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A SIMPLE METHOD FOR SUNFLOWER *IN VITRO* REGENERATION STARTING FROM MERISTEMATIC TISSUES

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Abstract

Sunflower, an important agronomic species, also considered the symbol of summer in gardens, is often the subject of biotic stress. The breeding for resistance involving biotechnological tools require well established protocols for in vitro regeneration. Sunflower is, however, well known as a species recalcitrant to in vitro regeneration. The best responsive tissues originate from immature embryos. Tissues of other sources are usually characterized by poor regeneration or require intermediary steps of culture for gaining morphogenetic potential. Here we present a new method for in vitro culture of sunflower involving meristematic tissues resulted from mature embryos. The advantages of this method stand out - it requires mature embryos which are easy to obtain and consist in only two steps of culture on simple media. The regeneration potential of this type of explants is high - up to 100% of the explants regenerate plants. We can conclude that the method developed here for in vitro sunflower regeneration holds promises for solving the regeneration problems of sunflower and can be applied, with slight optimization, for other plant species.

Key words*: meristem, organogenesis, rhizogenesis, sunflower, ungerminated mature embryo.*

INTRODUCTION

Sunflower is one of the most important oil-producing crops, ranking on the fourth place after soybean, rapeseed and groundnut in term of production per year (Dagustu, 2018).
The main interest in sunflower is the oil, used primarily as edible oil. In the near future, it is seen as a renewable source of biodiesel, a clean alternative to regular petroleum-based fuels. The seeds per se are also important for consumption or can be processed for different products. Although of less economic importance, sunflower hybrids are also highly appreciated by the general population for their ornamental attributes (Seiler & Gulya, 2016).
To fulfil all the requirements for such a high diversity of utilization and also to gain resistance to several biotic and abiotic stressors who jeopardise the crops, sunflower was the subject of a comprehensive genetic improvement during the time, mainly through conventional breeding (Seiler & Gulya, 2016).
However, the lack of genetic resources diminishes the chances for obtaining varieties with high resistance to diseases and pests, drought tolerance and salty soils, or to improve the oil quality (Dagustu, 2018). New technologies are needed to increase the genetic variability of sunflower. The biotechnologies involving tissues culture and genetic engineering could be a useful approach for creating genetic variation. Reliable *in vitro* morphogenetic techniques for improving sunflower qualities are necessary in order to fully exploit the information provided by the newly available genome assembly for this species combined with the new genome-editing techniques (Lewi et al., 2006).
Although a plethora of methods was established for sunflower *in vitro* culture, it continues to be considered a species with high recalcitrance to morphogenesis (Dagustu, 2018; Radonic et al., 2015). The morphogenetic potential is highly dependent on intrinsic factors like genotype, explant type and age or extrinsic factors such are culture media components, light and temperature (Deglene et al., 1997; Espinasse & Lay, 1989).
The only explants allowing reproducible results in a genotype-independent manner for sunflower are those resulting from immature

zygotic embryo although the technique requires considerable time and effort (Finer, 1987; Hewezi et al., 2003; Power, 1987; Sujatha & Prabakaran, 2001). Applying different methods, it was demonstrated that the explants resulted from germinated seeds or from plantlets lose their regeneration ability compared with those resulted from embryos (Finer, 1987; Power, 1987; Shin et al., 2000). The conclusion is that the morphogenetic capacity declines rapidly by increasing the age of the explants (Knittel et al., 1991).

The aim of our study was to establish a fast, simple and reliable method for *in vitro* regeneration of sunflower starting from meristematic tissue originating from ungerminated mature embryos. The role of the amount of nitrogen supplied in the culture media upon sunflower *in vitro* morphogenesis and growing was briefly tested.

MATERIALS AND METHODS

Sunflower seeds belonging to the hybrid Florina, produced by the National Agricultural Research and Development Institute - Fundulea, were randomly selected. After the hulls removal, the seeds were sterilized first, in 70% ethanol for one minute followed by 7% Domestos treatment for 20 minutes under agitation. Subsequently were rinsed for several times with sterile distilled water to completely get rid of the detergent. To facilitate the removal of the transparent and thin teguments they were kept for two hours in sterile, distilled water, in the dark.

The explants, having about 200-300 µm consist of apical meristem and leaf primordia, together (referring from now on as M-LP). They were carefully sectioned from the mature seeds, under the stereomicroscope, and placed on the culture media.

The culture media were based on Murashige and Skoog basal medium (1962, MS), supplemented with organics (Table 1). The pH was adjusted to 5.6.

For testing the role of nitrogen, two variants of medium were prepared, one containing the normal amount of ammonium nitrate of MS - 1.65 g l^{-1} (RJM-MS) and the other, containing a reduced amount - 1 g l^{-1} (RJM).

The cultures were kept at 27°C, in the dark during induction step and subsequently were transferred in light to a photoperiod of 16 h for stimulating the shoot growth.

Table 1. The constituents added to MS basal culture medium

Constituents	Amount (mg l^{-1})
Vitamins	
Myo-inozitol	200
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Growth regulators	
Indole-3-acetic acid (IAA)	0.5
Zeatin	1
6-Benzyladenine (BA)	0.5
GibberellinA3 (GA3)	0.2
Others	
Sucrose	10000
Sorbitol	15000
Agar	7000

For inducing rhizogenesis, the well-developed shoots, of approximately 5 mm in length (Figure 1), were placed on rooting media - MS salts and vitamins supplemented with 0.1 mg l^{-1} indole-3-butyric acid (IBA).

Observations were performed weekly using a stereomicroscope for assessing the time necessary for buds induction and for estimating the regeneration efficiency and development of the shoots.

RESULTS AND DISCUSSIONS

The regeneration potential of the hybrid Florina was tested previously using the method established by Paterson and Everett (1985) but did not produce the expected results (data not published). As a consequence, a preliminary study was started to assess the role of the type of explants for *in vitro* regeneration of sunflower (Aurori, 2012, doctoral thesis). This study revealed that mature ungerminated embryo represents the best source of explants in term of morphogenetic potential. Here we present a new method of plant regeneration in sunflower starting from the apical meristem connected with leaf primordia, originating from mature seed.

The culture consists in two steps (Figure 2). In the first one, involving a media which contain a cocktail of growth regulators (Table 1), the

shoot induction takes place, via callus. The same medium sustains the development of the shoots until they are suitable to be transferred, in the second step of culture, on medium containing 0.1 mg l^{-1} IBA, for induction and development of roots.

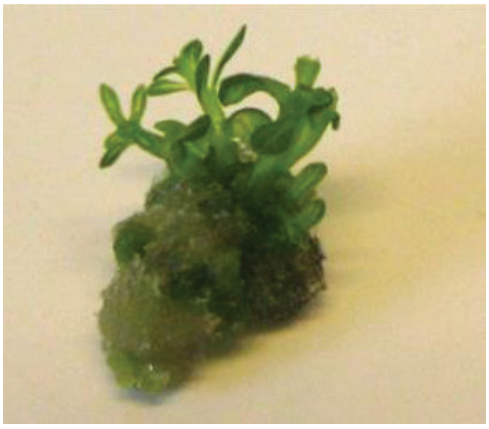

Figure 1. Plantlets regenerated on RJM-MS after 4 weeks of culture

After three weeks from initiation of the cultures all the explants started to develop callus on both induction media - RJM-MS and RJM, respectively. The callus has a vitrified appearance being white or translucent, with a hard consistency. At this stage of culture, the first signs of caulogenesis were observed but were restricted to the meristematic area. The foliar primordia did not regenerate buds.

Table 2. Plant regeneration on culture media - RJM and RJM-MS, respectively

Culture medium	Explants regenerating shoots (%)	Average no. of shoots/ explant	Explants regenerating roots (%)
RJM	100	2.1	82
RJM-MS	95	3.9	80

The source of nitrogen exerts an effect on enzyme activity, free amino acid composition and growth of sunflower (Weissman, 1972). We test here if the amount of ammonium nitrate affects the morphogenesis and efficiency of regeneration. Comparing the two culture media - RJM-MS and RJM - it was not found any significant difference in the morphogenetic potential in relation to the amount of ammonium nitrate (Table 2). During the induction phase, which takes place in the dark, the buds emerged on the 95% and 100% of the explants on RJM-MS and RJM, respectively. After the cultures were transferred to light the situation was reversed so the higher number of plantlets was harvested from RJM-MS. The average number of shoots per explant was of 2.1 and 3.9 on RJM and RJM-MS, respectively (Table 2). Although the plantlets exhibited mild vitrification (Figure 1), they had a normal morphology during growing. All the regenerated plants were transferred on rooting media. The percentage of plants that rooted did not differ significantly between the two culture media used in the previous step, being approximately 80%.

Particularly interesting and very useful in practice for *in vitro* culture of sunflower is the fact that these plants have a juvenile appearance - the flower bud has not been developed during culture as it happens so often for sunflower (Alibert et al., 1994; Baker et al., 1999; Power, 1987).

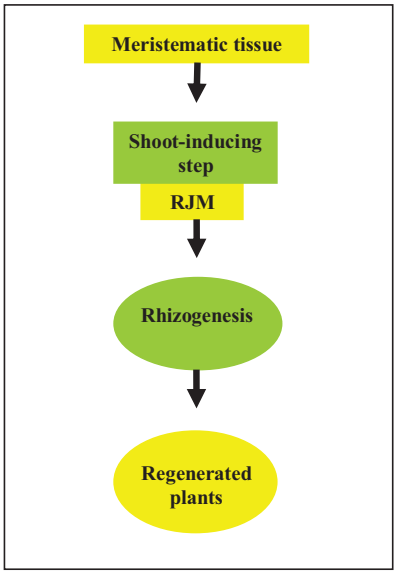

Figure 2. Schematic representation of the method for *in vitro* culture of the meristematic sunflower explants

CONCLUSIONS

For sunflower, as for other plants with poor *in vitro* response, the mature embryo proved to be

a reliable source of explants with high morphogenetic potential. It also has the advantage of being available all year round.
Particularly the apical meristem connected with the leaf primordia, resulted from ungerminated embryo, retains the entire capacity of regenerating shoots.
The regeneration occurred indirectly, from newly developed meristems (neomeristems).
The method is fast - it does not require intermediate steps - induction of the buds and plantlet growth takes place on the same culture medium. Rooted plants can be obtained in 6 weeks.
A reduced amount of ammonium nitrate, although not critical for induction, can decrease the number of shoots developed per explant.

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