Journal of Applied Botany and Food Quality 90, 68 - 75 (2017), DOI:10.5073/JABFQ.2017.090.010

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Embryo and callus induction by different factors in ovary culture of cucumber

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(Received June 23, 2016)

Summary

Haploid and doubled haploid lines can be obtained in a short time using *in vitro* methods. In this study, unfertilized ovaries of cucumber were harvested and placed on semi-solid MS media as explants in Petri dishes. The thermal shock pretreatment, cucumber genotypes, hormonal combinations and $AgNO_3$ were evaluated as experimental factors in three consecutive experiments. The results of first experiment revealed that the thermal shock pretreatment had a significant influence on embryo induction, and the highest rate of embryogenesis was produced in presence of thermal shock pretreatment and the NAA+2,4-D+KIN+BAP (0.5+0.7+1+1.8) mg/L hormonal combination. The highest rate of callus induction was recorded in combination of absence thermal shock and the NAA+2,4-D+KIN+BAP (1.5+0.7+1+1.8) mg/L hormonal combination. According to the second experiment results, genotypes and the other hormonal combinations in media culture had highly significant effects on embryo and callus induction. The NBDC6*6/32441 genotype had the highest effects on these traits. A combination of BAP (4 mg/L) and 2,4-D (1.5 mg/L) was found to be optimal for embryogenesis. In third experiment, the highest level of embryogenesis (24.93 %) and callus (24.19 %) induction were found in the local Iranian cultivar in comparison to NBDC6*6/32441 genotype. Silver nitrate treatment had significant effects on the embryo and callus induction. The highest rate of embryo induction was recorded in presence of silver nitrate; however the absence of $AgNO_3$ had a positive effect on the callus induction. In conclusion, the thermal shock pretreatment, silver nitrate, genotype and hormonal combination factors could play key roles in embryo and callus production, independently and simultaneously.

Keywords: Ovary culture, hormone, $AgNO_3$, genotype, cucumber

Introduction

Cucumber (*Cucumis sativus* L.) is one of the world's most important vegetable crops belonging to the family Cucurbitaceae and the fruit is rich in phosphorus, potassium and oxalic acid. It is also called "Khiar" and has been used for 3,000 years in particular Asia and Africa (KHAN et al., 2015).

One of important genetic materials in vegetable breeding programs is doubled haploid (DH) line. The use of completely homozygous DH lines would be considerably shortened breeding program. One of the most significant biotechnological advances in plant breeding has been the development of *in vitro* techniques to produce haploid plants that can be used to generate homozygous lines (EVANS et al., 2003).

There are several processes leading to haploid and DH cucurbits. These include primarily haploid parthenogenesis (induced primarily by pollination with irradiated pollen), *in vitro* gynogenesis (during *in vitro* culture of ovules and ovaries) and *in vitro* androgenesis (during *in vitro* culture of microspores and anthers) (GALAZKA and NIEMIROWICZ-SZCZYTT, 2013).

DH lines have been used successfully in plant breeding programs for many crops such as barley, wheat, maize, pepper, rice, and tobacco (THOMAS et al., 2003). The main advantage of double haploid lines is their complete homozygosis. This facilitates the phenotypic selection for qualitative and quantitative characters much easier. Thus, DHs can improve the efficiency and it is well known as time-consuming and labor-intensive. DH systems are widely applied in breeding (THOMAS et al., 2003) and genetic mapping (FORSTER and THOMAS, 2005).

Ovary culture has been one of the approaches used to generate homozygous lines for commercial production of F_1 hybrid varieties and genetic studies (DIAO et al., 2009). It has been pointed out in many experiments. On the other hand, successful regeneration of haploids and doubled haploids in ovary or ovule culture depend on several factors, i.e., pretreatment, genotype, female gametophyte development stage, and culture media composition (DIAO et al., 2009).

DIAO et al. (2009) examined the effect of several factors in detail, i.e. the duration of thermal shock pretreatment at 35 ºC, the concentrations of Thidiazuron (TDZ) and the presence of silver nitrate and investigated their effects on embryo formation in ovary culture of cucumber (*Cucumis sativus* L.). Their results showed that a thermal shock for 3 days at 35 °C at the start of the culture in contrast to 2 or 4 days would result in higher frequency of embryo formation. Thus the TDZ has a positive effect on embryo formation. The highest embryo formation frequency (72.7 %) was recorded when 0.04 mg/L TDZ into the induction medium. The results also revealed that was added to $AgNO_3$ to the induction medium had no significant effect on frequency of embryo formation, however embryo germination period was shortened and the number of embryos increased in each ovary slice.

MALIK et al. (2011) observed that thermal pre-treatment (4 °C) for 4 days increased embryo formation frequency (63.3 %) significantly in melon (*Cucumis melo* L.). Their experiment showed that TDZ (0.04 and 0.02) mg/L to induction medium significantly increased the number of responding ovaries (46.6 % and 65.83 %, respectively). The maximum number of plantlet regeneration (22.5 %) was achieved by culturing the ovary derived embryos on Murashige and Skoog medium (MS medium) supplemented with 0.6 mg/L 6-benzylaminopurine.

The objectives of this study were 1) to evaluate the effect of different concentrations of hormones and also hormone combinations on callus and embryo induction, 2) the influences of low and high temperature pre-treatment on callus and embryo induction, 3) the effect of silver nitrate on embryo and callus formation and 4) the influence of genotypes on callus and embryo induction, after all, as a local variety of cucumber in Iran in terms of taste and aroma, it is highly regarded and highly marketable for the Iranian consumers.

Material and methods

Plant material

The present experiment was carried out at the Plant Improvement and Seed Production Research Center of the Islamic Azad University, Isfahan (Khorasgan) Branch, Iran, (51° 36´ longitude and 32°

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63´ latitude) during 2013-2014. In this experiment one local variety of open field cucumber and two Breeding lines (NBDC20*15/53211 and NBDC6*6/32441) of greenhouse cucumbers, that have been released in this center, were used. All three genotypes were planted in a greenhouse and managed using standard agronomic practices. During the growing period, the diurnal greenhouse air temperature was kept within 25-30 °C and the nocturnal one within 19-21 °C with a relative humidity of about 60%.

Ovary culture

The female flowers were picked one day before opening and transferred to laboratory in the ice box. Most of the flowers were selected from first nodes (Shalabi, 2007). Unfertilized ovaries were isolated and surface sterilized by soaking in water for 3 min, followed by a 3 min washing in a water and detergent solution, and then rinsed three times in sterile distilled water. Ovaries were then placed in 10% sodium hypochlorite for 10 min, rinsed three or four times in sterile distilled water and finally, rinsed in 70% Ethanol for 5 min. The ovaries were sliced into 1 mm cross section under sterile conditions and placed carefully in a semi-solid medium in a petri dish. MS medium (Moqbeli et al., 2013) was used as basal medium in all experiments. All media were supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C, 1.1 kg/cm^2 for 20 min. All cultures were incubated at 24±2 °C under a 16/8 h (light/dark) photoperiod with light provided using cool-white fluorescent lamps with 3000 Lux.

Experimental factors

In this study, consecutive experiments were conducted with the individual results used for the preceding ones.

Experiment 1: This experiment was performed with respect to the two factors; factor I consisted of thermal shock pretreatment (35 °C pretreatment for 4 days and without pretreatment), and factor II consisted of various concentrations of cytokinins and auxins (2, 4-D, Kin, IBA, NAA, BAP, IAA from 0.1 to 3 mg/L). Immediately after placing the ovary slices on induction medium, cultures were exposed to thermal shock pretreatment at 35 °C for 4 days. After the thermal pretreatment, all cultures were transferred to the 25 °C growth chamber. Control petri dishes (without pretreatment) had been transferred to the 25 °C growth chamber from the beginning.

Experiment 2: According to the results of thermal shock pretreatment, the effect of genotype and different concentrations of hormones were investigated in the second experiment. The first factor consisted of two greenhouse inbred lines (NBDC515*20/3211 and NBDC6*6/32441) and the second factor consisted of 20 treatments of different concentrations and combinations of cytokinin and auxin plant growth regulators (2, 4-D, Kin, IBA, NAA, BAP, IAA from 0.1 to 3 mg/L).

Experiment 3: The most suitable greenhouse inbred line (NBDC6*6/32441) was selected and compared with the cucumber local cultivar as the first factor. The experiment carried out in presence and absence of silver nitrate in the culture medium as the second factor. Finally, seven selected concentrations and combinations of different hormones (2, 4-D, Kin, IBA, NAA, BAP, TDZ from 0.1 to 0.8 mg/L) were considered as the third factor. After thermal shock pretreatment, all treatments were transferred to the 25 °C growth chamber.

Statistical analysis

All cultures were observed when one month had elapsed from the first day of ovary slices incubation. The frequency of embryo and callus formation was recorded based on the ratio of the number of ovaries responding to the total number of treated ovaries. The results of all three experiments were analyzed based on the factorial experiment in a completely randomized design in three replications and three petri dishes in every replicate. Analysis of variance was performed by SAS (Ver. 9) software, and mean comparisons were assayed by an LSD (Least Significant Differences) test at $p \leq 0.05$ Proportion values were transformed with arcsine transformation before they were used in the statistical analysis. Interaction mean comparisons were carried out by MSTATC software.

Results and discussion

Effect of thermal pretreatment and growth regulators on callus and embryo induction (first experiment)

In the first experiment, analysis of variance indicated that thermal shock pretreatment (2 levels) and various concentrations of hormones (6 levels) had significant effects on callus induction and embryogenesis. Data presented in Tab. 1 showed that the most embryogenesis (38.98%) occurred during the thermal shock pretreatment. Dubas et al. (2014) revealed that the distribution of auxin changes during embryo development and this innovation depends on the temperature-inducible *in vitro* culture conditions. Many factors effect on successful ovary culture. For example, thermal treatment, (i.e. high or low temperature) applied to ovary or during *in vitro* culture, may have a strong influence on embryo formation (Yang, 1982). In cucumber, 35 ºC thermal shock pretreatment was effective on ovary culture (Gemes et al., 2002). Specific changes that occur in cellular architecture during the switching from gametophytic to embryogenic pathway period following a heat shock treatment in *Brassica napus* had been reported earlier (Satpute et al., 2005).

The Diao et al. (2009) results showed that with high temperature (35 °C) treatments, the amount of embryogenesis increased and the most embryogenesis observed in 3-day treatments. The main objective of the Koleva-Gudeva et al. (2007) study was to evaluate the response of anthers from different genotypes to various media and heat and cold-shock pre-treatments regarding the direct somatic embryogenesis. Their findings indicated that the cold and heat shocks (at 7 °C and 35 °C respectively, both in darkness) as a precondition will push the anther cultures towards direct somatic embryogenesis. Shalaby (2007) found that ovules exposed to 32 °C for 4 days produced the greatest number of gynogenic ovules in squash. Kurt and Evans (1998) revealed that the pretreatment of flower buds (at 4 °C for three days) significantly lowered the number of calli induced in solid medium but, it could not have that statistically significant effect in the liquid medium.

The fact that temperature can control the development of sexual explants such as isolated microspores has been well documented in *Brassica napus*. In this plant lower temperatures favor asymmetric divisions, while heat treatment favors symmetric divisions (Segul-Simarro et al., 2003). The Bueno et al. (1997) results were revealed that stress, particularly heat shock, sucrose starvation or a combination of both treatments could be the major and general sign of the inhibition of the normal gametophytic development of microspores and for the induction of the alternative embryogenic pathway.

Tab. 1: Effects of thermal pretreatment on embryo and callus induction in experiment 1.

Traits	Thermal shock pretreatment	Control
Embryogenesis (%)	38.98 [a]	7.20 [b]
Callus induction (%)	28.60 [b]	35.73 [a]

Means followed by a common letter are not significantly different at 5% level according to LSD test

Plant growth regulators such as cytokinin and auxin are the principal hormones in the plants involved in cell division and regulation of the differentiation (FEHER et al., 2003). Hormone combinations had a significant effect on callus induction and embryogenesis. According to Fig. 1, combination of 2,4-D+KIN (1.5+1) mg/L showed the highest level of embryogenesis (52.76%). However, this combination had no significant difference with that of NAA+2,4-D+KIN+BAP (0.8+0.8+0.8+2.5) mg/L.

Among the similar somatic embryogenesis production factors, the 2,4-D is one of the highest efficiency, and thus it is used in the majority of embryonic cell and tissue culture procedures (FEHER et al., 2003).

Mean comparison results showed that the highest rate of callus induction (47.76%) is observed in combination of 2,4-D+NAA+KIN+BAP (0.5+0.5+0.5+1.5) mg/L, however it didn't have any significant difference with NAA+2,4-D+KIN+BAP (1.5+0.7+1+1.8) mg/L combination (Fig. 1). The least embryogenesis and callus induction was observed in IBA+NAA+KIN+BAP (0.4+0.1+1+0.5) mg/L. In general, the medium containing 2,4-D and KIN had the highest rate of embryogenesis, and the medium containing NAA had the highest rate of callus induction. However, with increased BAP, the amount of callus induction decreases. Both auxins and cytokinins are essential for cell division and play a significant role in the meristem' establishment and activity, however fluctuation of cytokinin and auxin ratios favor development of either root or shoot meristems (SUGIMOTO et al., 2011). In general, increasing the ratio of cytokinin to auxin results in a shift from root to shoot organogenesis (HILL and SCHALLER, 2013). The KOLI and MURTHY (2013) results showed that the maximum callus induction observed in NAA+BAP (2+1) mg/L, 2,4-D+TDZ (2+2) mg/L and NAA+KIN (5+2) mg/L combinations and also reported that the more cytokinins and the less auxins the more callus induction occurs.

The analysis of variance indicated that there was a significant interaction between the thermal shock pretreatment and the hormonal combinations of the culture medium. The highest rate of embryogenesis was obtained in the T_4 hormone combination with thermal shock pretreatment, which is indicative of the fact that there is a significant difference between this treatment and other treatments (Tab. 2). The lowest rate of embryogenesis was observed in hormonal combinations IBA+NAA+KIN+BAP (0.4+0.1+1+0.5) mg/L and IAA+KIN+BAP (0.7+1.5+3) mg/L without thermal shock pretreatment. Hormonal combination of NAA+2,4-D+KIN+BAP (1.5+0.7+1+1.8) mg/L without thermal shock pretreatment showed the maximum callus production induction, although it did not have any significant difference with 2,4-D+NAA+KIN+BAP (0.5+0.5+0.5+1.5) mg/L hormonal combination (Tab. 2).

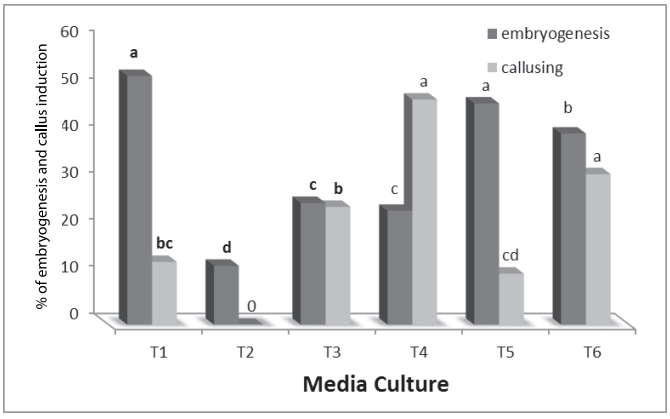

Fig. 1: Effects of different hormonal combinations on embryogenesis and callus induction in experiment 1.

For every trait, letters compared separately. Means followed by a common letter are not significantly different at 5% level according to LSD test
T1: 2,4-D+KIN (1.5+1 mg·l^{-1}); T2: IBA+NAA+KIN+BAP (0.4+0.1+1+0.5 mg·l^{-1}); T3: IAA+KIN+BAP (0.7+1.5+3 mg·l^{-1}); T4: NAA+2,4-D+KIN+BAP (0.5+0.5+0.5+1.5 mg·l^{-1}); T5: NAA+2,4-D+KIN+BAP (0.8+0.8+0.8+2.5 mg·l^{-1}); T6: NAA+2,4-D+KIN+BAP (1.5+0.7+1+1.8 mg·l^{-1})

Effect of genotypes and growth regulators on callus and embryo induction (second experiment)

According to the results of the first experiment, the thermal shock pretreatment compared to no heat treatment had a better effect on the traits in question. Therefore, thermal shock pretreatment was applied to all ovary cultures in this experiment. On the other hand, the second experimental hormonal compounds were designed on the basis of the best combination and concentration of PGRs in the first experiment.

As the results of ANOVA showed, genotypes in terms of embryogenesis and callus induction were significantly different. A comparison of means between genotypes (Tab. 3) showed that genotype 6*6/32441 resulted in the highest rate of embryogenesis and callus production induction. In a similar study RAKHI et al. (2010) cultured cotyledon explants from seven varieties of pumpkin. According to their results, percentage of callus production induction was different among various genotypes. The separate analyses of the KURT and EVANS (1998) demonstrated that cultivar significantly influenced callus production induction in both solid and liquid media.

Mean comparison for hormonal composition showed that combination of 2,4-D+BAP (1.5+4) mg/L had the highest rate of embryogenesis (i.e. 37.5%), although the difference among the four hormonal combinations NAA+2,4-D+BAP (0.7+0.1+2.5) mg/L, NAA+

Tab. 2: Effects of thermal pretreatment and different hormonal combinations on embryogenesis and callus induction in experiment 1.

Growth regulators		Embryogenesis (%)		Callus induction (%)	
Treatment	Concentration (mg/L)	Thermal shock pretreatment	Control	Thermal shock pretreatment	Control
2,4-D+KIN	1.5+1	57.20^{b}	48.33^{b}	0.00^{e}	26.66^{bc}
IBA+NAA+KIN+BAP	0.4+0.1+1+0.5	25.00^{c}	0.00^{d}	0.00^{e}	0.00^{e}
IAA+KIN+BAP	0.7+1.5+3	51.66^{b}	0.00^{d}	0.00^{e}	50.00^{b}
NAA+2,4-D+KIN+BAP	0.5+0.5+0.5+1.5	70.53^{a}	23.33^{c}	43.33^{bc}	52.20^{ab}
NAA+2,4-D+KIN+BAP	0.8+0.8+0.8+2.5	23.33^{c}	25.00^{c}	0.00^{e}	21.66^{de}
NAA+2,4-D+KIN+BAP	1.5+0.7+1+1.8	51.66^{b}	29.43^{c}	0.00^{e}	63.86^{a}

For every trait, letters compared separately. Means followed by a common letter are not significantly different at 5% level according to LSD test

Tab. 3: Effects of genotypes on callus and embryo induction in experiment 2.

Traits	Genotype	
	NBDC6*6/32441	NBDC20*15/53211
Embryogenesis (%)	17.44[a]	12.30[b]
Callus induction (%)	70.91[a]	38.91[b]

Means followed by a common letter are not significantly different at 5% level according to LSD test

BAP+KIN (1+1.5+2.5) mg/L, IBA+BAP+KIN (1+1.5+2.5) mg/L and NAA+2,4-D+BAP (1+2+1.75) mg/L was not significant (Fig. 2).

The most callus induction was observed in hormone combinations of IAA+KIN (1+1.5) mg/L (T_2) and 2,4-D+BAP (0.5+2) mg/L (T_3), although their difference with KIN+BAP+IAA+IBA (0.5 mg/L) (T_5) and 2,4-D (3 mg/L) (T_4) hormonal combinations was not significant (Fig. 2). These results show that the presence of auxin in the culture medium increased the callus formation rate. Auxins have several effects including cell enlargement, cell division, root initiation and apical dominance. In the culture medium high concentration of auxin inhibits root formation and leads to callus formation. Culture medium is a principal factor controlling the induction and development of intact plants (MUKHAMBETZHANOV, 1997). In some culture media, the presence of low levels of auxin is essential for stimulating explants to the embryos formation. This result is consistent with those of UGANDHAR et al. (2011), on the culturing of cucumber cotyledons. They showed that the presence of 0.5 mg/L IAA and 3 mg/L BAP or 3 mg/L KIN, helped to increase accountability and induce root formation from explants.

Effect of genotype, absence or presence of $AgNO_3$ and growth regulators on callus and embryo induction (third experiment)

Based on the results of ANOVA and comparison of the hormones and genotypes in the second experiment, seven hormonal combinations and genotype NBDC6*6.32441 were selected, optimized and prepared for the third experiment. In the third experiment, one local and one greenhouse genotypes of cucumber (NBDC6*6/32441) were compared.

The results showed that the genotypes used in this experiment (the local open field and greenhouse cucumber) made a significant difference in the percentage of embryogenesis and callus induction (Tab. 4). According to Tab. 4, the local genotype with the 24.93% had the highest rate of embryogenesis. It also had the highest callus induction rate with 24.19%. Therefore, the Iranian local variety was more successful in producing embryos and plantlets compared to the other genotype. SHALABY (2007) stated that differences among genotypes indicated that the genotype-specific results can be obtained depending on the genetic structure of the individually used hybrids. This agreed with the results obtained by DUMAS-DE-VALUX and CHAMBONNET (1986) in squash, LUX et al. (1990) in sugar beet, KOBAYASHI et al. (1993) in sweet potato and ALAN et al. (2004) in onion. Therefore, if the aim of tissue culture is to produce pure lines of local varieties, the results of this experiment will be applicable. The inbred line production in indigenous varieties could be used in order to transfer desirable traits such as fruit taste and quality of the commercial cultivars.

Tab. 4: Effects of genotypes on embryogenesis and callus induction in experiment 3.

Traits	Genotype	
	NBDC6*6/32441	Local cultivar
Embryogenesis (%)	11.93[b]	24.93[a]
Callus induction (%)	3.57[b]	24.19[a]

Means followed by a common letter are not significantly different at 5% level according to LSD test

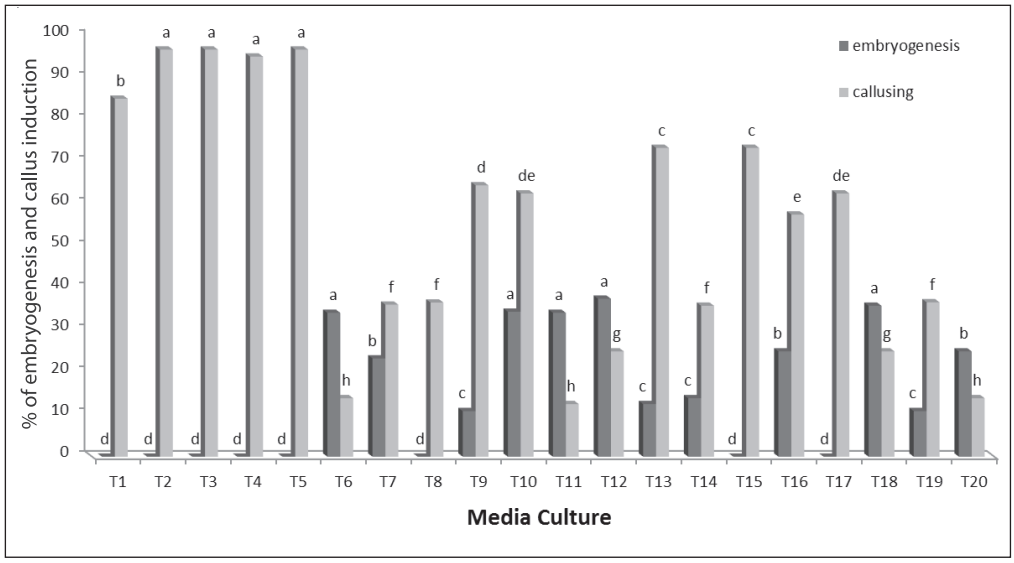

Fig. 2: Effects of different hormonal combinations on embryogenesis and callus induction in experiment 2.

For every trait, letters compared separately. Means followed by a common letter are not significantly different at 5% level according to LSD test
T1: KIN+2,4-D (1.25+0.5 mg·l^{-1}); T2: KIN+IAA (1.5+1 mg·l^{-1}); T3: BAP+2,4-D (2+0.5 mg·l^{-1}); T4: 2,4-D (3 mg·l^{-1}); T5: KIN+BAP+IAA+IBA (0.5+0.5+0.5+0.5 mg·l^{-1}); T6: BAP+2,4-D+NAA (1.75+2+1 mg·l^{-1}); T7: BAP+NAA (3+1.5 mg·l^{-1}); T8: KIN+BAP+IAA (2.5+1.5+1 mg·l^{-1}); T9: KIN+BAP+2,4-D (2.5+1.5+1 mg·l^{-1}); T10: KIN+BAP+NAA (2.5+1.5+1 mg·l^{-1}); T11: KIN+BAP+IBA (2.5+1.5+1 mg·l^{-1}); T12: BAP+2,4-D (4+1.5 mg·l^{-1}); T13: BAP+IBA (1+1.5 mg·l^{-1}); T14: KIN+BAP+2,4-D (0.75+1.5+0.5 mg·l^{-1}); T15:KIN+BAP+IBA (1.3+0.6+0.5 mg·l^{-1}); T16: KIN+BAP+2,4-D (0.5+2+1.8 mg·l^{-1}); T17: KIN+BAP+2,4-D (0.5+3.5+1.8 mg·l^{-1}); T18: BAP+2,4-D+NAA (2.5+0.1+0.7 mg·l^{-1}); T19: KIN+2,4-D+IAA+IBA (0.3+1.1+0.7+0.5 mg·l^{-1}); T20: KIN+BAP+IAA (3+0.75+0.5 mg·l^{-1})

Few characteristics of $AgNO_3$ such as stability, availability, water-solubility and specificity make it very useful for several applications in regulation used in the plant growth and morphogenesis *in vivo* and *in vitro* (KUMAR et al., 2009). In this research, silver nitrate had the significant effect on embryogenesis and induction of callus production. The highest rate of embryogenesis (15.71%) was observed in the medium with silver nitrate, but the highest rate of the callus production induction (18.34%) was obtained in the medium without silver nitrate (Tab. 5). Thus, the application of silver nitrate had a positive effect on the regeneration of ovary culture. Silver ions in the form of nitrate, such as $AgNO_3$, play a major role in somatic embryogenesis efficient shoot and root formation (BAIS et al., 2001). $AgNO_3$ is used in plant tissue culture as an ethylene antagonist. (BEYER, 1976a). The results of the KUMAR et al. (2009) were revealed that $AgNO_3$ promotes embryogenesis by blocking the inhibitory effect of endogenous ethylene on embryo formation (KUMAR et al., 2009). Embryo production were significantly increased in the majority of the morphotypes by addition of silver nitrate to the medium, and $AgNO_3$ treatment produced embryos in what would otherwise have been unresponsive morphotypes (DIAS and MARTINS, 1999). LI et al. (2013) conducted a study on the culturing of cucumber ovaries in two inbred lines cucumbers, four TDZ and three silver nitrate concentrations (0.5 and 10 mg/L). Their results showed that the biggest number of the regenerated plants produced in the presence of silver nitrate at a concentration of 10 mg/L. Their results also showed that the presence of silver nitrate in the culture media prolongs the time needed for the formation of the embryo. They stated that the presence of silver nitrate increased the number of the embryos, though it did not make a significance difference with culture without silver nitrate. There are various opinions and experimental documents on the silver subject. According to this hypothesis, the ethylene role in plants is inhibited by weak antagonists like CO_2 and strong antagonists such as Ag compounds. These procedures are possibly due to oxidation of ethylene by a metal-ion enzyme system. Another theory is that silver nitrate inhibits the ethylene role by means of silver ions by decreasing the receptor capacity to bind ethylene, thus inhibiting the earlier steps of its own pathway (KUMAR et al., 2009).

The culture media with different hormone concentrations and combinations used in this experiment made a significant difference with respect to the measured traits. The highest rate of embryogenesis was observed in 2,4-D+KIN+BAP (1.5+1+1.8) mg/L (i.e. 20.83%), and the difference between this treatment and other hormonal compounds like IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5) mg/L and 2,4-D+TDZ+KIN (1.5+0.5+1.5 mg/L) was significant (Tab. 6). The highest rate of callus induction (i.e. 20.83%) was detected in the hormonal compound NAA+2,4-D+TDZ+BAP (0.5+0.5+0.5+1.5) mg/L. However, the difference with hormonal compounds IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5) mg/L, NAA+KIN+BAP (0.1+1.5+1.5) mg/L and IBA+2,4-D+BAP (0.8+1+0.8) mg/L was not significant (Tab. 9). The least rate of callus induction (i.e. 3.33%) was observed in the hormonal compound 2,4-D+TDZ+KIN (1.5+0.5+1.5) mg/L.

Tab. 5: Effects of presence or absence of silver nitrate on embryogenesis and callus induction in experiment 3.

Traits	Silver nitrate	
	presence	absence
Embryogenesis (%)	15.71[a]	12.24[b]
Callus induction (%)	11.19[b]	18.34[a]

Means followed by a common letter are not significantly different at 5% level according to LSD test

The interaction between genotype and silver nitrate was not significant for embryogenesis and callus induction traits. This means that regardless of what genotype has been used (local or commercial), silver nitrate is able to increase the embryogenesis.

The interactive effect of genotype and hormonal combinations for these traits was significant. The local cultivar with combination of NAA+2,4-D+TDZ+BAP (0.1+0.3+1+2) mg/L, had the highest rate of embryogenesis, even though its difference with some other hormonal combinations and local and inbred line genotypes was not statistically significant (Tab. 7).

Tab. 6: Effects of different hormonal combinations on embryogenesis and callus induction in experiment 3.

Growth regulators	Embryogenesis (%)	Callus induction (%)
T_1	4.17[c]	20.83[a]
T_2	15.00[ab]	12.50[c]
T_3	12.50[b]	3.33[d]
T_4	16.66[ab]	20.83[a]
T_5	20.83[a]	10.83[cd]
T_6	15.16[ab]	20.16[ab]
T_7	13.63[ab]	14.54[ab]

Means followed by a common letter are not significantly different at 5% level according to LSD test

T_1: IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5 mg·l^{-1}); T_2: NAA+2,4-D+TDZ+BAP (0.1+0.3+1+2 mg·l^{-1}); T_3: 2,4-D+TDZ+KIN (1.5+0.5+1.5 mg·l^{-1}); T_4: NAA+2,4-D+TDZ+BAP (0.5+0.5+0.5+1.5 mg·l^{-1}); T_5: 2,4-D+KIN+BAP (1.5+1+1.8 mg·l^{-1}); T_6: NAA+KIN+BAP (0.1+1.5+1.5 mg·l^{-1}), T_7: IBA+2,4-D+BAP (0.8+1+0.8 mg·l^{-1})

The inbred line genotype showed the lowest embryogenesis rate with 3.33% in hormonal treatment of IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5) mg/L, the highest rate of callus induction was observed in local cultivar treated with NAA+2,4-D+TDZ+BAP (0.5+0.5+0.5+1.5) mg/L, although their difference from the two different hormone and genotype treatments was not significant (Tab. 7). The interactive effect of silver nitrate and hormone combinations was significant for embryogenesis and callus induction. The biggest percentage of embryogenesis (i.e. 25.88%) was observed in the presence of silver nitrate and hormonal combination NAA+2,4-D+TDZ+BAP (0.5+0.5+0.5+1.5) mg/L (Fig. 3). This treatment made a significant difference with some other treatments.

Silver nitrate has been used in several studies for its similar hormonal activity and transgender cucumber, which indicates the key role of this material in plant physiological changes in the vegetative and reproductive phases (STANKOVIC and PRODANOVIC, 2002; NAGAR et al., 2014; LAW et al., 2002).

The lowest rate of embryogenesis (i.e. 1.65%) was observed in the absence of silver nitrate and hormonal combination IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5) mg/L. The highest rate of callus induction was observed in the hormonal combination IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5) mg/L without silver nitrate, although it did not make any statistically significant difference with A_1T_6, A_2T_4 and A_2T_7 treatments (Fig. 3). In many crop species, TDZ stimulated embryogenesis with a higher efficiency and frequency than other cytokinins or the combined treatments of auxins and cytokinins (VISSER et al., 1992; ZHANG et al., 2001).

According to the analysis of variance results, interaction of genotype×silver nitrate×hormonal combinations was also significant for the rate of embryogenesis and callus induction. The highest rate of embryogenesis was observed in the treatment of local cultivar

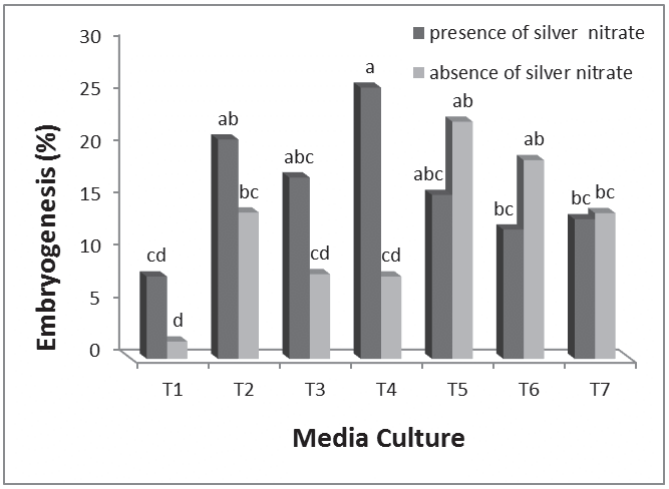

Fig. 3: Interaction effects of silver nitrate and different hormonal combinations on embryogenesis.

Means followed by a common letter are not significantly different at 5% level according to LSD test
T_1: IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5 mg·l^{-1}); T_2: NAA+2,4-D+TDZ+BAP (0.1+0.3+1+2 mg·l^{-1}), T_3: 2,4-D+TDZ+KIN (1.5+0.5+1.5 mg·l^{-1}); T_4: NAA+2,4-D+TDZ+BAP (0.5+0.5+0.5+1.5 mg·l^{-1}), T_5: 2,4-D+KIN+BAP (1.5+1+1.8 mg·l^{-1}); T_6: NAA+KIN+BAP (0.1+1.5+1.5 mg·l^{-1}), T_7: IBA+2,4-D+BAP (0.8+1+0.8 mg·l^{-1})

with 2,4-D+TDZ+KIN (1.5+0.5+1.5) mg/L in the presence of silver nitrate. However, the difference among treatments with $C_1A_1T_2$, $C_1A_1T_4$, $C_1A_2T_5$, $C_1A_2T_6$, $C_2A_1T_2$, $C_2A_1T_4$, $C_2A_1T_5$, $C_2A_1T_7$, $C_2A_2T_2$, $C_2A_2T_5$ and $C_2A_2T_7$ was not statistically significant (Tab. 8).
Callus production induction in the local cultivar with hormonal combination of IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5) mg/L in the absence of silver nitrate ($C_2A_2T_1$) resulted in the highest rate (i.e. 13.56%) of callus production which is significantly different from all of the other treatments with the exception of $C_2A_2T_5$ and $C_2A_1T_6$.

Conclusion

Haploids can be obtained using ovary as well as other sexual explants. The current study reveals different factors for the efficient ovary embryogenesis induction in cucumber using various treatments. Our findings demonstrated that thermal shock (at 35 °C) as a pretreatment favors an embryogenesis pathway. Other experiments in the present study showed that rate of embryo production induction was different among genotypes, and that silver nitrate had a significant effect on the embryogenesis and callus production induction.
In a nutshell, the results of the present assessment can be described and summarized as follows:

A- The thermal shock pretreatment compared to no heat treatment produced a better effect on embryo and callus production induction. Therefore, thermal shock pretreatment was applied to all ovary cultures.
B- The results showed that the genotype 6*6/32441 produced the highest rate of embryogenesis and callus production induction, and combination of 2,4-D+BAP (1.5+4 mg·l^{-1}) produced the highest rate of embryogenesis.
C- The application of silver nitrate had a positive effect on the regeneration of ovary culture.

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Tab. 7: Interaction effects of different hormonal combinations and genotype on embryogenesis and callus induction in experiment 3.

Growth regulators		Embryogenesis (%)		Callus induction (%)	
Treatment	Concentration (mg/L)	Greenhouse line	Local cultivar	Greenhouse line	Local cultivar
BA+2,4-D+TDZ+KIN+BAP	0.8+1+1+0.5+1.5	3.33^{f}	6.16^{ef}	1.65^{d}	38.33^{a}
NAA+2,4-D+TDZ+BAP	0.1+0.3+1+2	11.21^{b-f}	23.68^{a}	0.00^{d}	25.55^{b}
2,4-D+TDZ+KIN	1.5+0.5+1.5	6.10^{ef}	19.28^{abc}	0.00^{d}	6.60^{cd}
NAA+2,4-D+TDZ+BAP	0.5+0.5+0.5+1.5	15.71^{a-e}	18.01^{a-d}	0.00^{d}	42.66^{a}
2,4-D+KIN+BAP	1.5+1+1.8	20.08^{ab}	18.18^{a-d}	8.46^{cd}	11.53^{cd}
NAA+KIN+BAP	0.1+1.5+1.5	21.48^{a}	9.81^{c-f}	1.66^{d}	39.25^{a}
IBA+2,4-D+BAP	0.8+1+0.8	8.15^{d-f}	20.10^{ab}	8.85^{cd}	17.60^{bc}

For every trait, letters compared separately. Means followed by a common letter are not significantly different at 5% level according to LSD test

Tab. 8: Triple interaction effects of different hormonal combinations, genotypes and absence or presence of $AgNo_3$ on embryogenesis and callus induction in experiment 3.

Growth regulators	Embryogenesis (%)				Callus induction (%)			
	Greenhouse line (C1)		Local cultivar (C2)		Greenhouse line (C1)		Local cultivar (C2)	
	Presence Ag (A1)	Absence Ag (A2)	Presence Ag (A1)	Absence Ag (A2)	Presence Ag (A1)	Absence Ag (A2)	Presence Ag (A1)	Absence Ag (A2)
T1	6.66^{e-g}	0.00^{g}	9.03^{d-g}	3.30^{fg}	0.00^{h}	3.30^{gh}	23.43^{c-f}	56.13^{a}
T2	22.43^{a-d}	0.00^{g}	19.43^{a-c}	27.93^{ab}	0.00^{h}	0.00^{h}	22.13^{c-g}	28.96^{cd}
T3	3.16^{fg}	9.03^{d-g}	31.43^{a}	7.13^{e-g}	0.00^{h}	0.00^{h}	3.13^{gh}	9.80^{e-h}
T4	28.10^{ab}	3.33^{fg}	23.66^{a-c}	12.36^{c-g}	0.00^{h}	0.00^{h}	34.56^{bc}	50.93^{a}
T5	12.23^{c-g}	27.93^{ab}	19.03^{a-e}	17.33^{a-f}	0.00^{h}	16.93^{c-h}	5.53^{f-h}	17.53^{ch}
T6	14.53^{b-f}	28.43^{ab}	10.13^{c-g}	9.50^{d-g}	0.00^{h}	3.16^{gh}	50.10^{ab}	28.40^{c-e}
T7	6.46^{e-g}	9.83^{cg}	20.16^{a-e}	20.00^{a-e}	0.00^{h}	17.70^{c-h}	15.58^{d-h}	20.20^{c-e}

For every trait, letters compared separately. Means followed by a common letter are not significantly different at 5% level according to LSD test.
T_1: IBA+2,4-D+TDZ+KIN+BAP (0.8+1+1+0.5+1.5 mg·l^{-1}); T_2: NAA+2,4-D+TDZ+BAP (0.1+0.3+1+2 mg·l^{-1}); T_3: 2,4-D+TDZ+KIN (1.5+0.5+1.5 mg·l^{-1}); T_4: NAA+2,4-D+TDZ+BAP (0.5+0.5+0.5+1.5 mg·l^{-1}); T_5: 2,4-D+KIN+BAP (1.5+1+1.8 mg·l^{-1}); T_6: NAA+KIN+BAP (0.1+1.5+1.5 mg·l^{-1}); T_7: IBA+2,4-D+BAP (0.8+1+0.8 mg·l^{-1})

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