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Full Length Research Paper

Effect of sorbitol in callus induction and plant regeneration in wheat

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Six wheat genotypes were evaluated for their response to callus induction and regeneration on MS medium modified with different concentrations of sorbitol, that is, 0, 10, 20, 30 gL^{-1} along with optimum (3 mgL^{-1}) concentration of 2,4-D. Variability was observed among different genotypes for callus induction. Highest callus induction frequency was shown by Wafaq- 2001, which was about 85.62% followed by Inqalab-91 which showed 71.94% callus induction. While minimum callus induction frequency was shown by Saleem-2000 which was about 51.21%. Regarding sorbitol concentration highest average callus induction frequency (79.20%) was obtained at 20 gL^{-1} and lowest average callus induction frequency (59.20%) was observed at 30 gL^{-1}. In Wafaq-2001 and Inqalab-91 plant regeneration increased gradually by increasing the sorbitol concentration from 0 to 20 gL^{-1} but then it decreased. Similarly Auqab-2002 had no regeneration al all on non-sorbitol medium but showed regeneration on addition of sorbitol. Similarly time duration required for plant regeneration also decreased by increasing the concentration of sorbitol. It was also observed that sorbitol has given more strength to regenerated plant.

Key words: Callus induction, plant regeneration, wheat, 2,4-D, sorbitol.

INTRODUCTION

Plant breeding is the art and science of improving the heredity of crop plants. It results in the development of new varieties with improved characters. One of the examples of such achievements is green revolution, which resulted in 10-fold increase in wheat production. However, this revolution has already been exploited to its limits and alternative solutions are required to breed improved cultivars, in order to feed the rapidly growing population of the world. Although conventional breeding has made large improvements in different crops, these methods have some limitations such as long time required limited gene pool available for wheat breeders and presence of genetic barriers among different species To cope with these problems, biotechnology, integrated with classical breeding, is on the verge of creating the "evergreen revolution". For this purpose, a group of activities was focused on *in vitro* culture and regeneration as a tool of cereal breeding in the recent years. It is well documented that the genetic engineering of cereals currently depends on the use of tissue culture and plant regeneration (Mendoza and Kaeppler, 2002). Establishment of more efficient and less genotype dependent protocol is need of the day. MS medium developed by Murashige and Skoog (1962) is the most commonly used medium for wheat tissue culture. Growth regulator concentrations combinations used in culture media are critical for the induction of growth and morphogenesis. In wheat, 2,4-D alone or in combination with cytokinins has been used for callus initiation (Mathias et al., 1986 and Lazer et al., 1988). Different genotypes are reported to respond differently to callus induction under different 2,4-D concentrations (Elwafa and Ismail, 1999). High concentration of auxins and low concentration of cytokinins in the medium promote abundant cell proliferation. Shoot regeneration is better on hormone-free medium or that containing 2,4-D at low concentration than on a medium supplemented with IAA and BAP (Bennici et al., 1988; Chawala and Wenzel, 1987).

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; **BAP,** benzyl amino purine; **IAA,** indole-3-acetic acid; **MS,** Murashige and Skoog (1962) medium.

Present study was conducted to make certain modifications in MS medium to improve the efficiency of the existing protocol. For this purpose sorbitol was added in callus induction and regeneration medium.

MATERIALS AND METHODS

Explant source

Mature fresh seeds of the 6 wheat genotypes, that is, Auqab-2002, Inqalab-91, Saleem-2000, Sulman-96, Wafaq-2001, Zarlashta-99 were collected from the wheat programme, National Agriculture Research Centre (NARC), Islamabad, Pakistan.

Sterilization procedure

About 250 healthy seeds were chosen from the sample of each genotype and poured in a 100 ml Erlenmeyer flask. Seeds were washed with commercial detergent (zip) under tap water. Then under the laminar flow cabinet, these seeds were disinfested by a brief (40 s) rinse with 70% ethanol. The seeds were treated for 20 min with 40% chlorox (commercial bleach of 5.25% sodium hypochlorite solution) plus one drop of tween-80 (surfactant) followed by 5 times rinse with sterile distilled water. The surface sterilized seeds were then shifted under aseptic conditions separately to sterilized petri plates having filter papers for drying.

Callus induction media

For the preparation of callus induction medium, MS basal medium was supplemented with 2% (w/v) sucrose, 0.12% Gelrite and different concentrations, that is, 0.00, 10.0, 20.0 and 30.0 g/L of sorbitol with 3 mg/L 2,4-D (in a previous experiment it was found that best callus induction response was obtained at 3 mg/L of 2,4-D). The pH of the media was adjusted to 5.75 ± .02 before the addition of Gelrite. About 8 ml of medium was poured per test tube. The medium and other culturing instruments were autoclaved at 121°C for 15 min.

Inoculation

One explant (surface sterilized seed) per test tube was planted vertically under aseptic conditions. The scutellum side of seed was kept up in such a way that one-third portion of the seed remained above the surface of the media.

Growth conditions

The cultures were incubated in dark in an environmentally controlled chamber, where temperature was maintained at 25 ± 2°C throughout the growth period.

Growth period

The cultures were incubated for 4-5 weeks that was sufficient period for efficient callus formation. During this period, the contaminated cultures were removed and healthy cultures were checked from time to time for monitoring callus induction. Last one week was spent in data recording.

Growth measurement

Growth of callus cultures was monitored by taking number of calli and amount of callus produced.

Callus subculture

After 4 weeks of callus formation, the cultures were transferred to the subculture media. It was the same medium as for callus induction except that 2,4-D level was 2 $mgL^{-1.}$ For sorbitol supplemented media same amount of sorbitol was added in callus subculture media as that of callus induction media. The clumps of calli were divided into 3 - 4 mm sized pieces and transferred to the subculture medium. The cultures remained on this medium for 4 - 5 weeks before they were transferred to the regeneration media.

Regeneration media

MS salts and vitamins were used for regeneration with 3% sucrose and 0.2% (w/v) Gelrite supplemented with different concentrations of sorbitol, that is, 0.0, 10.0, 20.0 and 30.0 g/L along with 1 mg/L of BAP and 0.1mg/L of IAA (In a previous experiment it was observed that best germination is achieved on this hormone combination). Further procedure from pouring to autoclaving was same as that of callus induction. The cultured calli were transferred to the regeneration media under sterilized conditions in a laminar flow cabinet. The cultures were kept in controlled environment with 16 h light of approximately 1500 lux provided by general electric florescent tubes and 8 h dark period. When the regenerated plantlets reached height of 10 mm, they were transferred to new medium in 1000 ml flasks. Transfer of regenerated plantlet is essential to provide continuous supply of nutrients and further development. Fully developed plantlets were transferred to pots.

RESULTS

All the 6 genotypes responded positively to callus induction, however, variability was observed not only among the genotypes but also within all genotypes at different concentrations of sorbitol in the culture medium (Table 1, Figure 2). The effect of 0.0 and 10 g/L concentrations of sorbitol was not significant on callus induction however the effect of concentrations 20.0 and 30.0 gL^{-1} was significant (Table 2). After culture, the swelling of explant was observed within 2 to 3 days and initiation of callus was apparent as a white translucent tissue on the surface of the embryonic side of the seed within 3 to 7 days. The appearance of dense, translucent tissue was an indication of cell division activity, resulting in tissue clusters within 4 weeks of incubation (Figure 1). This time period was same for both in the absence and presence of the sorbitol. After 4 weeks of culture, 2 distinct types of calli were observed. Embryogenic callus is the desirable one that was visually judged by its white to pale yellow colour, compact, nodular and globular in shape and relatively dry in appearance. While nonembryogenic callus was yellow and brownish in colour, loose textured, irregular in shape and watery in appearance.

During the subculture period of 4 weeks, changes like greenish white colour, visible globular nodules and

Table 1. ANOVA for 2 factorial completely randomized design showing effect of sorbitol on callus induction in wheat.

Source	Degrees of freedom	Sum of squares	Mean squares	F value
Factor A (varieties)	5	8533.635	1706.727	596.696**
Factor B (sorbitol concentrations)	3	2746.366	915.455	320.056**
AB (interaction)	15	164.817	10.988	3.841**
Error	24	68.647	2.86	
Total	47	11513.465		

** Significant at P = 0.01.

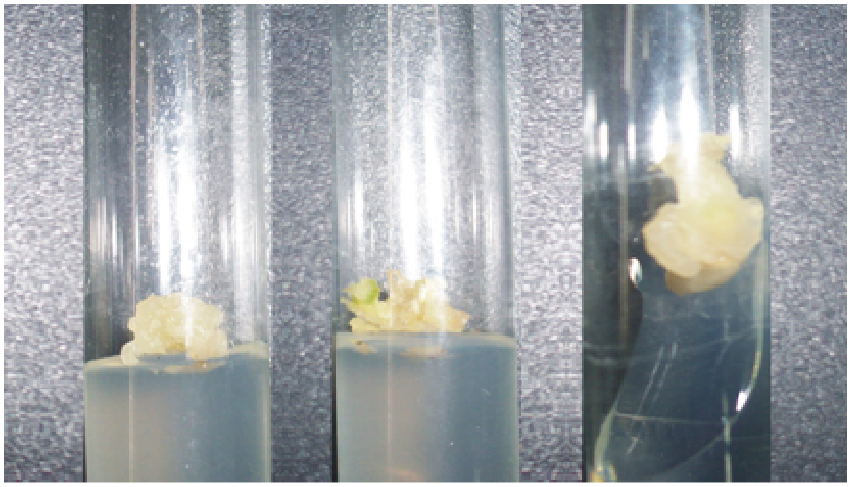

Figure 1. Callus proliferation at 3 mgL^{-1} 2,4-D with 20 gL^{-1} sorbitol in the induction media.

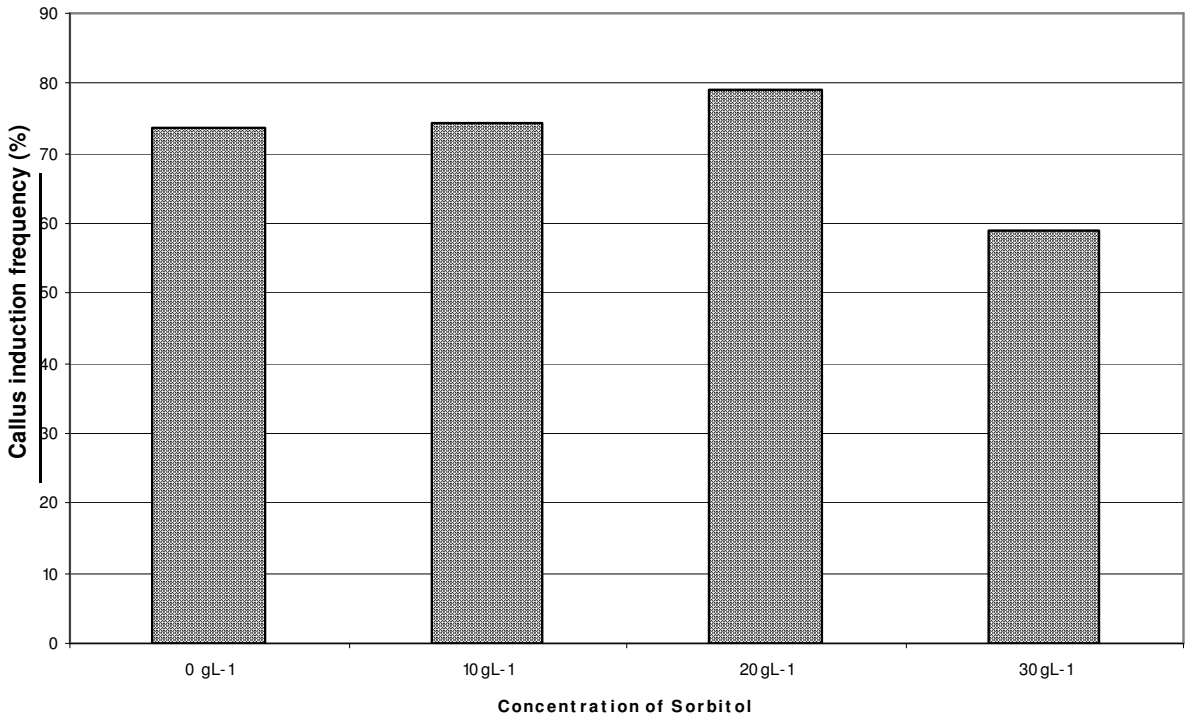

Figure 2. Effect of different concentration of sorbitol on callus induction in wheat.

Table 2. Effect of different concentrations of sorbitol on *in vitro* callus induction in wheat genotypes.

Original order				Ranked order			
Mean	1 =	73.68	B	Mean	3 =	79.20	A
Mean	2 =	74.32	B	Mean	2 =	74.32	B
Mean	3 =	79.20	A	Mean	1 =	73.68	B
Mean	4 =	58.97	C	Mean	4 =	58.97	C

Means denoted by different letters are significantly different at $P < 0.05$ according to Duncan's Multiple Range Test. Mean 1; 0.0 gL^{-1}, Mean 2; 10 gL^{-1}, Mean 3; 20 gL^{-1}, Mean 4; 30 gL^{-1}.

improved callus texture were observed and these changes were more in calli which were cultured on the media with sorbitol.

Effect of sorbitol on callus induction

Genotypes responded dfferently for all 4 (0.0, 10.0, 20.0 and 30.0 g/L) concentrations of sorbitol. It was observed that 0.0 and 10.0 gL^{-1} concentrations have non-significant effect on callus induction frequency, where it was about 73.68 and 74.32%, respectively. An increase in callus induction frequency was observed at 20.0 gL^{-1}, which was about 79.20% and then there was a decrease above 20.0 g/L, that is, at 30.0 g/L it was 58.97%.

Regardless of genotype it was also observed that calli on sorbitol supplemented media was better in quantity and quality than calli obtained on media without sorbitol (Tables 3 and 4).

Genotype effect on callus induction

Significant variability was observed among the genotypes for callus induction. Callus induction frequency was highest in Wafaq-2001 and Inqalab-91, which was about 98.52 and 90.40% respectively while minimum callus induction frequency was shown by Saleem-2000 which was 60.97% at best sorbitol concentration of 20gL^{-1} (Figure 3).

Plant regeneration

Green spot formation started after about 2 weeks on regeneration medium (Figure 4). In Wafaq-2001, Inqilab-91 and Auqab-2002 green spots went on increasing in number and after one month of culturing, shoot emerged from these green spots. It was also observed that green spot and shoot initiation started earlier and more frequently on sorbitol medium as compared to non-sorbitol medium (Table 5). Roots emerged late after shoot induction. In sorbitol supplemented media plantlet leaves were dark green in color and more in number while on non-sorbitol medium leave had light colour and they were less in number. In other genotypes, only green spot formation was observed much frequently but whole plant regeneration was not observed.

It was observed that callus induction and plant regeneration were independent of each other because, all the genotypes responded for callus induction while plant regeneration occurred only in Wafaq-01, Inqalab-91 and Aquab-2002.

Transplantation

When 3-4 plantlets developed from each callus clump and reached the height of 6 inches they were transferred to pots.

DISCUSSION

Callus induction

Effect of sorbitol on callus induction is reported for the second time. Firstly it was reported by Rashid et al. (2003) who evaluated its effect on callus proliferation and regeneration. In present study it was observed that sorbitol not only improves the quantity but also the quality of callus produced. Sorbitol acts as a hygroscopic element and creates osmotic stress as reported by Benkirane et al. (2000). This osmotic stresses have been shown to be important for induction of embryogenic cultures in wheat. The embryogenic callus was milky white in colour. The white colour of callus is reported to be due to abundant starch accumulation (Bhaskaran and Smith, 1990). In plant tissue culture, a desirable genotype is expected to possess high callus induction and plant regeneration capacity. However, numerous studies have shown the absence of such a relationship between callus induction and plant regeneration capacity. Cai et al. (1989) and Chowdhury et al. (1991) found that there is no significant relationship between plant regeneration and callus induction. It is known that callus induction and regeneration capacity may be controlled independently of each other (Sears and Deckard, 1982; Chowdhury et al., 1991 and Ozgen et al., 1996). Good embryoid production was seen during third and fourth weeks of sub-culture.

Plant regeneration

Only Wafaq-01 and Inqalab-91 responded positively for whole plant regeneration on non-sorbitol medium, however, their regeneration improved with addition of sorbitol. Aquab-2002 showed no plantlet regeneration on non-sorbitol medium but produced healthy plantlet on sorbitol medium. Rest of varieties showed no regeneration on sorbiot as well as non-sorbitol medium. However, green spot formation was improved in all the varieties with

Table 3. Fresh (FW) and dry weights (DW) (mg) of calli of different wheat genotypes at different concentrations of sorbitol in the induction media.

Sorbitol conc. (gL^{-1})	Genotypes											
	Aquab-2002		Inqalab-91		Saleem-2000		Sulman-96		Wafaq-2001		Zarlashta-99	
	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
0	317	39	323	40	296	36	347	43	374	46	374	46
10	326	40	336	41	287	35	369	45	386	48	384	47
20	364	45	374	46	314	38	381	47	416	51	426	52
30	350	43	367	45	284	35	368	45	391	48	415	50
Average	339.25	41.75	350	43	295.25	36	366.25	45	391.75	48.25	399.75	48.75
Correlation	0.999**		0.997**		0.995**		0.983*		0.991**		0.993**	

*Significance at P = 0.05; **significance at P = 0.01.

Table 4. Varietal response to callus induction frequency and callus quality at 3 mgL^{-1} of 2,4-D supplemented with 20 gL^{-1} sorbitol.

Varieties	Callus induction frequency (%)		Callus quality (%) (graded on a scale of 1 - 100 on the basis of visual observations)	
	Without sorbitol	With sorbitol	Without sorbitol	With sorbitol
Aquab-2002	77.14	82.05	33.33	37.5
Inqalab-91	84.61	90.4	63.63	68.42
Saleem-2000	52.5	60.97	28.57	32
Sulman-96	64.10	67.56	40	44
Wafaq-2001	94.73	98.52	66.66	73.13
Zarlashta-99	68.57	75	41.66	48.48

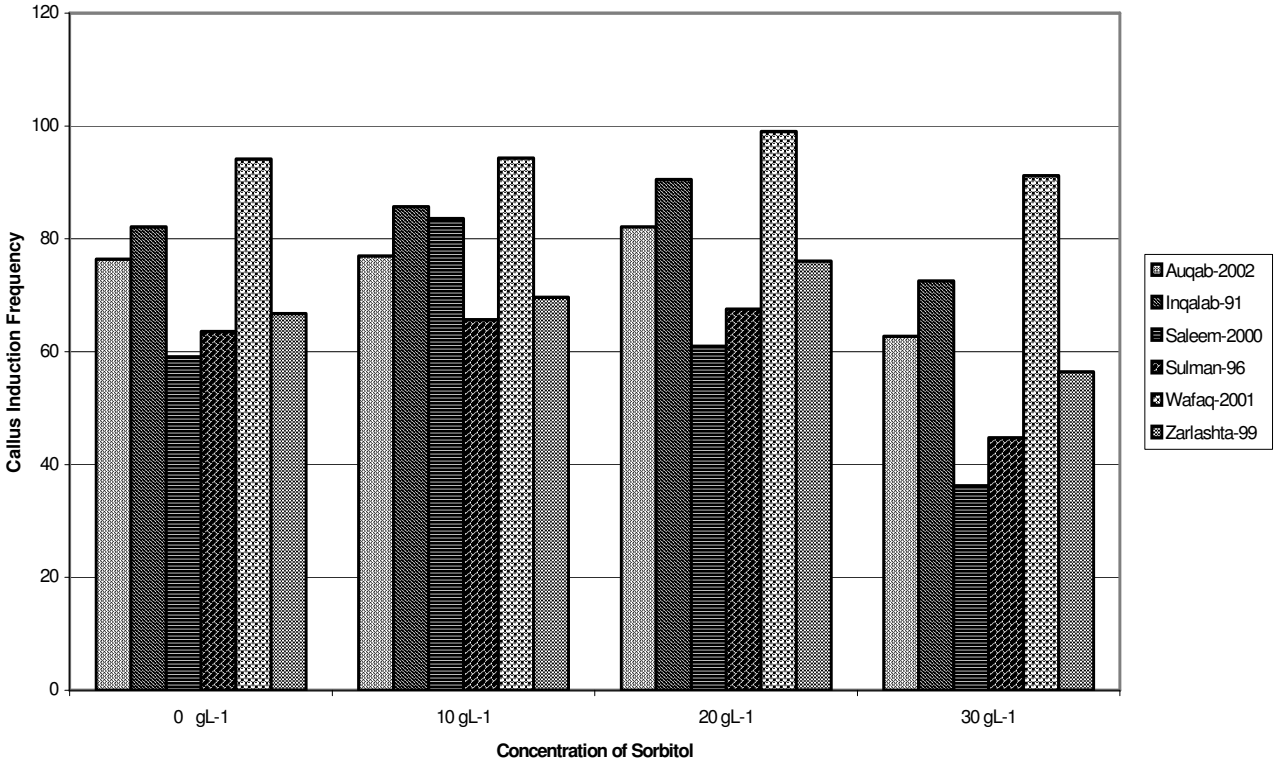

Figure 3. Response of different genotypes at different concentrations of sorbitol.

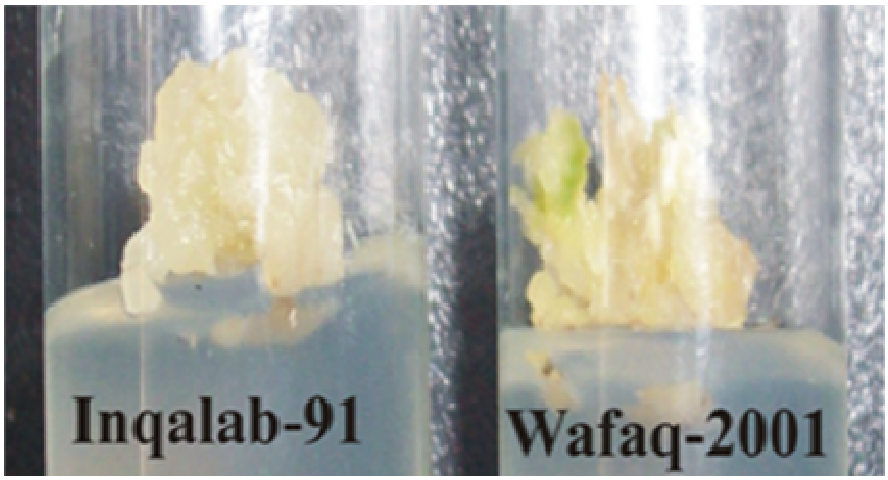

Figure 4. Green spots formation in Wafaq-01 and Inqalab-91.

Table 5. Response of genotypes towards plant regeneration at 1 mgL^{-1}BAP and 0.1 mgL^{-1} IAA with different concentrations of sorbitol

Sorbitol (gL^{-1})	Wheat genotypes	Green spots formation (%)	Root hairs formation	Plant regeneration (%)
0.0	Wafaq-01	72.73	+++	65
10.0		75	+++	72
20.0		100	+++	85
30.0		92.31	++	80
0.0	Inqalab-91	75	+++	70
10.0		77.78	+++	73
20.0		91.67	+++	80
30.0		88.89	+	75
0.0	Auqab-2002	66.67	+++	-
10.0		69.23	+++	-
20.0		90	+++	15
30.0		84.62	++	-
0.0	Saleem-2000	83.33	++	-
10.0		84.62	++	-
20.0		88.89	+++	-
30.0		87.5	++	-
0.0	Sulman-96	83.33	+++	-
10.0		87.5	+++	-
20.0		92.31	+++	-
30.0		90.91	++	-
0.0	Zarlashta-99	69.23	++	-
10.0		71.43	++	-
20.0		83.33	+	-
30.0		75	+	-

increase in sorbitol concentration upto 20 gL^{-1} and then it reduced slightly. This shows that these genotypes also have the potential for plant regeneration and by the manipulation of culture medium and growth conditions, their potential can be proved. AqGabor and Laszlo (1986) reported that sucrose medium with supplementation of mannitol almost doubled the amountof calli which formed shoot. This phenomenon indicates that wheat callus has an osmotic requirement for shoot formation. Satyavathi et al. (2004) and Ozgen et al. (1998) reported plant regene-

ration on media without growth regulators. It was observed that high cytokinin to auxin ratio (0.1 mgL-1 IAA plus 1 mgL-1 BAP) promoted plant regeneration. Only 3-4 plantlets were observed from each callus clump. It is important to mention here that the calli transferred on regeneration media were not whole calli developed from a mature embryo but were subdivided clumps of a whole callus.

Conclusion

All the genotypes responded positively towards callus induction, however, their response for plant regeneration was unsatisfactory except wafaq-2001 and Inqalab-91. So there is need to work more to reveal their response towards plant regeneration. This can be achieved by making modifications in culture medium and growth conditions. It was also proved by supplementation of sorbitol which not only enhanced callus induction but whole plant regeneration as well. This study revealed that addation of sorbitol in culture media can increase the efficiency of presently used MS medium. Wafaq-2001 and Inqalab-91 are suitable for application of modern biotechnological techniques for their genetic improvements. As presently Inqalab-91 is susceptible to rust hence transformation procedure can be applied to produce resistance in this variety against rust from any available source. This study also highlighted that endo-sperm supported mature embryos can be successfully utilized for the application of biotechnology in wheat.

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