Effects of zeatin riboside, mannitol and heat stress on eggplant (*Solanum melongena* L.) anther culture

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Abstract

In this research, the effects of zeatin riboside (0.6, 0.8, 1, 1.2 and 1.4 mg l^{-1}), duration of heat stress of 35 °C (6, 8, 10 and 12 days) and mannitol (0, 10, 20, 30 and 40 mg l^{-1}) on anther culture of four eggplant cultivars were investigated in independent experiments. These experiments were performed according to a factorial arrangement in a randomized complete blocks design layout. The results showed significant interaction effects between the cultivars and treatments. The highest percentage of embryo-derived plantlets (25%) was obtained in cultivar Chantal using 1 mg l^{-1} zeatin riboside and 8 days of the heat stress. The results also indicated that the use of 10 mg l^{-1} mannitol had the highest embryo-derived plantlets (66.6%) in cultivar Chantal. In cultivars Hadrian and Faselis, the ploidy levels of all regenerated plants from anther culture (10 and 21 plants, respectively) were determined through flow cytometry and it was found that 60% and 57.1% of the tested plants were haploid, respectively. In cultivars Chantale and Valentina, 30 regenerated plants were selected for determining their ploidy levels and the results showed that 40.2% and 51.5% of the plants were haploid, respectively.

Key words: Androgenesis, Haploidy, Osmotic stress.

INTRODUCTION

Solanum melongena (2*n*=24), eggplant, is an economically important vegetable crop throughout the world. In Asia, and the Mediterranean region, eggplant fruits are the main ingredient for national and regional cuisines (Lester and Hasan, 1991). Eggplant global production in 2013 was 49,418,212 t, with 1,867,350 ha harvested (FAOSTAT, 2015). Eggplant breeding is mainly focused on F1 hybrid cultivars, which have nearly replaced the open-pollinated cultivars, particularly in the intensive growing areas. In eggplant, crosses are relatively easy and a considerable number of seeds can be obtained by hand emasculation and pollination of the flowers; besides, some cross combinations may provide heterotic advantages (Quagliotti, 1979). Different researches have shown a significant heterosis on the yield, maturity, uniform harvest and other characteristics in eggplant (Dharmegowda, 1977 quoted from Wehner, 1999; Kaur *et al*., 2001; Singh *et al*., 2012). Selection and production of inbred lines is time-consuming and difficult to achieve. Even with 6 years of self-pollination, only about 98% homozygosity could be achieved (Germana, 2006; Ferrie and Möllers, 2010). At the present time, the pure inbred lines can be obtained by different haploidy methods. The doubled haploid (DH) lines are important for crop breeding programs because all characters are fixed without several self-pollinating generations. Haploidy can be envisioned as a method to accelerate the breeding program in many crops (Dunwell, 2010; Ferrie and Caswell, 2010; Germana, 2011). The first report on the androgenesis in eggplant by anther culture dates to 1973, although a gametophytic origin for the regenerated plants was not unambiguously demonstrated (Raina and Iyer, 1973). Several media and inductive treatments have been used

to produce doubled haploid through anther culture in different eggplant F1 hybrids and other cultivars under different experimental conditions (Sanguineti *et al.*, 1990; Rotino, 1996; Gémes Juhasz *et al.*, 2006; Alpsoy and Seniz, 2007; Salas *et al*., 2011). Various efforts to improve the quality of the embryo, using the most successful method of anther culture of Dumas de Vaulx *et al*. (1982) in eggplant resulted only in some minor improvements. There exist many factors which affect the *in vitro* embryogenic response of anthers. These include the type of genotype, physiological state and conditions of growth of donor plants, developmental stage of the microspore, culture media and conditions, together with their interactions (Smýkal, 2000; Wang *et al.*, 2000; Datta, 2005; Germanà, 2011). Among the five major solanaceous crops (tobacco, pepper, potato, tomato, and eggplant), the technology of the doubled haploids production sufficiently developed to produce DHs efficiently only in tobacco anther and isolated microspore cultures. It is necessary to continue the androgenesis researches on the other plants for achieving the considerable embryo production and plant regeneration.

The aim of this study was to evaluate the androgenesis in some eggplant genotypes and improve their androgenic responses by optimizing some factors affecting the haploid induction including the use of Zeatin riboside and applied heat and osmotic stresses in the anther culture induction medium. Also, the effects of the hormone combinations on shoot formation from eggplant anther-derived calli were investigated.

MATERIALS AND METHODS

Plant material

Four eggplant cultivars were used as donor plants: Chantale, Valentina, Faselis, and Hadrian. Plants were grown in the field conditions. Anthers were collected from the plants during mid-July to late-September.

Anther culture

DAPI staining was used for cytological determination of the microspore developmental stage. The anthers (4.5 mm) were collected from the flower buds containing greenish-yellow anthers when the microspores were at the early-mid uni-nucleate stage. In this stage, the petals were not visible. Collected buds were transported in melting ice and then immersed for 15 min. in sodium hypochlorite 1% (w/v). Rinsing was done 3 times in sterile distilled water. The anthers of control were cultured according to the method initially developed by Dumas de Vaulx and Chambonnet (1982) and then followed by modification with Rotino (1996). The anthers were cultured on C medium (Rotino, 1996) supplemented with NAA and Kin (5 mg l^{-1}), and sucrose (12%) and maintained in dark at 35 °C for 8 days and then at 25 °C with a photoperiod of 12 h for 4 days. Subsequently, the anthers were transferred to a solid R medium (Rotino, 1996) supplemented with Kin (0.1 mg l^{-1}) and 3% sucrose. They were cultured at 25 °C and the medium was refreshed every 3 weeks. After the appearance of embryos from the anthers, they were isolated and cultured on V3 medium (Rotino, 1996) for transformation into plantlets. The regenerated plantlets were transferred to 20 cm pots containing 1:1 mixture of peat moss and field soil, followed by acclimatization under high humidity conditions for 1 week. In all experiments, three traits including the percentages of responsive anthers, embryo-derived plantlets, and callus-derived shoots were analyzed. The responsive anther is defined as the anther that one or more plantlets or the callus emerge from it. Embryo-derived plantlets contained the embryos that were directly converted in to a complete plantlet. The shoots that were emerging from calli formed on the anthers were considered as indirect regenerated shoots. The percentages of responsive anthers, embryo-derived plantlets and callus-derived shoots were calculated as the number of responsive anthers, regenerated plantlets, and regenerated shoots per 100 cultured anthers.

Experiment 1: Effects of cultivar and Zeatin riboside on eggplant anther culture

In this experiment, the effects of Zeatin riboside concentrations (0.6, 0.8. 1, 1.2 and 1.4 mg l^{-1}) with 3 mg l^{-1} NAA in C medium were examined on anther culture of four eggplant cultivars (Chantale, Hadrian, Valentina and Faselis). This trial was conducted in a factorial experiment with randomized complete blocks design (RCBD) with 3 replications.

Experiment 2: Effects of cultivar and the duration of high temperature stress on eggplant anther culture

In this experiment, the effects of different durations of temperature stress (35 °C) (6, 8, 10, and 12 days) were investigated on androgensis induction in the four above mentioned cultivars in a factorial arrangement using RCBD with two factors and three replications. Heat treatments were applied on the anthers cultivated in the C medium.

Experiment 3: Effects of cultivar and osmotic stress on eggplant anther culture

In this experiment, the effect of osmotic stress (mannitol) was investigated in a factorial arrangement

using RCBD with two factors and three replications. The first factor was 3 cultivars (Chantale, Hadrian and Valentina) and the second factor was mannitol concentrations (0, 10, 20, 30, and 40 mg l^{-1}) added to the C medium.

Each replication had a unique Petri dish (60 x 15 mm) containing 9 ml modified C culture medium (Dumas de Vaulx and Chambonnet, 1982) supplemented with 120 g l^{-1} sucrose and NAA and Kin (5 mg l^{-1}) with 12 anthers.

Experiment 4: Effects of the hormone combinations on shoot induction in anther-derived calli in cultivar Chantale

In this research, the effects of the hormone combinations (Table 1) were investigated on the induction of shoot regeneration on the calli obtained from anther culture of cultivar Chantale. A completely randomized design (CRD) layout was used with 4 replications, each consisting of a glass bottle (90×60 mm) containing 50 ml MS medium (Murashige and Skoog, 1962) with 0.6 g callus and with 3 mg l^{-1} NAA.

Experiment 5: Effects of different medium compositions on the elongation of anther-derived shoots

In this experiment, the effects of some treatments on the elongation of the shoots obtained from the previous experiment were investigated. These treatments were hormone-free MS medium (Murashige and Skoog, 1962), hormone-free ½MS medium, MS medium supplemented with 1 and 1.5 mg l^{-1} GA_3. A CRD layout was used with 4 replications, each having a glass bottle (90×60 mm) containing 50 ml of medium with 7 explants.

Determination of ploidy level of regenerated plants

Two methods of chromosome counting and flow cytometry were used to determine ploidy level of plants regenerated from eggplant anther culture.

Chromosome counts

For chromosome counting, root tips (1 cm) were treated by alpha bromonaphthalene 1% at room temperature for 4 h. They were then fixed in absolute ethanol: glacial acetic acid (3:1) for 24 h and stored at 4 °C. Prior to chromosome counting, the root tips were hydrolyzed at 60 °C for 12 min and stained in 2% (w/v) acetoorcein at room temperature for 4 h and squashed in 45% (v/v) glacial acetic acid. Slides were investigated by BX50 Olympus microscope.

Flow cytometry

Flow cytometry has provided a rapid and precise tool to identify the ploidy level of a plant (Doležel and Bartoš, 2005). For this purpose, a BD FACSCantoTM-KE (BD: Biosciences, Bedford, MA, USA) flow cytometer and propidium iodide (PI) as the fluorescent stain, were used. Extraction of nuclei was done by chopping leaf samples from regenerated plantlets and *Solanum lycopersicum* cv. Stupicke as the reference standard in the Petri dishes containing about 1.0 ml Woody Plant Buffer (WPB) extraction buffer solution (Loureiro *et al.,* 2007). Nuclei suspension was filtered through a 50 and 30 μm Partec single tube filter to eliminate cell fragments and debris. Nuclei were stained with 50 μg ml^{-1} propidium iodide, and 50 μg ml^{-1} RNase was added to nuclei suspension to prevent staining of RNA. Ploidy status was determined by comparing a DNA C-value of the sample with that of a diploid and standard plant that was calculated by the following formula (Doležel and Bartoš, 2005):

(1) Sample 2C DNA (pg)=(sample G_1 peak mean/ standard G_1 peak mean)×standard 2C DNA content (pg)

Table 1. Hormone combinations (mg l^{-1}) for inducing shoot regeneration on the calli obtained from anther culture of cultivar Chantale.

Treatments	BAP	Kin
T_1	0.5	0.5
T_2	1	0
T_3	2	0
T_4	3	0
T_5	4	0
T_6	0	1
T_7	0	2
T_8	0	3
T_9	0	4
T_{10}	1	1
T_{11}	2	2
T_{12}	3	3

RESULTS AND DISCUSSION

In this research, the anther length was used as a morphological marker in order to describe the microspore developmental stage in each cultivar. The appropriate size of anthers for achieving the best embryogenesis response was determined in each cultivar. In cultivars Chantale, Faselis, and Valetina, the optimal size of anthers was 4-4.5 mm and that of cultivar Hadrian was 5-5.5 mm. Incubated anthers were discolored and turned brown after about 14 days. The first embryonic structures were visible in the swollen anthers after about 5 weeks of cultivation (Figure 1).

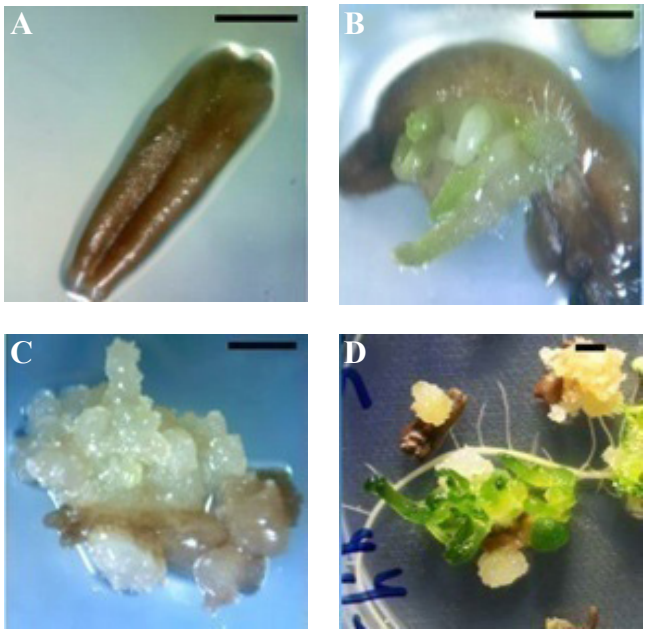

Figure 1. Anther culture in eggplant cv. 'Chantale'. **A:** anther at the optimal stage for anther culture, **B:** Swollen anther after 5 weeks of culture, with several produced embryos, **C:** Callus- inducing anther, and **D:** Shoot regeneration from callus produced on the anther. Bar 1 mm.

Table 2. Analysis of variance for Experiment 1 (Effects of cultivar and zeatin riboside) and Experiment 2 (Effects of cultivar and the duration of high-temperature stress) on eggplant anther culture.

Source of variation	df	Experiment 1 Mean of squares			Experiment 2 Mean of squares		
		Responsive anthers (%)	Embryonic regenerated plantlets (%)	Callus-derived shoots (%)	Responsive anthers (%)	Embryonic regenerated plantlets (%)	Callus-derived shoots (%)
Block	2	55.54**	28.91^{ns}	4.63^{ns}	149.01**	201.11*	40.51^{ns}
Cultivar	3	402.37**	121.52**	206.82**	287.40**	162.01*	212.28**
Treatment	4	171.95**	159.22**	176.52**	437.95**	312.54**	119.63*
Cultivar ×Treatment	12	160.32**	135.99**	82.36**	76.52*	96.46*	56.59^{ns}
Error	38	16.58	14.32	10.72	28.65	39.08	34.34

ns: non-significant,* and **: Significant at 0.05 and 0.01 probability levels, respectively.

Effects of cultivar and Zeatin riboside on eggplant anther culture

According to the results of the analysis of variance (Table 2), the interaction of cultivar and zeatin riboside concentration for percentages of responsive anthers, embryonic regenerated plantlets, and callus-derived shoots were highly significant at 0.01 probability level. Since cultivar and zeatin riboside interaction was found to be significant, mean comparison of the main effects was not accomplished. Mean comparison of treatment combinations (cultivar×zeatin riboside concentration) via LSD procedure determined the highest percentages of responsive anthers cultivar in Chantal with 1 mg l^{-1} zeatin riboside (27.8%) and cultivars Valentina and Faselis with 1.2 mg l^{-1} zeatin riboside (25% and 19.4%, respectively) (Table 3).

The results (Table 3) showed that the highest percentage of embryo-derived plantlets was obtained by the interaction between cultivar Chantal and 1 mg l^{-1} zeatin riboside (25%) and also by the interaction of cultivar Valentina and 1.2 mg l^{-1} zeatin riboside (19.4%). Comparison of the means for the percentage of callus-derived shoots (Table 3) showed that the highest number of indirectly regenerated shoots was obtained in cultivar Chantal using 1 mg l^{-1} zeatin riboside (22.2%).

The interaction of plant growth regulator with

Table 3. Mean comparison for the interaction of cultivar and zeatin riboside concentration on the responsive anther, embryonic regenerated plantlets and callus-derived shoots in eggplant anther culture.

Cultivar	Zeatin riboside (mg l^{-1})	Responsive anthers (%)	Embryonic regenerated plantlets (%)	Callus-derived shoots (%)
Chantale	0.6	8.3±0de	8.3±0^{cde}	0±0^{e}
	0.8	11.1±2.8^{de}	5.6±2.7^{def}	5.6±2.7^{d}
	1	27.8±2.7^{a}	25±4.8^{a}	22.2±2.7^{a}
	1.2	22.2±2.7^{ab}	13.9±2.8^{bc}	13.9±2.8^{b}
	1.4	13.9±2.8^{cd}	0±0^{f}	11.1±2.8^{bc}
Hadrian	0.6	13.9±2.8^{cd}	11.1±2.8^{cd}	8.3±0^{cd}
	0.8	8.3±0de	8.3±0^{cde}	0±0^{e}
	1	0±0^{f}	0±0^{f}	0±0^{e}
	1.2	0±0^{f}	0±0^{f}	0±0^{e}
	1.4	0±0^{f}	0±0^{f}	0±0^{e}
Valentina	0.6	5.6±2.7^{ef}	2.78±2.7^{ef}	0±0^{e}
	0.8	11.1±2.8^{de}	5.6±2.7^{def}	0±0^{e}
	1	11.1±2.8^{de}	8.3±0^{cde}	5.6±2.7^{d}
	1.2	25±4.8^{ab}	19.4±2.7^{ab}	11.1±2.8^{bc}
	1.4	13.9±2.8^{cd}	8.3±0^{cde}	5.6±2.7^{d}
Faselis	0.6	0±0^{f}	0±0^{f}	0±0^{e}
	0.8	5.6±2.7^{ef}	5.6±2.7^{def}	0±0^{e}
	1	13.9±2.8^{cd}	11.1±2.8^{cd}	8.3±0^{cd}
	1.2	19.4±2.7^{bc}	13.9±2.8^{bc}	13.9±2.8^{b}
	1.4	13.9±2.8^{cd}	5.6±2.7^{def}	5.6±2.7^{d}

Means followed by the same letter(s) are not significantly different at 0.01 level of probability.

the plant genotype and environmental factors play a crucial role in microspore embryogenesis, controlling microspore derived embryo differentiation and development as well as haploid/doubled haploid plant regeneration (Zur *et al.*, 2015). In this research, the androgenic response in eggplant significantly depended on cultivar, and it played a key role in anther culture. Different results have been reported on anther culture in eggplant varieties, although anther culture has been successful in other solanaceous plants such as potatoes, pepper, and tobacco species (Ellialtioğlu *et al.,* 2001). In an anther culture study on four genotypes of eggplant, haploid embryos at a rate of 0%–7.8% were obtained, depending on the varieties (Karakullukçu and Abak, 1992). In another report (Salas *et al.,* 2011) on the androgenic response of 12 accessions of common eggplant and related materials from the primary (eggplant complex) and secondary gene pools, anthers of 11 out of the 12 accessions produced somatic calli, whereas only 5 produced microspore-derived embryos, with variable results in terms of embryo quality, frequency of embryo induction, and plant germination.

Effects of cultivar and duration of high temperature stress on eggplant anther culture

According to the analysis of variance results (Table 2), the duration of high temperature stress and cultivar interaction for the percentages of responsive anthers and embryo-derived plantlets were highly significant at 0.01 probability level while the interaction effect was not significant for callus-derived shoots and the main effects of the duration of high temperature stress and cultivar were highly significant at 0.01 probability level for this trait. Mean comparison of treatment combinations (cultivar and duration of high temperature stress) indicated that, after 8 days of high temperature stress (35 °C), cultivars Chantal and Valentina had the highest percentages of responsive anthers (27.8% and 22.2%, respectively). High temperature stress did not affect this trait in cultivar Hadrian (Table 4). The results (Table 4) showed that the highest percentage of embryo-derived plantlets was obtained by the cultivar Chantal after 8 days of high temperature stress (25%). Comparison of the means for main effects of duration of high temperature stress and cultivar (Table 5) showed that the highest numbers of indirect regenerated shoots were obtained in cultivar Chantal (10.42%) in 10 days of the high temperature stress (8.3%).

Anther pretreatment is one of the main key factors to switch on androgenesis induction (Germanà *et al.,* 2011). The importance of pretreatment and the need

Table 4. Mean comparison for the interaction of cultivar and duration of high temperature stress on the responsive anthers and embryonic regenerated plantlets in eggplant anther culture.

Cultivar	Duration of high temperature stress (day)	Responsive anthers (%)	Embryonic regenerated plantlets (%)
Chantale	6	2.8±2.7^{de}	5.6±5.6^{cde}
	8	27.8±2.7^{a}	25±4.8^{a}
	10	16.7±4.8^{bc}	13.9±2.8^{bc}
	12	11.1±2.8^{cd}	5.6±2.7^{cde}
Hadrian	6	2.8±2.7^{de}	5.6±5.6^{cde}
	8	8.3±4.8^{cde}	11.1±5.6^{bcd}
	10	0±0^{e}	0±0^{e}
	12	0±0^{e}	0±0^{e}
Valentina	6	2.8±2.7^{de}	0±0^{e}
	8	22.2±2.7^{ab}	16.7±4.8^{ab}
	10	11.1±5.5^{cd}	5.6±5.6^{cde}
	12	2.8±2.7^{de}	0±0^{e}
Faselis	6	2.8±2.7^{de}	2.8±2.7^{de}
	8	8.3±4.8^{cde}	5.6±5.6^{cde}
	10	11.1±2.8^{cd}	13.9±2.8^{bc}
	12	8.3±4.8^{cde}	11.1±5.6^{bcd}

Means followed by the same letter(s) are not significantly different at 0.01 level of probability.

Table 5. Mean comparison for the main effects of cultivar and duration of high temperature stress on the callus derived shoot in eggplant anther culture.

Cultivar	Callus-derived shoots (%)	Duration of high temperature (day)	Callus-derived shoots (%)
Chantale	10.42±2.7^{a}	6	0.69±0.7^{b}
Hadrian	0.69±0.7^{b}	8	4.86±2.2^{ab}
Valentina	5.56±2.1^{ab}	10	8.33±2.7^{a}
Faselis	2.78±1.6^{b}	12	5.56 ±2.1^{ab}

Means followed by the same letter(s) are not significantly different at 0.01 level of probability.

for a specific stress treatment to trigger microspore development from gametophytic to sporophytic pathways has been highlighted in some studies (Zheng, 2003; Parra-Vega *et al.,* 2013; Eshaghi *et al.,* 2015). From the earlier reports on anther or microspore cultures and our results presented here, it can be concluded that heat shock can be used successfully as the stress factor for androgenesis. Several Heat Shock Proteins (HSP) are synthesized in heat-stressed microspores (Binarova *et al.,* 1997; Segui-Simarro *et al.,* 2003) of which HSP70 was suggested to inhibit apoptosis (Jaattela *et al.,* 1998). In Peruvian tomato (*Lycopersicon peruvianum*), the heat-shocked suspension cultures contained considerable amounts of the dominant small heat shock proteins in the cytoplasm, whose formation strictly depended on heat shock (37 to 40 °C) conditions (Nover *et al.,*1982).

Effects of cultivar and osmotic stress on eggplant anther culture

According to the analysis of variance (Table 6), the main effects of cultivar and osmotic stress were highly significant at 0.01 probability level for percentages of responsive anthers and callus-derived shoots traits while the interaction effect was not significant for these traits. However, the cultivar and osmotic stress interaction was highly significant at 0.01 probability level for embryo-derived plantlets. Mean comparison of treatment combinations (cultivar and mannitol concentration, Table 7) indicated that the use of 30 mg l^{-1} mannitol in cultivar Chantal resulted in high embryo-derived plantlets (66.7%). A comparison of the means for the main effects of mannitol and cultivar (Table 8) showed that the highest percentages of responsive anthers were obtained by cultivar Chantal (13.19%) and 10 mg l^{-1} mannitol (12.92%). The results showed

Table 6. Analysis of variance for the effects of cultivar and mannitol (gl^{-1}) on eggplant anther culture.

Source of variation	df	Mean of squares		
		Responsive anthers (%)	Embryonic regenerated plantlets (%)	Callus-derived shoots (%)
Block	3	103.02*	87.58^{ns}	44.75^{ns}
Cultivar	2	656.25**	3597.28*	223.44**
manitol	4	250.61**	1251.71**	148.73**
Cultivar×manitol	8	54.41^{ns}	854.14**	49.77^{ns}
Error	42	37.71	94.19	29.88

ns: non-significant, * and **: Significant at 0.05 and 0.01 probability level, respectively.

that the highest callus-derived shoots were obtained by cultivar Chantal (7.08%) and 10 mg l^{-1} mannitol (7.64%) (Table 8). Non-metabolizable osmotic agents such as PEG or mannitol have been described as effective factors for improving embryogenesis induction (Ferrie and Keller, 2007; Ilic-Grubor *et al.,* 1998).Our results regarding the positive effect of mannitol on adrogenesis accord with those reported by Bal *et al.* (2009) in eggplant, Gémes Juhasz *et al.* (2009) in pepper and Kaushal *et al*. (2014) in rice.

Effects of the hormone combinations on shoot regeneration from the calli obtained from eggplant anther culture

In responsive anthers, the wall tissue gradually turned brown and after 4 weeks they burst open due to pressures exerted by the growing calli or embryos with probably different origins (microspores or diploid cells of anther wall). Two types of response were observed; some anthers produced embryogenic calli, while some other produced embryos directly. The produced calli of the above mentioned experiment in cultivar Chantal were used as experimental material in this experiment. These calli were again multiplied and proliferated on the same medium and then were transferred to the media with different concentrations of BAP and Kin (Table 1). The results of ANOVA (Table 9) indicated a significant difference between hormone combinations at 1% probability level for the mean shoot number (0.5> and ≤0.5 cm). The means comparison (Table 10) showed that the use of 2 mg l^{-1} BAP and 2 mg l^{-1} Kin produced the highest number (4.3) of shoots ≤0.5 cm, while the use of 4 mg l^{-1} Kin produced the highest number (1.7) of shoots >0.5 cm.

Somatic embryogenesis and direct organogenesis are previously-studied methods for plant regeneration in eggplant, but the potential of shoot regeneration varies with genotype, explant, and culture media supplemented with different combinations of plant growth regulators. The genotype is the most important factor affecting somatic embryogenesis and organogenesis. Embryogenic competence occurs even within explant segments. According to the results provided from experiments 1 to 3 for the studied traits, cultivar Chantale had the best results. Therefore, the effects of the hormone combinations on shoot induction were investigated in this cultivar. Among growth regulators, auxins and cytokinins are of more significance as their ratio determines callogenesis, rhizogenesis, embryogenesis, and shoot regeneration in eggplant (Lakshman Naik and Ravali, 2016). We obtained considerable shoot regeneration by kinetin and 6-bemzylamino purine.

Table 7. Mean comparison for the interaction of cultivar and mannitol ($g.l^{-1}$) on the embryonic regenerated plantlets in eggplant anther culture.

Cultivar	Mannitol (gl^{-1})	Embryonic regenerated plantlets (%)
Chantale	0	16.7±5.9^{cde}
	10	22.9±3.9^{bc}
	20	33.3±5.9^{b}
	30	66.7±6.8^{a}
	40	4.1±4.2^{ef}
Valentina	0	12.5±5.4^{cdef}
	10	35.4±5.2^{b}
	20	22.9±7.8^{bc}
	30	18.7±7.1^{cd}
	40	0±0^{f}
Hadrian	0	6.2±3.9^{def}
	10	4.1±4.2^{ef}
	20	0±0^{f}
	30	0±0^{f}
	40	0±0^{f}

Means followed by the same letter(s) are not significantly different at 0.01 level of probability.

Effects of different medium compositions on the elongation of anther -derived shoots

Individual shoots were separated from shoot clusters

Table 8. Mean comparison for the main effects of cultivar and mannitol (gl^{-1}) on the callus derived shoot and responsive anthers in eggplant anther culture.

Cultivar	Responsive anthers (%)	Callus-derived shoots (%)	Mannitol (gl^{-1})	Responsive anthers (%)	Callus-derived shoots (%)
Chantale	13.19±2.1^{a}	7.08±2.1^{a}	0	9.72±2.5^{ab}	6.25±2.1^{a}
Valentina	9.17±1.9^{a}	3.33±1.3^{b}	10	12.92±2.8^{a}	7.64±2.9^{a}
Hadrian	1.67±0.9^{b}	0.42±0.4^{b}	20	8.33±2.7^{ab}	4.17±1.9^{ab}
			30	7.64±2.4^{b}	0±0^{b}
			40	0.69±0.6^{c}	0±0^{b}

Means followed by the same letter(s) are not significantly different at 0.01 level of probability.

and transferred onto ½MS and MS media supplemented with different concentrations of GA_3 (1 and 1.5 mg l^{-1}) for studying shoot elongation. The results of ANOVA (Table 11) indicated a significant difference between treatments at 1% probability for the percentage of elongated shoots. The means comparison (Table 12) showed that the highest percentage of elongated shoots (38.1%) was recorded on the MS medium containing 1.5 mg l^{-1} GA_3. These results are in agreement with other reports showing the positive effect of GA_3 on the shoot elongation (Hoque *et al.,* 2007; Shivaraj and Rao, 2011).

Determination of ploidy level and origin of the regenerated androgenic plantlets

In cultivars Hadrian and Faselis, the ploidy levels of all regenerated plants from anther culture (10 and 21 plants, respectively) were determined (Figures 2 & 3) and it was found that 60% and 57.1% of the tested plants were haploid, respectively. In cultivars Chantale and Valentina, due to the large number of the regenerated plants, 30 plants were randomly selected in each cultivar, and their ploidy levels were determined through flow cytometry. In cultivars Chantale and Valentina 40.2% and 51.5% of the plants were haploid, respectively.

CONCLUSION

Efficiency of anther culture can be affected by different factors. Our results showed that the androgenic embryo induction and frequency of haploid plant regeneration were strongly cultivar-dependent. The results indicated that cultivar Chantale had the best results for androgenic traits. Heat and osmotic stresses and also zeatin riboside were three important factors in eggplant anther culture and should be optimized in each cultivar. The number of androgenic plants can be increased through anther-derived callus culture in a medium containing higher concentrations of both BAP and Kin.

Table 9. Analysis of variance for the effect of hormone combination on shoot induction in the calli obtained from eggplant anther culture

Source of variation	df	Mean of squares	
		Shoots≤0.5 cm	Shoots>0.5 cm
Hormone combination	11	4.49**	0.96**
Error	24	0.72	0.33

**: Significant at 0.01 probability level.

Table 10. Mean comparison for the effect of hormone combination on shoot induction in the calli obtained from eggplant anther culture.

Hormone combination	Shoots obtained≤0.5 cm	Shoots obtained>0.5 cm
T1	1±0.6^{cd}	0.3±0.3^{c}
T2	0.3±0.3^{d}	0±0^{c}
T3	0.7±0.3^{cd}	0.7±0.3^{bc}
T4	1±0^{cd}	0±0^{c}
T5	0.3±0.3^{d}	0.3±0.3^{c}
T6	0.3±0.3^{d}	0±0^{c}
T7	0.7±0.6^{cd}	0.7±0.3^{bc}
T8	2±0.5^{bc}	1.3±0.6^{ab}
T9	1.7±0.9^{bcd}	1.7±0.3^{a}
T10	1.7±0.3^{bcd}	0.3±0.3^{c}
T11	4.3±0.3^{a}	1.3±0.3^{ab}
T12	3±0.5^{ab}	0.7±0.3^{bc}

Means followed by the same letter(s) are not significantly different at 0.01 level of probability.

Table 11. Analysis of variance for the effects of different medium compositions on the elongation of anther -derived shoots.

Source of variation	df	Mean of squares
Treatment	3	3.89**
Error	8	0.42

**: Significant at 0.01 probability level.

Table 12. Mean comparison for effects of different medium composition on the elongation of anther -derived shoots.

Treatment	Shoot elongation (%)
MS	0±0[b]
½ MS	19.1±0.3[ab]
MS + 1 mgl^{-1} GA_3	28.6±0.6[a]
MS + 1.5 mgl^{-1} GA_3	38.1±3[a]

Means followed by the same letter(s) are not significantly different at 0.01 level of probability.

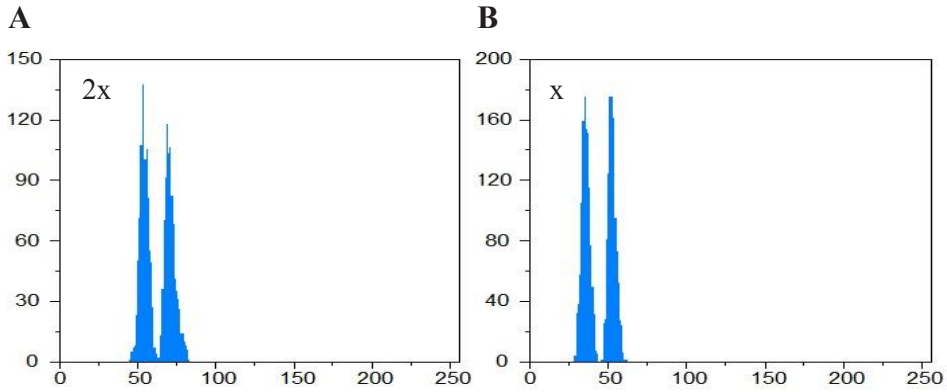

Figure 2. Flow cytometry histograms (2C DNA) of plants obtained from eggplant anther culture. **A:** Diploid plant (G1- x) and **B:** Haploid plant (G1- x). Peaks 50 refer to G1 phases of the cell cycle of standard plant (tomato).

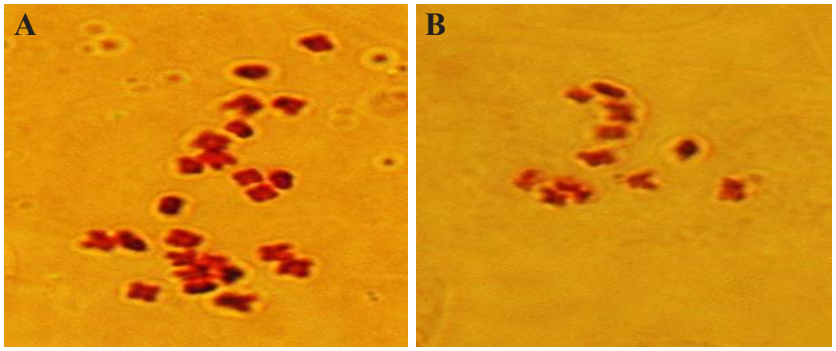

Figure 3. Chromosome counting of root tips: **A:** Diploid plant and **B:** Haploid plant.

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