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PLANT REGENERATION FROM COTYLEDONS DERIVED FROM MATURE SUNFLOWER SEEDS

Nestares, Graciela[1*]; Zorzoli, Roxana[1]; Mroginski, Luis[2] and Picardi, Liliana[1].

[1] *Cátedra de Genética, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario- C.I.U.N.R. - C.C. 14 - 2123 Zavalla - Argentina.*
[2] *Facultad de Ciencias Agrarias (U.N.N.E.) - Instituto de Botánica del Nordeste (I.B.O.N.E.) Sargento Cabral 2131 3400 Corrientes - Argentina*

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SUMMARY

The regeneration of whole fertile sunflower plants is highly variable and depends mainly on the genotype, though the nature of the explant and the hormone content of the culture medium are also important. Ten *Helianthus annuus* L. genotypes were evaluated *in vitro* for their morphogenic response: the inbred lines HA 300A, HA 89A, GP 762A, GP 762B, RHA 274, Rf 83-30, and the hybrids HPS-4, Morgan 733, ACA 884, and Contiflor 3. Cotyledons of mature seeds were used as explants. The basal medium was MS, to which different concentrations of growth regulators were added: *MI*, KIN 2 mg/l + IAA 1 mg/l ; *MII*, BA 0.5 mg/l + IAA 0.5 mg/l ; *MIII*, MI + 0.1 mg/l GA3 ; and *MIV*, *MII* + 0.1 mg/l GA3 . All media contained 200 mg/l of glutamine. Callus (C), regeneration (R), and hypertrophy (H) percentages were evaluated on the 30th day after initiation of the culture. The statistical analysis showed significant differences among genotypes and non-significant differences among media for R. Lack of independence for genotype-regeneration was also detected by the G-test.

Key words: *Helianthus annuus* L., in vitro culture, plant regeneration, cotyledon.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops worldwide. Consequently, there is interest in the development of genetic manipulation systems useful for the transfer of novel traits into the crop. The application of biotechnological methods for the improvement of sunflower is hampered by the difficulty of regenerating complete fertile plants. Reports have been published describing protocols such as particle bombardment (Hunold et al., 1995),

* Corresponding Author.

the use of *Agrobacterium* (Grayburn and Vick, 1995) or a combination of both (Knittel et al., 1994). One of the limiting factors for the application of any of these techniques resides in the availability of an efficient tissue culture regeneration system. Sunflower is considered to be a very "recalcitrant" species to tissue culture techniques (Freyssinet and Freyssinet, 1988). The first attempts to regenerate whole plants by direct organogenesis were made by Hendrickson (1954) using shoot tips. Paterson (1984) studied more than a hundred genotypes and demonstrated the requirement of cytokinin for shoot induction. She also showed that the optimum concentration of cytokinin varied with the genotype. Though several laboratories have obtained shoot regeneration from cultured hypocotyls, leaf pieces or cotyledons of young plantlets, the only explant allowing reproducible results with a large diversity of genotypes is the immature embryo. Its main drawback is that the use of this technique requires considerable time and effort.

At present the regeneration ability in sunflower is highly variable and depends on the nature of the explant, hormone content of the medium and mainly on the genotype. Ceriani et al. (1992) proposed the utilization of cotyledons as potential explants, since they have the advantages of being easy available and demanding little effort for their excision. The objective of the present study was to evaluate the *in vitro* morphogenic response of sunflower hybrids and the inbred lines frequently used in sunflower breeding programs.

MATERIALS AND METHODS

Ten genotypes, chosen because they sample types of material used in sunflower breeding, were used as source of explants. These genotypes were: three male-sterile inbred lines (HA 300A, HA 89A, GP 762A), one male fertile inbred line (GP 762B), two restorer inbred lines (RHA 274, Rf 83-30), and four hybrid varieties (HPS-4, Morgan 733, ACA 884, Contiflor 3). Cotyledons of mature seeds were used as explants. The enire seed material was sterilized for 30 s in 70 % ethanol and 15 min in 3 % sodium hypochlorite, and then rinsed three times with sterile distilled water. The basal medium was agar-solidified MS (Murashige and Skoog, 1962) supplemented with growth regulators at several concentrations: KIN 2 mg/l + IAA 1 mg/l (MI); BA 0.5 mg/l + IAA 0.5 mg/.l (MII); MI + 0.1 mg/l GA_3 (MIII); and MII + 0.1 mg/l GA_3 (MIV). All media also contained 200 mg/l of glutamine, and the pH was adjusted to 5.8 with 1 N NaOH prior to autoclaving. Cultures were grown at 25 ± 2ºC. Light was supplied by fluorescent bulbs with an 11-hr photoperiod. A minimum of 10 explants were cultured for each treatment and the experiments were repeated twice. The cultures were evaluated 30 days after their initiation. The regeneration ability of each genotype was scored by assessing the percentage of explants forming shoots (R). The percentage of explants developing callus (C) and the percentage of those showing hypertrophy of the tissue (H) were also scored . The X^2 test established the significance

of differences among genotypes and media. The G-test of Sokal and Rohlf (1986) was employed as test of independence for the factors genotype, medium, and regeneration ability.

RESULTS AND DISCUSSION

The response of the different genotypes was evident between days 20th and 25th of *in vitro* culture, when regeneration of shoots was observed. Plant regeneration was exclusively direct, without an intervening callus phase, irrespective of the culture medium analyzed. Some explants developed only callus but no plants could be regenerated from them since they had turned brown, whereas some other explants showed hypertrophy of the tissue. Table 1 shows the percentages of explants forming shoots for each treatment. Among the factors having an influence on the regeneration ability, the genotype is usually ranked high. Nine of the genotypes regenerated plants at least in one of the media utilized, and the response varied according to the genotype. The X^2 test showed significant genotypic differences for R in MI ($X^2 = 27.71$; $p< 0.01$), in MII ($X^2 = 21.36$; $p< 0.05$), in MIII ($X^2 = 31.72$; $p< 0.01$) and in MIV ($X^2 = 29.71$; $p< 0.01$). The male-sterile inbred line HA 300A demonstrated the highest regeneration potential. Knittel et al. (1991) observed a similar response for this genotype, using cotyledons of young plantlets as explants. The two lines GP 762A and GP 762B, male-sterile and male-fertile form, showed similar percentages of explants regenerating shoots (X2 = 0.23 ; ns, X2 = 1.23 ; ns, X2 = 1.46 ; ns, and X2 = 1.42 ; ns for MI, MII, MIII, and MIV, respectively). Consequently, presence or absence of the cms (cytoplasmic male sterility) trait had no visible influence on the regeneration ability under the conditions of the experiment.

Table 1: Percentages of explants forming shoots (R) for each genotype-media combination

Genotype	R_{MI}	R_{MII}	R_{MIII}	R_{MIV}
Morgan 733	0.0	17.6	0.0	0.0
Aca 884	17.4	24.1	7.1	17.2
Contiflor 3	8.0	10.7	10.3	23.0
HA 300A	41.7	35.0	36.7	42.8
HA 89A	22.2	27.3	29.4	22.2
GP 762A	9.0	0.0	10.0	9.1
GP 762B	4.8	8.3	0.0	0.0
Rh 274	28.6	36.8	13.6	31.6
Rf 83/30	11.8	16.7	5.0	0.0
HPS-4	3.4	0.0	0.0	7.1

MI : MS + 2 mg/l KIN + 1 mg/l IAA MIII: MI + 0.1 mg/l GA3MII: MS + 0.5 mg/l BA + 0.5 mg/l IAA
MIV: MII + 0.1 mg/l GA3

Non-significant differences were detected among the four media for the regeneration ability of each genotype, except for Morgan 733 ($X2 = 10.3$; $p< 0.05$). The effect of the addition of GA3 on R (MI versus MIII and MII versus MIV) was also analyzed. The only genotype that showed significant differences between MII and MIV was the restorer line Rf 83-30 ($X2=4.23$; $p< 0.05$), for which the GA3 diminished its regeneration ability. For the other inbred lines and hybrids the results were not statistically different indicating that GA3 had no effect on the regeneration ability of these genotypes.

Significant differences among genotypes were demonstrated for H ($X2 = 31.74$; $p< 0.01$, $X2 = 22.27$; $p< 0.01$, $X2 = 31.74$; $p< 0.01$, and $X2 = 21.09$; $p< 0.05$ for MI, MII, MIII, and MIV, respectively) and for C ($X2 = 24.91$; $p< 0.01$ and, $X2 = 28.33$; $p< 0.01$ for MI and MIII, respectively).

Table 2 shows the test of independence for the factors genotype, medium, and regeneration ability (G-test). The test demonstrated lack of independence for genotype-regeneration-medium; when each pair of factors was analyzed separately, genotype-regeneration showed dependency while genotype-medium and medium-regeneration were independent. No interaction among genotype-medium-regeneration was observed. These results, as those reported by others (Knittel et al., 1991 ; Kräuter and Friedt, 1991), evidence tight genotype dependence for plant regeneration in this species..

Table 2: **G -Test**: Test of independence for the factors genotype (G), medium (M) and regeneration ability (r)

Hypothesis	D.F.	G-value
G*M independence	27	14.52 ns
G*R independence	9	90.30 ***
M*R independence	3	2.76 ns
G*R*M interaction	27	31.96 ns
G*M*R independence	66	139.59 ***

ns: non-significant ; *** $p< 0.001$

Unfortunately, many shoots developed "vitreous" plants while others started to flower already *in vitro*. These two phenomena described by several authors (Witrzens et al., 1988 ; Pâques, 1991 ; Patil et al., 1993) are highly undesirable and lead to plant loss when transplanted to soil. The appearance of premature flower-heads generally does not allow the recovery of functional seeds while "vitreous" plants have very poor or missing root systems. Zorzoli et al. (1994) were able to reduce the premature flowering of plants obtained by embryo rescue by incubating them under 11-hr photoperiod. Although in the present study plants were grown under the same conditions, this problem could not be avoided.

These results suggest that sunflower cotyledons have potential for direct shoot organogenesis, though the regeneration ability is greatly influenced by the genotype. In order to make this system useful for transformation experiments,

this genotype-related regeneration potential could even be transferred from competent to non-competent lines by sexual crossing.

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REGENERACIÓN DE PLANTAS A PARTIR DE COTILEDONES DE SEMILLAS MADURAS DE GIRASOL

RESUMEN

La regeneración de plantas completas y viables en girasol es altamente variable dependiendo del tipo de explanto, de la composición del medio de cultivo y principalmente del genotipo. En el presente trabajo se analizó la respuesta morfogénica in vitro en 10 genotipos de Helianthus annuus L : las líneas HA 300A, HA 89A, GP 762A, GP 762B, RH 274, Rf 83-30, y los híbridos HPS-4, Morgan 733, ACA 884 y Contiflor 3. Como explanto se utilizaron cotiledones de semillas maduras. El medio basal fue MS con diferentes concentraciones de reguladores vegetales: 2 mg/l KIN + 1 mg/l AIA (MI) ; 0.5 mg/l BA + 0.5 mg/l AIA (MII) ; MI + 0.1mg/l AG3 (MIII) y MII + 0.1 mg/l AG3 (MIV). A todos los medios se les adicionó 200 mg/l de glutamina. A los 30 días de incubación in vitro se evaluó el porcentaje de regeneración (R), el porcentaje de callo (C) y el porcentaje de hipertrofia (H). En las pruebas de X2 se encontraron principalmente diferencias entre genotipos y no entre medios para R. La prueba de G detectó falta de independencia genotipo-regeneración. De estas pruebas se desprende que la capacidad de regeneración en esta especie evidencia una fuerte dependencia del genotipo.

RÉGÉNÉRATION DE PLANTES À PARTIR DE COTYLÉDONS DE SEMENCES MÛRES CHEZ LE TOURNESOL

RÉSUMÉ

La régénération de plantes complètes et viables chez le tournesol est hautement variable et dépend du type de l'éxplant, de la composition du milieu de la culture et principalement du génotype. Dans ce trevail-ci on enalysé la résponse morphogénique in vitro chez 10 génotypes de Helianthus annuus L: les lignées HA 300A, HA 89A, GP 762A, GP 762B, RH 274, Rf 83-30, et les hybrides HPS-4, Morgan 733, ACA 884 et Contiflor 3. Comme explant on a utilisé des cotylédons de semences mûres. Le milieu basal a été MS avec de différentes concentrations de régulateurs végétaux: 2 mg/l KIN + 1 mg/l AIA (MI) ; 0.5 mg/l BA + 0.5 mg/l AIA (MII) ; MI + 0.1mg/l AG3 (MIII) y MII + 0.1 mg/l AG3 (MIV). À tous les milieux on a ejouté 200 mg/l de glutamine. À 30 jours de l'incubation in vitro on a évalué le pourcentage de la régénération (R), le pourcentage de cal (C), et le pourcentage d'hypertrophie (H). Dans les épreuves de X2 on a trouvé principalement des différences entregénotypes et non entre les milieux pour R. L'épreuve de G a remarqué le manque d'indépendence génotype-régénération. De ces épreuves on remarque que la capacité de régénération dans cette espéce montre une forte dépendance du génotype.