HELIA, **19**, Nr. 25, p.p. 39-46, (1996) UDC 631.527.82:633.854.78

CALLUS INDUCTION AND SHOOT BUD FORMATION FROM CULTURED ANTHERS IN SUNFLOWER (*Helianthus annuus* L.)

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Received: March 1, 1996
Accepted: November 26, 1996

SUMMARY

Cold pretreated anthers with uninucleate microspores of "KBSH-1", "BSH-1" and "Morden" genotypes of sunflower, under dark incubation, resulted in maximum callus induction (>85%). The callus frequency from anthers of "KBSH-1" (92.80%) and "BSH-1" (92.70%) was almost similar on MS medium with 2 mg/l 2, 4-D and 1 mg/l BA, while in "Morden" it was the highest on medium with 1 mg/l each of NAA and BA. Uninucleate microspores of anthers divided symmetrically to give rise to multicellular proembryo-like bodies. Occasional shoot bud formation from cultured anthers of "BSH-1" and "KBSH-1" was observed on medium with 0.5 or 1.0 mg/l NAA and 1.0 mg/l BA. Calli of "BSH-1" subcultured on plain MS medium resulted in the formation of meristematic centres.

Key words: Sunflower, anther callus, multicellular proembryo, shoot bud formation.

INTRODUCTION

Haploid cells are of significance in the production of homozygous breeding lines, improved cultivars, genetic mapping (Snape, 1988), and efficient isolation of stable mutants (Ye et al., 1987) or transformants (Creissen et al., 1990). Of the several methods described in different species for production of haploid plants, anther culture is the most attractive and applicable to a wide range of plant species. Sunflower being variously self-incompatible and highly cross pollinated, haploids can be used to speed up the production of stable inbred lines. Production of androgenic haploids in sunflower has been reported (Assad et al., 1985; Mezzarobba and Jonard, 1986; Gurel et al., 1991; Thengane et al., 1994), but

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the responses are unsatisfactory due to genotype and explant specificity and very low level of plant regeneration from haploid tissues.

Keeping in view the specificity of androgenic responses, we report here callus induction, ontogeny and shoot bud formation from cultured anthers of two commercial hybrids "KBSH-1" (CMS-234Ax6D-1) and "BSH-1" (CMS-234A x RHA 274) and an open-pollinated variety, "Morden" (a selection from Cernianka-66 germplasm).

MATERIAL AND METHODS

Capitula of 2.0-2.5 cm diameter were collected from healthy field-grown plants. Peripheral rows (2-3) of disc florets of such capitula were examined for anther stage after they were squashed in acetocarmine (1.0%). The capitula containing anthers with uninucleate microspores were pretreated at 8°C for 48 h and surface sterilized in 0.2% (w/v) mercuric chloride. Intact anthers were dissected from 2-3 outer rows of disc florets and cultured on Murashige and Skoog (1962) agar medium (MS) with 30 g/l sucrose, pH 5.6-5.8 and supplemented with different auxins and cytokinins. Number of anthers placed in each treatment combination ranged from 80 to 90. The cultures were incubated at 25±2°C under 12 h photoperiod under fluorescent light (ca. 1000 lux) or in dark for two weeks before making observation on callus induction.

RESULTS AND DISCUSSION

Callus induction
Effect of cold pretreatment and incubation conditions

The medium combinations shown in Tables 1 and 2 are the same as in Table 3. Tables 1 and 2 were utilized to highlight the effect of cold pre-treatment and incubation condition. The frequency of callus induction presented in Table 1 was computed considering the number of responding anthers under cold pre-treatment over all media and incubation conditions. Similarly, Table 2 shows the effect of incubation condition over all media and cold pre-treatment. The frequency of callus induction from anthers varied due to pretreatment and incubation conditions. Cold pretreatment resulted in higher frequency of callus in all the three genotypes (Table 1). There may be an increase in endogenic auxin (IAA) content due to cold pretreatment as observed in barley anthers (Xu et al., 1993). Genotypic differences were also evident. Both positive (Thengane et al., 1994) and negative (Patil et al., 1993) effects of cold pretreatment on anther response have been noted earlier. According to Sunderland et al., (1984), cold pretreatment might disturb programmed microsporogenesis and allow embryogenesis by promoting senescence of anther walls and destroying the close association

between tapetum and pollen. Dark incubation had stimulatory effect on callus induction which resulted in increased callus to the extent of 13.6, 12.7 and 10.4 percent in "KBSH-1", "BSH-1" and "Morden", respectively, over light incubation (Table 2). Similar influence of dark incubation has been described not only on cultured anthers (Mix, 1985; Assad et al., 1985; Mezzarobba and Jonard, 1986) but also on hypocotyl explants (Paterson and Everett, 1985) of sunflower. The combination of cold pretreatment and dark incubation had synergistic effect on callus induction.

Effect of growth regulators

In the present study, MS medium with 2 mg/l 2,4-D alone induced callus in 63.7 percent of anthers, while addition of 1 mg/l BA resulted in substantial increase in callus frequency (93.2%). Very high callus frequency in "KBSH-1" (92.8%) and "BSH-1" (92.7%) anthers was found on medium with 2 mg/l 2,4-D and 1 mg/l BA. But for "Morden" responded best (97.2%) with 1 mg/l each of NAA and BA, indicating genotype specific requirement of growth regulators (Table 3). Effect of auxin-cytokinin balance, genotype specific interaction between them and incubation conditions on microspore cell division have been recognized in sunflower (Assad et al., 1985; Mezzarobba and Jonard, 1986; Patil et al., 1993; Thengane et al., 1994). Specific interactions were also noted in this study among genotypes, growth regulators and incubation conditions. "KBSH-1" produced maximum callus (97.2%) with 2 mg/l, 2,4-D and 1 mg/l BA under dark incubation, whereas in "BSH-1", the best response was obtained in 1 mg/l each of NAA and BA under light. However, irrespective of light or darkness, "Morden" had the highest callus frequency (97.6%) on 1 mg/l each of NAA and BA.

Table 1: Effect of cold* pretreatment on callus induction (%) in anthers of different sunflower genotypes.

Genotypes	Without cold	With cold	Mean (%) ±0.92
KBSH-1	69.8	82.1	77.8 (60.82)
BSH-1	72.0	78.6	76.0 (62.62)
Morden	77.4	84.5	81.8 (65.84)
Mean (%)	73.1	82.0	78.6 (63.09
±0.75	(63.23)	(62.96)	

* Anthers pretreated at 8°C for 48 h.
Figures in parantheses and S.Em are in arc sine values.

The type of callus varied depending on the genotype and growth regulators used. "KBSH-1" and "Morden" produced loose and translucent (Figure 1a) callus, while, "BSH-1" produced compact, organised and white callus (Figure 1b) on NAA containing media. The 2,4-D induced callus was compact and light yellow

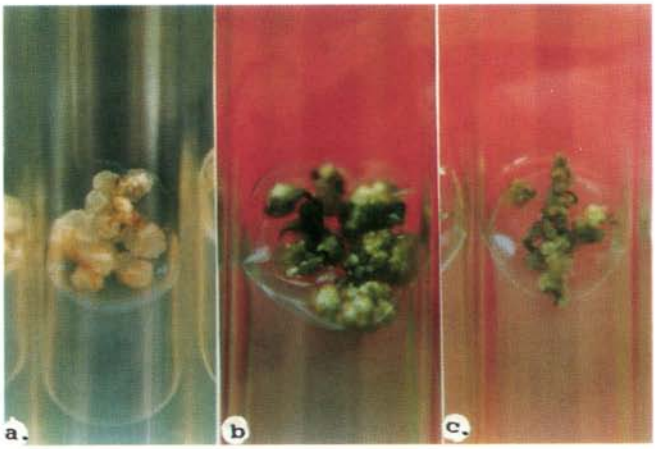

Figure 1. Callus induction from anthers in sunflower:
a. Loose translucent callus from "KBSH-1" on MS medium with NAA
b. Compact, organised and white callus from "BSH-1" on MS medium with NAA
c. Compact, light yellow-callus on MS medium with 2.4-D.

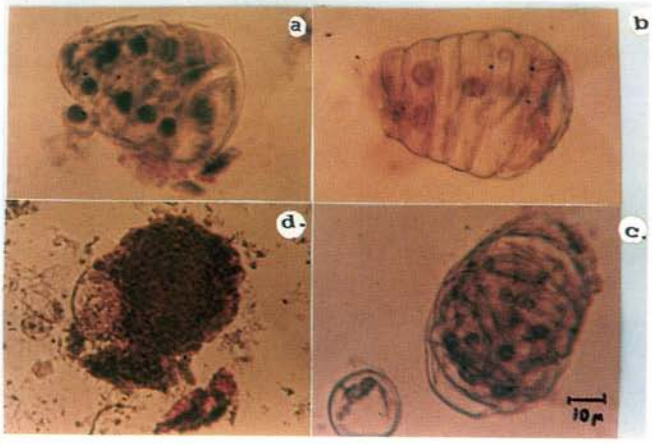

Figure 2. Ontogeny of cultured anthers of sunflower:
a. Multicellular pollen bodies in "Morden"
b. Multicellular pollen bodies in "BSH-1"
c. Multicellular pollen bodies in "KBSH-1" along with unresponded microspore
d. Early stage globular embryo in "KBSH-1".

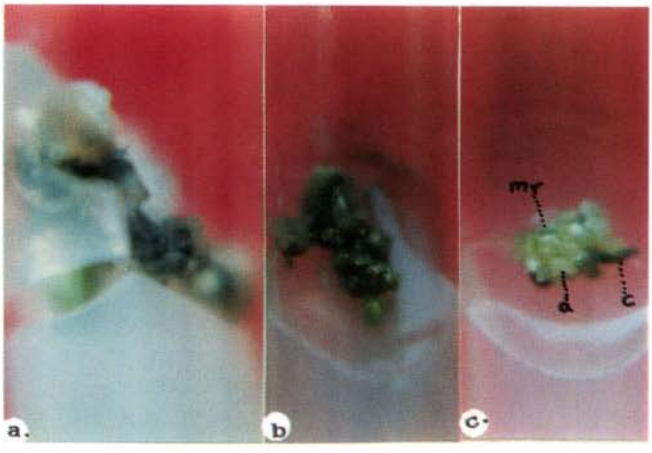

Figure 3. Plant regeneration in cultured anthers of sunflower:
a. Shoot bud formation in anther callus of "BSH-1" on MS medium with 1 mgl^{-1} NAA and BA
b. Shoot bud formation (leaf visible) in anthers of "KBSH-1" on basal MS medium
c. Development of meristematic centres from anthers of "BSH-1" on basal MS medium (mr -meristematic centre. a - anther, c - callus).

(Figure 1c) in all three genotypes. Thus, the type of callus depended more on growth regulators than on the genotype, as it was observed in the study of Novosa and Tuchin (1988).

Table 2: Mean (%) callus induction from anthers of sunflower incubated in dark and in light conditions.

Genotypes	Light	Dark	Mean (%) ±0.92
KBSH-1	70.2	83.8	77.8 (60.82)
BSH-1	68.4	81.1	76.0 (62.62)
Morden	75.8	86.2	81.8 (65.84)
Mean (%)	71.7	83.0	78.6 (63.09)
±0.75	(62.10)	(63.09)	

Figures in parantheses and S.Em are in arc sine values

Ontogeny

Cytological observations of microspore behaviour in cultured anthers indicated symmetric division of uninucleate microspores to give rise to two identical cells. Continued division of the two identical cells resulted in formation of multicellular structures, probably proembryos. This behaviour of microspores was found to be the same in all genotypes (Figures 2a-d), similar to the most common "B pathway" of pollen embryogenesis in tobacco (Sunderland and Wicks, 1971) and cassava anthers (Abraham et al., 1995).

Morphogenesis

Callus from cold pretreated anthers of "KBSH-1" and "BSH-1" on 0.5 mg/l NAA and 1 mg/l BA were cultured on MS medium without growth regulators. After five weeks on this medium, "KBSH-1" (5%) and "BSH-1" (5%) calli resulted in the formation of isolated shoot buds (Figure 3b) and meristematic centres (Figure 3c), respectively. In addition, cold pretreated anthers of "BSH-1" resulted in shoot bud formation in three percent of the cultures (Figure 3a) upon continuous incubation of callus for six weeks ind ark followed by four weeks in light on medium with 1 mg/l each of NAA and BA. Incidently, both "KBSH-1" and "BSH" hybrids have the same female parent (CMS-234A) indicating genotypic influences on regeneration as it has been shown by Assad et al., (1985), Mix (1985), and Thengane et al., (1994). Cold pretreatment is considered important for pollen embryo induction and plant regeneration (Thengane et al., 1994) as seen in the present study. Beneficial effects of combination of auxins and cytokinins have been recognized in many studies (Assad et al., 1985; Mix, 1985; Thengane et al., 1994) on sunflower. The response of anther-derived callus to morphogenic treatments with common auxins and cytokinins can be described as limited and eeratic. Studies with other synthetic growth regulators, levels of sucrose or other osmotically active compounds and their effect on endogenous levels of auxins and cytokinins are needed to understand the system and achieve better results.

Table 3: Frequency (%) of callus induction from anthers (with or without cold* pretreatment) in different sunflower genotypes under light and dark incubation.

Growth regulator (mg/l)	KBSH-1					BSH-1					Morden					Overall mean (%) ±1.19
	Without cold		With cold		Mean (%)	Without cold		With cold		Mean (%)	Without cold		With cold		Mean (%)	
	Light	Dark	Light	Dark		Light	Dark	Light	Dark		Light	Dark	Light	Dark		
2,4-D (1.0)	46.1	38.7	34.7	38.5	41.6	53.6	54.5	40.8	58.2	51.8	56.6	54.0	48.0	52.4	52.5	49.1 (43.67)
2,4-D (2.0)	59.3	54.0	68.8	59.8	60.6	40.8	72.4	66.3	69.3	62.0	70.0	66.3	69.1	67.0	68.2	63.7 (54.34)
2,4-D (2.0) + BA (1.0)	93.7	97.2	89.0	91.0	92.8	93.3	90.5	89.4	93.2	92.7	95.5	90.0	96.1	94.5	94.8	93.2 (77.15)
NAA (0.5) + BA (1.0)	71.2	81.1	77.8	89.5	85.7	75.0	71.0	71.1	89.1	82.7	75.0	90.0	76.0	97.0	91.7	87.0 (64.16)
NAA (1.0) + BA (1.0)	93.8	87.3	93.5	92.1	92.0	95.8	95.5	83.0	88.1	89.3	97.6	97.6	95.7	97.5	97.2	92.8 (76.13)
Mean (%)	69.1	70.7	71.6	85.2	77.8	68.5	75.7	68.3	83.6	76.0	76.4	78.5	75.2	89.6	81.8	78.6
±1.84	(60.94)	(60.87)	(59.83)	(61.62)	(60.82)	(63.35)	(63.74)	(57.84)	(65.55)	(62.62)	(65.97)	(64.48)	(64.64)	(68.26)	(65.84)	(63.09)

* Anthers pretreated at 8°C for 48 h.
Figures in parentheses and S.Em are in arc sine values.

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INDUCCION DEL CALLO Y FORMACIÓN DE YEMAS DE TALLO A PARTIR DE CULTIVO DE ANTERAS EN GIRASOL (*Helianthus annuus* L.)

RESUMEN

Anteras pretradas con frio con microsporas un nucleadas de los genotipos de girasol "KBSH-1", "BSH-1" y "Morden", en condiciones de incubación en la obscuridad dieron lugar a máxima inducción de callo (<85%). La frecuencia de callo de anteras de "KBSH-1" (92,80%) y "BSH-1" (92,70%) fue casi similar en medio MS con 2 mg/l de 2, 4-D y 1 mg/1 de NAA y BA. Las microsporas uninucleadas de anteras se divideron simétricamente para dar lugar a proembriones unicelulares. Se observó formación ocasonal de yemas de fallo de "BSH-1" y "KBSH-1" en medio con 0.5 a 1.0 mg de NAA y 1.0 mg/l BA. Loc callos de "BSH-1" cultivados en medio MS resultaron en la formación de centros meristemáticos.

INDUCTION DE CALS ET FORMATION DE POUSSES PAR CULTURE D'ANTHÈRES DE TOURNESOL (*Helianthus annuus* L.)

RÉSUMÉ

Des anthères au stade microspore uninuclé issues des génotypes "KBSH-1", "BSH-1" et "Morden", prétraitées par le froid, incubées à l'obscurité, conduisent à une induction callogène maximale (>85%). La fréquence des cals des anthères de "KBSH-1" (92.80%) et "BSH-1" (92.70%) était presque similaire sur le milieu MS avec 2 mg/l de 2-4D et l mg/l de NAA et BA., alors que chez "Morden" la fréquence était la plus forte sur un milieu avec 1 mg/l de NAA et de BA. Les microspores uninuclées des anthères se divisent symétriquement pour produire des structures proembryonnaires multicellulaires. On a observé des pousses occasionnelles chez "BSH-1" et "KBSH-1" par culture d'anthères sur milieux à 0.5 ou 1.0 mg/l de BA. Des repiquages successifs de cals de "BSH-1" sur milieu MS ont conduit à la formation de zones méristematiques.