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AUXIN EFFECTS ON SOMACLONAL VARIATION AND PLANT REGENERATION FROM MATURE EMBRYO OF BARLEY (*HORDEUM VULGARE* L.)

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Abstract

Crop improvement through genetic engineering depends on effective and reproducible plant regeneration systems. In barley (*Hordeum vulgare* L.), immature embryos are the most commonly used as explant source for *In vitro* regeneration and genetic transformation but, mature embryos are alternative to immature embryo due to the fact that they have advantages such as easy storage and ready availability throughout the year. The effects of different concentrations (2, 4, 6, 8, 10 and 12 mg/l) of three auxins (2,4-D, dicamba and picloram) were evaluated in mature embryo culture of barley. Calli, embryogenic calli and regenerated plants were observed in all of the studied auxins. The MS (Murashige & Skoog basal medium) containing 12 mg/l dicamba was found to be the most effective for embryogenic callus, responded embryogenic callus and regeneration efficiency. Plant tissue culture can bring about genetic changes that are known as somaclonal variation. Genetic and epigenetic changes were examined by RAPD (Randomly Amplified Polymorphic DNA) and CRED-RA (Coupled Restriction Enzyme Digestion-Random Amplification) techniques, respectively. Increased auxin concentration led to the decrease in the GTS (Genomic Template Stability) value. DNA hypermethylation occurred in higher concentrations of 2,4-D and picloram, while DNA hypomethylation was observed in dicamba.

Key words: Barley, RAPD, CRED-RA, 2,4-D, Dicamba, Picloram.

Introduction

Barley is one of the most important crops in the world after wheat, maize and rice. It is mainly used for animal feed, human consumption, and malting (Przetakiewicz *et al.,* 2003; Rostami *et al.,* 2013). Tissue culture is a powerful tool in plant gene transformation and molecular breeding. Callus induction and regeneration frequency of barley depend on some factors such as genotype, explant source and culture medium (Aguado-Santacruz *et al.,* 2011). Immature embryos are most commonly used as explant source for *In vitro* regeneration and genetic transformation of barley because of its higher regeneration potential (Gubišová *et al.,* 2011; Rikiishi *et al.,* 2008; Sharma *et al.,* 2005). However, mature embryos are alternative to immature embryos since they have advantages such as easy storage and ready availability throughout the year (Mendoza & Kaeppler 2002). Endosperm-supported mature embryos (He & Jia 2008) and excised mature embryos (or non-endosperm supported mature embryos) (Gurel *et al.,* 2009; He & Jia 2008; Yadav *et al.,* 2011) were used for callus induction in barley mature embryo culture. Composition of the culture medium, mainly including different amounts of growth regulators, is another important factor influencing callus initiation and plant regeneration from embryo (Gurel *et al.,* 2009; Jiang *et al.,* 1998). 2,4-Dichlorophenoxyacetic acid (2,4-D) and 3,6-Dichloro-o-anisic acid (dicamba) were successfully used for callus induction in barley (Vyroubalová *et al.,* 2011). 2,4-D, dicamba and 4-amino-3,5,6-trichloropicolinic acid (picloram) were tested to determine their effects on the callus induction in immature embryo culture of barley by Przetakiewicz *et al.* (2003); Šerhantová *et al.* (2004). Furthermore, there are researches that were conducted to determine the effects of these auxins on callus formation and plant regeneration in mature embryo culture of barley (Gurel *et al.,* 2009; Yadav *et al.,* 2011). However, excised mature embryos were used in these researches as explant sources. He & Jia (2008) showed that frequency of the shoot regeneration of endosperm supported mature embryos were higher than excised mature embryos in barley. Also, similar results have been found in mature embryo culture of wheat and triticale (Aydin *et al.,* 2011; Birsin & Ozgen 2004). Unlike our study, He and Jia (2008) investigated only one type of auxin in endosperm supported mature embryo culture of barley.

Plant tissue culture can bring about genetic changes that are known as somaclonal variation (Linacero *et al.,* 2011). Genetic change may involve in point mutations, activation of transposable elements, rearrangement of DNA, ploidy changes and epigenetic variations, especially methylation variations (Kaeppler *et al.,* 2000; Linacero *et al.,* 2011), although such variation is desirable in the creation of variants with desired characters in plant breeding. However, it is not desirable in plant transformation studies. Therefore, somaclonal variation should be detected during the early stage of plant tissue culture for plant transformation studies. Genetic changes induced by *In vitro* culture depend on plant species, genotype, the type of explant, and the culture media, plant growth regulators and duration of the culture (Temel *et al.,* 2008; Vyroubalová *et al.,* 2011). Temel *et al.* (2008) investigated genetic and epigenetic changes of the 24 weeks-old calli in mature embryo culture of barley. However, they determined the effect of culture time on the genetic and epigenetic changes. Some methods such as karyotype analysis, isozyme and molecular markers can be used to identify the genetic changes induced by *In vitro* culture (Roy *et al.,* 2012). RAPD (Random Amplified Polymorphic DNA), which amplifies different regions of the genome, has been widely used for the identification of genetic changes in the tissue culture since it allows better analysis of genetic changes (Rawat *et al.,* 2013). RAPD has been successfully used for the identification of somaclonal variation *In vitro* cultured barley (Temel *et al.,* 2008; Todorovska *et al.,* 1997). CRED-RA (Coupled Restriction Enzyme Digestion-Random Amplification) has been used

for the identification of DNA methylation that is one type of epigenetic variations and its availability is shown by (Temel *et al.,* 2008).

The aim of this study was to detect the effects of auxins type and concentration on tissue culture features and somaclonal variations in barley mature embryo culture.

Material and Methods

Endosperm supported mature embryo culture: Mature seeds of Tarm-92 cultivar (*Hordeum vulgare* L.) were used as sources of endosperm supported mature embryos. The seeds were washed with tap water for 15 min and then were dehusked by hand. These dehusked seeds were surface sterilized with 70% ethanol for 5 min, rinsed with sterile water and then with 5% solution of sodium hypochlorite with drops of TWEEN 80 for 20 min and afterwards sterilized seeds were rinsed with sterile water for three times and incubated at 4°C for 24 h in sterile distilled water. Imbibed seeds were prepared as described by Aydin *et al.* (2011); Filippov *et al.* (2006) for endosperm supported mature embryo culture. Fifteen prepared seeds in four replications were placed in furrow downwards in petri dishes containing MS (Murashige & Skoog 1962) medium supplemented with 20 mg/l sucrose, 2 g/l phytagel and different concentrations (2, 4, 6, 8, 10 and 12 mg/l) of auxins (2,4-D, dicamba and picloram). The media pH was adjusted to 5.8 with sodium hydroxide (NaOH) before the autoclaving and the media were autoclaved for 20 min at 121˚C and 105 kPa. Vitamins and auxin solutions were filter – sterilized and subsequently added to autoclaved medium. The petri dishes incubated at 25±1°C for 21 days in darkness for callus induction from endosperm supported mature embryos. After 21 days, callus induction (%) and embryogenic callus induction (%) were determined. Calli that were separated from the seeds were transferred to the same hormone-free callus initiation medium and incubated for 4 weeks in 16:8 h (day:night) photoperiod (light intensity of 50 µmol $m^{-2}s^{-1}$) at 25±1°C for plant regeneration. Responded embryogenic callus and regeneration efficiency were determined after 4 weeks. Regenerated plantlets were transferred to Meganta boxes containing the same regeneration medium and were grown under the same plant regeneration conditions until they reached 10-12 cm high. Tissue culture features were tested by using SAS 9.1 statistical software (Anon., 2003) according to completely randomized design with a factorial arrangement of 3 (auxins) x 6 (concentrations of auxins) = 12 treatment. Analysis of variance (ANOVA) was calculated using the general linear model procedure and mean separation was performed by Least Significant Difference (LSD) test at 0.05 level.

RAPD and CRED-RA analysis: After 21 days of incubation in callus induction medium, genomic DNA was isolated from bulked calli that were randomly collected and developed in MS medium supplemented with different concentrations of auxins and from control plant developed in hormone free MS medium using the method described by (Monsanto 2004) and stored at -20°C. The quality and concentration of the DNA were measured using a Nano-Drop (ND-1000) spectrophotometer and electrophoresis in a 0.8% (w/v) agarose gel. Forty RAPD primers were tested with control plant. Only 13 primers (OPW-20, OPW-13, OPA-1, OPA-2, OPA-4, OPA-12, OPA-13, OPH-18, OPY-6, OPY-11, OPY-15, OPY-16, OPB-8, OPW-4, OPW-7 and OPW-8) were amplified polymorphic amplicons and used in RAPD-PCR reactions.

PCR amplifications were carried out in QIAGEN® thermocycler in a total volume of 25 µl, containing 25 ng genomic DNA, 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH=8.3), 400 µM dNTP, 10 pmol primer, 2.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase. The amplification profile composed of an initial denaturation at 95°C for 5 min, followed by 42 cycles at 94˚C for 1 min, 36˚C for 1 min, 72˚C for 2 min and a final extension of 15 min at 72˚C. PCR products were separated by electrophoresis using 1% agarose gel in 0.5× TBE buffer with constant voltage of 70 V for 2 h. Gels stained by with ethidium bromide (10 mg/ml) visualized under UV light and photographed using gel visualization system. The sizes of the fragments were estimated based on a DNA ladder of 100 bp.

Genomic DNA samples from each treatment were separately digested with *Hpa*II and *Msp*I restriction endonucleases (which cut the sequence 5´-C/CGG-3´ with different sensitivity to cytosine methylation; MspI cuts if the inner C is methylated, whereas *Hpa*II cannot cleave in the presence of methyl groups). After checking the digestion on agarose gel, 1µl of each digestion product were amplified with 8 random primers (OPW-7, OPY-16, OPA-12, OPY-11, OPA-1, OPA-4, OPB-8 and OPA-13). Amplification and visualization conditions for CRED-RA are the same as described for RAPD analysis.

RAPD patterns were evaluated using the Total Lab TL120 computer software (Non-linear Dynamics, Total Lab Ltd., Newcastle Upon Tyne, United Kingdom). Genomic template stability (GTS, %) was calculated as follows: $GTS = 100-(100x\ a/n)$, where a is the average number of polymorphic bands detected in each treated sample, and n is the number of total bands in the control. Polymorphisms in RAPD profiles included disappearance of a normal band and appearance of a new band compared with the control. The average was calculated for each experimental group (Yildirim *et al.,* 2014). To compare the sensitivity of each parameter, changes in these values were calculated as a percentage of their control (set to 100%). The average number of polymorphisms (%) was calculated for each dose to realize CRED–RA analysis. To calculate the number of polymorphisms (%), the following formula was used: 100x *a/n* (Yildirim *et al.,* 2014).

Results

Variance analysis of the results showed that the main effect of the auxin type and concentration were significant on all evaluated parameters. However, responded embryogenic callus rate and regeneration efficiency were significantly affected by auxin type x concentration interaction (Table 1). Although the callus induction from endosperm supported mature started almost at the same time (2-3 day after culture) for all auxin types, rate of callus induction was higher on the medium containing dicamba than the medium containing 2,4-D and picloram. The highest callus induction was observed on MS medium supplemented with dicamba, whereas, the lowest callus induction occurred on the medium containing picloram. Callus induction ranged from 71.7% to 93.3% depending on the auxin concentrations. In addition, increasing of auxin concentration increased the rate of callus induction. 10 mg/l and 12 mg/l concentrations of dicamba were found to be best for callus induction.

Table 1. Callus induction (CI) (%), embryogenic callus induction (ECI) (%), responded embryogenic callus rate (REC) (%), regeneration efficiency (RE) (number) based on auxins and their concentrations.

Auxin	Concentration (mg/l)	CI (%)[a]	ECI (%)[b]	REC (%)[c]	RE (number)[d]
2,4-D	2	85.0	57.5	32.5	1.4
	4	95.0	57.5	30.0	1.6
	6	97.5	67.5	42.5	2.1
	8	95.0	82.5	65.0	2.9
	10	97.5	90.0	67.5	2.6
	12	97.5	95.0	57.5	3.7
	Mean	**94.6**	**75.0**	**49.2**	**2.4**
Dicamba	2	72.5	65.0	30.0	1.8
	4	85.0	75.0	35.0	1.8
	6	87.5	85.0	57.5	2.1
	8	95.0	92.5	70.0	2.4
	10	100.0	97.5	77.5	3.0
	12	100.0	100.0	80.0	4.1
	Mean	**90.0**	**85.8**	**58.3**	**2.5**
Picloram	2	57.5	50.0	17.5	0.0
	4	67.5	62.5	20.0	0.6
	6	72.5	72.5	25.0	1.4
	8	72.5	80.0	25.0	2.1
	10	77.5	87.5	32.5	2.6
	12	82.5	90.0	32.5	2.6
	Mean	**71.7**	**73.8**	**25.4**	**1.5**
Mean of auxins	2	71.7	57.5	26.7	1.1
	4	82.5	65.0	28.3	1.4
	6	85.8	75.0	41.7	1.8
	8	87.5	85.0	53.3	2.5
	10	91.7	91.7	59.2	2.7
	12	93.3	95.0	56.7	3.4
	Mean	**85.4**	**78.2**	**44.3**	**2.2**
F value (Auxin) (A)		20.45**	114.13**	79.57**	85.54**
F value (Concentration) (C)		99.0**	44.88**	223.30**	61.22**
F value (A xC)		1.57^{ns}	1.68^{ns}	9.99**	4.55**
$LSD_{(0.05)}$ (A)		3.5	2.8	3.2	0.2
$LSD_{(0.05)}$ (C)		4.9	3.9	4.6	0.3
$LSD_{(0.05)}$ (AXC)		-	-	7.9	0.5
CV %		6.9	6.2	5.6	9.5

**: Significant at p<0.01., ^{ns}: Non-significant at p>0.05

[a]Callus number / Explants number x 100;

[b]Embryogenic callus number / Explants number x 100;

[c]Responded embryogenic callus number / Explants number x 100;

[d]Regenerated plant number / Responded embryogenic callus number

The embryogenic callus was evaluated after 3 weeks of embryo culture on the callus induction media. The calli were classified into two types as non-embryogenic callus (NE) and embryogenic callus (EC), and both of them were observed in the culture. EC was characterized as compact, friable, irregularly shaped and creamy. NE was characterized as soft, loose and watery. Results presented in Table 1 indicate that embryogenic callus formation varied depending on the applied auxins type and the highest embryogenic callus induction (100%) was determined on the MS medium containing dicamba. Embryogenic callus increased when the concentration of auxin was increased. The highest callus was observed on the media with a high concentration of auxins. 12 mg/l dicamba was the most effective with the highest rate of embryogenic callus induction (100%).

Plant regeneration is correlated with the ability to form embryogenic calli. However, plant regeneration may not occur in all embryos on embryogenic callus and embryogenic callus. Therefore, embryogenic calli producing roots and shoots were recognized as responded embryogenic callus (REC) and regeneration efficiency (RE) was determined for responded embryogenic callus. After the embryogenic calli was transferred to the regeneration medium, green shoots were occurred within 10-12 days. Dicamba was more effective than 2,4-D and picloram for REC and regeneration efficiency. The highest RE was observed on the medium with dicamba (2.5), followed by 2,4-D (2.4) and picloram (1.5). Increasing auxins concentration is generally led to an increase in REC and RE. The analysis of variance for auxin x concentration interaction indicated that 12 mg/l dicamba was the most effective one for plant regeneration via endosperm supported mature culture in barley.

Somaclonal variations were examined by RAPD using 32 random primers. Only 16 primers produced amplified bands. The RAPD analysis of auxin induced calli showed polymorphisms based on using primers, and auxin type and concentrations of auxins. Polymorphic bands were detected in 7 primers (OPW-20, OPW-13, OPA-2, OPA-4, OPY-11, OPB-8 and OPW-8) and the highest polymorphic bands were scored in OPY-11 and OPA-4 primers (Table 2). Determined polymorphisms are characterized by variation in the loss of normal bands or the appearance of new bands. The molecular size of polymorphic bands range from 235 to 1145 bp. Changes in RAPD pattern were also measured as GTS (which is a qualitative measurement reflecting the changes in RAPD patterns) in relation to the pattern showed in the control plants. According to the average concentration of the used auxin, dicamba induced callus samples showed the highest GTS value (98.3%), followed by 2,4-D (97.0%) and picloram (96.5%). Increased auxin concentration led to a decrease in the GTS value and an increase in the polymorphism in all auxins. The highest (99.2%) and the lowest (94.7%) GTS values were determined in the formed calli in medium containing 2 mg/l dicamba and 12 mg/l picloram, respectively (Table 2). The polymorphism and GTS value were clearly dependent on the auxin type and the concentration of auxin type.

CRED-RA was performed to detect the level of DNA methylation and genomic DNA digested with *Hpa*II and *Msp*I enzymes. Eight oligonucleotide primers produced specific and stable results. DNA methylation value varied depending on the auxin type and the concentration of auxin (Table 2). DNA hypermethylation occurred in higher concentrations of 2,4-D and picloram, while DNA hypomethylation was observed in dicamba. It was observed that *Hpa*II polymorphism was higher than *Msp*I polymorphism and *Hpa*II polymorphism ranged from 0% to 66%, while *Msp*I polymorphism ranged from 0% to 50% based on the used primer and auxin concentration (Table 3).

Discussion

The development of a suitable regeneration system is a prerequisite for the application of genetic manipulation. It is widely known that the composition of culture medium, mainly growth regulators, is an important factor in plant regeneration response. Auxins from growth regulator are very significant for *In vitro* plant regeneration of higher plants. The effect of 2,4-D, picloram and dicamba were studied on callus induction and plant regeneration. The results of our study show that dicamba was better than 2, 4-D and picloram in callus induction and plant regeneration. These results are similar to the results obtained by other authors working with barley (Aguado-Santacruz *et al.,* 2011; Gurel *et al.,* 2009; Przetakiewicz *et al.,* 2003; Trifonova *et al.,* 2001) and wheat (Aydin *et al.,* 2011; Filippov *et al.,* 2006). Trifonova *et al*. (2001) reported that dicamba was superior in supporting long-term regeneration ability in barley. In addition, dicamba was generally superior to 2,4-D in promoting transient expression and subsequent stable transformation. According to Castillo *et al.* (1998) dicamba was superior to 2,4-D and picloram in the induction and maintenance of embryogenesis and regeneration capacity. Papenfuss & Carman (1987) reported that dicamba was readily used by metabolism and therefore, it increases the shoot formation. However, 2,4-D proves to be more resistance to enzymatic digestion and reaction and hence, stays in cells that are highly stable (Moore, 1989). Bregitzer *et al.* (1995) stated that the use of 2,4-D in the long-term maintenance of barley cultures cause a loss of the regenerative potential due to the loss of totipotency from barley. Our results are in contrast with those reported by Jiang *et al.* (1998), who reported that the frequencies of callus induction on media with dicamba or 2,4-D were similar and differences were insignificant. Zapata *et al.* (2004) also demonstrated that the J25-8 medium supplemented with 2.0 mg/l of 2,4-D is highly efficient for callus growth and plant regeneration in mature embryo culture of barley.

The results showed that the effects of auxin concentration on all evaluated parameters were significantly different. We observed that the increase in the auxin concentration increased the rate of all evaluated parameters. Similar results are presented in barley by Gurel *et al.* (2009) and in wheat by Filippov *et al.* (2006). Han *et al.* (2011) suggested that higher 2,4-D concentration (6 mg/L) could efficiently promote callusing response. Meanwhile, Chang *et al.* (2003) found no different in callus induction and plant regeneration by using concentrations of 2,4-D or dicamba higher than 3 mg/l in immature embryo culture of barley. However, Filippov *et al.* (2006) stated that mature embryos contain more old cells and more differentiated tissues than immature embryos and this necessitate the use of high concentration of auxin for re-differentiation. They also reported that endosperm can absorb the plant growth regulators in endosperm supported mature embryo culture and therefore, it is necessary to use higher concentration of auxin in endosperm supported mature culture than non-endosperm supported mature culture.

Table 2. Molecular sizes (bp) of bands (+: appearance / -: disappearance) and the average GTS values in RAPD profiles.

Primers	Control	+/-	Dicamba (mg/l)						2,4-D (mg/l)						Picloram (mg/l)					
			2	4	6	8	10	12	2	4	6	8	10	12	2	4	6	8	10	12
OPW-20	4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	673	673
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	763	-	-
OPW-13	4	+	-	-	-	-	-	-	946	-	946	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	235	-	-	-	-
OPA-1	4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA-2	6	+	-	-	-	-	-	-	-	-	-	-	-	-	494	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA-4	6	+	-	-	-	-	-	-	-	1054	-	664;881	664;881	664	-	-	354	-	-	-
		-	-	-	-	-	667	667	-	1145	-	-	-	-	-	-	978	-	-	467
OPA-12	5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA-13	6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPH-18	10	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPY-6	6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPY-11	7	+	-	930	-	930	930	-	-	-	759	759	-	373	543	543	381	381	381;543	381;543
		-	-	-	-	-	-	-	-	-	-	-	973;1021	973;1021	-	-		471	471	471
OPY-15	5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPY-16	6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPB-8	6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	830	830	-	830	-	-	-	-	-	772	-	-	-	-	-	-
OPW-4	5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPW-7	9	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPW-8	8	+	548	-	-	-	708	708	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	873;971	873;971	-	-	-	-	-	-	-	-	-	-	-	-
GTS	**100**		**99.2**	**99.1**	**98.9**	**98**	**97.2**	**97.1**	**98.4**	**97.9**	**97.5**	**97**	**96.1**	**95.2**	**98**	**97.5**	**97**	**96.6**	**95.7**	**94.7**

Table 3. CRED-RA band amounts and polymorphism %.

Primers	Dose (mg/l)	Dicamba						2,4-D						Picloram					
		Total band		Polymorphic bands		Polymorphism (%)		Total bands		Polymorphic bands		Polymorphism (%)		Total bands		Polymorphic bands		Polymorphism (%)	
		H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M
OPW-7	C	6	6	-	-	-	-	4	5	-	-	-	-	5	5	-	-	-	-
	2	7	5	1	1	14.2	20	6	5	1	2	16.6	40	4	4	1	0	25	0
	4	5	5	0	2	0	40	5	6	0	1	0	16.6	5	6	2	2	20	33.3
	6	7	8	1	0	14.2	0	5	5	1	0	20	0	4	6	1	1	25	16.6
	8	8	7	2	1	25	14.2	6	6	2	0	33.3	0	3	5	0	1	0	20
	10	6	6	1	1	16.6	16.6	6	4	0	0	0	0	5	7	2	2	40	28.5
	12	7	6	0	0	0	0	4	4	2	1	50	25	4	5	2	2	50	40
OPY-16	C	4	5	-	-	-	-	7	8	-	-	-	-	5	5	-	-	-	-
	2	6	7	0	1	0	14.2	5	6	3	0	60	0	4	5	1	0	25	0
	4	7	7	2	1	28.5	14.2	8	9	0	1	0	11.1	5	6	0	1	0	16.6
	6	7	8	2	2	28.5	25	6	6	2	3	33.3	50	6	5	1	1	16.6	20
	8	7	9	3	1	42.8	11.1	8	7	1	0	12.5	0	5	6	2	3	40	50
	10	6	5	0	1	0	20	9	9	1	2	11.1	22.2	4	5	0	1	0	20
	12	7	6	0	0	0	0	6	6	4	2	66.6	33.3	6	6	0	2	0	33.3
OPA-12	C	5	5	-	-	-	-	5	7	-	-	-	-	7	7	-	-	-	-
	2	5	5	1	1	20	20	6	4	0	1	0	25	5	7	0	0	0	0
	4	4	4	1	1	25	25	7	7	3	1	42.8	14.2	5	6	0	1	0	16.6
	6	5	6	0	1	0	33.3	5	6	2	2	40	33.3	5	5	2	1	40	20
	8	4	7	1	0	25	0	7	6	0	0	0	0	5	5	1	0	20	0
	10	5	5	1	1	20	0	9	7	4	1	44.4	14.2	5	5	1	0	20	0
	12	6	5	2	0	33.3	0	9	5	0	0	0	0	6	6	1	0	16.6	0
OPY-11	C	6	6	-	-	-	-	3	5	-	-	-	-	4	5	-	-	-	-
	2	5	6	2	2	40	33.3	5	5	0	0	0	0	6	5	1	1	16.6	20
	4	5	5	1	1	20	20	5	5	0	1	0	20	5	5	1	1	20	20
	6	4	6	2	2	50	33.3	3	2	1	0	33.3	0	5	6	2	1	40	16.6
	8	3	5	0	1	0	20	3	3	1	0	33.3	0	5	5	0	1	0	20
	10	5	5	1	2	20	40	3	5	1	0	33.3	0	4	6	2	2	50	33.3
	12	5	6	0	1	0	16.6	5	6	0	3	0	50	4	5	1	1	25	20

Table 3. (Cont'd.).

Primers	Dose (mg/l)	Dicamba						2,4-D						Picloram					
		Total band		Polymorphic bands		Polymorphism (%)		Total bands		Polymorphic bands		Polymorphism (%)		Total bands		Polymorphic bands		Polymorphism (%)	
		H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M
	C	6	6	-	-	-	-	5	5	-	-	-	-	4	5	-	-	-	-
	2	5	6	1	1	20	16.6	6	4	1	1	16.6	25	5	6	1	1	20	16.6
	4	5	5	0	0	0	0	5	5	0	0	0	0	5	6	0	0	0	0
OPA-1	6	5	5	1	2	20	40	4	5	1	1	25	20	4	5	0	0	0	0
	8	5	5	1	1	20	20	5	5	1	1	20	20	4	4	1	1	25	25
	10	6	4	1	0	16.6	0	5	4	1	0	20	50	5	5	0	0	0	0
	12	5	5	1	1	20	20	4	5	2	0	50	0	4	3	0	1	0	33.3
	C	4	5	-	-	-	-	3	5	-	-	-	-	9	8	-	-	-	-
	2	5	5	1	1	20	20	3	3	1	0	33.3	0	7	7	0	0	0	0
	4	3	4	2	1	66.6	25	3	7	2	1	66.6	14.2	7	8	2	0	28.5	0
OPA-4	6	5	7	0	2	0	28.5	4	4	0	0	0	0	8	7	1	1	12.5	14.2
	8	4	4	2	1	50	25	4	3	1	1	25	33.3	9	7	0	1	0	14.2
	10	4	5	1	0	25	0	6	8	0	1	0	12.5	9	7	1	1	11.1	14.2
	12	4	4	0	0	0	0	6	5	2	2	33.3	40	8	9	0	1	0	11.1
	C	3	4	-	-	-	-	4	4	-	-	-	-	5	8	-	-	-	-
	2	4	3	1	0	25	0	4	4	1	0	25	0	7	5	1	1	14.2	20
	4	4	4	1	1	25	25	3	3	0	1	0	33.3	6	7	1	1	16.6	14.2
OPB-8	6	3	3	1	0	33.3	0	3	4	0	1	0	25	7	6	0	0	0	0
	8	6	4	2	0	33.3	0	4	5	1	2	25	40	5	5	1	0	20	0
	10	3	4	1	1	33.3	25	4	3	1	1	25	33.3	6	5	2	1	33.3	20
	12	4	3	0	0	0	0	4	3	1	0	25	0	6	5	3	2	50	0
	C	7	7	-	-	-	-	8	8	-	-	-	-	5	7	-	-	-	-
	2	6	6	0	1	0	16.6	6	7	2	0	33.3	0	7	5	0	0	0	0
	4	7	6	1	1	14.2	16.6	8	8	2	0	25	0	5	4	0	0	0	0
OPA-13	6	5	3	0	0	0	0	8	9	0	2	0	22.2	6	8	0	0	0	0
	8	9	8	0	3	11.1	37.5	8	9	3	3	37.5	33.3	5	6	2	1	40	16.6
	10	5	5	0	0	0	0	7	9	4	2	57.1	22.2	7	8	0	2	0	25
	12	6	6	0	2	0	33.3	6	7	2	2	33.3	28.5	6	8	1	1	16.6	12.5
	C	**5**	**5**	**-**	**-**	**-**	**-**	**5**	**5**	**-**	**-**	**-**	**-**	**5**	**5**	**-**	**-**	**-**	**-**
	2	**5**	**5**	**1**	**1**	**17.4**	**17.5**	**5**	**5**	**1**	**1**	**23.1**	**11.2**	**5**	**5**	**1**	**1**	**12.6**	**7**
	4	**5**	**5**	**1**	**1**	**22.4**	**20.7**	**5**	**5**	**1**	**1**	**16.8**	**13.6**	**5**	**5**	**1**	**1**	**10.6**	**12.5**
Average	**6**	**5**	**5**	**1**	**1**	**18.2**	**20**	**5**	**5**	**1**	**1**	**18.9**	**18.8**	**5**	**5**	**1**	**1**	**16.7**	**10.9**
	8	**5**	**5**	**1**	**1**	**25.9**	**15.9**	**5**	**5**	**1**	**1**	**23.3**	**15.8**	**5**	**5**	**1**	**1**	**18.1**	**18.2**
	10	**5**	**5**	**1**	**1**	**16.4**	**12.7**	**5**	**5**	**1**	**1**	**23.8**	**19.3**	**5**	**5**	**1**	**1**	**14.9**	**17.6**
	12	**5**	**5**	**1**	**1**	**6.6**	**8.7**	**5**	**5**	**1**	**1**	**32.2**	**22.1**	**5**	**5**	**1**	**1**	**19.7**	**18.7**

Genetic factors are considered to be a major contributor to *In vitro* response of cultured tissues. Therefore, endosperm supported mature embryos of Olgun and Efes 98 cultivars cultured in MS medium that is determined as the best (12 mg/l dicamba) and the results obtained from these cultivars in line with the results are obtained from Tarm-92 cultivar (data not shown).

Somaclonal variation is a variation that occurs as a result of tissue culture. Somaclonal variation refers to genetic and/or epigenetic changes (Li *et al.,* 2007). In the present study, genetic and epigenetic changes were determined by means of RAPD and CRED-RA. We showed that RAPD was effective to detect variations that occur as a result of tissue culture. Similarly, RAPD has been successfully used for the identification of somaclonal variation by some researchers (Temel *et al.,* 2008; Todorovska *et al.,* 1997). Polymorphisms observed in RAPD pattern are characterized by variation in the loss of normal bands or the appearance of new bands. These polymorphisms may be due to sequence variations (point mutations, insertion, deletion or transposable elements) in loci by primer used. Increased auxin concentration led to a decrease in the GTS value and an increase in the polymorphism in all auxins. The concentrations of exogenously applied auxins are higher because mature embryos contain more old cells and more differentiated tissues than immature embryo. Synthetic auxins are also more effective than the endogenous because the artificial auxin cannot be metabolized in plant cells. Therefore, we have to use higher concentrations of the synthetic auxin. In a study performed on 2,4-D, (Pfeiffer & Hoftberger 2001) were found that 2,4-D often leads to an oxidative burst in the tissue through generating reactive oxygen species, including hydrogen peroxide. Much reactive oxygen does not appear to interact with DNA but they are precursors for hydroxyl radical. The reaction of hydroxyl radical with DNA generates a multitude of products, since it attacks sugar, pyrimidine and purine, including guanine residues to form 8-hydroxyde-oxyguanosine (8-OHdG). In addition, 8-OHdG predominantly produces transversion mutation (G to T) (Taspinar *et al.,* 2009). Filkowski *et al.* (2003) have reported that the same amount of dicamba induces point mutations and double-strand DNA breaks in *Arabidopsis* and single-strand DNA breaks in bean root nuclei. Furthermore, it has been recently suggested that dicamba-induced lesions on DNA could be accounted for the reactive oxygen species delivered *In vitro* (Gonzalez *et al.,* 2005; Grossmann *et al.,* 2001).

The result indicated that CRED-RA was effective to detect the DNA methylation in tissue culture, in line with Temel *et al.* (2008). However, Li *et al.* (2007) determined cytosine methylation by means of S-SAP and MSAP in the regenerates of wild barley (*Hordeum Brevisubulatum* (Trin.) Link). Additionally, the DNA methylation was detected with methylation-sensitive RFLP in *Elaies guineensis* Jacq. (Jaligot *et al.,* 2002). Our results showed that epigenetic change was more frequent and variable than genetic change. This result is in agreement with the result observed by Miguel & Marum (2011). In the present study, DNA hypermethylation was observed in 2,4-D and picloram except for dicamba. Similarly, Yildirim *et al.* (2014) reported that dicamba caused DNA hypomethylation in common bean.

These results suggested that dicamba would provide enough and suitable target material for genetic transformation studies without higher risk of mutations when compared to 2,4-D and picloram.

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