

The effect of auxins in inducing organogenesis or somatic embryogenesis in mature sunflower zygotic embryo derived apex

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Abstract

Induction of shoots or of somatic embryos is the key step for gaining the morphogenetic potential in sunflower (*Helianthus annuus* L.), species known as recalcitrant to *in vitro* regeneration. In the immature zygotic embryo derived tissues or in other juvenile tissues resulted from seedlings, the acquisition of the competence for regeneration can be achieved directly by cytokinin treatment or by preconditioning the explants on cytokinin containing medium. In this paper is presented a new type of explant for sunflower *in vitro* culture, consisting of the apex with primordial leaves, resulted from ungerminated mature zygotic embryo, in which a specific morphogenetic response was triggered by the exogenously applied auxins. Among the auxins tested, indole-3-acetic acid, indole-3-butyric acid and 1-naphthaleneacetic acid are inducers of an organogenetic response, apical/axillary shoots and adventitious buds being regenerated while 2,4-dichlorophenoxyacetic acid, 3,6-dichloro-2-methoxybenzoic acid and 4-amino-3,5,6-trichloropicolinic acid led to somatic embryo formation. Among the auxins tested only 4-amino-3,5,6-trichloropicolinic acid sustains the embryos development up to mature stage. A high amount of sucrose (120 g L⁻¹) supplied during the auxin treatment promotes the maturation of the embryos directly on the induction medium for all tested auxins with embryogenic effect. These findings show that regardless of the type of morphogenetic response aimed in sunflower meristematic tissues resulted from mature embryos, the presence of auxins is mandatory.

Keywords: apex with primordial leaves; ungerminated mature embryo

Abbreviations: 4-amino-3,5,6-trichloropicolinic (picloram); apex with primordial leaves (A-PL); auxins inducers of embryogenesis (AIE); auxins inducers of organogenesis (AIO); 3,6-dichloro-2-methoxybenzoic acid (dicamba); 2,4-dichlorophenoxyacetic acid (2,4-D); immature zygotic embryo (IZE); indole-3-acetic acid (IAA); indole-3-butyric acid (IBA); 1-naphthaleneacetic acid (NAA)

Introduction

In plant tissue culture the induction can be defined as the key step in which a group of cells, under the influence of external stimulus, become competent for morphogenesis (Potrykus, 1990). Further in the process the cell can become determined either for caulogenesis, during which unipolar structures are formed, named

adventitious buds, or for embryogenesis, in which bipolar structures arise, the somatic embryos (Christianson and Warnick, 1983). Both ways of regeneration are equally valuable for biotechnological application in crop improvement programs (Kumar and Srivastava, 2015).

Despite of the numerous regeneration protocols established, sunflower continues to be considered a recalcitrant species to *in vitro* culture (Radonic *et al.*, 2015). For improving sunflower traits by genetic transformation, gene editing techniques or other biotechnological tools, the induction of competent cells for efficient plant regeneration is mandatory (Lewi *et al.*, 2006). Very often, depending on the genotype, the only explants which responded in culture were derived from immature zygotic embryo (IZE) (Finer, 1987; Power, 1987). Even if their morphogenetic potential has been proven, the age and size of the IZE are considered critical for the efficiency of regeneration (Witrzens *et al.*, 1988; Espinasse *et al.*, 1989; Sujatha and Prabakaran, 2001). This requirement makes them even more difficult to obtain, the entire process being laborious and time consuming (Sujatha *et al.*, 2012). For one particular sunflower genotype, the inbred line HA 300B, the IZE has become a valuable experimental model, both types of regeneration can be alternatively induced by the interplays between the osmolarity of the medium due to the supplied carbon source (Bronner *et al.*, 1994; Jeannin *et al.*, 1995; Jeannin *et al.*, 1998) and the endogenous and exogenous growth regulators (Charriere and Hahne, 1998). However, in the routinely developed protocols for sunflower regeneration, IZE derived explants have the tendency to develop somatic embryos (Finer, 1987; Freyssinet and Freyssinet, 1988; Fiore *et al.*, 1997; Sujatha and Prabakaran, 2001). In the case of using explants from mature germinated seeds such are embryonic axis (Paterson, 1984; Power, 1987; Konov *et al.*, 1998; Hewezi *et al.*, 2003), cotyledons (Knittel *et al.*, 1991; Sujatha *et al.*, 2012) and hypocotyls (Ozyigit *et al.*, 2002) organogenesis predominates, despite of the numerous combinations and types of the growth regulators applied. Only a few protocols were established for inducing organogenesis in sunflower young leaves (Paterson, 1984; Power, 1987; Konov *et al.*, 1998; Shin *et al.*, 2000; Zhang and Finer, 2015), but to the best of our knowledge there is no method for inducing embryogenesis in leaves. In primordial leaves resulted from germinated seeds the age of explant was an important factor, the buds regeneration efficiency decreasing with the increase of the explant age (Shin *et al.*, 2000). Also, for inducing shoots in an efficient manner from primary leaves of the young seedlings (7 days old) (Zhang and Finer, 2015) or from the young leaves resulted from the embryonic axis (Konov *et al.*, 1998), the originating explants had to be preconditioned on cytokinin-containing medium.

Although morphogenesis was possible in sunflower tissues resulted from immature embryos (Freyssinet and Freyssinet, 1988; Bronner *et al.*, 1994), germinated mature seeds (Paterson, 1984) or seedlings (Konov *et al.*, 1998; Shin *et al.*, 2000), on medium free of auxins, these compounds were considered, sometimes empirically, without preliminary tests, as necessary for inducing caulogenesis or embryogenesis in *in vitro* culture of sunflower. The requirements for exogenously applied auxin may depend on: genotype (Espinasse *et al.*, 1989), the presence of other growth regulators (Power, 1987; Espinasse *et al.*, 1989; Sujatha and Prabakaran, 2001; Sujatha *et al.*, 2012), the osmolarity of the medium / sucrose supply (Finer, 1987; Charriere and Hahne, 1998), the age and type of the explant and the regeneration step (Fiore *et al.*, 1997; Freyssinet and Freyssinet, 1988; Dhaka and Kothari, 2002). The specificity of the auxin type, as the solely controlling factor, for inducing a certain response in sunflower tissue culture was not emphasized. To our knowledge, auxins lead to different responses in relation to the age and type of the explants and to the culture media. For instance, IAA led to somatic embryogenesis or organogenesis (Charriere and Hahne, 1998; Dhaka and Kothari, 2002), while dicamba triggered both types of responses (Finer, 1987; Charriere and Hahne, 1998).

The aim of this study was to investigate the morphogenetic potential of a new type of explant, the apex with primordial leaves (A-PL), resulted from ungerminated mature zygotic embryos (UMZE), under various auxin treatments. The presence of auxins was necessary for inducing the competence for regeneration, which is considered the most important feature for biotechnological improvement of sunflower. Regarding the type of

the triggered morphogenetic response, the auxins were grouped in inducers of organogenesis (AIO) and inducers of embryogenesis (AIE).

Materials and Methods

In vitro culture

Mature seeds of sunflower (*Helianthus annuus* L., cultivar 'Florina'), provided by the National Agricultural Research and Development Institute, Fundulea, Romania, were randomly selected for excising the A-PL. The peeled seeds were sterilized following a previously described method (Rakosy-Tican *et al.*, 2007). The aseptic seeds were soaked in distilled water for about 2 h. For preparing the explants, the translucent seed coat and the thin endosperm were carefully removed and one of the cotyledons was discarded with caution not to wound the apex (Figure 1a - d). With the tip of a blade scalpel the A-PL (approximately 300 μm in length), was carefully sectioned and placed on the surface of the culture media.

The basal culture medium consists of Murashige and Skoog (1962) supplemented with 1 mg L^{-1} B₁ vitamin, 100 mg L^{-1} myo-inositol, 100 mg L^{-1} peptone, 50 mg L^{-1} yeast extract, 0.1 mg L^{-1} kinetin, 0.05 mg L^{-1} GA₃, 100 mg L^{-1} ammonium succinate, 5 g L^{-1} KNO₃, 6 g L^{-1} agar, and 30 g L^{-1} sucrose (Nagy and Maliga, 1976). The auxins were added individually, after media sterilization, in different concentrations as follows: IAA (0.5, 1 and 2 mg L^{-1}), NAA (0.5, 1 and 2 mg L^{-1}), IBA (0.5, 1 and 2 mg L^{-1}), 2,4-D (1, 2, 5 and 10 mg L^{-1}), picloram (5 and 10 mg L^{-1}) and dicamba (1, 2, 5 and 10 mg L^{-1}), as shown in Table 1. Certain auxin treatments (0.5 and 1 mg L^{-1} IAA, 1 mg L^{-1} 2,4-D, 2 mg L^{-1} dicamba, 2 and 5 mg L^{-1} picloram) were also tested in combination with a high amount of sucrose (120 g L^{-1}) (Table 1). The medium without auxins was considered the control. The cultures were maintained in a climate chamber in the dark at 25 °C.

Table 1. The concentrations of the auxins used individually in the basal medium containing 30 or 120 g L^{-1} sucrose

Auxin \ mg L^{-1}	0.5	1	2	5	10
IAA	30*, 120**	30, 120	30		
IBA	30	30	30		
NAA	30	30	30		
2,4-D		30, 120	30	30	30
Dicamba		30	30, 120	30	30
Picloram			30	30, 120	

*30 g L^{-1} sucrose; **120 g L^{-1} sucrose; in grey area – AIO, in white area – AIE

Microscopic observations

For assessing the embryonic nature of the spherical structures resulted on culture medium with 2,4-D, dicamba and picloram, the accumulation of starch and lipid reserves was used as indicator. For staining the starch granules, randomly selected globular structures resulted from the initial explants were prepared after the method described by Yeung (1998) with a minor modification, the tissue was squashed between the microscope slide and the cover slip in a drop of Lugol solution instead of being sectioned. Alternatively, for staining of the oil bodies, Sudan IV was used following the method described by Yeung (1998). The observations were made using the inverted microscope (Olympus CK-2).

Data collection and analysis

The cultures were observed weekly and at the 2nd and 4th weeks pictures were taken under stereomicroscope using a digital camera (Canon PowerShot A400). The percentage of explants showing

morphogenesis (organogenesis or embryogenesis) was calculated for each treatment. To assess organogenesis the regeneration of buds from pre-existing meristems (apical/axillary shoots) or *de novo* induction of adventitious buds were recorded. Each treatment had tree replicates with the exception of treatments with 120 g L⁻¹ sucrose where only two replicates were performed and each replicate consisted in 10 explants. Data were analyzed by t-test using R statistical software (Free Software Foundation's GNU General Public License). Significance for all statistical comparisons was set at $p \leq 0.05$.

Results

A-PL, resulted from ungerminated sunflower mature zygotic embryo, was used for selective induction of organogenesis or somatic embryogenesis. This is a complex explant containing the apical meristem and the two primordial leaves (Figure 1d). The competence for regenerating apical / axillary shoots, adventitious buds or somatic embryos was acquired only in the presence of exogenously supplied auxins and only in the explants containing the apical meristem and primordial leaves together (A-PL), as a unit. The explant wounding prevents the induction of any morphogenetic response. A specific type of regeneration could be triggered by applying a certain auxin. According to the response induced in the explants, the auxins, regardless of their concentration in the culture medium containing 30 g L⁻¹ sucrose, were divided in two groups, AIO comprising IAA, IBA and NAA and AIE which include 2,4-D, dicamba and picloram. *Induction of apical / axillary shoots*

On AIO containing medium, the morphogenesis was possible from the pre-existing meristems, resulting one apical and / or two axillary primary shoots (Figure 2a - c). After two weeks in culture, the apical / axillary shoots (Figure 2c) showed regeneration with the highest efficiency on medium supplemented with 0.5 mg L⁻¹ for each auxin belonging to AIO type. Up to 70% of the explants produced shoots on medium containing IAA (Table 2).). It was important not to detach the apical / axillary shoots from the explants, for many of the treatments the adventitious buds being induced on their stem (Figure 2a).

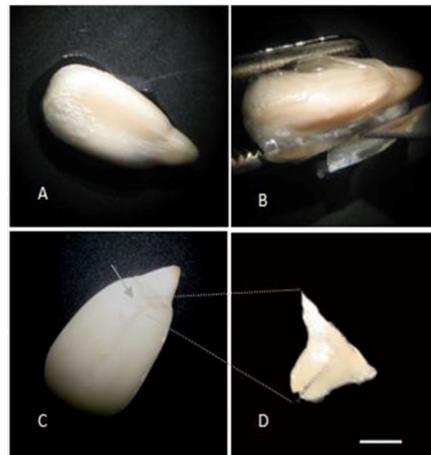


Figure 1. Preparation of the A-PL explant: mature sunflower seed (A), seed coat removal (B), seed after removal of one of the cotyledons, the A-PL is indicated by an arrow (C), A-PL, detail (D); bar – 200 μm

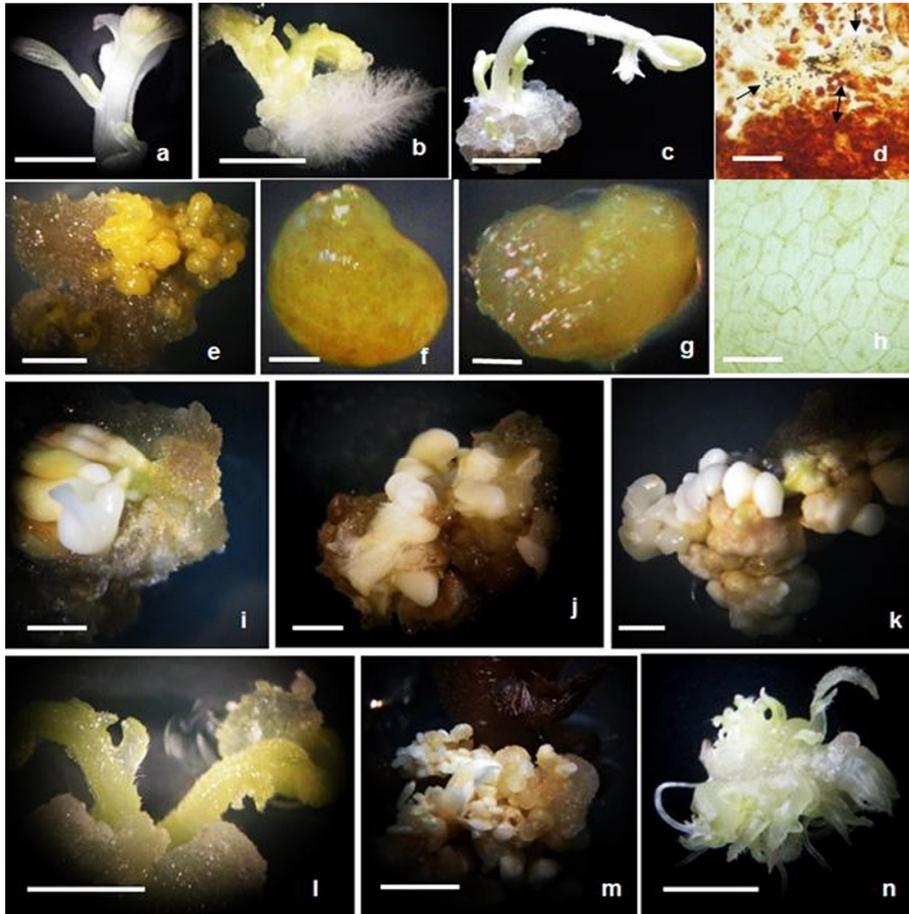


Figure 2. Morphogenesis of sunflower A-PL under different auxin treatments on normal (a-h) and high (i-n) sucrose concentrations: axillary shoot developed on the apical plantlet resulted after 4 weeks under 1 mg L^{-1} IAA treatment (a); adventitious buds on the primary leaves lamina for two weeks on 1 mg L^{-1} IBA (b); one apical and two axillary primary shoots developed after 4 weeks treatment with 2 mg L^{-1} IBA (c); embryonic cells presenting oil bodies stained in red with Sudan IV (double arrow) and large callus cells with blue-black starch granules stained with Lugol (arrow) (d); somatic embryos in early regeneration stage (two weeks) on 5 mg L^{-1} 2,4-D (e); detail of advanced globular (f) and cordiform embryos (g); epidermal cell monolayer of the cordiform embryo (h); mature embryos resulted after 4 weeks culture on high sucrose concentration under 1 mg L^{-1} 2,4-D (i), 5 mg L^{-1} picloram (j), and 2 mg L^{-1} dicamba treatments (k); adventitious buds on 1 mg L^{-1} IAA (l), cotyledons-like structures on 0.5 mg L^{-1} IAA (m), leaves on 0.5 mg L^{-1} IAA (n) resulted on medium containing 120 g L^{-1} sucrose; *Bar* $100 \mu\text{m}$ (d, h), $300 \mu\text{m}$ (f, g), 3 mm (i, j, k), 5 mm (c, e, l, m, n), 1 cm (a, b)

Induction of adventitious buds

Two weeks later, the induction of the adventitious buds was assessed. Among the AIO, IAA gave the best response regarding the frequency of explants regenerating adventitious buds for all the tested concentrations (0.5 , 1 , and 2 mg L^{-1}), followed by 0.5 and 1 mg L^{-1} IBA. These results were significantly better (t -test, $p < 0.05$) compared with those obtained on medium with NAA (Table 2). The buds were located also on the leaf lamina (Figure 2b) or on the petiole of the grown primordial leaves (Figure 2l) with no specificity in regard to auxin type, excepting the adventitious buds induced by NAA which were not regenerated on the leaf lamina at all. Although callus was obtained under different AIO treatments (results not presented here) no indirect shoot regeneration occurred.

Table 2. The effect of AIO (IAA, IBA, NAA) on sunflower A-PL organogenesis – from pre-existing meristems and induced *de novo*

Auxin concentration (mg L ⁻¹)	Explants forming apical / axillary shoots (% ± SE)	Explants forming adventitious buds (% ± SE)
Control (no auxin)	0 a	0 a
IAA		
0.50	70.0 ± 0.0 b <u>19.5 ± 0.0</u>	52.5 ± 9.6 b <u>0</u>
1.00	55.0 ± 2.8 c <u>19.5 ± 0.0</u>	40.0 ± 12.2 b <u>5.0 ± 0.0</u>
2.00	25.0 ± 2.8 d	45.0 ± 2.5 b
IBA		
0.5	37.5 ± 9.6 cd	37.5 ± 7.3 b
1.0	35.0 ± 8.4 cd	40.0 ± 3.5 b
2.0	22.5 ± 7.5 d	20.0 ± 4.7 c
NAA		
0.5	66.6 ± 9.4 b	10.0 ± 0.0 c
1.0	36.6 ± 4.7 cd	3.3 ± 2.7 d
2.0	23.3 ± 8.4 d	6.6 ± 5.4 cd

Note: The underlined values resulted on 120 g L⁻¹ sucrose treatment. Different letters show significant differences between auxin treatments (t test, p < 0.5)

Induction of somatic embryos

Supplementation of AIE (2,4-D, dicamba or picloram) in media containing 30 g L⁻¹ sucrose, led to the same type of response, the induction of somatic embryos (Table 3, Figure 2e - g). The induction of the embryos occurred mainly indirectly after 3 - 4 weeks of culture from the primordial leaves (Figure 2e). Different concentrations for each AIE were required for embryo induction; on 2,4-D the best response was for the lowest concentrations (0.5 and 1 mg L⁻¹, p < 0.05), while for dicamba a higher amount (10 mg L⁻¹) was needed for an efficient embryo induction. Picloram was highly effective at 2 and 5 mg L⁻¹, 100% of the explants regenerating somatic embryos. On 2,4-D and dicamba supplementation, only embryos in the initial developmental stages were obtained (globular and cordiform, Figure 2e - g), while on picloram the embryos developed up to the cotyledonary stage.

The role of supplementation with sucrose

Based on the fact that in sunflower IZE culture a high sucrose concentration was able to change the fate of morphogenesis (Jeannin *et al.*, 1995) alternatively, for IAA, 2,4-D, dicamba and picloram in concentrations which gave the best results, the role of a high amount of sucrose (120 g L⁻¹) on the morphogenetic response of the A-PL was also evaluated. In the presence of IAA (0.5 and 1 mg L⁻¹) which was the most suitable for inducing apical / axillary shoots development on media containing 30 g L⁻¹ sucrose, shoots resulted only sporadically on medium containing high amount of sucrose (Table 2). In the same time, the induction of the adventitious buds was almost inhibited (Table 2). A high sucrose concentration led instead to the formation of leaves or to structures similar in appearance with the cotyledons (white and thickened) (Figure 2l - n). No embryos with a normal structure were obtained (Figure 2l - n). When a high sucrose concentration was used simultaneously with AIE treatment, the development of embryos up to the cotyledonary stage was promoted (Figure 2i - k, Table 3).

Table 3. The effect of AIE (2,4-D, dicamba, picloram) on sunflower A-PL embryogenesis after 4 weeks in culture

Auxin concentration (mg L ⁻¹)	Explants forming embryos (% ± SE)
Control (No auxin)	0 a
2,4-D	
1.0	97.5 ± 2.1 b <u>85.0 ± 14.5</u>
2.0	93.3 ± 5.4 b
5.0	67.5 ± 8.1 c
10.0	40.0 ± 15.8 c
Dicamba	
1.0	10.0 ± 5.7 d
2.0	55.0 ± 25.9 bc <u>40.0 ± 10.5</u>
5.0	60.0 ± 23.0 bc
10.0	85.0 ± 2.8 bc
Picloram	
2.0	100 ± 0.0 b
5.0	100 ± 0.0 b <u>100 ± 0.0</u>

Note: The underlined values resulted on 120 g L⁻¹ sucrose treatment. Different letters show significant differences between auxin treatments (t test, p < 0.5)

Discussion

In preliminary trials the induction of a morphogenetic response in the sunflower A-PL resulted from ungerminated mature embryo was tested on culture media supplemented with different cytokinins (6-benzylaminopurine, kinetin or zeatin). No morphological structures compatible with plant regeneration (buds or somatic embryos) were obtained; in the best case only, leaves were regenerated on medium containing cytokinins (data not shown).

This study shows that A-PL requires exogenously applied auxins for gaining the morphogenetic potential. Organogenesis or somatic embryogenesis could be selectively induced by certain auxin treatments. In the experimental system established in this study IAA, IBA and NAA were AIO and 2,4-D, dicamba and picloram were AIE. On AIO, depending on the type of auxin the growth of apical and/or two axillary primary shoots was obtained, most probably directly from the pre-existing meristems similar to the culture of embryonic axis in which shoot morphogenesis was induced by preconditioning the explants on medium containing cytokinin (Konov *et al.*, 1998), or directly in the presence of cytokinin (Hewezi *et al.*, 2003). The most important aspect is the induction of adventitious shoot which was achieved in over 50% of the explants, on the best regenerating media containing 0.5 mg L⁻¹ IAA. This value can be considered very good in term of efficiency for sunflower morphogenesis, in several reports only a reduced number of explants responding in culture (Finer, 1987; Espinasse *et al.*, 1989). On the contrary, on picloram treatment the embryo's induction frequency reached 100%. Maintaining continuously the explants on the AIE led to proliferation of the embryos, which remained in the globular or cordiform stages when 2,4-D and dicamba were used, and developed up to the cotyledonary stage in case of using medium with picloram.

Until now, the only sunflower explants in which a specific type of regeneration could be induced in a controlled manner were represented by those resulted from IZE (Bronner *et al.*, 1994; Jeannin *et al.*, 1995; Jeannin *et al.*, 1998). The main factor involved in targeting a specific type of regeneration in IZE, was the osmolarity of the medium due to the concentration of sucrose. On high osmotic condition somatic embryos were induced, while on low osmotic medium shoots were obtained (Jeannin *et al.*, 1995). Compared to our results when regeneration was possible only in the presence of exogenously auxin, in the IZE culture the exogenously supplied auxin was not required for inducing a certain response, the presence of cytokinin as a solely growth regulator being mandatory for regeneration (Charriere and Hahne, 1998). By adding auxin, the response could be modified in relation to the osmolarity of the medium in the IZE (Charriere and Hahne, 1998). The requirement for auxin can be related to the developmental stage of the explants. In early stages of the IZE the endogenous auxin is synthesized in high concentrations, whereas during the embryo maturation lower concentrations were reported (Michalski, 1969). This could explain why in IZE the morphogenetic potential can be achieved without exogenous auxin supply, while the auxin is needed for getting similar results in mature embryo derived tissues. Indeed, the endogenous auxin participation was directly (Thomas *et al.*, 2002) or indirectly (Charriere and Hahne, 1998) proven in somatic embryo induction in sunflower IZE culture on media free of auxins.

Gaining the regeneration potential in sunflower was a continuous challenge and during time several protocols were established. For the majority of the methods the auxin was an important component of the culture media. Regardless of their type, somatic embryos or shoots were induced, the regeneration pathway being more related to other factors, the type of the explant having the highest influence on the way of the morphogenesis. Somatic embryos were induced by different auxins, IAA (Charriere and Hahne, 1998), NAA (Patterson and Everett, 1985; Pelissier *et al.*, 1990; Fiore *et al.*, 1997; Charriere and Hahne, 1998), 2,4-D (Finer, 1987; Sujatha and Prabakaran, 2001), dicamba (Finer, 1987) and picloram (Jayabalan and Chandrasekar, 2007), mostly in IZE derived tissues. In the same way, buds were developed under different auxin treatment: IAA (Nataraja and Ganapathi, 1989; Sujatha *et al.*, 2012), NAA (Power, 1987; Knittel *et al.*, 1991; Sujatha and Prabakaran, 2001; Hewezi *et al.*, 2003; Zhang and Finer, 2015), IBA (Nataraja and Ganapathi, 1989) and 2,4-D (Ozyigit *et al.*, 2007). Among the AIE, 2,4-D and dicamba were more appropriate for inducing somatic embryos from IZE (Finer, 1987). On the contrary, in the present study picloram led to the best results regarding both, the efficiency of regeneration (100%) and the development of somatic embryos, mature embryos being obtained on the induction medium. Among the AIO, IAA led to the best response regarding the both processes involving organogenesis - induction of the adventive meristems and shoot development, when primary shoots grow from the pre-existing meristems.

Although *in vitro* sunflower regeneration was induced in tissues of different origin, there is only one reference for using primordial leaves as explants resulted directly from germinated seeds with no other pre-treatments (Shin *et al.*, 2000). In that case the regeneration occurred via organogenesis on auxin free medium, while the presence of auxin significantly decreased the morphogenetic response (Shin *et al.*, 2000). The explants used in the present study become competent for regeneration only if auxin was supplemented in the culture medium. In this case, adventitious buds were induced on the primordial leaves which are part of a more complex explant, A-PL, derived from ungerminated embryos. This difference in response can be related to the developmental stage of the embryos. It could be explained by the fact that the germinated embryos have a high amount of endogenous auxin (Ni *et al.*, 2001) which can promote the first steps of regeneration. Another way of inducing the potential for regeneration in sunflower young leaves was by preconditioning the growth of the generating explants on medium containing cytokinin (Konov *et al.*, 1998; Zhang and Finer, 2015).

The high osmolarity of the culture medium, modulated by sugar supply can be one of the most important factors responsible for inducing somatic embryogenesis in sunflower (Jeannin *et al.*, 1995). Among the sugars tested sucrose showed the highest regeneration efficiency (Jeannin *et al.*, 1995; Sujatha and Prabakaran, 2001). In our case, on low sucrose concentration (30 g L^{-1}), either shoots or somatic embryos were

obtained, the auxin being the key factor for the determination of the morphogenetic response. On high sucrose concentration medium (120 g L^{-1}), although the type of regeneration wasn't changed in relation to AIO or AIE, the embryogenesis was sustained in the sense that the embryos developed on AIE up to the cotyledonary stage. On the IAA, even if no embryo was obtained, several cotyledon-like structures were induced, which suggests that using A-PL, a high sucrose concentration works exclusively as promoter of the embryo maturation by increasing the accumulation of the reserve substances rather than as an inducer of embryogenesis (Businge *et al.*, 2013).

The accumulation of the reserve substances is a prerequisite for somatic embryogenesis (Merkle *et al.*, 1995). To assess if the spherical structures that were obtained are true somatic embryos we looked for the presence of the starch granules since, in sunflower protoplasts, a positive correlation between the proportion of cells containing starch and the subsequently morphogenetic callus was found (Laparra *et al.*, 1997). No starch granules were present in the cells of the embryos, but they were rich in oil bodies (Fig. 2d). This was not surprising since during sunflower in planta IZE development the sugars are readily directed toward the fatty acid synthesis (Alonso *et al.*, 2007). The oil bodies weren't present in the callus cells surrounding the group of the embryos. Moreover, starch granules were spotted in the non-morphogenetic large cells of the callus, only. The epidermis of the embryos (Figure 2h), one of the first sign of embryonic organization (Finer, 1987), was also distinguished. Based on our findings, the presence of the oil bodies can be considered an indicator of the early embryogenesis stages in sunflower and they can be easily seen even without a specific staining. Squashing technique presents the advantage that is fast and informative enough, allowing the study of unicellular layers but the quality of the images is not that high (Figure 2d). The maintenance of the explants in the dark was beneficial for inducing embryogenesis as was highlighted already for sunflower *in vitro* culture (Fiore *et al.*, 1997).

By achieving the selective induction of the somatic embryos or buds in sunflower tissue culture this method may offer a good starting point for the development of an efficient protocol for plant regeneration. Further studies are necessary to demonstrate if this plasticity in the response which appears to be under the control of auxins is genotype dependent or is more related to the explant type.

Conclusions

Using a new type of explants for sunflower *in vitro* culture - A-PL, resulted from ungerminated mature embryos, the competence for somatic embryogenesis or caulogenesis can be efficiently induced by certain auxins. IAA and IBA were the most favourable auxins for inducing organogenesis, whereas picloram was the most efficient for inducing embryogenesis. A high sucrose concentration used in combination with EIA promoted the development and maturation of the embryos. The advantage of starting from ungerminated mature embryos is that the method is simple and fast – no germination or preconditioning is needed for acquiring the competence for morphogenesis. The mature seeds are available all year round, so the drawback of using IZE, which are difficult to obtain is overcome.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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