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THE EFFECT OF EXPLANT SOURCE AND CYTOKININ CONCENTRATION ON THE DIRECT BULB FORMATION OF TULIP (*Tulipa gesnerina* L.) BY PLANT TISSUE CULTURE TECHNIQUE

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ABSTRACT

The study was conducted in the Plant Tissue Culture Laboratory, Faculty of Agriculture, University of Basrah, Iraq during the 2018/2019 growth season. The results indicate that the Arma cultivar has significant superior in the percentage of healthy cultures compared to the Flaming flag, it was recorded the highest percentage of survival of 77.67%. The Flaming flag had the lowest healthy percentage of 76.00%. Mercuric chloride treatment was significantly superior, which recorded the highest percentage of healthy cultures, which reached 96.00%. But, the sodium hypochlorite recorded the lowest percentage of healthy cultures, which was 57.67%. The apical and lateral buds, disc stem+ apical bud of Arma cultivar have recorded positive results in their response to the shoot proliferation. Whereas, the fleshy leaf segment and disc stem, did not record any response to the shoot proliferation. The lateral bud was significantly superior to the other explants in recording the highest percentage of response to shoot proliferation, which was 83.33%. But the apical bud recorded the lowest response of 58.67%. The higher significant superiority of the Arma cultivar compared to the Flaming flag cultivar in the percentage of the response of the shoots to the bulb formation, the bulb diameter and weight of the tulip plant after eight weeks of culturing, as it reached 70.67%, 1.19 cm and 1.98 g, respectively. The shoots that were cultured on the medium supplemented with 1.5 mg L^{-1} BA were significantly superior in their response to the bulb formation and the number of bulbs, which reached 72.17% and 2.67 bulbs per shoot, respectively. Whereas, the shoots cultured on the MS medium supplemented with 1.0 mg L^{-1} BA were significantly higher in diameter and bulb weight, which reached 1.18 cm and 1.86 g, respectively.

Keywords: Apical bud; benzyl adenine; *in vitro*; proliferation; sterilization.

INTRODUCTION

The tulip (*Tulipa gensnerina* L.) plant is one of the most beautiful flowering bulb plants that are belonged within the cut flowers. The genus of this plant belongs to the family of Liliaceae [1]. The tulip plant is vegetative propagation by bulbs [2]. The number of tulip bulbs produced from one bulb is limited when propagated by traditional methods (2-3 bulbs). Nowadays, plant tissue culture can be considered as a powerful method for shoot organogenesis [3-6]. Agricultural researchers resorted to propagating this plant by tissue culture to produce true to type plants, free of viruses, and obtaining large numbers of bulbs [7,8]. The source of the explant, its physiological condition, age, and size has a great effect on the success and failure of tissue culture [9]. In a study by Maslanka and Bach [10] for *in vitro* propagation of tulips of the Apeldoorn cultivar by culturing axillary and apical buds on MS media supplemented with 0.93 mg L^{-1} naphthalene acetic acid (NAA) and 1.10 mg. L^{-1} thidiazuron (TDZ), as there were obtained shoots 12 weeks after culturing. Maślanka and Bach [11] used the scale explants to micropropagation of the tulip (*Tulipa tarda*) plant by culturing them on MS media supplemented with 3% and 6% sucrose without adding growth regulators, 0.11 mg L^{-1} benzyl adenine (BA) or 6.89 and 34.47 mg L^{-1} abscisic acid (ABA).

Sterilization means the elimination of all contaminations, including bacteria, fungi, and others on the surfaces of the explants before culturing them on the culture media. The sterilization process is one of the important factors on which the success or failure of culturing explants depends, as the survival of these contaminations on the explant which lead to failure of its growth as a result to compete with it for nutrient or secrete toxic substances in the medium [12]. Podwyszynska and Marasek [13] indicated that the explants of tulip were sterilized with 0.1% mercuric chloride solution and then washed with sterile distilled water several times. Kabir et al. [14] showed that sterilization of the gladiolus corms is done by first removing the outer leaves of the corms, then washing them well with detergent (Trix) and tap water for 20 minutes. After that, it is sterilized with a 1% mercuric chloride solution with two drops of Tween-20 for ten minutes and then washed with sterile distilled water 4 times. Kumar et al. [15] indicated to the best sterilization for buds of the gladiolus plant by washing them with 3-4 drops of Tween-20 and then soaking them with the fungicide Bavistin at a concentration of 0.1% with 70% ethanol for 4-5 minutes. The explants transferred them to 0.1% mercuric chloride for 10 minutes. Then the buds are washed 3-4 times with sterile distilled water. Podwyszyn´ska and Sochack [16] indicated that tulip plants differ in their ability to bulb formation depending on the difference between the cultivars. The two cultivars Fringed Black and Mead-Season Black have a high ability to bulb formation without the need for low temperatures. As for the Lily-Flowered Group cultivar, it was less able to bulb formation. The New Beet cultivar produces small bulbs in large numbers. In a study by Podwyszyn´ska et al. [17] on the micropropagation of a tulip by culturing the two cultivars (Prominence and Blue Parrot) found that MS medium supplemented with 8 mg L^{-1} isopentenyl adenine (2iP) recorded the highest percentage of bulb formation.

Kizil et al. [18] reported on micropropagation of *Hyacinthus orientalis* L., by culturing the leaf sheath on MS medium supported with different concentrations of 0.5, 1.0, and 1.5 mg L^{-1} with 0.1 mg L^{-1} NAA. They found that the treatment 1.5 mg L^{-1} Ba + 0.1 mg L^{-1} NAA recorded the highest number of bulbs, reached 4.91 bulbs per explant. While the highest mean of bulb diameter was recorded when treatment was 1.0 mg L^{-1} BA + 0.1 mgL^{-1} NAA, which was 0.69 cm. The present study aims to determine the best sterilizer solution, explant source and concentration of the growth regulator benzyl adenine that can be used in the shoot proliferation and bulb formation of tulips by *in vitro* culture technique.

MATERIALS AND METHODS

The study was conducted in the Laboratory of Plant Tissue Culture, Faculty of Agriculture, University of Basrah, Iraq during the 2018/2019 growth season. The bulbs of two cultivars of tulip plant (*Tulipa gensnerina* L.) were the Arma and Flaming flag generation (F1) produced by the Dutch Company Holland Bulb Market Company.

In this study, explants were used, which are the apical and lateral buds, disc stem, fleshy leaf segment, and disc stem + apical bud were taken from two cultivars of tulip bulbs: Arma and Flaming flag (Plate 1, A- F). They were washed with tap water and liquid soap. The explants were excised from the bulbs were sterilized after soaking them with the fungicide TOPSIN-M, which was prepared by adding 5 g of the fungicide to one litre of sterile distilled water. After that, the explants were placed with fungicide for 15 minutes, and then were washed with sterile distilled water three times. Then the explants were divided into two equal groups. The first group was immersed in a 1.05% sodium hypochlorite solution + 2-3 drops of Tween-20 for 15 minutes. The second group was immersed in 0.1% mercuric chloride solution with 2-3 drops of Tween-20 for 15 minutes. After the sterilization period ended, the explants of both groups were removed from the sterilization solution and washed with sterile distilled water three times. The percentage of healthy cultures (free of contamination) was calculated after 4 weeks of culturing.

The MS medium used in the experiments was prepared from ready-made MS salts [19] that produced by Phytotechnology Lab.com, supplied with vitamins: 4.43 g L^{-1} MS salts, 100 mg L^{-1} Myo-inositol, 10 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} Pyrodoxine, 10 mg L^{-1} Thiamin-HC, 1 mg L^{-1} Glycine, 170 mg L^{-1} Sodium hydrogen orthophosphate, 40 mg L^{-1} adenine sulfate, 1 g L^{-1} polyvinyl pyrrolidone (PVP) and 30 g L^{-1} sucrose. Cytokinin BA was added at different concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 mg. L^{-1} with a constant concentration of 0.5 mg. L^{-1} NAA to the MS media. The 7.5 g L^{-1} Phyto agar was added to the MS medium after adjusting pH to 5.8 by hydrochloric acid and sodium hydroxide solution at a concentration of 1 N for each of them. The MS medium was poured in an amount of 15 ml into the culture tube of 2.5 x 18 cm or jar of 6.5 x 14 cm dimensions, and then the nozzles of the culture vessels were blocked with medical cotton and aluminium foil. The culture tubes or jars were sterilized with the Autoclave under the pressure of 1.04 kg cm^{-2} and at 121°C for 20 minutes. The cultures were incubated at a temperature of 25 ± 2°C and a light density of 1000 lux. The following indicators were calculated after eight weeks of culturing:-

1. The explant response to shoot proliferation (%).
2. The number of shoots per explant.
3. The percentage of response to bulb formation.
4. The number of bulbs per explant.
5. The bulb diameter (cm)
6. The bulb weight (g).

Experimental Design and Statistical Analysis

The experiment of the study was designed using a complete randomized design. Data were analyzed statistically using analysis of variance. The revised least significant difference test was used to compare the means of treatments at a 1% probability level [20].

RESULTS AND DISCUSSION

Table 1 shows the effect of the cultivar and the sterile material type on the percentage of healthy cultures of the explants of the tulip plant after four weeks of *in vitro* culture. The results indicate that the Arma cultivar has significant superior in the percentage of healthy cultures compared to the Flaming flag, it was recorded the highest percentage of survival of 77.67%. The Flaming flag had the lowest healthy percentage of 76.00%. The results of the same Table also indicate that there were significant differences in the effect of the sterile type on the percentage of survival of the explants of the tulip plant after four weeks of culturing. Mercuric chloride treatment was significantly superior, which recorded the highest percentage of healthy cultures, which reached 96.00%. But, the sodium hypochlorite recorded the lowest percentage of healthy cultures, which was 57.67%. The results from Table 1 also showed that there were no significant differences between the effect of the cultivar and the type of sterile material in the percentage of survival of the cultures after four weeks of culturing.

Table 1. The effect of the cultivar, sterile material type and the interaction between them on the percentage survival of the tulip explants after four weeks of culturing

Cultivar	Sterile material type		Cultivar effect
	$HgCl_2$	NaOCl	
Arma	96.67	58.67	77.67
Flaming flag	95.33	56.67	76.00
Sterile material effect	96.00	57.67	
R-LSD (0.01)	Cultivar effect High significant	Sterile material effect High significant	Interaction Cul×Ste Not significant

The reason for the significant difference between the two cultivars is due to the genetic difference between them [16]. The reason for the significant superiority of $HgCl_2$ is due to the active effect of mercuric chloride in the surface sterilization of explants and the elimination of the micro-organisms that cause contamination and fungal, bacterial and viral diseases present on their surfaces due to its containment of free radicals useful in eliminating them [21]. Or perhaps the reason for this is due to the interaction between the mercuric chloride and the fungicide TOPSIN-M, which made both of them the optimum combination to eliminate contaminants, which led to an increase in the percentage of survival of the cultures of the tulip plant. The results of this study agreed with the results of many researchers that the use of mercuric chloride had an effective role in obtaining healthy and contaminant-free plants [14,15,22].

Fig. 1 shows the effect of the explant source of the tulip plant Arma cultivar on shoot proliferation when cultured on the MS medium supplemented with 1.0 mg L^{-1} BA after eight weeks of culturing. The explants of the apical and lateral buds, disc stem+ apical bud have recorded positive results in their response to the shoot formation (Plate 1, G, H, I). The same Fig. 2 also shows that the lateral bud was significantly superior to the other explants in recording the highest percentage of response to shoot formation, which was 83.33%. While the disc stem+ apical bud explant recorded 62.77% response to the shoot proliferation, which was significantly superior to the apical bud, which gave the lowest response of 58.67%. Whereas, the other explants, the fleshy leaf segment and disc stem, did not record any response to the shoot proliferation.

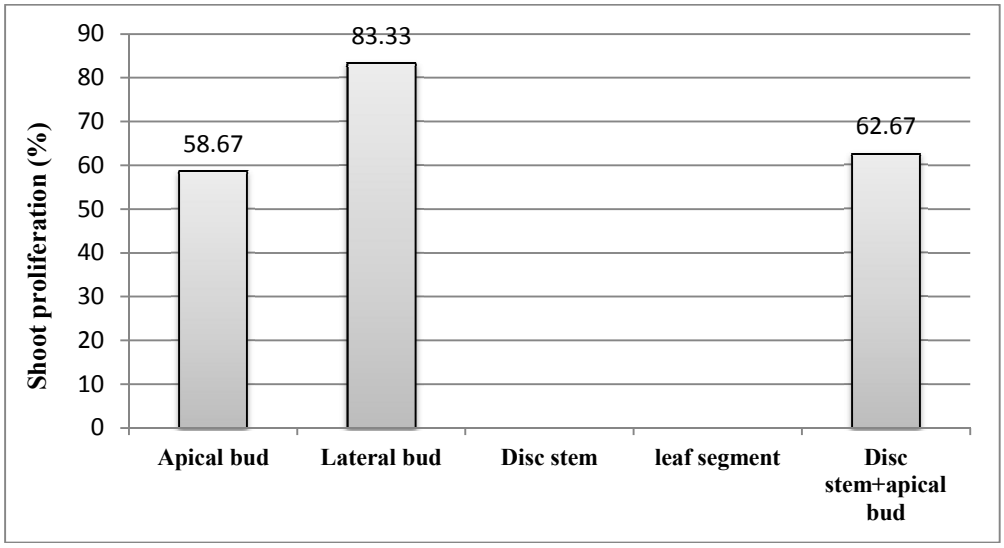

Fig. 1. Effect of explant source on shoot proliferation of tulip plant cv. Arma after eight weeks of culturing

The response of the apical and lateral buds and the bulb disc+ apical bud to the shoot proliferation was due to the presence of active meristematic tissues that were stimulated by cytokinin, which was added to the medium that interacted with endogenous hormones that led to cell division, differentiation and growth that led to the shoot regeneration. The reason for the success or failure of the explants in shoot proliferation depends on the presence of active meristematic cells in them, or the ability of their cells to dedifferentiation and the direct and indirect shoot initiation, or their content of endogenous and exogenous hormones that there has a role in the shoot regeneration. The results of the current study agree with the results of other studies [15,23,24]. These results are in agreement with Abdul Ghaffoor et al. [25] when they cultured the axillary and apical buds of the tulip by in vitro culture.

The results in Table 2 show that the lateral bud that cultured on MS medium supplemented with 1.0 mg L^{-1} BA was significantly superior the other explants in the mean number of shoots, shoot length and number of leaves after eight Weeks of culturing, which amounted to 4.67 shoots per explant, 6.00 cm and 3.33 leaves per shoot, respectively. The disc stem+ apical bud was significantly superior the apical bud in the number of shoots, as it reached 2.67 shoots per explant. As for the apical bud, it gave the lowest means for the shoot number and length and leaf number, which amounted to 1.33 shoots per explant, 2.67 cm and 2.00 leaves per shoot, respectively.

The results in Tables 3, 5 and 6 indicate the higher significant superiority of the Arma cultivar compared to the Flaming flag cultivar in the percentage of the response of the shoots to the bulb formation, the bulb diameter and weight of the tulip plant after eight weeks of culturing, as it reached 70.67%, 1.19 cm and 1.98 g, respectively. However, the increase in the number of bulbs was not significant for the Arma cultivar, which formed from the shoots, compared to the Flaming flag cultivar (Table 4). The shoots that were cultured on the medium supplemented with 1.5 mg L^{-1} BA were significantly superior in their response to the bulb formation and the number of bulbs, which reached 72.17% and 2.67 bulbs per shoot, respectively. Whereas, the shoots cultured on the MS medium supplemented with 1.0 mg L^{-1} BA were significantly higher in diameter and bulb weight, which reached 1.18 cm and 1.86 g, respectively (Plate 1, J, K).

The interaction treatment between the Arma cultivar and 1.5 mg L^{-1} BA concentration was significantly superior to the other treatments in the percentage of response to the bulb formation, which amounted to 82.67%. While the interaction treatment between the Arma cultivar and the 1.0 mg L^{-1} BA concentration recorded the highest bulb diameter was 1.65 cm (Plate 1, L). The effect of the interaction between cultivar and BA concentration was not significant in the number and weight of bulbs. As for the interaction treatment between the Flaming flag cultivar and the concentration of 1.0 mg L^{-1} BA recorded the lowest percentage response to the bulb formation and the number of bulbs was 51.33% and 1.0 bulb per shoot, respectively. The results also show that the interaction treatment between the variety Flaming flag and the concentration of 1.5 mg L^{-1} BA recorded the lowest value in the diameter and weight of the bulb, which was 0.51 cm and 0.46 g, respectively.

Table 2. The effect of the explant source on the some vegetative characteristics of the tulip shoots cv. Arma after eight weeks of culturing

Explant source	Number of shoots per explant	Shoot length (cm)	Number of leaves per shoot
Apical bud	1.33	2.67	2.00
Lateral bud	4.67	6.00	3.33
Disc stem	-	-	-
Fleshy leaf segment	-	-	-
Disc stem+ apical bud	2.67	3.67	2.00
R-LSD (0.01)	0.965	1.325	1.268

Fig. 2. Effect of explant source and BA concentration on the shoot proliferation and bulb formation of tulip (*Tulipa gensnerina* L.) Arma and Flaming flag cultivars. Tulip bulb (A); Apical bud (B); Lateral bud (C); Disc stem+ apical bud (D); Fleshy leaf segment (E); Disc stem (F); Apical bud growth after 4 weeks of culturing (G); Lateral bud growth after 4 weeks of culturing (H); Disc stem+ apical bud growth after 4 weeks of culturing (I); Bulb formation of Arma cultivar after 8 weeks of culturing (J); Bulb formation of Flaming flag cultivar after 8 weeks of culturing (K); Bulb formation of Arma cultivar

Table 3. The effect of the cultivar, BA concentration and the interaction between them on the percentage response to bulb formation after eight weeks of culturing

Cultivar	BA concentration (mg L^{-1})					Cultivar effect
	0.5	1.0	1.5	2.0	2.5	
Arma	-	58.67	82.67	-	-	70.67
Flaming flag	-	51.33	61.67	-	-	56.50
BA effect	-	55.00	72.17	-	-	
R-LSD (0.01)	Cultivar effect		BA concentration effect			Interaction Cul+ BA
	High significant		High significant			3.73

Table 4. Effect of the cultivar, BA concentration and interaction between them on the number of the tulip bulbs after eight weeks of culturing

Cultivar	BA concentration (mg L^{-1})					Cultivar effect
	0.5	1.0	1.5	2.0	2.5	
Arma	-	1.33	3.33	-	-	2.33
Flaming flag	-	1.00	2.00	-	-	1.50
BA effect	-	1.17	2.67	-	-	
R-LSD (0.01)	Cultivar effect		BA concentration effect			Interaction Cul+ BA
	Not significant		High significant			Not significant

The reason for the high significant superiority of the Arma cultivar than the Flaming flag cultivar in response percentage to the bulb formation and the diameter and weight of the bulb formed from the shoot of the tulip plant is due to the genetic difference between the two cultivars [16]. The reason why bulbs are not formed at concentrations 0.5, 2.0 and 2.5 mg L^{-1} of benzyl adenine that have been added to the MS medium may be due to the concentration being 0.5 mg L^{-1} of Benzyl adenine was insufficient to interact with the auxin in order to induce nutrient accumulation and transferred to the bases of leaves for bulb formation. The operation was reverse in high concentrations at 2.0 and 2.5 mg L^{-1} BA, which had an effect than the optimum effect on stimulating the shoots to formation of bulbs, which led to a negative effect on cell division and enlargement. The reason for the significant superiority of the concentration of 1.5 mg L^{-1} of BA, which was added to the MS medium with a constant concentration of 0.5 mg L^{-1} of naphthalene acetic acid, because it is the optimum combination of cytokinin and auxin that induce the nutrient accumulation in the bases of the leaves, which led to the swelling of leaf bases and the formation of bulbs.

Table 5. Effect of the cultivar, BA concentration and the interaction between them on the diameter of the tulip bulb after eight weeks of culturing

Cultivar	BA concentration (mg L^{-1})					Cultivar effect
	0.5	1.0	1.5	2.0	2.5	
Arma	-	1.65	0.73	-	-	1.19
Flaming flag	-	0.70	0.51	-	-	0.61
BA effect	-	1.18	0.62	-	-	
R-LSD (0.01)	Cultivar effect		BA concentration effect			Interaction Cul+ BA
	High significant		High significant			0.070

Table 6. Effect of the cultivar, BA concentration and the interaction between them on the weight of the tulip bulb after eight weeks of culturing

Cultivar	BA concentration (mg L^{-1})					Cultivar effect
	0.5	1.0	1.5	2.0	2.5	
Arma	-	2.33	1.63	-	-	1.98
Flaming flag	-	1.39	0.46	-	-	0.93
BA effect	-	1.86	1.05	-	-	
R-LSD (0.01)	Cultivar effect		BA concentration effect			Interaction Cul+ BA
	High significant		High significant			Not significant

CONCLUSION

Mercuric chloride is more active and efficient in surface sterilization of explants that were taken from tulip bulbs. The lateral bud explants that were taken from the tulip bulbs of the Arma and Flaming cultivars are more responsive to the shoot proliferation, while the fleshy leaf segments and the disc stem did not respond to it. Benzyl adenine concentration at 1.5 mg L^{-1} led to the highest percentage of bulb formation and their numbers increased after eight weeks of culturing.

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