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FLORET PRIMORDIA DIFFERENTIATION FROM *In vitro* CULTURED SUNFLOWER CAPITULA

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SUMMARY

In vitro culture has become a useful tool for studies concerning the assessment of physiological and biochemical factors controlling floral morphogenesis. For sunflower, *in vitro* techniques starting from different explants yield good results on culture initiation and plant regeneration. Although inflorescence generation on cultured shoots is sometimes achieved, there are no reports about *in vitro* florets developed from cultured young capitula. This work describes a protocol for the *in vitro* culture of sunflower reproductive meristems where floral organogenesis can be expressed.

Experiments were carried out using undifferentiated sunflower capitula excised from plants grown under greenhouse conditions. White (W) and Linsmaier and Skoog (LS) culture media were compared. LS medium induced a comparatively large capitula receptacle area expansion, reduced the explant hyperhydration and calli formation and was chosen for further experiments. The presence of casein hydrolysate (CH) in LS culture media was tested, resulting in reduced surgical stress and recovery of explants.

The addition of Kinetin to LS medium plus CH proved to induce differentiation of new primordia at the capitulum generative front. Explants cultured with 0.1 mg l^{-1} Kinetin only differentiated mother bract primordia. Explants cultured with 1 mg l^{-1} Kinetin showed larger expansion of the receptacle area and new floret primordia did not differentiate their mother bract. Intermediate levels of kinetin (0.5 and 0.8 mg l^{-1}) were tested in addition to two levels of IAA (1.3 and 2.5 mg l^{-1}). The combination 0.8 mg l^{-1} Kinetin and 2.5 mg l^{-1} IAA was capable of differentiating floret primordia that closely resembled those formed in the capitula developed *in vivo*.

Key words: **capitulum, casein hydrolysate, floral morphogenesis, *Helianthus*, *in vitro*, kinetin, sunflower**

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INTRODUCTION

In vitro culture of floral explants constitutes a useful tool for studying the physiological and biochemical process that control flower morphogenesis (Konar and Kitchlue, 1982). This technique is also useful for studying the influence that growth regulators, nutrients and the presence of vegetative structures might have in this process (Yaneheva *et al.*, 2003).

In vitro culture techniques have been widely applied to improve several features of many economically important crops (Jennings, 1976, and Borlaug, 1983, cited by Bidney and Scelonge, 1997). For sunflower, there has been progress in the establishment and *in vitro* regeneration of the plant from different vegetative explants (cotyledons, hypocotyls, immature embryos, etc.). At present, there is a limitation to complete, by means of this technique, the flowering process (Bidney and Scelonge, 1997).

There are evidences of *in vitro* culture in herbaceous species where fertile flowers have been successfully obtained, which were identical to those developed under natural conditions. Flowers were obtained after the regeneration of the whole plant (Pande *et al.*, 2002), from the culture of flower buds (Hicks and Sussex, 1970) or inflorescence segments (Ma *et al.*, 1996; Slawinska and Obendorf, 2001). Presently, there is no evidences of *in vitro* floret primordial differentiation from undifferentiated sunflower capitula.

It is assumed that, in plants, every organogenic process is influenced by the internal hormonal balance that, in *in vitro* systems, depends on the levels of growth regulators present in the culture medium (Vengadesan *et al.*, 2002; Yaneheva *et al.*, 2003). Accordingly, the level of carbon sources in the culture media, such as sucrose, greatly influences the organogenic response of the explant (Charrière *et al.*, 1999). Therefore, defining the correct balance of growth regulators and nutrients is one of the key issues to develop a protocol for good explant development (Charrière and Hahne, 1998).

The objective of this work was to develop an appropriate protocol to complete *in vitro* flower morphogenesis of sunflower partially differentiated capitula.

MATERIALS AND METHODS

Plant material

Experiments were carried out using *Helianthus annuus* L. plants of the commercial hybrid Dekasol 4030 (Monsanto®, Argentina). Plants were grown under semi controlled environmental conditions, with a light/dark regime of 18/6 h, air temperature of 25±3°C, photon flux of 280 μmol $s^{-1}m^{-2}$ PPFD and a relative humidity of about 60% in 2 l plastic pots that contained garden soil appropriately fertilized and maintained at field water capacity.

Culture media

Three culture media were used: White's basal medium (W) (White, 1963) supplemented with myo-inositol (100.0 mg l^{-1}), sucrose (20.0 g l^{-1}) and agar (6.0 g l^{-1}) at pH 5.5; Linsmaier and Skoog's basal medium (LS) (Linsmaier and Skoog, 1965) with half of major salts, myo-inositol (100.0 mg l^{-1}), sucrose (40.0 g l^{-1}) and agar (10.0 g l^{-1}) at pH 5.5; a control medium (C) formed by an agar support (6.0 g l^{-1}) at pH 6. The purpose of the last medium was to detect a post-dissection development of explants as a result of reserves accumulated in their tissues.

In later tests, the LS medium was supplemented with casein hydrolysate (CH, Sigma) at a concentration of 0.5 g l^{-1} (LS+CH) and pH 5.5.

Growth regulators

Using the basal media of LS+CH, treatments were carried out with growth regulators kinetin (Kin) and indoleacetic acid (IAA). Firts, two levels of Kin (1.0 and 0.1 mg l^{-1}) were used, and then four treatments were made combining two levels of Kin (0.5 and 0.8 mg l^{-1}) with two levels of IAA (1.3 and 2.5 mg l^{-1}), defined as: Kin+IAA-1 (0.5 mg l^{-1}+ 1.3 mg l^{-1}, respectively); Kin+IAA-2 (0.5 + 2.5); Kin+IAA-3 (0.8 + 1.3) and Kin+IAA-4 (0.8 + 2.5). The pH of all media was adjusted at 5.5 in each case. In all experiments, the culture media were sterilized by autoclaving at 1 atm during 20 minutes at 121°C and poured into 14 cm diameter Petri dishes, inside the laminar flow chamber.

Explant culture

The explants consisted of partially differentiated capitula dissected when they reached the floral stage (FS) 5 (Marc and Palmer, 1981), 19-21 days from seedling emergence and when the floral primordia started to differentiate over the edge of the receptacle rim (Figure1A). Also at this stage, the central meristematic region of the receptacle remains uncommitted but with the potentiality of differentiating floral primordia (Hernandez and Palmer, 1988; Hernandez and Green, 1993). The apices were sterilized by immersion in a Na hypochlorite solution (4.0 g l^{-1}active Cl) during 20 minutes followed by three rinses with sterile distilled water. The leaves and phyllaries that cover each meristem and the peduncle of the inflorescence were removed inside the laminar flow chamber.

Six capitula were inoculated in each box. The boxes were sealed and maintained at 25±1°C during a 16/8 hrs light/darkness photoperiod, under a fluorescent light that supplied 165 μmol s^{-1} m^{-2} PPFD.

Observations

From the beginning of the experiment and at 3-4 day intervals during 4-7 weeks, the diameter of the receptacles was measured and all explants had their FS evolution determined. Every morphogenic change was also recorded. The relative expansion rate of the capitulum meristematic surface (CRER) was calculated throughout the experiment from the diameter values of the receptacle according to equation 1.

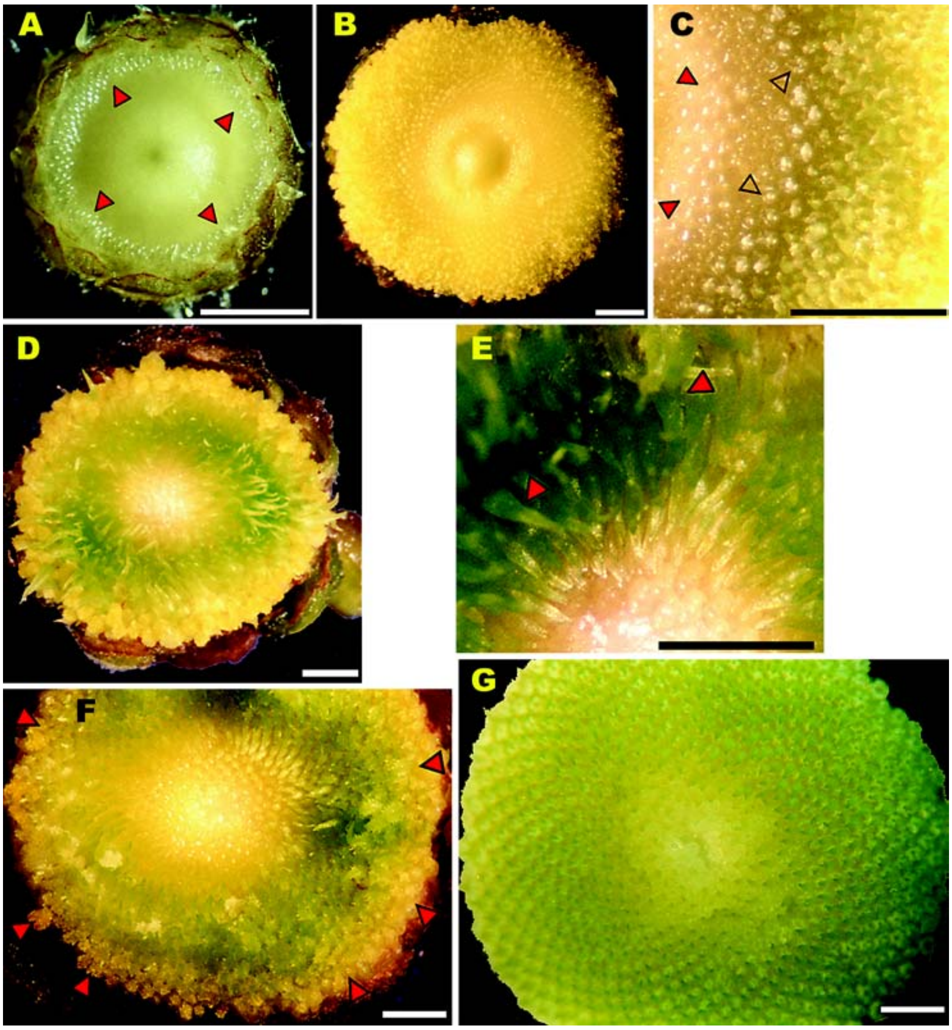

Figure 1: In vitro floret primordia differentiation in cultured capitula. ***A.*** *Capitulum at FS 5 after the initial inoculation with incipient primodia development at the receptacle rim (darts).* ***B.*** *Capitulum grown in 1.0 mg* l^{-1} *Kin media culture, showing the primordia differentiation up to the completion of FS 7.* ***C.*** *Detail of B, showing new bractless floret primordia (closed darts) and old primordia developing pentalobed corollas (open darts).* ***D.*** *Capitulum grown in 0.1 mg* l^{-1} *Kin media culture showing excessive development of primordia bracts (the corolla is absent) up to the completion of FS 7.* ***E.*** *Detail of* ***D.*** *Chlorophyll synthesis in primordia bracts is also observed at the receptacle center (arrows).* ***F.*** *Capitulum grown in 1.3 mg* l^{-1} *IAA+0.5 mg* l^{-1} *Kin media culture showing the differentiation of vegetative primordia up to the completion of FS 8 and the hyperhydration of the peripheral florets (darts).* ***G.*** *Capitulum grown in a 2.5 mg* l^{-1} *IAA+0.8 mg* l^{-1}*Kin culture media showing the normal development of floret primordia up to the completion of FS 8. Scale bars=1 mm.*

$$CRER = [RAt2 - RAt1] / \Delta t \qquad (1.)$$

Where: RAt1 and RA t2 correspond to the receptacle area (RA) in each measuring time (t1, t2, etc.) respectively and Δt corresponds to the time interval (in days) between two consecutive measurements.

RESULTS

Culture media comparison

The final area of the receptacle attained by the capitula inoculated in LS medium was significantly larger than those inoculated in W medium (Figure 2). The development of capitula in C medium, without growth regulators or nutrients, was negligible. This response demonstrated the impossibility of initial development of explants controlled by their own carbon or hormonal reserves present prior to dissection.

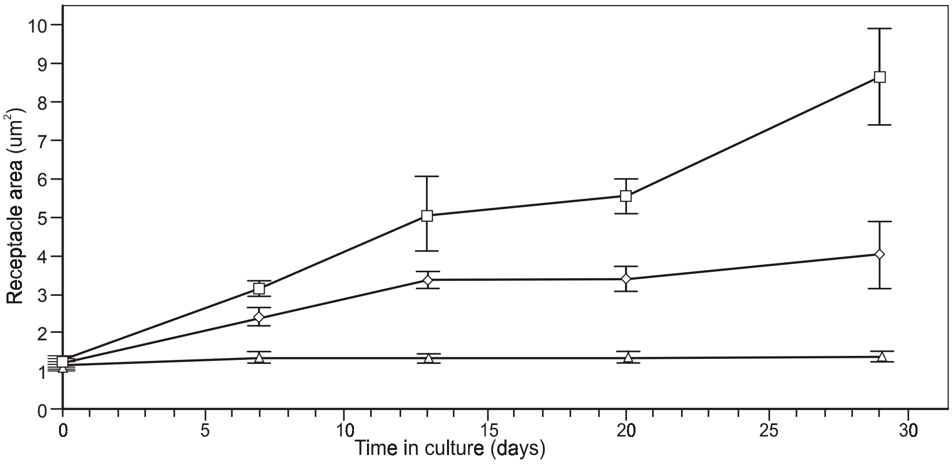

Figure 2: Effect of different culture media on undifferentiated capitula development. (▲) Control; (◆) White; (■) Linsmaier and Skoog. Vertical bars: ±1 SD.

The explants grown in LS medium presented, as compared with those in W and C media, a low percentage of callus formation and hyperhydration, remaining hydrated and with low oxidation throughout the observation period (Table 1). Even though these explants presented the growth of mother bracts, this development was moderate as compared with that of the capitula cultured in C and W media. Rooting was observed in none of the explants and none of the culture media.

Table 1: Morphological responses and quantification (%) in explants grown in different culture media

	Culture medium		
	Control	White	LS
Calli formation	91.7	91.7	25.0
Hyperhydration/Oxidation	75.0	54.0	16.6

Casein hydrolysate effect

The presence of CH in the LS basal medium proved to be adequate for the explant development. As from 30 days post culture, when the subculture was made, CRER of the explants grown in the CH media was significantly higher than that of the explants growing in a medium without this protein source (Figure 3).

Also, the general appearance of the explants grown in CH medium was highly acceptable. The meristems looked well hydrated, the mother bracts of the floral primordia had grown proportionally to the receptacle expansion and neither hyperhydration nor oxidation was noticed.

Growth regulators treatments

Kinetin

Kin addition to the media in the two concentrations tested resulted in the differentiation of floral primordia in the generative front (Palmer and Steer, 1985), which maintained the phylotactic order (Figure 1B and F). However, a differential response was observed between the treatments as regards receptacle expansion.

The capitula grown in media with 1 mg $l^{-1}$of Kin exhibited an important growth within the first 10 days of culture, which decreased but did not cease until the end of the experiment (Figure 4, Figure 1F). Conversely, the expansion of the receptacles in the capitula grown in media with 0.1 mg $l^{-1}$of Kin was significantly lower but almost constant during the whole experiment (Figure 4).

In both treatments, the expansion of the receptacles was accompanied by the formation of primordia. However, this morphogenic response had different characteristics according to Kin concentration in the culture media. In the capitula grown in media with 1 mg $l^{-1}$of Kin, the primordia already differentiated at inoculation time and they continued their development until they formed pentalobed corollas, but growth of mother bracts of flowers ceased (Figure 1C). The primordia differentiated *de novo* over the generative front did not elongate, the mother bract primordia were absent and they acquired a crown shaped appearance (Figures 1B and C).

Contrarily, in the capitula grown in media with 0.1 mg $l^{-1}$of Kin, the previously differentiated primordial mother bract showed an excessive development in detriment of the floret corolla differentiation (Figure 1D). It was also observed that developed bracts showed an intense green color (Figure 1D and E), assuming chlorophyll synthesis and reversion to the vegetative condition in that area of the explant. Also, the primordia differentiated *de novo* over the generative front developed a cone shape, becoming similar to the mother bract primordia (Figure 1E).

IAA - kinetin

The presence of IAA in the LS basal medium, supplemented with CH and Kin produced a good development of the explants. The explants grown in media with 2.5 mg l^{-1} of IAA were the ones that showed the larger receptacle expansion (Figure

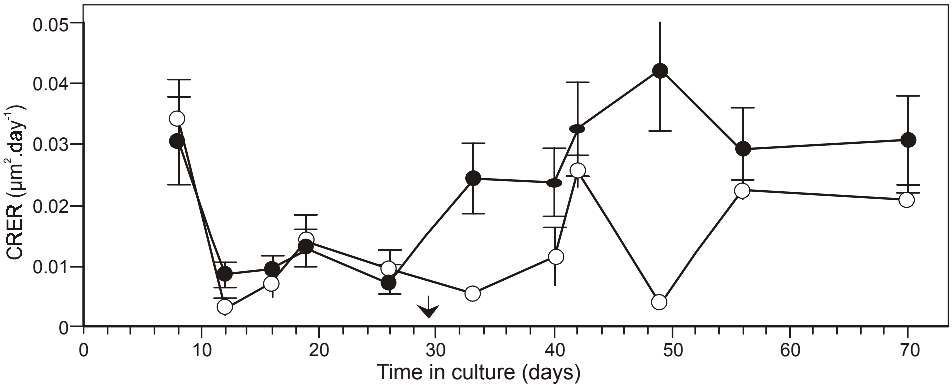

Figure 3: Capitula relative expansion rate (CRER = μm^2 day^{-1}) in LS basal medium with (●) and without (○) casein hydrolysate. Arrow indicates subculture time. Vertical bars:±1 SD

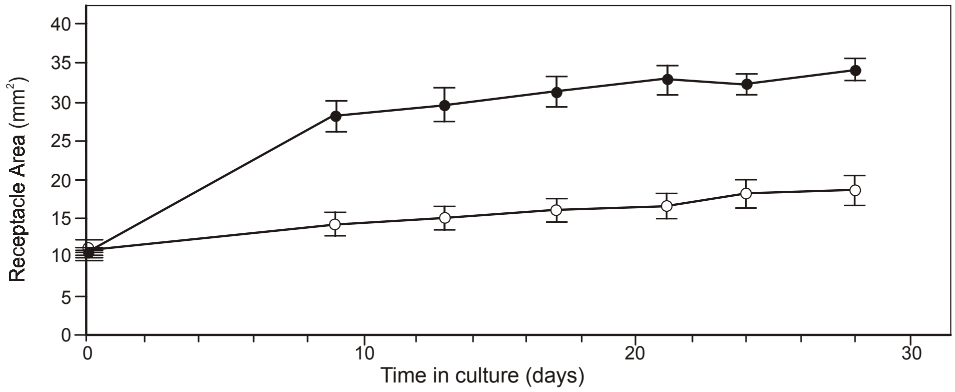

Figure 4: Effect of different doses of kinetin in LS culture medium on the receptacle area. (●) 1.0 mg l^{-1} Kin (○) 0.1 mg l^{-1} Kin. Vertical bars:±1 SD.

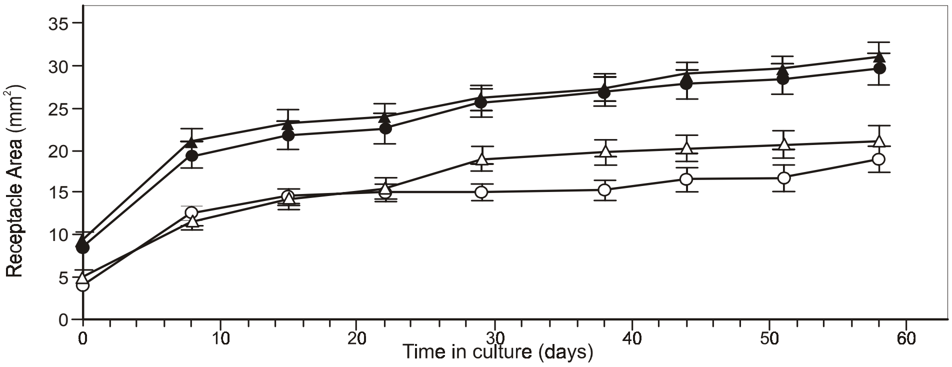

Figure 5: Combination of different levels of IAA and kinetin on the receptacle area. (○) Kin+IAA-1 (0.5 mg l^{-1}+1.3 mg l^{-1}); (●) Kin+IAA-2 (0.5+2.5); (○) Kin+IAA-3 (0.8+1.3); (▲) Kin+IAA-4 (0.8+2.5). Vertical bars:±1 SD

1G); at the same time, a complete formation of primordia in the generative front could be observed (Figure 1G).

The explants grown in Kin+IAA-4 media showed larger receptacle expansion and they were the only ones able to promote a complete development of the pair corolla-mother bract in each floret primordia (Figure 1F and G).

In the other treatments, the development of vegetative structures was predominant, especially of mother bracts of flower, though in the treatment with Kin+IAA-1, the corollas of the primordia previously formed showed an excessive development, acquiring a hyperhydrated appearance (Figure 1F).

DISCUSSION

The purpose of this work was to develop a protocol for *in vitro* culture of partially differentiated sunflower capitula, in which they could continue their growth and floral differentiation process started *in vivo*. The abundance of literature on this subject proves the conspicuous reversion of reproductive explants to the vegetative stage with the subsequent callus formation and generation of new shoots, as well as the most frequent morphogenic responses of *in vitro* culture (Konar and Kitchlue, 1982; Pierik, 1990; Bhojwani and Razdan, 1996). However, for our purpose, it was indispensable that the culture media promoted no callus or rooting in the explants.

The *in vitro* organogenic process typically has three steps. Changes in tissue organization prior to the emergence of a new organ in the explant must follow the sequential stages of induction, determination and morphogenesis (Yaneheva *et al.*, 2003). The technique of *in vitro* culture takes advantage from genetic stability characteristic of meristem cells to show, through the organogenic process, the intrinsic genetic formation of each taxon (Kieffer *et al.*, 2001; Vengadesan *et al.*, 2002).

The potentiality of floral buds to grow and differentiate in *in vitro* systems varies among species (Konar and Kitchlue, 1982) and depends, among other factors, on the development status of the explants (Saini and Jaiwal, 2002), on the presence of vegetative structures at the inoculation time (Kieffer *et al.*, 2001), on the nutritional and hormonal characteristics of the media (Charrière *et al.*, 1999; Yaneheva *et al.*, 2003) and on the culture conditions (Konar and Kitchlue, 1982).

In this sense, the use of sunflower capitula in FS 5 (Marc and Palmer, 1981) as explants, with no phyllaries nor peduncles attached to them, was based in previous studies that showed that these vegetative structures had, after a short period of *in vitro* culture, an excessive development in detriment of reproductive structures, posing a strong relationship, probably with nutrients in the culture media (Pellegrini and Hernandez, 2002).

When the explants were grown in an agar-water support media, they showed neither growth of the receptacle nor floret differentiation. After 15-day culture, the formation of compact callus was observed at the base of the receptacles. This experience allows to discard the action of reserves accumulated in the explants' parenchymatic tissues that might mask the action of the culture in their growth and development. Also, the lack of vegetative structures (e.g., phyllaries) was probably a determinant thereof. Many authors point out the importance of the partial presence of vegetative structures such as leaves (Caplin and Griesel, 1967) or stems segments (Nitsch and Nitsch, 1967) in the *in vitro* development of floral buds (Hicks *et al.*, 1981).

Literature states that W and LS are the most extensively used basal media for *in vitro* culture of flowers (Bhojwani and Razdan, 1996). In our case, the comparison of the explants response grown in both media with the appearance of important characteristics in the development of explants (callus formation capacity, hyperhydration and receptacle expansion) allowed us to define the LS basal medium as adequate for our purposes. These differential responses of the explants would be supported by the differences that these two basal media show in their composition.

At present, the causes of physiological disorders associated with hyperhydration remain undiscovered, but, as confirmed by our observations, some authors point out that the concentration of carbohydrate and gelling agents, pH variations and water content in the medium can be possible factors that contribute to this process (Yao *et al.*, 1996; Ueno *et al.*, 1998). In spite of agar being considered an inert substance which would apparently exert no osmotic effect over the system (Kyte, 1987), a high concentration of agar in the medium leaves less water available for explants through the increase of the matrix potential (Pierik, 1990), thus reducing the hyperhydration risk of explants (Bhojwani and

Razdan, 1996). Anyhow, there are references that showed successful cultivation of floral buds in liquid culture media (Ma *et al.*, 1996; Slawinska and Obendorf, 2001). Sucrose would act in the same way, though this would influence the system osmotically. The fact that the LS medium has twice the sucrose in the W media as well as a higher percentage of agar (1.0% and 0.6%, respectively) seems to explain, at least partly, the reduction of the hyperhydration phenomenon in the explants grown in the LS media (Pierik, 1990). When an explant is placed in an artificial medium, it becomes heterotrophic until it adapts to that medium and supplies itself. The role of certain organic substances in a culture medium, such as vitamins, hormones and proteins, is associated with the explant intrinsic requirement to achieve development. Adaptation and morphogenic response of the explants will depend on their presence (Kyte, 1987) as well as on the sucrose content (Charrière *et al.*, 1999). In this sense, the differences between sucrose content and type and quantity of vitamins in the media used in this work could explain the differential formation of callus in the explants grown in each medium (Table 1). It is also probable that the lack of other organic nutrients in both culture media would result in the absence of *de novo* formation of structures in the generative front of the capitula.

The incorporation of CH in the LS media, as protein source, was important and was reflected in good growth and development of the explants. The results obtained in our work confirm that the presence of CH in the media reduces post-dissection of the explants, thus improving the culture establishment (Raghavan, 1976). In this sense, it could be observed in this work that the expansion rate of the receptacle decreased after the inoculation in the media but the decrease was significantly lower than that observed in the explants grown in the media without CH (Figure 3). The same response was obtained after subculture (Figure 3). Also, the degree of development shown by mother bracts of the floret primordia as well as good hydration of the whole meristem are morphological characters which seem to confirm that CH presence improved the establishment of explants in the culture media.

The presence of growth regulators in an in vitro culture medium and their participation in floral initiation have been widely discussed (Bhojwani and Razdan, 1996). Their action mode is generally species-dependent (Konar and Kitchlue, 1982). For example, in Aquilegia, the presence of IAA is required for floral organs initiation (Tepfer et al., 1966), while Kin increases the number of initiated floral pieces (Bilderback, 1972). Hicks and Sussex (1970) proved that Kin was not necessary to promote the development of flower pieces in *Nicotiana*, but it was at later development stages to obtain *in vitro* culture flowers equal to those grown in natural conditions. In our work, the inclusion of Kin in the culture media was fundamental to induce the morphogenic process over the generative front of the receptacle. Both Kin concentrations compared promoted different responses in the explants. At low Kin concentrations, the development of vegetative structures overrides that of the reproductive ones. This situation was opposite to that observed with high concentrations of this growth regulator. These results agree with those found by Nitsch and Nitsch (1967) and Hicks *et al.* (1981), who pointed out the direct effect of Kin in the induction and *in vitro* floral development. Konov *et al.* (1998) also used the inducing effect of cytokinins to promote the formation of *de novo* primordia of sunflower leaves.

It has been widely proved that, in higher plants, morphogenic processes depend on the balance of growth regulators, especially cytokinins/auxins (Charrière *et al.*, 1999). However, the mechanism by which these relationships are translated into morphogenic responses still remains unknown (Charrière and Hahne, 1998). The partial vegetative reversion observed under some culture conditions here, is a phenomenon frequently observed in the *in vitro* culture of reproductive structures (Konar and Kitchlue, 1982). Some authors attribute this phenomenon to a response of the explants to a severe or very early dissection, or to an inadequate photoperiodic regime (Hicks and Sussex, 1970). The combination of Kin intermediate values with two levels of IAA offered better results than those obtained with only one growth regulator, even though the combination with high Kin and IAA concentrations showed to be most adequate. Floret primordia differentiated in the whole meristem while the development of the mother bracts and the primordial corollas already differentiated at the inoculation time was not altered. The differentiation of floret primordia in the generative front was similar to that of the floret primordia formed in the capitula developed *in vivo*.

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DIFERENCIACIÓN DE PRIMORDIOS FLORALES EN CAPÍTULOS DE GIRASOL CULTIVADOS *In vitro*

RESUMEN

El cultivo *in vitro* se ha transformado en una herramienta importante en los estudios concernientes a los factores fisiológicos y bioquímicos que controlan la morfogénesis floral. Para el girasol, las técnicas *in vitro*, a partir de diferentes explantos, han producido buenos resultados en la iniciación del cultivo y en la regeneración de plantas. Sin embargo, aunque se ha logrado generar inflorescencias en plantas cultivadas *in vitro*, no existen informes acerca del desarrollo de primordios florales diferenciados a partir de capítulos jóvenes cultivados *in vitro*.

Este trabajo describe un protocolo para el cultivo *in vitro* de meristemas reproductivos de girasol en los que pueda expresarse la organogenesis floral.

Las experiencias se realizaron utilizando capítulos indiferenciados de girasol, disectados de plantas crecidas en invernáculo. Se compararon los medios de cultivo de White (W) y de Linsmaier y Skoog (LS). El medio LS indujo a una mayor expansión del área del receptáculo de los capítulos, redujo la vitrificación de los explantos y la formación de callos, y fue seleccionado para las siguientes experiencias. El agregado de hidrolizado de caseína al medio LS resultó beneficioso en la recuperación de los explantos y redujo el estrés de la disección.

La incorporación de kinetina al medio LS con hidrolizado de caseína indujo a la diferenciación de nuevos primordios en el frente generativo. Los explantos cultivados con 0.1 mg l^{-1} de kinetina sólo diferenciaron primordios de brácteas. Los explantos cultivados con 1 mg l^{-1} de kinetina mostraron mayor expansión del área del receptáculo y los nuevos primordios no diferencairon la bráctea madre. Se probaron niveles intermedios de kinetina (0.5 y 0.8 mg l^{-1}) en combinación con dos niveles de IAA (1.3 y 2.5 mg l^{-1}), resultando que 0.8 mg l^{-1} de kinetina y 2.5 mg l^{-1} de IAA, fue la combinación capaz de diferenciar primordios florales que se asemejan fielmente a los desarrollados en capítulos normales.

DIFFÉRENTIATION DES PRIMORDIA DES FLEURONS SUR DE CAPITULES DU TOURNESOL CULTIVÉS *In vitro*

RÉSUMÉ

La culture de tissus *in vitro* est devenue un outil important pour étudier les facteurs physiologiques et biochimiques qu'interviennent dans le contrôle de la morphogenèse florale. Chez le tournesol, les techniques *in vitro* initié avec différents explantats donnent des bons résultats pour l'initiation des cultures et la régénération de plants. Bien que la génération d'inflorescences sur des tiges cultivées *in vitro* a été achevée, il n'y a pas de registres sur le développement des primordia des fleurons sur de jeunes capitula cultivés *in vitro*.

Ce travail décrit un protocole expérimental concernant la culture *in vitro* de méristèmes reproductives du tournesol dans les quels l'organogenèse peut être exprimée.

Les essais ont été réalisés fait avec des capitules indifférenciés de tournesol, prélevés des plantes cultivées dans une serre. Le milieu de culture de White (W) et celui de Linsmaier et Skoog (LS) ont été comparés. Le milieu LS induit une plus grande expansion de la surface du réceptacle des capitules, réduit la vitrification des explantats et la formation des cals et il a été choisi pour les essais suivants. L'effet d'ajouter un hydrolysé de caséine au milieu LS a eu un effet avantageux pour la récupération des explantats et la réduction du stress souffert par la dissection.

L'incorporation de kinétine au milieu LS avec hydrolysé de caséine induit la génération de nouvelles ébauches dans la surface générative. Les explantats cultivés avec 0.1 mg l^{-1} de kinétine ont différencié seulement des ébauches de bractées. Les explantats cultivés avec 1.0 mg l^{-1} de kinétine ont montré une plus grande expansion de la surface du réceptacle et les nouvelles ébauches n'ont pas différencié la bractée de la base. Des niveaux intermédiaires de kinétine (0.5 et 0.8 mg l^{-1}) en association avec deux niveaux d'AIA (1.3 et 2.5 mg l^{-1}) ont été testés. L'association de 0.8 mg l^{-1} de kinétine et 2.5 mg l^{-1} d'AIA conduit à la différentiation des ébauches de fleurons fidèlement semblables a ceux des capitules normaux.