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RESEARCH ARTICLE

PROMOTION OF *IN VITRO* SHOOT FORMATION FROM SHOOT TIP OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL. CV. OMDURMAN) BY ETHYLENE INHIBITORS

Magdoleen G. Osman, and Mutasim M. Khalafalla*

Commission for Biotechnology and Genetic Engineering, National Center for Research, P. O. Box 2404, Khartoum, Sudan

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ABSTRACT

The promotive effect of ethylene inhibitors, silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$) on *in vitro* shoot regeneration of tomato (*Lycopersicon esculentum*Mill. cv. Omdurman) was investigated. Shoot development was induced on shoot tip explant cultured on MS (Murashige and Skoog, 1962) medium supplemented with kinetin (Kn) at 4.0 mg/l. Addition of $CoCl_2$ and $AgNO_3$ to the medium enhanced regeneration frequency as well as number of shoots obtained per explant. The best results (2.4 and 2.3) for the number of shoot per explant were obtained by using $CoCl_2$ at 3.0 mg/L and $AgNO_3$ at 5.0 mg/l, respectively. 100% of the *in vitro* induced shoots produced roots when cultured on half and full strength MS medium without growth regulators. The highest number of rooted micro shoots (22± 0.9) was obtained on half strength MS media agar-solidified and supplemented with 0.5 mg/l Naphthalene Acetic Acid (NAA). On the other hand, the longest root (7.4 ± 0.9 cm) was obtained on the same medium supplemented with 0.1 or 0.5 mg/l IBA. Rooted plants were hardened and 95% survived under greenhouse conditions.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is the second most popular vegetable crop next to potato in the world (Bahatia *et al.,* 2004). In the Sudan, tomato is an important vegetable crop that account, for over 40% of the vegetable production area (Ahmed *et al.,* 2001) and grown as an irrigated winter crop.

Pests, diseases incidences and heat stress are the major factors that limit tomato production in Sudan. The high temperatures during summer accompanied by low humidity limit the production of tomato to the cooler part of the year and leads to the seasonality of the crop production (Abdalla and Verkerk, 1968). A variety of control measures and techniques including cultural practices in the field have been tested previously under Sudan conditions. However, the use of these techniques to improve this crop is limited. Recognizing its economic importance, still there is a vast scope to utilize modern biotechnology to develop cultivars resistant to various biotic and abiotic stresses of this valuable crop. Success of utilizing such technology, largely rely on efficient *in vitro* regeneration techniques.

The gaseous environment in tissue culture sealed containers is one of the important factors controlling regeneration of shoots *in vitro*. Varying amounts of ethylene is released in culture vessels during *in vitro* regeneration (De Proft *et a*l., 1985).

***Corresponding author:** *Mutasim@myway.com; mkhalafalla@ncr.sd*

Ethylene is recognized as a ubiquitous plant hormone (Lieberman, 1979; Yang, 1985), which influences growth and development of plants (Abeles, 1973; Yang and Hoffman, 1984; Mattoo and Suttle, 1991). Thus, by regulating the production or action of ethylene, the growth and development of some tissue cultures can be controlled to a certain extent (Beyer, 1976c; Davies, 1987; Purnhauser *et al.*, 1987; Songstad *et al.* 1988; Chi and Pua, 1989; Bais *et al.,* 2000a; Giridhar *et al*., 2003). $AgNO_3$ and $CoCl_2$ has been known to inhibit ethylene action and ethylene synthesis (Beyer, 1976a; Lau and Yang, 1976). Silver ion is capable of specifically blocking the action of exogenously applied ethylene in classical responses such as abscission, senescence and growth retardation (Beyer, 1976c). These observations led to its application in tissue culture with the aim of improving shoot regeneration. Addition of $AgNO_3$ to the culture media greatly improved the regeneration of both dicot and monocot plant tissue cultures (Beyer, 1976c; Duncan *et al*., 1985; Davies, 1987; Purnhauser *et al.,* 1987; Songstad *et al.,* 1988; Chi and Pua, 1989; Veen and Over Beek, 1989; Bais *et al*., 2000a; Giridhar *et al.,* 2003). Moreover, the addition of CoCl2, promoted shoot regeneration from callus cultures of *Nicotiana plumbaginifolia and Triticum aestivum* (Purnhauser *et al.,*1987) and enhanced somatic embryogenesis of *Daucus carota* (Roustan *et al.,*1989).The exact mechanism of ethylene inhibitors action on plants is unclear. However, few existing evidences suggest its interference in ethylene perception mechanism (Beyer, 1976c).

Table 1. Effect of ethylene inhibitors on tomato multiple shoot induction from shoot tip explants after six weeks of culture on MS medium supplemented with 4.0mg/l Kin.

$AgNo_3$	$CoCl_2$	Regeneration response %	No of shoots / explants (mean ± SE)	Shoot length / explants (mean ± SE)
0.0	0.0	100	2.0 ± 0.0	3.3 ± 0.1
1.0	0.0	100	1.2 ± 0.1	3.1 ± 0.1
2.0	0.0	100	1.3 ± 0.1	3.1 ± 0.1
3.0	0.0	100	1.9 ± 0.1	3.2 ± 0.1
4.0	0.0	100	1.8 ± 0.1	3.2 ± 0.1
5.0	0.0	100	2.3 ± 0.1	3.3 ± 0.1
10.0	0.0	88	1.2 ± 0.1	2.9 ± 0.1
0.0	1.0	100	1.1 ± 0.1	3.4 ± 0.1
0.0	2.0	100	1.4 ± 0.1	3.5 ± 0.1
0.0	3.0	100	2.4 ± 0.1	3.6 ± 0.1
0.0	4.0	100	1.5 ± 0.1	3.4 ± 0.1
0.0	5.0	100	1.3 ± 0.1	3.4 ± 0.1
0.0	10.0	85	1.2 ± 0.1	3.3 ± 0.1

Table 2. Effect of auxins on rooting of *in vitro* derived shoots after six weeks of culture on full-strength and half –strength MS media.

PGR.	Con.	Full MS			1/2 MS		
		Reg. response (%)	No of roots / shoot (mean ± SE)	Root length (mean ± SE) (cm)	Reg. response (%)	No of roots / shoot (mean ± SE)	Root length (mean ±SE) (cm)
IBA	0.0	100	9.3 ± 0.8	4.2 ± 0.2	100	12.0 ± 0.8	5.4 ± 0.3
	0.1	100	7.7 ± 1.2	5.7 ± 0.3	100	6.0 ± 0.7	7.4 ± 0.2
	0.5	100	8.4 ± 1.3	5.9 ± 0.2	100	7.0 ± 0.6	7.4 ± 0.1
	1.0	100	15.0 ± 1.5	5.1 ± 0.2	100	7.0 ± 0.5	7.2 ± 0.2
IAA	0.1	100	10.0 ± 1.2	4.8 ± 0.2	100	13.0 ± 1.6	5.5 ± 0.2
	0.5	100	11.0 ± 1.8	5.2 ± 0.2	100	21.0 ± 1.7	6.1 ± 0.2
	1.0	100	16.0 ± 1.5	4.7 ± 0.2	100	21.0 ± 0.7	6.3 ± 0.2
NAA	0.1	100	19.0 ± 1.6	4.0 ± 0.2	100	20.0 ± 1.5	5.0 ± 0.2
	0.5	94	17.0 ± 1.7	3.4 ± 0.1	100	22.0 ± 0.9	4.3 ± 0.2
	1.0	85	9.6 ± 1.0	2.3 ± 0.1	90	11.0 ± 1.1	2.9 ± 0.1

This paper describes the effect of silver nitrate and cobalt chloride, ethylene inhibitors, on the frequency of adventitious shoot regeneration and number of shoots per explant of *in vitro* culture of shoot tip of tomato cultivar Omdurman.

MATERIALS AND METHODS

Plant material

Mature seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar Omdurman were obtained from National Institute for Promotion of Horticultural Exports, University of Gezira, Sudan.

Surface sterilization and seed germination:

Seeds were washed by continuously running tap water for 15 minutes then washed by sterile distilled water. Under laminar flow cabinet seeds were disinfected with clorex 25% (0.5% free chlorine) v\v for 15 minutes with continuous shaking then rinsed five times with sterile distilled water. After surface sterilization, twenty seeds were directly transferred to culture bottle containing-half-strength MS basal media solidified with 0.7% agar and incubated for 12-14 days at 25 °C ± 2 with a 16 hr photoperiod for germination.

Explants preparation

In vitro produced seedling 14 days- old were used as a source of explants. Cotyledon and hypocotyls were removed and discharged, shoot tip of 1.0 cm length was used as explant for multiple shoots induction.

Effect of ethylene inhibitors on multiple shoot induction

Shoot tip explants were cultured in MS (Murashige and Skoog, 1962) medium containing Kin at 4.0 mg/L and supplemented with silver nitrate ($AgNO_3$) and cobalt chloride ($CoCl_2$) at different concentrations (1, 2, 3, 4, 5 and 10 mg/L). Cultures were incubated for six weeks at 25°C±2 under cool white fluorescent light and 16 photoperiod.

***In vitro* rooting of regenerated shoots**

Shoots developed from the explants were excised and transferred to full and half-strength of MS media supplemented with different concentrations of auxins (IAA, NAA and IBA) at different concentrations (0.0, 0.1, 0.5 and 1.0 mg/L) to evaluate their effects on rooting of directly regenerated plantlets. The effect of auxins was evaluated on the number of roots/shoot, root length and rooting percentage.

Acclimatization

Rooted plantlets were taken out from culture tubes and washed thoroughly with tap water to remove the culture medium from the roots. Plants were transferred to plastic pots containing a mixture of autoclaved soil and sand at the rate 1:1 . The pots were covered with glass bottles to prevent rapid loss of humidity and were kept under culture room conditions . The plants were watered three times a week. After 3-4 weeks the glass bottles were removed and the plants were transferred to a green house and placed under shade .

Culture condition and data analysis

All the media used were supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar, the pH was adjusted to 5.8±0.02 prior to the addition of agar and culture media were autoclaved at 121 ºC for 20 min. The experiment was repeated 3 times and all parameters were collected after 6 weeks of incubation then standard error was calculated by excel computer program. Means were separated by Duncan's multiple range test (DMRT) (Duncan, 1955).

RESULTS AND DISCUSSION

Ethylene production in tissue culture stimulated either by mechanical wounding (Nakajima *et al.,* 1990) and/or though gaseous exchange in sealed containers (Chi *et al.,* 1991). Its accumulation inhibits *in vitro* regeneration in several plant species (Gong and Pua, 2004). Here in this study, with the aim of optimizing previous protocol developed in our laboratory (Ishag *et al.,* 2009), the effect of ethylene inhibitors on tomato multiple shoot induction was addressed by adding different levels of silver nitrate and cobalt chloride to the culture media (MS) containing Kin at 4.0 mg/l. The result showed that the addition of ethylene inhibitors improved the regeneration frequency by 20%. The best result for the number of shoots per explant (2.4) was obtained by using $CoCl_2$ at 3.0 mg/l followed by 2.3shoots/explant which was obtained by adding AgNO3 at 5.0mg/l (Table 1).

The positive effect of $AgNO_3$ on shoot regeneration was already reported for numbers plants including Cotton (Abdellatef and Khalafalla, 2008), faba bean (Khalafalla and Hattori, 2000), Sesame ((Abdellatef *et al,*. 2010) , *nicotiana plumbaginifolia* and *Triticum aestivum* (Purnhauser *et al.*, 1987), Zea mays (Songstad *et al.*,1991), *Brassica campestris* sp.Oleifera (Burnett *et al,,*1994).*Pereinensis* (Chi *et al*.,1990) and *Raphanus sativus* (Pua *et al*., 1996). Moreover, in other study (Chraibi *et al.*, 1991) it was reported that $CoCl_2$ was equally effective as $AgNO_3$ in eliciting morphogenesis. Roustan *et al.,* 1992, Mhatre *et al*., 1998 report that $CoCl_2$ induced multiple shoots but a lower frequency of shoot per explant in melon and cucumber respectively.

In some cases, $CoCl_2$ was equally effective as $AgNO_3$ in eliciting morphogenesis (Chraibi *et al*., 1991). Regenerated shoots were excised and rooted on full and half-strength MS medium without or with different levels of NAA, IAA, or IBA (Table 2). Our result showed that 100% rooting was obtained on shoot cultured in both full and half-strength MS medium without growth regulators (Table 2). Similarly, (Mensuali-Sodi *et al*., 1995) reported that tomato *in vitro* rooting does not require any exogenous plant growth regulators.

However, during our study, cultivation of *in vitro* induced shoots on half-strength MS medium supplemented with 0.5 and 2.0 mg/l NAA resulted in a large number of rooted micro shoots (22± 0.9) and longer root (7.4 ± 0.9 cm), respectively. These results can be explained by the promotive effect of auxins on root

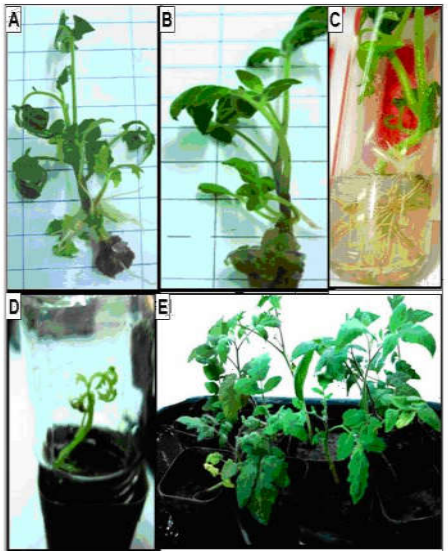

Figure 1. *In vitro* multiple shoot induction and plant regeneration in tomato (*Lycopersicon esculentum*Mill. cv. Omdurman) **A**: Multiple shoots bunches induced from shoot tip explants on shoot formation media containing Kin at 4.0 mg/l supplemented with $AgNO_3$ at 5.0mg/l. **B**: Multiple shoots bunches induced from shoot tip explants on shoot formation media containing Kin at 4.0 mg/l supplemented with $CoCl_2$ at 3.0mg/l. C. Rooted shoot on half-strength MS basal medium supplemented with 1.0 mg/l IAA. D. Acclimatization of *in vitro* regenerated tomato plant **E**. Tomato plant established in soil under green house conditions

initials, as observed by (De Klerk *et al*., 1999). The beneficial effect of using half-strength MS medium for rooting of *in vitro* induced shoots has already been reported for tomato. (Devi *et al*., 2008) reported that the best rooting was found to be in half-strength medium supplemented with 0.2mg/l IBA.

For acclimatization plantlets were removed from rooting medium after six weeks of incubation and transferred to plastic pots containing autoclaved soil and sand at the rate 1:1 covered with glass bottle to maintain humidity and was kept under culture room conditions for one week similar procedure was done by (Roussos *et al*., 1999). After three weeks, glass bottles were removed and transferred to green house and placed under shade until growth was observed. 95% of plants survived and all were morphologically normal (Fig 1).

In conclusion, development of an efficient tissue culture and plant regeneration protocol for tomato cultivars is the first step towards the application of transgenic technology to improve tomato breeding and is, thus, the foundation of tomato biotechnology. Furthermore, the present finding of enhancement of multiple shoot induction by the addition of various additives will promote the application of plant tissue culture technology in the area of selection resistance and production of tomato artificial seeds.

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