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RESEARCH ARTICLE

Silver nitrate is essential for successful regeneration of Egyptian sunflower (*Helianthus annus* L.) cv. Giza 102

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Abstract

Sunflower (*Helianthus annus* L.) is an important source of vegetable oil. Egypt relies almost entirely on importation of vegetable oils due to limitation of land and competition with other warm-season more profitable crops. In the present study, cotyledons dissected from 24 hr germinating seeds were tested on 16 different media. The highest shoot-buds formation was on medium fortified with 0.5 mg/l naphthalene acetic acid and 1.0 mg/l benzyl adenine, yet no further development was observed. Healthy shoots only developed after adding 5.0 mg/l silver nitrate to the regeneration medium; while shoot elongation was achieved on a medium fortified with 0.05 mg/l indol acetic acid and 5.0 mg/l silver nitrate. Root formation was possible when using a low-salt medium with indol acetic acid and charcoal. The current work is a step in the production of genetically enhanced dehydration-stress tolerance varieties suitable to new reclaimed area in Egypt.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is a North America originated crop, which spread into Europe and the rest of the world through Spain. It is a member of the family *Asteraceae* (Compositae), a family with more than 23,000 species, where the majority of species are of ornamental importance. Sunflower oil ranks 4th in the world total oil consumption after palm oil, soybean oil and rapeseed oil (about 15% of the world total vegetable oil). Over the past decade, world production of sunflower oil has doubled, from close to 7.5 million tons in 2002 to more than 15.2 million tons in 2012 (FAOSTAT, 2014), reflecting the increasing value of sunflower oil for its quality and high health benefits, as well as for its multiple usages for seeds, seed-meals, and petals for human and livestock worldwide

The continuous increase in world's population that is coupled with environmental changes, limitations in agricultural land, and the need to develop new crops that are economically productive in marginal lands, are important factors that forcing scientists to incorporate certain traits into known crops to improve their productivity especially under unfavorable conditions.

In general, sunflower is a highly recalcitrant plant for regeneration (Mohamed et al., 2006; Moghaddasi, 2011), yet regeneration success was reported to range from 0 up to 95% and was influenced by several factors, as in genotype (Paterson and Everett, 1985; Knittel et al., 1991; Ceriani et al., 1992; Fiore et al., 1997; Weber et al., 2000; Sujatha and Prabakaran, 2001; Azadi et al., 2002; Shin et al., 2000; Hewezi et al., 2002; Abdoli et al., 2003; Ozyigit et al., 2007), and explant type (Greco et al., 1984; Bohorova et al., 1986; Lupi et al., 1987; Freyssinet and Freyssinet, 1988; Espinasse and Lay 1989; Pelissier et al., 1990; Prado and Berville, 1990; Burrus et al., 1991; Knittel et al., 1991; Power et al., 1991; Chraibi et al., 1992; Bidney et al., 1992; Ceriani et al., 1992; Krasnyanski and Menczel, 1993; Thengane et al., 1994; Wingender et al., 1996; Baker et al., 1999; Weber et al., 2000; Shin et

al.,2000; Azadi et al., 2002; Dhaka and Kothari, 2002; Abdoli et al., 2003; Hewezi et al., 2002; Ozyigit et al., 2007; Sujatha et al., 2012).

In Egypt, sunflower is cultivated in small area (around 60 thousand acer) and used as human snack and for bird-feeding, mainly because it is not profitable for farmers to grow compared to other summer crops. Egypt grows less than 5.0 % of its need from vegetable oils, and depends entirely on importation of crushed oil-seeds or partially processed oil. To our knowledge, no publication on establishment of reliable regeneration system for Egyptian varieties has ever been attempted before. Therefore, and in an effort to establish sunflower as an oil crop in Egypt, we investigated the possibility to establish a reliable regeneration system in sunflower cultivar Giza 102 using cotyledons as explant source as a step on the way to genetically improve existing cultivars.

Material and Methods

Plant materials: The seeds of sunflower (*Helianthus annuus* L. cv. Giza 102) were kindly obtained from Field Crops Research Institute, (FCRI), Agriculture Research Center (ARC), Giza, Egypt.

Methods: Sunflower seed germination, seeds of sunflower var. Giza 102 were surface sterilized under complete aseptic conditions by rinsing in 70% ethanol solution for 30 sec followed by 20 min soaking in a 20% Clorox® (commercial bleach) with two drops of Tween 20, with frequent stirring. Seeds were washed 5-6 times with sterile distilled water to ensure the removal of disinfecting residues. Seeds were germinated for 24 hr on half strength MS basal salt mixture medium (Murashige and Skoog, 1962), supplemented with Gamborg's B_5 vitamins (Gamborg et al., 1968), 10.0 g/l sucrose and solidified with 6.5 g/l agar. The pH of all tissue culture media was adjusted at 5.7 and sterilized in an autoclave at 121ºC/15 psi for 20 min.

Explant preparation: The pericarp of the overnight germinated seeds was removed using two forceps then the entire embryo section was removed using scalpel. The two cotyledons were separated using a forceps and scalpel, each cotyledon was divided longitudinally and placed on regeneration media.

Regeneration: Sixteen different regeneration media were tested (Table 1), media consisted of MS basal salt mixture supplemented with Gamborg's B_5 vitamins, 30.0 g/l sucrose, benzyl adenine (BA, at concentrations of 0, 0.5, 1.0 or 2.0 mg/l), naphthalene acetic acid (NAA, at concentrations of 0.0, 0.5, 1.0, or 2.0 mg/l) and 6.5 g/l agar. The explants were placed upside down on media surface and the plates were incubated in a growth chamber at 22 ± 2 ºC with 16/8 hr light/dark photoperiod. Each treatment had 5 replicates with a total of 50 explants per treatment; the experiment was repeated three times. Shoots primordia were scored after 4 weeks.

Shoot development and elongation: longitudinally sectioned sunflower cotyledons, prepared as described before were placed on nine T_{S7}-based media supplemented with different concentration of gibberellic acid (GA_3) and silver nitrate ($AgNO_3$) (Table 2). Each treatment consisted of 5 replicates with 50 explants. The experiment was repeated 3 times, and shoots development were observed at 23 days (Table 2). Well-developed shoot-clusters were placed on shoot elongation medium {MS basal salt mixture supplemented with Gamborg's B_5 vitamins + 0.05 mg/l Indol acetic acid (IAA) + 5.0 mg/l $AgNO_3$ + 10.0 g/l sucrose and 6.5 g/l agar} for 3-4 weeks, before transferring the shoots to rooting medium consisting of $_{1/2}$ strength MS basal salt mixture supplemented with Gamborg's B_5 vitamins, 0.1 mg/l IAA, 2.0 g/l activated charcoal, 10.0 g/l sucrose and 6.5 g/l agar. Healthy plantlets with strong rooting system were transferred into pots containing a soil mixture (clay: sand: peat moss) (1:1:1) in a Conviron® growth chamber at 25 $_{\pm}$ 2˚C and 16/8hr light/dark photoperiod.

Results and Discussion

In this work, dissected cotyledons coming from 24 hrs germinated seeds were used as an explant source. Two weeks after placing the dissected cotyledons on different media, cotyledons enlarged 4-5 times and turned green (Figure 1a); later on we observed the beginning of callus formation which later on (3-4 weeks) started to develop into bud clusters and shoot primordia (Figure 1b). In general, shoot clusters in all treatments (T_{S1} to T_{S16}) failed to show further development (even after placing or fresh media). We also observed that, prolonging exposure to regeneration media led to vitrification and further callus formation (data not shown).

In general, media supplemented with 0.5 mg/l NAA (with or without cytokinin) had a higher average of shoot primordia. The addition of 0.5 mg/l NAA caused ≥4 folds increase in total shoot primordia number compared to media without NAA. Medium T_{S7} produced the highest number of shoot primordia, followed by T_{S6} and T_{S8} (47.4, 34.3 and 23.3, respectively; Table 1). Interestingly, the same range of concentration of 1.0 mg/l BA combined with 0.5 mg/l NAA was reported by other researchers to give the highest shoot regeneration (Knittel et al. 1991; Baker et al. 1999; Ozyigit et al. 2007), indicating the importance of auxins/cytokinin balance for in sunflower regeneration.

Some researchers suggested the need for nitrogen enriched media (*via* the addition of 5.0 g/l KNO_3) to achieve successful shoot regeneration in sunflower (Paterson and Everett, 1985; Knittel et al., 1991; Power et al., 1991;

Ceriani et al., 1992; Fiore et al., 1997; Baker et al., 1999). In the present study, we conducted some experiments and noticed that the addition of 5.0 g/l KNO_3 to regeneration medium did not result in any increase in buds formation. At the contrary, none of these bud clusters developed into shoots, they simply started to form more callus (Data not shown).

As mentioned earlier, shoot-bud clusters coming from different media (T_{S1} to T_{S16}) failed to show any further development, we anticipated that this problem could be a result of ethylene production inside the tissue culture vessels; therefore we decided to conduct another set of experiments by adding $AgNO_3$ at different concentrations along with GA_3 using T_{S7}-based medium (the medium with the best shoot-bud formation; Table 2). Out of the nine different media combinations used, only medium T_{Sd2} (T_{S7} medium supplemented with 5.0 mg/l $AgNO_3$) was successful for the development of healthy non-vitrified shoots (Table 2; Figure 1c).

Different researchers suggested the usage of GA_3 in regeneration medium to produce healthy shoots in sunflower (Power et al., 1991; Fiore et al., 1997), and suggested that the usage of high concentration of GA_3 (0.5 mg/l or higher) resulted in production of elongated pale green shoots. In this study, GA_3 addition in low concentrations (0.0, to 0.3 mg/l) with or without the addition of $AgNO_3$ resulted in production of unhealthy, pale green malformed shoots, which failed to produce roots when transferred to rooting medium (Figure 2A).

On the other hand, and in agreement with Baker (1999), the addition of $AgNO_3$ had a dramatic impact on the morphological characteristics of the shoots and the production of un-vitrified normal looking shoots. Although higher concentrations of $AgNO_3$ promoted more shoots and leaves development, yet it caused some dwarfism in shoots. Our previous work with other oil-crops also revealed that adding $AgNO_3$ to regeneration media had a dramatic improvement on regeneration frequency (Al-Shafeay et al., 2011; Khalil et al., 2015).

Interestingly, the formation of vitrified or etiolated pale green shoots or shoot primordia which never developed any further has been a well-documented problem in sunflower regeneration, a problem that different research groups tried to solve. Some researchers suggested lowering plant hormone concentration in regeneration media (Baker et al., 1999; Dhaka and Kothari, 2002), while others completely omitting plant hormones from media (Shin et al. 2000; Weber et al. 2000; Azadi et al. 2002).

Clusters containing 0.5-1.0 cm long shoots, resulting from T_{Sd2} medium were transferred to elongation medium for 2-3 weeks (some even started to form roots on this medium; Figure 2B), and well-elongated shoots were then transferred to rooting medium fortified with activated charcoal (Figure 2C). The majority of shoots (more than 95%) once placed on rooting medium were able to form rooting system.

From our previous experience with other oil crops tissue culture, we found that decreasing sucrose concentration in rooting media, when coupled with using $_{1/2}$ strength MS salts mixture, generally results in improvement in roots formation (Al-Shafeay et al., 2011; Khalil et al. 2015). Low concentration of IAA was used to promote roots formation, while activated charcoal was added to further absorb any ethylene production inside the tissue culture vessel and because it has been suggested by several research groups (Baker et al., 1999; Azadi et al. 2002). On the other hand, Fiore et al. (1997) used a mixed amino acid (glutamine, aspartic acid, L-arginine and glycine) in addition to supplementing regeneration media with 0.2 mg/l GA_3 to elongate healthy non-vitreous shoots.

Table 1. Media combination used in the establishment of regeneration system in sunflower.

Shoot formation media for sunflower (5 reps, 50 explant/treatment, repeated 3 times)		
Media no.	**Media composition (mg/l)**	**Number of shoot primordia**
T_{S1}	0.0 BA + 0.0 NAA	0.14 ± 0.14 h
T_{S2}	0.5 BA + 0.0 NAA	2.73 ± 1.40 g
T_{S3}	1.0 BA + 0.0 NAA	11.9 ± 1.60 e
T_{S4}	2.0 BA + 0.0 NAA	10.1 ± 2.10 e
T_{S5}	0.0 BA + 0.5 NAA	0.10 ± 0.10 h
T_{S6}	0.5 BA + 0.5 NAA	34.3 ± 1.90 b
T_{S7}	1.0 BA + 0.5 NAA	47.4 ± 6.40 a
T_{S8}	2.0 BA + 0.5 NAA	23.3 ± 5.60 c
T_{S9}	0.0 BA + 1.0 NAA	0.50 ± 0.50 h
T_{S10}	0.5 BA + 1.0 NAA	18.4 ± 3.50 d
T_{S11}	1.0 BA + 1.0 NAA	5.10 ± 2.30 f
T_{S12}	2.0 BA + 1.0 NAA	17.0 ± 5.30 d
T_{S13}	0.0 BA + 2.0 NAA	1.95 ± 1.70 g
T_{S14}	0.5 BA + 2.0 NAA	11.4 ± 1.80 e
T_{S15}	1.0 BA + 2.0 NAA	16.6 ± 2.60 d
T_{S16}	2.0 BA + 2.0 NAA	6.90 ± 3.40 f

The experiments were repeated three times, with 50 explant per treatment; numbers represents mean value ± SE; numbers followed with the same letter are non-significant at P≤ 0.05

Table 2. Media combinations used in sunflower shoot development.

Shoot development media (5 reps, 50 explant/treatment, repeated 3 times)			
Media	**T_{S7} medium + $AgNO_3$ + GA_3 (mg/l)**	**Shoot primordia**	**Well-developed shoots**
T_{Sd1}	0.00 $AgNO_3$ + 0.0 GA_3	43.6 ± 6.9	1.20 ± 0.7 b c
T_{Sd2}	5.00 $AgNO_3$ + 0.0 GA_3	39.1 ± 5.3	15.0 ± 3.6 a
T_{Sd3}	10.0 $AgNO_3$ + 0.0 GA_3	31.0 ± 5.9	4.60 ± 1.6 b
T_{Sd4}	0.00 $AgNO_3$ + 0.1 GA_3	17.0 ± 8.9	1.60 ± 1.0 b c
T_{Sd5}	5.00 $AgNO_3$ + 0.1 GA_3	13.6 ± 6.4	2.40 ± 1.1 b c
T_{Sd6}	10.0 $AgNO_3$ + 0.1 GA_3	25.8 ± 7.0	3.20 ± 1.1 b
T_{Sd7}	0.00 $AgNO_3$ + 0.3 GA_3	1.00 ± 0.0	0.00 ± 0.0 c
T_{Sd8}	5.00 $AgNO_3$ + 0.3 GA_3	21.5 ± 6.3	3.50 ± 0.6 b
T_{Sd9}	10.0 $AgNO_3$ + 0.3 GA_3	21.3 ± 1.8	4.30 ± 0.5 b

The experiments were repeated three times, with 50 explant per treatment; numbers represents mean value ± SE; numbers followed with the same letter are non-significant at P≤ 0.05

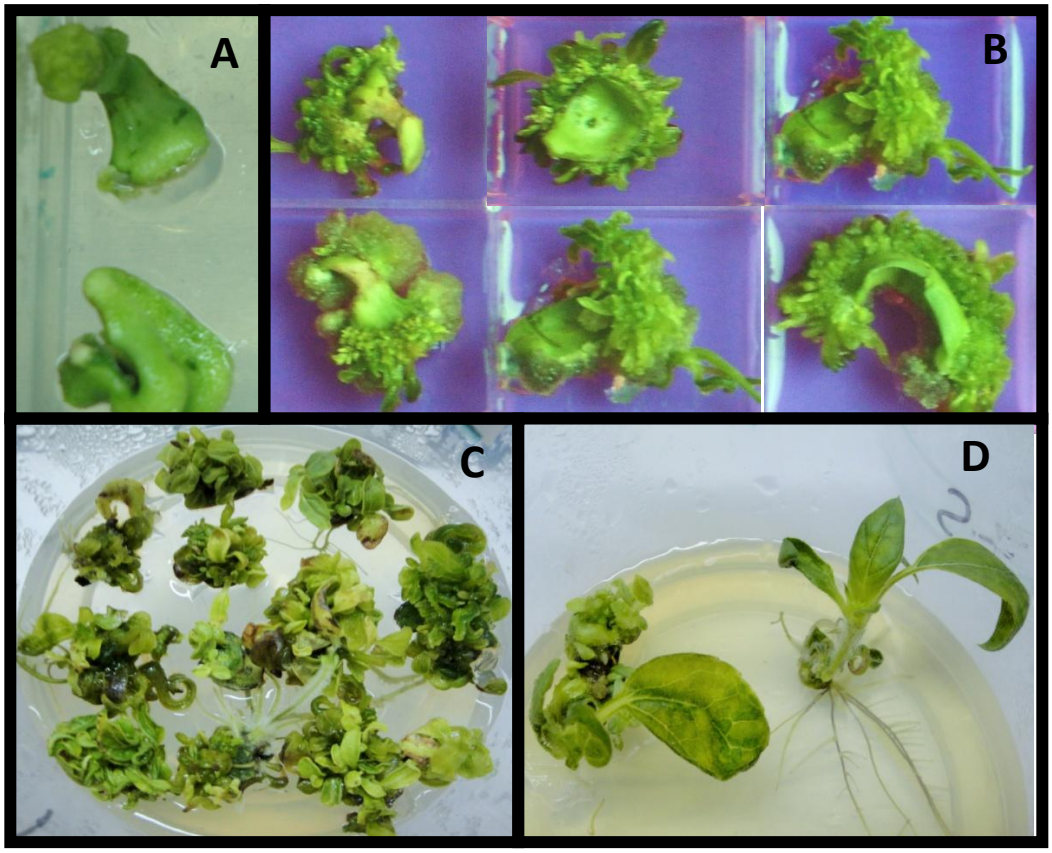

Figure 1. Regeneration steps of sunflower (*Helianthus annus* L.) cv. Giza 102. (A) 14 days old cotyledons on regeneration medium, (B) initiation of shoot primordia 4-6 weeks post establishment of culture without the adding AgNO3. (C) Two months old cultures showing shoot formation on medium supplemented with AgNO3. (D) Well-developed sunflower shoots forming roots on elongation medium.

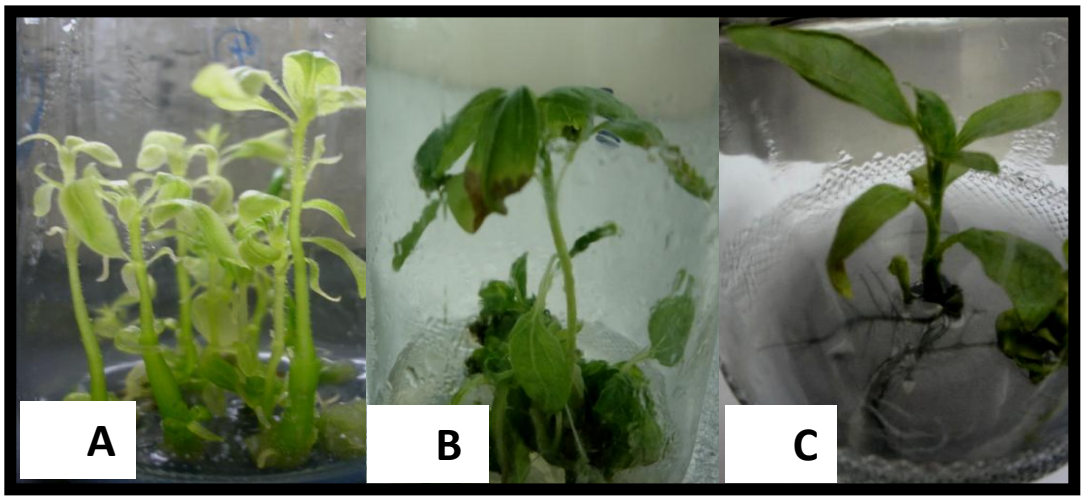

Figure 2. Sample of regenerated sunflower shoots (*Helianthus annus* L.) coming through tissue culture treatments. (A) Vitrified shoots growing from shoot-clusters on media with different GA3 concentrations. (B) Well-developed shoots of sunflower from media supplemented with silver nitrate. (C) normal-looking healthy sunflower shoots developing roots on rooting medium.

Conclusion

We successfully established a regeneration protocol for Egyptian sunflower as a step required to genetically enhance sunflower to be grown in marginal lands. We observed that NAA had a significant effect on formation of shoot primordia, regardless of cytokinin presence or absence. Development of normal non-vitrified shoots was only possible when adding $AgNO_3$ to the regeneration medium. In general, ethylene is a critical factor determining the successful regeneration of sunflower.

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