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Improvement of plant regeneration efficiency using polyamines for double haploid production in wheat (*Triticum aestivum*)

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Haploid plant production followed by chromosome doubling offers the fastest method for developing homozygous lines. Bread wheat double haploids are produced by various intergeneric crosses e.g. wheat × maize, wheat × pearl millet, wheat × tripsacum, wheat × teosinte, wheat × barely, wheat × job's tears, wheat × congongrass and wheat × sorghum (Puja *et al.* 2011). Out of these, the wheat × maize cross is the system of choice for production of doubled haploids in wheat. Major steps of this method such as emasculation of wheat ears, pollination of emasculated ears with maize, post pollination application of colchicine along with 2,4-D followed by embryo rescue and culture at 15–18 days after pollination, have been standardized at Punjab Agricultural University as a result of series of studies (Bains *et al.* 1998). Out of which, post pollination hormonal application and embryo rescue was identified as two critical steps for success of wheat × maize cross. The low embryo germination rate is found to be major limiting factor for broader use of wheat × maize system.

Efficient *in vitro* plant regeneration is of paramount importance for success of double haploid production through wheat × maize system. Among them, the composition of culture medium could be easily manipulated and this provides ensure option to improve *in vitro* plant regeneration. Kaur (1998) used two additives casein hydrolysate (200 mg/l) and a set of amino acids (L-glutamine 400 mg/L + L-cysteine 20 mg/L + L-arginine 10 mg/L + L-leucine 10 mg/L) and recorded enhanced plant regeneration frequency (38.09% and 23.72% respectively) as compared to control (10.32%). Puja *et al.* (2011) used four additives (casein, activated charcoal, kinetin and BAP) in five combinations, and found that kinetin+activated charcoal based medium gave highest plant regeneration frequency (66%). The medium containing casein hydrolysate + activated charcoal further improved plant regeneration frequency (76.7%), but still embryo rescue medium needs some modifications to enhance plant regeneration frequency to desired level. In this context, the polyamimes, based embryo rescue medium was investigated. Besides auxins and cytokinins, polyamines are also important for *in vitro* plant developmental processes such as embryogenesis, callus induction, organogenesis and morphogenesis (De-la-Pana *et al.* 2008, Redha and Suleman 2013). The present study evaluates the effect of putrescine (PUT), spermidine (SPD), and spermine (SPM) in embryo rescue medium on plant regeneration.

The present study was carried out at the Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana and at off-season (June-September) research station, Keylong, Himachal Pradesh during 2015–16 and 2016–17. F_1s of wheat were pollinated with maize pollen. The immature caryopses, (15–18 days old) were removed carefully from the spikes. The self-seed was discarded and only hybrid caryopses were used for further studies. The caryopses having embryos were sorted out using inverted light method (Bains *et al.* 1998), and then washed thoroughly with tilt (1 g/l), bavistin (1 g/l), omnatax (0.5 g/l) and tween-20 (TOBT solution) for 20 min followed by 4–5 washings with distilled water. After that, these were surface sterilized with $HgCl_2$ (0.1%) for 8 min and ethanol (70%) for 30 sec followed by 2–3 rinsings with autoclaved water under laminar air flow. The caryopses were dissected using autoclaved forceps and scalpel (Fig 1a). The free floating embryos were taken out (Fig 1b) and put in glass test tubes (15 mm × 125 mm) containing 4–5 ml of solidified plant regeneration medium. About 20 embryos were used per replication and cultured at 25°C in darkness until germination. After embryo germination, the tubes were placed in racks having 8/16 h day/night and 40–50% humidity. The data regarding number of plants regenerated were recorded.

The regeneration medium consists of MS pre-mix 50 ml/l, sucrose 20 g/l, myo-inositol 100 mg/l, Kinetin 0.2 mg/l along with gelrite 2.6 g/l, and *p*H was adjusted to 5.8. Three polyamines (putrescine, spermidine and spermine) individually and in combination were used (Table 1).

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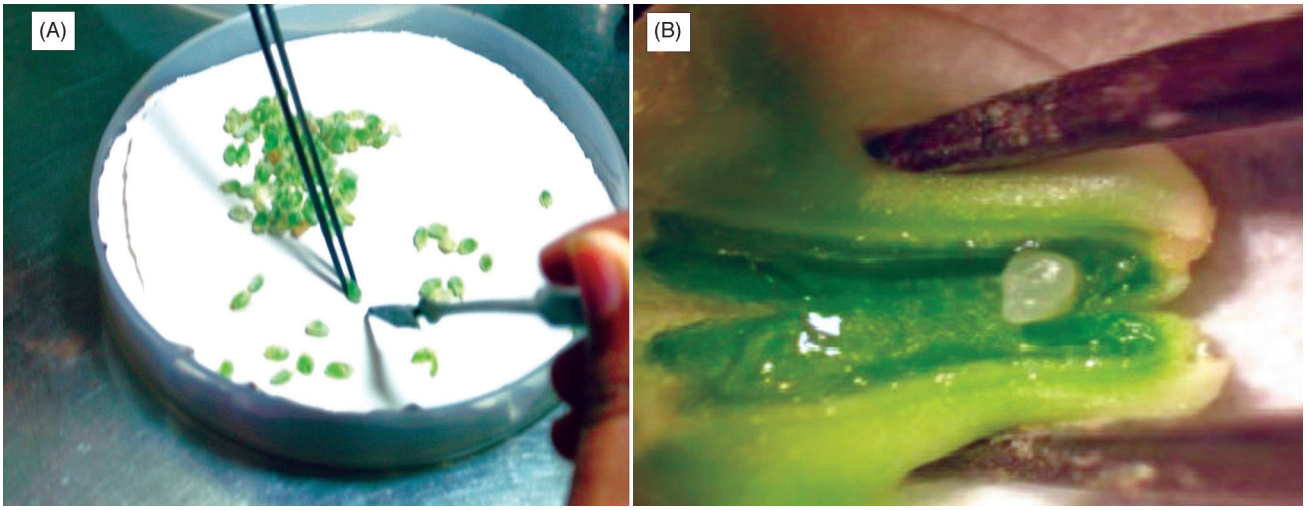

Fig 1a) Embryo rescue under laminar flow 1b) caryopses carrying haploid embryo.

The stock solution of different polyamines (100 ppm) was filter-sterilized by using 0.22 µm syringe-filters under laminar flow. The autoclaved standard embryo rescue medium was kept in laminar flow and pre-sterilized polyamines stock solution was added as required.

Table 1 List of treatments

Treatment	Medium composition	Polyamines concentration
T_1	SM as control	-
T_2	SM + PUT	1 ppm
T_3	SM + SPD	1 ppm
T_4	SM + SPM	1 ppm
T_5	SM + PUT + SPD	0.5 + 0.5 ppm
T_6	SM+ PUT + SPM	0.5 + 0.5 ppm
T_7	SM + SPD + SPM	0.5 + 0.5 ppm
T_8	SM + PUT + SPD + SPM	0.5+ 0.25 + 0.25 ppm

SM, Standard Medium; PUT, Putrescine; SPD, Spermidine; SPM, Spermine.

The pilot study included the use of three polyamines putrescine, spermidine and spermine at low concentration (1 ppm) to understand their response on plant regeneration frequency. The highest plant regeneration frequency (58.7%) was observed on medium containing PUT which was near about double than control (33.5%) (Table 2).The polyamine SPD and SPM also enhanced plant regeneration frequency, but far less increase was recorded for SPM based medium (35.0%) as compared to control (33.5%). The PUT+ SPD based medium further enhanced plant regeneration frequency (69.3%) followed by medium containing PUT + SPM (51.3%). Further, the medium containing combination of three polyamines (PUT + SPD + SPM) showed negative effect on plant regeneration frequency (31.8%) although non-significant difference was observed as compared to control (33.5%). But the plants regenerated on PUT+SPD+SPM based medium was found to be more vigorous and healthy.

The above results necessitated the evaluation of higher concentration of polyamines. So the higher concentration of

Table 2 Effect of different polyamines on plant regeneration frequency in wheat × maize system at Keylong and Ludhiana

Polyamine	Locality	Replication	No. of embryos	No. of plants	Plant regeneration frequency (%)	Callus obtained	Callus formation frequency (%)
Control	Keylong	R1	43	15	34.8	4	9.3
		R2	31	10	32.2	3	9.6
		Mean	37	12.5	33.5	3.5	9.4
	Ludhiana	R1	13	4	30.7	1	7.6
		R2	23	7	30.4	2	8.6
		Mean	18	5.5	30.5	1.5	8.1
PUT	Keylong	R1	40	24	60.0	5	12.5
		R2	33	19	57.5	5	15.1
		Mean	36.5	21.5	58.7	5	13.8

Contd.

Table 2 (*Concluded*)

Polyamine	Locality	Replication	No. of embryos	No. of plants	Plant regeneration frequency (%)	Callus obtained	Callus formation frequency (%)
	Ludhiana	R1	19	11	57.8	2	10.5
		R2	16	9	56.2	2	12.5
		Mean	17.5	10	57.0	2	11.5
SPD	Keylong	R1	37	21	56.7	4	10.8
		R2	23	13	56.5	2	8.6
		Mean	30	17	56.6	3	9.7
	Ludhiana	R1	20	11	55.0	2	10.0
		R2	24	13	54.1	3	12.5
		Mean	22	12	54.5	2.5	11.2
SPM	Keylong	R1	38	14	36.8	9	23.6
		R2	24	8	33.3	6	25.0
		Mean	31	11	35.0	7.5	24.3
	Ludhiana	R1	20	6	30.0	4	20.0
		R2	19	6	31.5	4	21.0
		Mean	39.5	6	30.7	4	20.5
PUT+SPD	Keylong	R1	40	28	70.0	0	0
		R2	32	22	68.7	0	0
		Mean	36	25	69.3	0	0
	Ludhiana	R1	20	14	70.0	0	0
		R2	23	16	69.5	0	0
		Mean	21.5	15	69.7	0	0
PUT+SPM	Keylong	R1	38	20	52.6	7	18.4
		R2	24	12	50.0	5	20.8
		Mean	31	16	51.3	6	19.6
	Ludhiana	R1	20	10	50.0	3	15.0
		R2	25	13	52.0	4	16.0
		Mean	22.5	11.5	51.0	3.5	15.5
SPD+SPM	Keylong	R1	41	19	46.3	4	9.7
		R2	30	13	43.3	2	6.6
		Mean	35.5	16	44.8	3	8.1
	Ludhiana	R1	20	9	45.0	1	5.0
		R2	23	10	43.4	1	4.3
		Mean	21.5	9.5	44.2	1	4.6
PUT+SPD+SPM	Keylong	R1	40	13	32.5	1	2.5
		R2	32	10	31.2	0	0
		Mean	36	11.5	31.8	0.5	1.2
	Ludhiana	R1	19	5	26.3	0	0
		R2	16	4	25.0	0	0
		Mean	17.5	4.5	25.6	0	0
		CD (5%)			2.039		1.816

PUT, Putrescine; SPD, Spermidine; SPM, Spermine.

Table 3 Effect of different polyamines and their concentrations on plant regeneration frequency in wheat × maize system (Ludhiana)

Polyamine	Conc. (ppm)	Replication	No. of embryos	No. of plants	Plant regeneration frequency (%)
Control	0	R1	26	8	30.7
		R2	21	7	33.3
		Mean	23.5	7.5	32
PUT	1	R1	23	12	52.1
		R2	21	11	52.3
		Mean	22	11.5	52.2
	2	R1	21	10	47.6
		R2	23	11	47.8
		Mean	22	10.5	47.7
	5	R1	23	5	21.7
		R2	18	4	22.2
		Mean	20.5	4.5	21.9
SPD	1	R1	26	13	50.0
		R2	22	11	50.0
		Mean	24	12	50.0
	2	R1	22	10	45.4
		R2	18	8	44.4
		Mean	20	9	44.9
	5	R1	24	5	20.8
		R2	28	5	17.8
		Mean	26	5	19.3
SPM	1	R1	23	8	34.7
		R2	24	8	33.3
		Mean	23.5	8	34.0
	2	R1	24	8	33.3
		R2	21	7	33.3
		Mean	22.5	7.5	33.3
	5	R1	23	6	26.0
		R2	19	5	26.3
		Mean	21	5.5	26.1
	CD (5%)				1.178

PUT, -Putrescine, ; SPD, -Spermidine,; SPM-, Spermine.

polyamines (2 and 5 ppm) along with lower concentration (1 ppm) was studied afterwards in main season at Ludhiana. The results showed that polyamines PUT, SPD and SPM at concentration 2 ppm (41.9%) showed positive effect on plant regeneration frequency, but increase was found to be significantly less than polyamines concentration at 1 ppm (45.5%) (Table 3). Further, the higher concentration of polyamines (5 ppm) showed negative effect on plant regeneration frequency significantly (22.4%), for PUT (21.9%), SPD (19.3%) and SPM (26.1%) as compared to control (32%). It was concluded that polyamines at lower concentration (1 and 2 ppm) gave higher plant regeneration frequency, and further higher concentration (5 ppm) lowered plant regeneration frequency. This promising concentration of polyamines alone (1 ppm) and in combination (not exceeding than 1 ppm) was retested during main-season in Ludhiana. The results confirmed that PUT+SPD (69.7%) based medium enhanced plant regeneration frequency followed by PUT (57%) based medium. The PUT+SPD+SPM (25.6%) based decreased the plant regeneration frequency as compared to standard medium (30.5%). This study is in agreement with the observations of other researchers also (Redha and Suleman 2013, Aydin *et al.* 2016).

SUMMARY

Polyamines are low molecular weight nitrogen containing compounds present in all living organisms. These are considered to be a new class of plant hormones, and play a positive role in plant growth and developmental processes including cell division, *in vitro* organogenesis and somatic, and zygotic embryogenesis. In our study, three polyamines PUT, SPD and SPM were used alone and in combinations in plant regeneration medium for *in vitro* plant regeneration from embryo developed through wheat × maize cross for double haploid production in wheat. Usually, these embryos have low regeneration efficiency but polyamines addition alone and in combinations in plant regeneration medium enhanced plant regeneration frequency as compared to control. Lower concentration of polyamines (1 and 2 ppm) has been found to be effective as compared to higher concentration (5 ppm). The polyamine concentration 1 ppm found to be promising concentration to improve plant regeneration. The combination of polyamines PUT+SPD (0.5 ppm+0.5ppm) proved better than other combinations to enhance plant regeneration frequency. The combination of PUT+SPD+SPM (0.5 ppm + 0.25 ppm + 0.25 ppm) although reduced plant regeneration frequency but plants were more vigorous and healthy.

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