The effect of different hormone combinations on direct and indirect somatic embryogenesis in Agave *americana*

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Abstract

Direct and indirect somatic embryogenesis in Agave *Americana* through basal parts of micropropagated shoots were considered in this study. For micropropagation, 2 factors (BA and NAA) were utilized in different concentrations. A factorial experiment based on a completely randomized design (CRD) was accomplished with four replicates in a modified Murashige and Skoog medium (MSB). BA in 3 mg/L along with NAA in 0.1 mg/L was the best treatment for multiplication. Callus and embryo induction were evaluated using different auxins in a CRD experiment with three replicates in MS medium supplemented with L2 vitamins. Picloram 2.5 and 2 mg/L and also Dicamba 1 mg/L induced 66.7%, 60%, and 53.3% of explants to somatic embryo, respectively. Also, 2,4-D 2 mg/L had the highest impact on callus induction (95.6%). Embryogenesis was studied using Dicamba and Picloram in the same culture medium in a factorial experiment based on CRD with three replicates. The results of orthogonal polynomial analaysis indicated that high concentrations of Dicamba from 0 to 1.5 mg/L had a significant effect on somatic embryo induction. However, higher concentrations had the opposite effect on somatic embryo numbers. Germination of somatic embryos was performed in MSB medium without growth regulators.

Keywords: Agave, Micropropagation, Somatic embryogenesis, Callus, Hormones

Naziri, M., Sh. Sadat and **M. Soltani Howyzeh .**2019. 'The effect of different hormone combinations on direct and indirect somatic embryogenesis in Agave *Americana*'. *Iranian Journal of Plant Physiology*, 9 (2),2739-2747.

Introduction

Among all medicinal plants, Agaves are a very important group with renewable source for food, beverages (tequila), fibbers (sisal), silage for livestock and drugs (saponins, sterols, steroidal alkaloids, alkaloidal amines). Some of them such as *Agave Americana* are also considered as ornamental plants (due to their distinctive leaf form and color) (Kulus, 2014). Despite its economic importance, the *Agave* spp. has not

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Received: October, 2018
Accepted: December, 2018

been genetically improved. This is probably owing to the fact that they have a very long life cycle (8-20 year) and many of them have an inefficient sexual reproduction mechanism (Robert et al., 2005) creating an obstacle for breeding programs. Moreover, new promising cultivars need to be propagated in large scales to establish a continuous program of selection. In nature, however, a plant will only produce some 25 healthy rhizomes over a period of 5 years, which is not sufficient to establish a continuous program of selection (Robert et al., 2005). In addition, a great deal of variability is observed in the size and vigor of the offspring from a single mother. Another problem associated with the use of vegetative propagated plants is that microbial diseases are easily transmitted to the next generation and are dispersed by men. In the last decade pathogenic bacteria and fungi have been an important problem causing economic losses to the agave growers with the greatest infestation in 1997 when 20% of plants were affected to varying degrees (Fucikovsky, 2002). Therefore, the absence of healthy and horticulturally improved varieties which are morphologically and genetically homogeneous strongly requires a clonal propagation method. The use of tissue culture to propagate elite individuals has proved to be an alternative strategy to improve yields through selection and cloning of elite materials (Robert et al., 1987, 2004, 1992). However, the cost of in vitro micropropagated plants is still high compared to those naturally propagated. Hence more efficient and cost-effective methods are needed for large scale production for commercial planting. Somatic embryogenesis might offer such an option. As a method for micropropagation, it increases the multiplication efficiency of the system, decreases the costs of production, and permits partial automation of the process through the use of bioreactors (Monja-Mio and Robert, 2013). Moreover, somatic embryogenesis has a great potential for in vitro germplasm conservation, genetic transformation, and synthetic seed production. Although there have been a number of reports for somatic embryogenesis of different Agave species (Rodríguez-Garay et al., 1996; Nikam et al., 2003; Martínez-Palacios et al., 2003;Tejavathi et al., 2007;Portillo et al., 2007; Monja-Mio and Robert 2013;Oliva et al., 2014), there is only one report on direct somatic embryogenesis of genus. A. *Fourcroydes*, (Monja-Mio and Robert, 2013). Moreover, there is only one report on indirect somatic embryogenesis of Agave *Americana* in Mexico (Oliva et al., 2014) which cannot guarantee reproducibility of the introduced protocol for other ecotypes of this species in different regions. Because genetic properties of the different clonal lines and the seasonal or environmental conditions in which they were grown can affect endogenous hormones leading to different responses to in-vitro growth regulators, the same genotypes in different regions may have different responses to plant growth regulators. Accordingly, the aim of this study is to introduce new protocols for direct and indirect somatic embryogenesis of Agave *Americana* in southern Iran to accelerate breeding programs in this region.

Material and Methods

Plant material

Shoots, extracted from the rhizomes in the plantations, were selected as a source of explants. Before taking the plant tissues into the laboratory, all leaves of rhizomatous offshoots were removed and only a segment of the basal part was left. This remaining part was thoroughly washed with soap and a brush to remove all the soil around. The external tissues were removed with a sharp butcher knife, leaving a block of tissue of 6–8 cm per side. The basal parts were dipped in 70% ethanol for 1 minute as a pre-sterilization treatment. The explants were then sterilized with 40% (v/v) sodium hypochlorite (containing 5% active sodium hypochlorite) for 30 minutes under the fume hood followed by three rinses with sterile water (Robert et al., 2005). Finally, the fibrous tissues at the base were eliminated in a single cut, leaving a meristematic slice of tissue about 1-cm thick. The blocks were cut into smaller (0.8 cm3) cubes that constitute the explants to be cultured. Explants were placed in jars containing 50 ml MSB medium: Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with reduced nitrogen (10 mM KNO_3 and 5 mM NH_4NO_3), supplemented with 0.024 mg/L 2,4-diclorophenoxyacetic acid (2,4-D), and 10 mg/L 6-benzyl aminopurine (BA) as described by Robert

et al. (2005). The medium was adjusted to pH 5.75 and with a 0.1 N solution of either HCl or KOH and sterilized at 121 °C for 15 min. The jars were incubated in a growth room at 27 ± 2 °C under a 16-h photoperiod (45 µmol/m2/s). After 5 to 7 weeks, new plantlets formed on the surface of the explants but did not grow or multiply anymore in the later weeks. It seemed that BA with high concentrations (10 mg/L) was suitable for shoot induction but excessive for later growth and multiplication. In order to optimize the best protocol for micropropagation, two hormones, BA and NAA, in different concentrations were utilized in a factorial experiment based on CRD (completely randomized design) (BA=1, 2, 3 mg/L and NAA = 0.1, 0.3, 0.5 mg/L). Four replicates (jars) per treatment with ten single shoots were evaluated and the number of multiplicities shoots for each treatment was recorded for data analysis. In order to select the best auxin concentration to induce callus and direct somatic embryos from basal segments, a medium consisting of MS salts supplemented with 3% (w/v) sucrose, L2 vitamins (Phillips and Collins, 1979) and different amounts of auxins were utilized for the experiment. The culture medium was adjusted to pH 5.75 and sterilized at 121° C for 15 min. In this experiment five different auxins (NAA, 2,4-D, Dicamba, Picloram, and IBA) were evaluated at five different concentrations (0.5, 1, 1.5, 2, and 2.5 mg/L) in a CRD experiment with three replicates. Each replicate consisted of fifteen segments all cut from the basal parts. Explants were placed in disposable sterile plastic Petri dishes (100 × 15 mm) containing 25 ml of freshly prepared medium. The cultures were incubated in darkness at 25 ± 2 °C. The germination phase was carried out under a 16- h photoperiod (45 µmol m^{-2} s^{-1}) at 25 ± 2 °C for 60 d. In another experiment, indirect somatic embryogenesis from callus cultures was considered. The auxins with the highest effect on direct somatic embryogenesis from the previous experiment were selected. Different concentrations of the two auxins (0.5, 1, 1.5, and 2 mg/L) of Dicamba and Picloram in the same culture medium were utilized in a CRD (completely randomized design) factorial experiment. Three replicates (jars) per treatment with ten single calluses were evaluated. Embryos started to appear 58 days after subcultures in the medium, and the number of embryogenic calluses was recorded after 90 d.

Embryo conversion to plantlets and acclimatization

The somatic embryos that reached advanced stages of development were isolated and cultured in jars containing 50 ml of germination medium consisting of MSB medium without growth regulators (Robert et al., 2005). Well-developed plantlets were washed to remove residual nutrients and agar. They were then planted in plastic pots filled with a mixture of vermiculite and soil (3:1). The pots were transferred to a greenhouse and survival rates were evaluated after one month.

Statistical Analysis

All the data were analyzed with the SAS statistics program. The analysis of variance (ANOVA) was used to calculate statistical significance and polynomial regression. The mean ± SE (standard error) were determined using Duncan test at $p \leq 0.01$ level. Graphs and pie charts were prepared using Excel program.

Results

Micropropagation

Table 1 shows the results of variance analysis for micropropagation, callus formation, direct somatic embryogenesis (DSE), and indirect somatic embryogenesis (ISE) for Agave *Americana*. Significant differences ($p \leq 0.001$) were observed for A (BA), B (NAA), and AB (interaction effect) for micropropagation. Duncan test (Figure I) indicated that treatment a3b1 (BA=3 and NAA= 0.1 mg/L) had the highest effect on multiplication of a single shoot significantly.

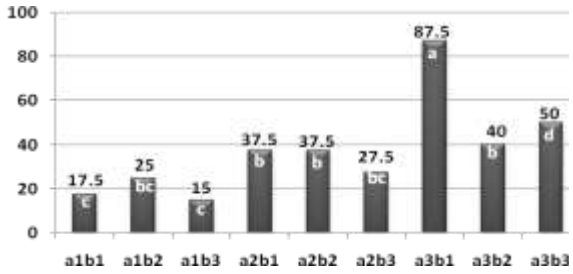

Fig. I. Results of Duncan test for 9 micropropagation treatments; percentages with the same letter are not significantly different.

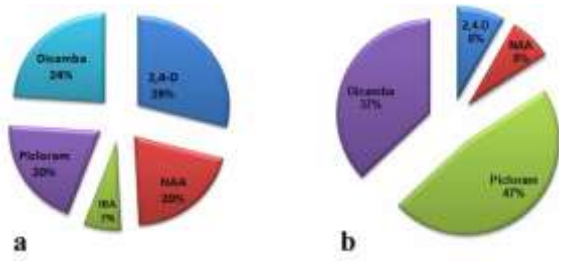

Fig. II. The portion of each auxin in a: callus induction and b: somatic embryogenesis

Table 2
The results of Duncan test for callus formation (Callus), DSE (Direct Somatic Embryogenesis), and no response (No RSP) by using different auxins (Column 1) at different concentrations (Column 2).

Auxin	mg/L	Callus%	DSE%	No RSP%
2,4-D	0.5	28.9 ^{e}	11.1 ^{fg}	60
	1	51^{bc}	4.5 ^{hij}	44.5
	1.5	53.3 ^{b}	26.7 ^{de}	20
	2	95.6 ^{a}	2.2 ^{ij}	2.2
	2.5	53.3 ^{b}	0 ^{j}	46.7
NAA	0.5	24.5 ^{ef}	4.4 ^{hij}	71.1
	1	48.9 ^{bc}	17.8 ^{ef}	33.3
	1.5	51.1 ^{bc}	8.9 ^{gh}	40
	2	44.4 ^{bc}	6.6 ^{ghi}	49
	2.5	26.7 ^{e}	2.2 ^{ij}	71.1
IBA	0.5	8.9^{h}	0^{k}	91.1
	1	8.9^{h}	0 ^{k}	91.1
	1.5	11.1^{gh}	0 ^{k}	88.9
	2	13.3 ^{fgh}	0 ^{k}	86.7
	2.5	22.2 ^{efg}	0 ^{k}	77.8
Picloram	0.5	22.2 ^{efg}	35.6 ^{cd}	42.2
	1	51.1 ^{bc}	46.7 ^{bc}	2.2
	1.5	51.1^{bc}	40.7 ^{bc}	2.2
	2	40 ^{cd}	60 ^{ab}	0
	2.5	31.1 ^{de}	66.7^{a}	2.2
Dicamba	0.5	48.9 ^{bc}	46.7 ^{bc}	4.4
	1	42.2 ^{bcd}	53.3 ^{ab}	4.4
	1.5	42.2 ^{bcd}	48.9 ^{bc}	8.9
	2	48.9 ^{bc}	22.2 ^{e}	28.9
	2.5	51.1 ^{bc}	24.4 ^{e}	24.4

Callus induction and direct somatic embryogenesis

Statistical analysis (Table 1) showed a high significant difference for callus and DSE, respectively. Table 2 also indicates the response of explants to various auxin concentrations in the culture medium that varies from no response in explants, to callus formation and embryogenic structures. Figs. II, a and II, b show two pie charts illustrating the portion of each auxin in callus induction and somatic embryogenesis, respectively. According to Fig. II a, auxin 2,4-D had the highest portion (29%) among other auxins for callus induction which was followed by Dicamba, Picloram, NAA, and IBA. Table 2 also indicates that 2,4-D in 2 mg/L has resulted in 95.6% callus formation in explants. Fig. II, b also indicates the portion of each auxin in direct somatic

Table 1
Results of analysis of variance for micropropagation, callus formation, DSE (Direct somatic embryogenesis) and ISE (Indirect somatic embryogenesis)

S.O.V		DF	MS
Micropropagation	A	2	0.87**
	B	2	2.06**
	AB	4	1.23**
Callus formation		24	25.4**
Direct SE induction		24	33.8**
Indirect SE induction	A	3	8.63^{ns}
	B	3	13568**
	AB	9	14.28^{ns}
Contrasts	Linear	1	10205**
	Quadratic	1	20295**
	Cubic	1	10205**

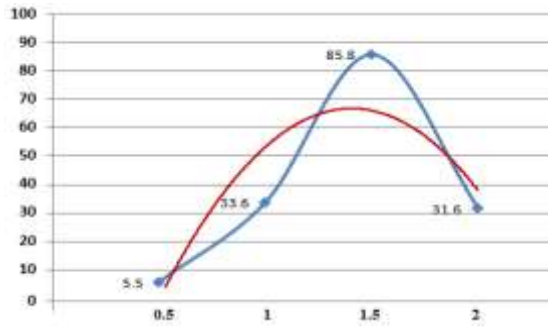

Fig. III. Response curve of factor B (Dicamba) in blue line after orthogonal polynomial analysis; the red curve line also indicates the general trend-line for factor B.

embryogenesis. Accordingly, Picloram had the highest portion on somatic embryogenesis followed by Dicamba, NAA, and 2,4-D. IBA induced no somatic embryos and was not shown in Fig. II, b.

Indirect somatic embryogenesis

Statistical analysis showed a high significant difference for factor B (Dicamba), but no significant difference for factor A (Picloram). Moreover, the interaction effect between these two factors was not significant with regard to embryo numbers. Accordingly, a response curve was prepared for factor B. The results of orthogonal polynomial analysis are presented in Table 1 indicating significant differences for linear, quadratic, and cubic regression for this factor. Fig. III illustrates response curve of factor B in a blue line. Based on the blue line, the trend was linear at first (0.5- 1 mg/L), but with increasing concentration (1 to 1.5 mg/L), it increases with sharper trend turning out to an increasing curve (quadratic). By increasing the concentration (1.5 to 2 mg/L) the trend decreases dramatically presenting the cubic component of the regression. The red line (curve line) also indicates the general trend line showing that increasing concentration of Dicamba from 0.5 to 1.5 mg/L has resulted in an increase and then a decrease in somatic embryo induction forming a bell-shaped curve. Figs. IV, a-i show different stages from achieving micropropagated plantlets, callus formation, somatic embryogenesis, and differentiation to shoot and root.

Germinated somatic embryos gave rise to plantlets of a normal morphology (Fig. IV, f). Well-developed plantlets were then transferred to a greenhouse for acclimatization. Eighty-seven percent of plantlets survived acclimatization successfully.

Discussion

Micropropagation

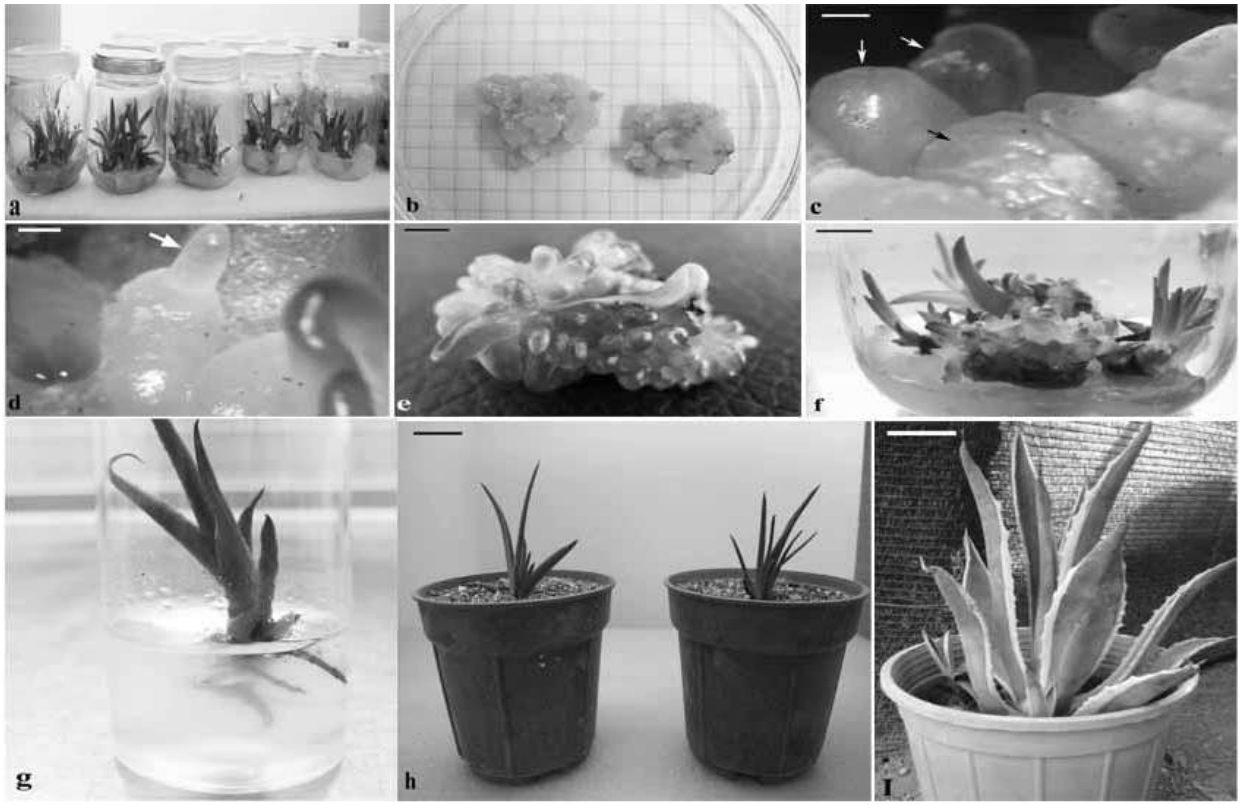

Fig. IV, a-i. Different stages from achieving micropropagated plantlets: (a) callus formation, (b) globular-shaped somatic embryo (bar= 1.5 mm), (c) torpedo-shaped somatic embryo (bar= 1.2 mm), (d) Embryo at cotyledonary stage, (e) (bars = 3 mm), and (f) (bars= 1 cm), Rooted plantlets, (g) Acclimatized plantlets (bar= 3cm), (h), and (i) an eleven-month plant in a pot (bar= 9 cm)

Although MSB supplemented with 0.024 mg/L 2,4-diclorophenoxyacetic acid (2,4-D), and 10 mg/L 6-benzylaminopurine (BA) as described by Robert et al. (2005) was suitable for shoot induction, it does not seem an appropriate protocol for later growth and multiplication. Moreover, most of the proliferated shoots showed abnormal hyperhydricity which is a physiological malformation that results in excessive hydration, low lignifications, impaired stomatal function and reduced mechanical strength of tissue culture-generated plants. Robert et al., (2005) also reported the same phenomenon in different Agave species. Hence, it was decided to reduce cytokinin to a lower level and add another useful auxin (NAA) to the medium. However, different species show some degree of variation in their culture requirements and their propagation efficiency. Monja-Mio and Robert (2013) suggested that 10 mg/L BA in modified MS (with reduced nitrogen containing 10 mM KNO_3 and 5 mM NH_4NO_3) was suitable for multiplication of *Agave fourcroydes* while Depnath et al., (2009) reported that 6 mg/L of BAP in MS medium was appropriate for shoot elongation and multiplication of *Agave sisalana*. In another study Robert et al.,(2005) suggested 1 mg of BA along with 0.024 mg/L 2,4-D for shoot proliferation of most Agave species which was different from our results (BA=3 and NAA= 0.1 mg/L). The varying results from different reports depend not only on the species in question, but also on the genetic properties of the different clonal lines and the seasonal or environmental conditions in which they were grown which can affect endogenous hormones leading to different responses to in-vitro growth regulators. It seems that hormone adjustments will have to be made for each genotype to make the system as efficient as possible.

Callus induction and direct somatic embryogenesis

In this study different kinds of auxins in different concentration were utilized for callus formation and direct somatic embryogenesis. It has been proved from the previous reports that an auxin is generally required for the induction of callus and somatic embryogenesis from explants. Auxins seem to cause DNA to become more methylated than usual and this might be necessary for the re-programming of differentiated cells (Terzi, and Lo Schiavo, 1990). Thus, tissue-specific programs specifically associated with differentiation would be eradicated by hypermethylation, with perhaps a small fraction of the cells reaching an ultimate state of dedifferentiation and finally would be capable for morphogenesis, or embryogenesis (Terzi, and Lo Schiavo, 1990). Although there are a number of reports on using different regions of a plantlet as explants (apical, middle, or basal part), the reason for choosing leaf base for callus formation or DSE in this study was the satisfactory results of some previous reports on utilizing leaf base in different Agave species including *victoriae-reginae* (Rodríguez-Garay et al., 1996), *A. tequilana*, *Yucca valida*, *A. sisalana* and *A. amaniensis* (Robert et al., 2005), *Agave vera-cruz* (Tejavathi et al., 2007), and *Agave tequilana* (Portillo et al., 2007). The use of NAA for somatic embryogenesis has previously been reported in *A. vera-cruz* (Tejavathi et al., 2007) and *A. fourcroydes* (Monja-Mio and Robert,2013). The use of auxin 2,4-D has also been reported in several Agave species such as *A. victoriae reginae* (Rodríguez-Garay et al., 1996; Martínez-Palacios et al., 2003), *A. vera-cruz* (Tejavathi et al., 2007), *A. Tequilana* (Portillo et al., 2007), and *A. fourcroydes* (Monja-Mio and Robert, 2013). There are also several reports on somatic embryogenesis in different plant species using IBA alone or along with a cytokinin including *Ceratonia siliqua* L. (Canhoto et al, 2006), *Querem robur* L. (Chalupa, 1990), and *Echinacea purpurea* (Jain and Gupta, 2005). The use of Picloram and Dicamba in the embryogenic induction of *Agave fourcroydes* has also been reported by Monja-Mio and Robert (2013). The results of Fig. II, a, and Table 2 which have introduced 2,4-D as a good candidate for explant callugenesis are in accordance with the results of a number of reports on using 2,4-D alone or in combination with other phytohormones for callus induction in different Agave species (Groenewaldet al., 1977; Powers and Backhaus 1989;Monja-Mioand Robert, 2013; Oliva et al., 2014). Fig. II, b also indicated that Picloram had the highest portion on direct somatic embryogenesis followed by Dicamba, NAA and

2,4-D. Monj-Mio and Robert (2013) also suggested Picloram and Dicamba for direct somatic embryogenesis of *Agave fourcroydes* which is consistent with our findings in using the two mentioned auxins for DSE, though with different concentrations . The previous report suggested 0.5 mg/L Dicamba or 0.5 mg/L Picloram for DSE which is not in accordance with our suggested concentrations (Picloram with 2.5 and 2 mg/L and Dicamba with 1 mg/L) in DSE. Although the use of 0.5 mg of each auxin in our culture medium induced somatic embryos, the percentage (36-47%) was significantly lower than that of 2 and 2.5 mg/L for Picloram and 1 mg/L for Dicamba (53.3-66.7%). It should be considered that the explant type used for DSE in this study was leaf base while the explant type from the previous report (Monja-Mio and Robert, 2013) was thin tissue segments cut transversally from stems. The proportion of meristematic tissue in different explants varies according to the region (apical, middle, or basal), which influences the number of embryos produced by segment (Monja-Mio and Robert, 2013). In addition to different utilized tissues, the species which were evaluated in current study and the previous report were different leading to different DSE.

Genotype is one of the most important factors that determine the capacity for somatic embryogenesis (Fehér, et al., 2003) and it is one of the main reasons for the lack of reproducibility of many protocols. The genotypic differences in the embryogenic response could be due to variations in the endogenous levels of growth regulators (Jiménez, 2005), quantitative or qualitative genetic differences (Henry et al., 1994; Fehér, 2008), or epigenetic differences, particularly the pattern of chromatin condensation (Fehér, 2005).

Indirect somatic embryogenesis

George et al. (2008) reported that in most auxin effects, a bell-shaped concentration/activity curve can be observed which is consistent with our results in general. They suggested that at low concentrations (0.1 - 10 µM) the effect usually increases with concentration, but concentrations higher than 10 µM are often inhibitory. This inhibitory effect is usually due to an increase in ethylene production at higher auxin concentrations. However, the results of this study for Dicamba indicated that concentration over 6.78 µM was inhibitory and resulted in lower somatic embryo production. This small difference is due to the fact that the mentioned range can vary from auxin to auxin, plant to plant, or developmental stage to stage. Accordingly, it is concluded that high concentrations of Dicamba (over 1.5 mg/L) will have an undesirable effect on somatic embryogenesis in *Agave americana*. Embryogenic calluses were friable containing elongated and small globular creamy cells from which the somatic embryos arose. This callus appearance was similar to that reported by Portillo et al., (2007) in somatic embryogenesis of *Agave tequilana* Weber cultivar azul, but differed to that reported for the monocot species *Gasteria verrucosa* and *Haworthia fasciata*, closely related to Agaves where the somatic embryos were originated from yellow and compact calluses (Beyl and Sharma, 1983). These observations also differ from those references on indirect somatic embryogenesis in other Agave species, where the embryogenic calluses were snowy, compact, and nodular (Martínez-Palacios et al., 2003; Nikam et al., 2003). The somatic embryos that reached advanced stages of development started chlorophyll synthesis under the light. Some studies reported that this characteristic is most likely because of the effect of high concentrations of cytokinins that enhance chlorophyll synthesis in plants (George, 1993; Zaffari et al., 1998; Portillo et al., 2007). Since there was no cytokinin in regeneration medium in our study, it seems that the presence of light and not cytokinins stimulates chlorophyll synthesis. In other words, once the maturation process is completed, somatic embryos acquire the normal green pigment to be similar to zygotic embryos (Portillo et al., 2007) which will be simply attained under the light conditions. Robert et al. (Robert et al, 2005) also suggested that somatic embryogenesis is enhanced when germination medium consisted of MSB medium without growth regulators.

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