

VERIFYING THE INFLUENCE OF THE CYTOPLASM ON THE REGENERATION ABILITY IN SUNFLOWER

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SUMMARY

Sunflower *in vitro* culture regeneration is highly dependent on the genotypic context and culture conditions. However, there is insufficient information about the effect of the cytoplasm on the regeneration ability of this species. The aim of this experiment was to evaluate the effect of different cytoplasmic background on the regeneration ability of HA89, a well known recalcitrant inbred line. Cotyledons of seven HA89-alloplasmic lines and the fertile HA89 inbred line were used as explants on four different culture media. After 36 days of incubation, the regeneration percentage, as well as the productivity and proliferation rates, was recorded. Genotype \times culture media interaction was detected in order to obtain regeneration percentage. Further analysis of this interaction demonstrated that the effect of the cytoplasm and the resulting interactions with the culture medium are key factors to be taken into account for better understanding of the *in vitro* culture performance of this species. Furthermore, non-nuclear genome could be considered as another source of variability modifying the regeneration ability of recalcitrant sunflower genotypes.

Key words: alloplasmic lines, AMMI models, *Helianthus annuus* L., *in vitro* culture

INTRODUCTION

Cytoplasmic male sterility (*cms*) in plants is defined as the maternally-inherited inability to produce functional pollen due to the mitochondrial (*mt*) genome constitution (Schnable and Wise, 1998). Male sterility is very useful for the production of hybrid seeds in cultivated species. Commercial sunflower hybrids are generally obtained using a single source of *cms*, PET1, which originates from cross-breeding between *Helianthus petiolaris* and *Helianthus annuus* (Leclercq, 1969). Recently, several *cms* backgrounds have been developed by intraspecific and interspecific

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crosses, which resulted in more than 70 *cms* sources being available at the moment (Serieys, 2002). Since these *cms* sources were identified, several experiments to estimate the influence of the cytoplasmic effect on important agronomic traits have been developed before their introgression into commercial breeding programs.

In vitro tissue culture has a great potential to be used in the process of breeding several species, given the availability of an efficient *in vitro* tissue culture protocol. Even though sunflower is known as recalcitrant to *in vitro* regeneration techniques (Rákosy-Tican *et al.*, 2007), fertile plants have been regenerated by organogenesis or somatic embryogenesis using different explants such as hypocotyls, leaves, cotyledons, meristematic apices and immature zygotic embryos (Kallerhoff and Alibert, 1996; Fambrini *et al.*, 2001; Vega *et al.*, 2007; Mayor *et al.*, 2010). Furthermore, several studies demonstrated that regeneration ability of these species depends on the genotype and the culture conditions (Nestares *et al.*, 1996; Baker *et al.*, 1999).

The effect of the nuclear genome on the regeneration ability in sunflower has been well documented (Nestares *et al.*, 1996; Nestares *et al.*, 2001; Mayor *et al.*, 2006). However, there are few reports about the cytoplasmic effect on the *in vitro* organogenesis. Nestares *et al.* (1998) found significant differences for regeneration ability between the male-sterile and male-fertile cytoplasmic states of a representative set of sunflower inbred lines, suggesting that cytoplasmic genes affect the expression of this trait.

The effect of different cytoplasmic sources in the *in vitro* response has been described in other crops as well, such as maize, barley, wheat, and potato (Nesticky *et al.*, 1983; Powell, 1988; Orlov and Pavilova, 1989; Singsit and Veilleux, 1989; Frei, 2003) suggesting that, besides environmental conditions, the nuclear and maternal inherited genome may modify the regeneration ability of sunflower. In this study, the effect of different cytoplasmic background on the regeneration ability in sunflower was assessed under different *in vitro* culture conditions.

MATERIALS AND METHODS

Plant material

Seven alloplasmic lines with the nuclear genotype HA89 were evaluated for their *in vitro* performance. The evaluated cytoplasmic sources and their origins were as follows: RES1 (*H. resinosus*), PEF1 (*H. petiolaris* ssp. *Fallax*), RIG (*H. rigidus*), MAX1 (*H. maximiliani*); fertile cytoplasm (*H. annuus*), PET1 (*H. petiolaris*), PET2 (*H. petiolaris*) and GIG1 (*H. giganteus*). The alloplasmic lines were developed by implementing introgression of different sources of cytoplasm into an elite inbred line (HA89) using backcrossing (Serieys, 2002). The fertile HA89 inbred line was used as a tester.

***In vitro* culture**

Seeds without pericarps were surface-sterilized in 98% ethanol for 30 sec, soaked in 5% sodium hypochlorite for 15 min, and then rinsed three times in sterile distilled water. The sterile seeds were germinated on the half-strength Murashige and Skoog's (MS) (1962) medium supplemented with 10 g l^{-1} of sucrose and solidified with 9 g l^{-1} of agar. Seeds were kept in darkness at $25 \pm 2^\circ\text{C}$ for 2 days before being transferred to light ($25 \pm 2^\circ\text{C}$ and 12 h photoperiod) for another 2 days.

Cotyledons were used as explants and placed on four different culture media. Each medium contained base salt, vitamins and iron chelates MS medium supplemented with of 30 g/l of sucrose, 6 g/l of agar and one of four different growth-regulating combinations:

- A: MS + 1 mg/l IAA (indol-3-acetic acid) + 2 mg/l Kinetin (6-furfurylaminopurine)
- B: MS + 1 mg/l IAA + 2 mg/l BAP (6-benzylaminopurine)
- C: MS + 1 mg/l NAA (α -naphthylacetic acid) + 2 mg/l Kinetin
- D: MS + 1 mg/l NAA + 2 mg/l BAP

Explants were incubated for 36 days in a growth chamber at $25 \pm 2^\circ\text{C}$, under 12 h daily illumination ($50 \mu\text{M m}^{-1}\text{s}^{-1}$), in a completely randomized design with 2 replications and 20 explants per replication.

Once the incubation period was completed, the following traits were evaluated:

- Regeneration percentage (RP): (explants that regenerated shoots/total explants) $\times 100$
- Productivity Rate (PROD): number of shoots/total explants
- Prolificity Rate (PROL): number of shoots/explants that regenerated shoots

Data analysis

What the data did not show was a normal distribution, so consequently they were transformed by $(x+0.5)^{1/2}$. The transformed data were analyzed by a two-way ANOVA using the following model:

$$X_{ij} = \mu + g_i + \varepsilon_j + (g\varepsilon)_{ij} + e$$

where μ is the experimental mean, g_i is the cytoplasm effect, ε_j is the culture medium effect, $(g\varepsilon)_{ij}$ is the cytoplasm-medium interaction effect and e is the experimental error.

The effect of the cytoplasm and different culture media on the regeneration ability were compared using Duncan's Multiple Range Test ($\alpha=0.05$) on the transformed data. The Additive Main effects and Multiplicative Interaction models (AMMI) were used for further analysis of the genotype by environmental interaction (Gauch, 1988; Zobel *et al.*, 1988; Gauch and Zobel, 1996). AMMI is a particularly relevant analysis used to understand the factors underlying two-way interactions better. In this study, each cytoplasm source was considered as a "genotype" (G) and different culture media were considered as "environments" (M) and both, AMMI1 and AMMI2

models, were used to analyze the cytoplasm \times culture medium ($G \times M$) interactions. The general AMMI model equation was as follows (Gauch, 1992):

$$Y_{ger} = \mu + g_g + \varepsilon_e + \sum n^{\lambda} n^{\gamma} \delta_{en} + \rho_{ge} + \varepsilon_{ger}$$

where the additive part is an ordinary ANOVA that leaves the interaction ($g\varepsilon$) as a non-additive residual. The multiplicative part of the AMMI model uses PCA (Principal Component Analysis) to decompose the interaction into orthogonal linear combinations (principal components). In this case we use AMMI1 and AMMI2 models for the first and second principal components respectively (Gauch, 1992). ANOVA, AMMI1 and AMMI2 analysis were performed using InfoGen Software (Balzarini and Di Renzo, 2003).

RESULTS AND DISCUSSION

The alloplasmic and fertile lines differed in prolificity rate (Table 1A). On average, the RES1 line produced 3.25 shoots per regenerating explants, two and half times more than the mean of others cytoplasms evaluated.

The environmental effect was assessed by different growth-regulating concentrations in the culture media. All genotypes in medium A had higher prolificity and productivity rates (Table 1b). These results suggest that indol-acetic acid and kinetin in the basal medium had a positive effect on the amount of regenerated shoots, regardless of the cytoplasm source considered.

Table 1: Mean values (data no transformed) of Regeneration Percentage (RP), Productivity Rate (PROD) and Prolificity Rate (PROL) of each cytoplasm through all media (section A) and mean values (data no transformed) of all cytoplasm in each medium (section B)

Section A			
Cytoplasm	RP	PROD	PROL
RES1	23.13 a	0.66 a	3.25 a
PEF1	28.29 a	0.48 a	2.46 ab
RIG	25.00 a	0.30 a	1.16 b
MAX1	41.56 a	0.24 a	0.84 b
Fertile cytoplasm	30.10 a	0.14 a	0.84 b
PET1	27.50 a	0.13 a	1.75 ab
PET2	31.28 a	0.50 a	1.33 ab
GIG1	28.75 a	0.37 a	1.14 b
Section B			
Culture media			
A	25.33 a	0.74 a	2.39 a
B	31.61 a	0.36 ab	1.76 ab
C	29.46 a	0.09 b	1.25 ab
D	31.41 a	0.21 b	0.98 b

Values followed by the same letter are not significantly different at $p=0.05$ (Duncan's multiple range test)

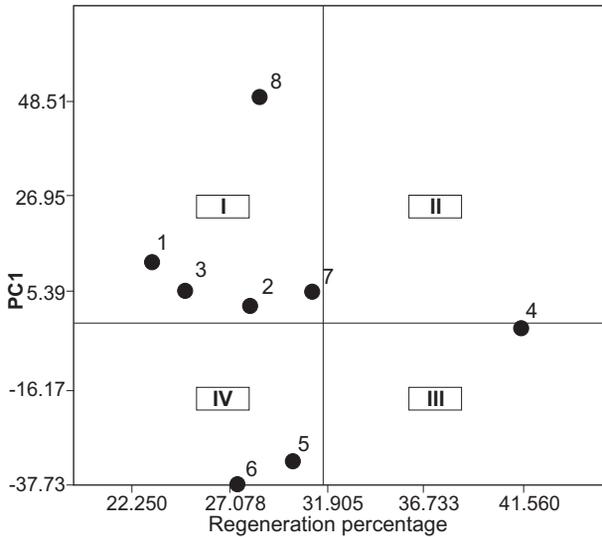


Figure 1: Biplot of the AMMI1 model for the Regeneration Percentage of the eight sunflower lines carrying different cytoplasms and cultured on four regeneration culture media. 1- RES1; 2- PEF1; 3- RIG; 4- MAX1; 5- Fertile cytoplasm; 6- PET1; 7- PET2; 8- GIG1

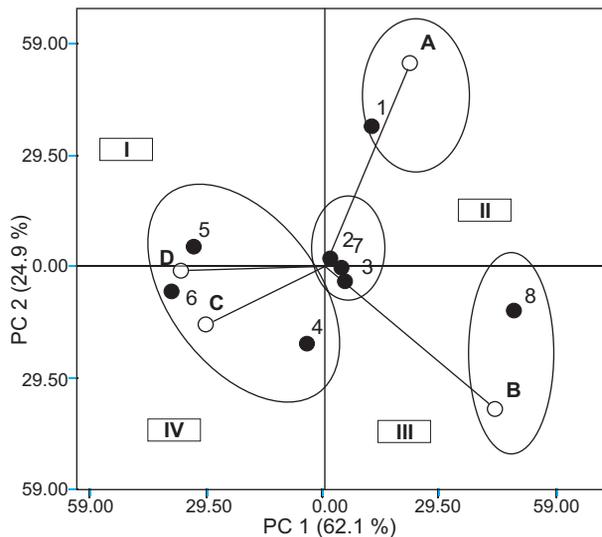


Figure 2: Biplot of the AMMI2 model for the Regeneration Percentage of eight sunflower lines carrying different cytoplasms and cultured on four regeneration culture media. 1-RES1; 2- PEF1; 3- RIG; 4- MAX1; 5- Fertile cytoplasm; 6- PET1; 7- PET2; 8- GIG1
 A: Medium A (1 mg/l IAA (indol-3-acetic acid) + 2 mg/l Kinetin (6-furfurylaminopurine)
 B: Medium B (1 mg/l IAA + 2 mg/l BAP (6-benzylaminopurine)
 C: Medium C (1 mg/l NAA (α -naphthylacetic acid) + 2 mg/l Kinetin)
 D: Medium D (1 mg/l NAA + 2 mg/l BAP)

The cytoplasm by culture media interaction was detected for the regeneration percentage ($F=3.14$, $p<0.001$). Also, the culture medium had an effect on the productivity rates ($F=6.74$, $p<0.001$).

For further understanding of the $G \times M$ interaction for RP, the AMMI1 and AMMI2 models were used. Biplot display of the AMMI1 analysis is shown in Figure 1 in which the overall mean RP values for the 8 cytoplasmic lines versus the PC1 (First Principal Component) scores were plotted. Genotypes with low regeneration capacity are in quadrants I (RES1; PEF1; RIG; PET2 and GIG1) and IV (Fertile line and PET1) and the genotype with high regeneration ability are in quadrants II and III (MAX1).

The magnitude and the signal of the PC1 scores also must be considered, *i.e.*, scores that are close to zero belong to cytoplasmic lines that have contributed very little to the interaction ($G \times M$). Thus MAX1 presented the higher regeneration ability (more than 40%) and performed as the most stable genotype of the set. Although PEF1, PET2, RIG and RES1 lines have a stable behaviour, they showed weaker regeneration ability than MAX1. The fertile cytoplasm, PET1 and GIG1 lines, had PC1 scores higher than the others cytoplasmic lines. However, they were mostly affected by the culture media growth regulators and therefore should be considered as specifically adapted to specific growth-regulating combinations.

The AMMI2 method not only allows the identification of stable cultivars by visual inspection, but also provides information on the environments. The information obtained from AMMI 2 biplot clearly showed the association between cytoplasmic lines and the culture media presented on the same graph. The first two axes of PC analysis of AMMI 2 explained 87% of interaction (Figure 2). As Crossa (1990) suggested, the PC1 represents the replicable effects of the $G \times M$ interaction, while the PC2 captures those effects with no defined pattern that may be otherwise considered as "noise". The GIG1 line was placed in quadrant III showing that it has good adaptation to culture medium B, as well as RES1 that has good adaptation to medium A. Both media were supplied with the same auxin (IAA) but they differed in the content of cytokinin. Thus, the type of cytokinins and its interaction with cytoplasmic background play a key role in the regeneration of these two alloplasmic lines. On the other hand, MAX1, PET1 and the fertile cytoplasm line had a good response to a greater range of culture media, specially with those that had the auxin NAA, independently of the level of cytokinin used. The most stable lines in this analysis were PEF1, RIG and PET2, followed by MAX1, HA89B and PET1. The lines with the greatest $G \times M$ effects were RES1 and GIG1.

Several reports about sunflower regeneration were focused on establishing the ideal concentration of growth regulators and the relationship between auxins and cytokinins (Paterson, 1984; Pugliesi *et al.* 1991). Also, several results about the interaction between the genotypes and the culture medium in the *in vitro* culture systems were reported (Barandiaran *et al.*, 1999; Nestares *et al.*, 2002). However, no reports of possible interaction between cytoplasm and culture media have been

published yet. This study demonstrates that the effect of cytoplasm, together with the nuclear genome and their resulting interactions with the culture medium are key factors to be considered for a better understanding of the *in vitro* culture in this species.

It is worth noting that the *in vitro* performance was not influenced by the origin of *cms* in this experiment. All the *cms* sources evaluated were originated by inter-specific cross-breeding between *H. annuus* and other wild relatives such as: *H. petiolaris* (PET1 and PET2), *H. petiolaris* ssp. *fallax* (PEF1), *H. resinosus* (RES), *H. rigidus* (RIG), *H. giganteus* (GIG1) and *H. maximiliani* (MAX1). The *in vitro* response observed had little to no relation with the origin of these cytoplasm. It is a well known fact that the PET1 and PET2 come from the same original cross-breeding, whereas PEF originated from the cross-breeding between different subspecies. Horn (2002) demonstrated that PEF1 and PET2 mitochondrial DNA had a clearly different organization than PET1. This fact might be associated with different responses observed in the *cms* sources used in this experiment. A similar response was observed with PEF1 and PET2, whereas the PET1 response seems to be similar to that observed in the fertile cytoplasm line.

Nestares *et al.* (1998) have pointed that cytoplasm substitution alone would not enhanced the plant regeneration capacity of recalcitrant genotypes. Nevertheless, the examination of interactions among the cytoplasm, nuclear genome and medium might be necessary for improving the regeneration ability in sunflower.

CONCLUSIONS

Our results have demonstrated that cytoplasm substitution in a genotype, together with a suitable formulation of the culture medium, must be considered to increase the regeneration ability of recalcitrant genotypes.

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RESUMEN

La regeneración *in vitro* del girasol es altamente dependiente del contexto genotípico y de las condiciones de cultivo. Sin embargo, existe muy poca información acerca del efecto del citoplasma sobre la capacidad de regeneración en esta especie. El objetivo de este trabajo fue evaluar el efecto de diferentes fondos citoplásmicos sobre la capacidad de regeneración de la línea endocriada recalcitrante conocida como HA89. Como explantos se utilizaron cotiledones de la línea endocriada HA89 fértil y de siete líneas aloplásmicas, los cuales fueron colocados en cuatro medios de cultivo diferentes. Luego de 36 días de incubación, se calcularon el porcentaje de regeneración, la productividad y la prolificidad. Se determinó la existencia de interacción genotipo \times medio de cultivo para el porcentaje de regeneración. El análisis de esta interacción demostró que el efecto del citoplasma y la interacción con el medio de cultivo son factores clave a considerar para lograr una mejor comprensión del desempeño de esta especie en cultivo *in vitro*. Además, el genoma no nuclear podría ser considerado como otra fuente de variabilidad que modifica la capacidad de regeneración de genotipos recalcitrantes de girasol.

RÉSUMÉ

La régénération *in vitro* du tournesol est très dépendante du génotype et des conditions de culture. »Cependant, il y a très peu d'informations sur l'effet du cytoplasme sur la capacité de régénération de cette espèce. L'objectif de ce travail était d'évaluer l'effet de différents fonds cytoplasmiques sur la capacité de régénération de la ligne consanguine récalcitrant connu comme HA89. Comme explants ont été utilisés les cotylédons de la ligne HA 89 fertile et sept lignes alloplastiques, qui ont été placés dans quatre milieux de culture différents. Après 36 jours d'incubation, nous avons calculé le pourcentage de régénération, de la productivité et de la prolificidad. Nous avons déterminé l'existence de l'interaction génotype \times moyenne de culture pour le pourcentage de régénération.

L'analyse de cette interaction a montré que l'effet du cytoplasme et l'interaction avec le milieu de culture sont des facteurs clés à considérer pour atteindre une meilleure compréhension du comportement de cette espèce dans la culture *in vitro*. Le génome no nucléaire pourrait être considéré comme une autre source de variabilité qui modifie la capacité de régénération des génotypes récalcitrants du tournesol.