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Influence of silver nitrate ($AgNO_3$) and cold pretreatment on the doubled haploid production of tomato using anther culture

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Summary. The responses of tomato (cv. MSK8) anthers to different concentrations of silver nitrate and cold pretreatment were studied for producing doubled haploid (DH) plants via anther culture. Flower buds were collected from the plants grown in greenhouse conditions and isolated anthers were incubated on two cold pretreatments (36 and 72 h) at 4°C under the dark condition in four different silver nitrate (AgNO3) solutions with 0, 5, 10, 15 mg/l concentrations. Analysis of variance showed that interaction between AgNO3 and cold pretreatment was not significant for androgenic traits. However, cold pretreatment had significant effects on measured traits. Achieved results revealed that 36h cold pretreatment was more effective than 72h for studied traits. Callus and shoot induction, plantlet regeneration as well as green plants production were obtained from all the concentrations tested, but with significantly different rates. However, the percentage of DH plant was not influenced by AgNO3 concentrations. The highest callus and shoot induction percentage and the number of plant regeneration were obtained from 5 mg/l AgNO3. The anthers pretreated by 10 mg/l AgNO3 produced more percentage of green plant production than other concentrations. It seems that the application of AgNO3 pretreatment had positive effects and can be proposed.

Key words: Anti-ethylene, callus induction, green plants, haploid production, shoot induction

Introduction

Anther culture provides a rapid way to produce haploid lines and these lines can be easily used for subsequent production of doubled haploids (DH) and F1-hybrids. DHs have proved to be particularly valuable in the analyses of the genetically complex basis of QTL mapping (1, 2). Anther culture to produce DH lines has been studied widely in tomato (3). However, the production of haploid and DH lines with anther culture has a lot of problem in plant tissue culture (4). Androgenesis rate was affected by various factors such as genotype (3, 5), culture conditions of donor plant (3), developmental stage of microspore (6), pretreatment of flower bud and anthers (2), colchicine pretreatment (2, 7) and culture medium (3, 8). The cold temperature pre-treatment widely used for androgenesis. Several studies have recently worked on androgenesis from flower buds of tomato by cold pre-treatment techniques but the precise temperature and treatment time has been varied in previous reports (8).

It has been reported that ethylene inhibits somatic embryogenesis (9, 10-12) and shoot regeneration (13-15), and callus growth (16). Ethylene produced during in all plant tissue culture process but there are two important reasons for ethylene production: the stress during explant excision (1, 17) and the presence of auxin in the culture medium (18). Application AgNO3 could be improved the regeneration in *Brassica campestris* (17, 19) and *Coffea canephora* (20), the

somatic embryogenesis in *Daucus carota* (21), and Rotula aquatica Lour (22). It has been reported that no embryo was obtained in some *B. oleracea* morphotypes without the addition of AgNO3 (23). Adding AgNO3 into the callus induction medium significantly enhanced the embryogenic callus production of male immature inflorescence cultures of buffalograss (11). Fernandez et al. (24) reported that AgNO3 affected both the percentage of embryogenic explants and the number of somatic embryos per explant. Furthermore, this was explained with the possible involvement of AgNO3 in ethylene metabolism. Root initiation and elongation of in vitro axillary bud cultures of *Vanilla planifolia* were improved when AgNO3 was added. These beneficial effects of AgNO3 on rooting may result from inhibition of ethylene action (13). Silver ions also employed in the form of silver thiosulphate in several tissue culture studies (10, 17).

The aim of the present work was to evaluate the effects of AgNO3, anti-ethylene compound, at four concentrations as well as two cold pretreatments (4°C) (36 and 72 h) on prevention of browning of cultured anthers and androgenic responses of tomato cv MSK8 (through anther culture) as well as doubled haploid (DH) plants production.

Material and Methods

Donor plants

The seeds of tomato cv. MSK8 obtained from Tomato Genetic Resource Center of the University of California, Davis. The Seeds were sown on Jan. 10, Jan. 30 and Feb. 20, 2015, in the greenhouse of Faculty of Agriculture, Tabriz University (East Azerbaijan, Iran) and irrigated once per week and fertilized if needed. The anther donor plants were grown at about 25°C during days and about 18°C during nights under a long day condition (16 hours). Floral buds were collected during 25 days from the beginning of flowering as proposed by Motallebi-Azar et al. (8) and Park et al. (4). Floral buds (4–5 mm) containing anthers (1.7–2.5 mm) with pollen mother cell at prophase I to metaphase II were harvested (8) in the morning, surface sterilized in 70% ethanol for 5 min, followed by immersion in a 2.5% solution of sodium hypochlorite for 15 min and rinsed three times with sterilized distilled water. The anthers were removed aseptically and placed onto the nutrient medium in a 6 × 1.5 cm Petri dish containing 10 ml of induction medium. The dishes were wrapped in parafilm and placed in a dark growth chamber at 4°C for 36 or 72 h and then transferred to 26 ± 2°C and kept them for 4 weeks under dark condition. After the treatment in darkness, each plate was exposed to 4–7 weeks of 16d/8n photoperiod (with the light intensity of about 80 μmol m^{-2} s^{-1}) provided by cool white fluorescent lamps.

Culture Media

The culture medium, used in this experiment, were as the following: induction medium: MS basic + 2 mg/l IAA + 1 mg/l 2ip + 20 g/l glucose + 7 g/l agar according to Motallebi-Azar et al., (21). Finally, for rooting, the shoots >2 cm transferred to root induction medium including half straight MS + 2 mg/l IBA + 0.5 mg/l GA3 for 2 weeks and then plantlets transferred to perlite in a mist system (8).

$AgNO_3$ treatment

The anthers dissected under sterile conditions were dipped in 0, 5, 10 and 15 mg/l aqueous solution of AgNO3 for 36 and 72 h at 4°C under dark condition, to inhibit ethylene synthesis of the microspores and its other useful effects, and then they were rinsed in sterile distilled water. After AgNO3 pretreatments, the anthers were transferred to AgNO3 free semisolid shoot induction medium (8). The percentage of callus and shoot induction, the number of regenerated plants as well as the percentage of DH plants and green plant production were calculated.

Statistical Analysis

The experiment was conducted as a factorial experiment based on a completely randomized design with three replications. Each experimental unit had three Petri dishes with 10 anthers. Analysis of variance was carried out by SPSS statistical software (version 16.0) and mean comparisons were accompanied by Duncan's New Multiple Range Test at 5% probability level.

Results

The first callus induction was obtained in the 2nd week of culturing (Figure 1A), but intensive callus production was observed in the 4th week (Figure 1B). First shoot induction was taken place after 4th week (Figure 1C). Shoots with 2 cm in length (Figure 1D) were cut and placed the root induction for shoot growth and root development (Figure 1E). Plantlets (Figure 1F) were obtained 4 weeks after translocating of the shoot to the root induction medium.

Analysis of variance showed that differences among AgNO3 concentrations were significant for callus induction percentage, but cold pretreatment and the interaction between cold pretreatment and AgNO3 concentration were non-significant. The maximum callus induction was obtained on 5 mg/l AgNO3. However, in low (without AgNO3) or high this concentration, callus induction was decreased. When both cold pretreatment conditions were considered, the results of the experiment showed that the optimum concentration of AgNO3 for callus induction in tomato anther culture is 5 mg/l (Figure 2).

AgNO3 concentrations and cold pretreatment have significantly influenced the shoot induction but the interaction between them was not significantly different. This result showed that the effects of AgNO3 did not depend on cold pretreatment for shoot induction. The shoot induction percentage in 36h cold pretreatment was higher than 72h cold pretreatment. In non-pretreated anthers by AgNO3 (control), Shoot induction was lower than that on pretreated anthers. The shoot induction was the maximum at 5 mg/l and with increasing AgNO3 concentrations resulted significantly decreased. It showed that optimum concentration for shoot induction was 5 mg/l and more than that concentration had negative effects (Figure 3).

The regenerated plant number was influenced significantly by AgNO3 concentrations. However, cold pretreatment and interaction between cold pretreatment and AgNO3 concentrations were not significant in term of this trait. Therefore, with respect to shooting induction, the addition of AgNO3 to pretreatment solution was a positive effect on regenerated plants number. However, only 5 mg/l AgNO3 was higher regenerated plants number than control due to high shoot induction in this concentration (Figure 4).

Four different AgNO3 concentrations (0, 5, 10, and 15 mg/l) were tested. DH plantlets were obtained from all the concentrations tested (20-25%), but with same production rates. Therefore, AgNO3 did not effect on doubling chromosome on the early stage of

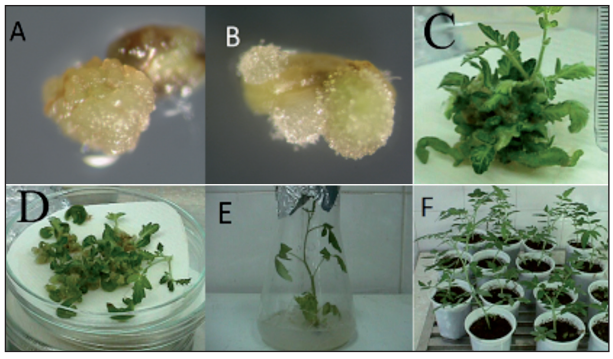

Figure 1. *Tomato cv MSK8* anther culture stages: A: The first callus induction in 2th week of culturing; B: intensive callus production in 4th week; C: Shoot induction; D: Shoots with 2 cm in length; E: Root induction; F: Plantlets.

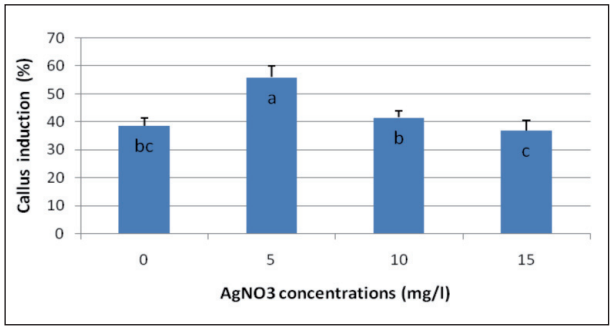

Figure 2. The effects of different $AgNO_3$ concentrations (mg/l) and cold (hour) pretreatments on percentage of callus induction from anther cultures of Tomato cv MSK8.

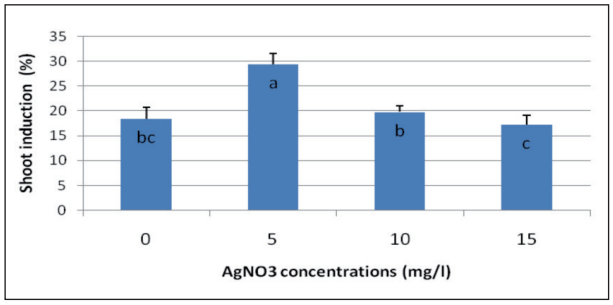

Figure 3. The effects of different AgNO3 concentrations (mg/l) and cold (hour) pretreatments on percentage of shoot induction from anther cultures of Tomato cv MSK8.

microspore development. However, the percentage of DH plantlets was significantly influenced by cold pretreatment and it was higher in 36h than 72h cold pretreatment.

AgNO3 concentrations and cold pretreatment have significantly influenced the percentage of green plantlet production. The same as other androgenic traits, production of green plant in 36h was higher than 72h CDP. The lowest green plant production was obtained on without pretreated anthers (control). AgNO3 improved green plant production in all concentrations, but with different rates. The maximum green plant production was observed on 10 mg/l AgNO3. However, the green plants' production was decreased in other concentrations; nevertheless, all treatment was higher than control (Figure 5).

Discussion

The Ag+ ions inhibit ethylene action in a wide variety of plants. The ethylene inhibiting effect of Ag+ related to an interference with ethylene binding. The

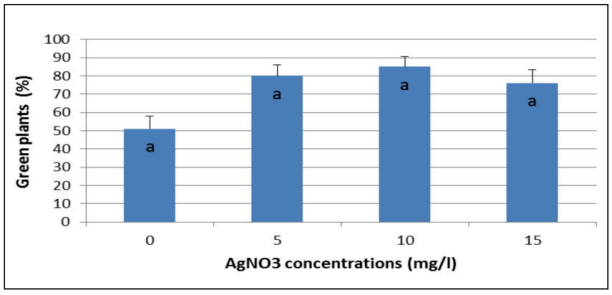

Figure 5. The effects of different AgNO3 concentrations (mg/l) and cold (hour) pretreatments on percentage of green plant production from anther cultures of Tomato cv MSK8.

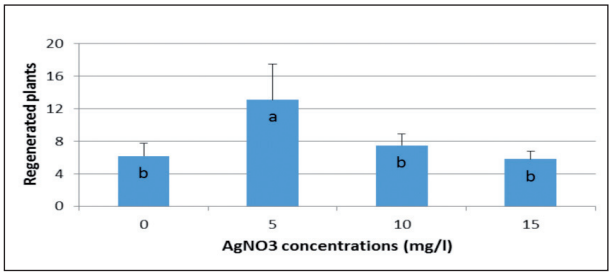

Figure 4. The effects of different AgNO3 concentrations (mg/l) and cold (hour) pretreatments on number of regenerated plantlet from anther cultures of Tomato cv MSK8.

positive effect of Ag+ ions in callus and shoot organogenesis suggests that ethylene produced by cultured explants might be inhibiting shoot organogenesis of those explants (25). This experiment was conducted to study the effect of different concentrations of AgNO3 on callus and shoot induction as well as the production of DH plantlet in tomato cv. MSK8. The results showed a positive effect of AgNO3 on callus induction. Among the varying AgNO3 concentrations used for callus induction (when both cold pretreatment conditions were considered), the highest frequency of callus induction of 55% was obtained at 5 mg/l AgNO3 pretreatment. Silver ions in the form of nitrate, such as AgNO3, play a major role in influencing callus induction (16, 26). Addition of AgNO3 to modified N6 medium has been reported to promote callus development and green plant regeneration from indicia rice anthers (19). Application of AgNO3 caused to decrease in callus induction; it seems that ethylene production by injured anther cells has been inhibited by AgNO3 treatment. When anthers did not pretreat by AgNO3 (control), callus induction was the lower than pretreated anthers, it shows that AgNO3 inhibited ethylene production by injured anther cells (21).

Thermal pretreatments, especially cold pretreatments, are one of the most important factors influenced shoot induction via anther culture in tomato and other plants (2). The results showed short cold pretreatment was more effective than the long period for inducing shoot from calli. Therefore, for blocking the gametophytic stage of microspore and inducing saprophytic stage, short cold pretreatment is essential. Motallebi-Azar et al., (8) showed the highest shoot induction occurred when isolated anthers were pretreated at 4oC for 48h. In addition, cold pretreatment was depended to other factors influenced shoot induction from anthers (2). Shoot induction was influenced by AgNO3 concentration and optimum concentration was observed on 5 mg/l. The higher AgNO3 concentrations showed negative effects on shoot induction. Ethylene increased shoot induction percentage. AgNO3 promoted shoot regeneration in terms of shoot inhibitor such as AgNO3, in a specific concentration, promoted cytokinin effects and caused number/explant and shoot multiplication percentage (19). In a similar way, AgNO3 enhanced shoot regeneration of watermelon

(2), Vanilla planifolia (12), and Chinese radish (15). AgNO3, which also stimulated shoot regeneration, inhibited rooting in wheat and triticale (24).

The results showed that AgNO3 pretreatment application improved the number of regenerated plantlets when anthers pretreated by 5 mgl-1 AgNO3. The higher concentration of AgNO3 than 5 mg/l had negative effects on improving the number of regenerated plantlets, the same as shoot induction. AgNO3 application improved the regeneration in *Brassica campestris* (19), *Coffea canephora* (20), *Daucus carota* (21) and *Rotula aquatica* Lour (22). It has been reported that embryo was not obtained in some B. oleracea morphotypes without the addition of AgNO3 (23). AgNO3 was also essential for androgenic response in the cytoplasmic male sterile lines of *Brassica juncea*, and it markedly increased the frequency of androgenesis in the cultivated species (27).

Haploid plant production was influenced by cold pretreatment (2), but it was not affected by AgNO3 concentrations and interaction between them. It seems that 36h cold pretreatment was the effective cold pretreatment for tomato anther culture to produce haploid plants (8). AgNO3 had effects on cell growth and development, but it did not involve chromosome doubling (22).

Application of AgNO3 as a pretreatment of isolated anther had positive effects on green plants production. According to our results, the highest green plant production was obtained on 10 mg/l (when both cold pretreatment conditions were considered). With using AgNO3, the frequency of green plant differentiation doubled (2). It is also known to promote pollen embryo production in anther cultures of Brussels sprouts (9). AgNO3 as an anti-ethylene agent to delay anther positive impact in anthers response in the present study. It seems that AgNO3 had a positive effect on callus and shoot induction by blocking the inhibitory effect of endogenously produced ethylene in culture vessels. Lentini et al. (19) reported that application of 10mg/l of AgNO3, anti-ethylene compound to callus induction medium promoted 2 fold increase in pollen callusing frequency and green plant regeneration. The similar positive effect of AgNO3 was reported in anther culture of Brassica (28).

Conclusions

Cold pretreatments and anti-ethylene compounds like AgNO3 are more important factors influenced androgenic responses in plants such as tomato. Achieved results showed significant positive effects of cold pretreatment at 4oC for 36h on shoot induction and DH plantlets production. In this case, the best AgNO3 concentration was 5 mg/l for callus and shoot induction. The most considerable effects of AgNO3 were its effect on plant regeneration and green plantlets production, so with using AgNO3, in addition to increasing plant regeneration, high increasing of green plantlets production were observed. It seems that 5 mg/l of AgNO3 was the best concentration of AgNO3 to improve androgenic responses.

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