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## **RAPID AND ROBUST *in vitro* REGENERATION IN WHEAT (*Triticum aestivum* L.) USING SORBITOL**

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**W**heat is one of the most major important crops in world and Egypt. It forms a staple part of the diet in over sixty countries, serving as a major food for about six billion people being 10-20% of the daily calorific intake and unique in its ability to leaven bread (Bhalla, 2006).

Application of biotechnology is critical to the world's fight against hunger. The effective and complementary use of biotechnological tools will be required for meeting the challenge posed by the world's expanding demand for food.

The fundamental discoveries made in the field of plant biotechnology had

initiated a biological revolution in plant breeding and agricultural production. Even though wheat breeding had been developed by classical breeding methods for a long time, biotechnology as breeding implementation attract the breeder's attention (Rashid *et al.*, 2011). More recently, through the introduction of biotechnology tools, crossing barriers had been overcome, and genes from unrelated sources had become available to be introduced asexually into plants. However, advances in plant biotechnology make it possible to use exogenous genes (Wada *et al.*, 2009).

Carbohydrates are necessary and it considered as an energy provenance and a substrate of carbon for biosynthesis

(Amiri and Kazmitbar, 2011). Sorbitol also known as glucitol, is a sugar alcohol, and is supposed to be metabolized sometimes by plant tissues and consequently unavailable as carbon sources (George *et al.*, 2008).

According to the reported literature, enriched media with sorbitol or other carbohydrate sources may well promote callus induction, somatic embryogenesis, specific compounds metabolism and shoot regeneration rates significantly (Marezkiet *al.*, 1972; Klenovska, 1973; Lai and Liu, 1986 & 1988; Liu and Lai, 1991; Swedlund and Locy, 1993; Huang and Liu, 2002; Geng, 2008; Gerdakaneh *et al.*, 2009; Aazami *et al.*, 2010; Wani *et al.*, 2010; Ghobeishavi *et al.*, 2015; Mishra and Singh, 2016), since enriched carbohydrates supplied to a medium not only acts as a source of carbon and energy, but also has an osmotic role during induction and differentiation (Thorpe and Murashige, 1970; Verma and Dougall, 1977). Moreover, it also provides an alternative conception that the induction, growth and cell differentiation could be improved by the cellular physiological water status (Huang and Liu, 2002).

The progress made towards cereal regeneration/transformation systems was slow, mainly because of difficulties faced in the long period for callus induction and plant regeneration. Success in plant genetic manipulation necessitates the capability of transformed cells to regenerate; also, the recovery of transgenic plants requires a fine tuning for a short period regenera-

tion strategy. Therefore, it is necessary to establish a new fast reliable *in vitro* regeneration system for our Egyptian wheat cultivars. Our objective was to establish rapid, robust and efficient wheat regeneration system for two local Egyptian wheat cultivars (Giza 164 and Sids 1) using different concentrations of sorbitol to realize its effect on callus induction and cell differentiation.

## MATERIALS AND METHODS

### *Sterilization and isolation of explants*

Immature seeds of two local wheat cultivars (*Triticum aestivum* L.) cv. Giza 164 and Sids 1 were obtained from Wheat Department, Field Crops Institute, Agricultural Research Center (ARC), Ministry of Agriculture and Land Reclamation, Egypt. Wheat spikes were collected from field grown plants after 10-12 days post anthesis approximately, immature caryopsis were removed from spikelet's under aseptic condition and grains were surface sterilized with 20% (v/v) commercial Clorox<sup>®</sup> (5.25% Sodium hypochlorite) with few drops of Tween 20 for 20 min., followed by rinsing five times in sterile ddH<sub>2</sub>O. Immature embryos were then aseptically dissected.

### *Callus Initiation*

Immature embryos were cultured onto callus induction medium (Weeks *et al.*, 1993) basically contains Murashige and Skoog salts (Murashige and Skoog, 1962) supplemented with 0.15 g of L-Asparagine, 0.1 g of myo-inositol, 20 g

sucrose, 2.5 g Phytigel as a solidifying agent and 2.0 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) as an auxin source and pH at 5.7 with different concentrations of sorbitol (control, 10, 20 and 30 g/l). The four tested media cultures were then incubated in dark for three weeks in controlled growth chamber (Shel-Lab, USA) at temperature of 25°C.

### ***Plant Regeneration***

Induced three weeks-old immature derived callus were subcultured onto regeneration medium basically contains Murashige and Skoog salts and vitamins, 20 g/l sucrose and 2.2 g/l Phytigel, fortified with 0.1 mg/l thidiazuron and supplemented with the four different sorbitol concentrations previously mentioned (control, 10, 20 and 30 g/l). Subcultured callus were then kept in the growth chamber under cool white fluorescent light for 16 h light/8 h dark cycle for three weeks and subcultured once more for additional three weeks.

### ***Acclimatization***

Obtained plantlets were transferred into pots in controlled growth chamber incubator and successfully established at 25°C temperature and 16 h light/8 h dark cycle in the greenhouse.

### ***Statistical Analysis***

Data obtained were exposed to the proper statistical analysis of complete randomized design as described by Snedecor and Cochran (1969) in three

replicates. Means obtained were differentiated using Duncan's new multiple range test as described by Duncan (1955).

## **RESULTS AND DISCUSSION**

For conducting genetic transformation of any crop, a rapid and robust tissue culture system is a prerequisite. Production of embryogenic callus with high regeneration potential is critical step for efficient genetic transformation in wheat. Age of callus plays a vital role in differentiation process and regeneration capacity. More than five month old calli start losing their regeneration capacity, so calli age is an important factor that influence callus regeneration. Thus, modifying medium is an important step towards regeneration. It was found that frequency of regeneration is genotype dependent. Here, we report successful rapid and robust regeneration *via* embryogenesis in two Egyptian wheat cultivars i.e. Giza 164 and Sids 1. A protocol has been developed using different sorbitol concentrations as osmotic regulator to enhance embryogenic callus formation and its subsequent regeneration.

### ***Cultivars effect***

Results in Table (1) show regeneration characteristics as measured by callus induction percentage and number of regenerated shoots per callus. Data indicate that callus induction percentage was not affected significantly among the studied cultivars i.e. Giza 164 and Sids 1, and in contrary number of regenerated shoots per callus was highly significantly affected.

One of the main factors in regeneration of cereals is the genotype. The effect of cultivar on plant regeneration has been observed in our studies. The genotypic differences among studied cultivars in plant regeneration may be regarded to the effect of gene action of the plant genotype, and also may be related to the variations among the two cultivars in the endogenous hormone levels affecting regeneration process. Many previous studies reported that callus induction and plant regeneration were controlled genetically and also indicated that callus ability to differentiate is a genotype dependent. The genotypic differences were reported by many investigator as Agarwal and Tiwari (1995), Varshney and Altpeter (2001), Yadava and Chawla (2001), Mzouri and Amssa (2002), Yadava and Chawla (2002), Li *et al.* (2003), Yu *et al.* (2003), Fahmy and El-Shihy (2006), Chauhan *et al.* (2007), Monostori *et al.* (2008), Yu *et al.* (2008) and Munazir *et al.* (2010).

### ***Sorbitol concentrations effect***

Data presented in Table (2) show the effect of four sorbitol concentrations levels (control, 10, 20 and 30 g/l) on plant regeneration characteristics i.e. callus induction percentage and number of regenerated shoots/callus. By increasing sorbitol levels up to 20 g/l, callus appeared to be entirely embryogenic; moreover, callus induction percentage and number of regenerated shoots per callus were increased as shown in Table (2). These results were in agreement with those previously obtained by Edyta *et al.* (2008) and

Ghobeishavi *et al.* (2015). Contrarily, high sorbitol concentration (30 g/l) scored the lowest response giving a negative correlation with plant regeneration characteristics as shown as in Table (2) which was synchronized with data obtained by Swedlund and Locy (1993) and Grewal *et al.* (2005). They noted that high carbohydrate concentration decreased growth of callus, caused callus necrosis, somatic embryos development decrements and thus blocked plant regeneration. These effects may be due to that 20 g/l treatment act with a specific manner to promote cell differentiation and thus plant formation and also acts as appropriate concentration with carbon source towards the promotion of cell differentiation. It was observed that sorbitol proper levels shorten culture period and enhance both embryogenic calli production and differentiation compared to the sorbitol-free medium (control). It was also noticed that after three weeks culture on sorbitol-free medium there were no meristematic centers on the callus surface, on the other hand, sorbitol addition enhanced meristematic centers and subsequently differentiation. Carbohydrates functions as a carbon source and an osmotic regulator affected critically regeneration efficiency (Ghobeishavi *et al.*, 2015), moreover, it is translocated and metabolized in plants (Loescher, 1982), also, it support effectively callus growth and differentiation (Jain *et al.*, 1995). Additionally, carbohydrates continuous supplies for *in vitro* cultured cells are essential, since photosynthetic activity of tissue grown *in vitro* is usually reduced (Amiri

and Kazmitbar, 2011). However, several reports indicate that carbohydrates function as osmotic regulators and this function is critical also for somatic embryogenesis in wheat (Zhou *et al.*, 1991; Ball *et al.*, 1992), whereas, pre-treatment of callus at osmotic pressure facilitated the uptake of material into cells, additionally, influenced differentiation type and degree, and thus plant regeneration (Strickland *et al.*, 1987; Chu *et al.*, 1990; Ryschka *et al.*, 1991; Ghosh and Zapata, 1993; Swedlund and Locy, 1993; Navarro-Alvarez *et al.*, 1994; Barakat and Abdel-Latif, 1995; Jain *et al.*, 1995; Lou and Kako, 1995; Jain *et al.*, 1996; Choi and Soh, 1997; Jain *et al.*, 1997; Blanc *et al.*, 1999 & 2002; Slesak and Przywara, 2003; Li *et al.*, 2005; Hassan *et al.*, 2009; Gerdakaneh *et al.*, 2009; Aazami *et al.*, 2010; Wani *et al.*, 2010; Ghobeishavi *et al.*, 2015). Also, Hassan *et al.* (2009) reported that osmotic stresses in wheat have a main key factor in callus and embryogenesis induction. Furthermore, Several reports pointed that the carbon source can enhance somatic embryogenic induction and globular somatic embryos development, degree of differentiation, and thus the efficiency of plant regeneration (Strickland *et al.*, 1987; Lai and Liu, 1988; Chu *et al.*, 1990; Liu and Lai, 1991; Ghosh and Zapata, 1993; Swedlund and Locy, 1993; Navarro-Alvarez *et al.*, 1994; Jain *et al.*, 1995; Slesak and Przywara, 2003; Gerdakaneh *et al.*, 2009). In this work, adding sorbitol enhanced callogenesis and differentiation (Table 2) and was similar to early data obtained by Rashid *et al.* (2003), as they found that

using sorbitol-sucrose mixture as a carbon source is better for callogenesis and thus for regeneration improvements, while low response of sucrose (control) in shoot differentiation may be due to the slow break up of sucrose into glucose and fructose as mentioned by Pua and Chong (1984). Wang *et al.* (1999) pointed that sorbitol is used as an osmotic agent since calli cannot metabolize it, and accordingly sucrose with an appropriate amount must be applied for providing cells with carbon source (Shahsavari, 2011). Therefore, it could be concluded that contribution of sorbitol is based on two main functions: first, acts as a primary carbon source enhancing callogenesis and regeneration; and second, as an osmotic regulator which have positive impact on callus ability for regeneration (Maretzki *et al.*, 1972; Klenovska, 1973; Swedlund and Locy, 1993; Geng *et al.*, 2008).

#### ***Cultivars X Sorbitol concentrations interaction effect***

Effect of interaction between cultivars and sorbitol concentrations were presented in Figs (1, 2 and 3). Significant effects on regeneration characteristic as measured by callus induction percentage were obtained. 20 g/l sorbitol treatment scored the highest values with both tested cultivars (Fig. 2). Giza 164 scored its lowest value when cultured on 30 g/l sorbitol. In contrarily, Sids 1 scored its lowest value when cultured on control media (Fig. 2). Therefore, it could be concluded that adding 20 g/l sorbitol is considered the optimum concentration that acts as com-

plementary factor with carbon source in promotion of callogenesis in both cultivars. Whereas, regeneration characteristic i.e. number of regenerated shoots/callus obtained were affected significantly by the interaction between cultivars and sorbitol concentrations. Giza 164 cultivar scored its highest value and Sids 1 cultivar showed the same attitude when cultured onto media supplemented by 20 g/l sorbitol (Fig. 3). Giza 164 had a tendency in superiority over Sids 1 through number of regenerated shoots/callus. Moreover, high sorbitol concentration and control produced less value for both cultivars; respectively (Fig. 3). These results may be due to that the addition of 20 g/l sorbitol considered as specific and optimum sorbitol concentration for each cultivar, which acts as complementary factor with carbon source for production the highest number of regenerated shoots/callus and may also retain to the presence of genotypic differences in endogenous carbon balance in cell that switch significantly differentiation process. Data is synchronized with Sah *et al.* (2014) as they mentioned that regeneration depends on genotype and its interaction with osmotic medium and as well as Shamsavari (2011) who observed significant differences in interaction between varieties and sorbitol treatments where sorbitol not only increased callus induction but also promoted plant regeneration in all tested cultivars. Wetherell (1984) proposed that osmotic stress may disrupt the plasmodesmatal connections between pre-embryonic cells, thus enhance the cells physiologically to be isolated then consequently allow greater

number of cells to differentiate. Blanc *et al.* (2002) reported that the carbohydrates synthesis and accumulation in callus cells were followed by enhancing differentiation process. Also, Iantcheva *et al.* (2005) mentioned that osmotic treatment had a primary effect in embryogenic potential enhancement of tissues and also in regeneration phase shortening. Moreover, Edyta *et al.* (2008) pointed out that existence of sugar-alcohol in medium caused meristematic centers formation and shoots on wheat callus much earlier. Furthermore, Mishra and Singh (2016) stated that sorbitol induced osmotic stress and thus enhanced callogenesis and shoot regeneration in wheat callus.

## SUMMARY

Two local wheat cultivars were evaluated for their callus induction and regeneration response on MS medium supplemented with different concentrations of sorbitol, (control, 10, 20, 30 g/l). Callus induction and regeneration variabilities were observed among the two tested cultivars. Giza 164 cultivar surpassed Sids 1, where Giza 164 scored 8.08 regenerated shoots/callus, while Sids 1 scored 5.49. Regarding sorbitol concentration, highest regeneration record (9.85) as number of regenerated shoots per callus was obtained at 20 g/l sorbitol, while the lowest (3.03) was recorded at 30 g/l. In both cultivars, plant regeneration was increased gradually by increasing the sorbitol concentration from zero to 20 g/l, whereas at 30 g/l regeneration in both tested cultivars decreased. Establishment

of a fast, efficient and reliable *in vitro* regeneration system is critical step for efficient genetic transformation in wheat. Herein we report that sorbitol addition to callus and regeneration medium enhanced effectively and rapidly regeneration process in wheat cultivars under this study.

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Table (1): Effect of cultivar on callus induction percentage and number of regenerated shoots per callus.

Cultivars	Callus induction percentage	Number of regenerated shoots/callus
Giza 164	90.00 A	8.08 A
Sids 1	91.00 A	5.49 B

Means followed by different capital letters in columns are significantly different at  $P = 0.05$  according to Duncan's multiple range test.

Table (2): Effect of sorbitol concentrations on callus induction percentage and number of regenerated shoots per callus.

Sorbitol concentrations	Callus induction percentage	Number of regenerated shoots/callus
Control	82.15 B	6.81 AB
10	92.20 AB	7.45 AB
20	98.88 A	9.85 A
30	86.03 AB	3.03 B

Means followed by different capital letters in columns are significantly different at  $P = 0.05$  according to Duncan's multiple range test.

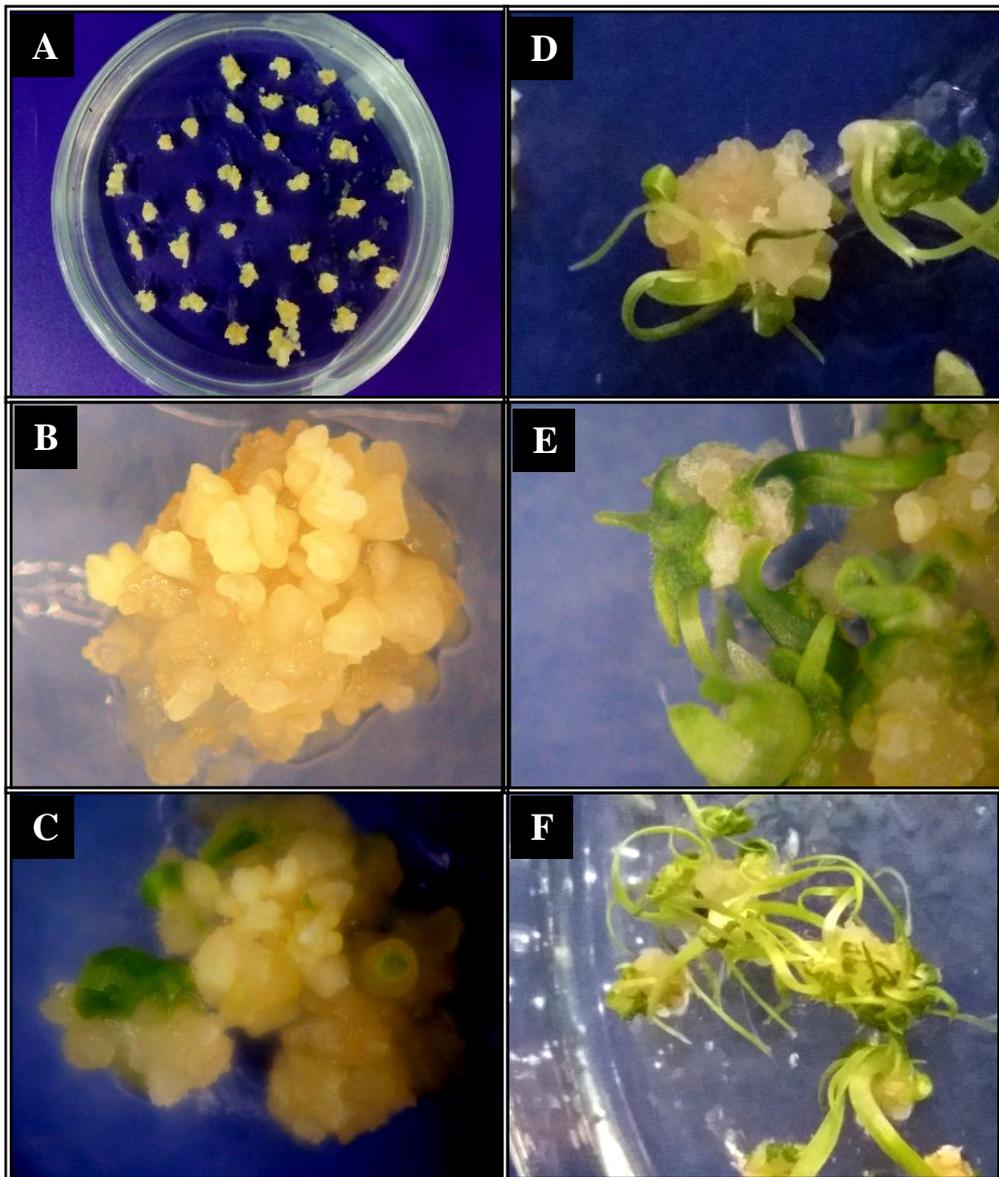


Fig. (1): (A). Immature embryo-derived calli on the induction medium. (B). Callus showing embryogenesis on induction medium. (C). Callus initiated on induction medium showing dark green meristematic domes (arrow). (D). Callus showing multiple primordial shoot regeneration. (E). Multiple shoot regeneration on regeneration medium showing multiple shoots. (F). Multiple shoots and growth on regeneration medium.

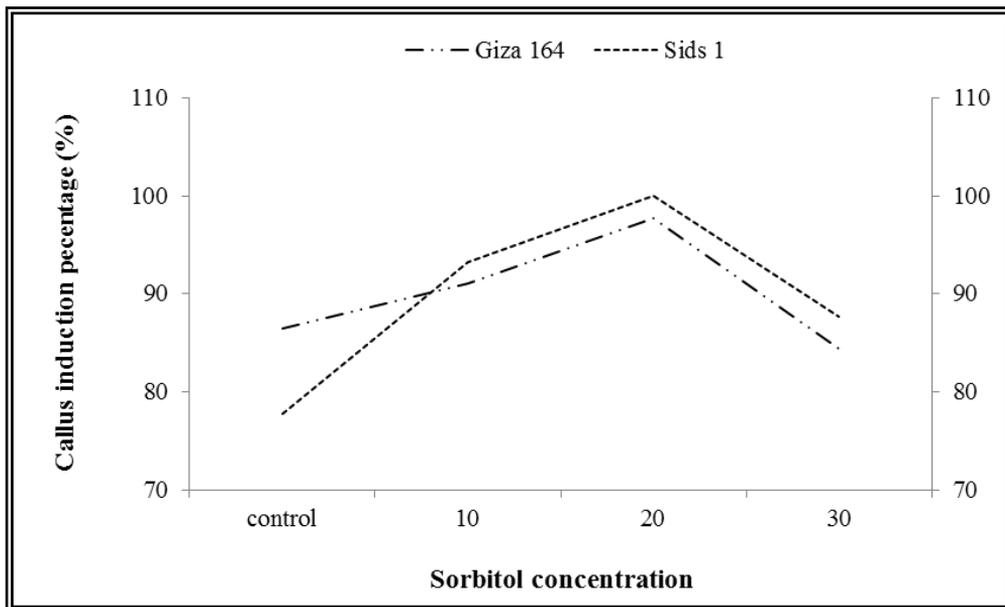


Fig. (2): Effect of interaction between cultivars and sorbitol concentrations on callus induction percentage.

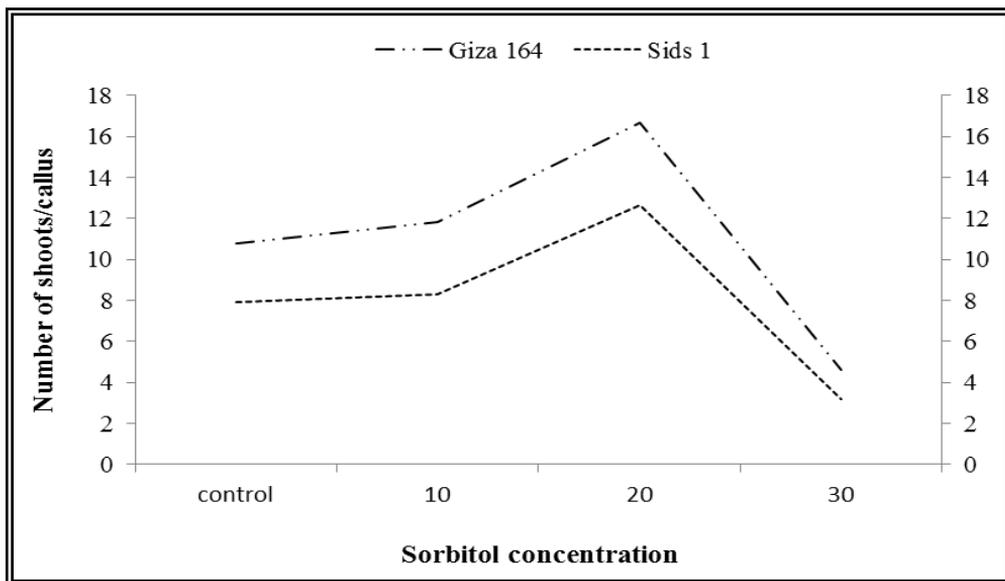


Fig. (3): Effect of interaction between cultivars and sorbitol concentrations on number of regenerated shoots per callus.