabela 1: Algumas citações bibliográficas do potencial morfogênico dos estratos parietais da antera e do conectivo, de acordo com o ano da observação.

Ano	Espécie	Evento morfogênico	Referência
1963	*Cajanus cajan*	calogênese	Vasil (1967)
1965	*Ranunculus sceletarus*	embriogênese somática	Konar e Nataraja (1965)
1972	*Medicago sativa*	embriogênese somática	Saunders e Bingham (1972)
1972	*Petunia* sp	não mencionado	Sangwan e Norreel (1975)
1980	*Hevea brasiliensis*	embriogênese somática	Michaux-Ferrière *et al*. (1992)
1992	*Vitis rupestris*	embriogênese somática e organogênese	Altamura *et al*. (1992)
1995	*Manihot esculenta*	embriogênese somática	Mukherjee (1995)
1996	*Malus* sp	calogênese	Ochatt e Zhang (1996)
2000	*Oenothera hookeri*	organogênese	Martinez e deHalac (2000)
2000	*Pometia pinnata*	embriogênese somática	Sudarmonovati *et al*. (2000)

Quando a planta sofre a indução floral e destina recursos para a diferenciação de órgãos reprodutivos, forma as anteras como um sítio extremamente especializado para a microsporogênese e a microgametogênese. A antera constitui-se de vários tecidos, destinados à senescência combinada à gametogênese (Mariath *et al*., 2003). No momento em que é estabelecida *in vitro*, a antera deixa de fazer parte do botão floral e de receber o estímulo nutricional e hormonal específico à conclusão da rota gametofítica. Considerando as funções especializadas e o momento específico da microgametogênese em que inicia a degradação de cada tecido, a antera pode ter diversas respostas às condições de cultivo. Isso pode explicar a ocorrência simultânea dos distintos eventos morfogênicos, listados nos capítulos III e VI.

O cultivo pode exercer um estímulo diferente sobre cada uma destas células e a

resposta pode depender de inúmeras condições, além daquelas mencionadas nos textos clássicos, como a constituição do meio e as condições físicas *in vitro*. A resposta morfogênica pode depender, inclusive, da posição da célula em relação às demais e em relação ao meio, da resposta dos tecidos adjacentes e da liberação de compostos pelas células adjacentes.

Os estratos parietais e o conectivo podem ter efeito benéfico ou inibitório sobre a embriogênese do pólen, dependendo das circunstâncias (Maheshwari *et al*., 1982), dentre as quais, a expressão do potencial morfogênico (capítulo III).

No presente estudo, foi demonstrada a ocorrência de embriogênese a partir dos micrósporos e dos tecidos diplóides no mesmo cultivo de anteras (capítulo II). A embriogênese somática pode ter origem multicelular (a partir de calos conectivais) e unicelular (a partir de células epidérmicas não reabsorvidas e células da camada média invasivas ao espaço locular) (capítulo III).

Registrada em calos velhos, depois de retirados do contato com auxina, a rizogênese em anteras de soja, a princípio, não era explicada (Hu *et al*., 1996). Porém, alguns trabalhos comprovaram que a formação de raízes é comum em calos embriogênicos tardios, a partir dos mesmos complexos celulares que inicialmente originaram embriões somáticos de origem unicelular, principalmente relacionada ao decréscimo da concentração de auxina (Matsuoka e Hinata, 1979; Ho e Vasil, 1983; Buffard-Morel *et al*., 1992; Tarré *et al*., *in press*).

Por um lado, a ocorrência de embriogênese somática no cultivo de anteras de soja é uma resposta desanimadora: a simultaneidade de eventos morfogênicos dificulta os trabalhos visando desencadear androgênese e propõe uma releitura criteriosa de todos os trabalhos em que foram obtidos embriões a partir de anteras de soja nas condições até então consideradas favoráveis à androgênese.

Por outro lado, a resposta embriogênica dos tecidos estaminais traz a possibilidade de regeneração clonal a partir de plantas cujo desempenho vegetativo já é conhecido, incluindo indivíduos segregantes de uma F_1. O estame serviria como explante a partir de uma planta madura, quando a resposta morfogênica não tem sucesso em outros sistemas. Esta estratégia atenderia demandas muito específicas em trabalhos de manipulação genética.

3. Células dos tecidos diplóides de anteras *in vitro* podem originar proliferações

invasivas ao espaço intralocular

No cultivo de anteras de soja, células conectivais e da camada média podem proliferar-se de forma invasiva ao lóculo da antera (capítulo III). Na sua fase inicial, estas proliferações são arredondadas e parecidas com um grão de pólen multicelular, o que pode gerar confusão na interpretação de secções histológicas. Observações histológicas pontuais como as de Kaltchuk-Santos *et al.* (1997), em soja, e de Peixe *et al*. (2004), em *Prunus armeniaca*, associaram estas proliferações à resposta androgênica.

Somente observações histológicas seriais permitiram o esclarecimento quanto à origem destas proliferações. Em secções histológicas nas quais a ligação das proliferações iniciais com os tecidos diplóides não foi identificada, executamos um teste com o fluorocromo auramina O (não mostrado). A esporopolenina, proteína que integra a exina, reage na presença da auramina, fluorescendo em amarelo (Vithanage e Knox, 1979). A presença de esporopolenina no entorno destas proliferações intraloculares constituídas de poucas células indicaria a origem androgênica. Porém, não houve reação fluorocromática em nenhuma das seis secções submetidas ao teste, confirmando a origem diplóide.

4. A variação atribuída à genótipo-dependência incluiu as condições fisiológicas e o estádio de desenvolvimento da antera no momento do estabelecimento *in vitro*

Na maioria dos cultivos de antera, foram empregadas as cultivares listadas na tabela 2. A escolha destas cultivares visou testar genótipos o mais distintos possível, dentro do material disponibilizado pela Empresa Brasileira de Pesquisa Agropecuária (Embrapa). Apesar disso, somente comprovamos irrefutavelmente a ocorrência de androgênese em dois indivíduos da F2 do cruzamento BRQ96-3065 X BRSMG – Liderança.

A genótipo-dependência da resposta embriogênica foi comprovada por inúmeros autores (Ouédraogo *et al*., 1998; Barro e Martín, 1999; Rudolf *et al*.,1999; Silva *et al*., 2000). Mas ao longo dos nossos experimentos, a cultivar mais responsiva de um ano não repetiu a resposta superior no ano seguinte (dados não mostrados). Posteriormente, o estudo histológico (capítulo III) não evidenciou diferença nos eventos embriogênicos entre as quatro cultivares.

Efeitos genotípicos e ambientais que causam variação entre anteras do mesmo botão (heterocronia), entre botões do mesmo tamanho e entre ciclos de diferentes cultivares induzem ao estabelecimento *in vitro* de anteras em diferentes estádios de desenvolvimento, pois a seleção dos botões florais é feita pelo comprimento. Botões florais com 3 a 3,5 mm

apresentam anteras em estádios desde o final da meiose até pólen maduro (Lauxen *et al*., 2003). Uma vez que as diferenças fisiológicas entre anteras jovens e maduras podem ser responsáveis por diferentes respostas ao cultivo (capítulo III), podemos propor que a variação quantitativa significativa encontrada entre cultivares sofreu um efeito importante das condições fisiológicas do explante ao início do cultivo.

Tabela 2: Descrição sucinta das cultivares doadoras de anteras mais empregadas neste trabalho. Fonte: Reunião de Pesquisa da Soja da Região Sul (2003)

	Bragg	**IAS 5**	**MG/BR-6 – Conquista**	**BRS133**
Instituição de origem	Universidade da Flórida	Universidade da Carolina do Norte	Embrapa Soja, Epamig, Copamil, ABF	Embrapa Soja
País	EUA	EUA	Brasil	Brasil
Ano	1966	1973	-	1998
Área de cultivo	RS	RS e SC	SP, MG, GO, DF, MT, RO, TO, BA, RR	SC
Ciclo	Médio (140 dias)	Precoce (132 dias)	Precoce (128 dias)	Médio (136 dias)

As observações histológicas corroboraram as afirmações de Lauxen *et al*. (2003): enquanto o comprimento do botão for critério para seleção das anteras, não é possível testar isoladamente o efeito do genótipo. Assim, um teste com um grande número de cultivares, como sugerido por Kaltchuk-Santos (1999), forneceria informação limitada sobre os genótipos mais responsivos.

5. Eventos androgênicos não dependeram da presença da auxina 2,4-D

A auxina 2,4-D foi considerada essencial para a indução à androgênese em soja (Jian *et al*., 1986; Kaltchuk-Santos *et al*., 1997; Oliveira *et al*., 1998). Um teste com concentrações mais elevadas de 2,4-D foi sugerido para futuras tentativas de otimização da resposta androgênica (Oliveira *et al*., 1998; Kaltchuk-Santos, 1999).

Em explantes cotiledonares de soja, o 2,4-D é usado a 40 mg L^{-1} para induzir embriogênese somática (Bailey *et al*., 1993; Droste *et al*., 2001). Em tecidos estaminais, o

2,4-D foi capaz de desencadear embriogênese somática na concentração de 2 mg L^{-1} (capítulo III). Na concentração de 10 mg L^{-1}, o 2,4-D teve efeito significativo sobre a percentagem de explantes que originam calos e sobre a inviabilização dos micrósporos (capítulo IV).

Grãos de pólen com núcleos simétricos e extranumerários foram observados em botões florais armazenados a 4ºC, em proporções que não diferiram significativamente dos observados em anteras cultivadas na presença de 2 mg 2,4-D L^{-1} (capítulo V).

No cultivo de micrósporos, eventos claramente androgênicos foram observados na presença de 1 mg mL^{-1} do ácido triclorofenoxiacético (2,4,5-T) combinado a 1 mg mL^{-1} de cinetina, integrantes do meio PTA-15 modificado (Skinner e Liang, 1996) (capítulo VII). Em cultivos adicionais (dados não mostrados) micrósporos isolados nos meios B5 e MS contendo 2 mg 2,4-D L^{-1}, apresentaram intenso decréscimo de viabilidade e não foram registrados eventos androgênicos.

Assim, nossos resultados não confirmam que a androgênese em soja é favorecida pela presença de 2,4-D, tanto no cultivo de anteras quanto no cultivo de micrósporos isolados.

6. Divisões celulares associadas à androgênese ocorrem também *in vivo* e em botões florais armazenados a 4ºC

Inúmeros fatores desencadeadores de estresse são apontados na literatura como responsáveis por acionar a rota androgênica (Smýkal, 2000; Pechan e Smýkal, 2001). Em várias espécies vegetais, o pré-tratamento a 4ºC é comprovadamente predisponente à androgênese (Immonen e Antilla, 1999; Gu *et al*., 2004). No cultivo de anteras de soja, este pré-tratamento aumentou a freqüência de grãos multinucleados ao longo do cultivo (Kaltchuk-Santos *et al*., 1997). Entretanto, não há registro da ocorrência de divisões celulares atípicas, resultando em grãos de pólen com núcleos simétricos ou extranumerários, fora do ambiente *in vitro*.

A observação de eventos associados à androgênese em botões florais, tanto *in vivo* quanto armazenados a 4ºC (capítulo V) levantou hipóteses instigantes. O cultivo de anteras e o tratamento a 4ºC foram capazes de acionar o mesmo evento celular em alguns micrósporos, por isso, é possível que ambos atuem como fatores de estresse, e, como tal, precisariam cessar para viabilizar a continuidade do desenvolvimento esporofítico.

Em anteras de *Hordeum vulgare*, foi demonstrada uma forte correlação entre a

concentração de ácido abscísico (ABA) induzida por estresse e a viabilidade dos micrósporos (Wang *et al*., 2000), por isso, a degradação significativamente menor em botões armazenados a 4ºC pode ser resultante, tanto da ausência de proliferação dos estratos parietais e do conectivo, quanto da produção de ABA endógeno estimulado pelo estresse térmico.

De acordo com esta hipótese, é possível que as condições propostas anteriormente para a androgênese através do cultivo de anteras tenham dificultado a continuidade do desenvolvimento esporofítico em grãos de pólen multinucleados. Por isso, foram tão poucos os registros de grãos de pólen multicelulares neste sistema (Yin *et al*., 1982; Cardoso, 2002).

No cultivo de micrósporos e grãos de pólen isolados, foram testados regimes térmicos de incubação em meio B5 líquido. Um dos tratamentos foi a 4ºC por 20 horas mas não apresentou diferença em relação aos demais tratamentos (dados não mostrados). O tratamento a baixa temperatura também não teve sucesso quando aplicado em micrósporos isolados de *H. vulgare* (Wang *et al*., 2000).

7. Grãos de pólen podem formar núcleos extranumerários de formato fragmentado, parecendo células em morte celular programada

A análise de material corado com carmim foi utilizada por inúmeros autores para o acompanhamento de cultivos androgênicos (Guha e Maheshwari, 1966; Willcox *et al*., 1991; Kaltchuk-Santos *et al*., 1997). No caso da soja, o emprego do carmim é dificultado pelo pequeno tamanho do micrósporo e pela variação na intensidade de coloração.

Em análises do mesmo material com carmim e com o fluorocromo mitramicina (dados não mostrados), observamos que a reação fluorocromática oferece maior visibilidade quanto à morfologia dos núcleos: uma mesma amostra de micrósporos isolados foi dividida em duas partes e, naquela analisada em carmim, a proporção de grãos de pólen com núcleos simétricos foi inferior. Ou seja, em uma extensa contagem ao microscópio, núcleos atípicos podem não ser contabilizados como tal, quando corados com carmim.

Além de oferecer menor visibilidade da morfologia do núcleo, o carmim ainda não permite estimativa de viabilidade dos grãos de pólen.

O emprego de três técnicas de análise com o mesmo material (capítulo V) permitiu o registro de grãos de pólen com núcleos extranumerários de formato fragmentado, cuja proporção aumentava ao longo do tempo de cultivo e de armazenamento a 4ºC. Estes

núcleos atípicos são bastante similares aos que ocorrem durante a morte celular programada (Wang *et al*., 1996; Pennell e Lamb, 1997; Wang *et al*., 1999), e, por isso, associados a um evento degradativo. A morte celular programada foi registrada em células diplóides da antera *in vivo* durante a microgametogênese de *H. vulgare* (Wang *et al*., 1999). Na célula vegetativa do grão de pólen de várias espécies, também foi registrada a degradação de DNA plastidial *in vivo* (Clément e Pacini, 2001).

Desta forma, consideramos a hipótese de que, ao longo de muitos anos de pesquisa, a resposta androgênica de soja não foi otimizada, mesmo com a identificação de tratamentos que desencadeavam maior proporção de grãos de pólen multinucleados, porque estes tomavam uma rota degradativa. Independentemente da proporção de grãos de pólen responsivos, as condições de cultivo desfavoreciam a continuidade do desenvolvimento androgênico.

8. A comprovação da origem androgênica é mais segura por meio de marcadores moleculares codominantes do que por determinações do nível de ploidia

Em muitos estudos, a determinação do nível de ploidia é utilizada para atribuir origem androgênica a tecidos, brotações ou embriões regenerados do cultivo de anteras (Hughes *et al*.,1975; Zhao *et al*., 1998). Entretanto, o estudo de mais de duzentas espécies de plantas mostrou que variações no número cromossômico, incluindo diploidia, poliploidia e aneuploidia, são comuns entre plantas androgênicas obtidas de um mesmo cultivo (Henry, 1998).

A presença de células haplóides foi registrada anteriormente no cultivo de anteras de soja como um indicativo da origem androgênica de embriões (Zhao *et al*., 1998). Porém, em secções histológicas de calos conectivais (capítulo VI) observamos quatro células com número cromossômico bastante inferior a 40 (2n), aparentemente haplóides. Foi possível confirmar o nível haplóide (20 cromossomos) em apenas uma destas células através da contagem cromossômica, pois, nas demais, os diferentes planos de corte dificultaram distinção entre cromossomos.

Células haplóides também foram registradas durante a embriogênese somática no cultivo de anteras de *M. esculenta* (Woodward e Puonti-Kaerlas, 2001) e de *V. vinifera x V. rupestris* (Altamura *et al*., 1992) e em calos embriogênicos derivados de raízes de *Arabidopsis thaliana* (Yihua *et al.,* 2001). Yihua *et al.* (2001) comprovaram que tais células originaram-se de reduções similares às da meiose, também registradas *in vivo*, em

inúmeros casos, por exemplo, em pontas de raízes de *Vicia faba* (Chen *et al*., 2000).

Assim, determinações do nível de ploidia não oferecem inferências seguras sobre a origem androgênica. Por isso, é recomendável o emprego de marcadores moleculares codominantes para confirmação irrefutável da androgênese (Chani *et al*., 2000; Höfer *et al*., 2002; Wang *et al*., 2002).

9. O cultivo de micrósporos e grãos de pólen isolados é uma alternativa promissora para o estudo da androgênese em soja

A análise conjunta dos resultados deste trabalho permite a proposição de uma seqüência de fatos para explicar as limitações do cultivo de anteras de soja:

1º Eventos celulares tipicamente androgênicos ocorreram em baixa proporção dos micrósporos e grãos de pólen de soja *in vitro* (Yin *et al*., 1982; Kaltchuk-Santos *et al*., 1997).

2º As condições de cultivo desenvolvidas ao longo destas três décadas favoreceram a proliferação das células dos estratos parietais e do conectivo. Dentre estas condições, destacam-se o emprego da auxina 2,4-D e a indução sob a luz .

3º Além de competir *in vitro* por nutrientes, invadir ou comprimir o espaço intralocular e produzir grande quantidade de compostos fenólicos, a proliferação das células diplóides originou estruturas embriogênicas.

4º O estudo citológico em anteras fixadas em Farmer e coradas com carmim propiônico não permitiu inferências seguras sobre a resposta do explante como um todo, pois o esmagamento destruiu os tecidos que estavam se organizando e o padrão de coloração não permitiu inferências quanto à viabilidade dos micrósporos e grãos de pólen.

5º Calos conectivais não foram testados quanto à formação de estruturas embriogênicas, apesar da discordância entre autores. Enquanto alguns autores afirmaram que os tecidos da planta madura eram recalcitrantes (Saka *et al*., 1980), outros afirmaram que os calos primários de anteras eram diplóides (Yin *et al*., 1982; Ye *et al*., 1994).

6º Estudos que comprovaram respostas morfogênicas da antera diferentes da androgênese (Tabela 1) não foram incluídos nas revisões bibliográficas.

7º Ocorrendo em baixa proporção, tanto eventos celulares androgênicos, quanto estruturas embriogênicas, foram associados a uma mesma rota: os precursores dos embriões obtidos do cultivo de anteras seriam alguns dos grãos de pólen multinucleados (Kaltchuk-Santos *et al*., 1997). Entretanto, a intensa proliferação dos estratos parietais e do

conectivo foi o fator limitante à continuidade do desenvolvimento esporofítico dos grãos de pólen multinucleados.

Assim, os resultados apontaram que o cultivo de micrósporos isolados ofereceria condições mais adequadas ao estudo da androgênese em soja, eliminando os efeitos dos tecidos diplóides da antera. Entretanto, a troca de sistema parecia um obstáculo intransponível, tanto pela inexistência de publicações consistentes a respeito, quanto pela falta de equipes trabalhando com este sistema no país. Ainda acrescentou-se a estas limitações, as deficiências estruturais do laboratório.

Hu *et al*. (1996) referiram-se ao trabalho de D.P. Liu e G.L. Zhao (1986), os quais declararam ter obtido calos haplóides a partir do cultivo de micrósporos de soja isolados. Esta única referência, de três páginas, fez parte de uma publicação em chinês, não-indexada. Doze anos depois, em 1998, G.L. Zhao publicou em inglês, junto com outros pesquisadores, um artigo declarando a obtenção de plantas a partir do cultivo de anteras, as quais ainda estavam sendo analisadas. Dada a importância econômica da soja, o abandono do cultivo de micrósporos sugere que D.P. Liu e G.L. Zhao não tiveram sucesso com a técnica, uma vez que, na década de 1980, poucos laboratórios tinham condições de isolar adequadamente micrósporos de espécies que não apresentam abertura estomial espontânea ou induzida *in vitro*.

A época e as condições políticas da China em que D.P. Liu e G.L. Zhao executaram estes cultivos, a obtenção não-procedente de calos (discordante da maioria dos trabalhos em que não há mediação de calos), a falta de registro fotográfico e a própria descontinuidade da pesquisa comprovam que o trabalho destes pesquisadores chineses teve limitações, principalmente pelo desconhecimento do potencial morfogênico das células diplóides da antera. Lamentavelmente, não foi possível estabelecer contato direto com estes pesquisadores, nem mesmo por meio da "Internet".

As tentativas de contato com pesquisadores europeus que trabalham com este sistema foram infrutíferas. Apenas os doutores João Carlos Silva Dias (Instituto Superior de Agronomia – Universidade Técnica de Lisboa - Portugal) e Jan B. M. Custers (Plant Research International B.V. – The Netherlands) responderam a algumas mensagens. O Dr Jan B. M. Custers informou que estava cultivando plantas de soja em casa de vegetação para dar início ao cultivo de micrósporos isolados, dentro de um grande projeto de pesquisa que incluía outras espécies. Entretanto, ele deixou de responder nossas mensagens. Assim, outro obstáculo ao avanço dos nossos cultivos foi a falta de contato com pesquisadores

experientes no assunto.

Por isso, o estabelecimento de uma técnica eficiente para o isolamento dos micrósporos foi um desafio muito grande, pela falta de apoio de pesquisadores com experiência neste sistema. As estratégias de isolamento testadas fundamentaram-se apenas em artigos científicos. Exceto a malha de náilon de 37 μm, todo o material para isolamento e cultivo foi improvisado de acordo com as condições do laboratório.

Os testes iniciais para o isolamento de micrósporos e grãos de pólen de soja confirmaram observações feitas em outras espécies. Entretanto, a preparação do isolado é muito mais trabalhosa em soja do que nas espécies para as quais a técnica de isolamento está publicada (capítulo VII).

A partir da técnica de isolamento desenvolvida, diversas combinações de cultivares, meios de cultivo e regimes térmicos de incubação foram testados. Apesar dos meios B5 e MS serem recomendados para androgênese em cultivo de anteras de soja, grãos de pólen multicelulares foram observados apenas em meio PTA-15 modificado (Skinner e Liang, 1996).

PERSPECTIVAS

Por meio deste trabalho, é proposta uma revisão criteriosa dos conceitos estabelecidos nos trinta anos de pesquisa em cultivo de anteras de soja. Os resultados trazem um questionamento ao paradigma vigente para a resposta androgênica desta espécie.

Uma vez que este trabalho trouxe mais retificações aos conhecimentos anteriores do que novas constatações, os capítulos IV, V, VI e VII desta tese deixam problemas de pesquisa que poderão fundamentar futuros projetos.

O capítulo IV indica condições de cultivo que desfavorecem a proliferação dos tecidos diplóides da antera. Neste caso, condições de cultivo mais favoráveis à resposta dos micrósporos podem ser testadas.

O capítulo V demonstra de maneira inédita a ocorrência de eventos associados à androgênese fora do ambiente de cultivo, levantando hipóteses para uma nova discussão conceitual.

O capítulo VI propõe o emprego das anteras como explantes para a embriogênese somática, desde que sejam testados ajustes das condições de cultivo até o aperfeiçoamento

de um protocolo, com base no meio B5 e no emprego da combinação de 2,4-D e BA. Neste caso, a obtenção de plantas via embriogênese primária de origem multicelular serviria apenas para clonagem de indivíduos segregantes. A viabilização da embriogênese secundária de origem unicelular teria emprego mais amplo, permitindo trabalhos de manipulação genética.

O capítulo VII constitui-se em um primeiro registro do cultivo de micrósporos e grãos de pólen isolados, que, esperamos, subsidie futuros trabalhos de pesquisa de maior sucesso. Os poucos cultivos que executamos não permitem inferências mais consistentes. Devido à indisponibilidade de trabalhos a respeito, o estabelecimento de um protocolo requererá o teste de uma infinidade de condições de cultivo sobre inúmeros genótipos. Salientamos que o cultivo de micrósporos isolados de soja é muito mais trabalhoso do que de espécies para as quais os protocolos já estão disponíveis há décadas, como *Zea mays* e *Brassica* sp.

Tal como no início deste trabalho, não podemos afirmar quanto esforço de pesquisa ainda será necessário até a obtenção de haplóides e duplo-haplóides de soja. Entretanto, agora temos maior clareza quanto às limitações e às potencialidades desta espécie nos cultivos que visam desencadear androgênese.

CAPÍTULO IX

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