YYÜ TAR BİL DERG
(YYU J AGR SCI)
2015, 25(2): 113-118

Geliş Tarihi (Received): 11.07.2014
Kabul Tarihi (Accepted): 18.04.2015

Araştırma Makalesi/*Research Article (Original Paper)*

Stimulation Effect of $AgNO_3$ and $CoCl_2$ as Ethylene Inhibitors on in-Vitro Organogenesis of Sunflower (*Helianthus annuus* L.)

Fahimeh MIRZAI, Ebrahim Dorani ULIAIE*, Ali Bandeh HAGH

Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Tabriz University, Tabriz, Iran
* e-mail: uliaie@yahoo.com; Tel: +98(4113292031) ; Fax: +98(4113356003)

Abstract : To achieve an effective and reliable shoot organogenesis protocol in *Helianthus annuus* L., effect of ethylene inhibitors including $AgNO_3$ and $CoCl_2$ were investigated. Cotyledonary explants of two cultivars including CMS19 and Progress were cultured in MS medium with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} IAA supplemented with different concentrations of $AgNO_3$ (0, 1.5 and 3 mg L^{-1}) and $CoCl_2$ (3 and 6 mg L^{-1}). Statistical analysis of data showed a significant difference among cultivars and ethylene inhibitors concentrations for organogenesis parameters including shoot regeneration and number of shoots per explant. Addition of ethylene inhibitors to culture medium improved regeneration frequency and number of shoots per explant. Shoot generation was improved with increasing concentrations of $CoCl_2$ but in the case of $AgNO_3$, increasing the concentration gave adverse results. Treatment of explants with $AgNO_3$ in low concentration (1.5 mg L^{-1}) improved organogenesis in *Helianthus annuus*. The highest shoot regeneration was obtained in media supplemented with 6 mg L^{-1} $CoCl_2$ (R/E= 63% and S/RE= 3) and 1.5 mg L^{-1} $AgNO_3$ (R/E= 73% and S/RE= 2).This study suggests that ethylene inhibitors, particularly $CoCl_2$, could be used to improve the efficiency of *in vitro* shoot regeneration and plant transformation protocols of sunflower.

Key words: Cotyledon, Silver nitrate, Cobalt chloride, *Helianthus annuus* L., Sunflower, Plant regeneration

Ayçiçeği (*Helianthus annuus* L.)'nin in vitro Organogenezi Üzerine Etilen İnhibitörleri Olarak $AgNO_3$ ve $CoCl_2$'nin Uyarıcı Etkisi

Özet: Ayçiçeğinde (*Helianthus annuus* L.) etkili ve güvenilir bir organogenez protokoluna ulaşmak için, etilen inhibitörleri olarak $AgNO_3$ ve $CoCl_2$'nin etkisi araştırılmıştır. CMS19 ve Progress çeşitlerinden elde edilen kotiledon eksplantları 2.0 mg/L BAP ve 1.0 mg/L IAA içeren MS ortamına farklı konsantrasyonlarda $AgNO_3$ (0, 1.5 ve 3 mg/L) ve $CoCl_2$(3 ve 6 mg/L) ilave edilerek kültüre alınmıştır. Yapılan istatistik analizine göre; eksplant başına sürgün sayısı ve sürgün rejenerasyonunu içeren organogenez parametrelerinde etilen inhibitörlerinin konsantrasyonu ve çeşitler arasında istatistiksel olarak önemli farklılıklar bulunmuştur. Kültür ortamına etilen inhibitörlerinin eklenmesi, eksplant başına sürgün sayısını ve rejenerasyon frekansını artırmıştır. Sürgün rejenerasyonu $CoCl_2$ konsantrasyonun artışıyla artmış, ancak $AgNO_3$ konsantrasyonunun artışına bağlı olarak azalmıştır. Düşük konsantrasyonda (1.5 mg/L) $AgNO_3$ uygulaması ayçiçeğinde (*Helianthus annuus*) organogenez artırmıştır. En yüksek sürgün rejenerasyonu ortama 6 mg/l $CoCl_2$ (R/E=% 63 ve S/RE= 3) ve 1.5 mg/L AgNO3 (R/E=% 73 ve S/RE= 2) eklendiğinde elde edilmiştir. Bu araştırmada etilen inhibitörlerinin, özellikle $CoCl_2$'nın ayçiçeğinde bitki transformasyon protokolu ve in vitro sürgün rejenerasyon etkinliğinin iyileştirilmesinde kullanılabileceği önerilmiştir.

Anahtar kelimeler: Kotiledon, Gümüş nitrat, Kobalt klorür, *Helianthus* annuus L., Ayçiçeği, Bitki rejenerasyonu

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops and has a potential value as a source of oil, protein, vitamin and energy. The use of biotechnological methods for breeding of sunflower is limited due to lack of an efficient and reproducible protechols for plant regeneration from transformed cells (Ceriani et al., 1992). Like all spicese regeneration frequency in sunflower strongly

depends on genotype, interaction genotype with culture conditions (Sarrafi et al. 1996) and environmental and physiological factors (Paterson and Everett 1985; Power 1987;Sarrafi et al. 2000). Accumulation of ethylene is one of important limiting factor in *in vitro* regeneration of plants (Biddington 1992). Ethylene (C_2H_4) plays an important role in plant growth and development (Yang and Hoffman 1984) and also inhibits plant growth and morphogenesis depending upon the species and culture stage (Kumar et al. 1998). Ethylene can affect callus growth; shoot organogenesis and somatic embryogenesis (Biddington 1992; Huang et al. 2001; Jha et al. 2007; Chatfield and Raizada 2008). Ethylene has certain positive effects on callus culture and root growth but it inhibit *in-vitro* differentiation and growth of the shoots. Hence, ethylene inhibitors can be added to culture medium to prevent the negative effects of it (Chae et al. 2012; Park et al. 2012).

$AgNO_3$ and $CoCl_2$ are known as effective ethylene inhibitors that promote shoot growth when added to the media (Biddington 1992). $AgNO_3$ has been known to inhibit ethylene action (Beyer 1976a) and cobaltous ions are known to inhibit ethylene synthesis (Lau and Yang 1976). So the addition of certain chemicals such as cobalt chloride or silver nitrate to the culture medium can inhibit ethylene production or its function by blocking certain steps in the biosynthesis or signaling pathways respectivily (Pua and Chi 1993) and increases *in-vitro* regeneration in monocots (Purnhauseret al. 1987; Songstad et al. 1988) and dicots (Chi et al. 1991; Roustan et al. 1989). In the present study the effect of ethylene inhibitors on the efficiency of shoot organogenesis in sunflower is reported.

Material and Methods

Plant material and prepering the explants

Seeds of *H. annuus* (CMS19 and Progres) were prepared from Seed and Plant Improvement Institute (Karaj, Iran) and stored at 4°C. The seeds without pericarps were surface-sterilized with 70% (v/v) ethanol for 30 s and 3% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Ten seeds were placed on 30 mL of agar-solidified culture medium in Petri dishes. The basal medium consisted of hormone free half strength MS medium (Murashige and Skoog, 1962), supplemented with 3% sucrose, B5 vitamins and solidified with 0.7% (w/v) agar. The pH of medium was adjusted to 5.7 before adding agar, and then sterilized by autoclaving at 121°C for 20 min. Cultures were maintained at 25±1°C under 16 h light/ 8 h dark cycle with a light flux of 80 μmol m^{-2} s^{-1}.

In vitro shoot organogenesis

Two days seedlings cotyledons were excised by using sharp scalpel. Seeds were cut twice perpendicular to the long axis of the seeds. The first cut include 2 mm of the proximal end of cotyledon. This part harboring the entire embryo, which was discarded. Across the middle of the cotyledons cuted in the second step. Explants of proximal sections were placed abaxial side down onto the regeneration medium in Petri dishes. Each Petri dish, sealed with a Parafilm strip to prevent desiccation, contained five explants of cotyledons. Regeneration medium consisted of full strength MS medium supplemented with 3% sucrose, 2 mg L^{-1} BAP and 1 mg L^{-1} IAA. To determine the effects of ethylene inhibitors the regeneration medium was supplemented with 0, 1.5 and 3mg L^{-1} silver nitrate ($AgNO_3$) and 3 and 6 mg L^{-1} cobalt chloride ($CoCl_2$).

The experiment was done in a randomized completely design with three repeat, two genotypes and five treatments. When Shoots were achieved 4 mm or longer in length, the regeneration ability of genotypes was scored by assessing percentage of explants forming shoots (%R/E) and average number of shoots per regenerated explants (S/RE). Statistical analysis performed using SPSS for Windows release 16.0 (SPSS, Chicago, IL, USA). Differences between means were scored with Duncan's multiple range tests.

Rooting of in vitro produced shoots

For root induction, regenerated shoots (2-3 cm in length) were transferred to rooting media consist of half strength MS medium supplemented with 2 and 4 mg L^{-1} of Naphthalene Acetic Acid (NAA), Indole Acetic Acid (IAA) and Indole Butric Acid (IBA). The medium was solidified with 7 g L^{-1} Agar. One shoot were cultured in each culture vessel. Three repetitions were performed for each treatment. Regenerated shoots were incubated at 25 ± 1 °C in a growth chamber with a 16-h photoperiod under

standard cool white fluorescent tubes (35 µmol s-1·m-2) for 3 weeks. After 3 weeks, Root quality was evaluated. The rooted plants were carefully washed with water to remove agar, transferred to pots containing mixture of autoclaved soil and Perlite, and covered with plastic bag to maintain high humidity and were kept under culture room conditions for one week. The plants were then transferred to the greenhouse.

Results and Discussion

Analysis of variance shows that there was not any significant difference between genotypes on percentage of explants forming shoots (%R/E), but the average number of shoots per regenerated explant (S/RE) had significant difference at 1% level. Use of ethylene inhibitors had significant effect on %R/E at the 5% level and on S/RE at 1% level. The interaction between genotype and ethylene inhibitors was not significant for the organogenesis traits which indicate that these two factors are acting independently.

The average number of shoot per regenerated explant at the Progress (2.43) was more than CMS19 that show organogenesis is dependent on the genotype. Percentage of regeneration significantly increased in media containing3 mg L^{-1} cobalt chloride and 1.5 mg L^{-1} silver nitrate. The average number of shoots per regenerated explant in presence of 6 mg L^{-1} cobalt chloride significantly increased, but decreased at 3 mg L^{-1}silver nitrate containing medium. Effect of other concentrations of ethylene inhibitors were not significant whit control (Table 1).

The shoot growth was increased with increasing concentrations of $CoCl_2$ and was declined with increasing concentrations of $AgNO_3$. The opposite was seen in the case of regeneration frequency. The greatest shoot growth was found when the regeneration medium was supplemented with 6 mg L^{-1} $CoCl_2$, obtaining 63% regeneration frequency with the largest number of shoots (3.03) in each explant. Treatment with 6 mg L^{-1} $CoCl_2$ produced 44% more shoots per explant compared to the control. Regeneration frequency was also 13% higher in compared to the control (Table 1). Treatment with $AgNO_3$ did not give good result in shoot growth but increased regeneration frequency.

Table 1. Effect of genotypes and different concentrations of ethylene inhibitors on shoot regeneration of sunflower after 4 in regeneration medium (Murashige and Skoog medium with 2.0 mg/L BAP and 1.0 mg/L IAA)

Ethylene inhibitors (mg/L)	Regeneration %	No of shoot/Explant (Mean ± SE)
Control	56.66 b*	2.1 ± 0.1 b
$CoCl_2$		
3	76.66 a	2.37 ± .01 b
6	63.33ab	3.03 ± 0.1 a
$AgNO_3$		
1.5	76.66 a	2.04 ± 0.1 b
3	60.00 b	1.52 ± 0.1 c

*Mean values with the same letter are not significantly different at the 0.05 probability level (Duncan's test)

Positive effects of silver nitrate and cobalt chloride on plant regeneration and number of shoots per explant have been reported in some plants, including *Helianthus annuus, Papaya axillary*, *Lycopersicon esculentum* and *Capsicum frutescens* (Chraibi et al.,1991; Biddington, 1992; Lai et al., 2000; Sharma et al., 2008; Osman and Khalafalla, 2010). The growth and development of tissue cultures can be controlled to a certain extent by regulating the production or action of ethylene (Beyer, 1976b; Purnhauser et al. 1987; Songstad et al. 1988; Chi and Pua, 1989; Bais et al. 2000; Giridhar et al. 2003). However in this experiment silver nitrate increased explants forming shoots compared to the control but did not have any significant effect on number of shoots per explant and caused abnormal shoots. It is assume that use of $AgNO_3$ in high concentration have negative effects on shoot regeneration (Fig. 2E). These results are consistent with the results of Baker et al (1999). They reported when silver nitrate added to development media at 0.4 – 1.7 mg L^{-1}, callus induction, regeneration capacity and number of shoots per explant were not affected. Although shoot verification occurred slightly, but there was no improvement in rooting or survival and higher concentrations of $AgNO_3$ was produced pale green, over-elongated or malformed shoots. In contrast, increase the concentration of cobalt chloride increases number of shoots and it has better effect than silver nitrate to obtain shoots with normal morphogenesis. For reduction of shoots hyperhydricity silvernitrate had better effect than cobalt chloride.

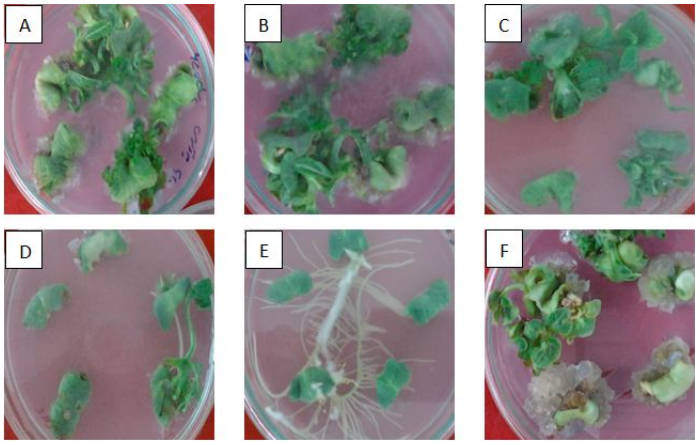

Figure 2. Effects of ethylene inhibitors on shoot regeneration from cotyledonary explants in medium supplemented with: (A) 6 mg L^{-1} $CoCl_2$; (B)3 mg L^{-1} $CoCl_2$; (C) 1.5 mg L^{-1} $AgNO_3$; (D) 3 mg L^{-1} $AgNO_3$. (E) 5 mg L^{-1} $AgNO_3$; (F) control medium without ethylene inhibitors.

For in vitro rooting, shoot of Progress subjected to rooting in MS medium supplemented with 2 and 4 mg L^{-1} of NAA, IAA and IBA. After a few days callus and root buds appeared on cut end of shoots. Fig. 2 shows the effect of different concentration of the tested auxins on rooting after three weeks. All shoots in all auxins in both concentrations give raise the roots but there was phenotypically difference between treatments according to type of ouxins and its concentrations (Fig.3). Shoots on media containing NAA had less root growth than others. Quality of roots obtained from IAA and IBA containing medium was better than NAA. Roots obtained from NAA were short, thick and with increasing concentration of NAA amount of callus-like roots was increased. Thick and thin roots were induced in IBA containing medium but thin and long roots were produced in IAA supplemented medium. The shoot rooted in IBA containing medium showed better compatibility with the soil.

Figure 3. In-vitro rooting quality of sunflower shoots on medium containing different auxin in tow concentrations.

References

Alibert G, Aslane-Chanab JC, Burns M (1994). Sunflower tissue and cell cultures and their use in biotechnology. Plant Physiol. And Bioch. 32: 31-44

Bais HP, Sudha G, Suresh B,Ravishankar GA(2000). $AgNO_3$ influences in vitro root formation in *Decalepisha miltonii* Wight and Arn. Curr. Sci. 79: 894-898.

Baker CM, Munoz-Fernandez N,Carter CD (1999).Improved shoot development and rooting from mature cotyledons of sunflower. Plant Cell, Tiss. and Org. Cult. 58: 39-49.
Beyer EM (1976a). A potent inhibitor of ethylene action in plants. Plant Physiol. 58 (3): 268-271.
Beyer EM (1976b). Silver ion: a potent anti-ethylene agent in cucumber and tomato. HortScience. 11 (3): 175-196.
Biddington NL (1992). The influence of ethylene in plant tissue culture. Plant Growth Regulation, 11 (2): 173-187.
Ceriani MF, Hopp HE, Hahne G, Escandon AS (1992). Cotyledons: an explant for routine regeneration of sunflower plants. Plant Cell Physiol. 33: 157-164.
Chae SC, Kim HH, Park SU (2012). Ethylene Inhibitors Enhance Shoot Organogenesis of Gloxinia (*Sinningia speciosa*). The Scientific World Journal.
Chatfield SP, Raizada MN (2008). Ethylene and shoot regeneration: hookless1 modulates de novo shoot organogenesis in *Arabidopsis thaliana.* Plant Cell Rep. 27: 655-666.
Chi GL, Pua EC (1989). Ethylene inhibitors enhanced de novo shoot regeneration from cotyledons of *Brassica campastrisspp* in vitro. Plant Sci. 64: 243-250.
Chi GL, Pua EC, Goh CJ (1991). Role of ethylene on de novo shoot regeneration from cotyledons of *Brassica campastris spp* Pekinensis (Lour) Olsson in vitro. Plant Physiol. 96 (1): 178-183.
Chraibi KMB, Latche A, Roustan JP, Fallot J(1991). Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. Plant Cell Rep. 10 (4): 204-207.
Giridhar P, Indu EP, Vijayaramu D, Ravishankar GA (2003). Effect of silver nitrate on in vitro shoot growth of Coffee. Trop. Sci. 43 (3): 144-146.
Huang X L, Li XJ, Li Y, Huang LZ (2001). The effect of AOA on ethylene and polyamine metabolism during early phases of somatic embryogenesis in *Medicago sativa*. Physiol Plant. 113: 424-429.
Jha AK, Dahleen LS, Suttle JC (2007). Ethylene influences green plant regeneration from barley callus. Plant Cell Rep. 26: 285-290.
Kumar P, Dimps RC, Goh CJ (1998). Influence of petiole and lamina on adventitious shoot initiation from lamina explants of *Paulownia fortunei*. Plant Cell Rep. 17: 886-890
Lai CC, Yeh SD, Yang JS (2000). Enhancement of *Papaya axillary* shoot proliferation in vitro by controlling ethylene. Bot. Bull. Acad. Sin. 41: 203-212.
Lau OL, Yang SF(1976). Inhibition of ethylene production by cobaltous ion. Plant Physiol. 58 (1): 114-117.
Osman MG, KhalafallaMM (2010). Promotion of in vitro Shoot Formation From Shoot Tip of Tomato (*LycopersIcon esculentum* mill. cv. Omdurman) by Ethylene inhibitors. Int. J. Curr. Res. 4: 082-086.
Park EH, Bae H, Park WT, Kim YB, Chae SC, Park SU (2012). Improved shoot organogenesis of gloxinia (*Sinningia speciosa*) using silver nitrate and putrescine treatment. Plant Omics. 5: 6-9.
Paterson KE, Everett NP (1985). Regeneration of *Helianthus annuus* in bred plants from callus. Plant Sci.42: 126-132.
Power CJ(1987). Organogenesis from *Helianthus annuus* inbreds and hybrids from the cotyledons of zygotic embryos. Am. J. Bot. 74: 497-503.
Pua EC, Chi GL (1993). De novo shoot morphogenesis and plant growth of mustard (*Brassica juncea*) in vitro in relation to ethylene. Physiol. Plant. 88 (3): 467-474.
Purnhauser L, Medgyesy P, Czako M, Dix PJ, Marton L (1987). Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv tissue cultures using ethylene inhibitor $AgNO_3$. Plant Cell Rep. 6 (1): 1-4.
Roustan J, Latche A, Fallot J (1989). Stimulation of Daucuscarota somatic embryogenesis by inhibitors of ethylene synthesis: Cobalt and nickel. Plant Cell Rep. 8 (3): 182-185.
Sarrafi A, Bolandi AR, Serieys H, Berville A,Alibert G, (1996). Analysis of cotyledon culture to measure genetic variability for organogenesis parameters in sunflower (*Helianthus annuus* L.). Plant Sci. 121: 213-219.
Sarrafi A, Kayyal H, Rachid Al-Chaarani G, Cantin F,Chaline AS, Durielle E(2000). Inheritance of organogenesis parameter in cotyledons of sunflower (*Helianthus annuus* L.). J. Genet. Breeding. 54: 227-231.
Sharma A, Kumar V, Giridhar P, Ravishankar GA (2008). Induction of in vitro flowering in *Capsicum frutescens* under the influence of silver nitrate and cobalt chloride and pollen transformation. Electron. J. Biotechnol. 11(2):84-89.

Songstad DD, Duncan DR, Widholm JM (1988). Effect of 1-aminocyclopropane-1carboxylic acid, silver nitrate and non- bornadiene on plant regeneration from maize callus cultures. Plant Cell Rep. 7 (4): 262-265.

Witrzens B, Scowcroft WR, Downes RW, Larkin PJ (1988). Tissue culture and plant regeneration from sunflower (*Helianthus annuus*) and interspecific hybrids (*H tuberosus* × *H. annuus*).Plant Cell, Tiss. and Org. Cult. 13: 61-76.

Yang SF, Hoffman NE (1984). Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant Physiol. 35: 155-189.