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Highly Efficient Regeneration *via* Somatic Embryogenesis from Immature Embryos of Egyptian Wheat Cultivars (*Triticum aestivum* L.) Using Different Growth Regulators

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Abstract: Two wheat cultivars (Giza 163 and Giza 164) were used to study the effect of three different growth regulators i.e. Thidiazuron (TDZ), Zeatin riboside (ZR) and Dicamba on regeneration efficiency. In comparing results of regeneration efficiency for the three growth regulators across the two cultivars, it is concluded that the highest regeneration efficiency was observed by using the highest TDZ concentration (0.2 mg L^{-1}) as compared with other used concentrations of the two other growth regulators. On the other hand, it was clear that ZR had higher influence on regeneration efficiency than Dicamba. Moreover, Giza 164 was better than Giza 163 in regeneration efficiency across all growth regulators and all concentrations. This highly efficient regeneration system is considered a new addition that will open the door for improving wheat crop by *in vitro* techniques.

Key words: Regeneration · immature embryo · wheat cultivars

INTRODUCTION

Wheat is the most critical agricultural crop worldwide, where the stability of the community and regimes depends mainly on the availability of the strategic commodities. The efficient regeneration of normal and fertile plants from single cells, a basic prerequisite for molecular genetic improvement of plants, proved to be rather difficult for different wheat varieties because of their extreme recalcitrance to manipulation *in vitro*. These constraints were overcome by the culture of immature embryos at defined stage of development onto a defined nutrient medium supplemented with defined concentrations of hormones. Establishment of a highly efficient regeneration system for the input use of efficient varieties with unique quality profiles is, however, a prerequisite. Wheat regeneration *via* tissue culture varies with the genotype [1]. Immature zygotic embryos of two wheat (*Triticum aestivum* L.) genotypes, known for their different ability to generate embryogenic callus, were used as initial explants to establish callus cultures [2]. Thidiazuron was first reported to have cytokinin activity in 1982. It has been used successfully *in vitro* to induce shoot formation and to promote auxillary shoot proliferation. Thidiazuron is especially effective with recalcitrant woody species. Shoot number produced on medium containing Thidiazuron is equivalent to or greater than the number initiated on medium with purine type cytokinins and low concentration of Thidiazuron is too effective for micropropagation [3] and for induced tobacco plant regeneration [4]. Dicamba also, induced callus formation in dark grown wheat embryos cultivated on modified MS medium [5]. Dong and Jia [6] reported that high frequency shoot regeneration was induced from water melon cotyledons cultured on MS medium containing Zeatin riboside and it was significantly more efficient than 2ip and Kinetin. Mee-Sook *et al.* [7] stated that stable auxiliary shoot establishment was achieved on MSB5 medium containing a combination of 5 μM Thidiazuron (TDZ), 5 μM (BA) and 1 μM (IBA). The objective of this research is to establish an efficient regeneration system which is a basic prerequisite for the molecular genetic improvement of plants, through studying the effects of different hormones, i.e., Thidiazuron, Zeatin riboside and Dicamba on regeneration frequency of two local wheat cultivars, (Giza 163 and Giza 164).

MATERIALS AND METHODS

Two local wheat (*Triticum aestivum* L.) cultivars; Giza 163 and Giza 164 were tested for their performance during *in vitro* regeneration. Immature embryo was the system of tissue culture used in the present study.

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Table 1: Effect of different concentrations of TDZ on number of shoots (Y1) and leaf-like structure (Y2) per callus for two Egyptian wheat cultivars Giza 163 and Giza 164

			TDZ concentration mg L^{-1}				
Trait	Cultivar	Control	0.1	0.15	0.2	Cultivar mean	Trait mean
Y1	Giza163	2.50 D	6.75 bC	9.82 bB	13.76 bA	8.21 b	
	Giza164	3.02 D	9.48 aC	13.19 aB	17.12 aA	10.70 a	
	Average	2.76 D	8.11 C	11.51 B	15.44 A		9.46
Y2	Giza163	4.85 D	21.74 a A	18.88 aB	15.81 aC	15.32 a	
	Giza164	6.40 D	17.57 bA	14.50 bB	7.22 bC	11.42 b	
	Average	5.62 D	19.65 A	16.69 B	11.51 C		13.37

- Means followed by different capital letters in columns and those followed by different small letters in rows are significantly different at p=0.05 according to Duncan's multiple range test

Immature caryopsis of the two cultivars were collected approximately two weeks post-anthesis. Seeds were surface sterilized with 20% commercial Clorox (5.25% Sodium hypochlorite) supplemented with few drops of Tween 20, then washed five times with sterile D.D.H_2O. Immature embryos for each cultivar were aseptically isolated. Fifty immature embryos were cultured with the scutellum side up onto the callus induction medium modified, for wheat cell culture medium containing MS [8] salts (Sigma, M5524), supplemented with 2 mg L^{-1} 2,4-D as a source of auxin, 0.15 g L^{-1} of L-Asparagine, 0.1 mg L^{-1} of myo-inositol, 20 g L^{-1} sucrose and 2.5 g L^{-1} phytagel. Calli were maintained in dark at 25°C and subcultured two times onto a fresh medium at 2 weeks intervals. After four to six weeks from culturing, calli were transferred to a fresh medium at the rate of 10 calli per Magenta box (Sigma, GA7) containing 50 mL of Phytagel-solidified MST basal medium (Sigma, M5519) supplemented with 3% sucrose and different growth regulators with different concentrations i.e. Thidiazuron (TDZ) (0.1, 0.15 and 0.2 mg L^{-1}), Zeatin riboside (0.5, 1.0 and 1.5 mg L^{-1}) and Dicamba (0.05, 0.1 and 0.2 mg L^{-1}). Calli were maintained on MST for six weeks at 25°C temperature, 25-50 μL E/m^2 light intensity and 16 h photoperiod. Number of shoots containing at least one expanded leaf per calli, number of leaf like structures (not containing at least one expanded leaf) and number of regenerated calli containing at least one shoot were recorded six weeks post-culturing onto the medium, using dissecting microscope. Data obtained were exposed to the proper statiscal analysis of complete randomized design described by Snedecor and Cochran [9], in three replicates. Differences between means were tested by using Duncan's new multiple range test as described by Duncan [10].

RESULTS AND DISCUSSION

In the present study, we have made attempts to improve the regeneration efficiency of two local wheat cultivars i.e., Giza 163 and Giza 164. Different growth regulators with different concentrations were used to identify the best growth regulator and its concentration that gives the highest regeneration frequency; to be utilized after then in transformation experiments for these two wheat cultivars. Regeneration efficiency is scored as the number of shoots/callus (Y1) and number of leaf-like structures/callus (Y2). Comparison between the two cultivars for their reaction to TDZ showed significant differences in their regeneration efficiencies. The mean Y1 of Giza 164 (10.7) was significantly higher than that of Giza 163 (8.21) across all treatments. As expected, opposite results were given for Y2 means, where Giza 163 showed higher number of leaf-like structures/callus than Giza 164 across all treatments as shown in Table 1 and Fig. 1 & 2. Comparison among different concentrations of TDZ indicated that the higher the concentration of cytokinin, the higher the number of shoots/callus across the two cultivars. As far as results of leaf-like structures/callus, it was shown that TDZ gave higher values than shoot/callus either within or across cultivars. In general, it can be concluded that the cytokinin activity of TDZ was highest, when using 0.2 mg L^{-1}, where the number of shoots/callus and consequently the no. of plantlets was highest either for each or across the two wheat cultivars. The ability of TDZ to stimulate cell division has also been demonstrated in many plant species [4].

Apart from stimulating cell division, TDZ had also been shown to induce adventitious shoot formation from tobacco leaf discs [4]. Also, it was reported that TDZ induced auxillary shoot proliferation in several plant species, i.e. carnation [11, 12], apple [13], pear [14], peach [15] and azalea [16]. Moreover, TDZ promotes conversion of cytokinin ribonucleotides to the biologically more active ribonucleotides [17]. Furthermore, TDZ encourages the synthesis of endogenous purine cytokinins and inhibits their degradation [4]. An advantage of using TDZ is the resistance to degradation by cytokinin oxidize [18]. Therefore, TDZ is considered as quite stable in tissue

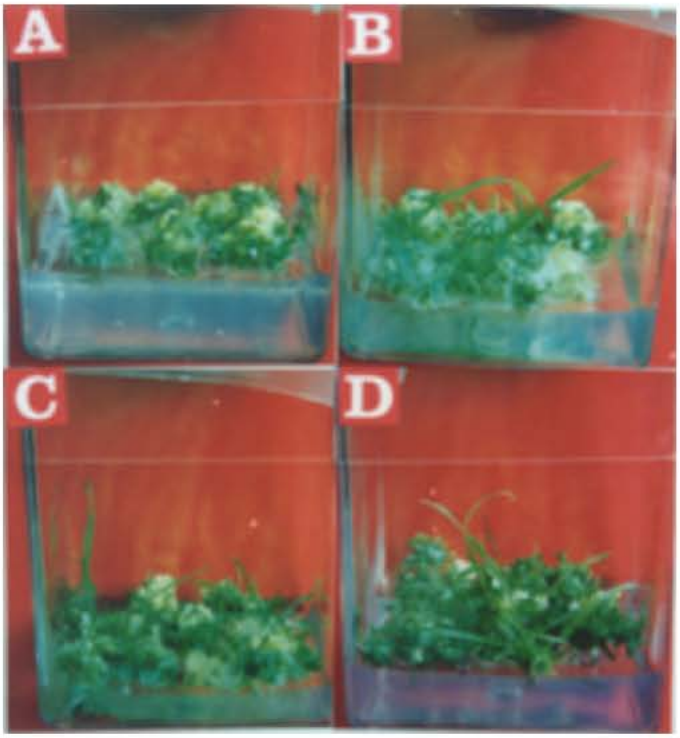

Fig. 1: Effect of different concentrations of TDZ on regeneration efficiency of Giza 163 cultivar
A: Control; B: 0.1 mg L^{-1}; C: 0.15 mg L^{-1} and D: 0.2 mg L^{-1}

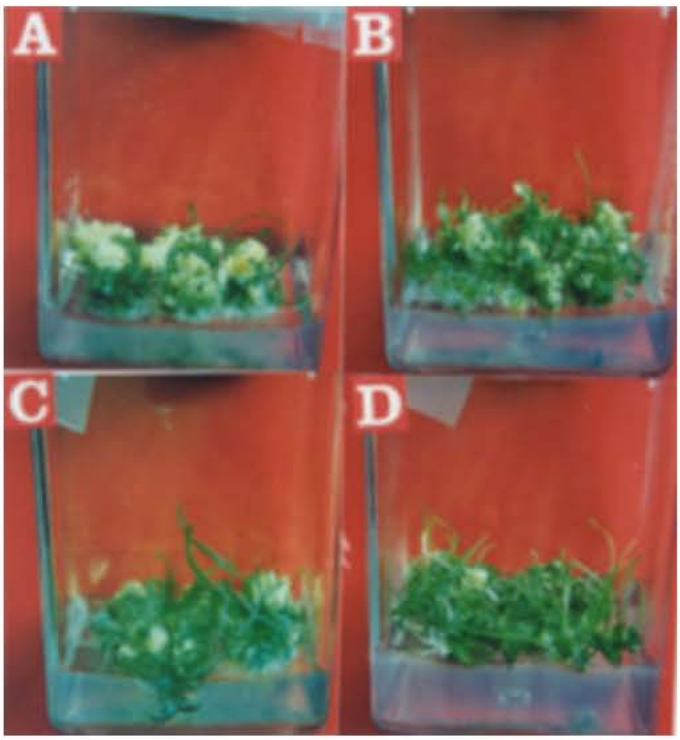

Fig. 2: Effect of different concentrations of TDZ on regeneration efficiency of Giza 164 cultivar
A: Control; B: 0.1 mg L^{-1}; C: 0.15 mg L^{-1} and D: 0.2 mg L^{-1}

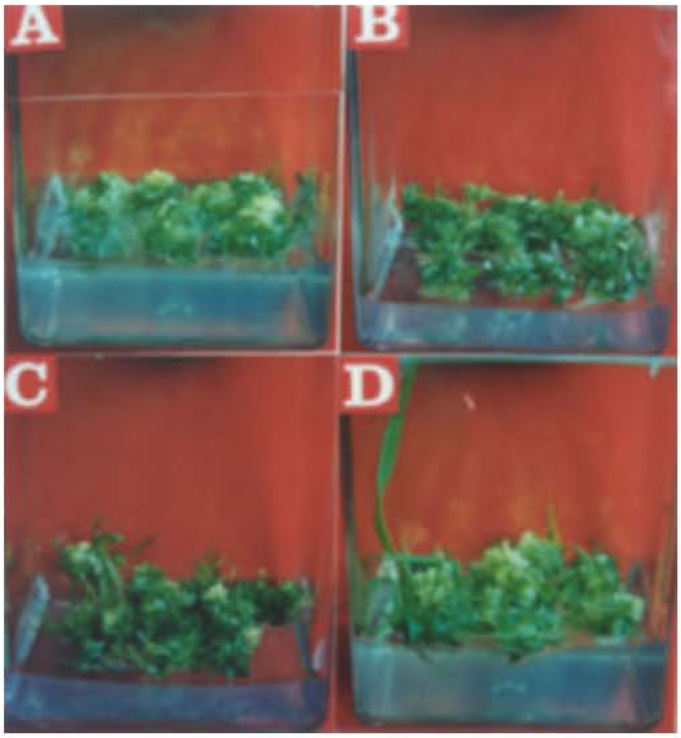

Fig. 3: Effect of different concentrations of Zeatin riboside on regeneration efficiency of Giza 163 cultivar
A: Control; B: 0.5 mg L^{-1}; C: 1.0 mg L^{-1} and D: 1.5 mg L^{-1}

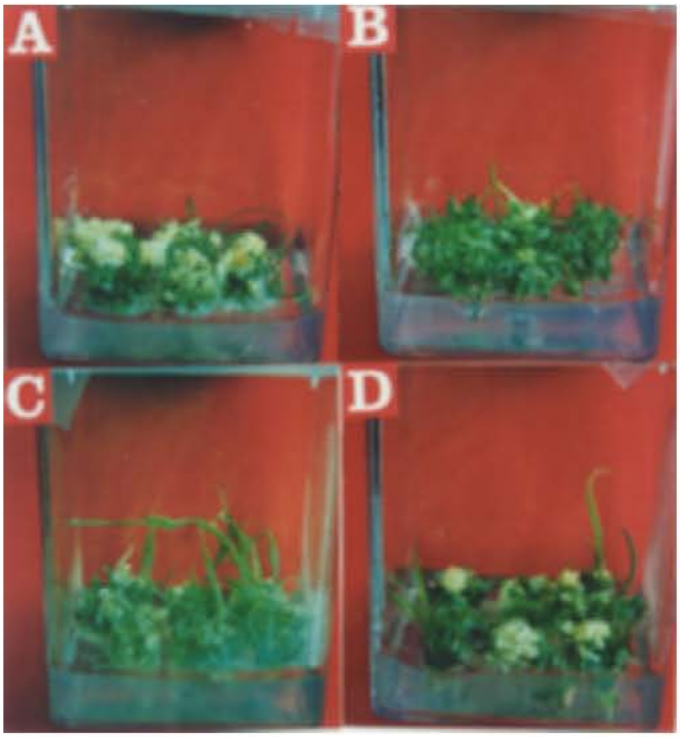

Fig. 4: Effect of different concentrations of Zeatin riboside on regeneration efficiency of Giza 164 cultivar
A: Control; B: 0.5 mg L^{-1}; C: 1.0 mg L^{-1} and D: 1.5 mg L^{-1}

Table 2: Effect of different concentrations of Zeatin riboside on number of shoots (Y1) and leaf-like structures (Y2) per callus for two Egyptian wheat cultivars Giza 163 and Giza 164

			Zeatin riboside concentration mg L^{-1}				
Trait	Cultivar	Control	0.1	0.15	0.2	Cultivar mean	Trait mean
Y1	Giza163	2.50 D	9.18 bB	10.88 bA	4.96 bC	6.88 b	
	Giza164	3.02 D	10.19 aB	14.40 aA	7.12 aC	8.68 a	
	Average	2.76 D	9.69 B	12.64 A	6.04 C		7.78
Y2	Giza163	4.85 C	8.45 aA	7.32 aB	3.53 aD	6.04 a	
	Giza164	6.40 C	7.03 bA	6.50 bC	3.20 bD	5.78 b	
	Average	6.63 C	7.88 A	6.91 B	3.37 D		5.91

Table 3: Effect of different concentrations of Dicamba on number of shoots (Y1) and leaf-like structures (Y2) per callus for two Egyptian wheat cultivars Giza 163 and Giza 164

			Dicamba concentration mg L^{-1}				
Trait	Cultivar	Control	0.1	0.15	0.2	Cultivar mean	Trait mean
Y1	Giza163	2.50 B	7.87bA	2.01 C	0.69 D	3.26 b	
	Giza164	3.02 B	9.66 aA	2.30 C	0.80 D	3.95 a	
	Average	2.76 B	8.77 A	2.16 C	0.75 D		3.61
	Giza163	4.85 C	11.23 aA	7.68 aB	3.43 D	6.80 a	
Y2	Giza164	6.40 C	8.93 bA	6.97 bB	2.35 D	6.16 b	
	Average	5.62 C	10.08 A	7.32 B	2.89 D		6.48

- Means followed by different capital letters in columns and those followed by different small letters in rows are significantly different at p=0.05 according to Duncan's multiple range test

culture. Moreover, TDZ is more biologically active than BA or Zeatin since lower concentrations are sufficient in tissue culture, which agree with the present findings. In grain legumes, TDZ has also been used for shoot formation originated from multi-cellular explant [19, 20] or from protoplast [21]. In comparing results of TDZ with those of 2,4-D or picloram, it was shown that TDZ induced higher somatic embryogenesis in protocalli [22]. In studying the effects of TDZ against other hormones, Mee-Sook *et al.* [7] revealed that shoots biomass, which is the determinant of both auxiliary shoot number and dry weight, is shown to be as a more accurate indicator of effective shoot proliferation than auxillary shoot number, only. However, the present finding disagrees with Mee-Sook *et al.* [7], where shoot biomass takes into account the leaf-like structures, which results in no regenerated plants. Therefore, we think that number of auxillary shoots only is a better indicator for the regeneration efficiency of any given plant genotype.

The influence of different concentrations of ZR on Y1 and Y2 of the two local wheat cultivars, Giza 164 and Giza 163, is presented in Table 2 and Fig. 3 & 4. Comparison between the two cultivars showed significant differences in their number of shoots/callus, where the mean Y1 of Giza 164 (8.68) was higher than that of Giza 163 (6.88) across all ZR concentrations. Opposite results were given for Y2 means, where Giza 163 showed higher number of leaf-like structures/callus than Giza 164 across all treatments. The comparison among different concentrations of ZR for number of shoots/callus showed that 1 mg L^{-1} ZR gave the highest mean, while for leaf-like structures indicated that 0.5 mg L^{-1} ZR exhibited the highest means.

In general, it can be concluded that the cytokinin activity of Zeatin riboside was high, when using 1 mg L^{-1}, where the number of shoots/callus and consequently the number of plantlets, was highest either for each or across the two wheat cultivars. Zeatin riboside, that has cytokinin activity, was used for many species to enhance the plant regeneration efficiency. Yadav and Sticklen [23] reported that the culture of leaf explants in medium containing zeatin or zeatin riboside for six days and then subcultured to medium containing zeatin riboside (1 mg L^{-1}) only caused shoot regeneration in high number. Also, Perl *et al.* [24] reported that zeatin riboside in the concentration of 1 mg L^{-1} promoted the shoot initials to plantlets in wheat. The influence of different concentrations of Dicamba as the only source of auxin on the regeneration efficiency of the two local wheat cultivars Giza 163 and Giza164 is shown in Table 3 and Fig. 5 & 6. Comparison between the mean Y1 of the two cultivars showed significant differences, where it was higher for Giza 164 than Giza 163 across treatments. Comparison between the two wheat cultivars for Y2 indicated opposite results, where Giza 163 showed higher number of leaf-like structures/callus than Giza 164 across

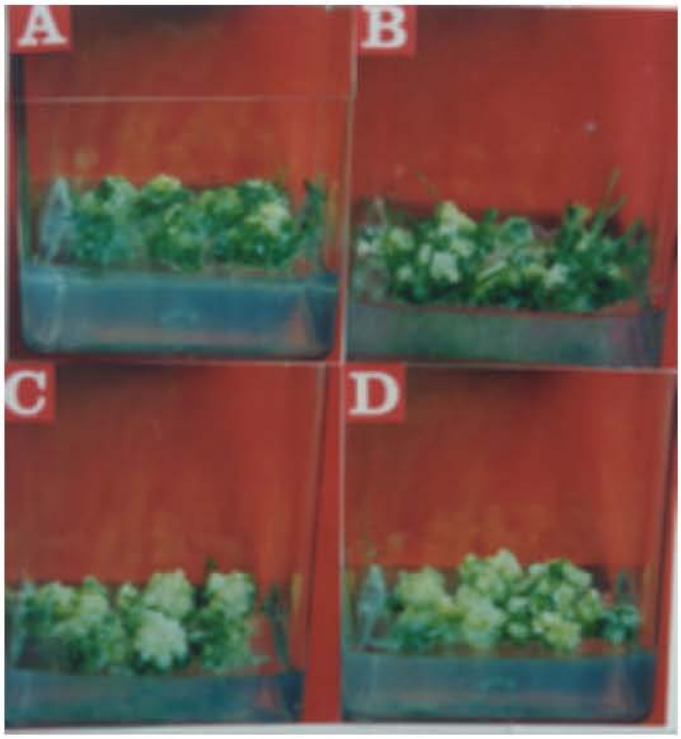

Fig. 5: Effect of different concentrations of Dicamba on regeneration efficiency of Giza 163 cultivar
A: control; B: 0.05 mg L^{-1}; C: 0.1 mg L^{-1} and D: 0.2 mg L^{-1}

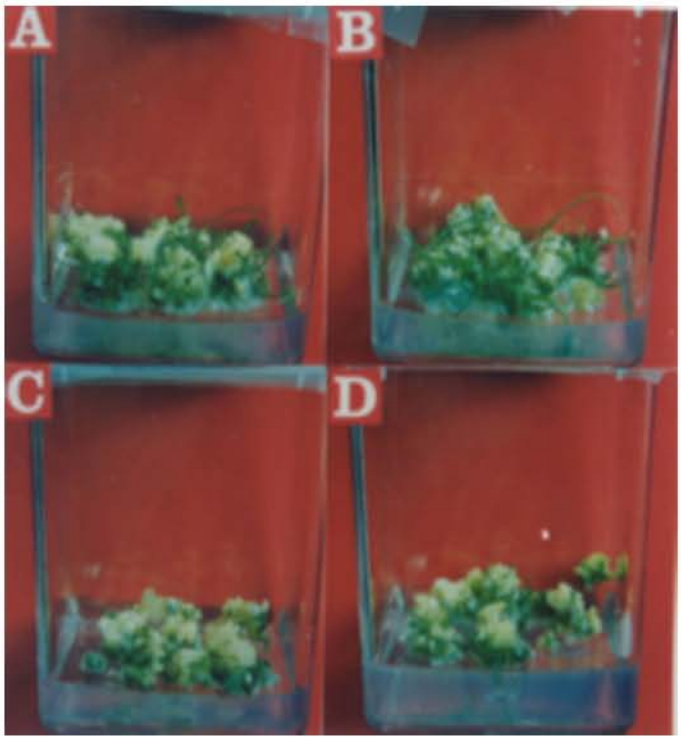

Fig. 6: Effect of different concentrations of Dicamba on regeneration efficiency of Giza 164 cultivars
A: control; B: 0.05 mg L^{-1}; C: 0.1 mg L^{-1} and D: 0.2 mg L^{-1}

treatments. The lowest concentration (0.05 mg L^{-1} Dicamba) gave the highest Y1 and Y2 means for the two cultivars. The highest concentration of Dicamba (0.2 mg L^{-1}) was shown to give the lowest means for both studied traits for each or across the two cultivars. In other words, the lower the concentration of Dicamba, the higher the regeneration efficiency for both wheat cultivars. Auxin activity of Dicamba was first reported by Keitt and Baker [25] and its use in plant tissue culture was first reported for wheat regeneration by Dudits *et al.* [26]. Dicamba was lately shown to be effective for plant regeneration from tissue cultures of *Dactylis glomerata* [27], *Poa pratensis* [28] and *Zea mays* [29]. As a substitute to the cytokinin TDZ, the auxin Dicamba was used in many reports in the regeneration medium but with high concentrations. Weeks *et al.*, [30] reported that the concentration of 0.5 mg L^{-1} Dicamba improved wheat regeneration. Dicamba is successfully used for regeneration of Bobwhite wheat cultivar, which is considered as the model wheat cultivar worldwide. Also, Papenfuss and Carman [5] reported an increased shoot formation rate from wheat callus cultures when incubated on medium containing Dicamba.

In comparing the results of the regeneration efficiency for the three growth regulators across the two cultivars, it can be shown that the influence of TDZ for the two characters, i.e., Y1 and Y2 was more potent when compared with the other two hormones. In comparing the results of the two hormones Zeatin riboside and Dicamba. ZR was much better than Dicamba because number of shoots/callus reflects number of plantlets developed through this regeneration regime, while number of leaf-like structures usually represent a dead-end plant regeneration system. A reason for the high efficiency of TDZ can be the potential of expressing a cytokinin activity as well as an auxin activity [31]. In other reports, Huetteman and Preece [32] indicated that TDZ shows a strong cytokinin-like activity and supposed to be the most effective plant growth regulator for apple tree [33] and in *Geranium* [34]. In this concern, chemical structure of TDZ was found to be different from common cytokinins [32]. In general, TDZ was shown to satisfy both cytokinin as well as auxin requirements in many plant species [34]. However, it was reported that such a cytokinin was used in many reports in a very high concentration (up to 10 mg L^{-1}) [35]. It is well known that the higher the regeneration efficiency for a given genotype, the higher the possibility of getting transgenic plants.

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