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Full Length Research Paper

Establishment of efficient *in vitro* culture protocol for wheat land races of Pakistan

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The reliability of the production and presence of disease resistance especially rust has sparked a renewed interest in improving landraces and exploiting these in wheat variety development programs. *In vitro* culture is a pre-requisite for most of the tools of biotechnology. In this context, three Pakistani wheat (*Triticum aestivum* L.) land races viz., LLR-13, LLR-15 and LLR-16, having leaf rust resistance were assessed for *in vitro* plant regeneration response. Immature embryos were cultured on Murashige and Skoog medium supplemented with 2, 4, 6 and 8 mg/L of 2,4-Dichlorophenoxyacetic acid (2,4-D) for callogenesis and MS + zeatin riboside (1.0 mg/L) medium for regeneration of these calli. All three land races produced callus on all 2,4-D concentrations; higher doses (8 mg/L) being the most effective for callus fresh weight. However, there was a strong carry over effect of 2,4-D on regeneration capacity. Calli induced at 2 mg/L of 2,4-D showed the highest regeneration frequency for LLR-16, LLR-13 and calli of LLR-15 induced on 4mg/L were more regenerative. Overall, LLR-16 produced the maximum regenerants. Rooted plantlets were transferred to glasshouse for further evaluation.

Key words: Leaf rust, immature embryos, wheat land races, regeneration, Murashige and Skoog (MS) medium.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is known as the “king of cereals” being the most valuable staple food world-wide. In Pakistan, it is grown on an area of 9.06 million hectares with total production of 23.42 million tons and an average yield of 2585 kg ha^{-1}. It contributes 13.1% to the value added in agriculture and 2.8% to Gross Domestic Product (GDP) in Pakistan (Anonymous, 2009).

Wheat rusts are not new to Pakistan. Since inde-pendence, the country has encountered six leaf rust epidemics so far (Herrera-Foessel et al., 2007). Today, most Pakistani wheat varieties can resist one or several rusts, but that is not always enough to guarantee their survival. The wide distribution of pathogen virulence, therefore, requires identification of new resistance genes from fresh sources and their incorporation into our wheat cultivars.

Wheat landraces are intermediaries between the wild populations and modern varieties. They usually exhibit variation for qualitative and quantitative traits as well as biotic and abiotic stress resistance, owing to their heterogeneity. Hence, wheat landraces can surely be exploited for the creation of broad genetic base germplasm pools.

The establishment of an efficient regeneration protocol is a pre-requisite to effective exploitation of most biotechniques. Various explants that have been used for *in vitro* regeneration of wheat regeneration is being established using various explants viz; shoot meristems (Ahmad et al., 2002), leaf bases (Wang and Wei, 2004), microspores (Folling and Olesen, 2001; Liu et al., 2002), mature seeds (Zale et al., 2004),

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Figure 1. Growing field of wheat landraces (LLR-13).

immature scutellum (Pastori et al., 2001) and immature inflorescences (Sparks et al., 2001). Various tissue culture techniques exploit heritable somaclonal variation for wheat improvement. It is the variation induced in the plant tissues under axenic conditions (Larkin and Scowcroft, 1981). Regeneration in wheat follows either somatic embryogenesis or organogenic pathways. Callus induction and plant regeneration both are independent phenomena in wheat (Yasmin et al., 2009), being genotype-dependent and strongly influenced by the culture medium composition. Therefore, selection of appropriate genotype for *in vitro* manipulation is the primary task for any *in vitro* study.

At present, a large number of wheat land races with important economic traits have been reported in Pakistan, which still needs detailed evaluation for their efficient exploitation in wheat breeding programs. There is no report on the *in vitro* exploitation of Pakistani wheat land races, so far. Hence, this pioneering attempt was aimed at the establishment of *in vitro* culture protocols for three leaf rust resistant Pakistani wheat land races, for possible future exploittation in wheat improvement via somatic hybridization, etc.

MATERIALS AND METHODS

The proposed research was carried out at the Center of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad and Agricultural Biotechnology Research Institute (ABRI) AARI, Faisalabad, Pakistan.

Source of germplasm

Three leaf rust resistant wheat landraces LLR-16, LLR-15 and LLR-13 used were kindly provided by Wheat Research Institute (WAI), AARI, Faisalabad, Pakistan.

In vitro plant regeneration studies

Sterilization of explants

Immature seeds were collected from spikes of selected wheat germplasm 14 to18 days after anthesis, rinsed several times in distilled water, and surface sterilized with 70% ethanol (v/v) for 2 to 3 min followed by three washings with autoclaved distilled water in the laminar flow cabinet. The immature seeds were then sterilized with 10% sodium hypochlorite (v/v) for 5 to 8 min followed by three rinses with sterilized water. Using a stereoscope in the flow hood, the sterilized seeds were dissected, immature embryos excised and cultured on nutrient medium.

Callus induction and maintenance

For callus induction, immature embryos were placed with the scutellum upwards on a solid agar medium in test tubes. The basal culture medium consisted of MS mineral salts supplemented with 2 to 8 mg/L 2,4-D, 30 g/L sucrose, solidified with 8 g/L agar. Medium pH was adjusted to 5.8 and autoclaved for 20 min, at 121°C and 1.1 kg/cm^2 pressure. Immature embryos were cultured in 10 ml aliquots of MS basal medium contained in 25 ml capacity Pyrex test tubes (18 × 150 mm). The cultures were incubated in dark for two weeks at 24 ± 2°C, followed by transfer to a 16 h photoperiod (19.5 µ mol m^{-2} s^{-1}, cool white fluorescent tubes) (Figures 1 to 3).

The induced calli (with the original explants still attached) were routinely subcultured (two to three times) onto their

Figure 2. Growing field of wheat landraces (LLR-15).

Figure 3. Growing field of wheat landraces (LLR-16).

respective fresh medium every two weeks for further proliferation. Callus cultures that showed slow growth or browning were discarded.

Plant regeneration from immature embryo-derived callus

Immature embryo-derived calli (six to eight weeks old) induced on all five callus induction media, were transferred to MS-based regeneration medium (MS + Zeatin riboside 1.0 mg/L) as reported by Fahmy et al. (2006). Shoot clumps were separated into single shoots. Regenerated shoots (3 to 5 cm long) were rooted on hormone free MS medium. Each medium treatment was replicated ten times. The cultures were incubated under growth conditions as described for callus maintenance.

Rooting and acclimatization

Single shoots were rooted on simple MS medium without any growth regulator. Regenerated plants bearing well developed roots were removed from the test tubes and agar medium rinsed off the root system. These were transferred to polythene bags containing sterilized sandy loam soil. The bags were incubated in the growth room at the same growth conditions as described for regenerated plantlets. After 15 to 20 days of hardening, the plantlets were subsequently transferred to the 15 cm diameter pots. The plants were covered with transparent propagator lids to avoid loss of water due to evapotranspiration. The propagator vents were opened after five days, and the lids removed after 14 days. The well developed plantlets were transplanted in the field for further evaluation.

Table 1. Effect of 2,4-D on callus induction response of different wheat landraces.

MS + 2,4-D (mg)	Callus induction percentage		
	LLR-16	LLR-15	LLR-13
Control	0.00[d]	0.00[e]	0.00[e]
2.0	20.00[c]	56.00[d]	63.33[d]
4.0	40.00[b]	66.00[c]	71.11[c]
6.0	32.00b[c]	76.00[b]	80.00[b]
8.0	65.56[a]	86.00[a]	88.00[a]
Mean	31.4b[c]	56.8[d]	60.48[d]

Each medium treatment was replicated ten times. Values are means of 100 observations per treatment per genotype. Means sharing the similar letter in a column are statistically non-significant (P > 0.05).

Experimental lay out and data analyses

The experiment was laid according to completely randomized design (CRD). There were five treatments. Each treatment was repeated ten times with 100 explants per treatment. The means and standard deviations were computed from each treatment. Statistical procedure adopted to determine the differences among means included LSD test at 5% level of significance (Steel et al*.,* 1997). Data were collected for percent callus induction, callus morphology, fresh weight of callus, regeneration frequency, frequency of rooting, number of regenerated plants per genotype, and number of rust resistant somaclones per genotype.

RESULTS

Callus initiation and maintenance

Five different levels of 2,4-D (2, 4, 6 and 8 mg/L) were used for callus induction, along with control (MS0 medium). Callus started appearing two to three days of incubation. All three wheat landraces showed varying *in vitro* culture response for callus formation when cultured on MS medium supplemented with different concentrations of 2,4-D (2 to 8 mg/L).

Callus induction response

LLR-16

Statistical analysis of immature embryo-derived callus data for LLR-16 revealed that there was a significant positive correlation between the callus induction percentage and the level of 2,4-D in the culture medium. Callus induction response ranged from 20 to 65.5% for MS + 2 and 8mg/L of 2,4-D, respectively. The mean callus induction in LLR-16 was found to be 31.4 (Table 1).

LLR-15

Immature embryos of LLR-15 produced callus on all four concentrations of 2,4-D; 8mg/L being the best dose supporting the maximum callus induction response (86%) followed by 76% and 66% at 6 and 4 mg/L of 2,4-D, respectively. The lowest value for callus induction was observed in immature embryos cultured on MS + 2 mg/L of 2,4-D. Like LLR-16, there was a significant positive correlation between callus induction percentage and the concentration of 2,4-D in the culture medium (Table 1). The mean callus induction value was recorded as 56.8%.

LLR-13

Callus induction response from immature embryos as explants at various levels of 2,4-D showed highly significant results of 63 to 88%. The lowest callus formation was recorded for 2,4-D concentration of 2 mg/L (63%). Immature embryos of LLR-13 produced higher callus masses with increase in the concentrations of 2,4-D, (Table 1). The overall mean callus induction value was 60.48% for all the treatments of this genotype.

Callus morphology

Calli induced from immature embryos of the three wheat landraces were subcultured to same respective fresh callus induction medium after every two weeks. The callus morphology of all landraces was influenced by different concentrations of 2,4-D.

Callus cultures of LLR-16 and 15 had yellowish green appearance and were hard and compact in texture when induced on all 2,4-D concentrations

Table 2. Morphological characterization of immature embryo-derived calli of wheat land races as influenced by various 2,4-D concentrations.

Culture medium MS +2,4-D mg/L	LLR-16			LLR-15			LLR-13		
	Quality	Color	Type	Quality	Color	Type	Quality	Color	Type
Control	-	-		-				-	
2.0	+	Yellowish green	Hard, compact	+++	Creamy yellow	Compact	+++	Light Yellowish	Hard, compact
4.0	++	Yellowish green	Hard, compact	+++	Creamy yellow	soft	++++	Light Yellowish	Soft
6.0	++	Yellowish green	Hard, compact	++++	Yellowish green	Soft	++++	Light green	Loose and watery
8.0	+++	Yellowish green	Hard, compact	++++	Yellow	friable	++++	Yellowish green	Loose and watery

n = 100 observations per treatment per genotype.

(Table 2). Likewise, the compact texture of calli of LLR-13 was observed on all four concentrations of 2,4-D. However, the calli of various shades from light yellowish to light green and yellowish green were observed on 2, 6 and 8 mg/L of 2,4-D, respectively (Table 2). The calli of two landraces (LLR-16 and LLR-15) showed similar appearance and texture at all 2,4-D concentrations. The immature embryos of LLR-13 developed hard and compact textured calli similar to the rest of the two land races on all except 6 mg/L dose of 2,4-D. This particular 2,4-D concentration produced loose and watery callus which was light green in color (Table 2).

Fresh weight of callus

LLR-16

The data analysis for callus fresh weight from immature embryos revealed highly significant results for treatments and their fresh weight (Table 3).

A comparison of all the wheat land races for mean fresh weight values of callus on all the culture media indicated a general trend as far as the minimum value was concerned; 8 mg/L of 2,4-D proved to be the least effective concentration in this regard for all the land races. However, 4 mg/L of 2,4-D was found to be the most effective 2,4-D concentration for callus cultures of LLR-16 and LLR-15 which resulted in the highest average callus fresh weight values of 0.59 and 0.46, respectively. However for LLR-13, 6 mg/L proved to be the 2,4-D concentration which favored the highest fresh weight of callus (0.59).

Overall, the lower concentrations favored the fresh weight of callus values. A comparison of average fresh weight of callus showed no significant differences among the wheat land races with 0.21, 0.23 and 0.28 g for LLR-16, LLR-15 and LLR-13, respectively (Table 3).

Plant regeneration

Regeneration frequency in LLR-16

The callus cultures induced at different 2,4-D concentrations were transferred to shoot regeneration medium (MS + zeatin riboside 1.0 mg/L). Data analysis for the regeneration frequency of callus revealed highly significant results for treatments and regeneration frequency (Table 4). Moreover, it also indicated that callus cultures originally induced on higher dose of 2,4-D had lower regeneration frequency, when transferred to regeneration medium (Plate 1).

The average values for the regeneration frequency ranged between 15.56 to 73.33, from transfer of callus originally induced on MS + 8 and 2 mg/L of 2,4-D on the regeneration medium, respectively. Mean regeneration frequency value for all the callus cultures induced on all the four 2,4-D concentrations was 32.88 for LLR-16.

LLR-15

Shoot regeneration response was higher in callus cultures of LLR-15, originally induced on lower dose of 2,4-D, when transferred to the

Table 3. Effect of 2,4-D on immature embryo-derived callus fresh weight of wheat land races.

MS + 2,4-D mg/L	LLR-16	LLR-15	LLR-13
Control	0.00^{e}	0.00^{e}	0.00^{e}
2.0	0.17^{c}	0.13^{d}	0.48^{b}
4.0	0.59^{a}	0.46^{a}	0.19^{c}
6.0	0.20^{b}	0.33^{b}	0.59^{a}
8.0	0.11^{d}	0.22^{d}	0.14^{d}
Mean	0.21	0.23	0.28

Each medium treatment replicated ten times. Values are means of 100 observations per treatment per genotype. Means sharing the similar letter in a column are statistically non-significant (P > 0.05).

Table 4. Plant regeneration response of callus cultures of different wheat land races as influenced by original 2,4-D concentrations in callus medium.

Initial callus source MS + 2,4-D	Regeneration frequency		
	LLR16	LLR15	LLR13
Control	0.00^{d}	0.00^{c}	0.00^{d}
2.0	73.33^{a}	13.33^{a}	65.55^{a}
4.0	43.33^{b}	17.78^{a}	48.89^{b}
6.0	32.22^{bc}	7.78^{b}	38.89^{b}
8.0	15.56^{cd}	5.55^{b}	18.89^{c}
Mean	32.88^{bc}	8.89^{b}	34.44^{b}

Each medium treatment was replicated ten times. Values are means of 50 observations per treatment per genotype. Means sharing the similar letter in a column are statistically non-significant (P > 0.05).

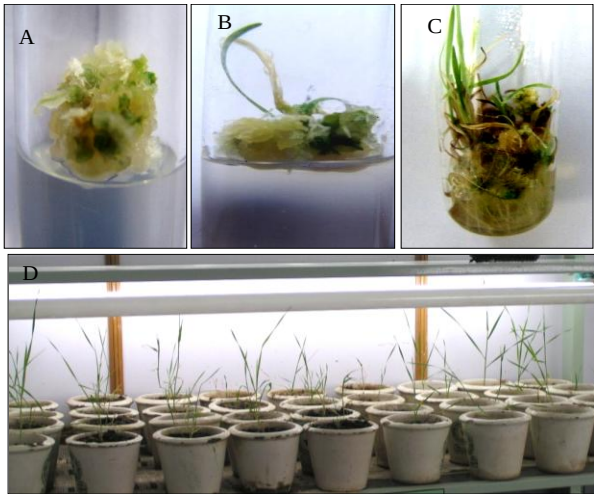

Plate 1. Immature embryo culture of LLR 16**.** A to C, Callus cultures originating on MS + 8, 6 and 4 mg/L 2,4-D after transfer to regeneration medium; D, regenerated plantlets two week after transfer to pots.

regeneration medium (Plate 2). Here, 4 mg/L of 2,4-D proved to be the most effective concentration for shoot regeneration with the highest average value of 17.78, while the minimum number of shoots were produced from callus cultures originally induced on MS medium supplemented with the highest dose of 2,4-D (8 mg/L)

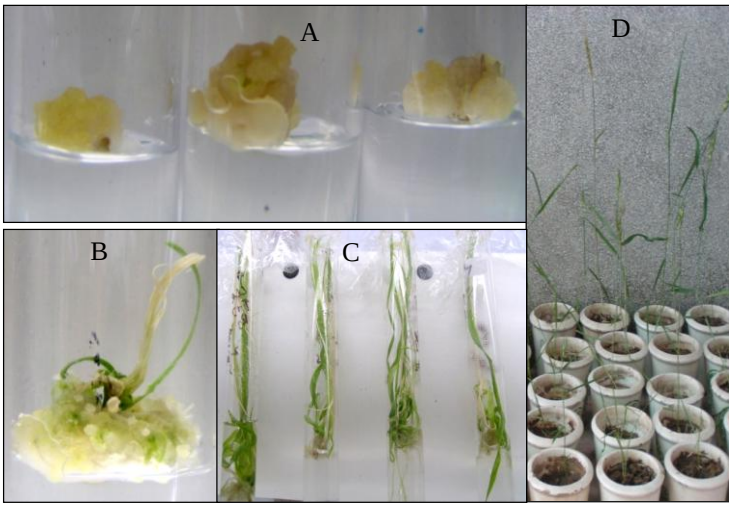

Plate 2. Immature embryo culture of LLR 15. A, four weeks old callus cultures originating on MS + 2, 8 and 4 mg/L 2,4-D; B, regenerated callus, induced on 6 mg/L of 2,4-D; C, regenerated shoots four weeks after transfer to rooting medium; D, regenerants three weeks after transfer to pots.

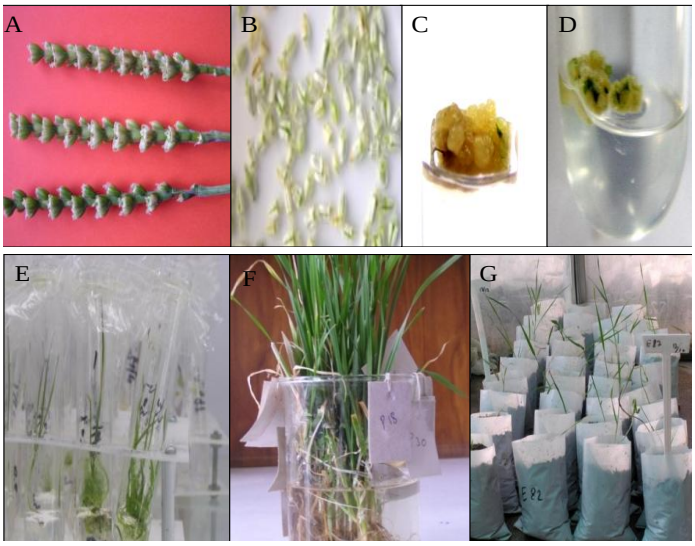

Plate 3. Immature embryo culture of LLR 13**.** A, Emasculated spikes; B, immature embryos; C, four weeks old callus induced at MS + 6 mg/L 2,4-D; D, initiation of regeneration in callus three weeks after transfer to regeneration medium; E, regenerated shoots two weeks after transfer to rooting medium; F, rooted regenerants ready to transfer to pots; G, regenerated plantlets one week after transfer to polythene bags.

LLR-13

Callus cultures of LLR-13 responded to regeneration medium in an inverse proportion to the concentrations of 2,4-D in the initial callusing medium (Plate 3). Increasing the 2,4-D concentrations from 2 to 8 mg/L gradually decreased the average regeneration value from 65.55 to 18.89, respectively (Table 4). The regeneration response was highly genotype and hormone dependent. Wheat land race LLR-13 exhibited the highest regeneration potential with average value of 34.44, followed by LLR-16 with 32.88 mean regeneration frequency, whereas LLR-15 showed a minimum regeneration capacity (8.89).

Table 5. Rooting response in shoot cultures of different wheat land races as influenced by original 2,4-D concentrations in callus medium.

Initial callus source MS + 2,4-D	Frequency of rooting		
	LLR16	LLR 15	LLR13
Control	0.00[c]	0.00[b]	0.00[c]
2.0	76.67[a]	56.67[a]	86.67[a]
4.0	76.67[a]	50.00[a]	76.67[b]
6.0	76.67[a]	53.33[a]	86.67[a]
8.0	70.00[a]	70.00[a]	86.67[a]
Mean	60.00[b]	46[a]	67.34[b]

Each medium treatment was replicated ten times. Values are means of 50 observations per treatment per genotype. Means sharing the similar letter in a column are statistically non-significant (P > 0.05).

Rooting of regenerants

Frequency of rooting in LLR-16

The regenerated shoot clumps were separated into single ones and transferred to half strength MS medium for root formation. Rooting frequency estimates were made three weeks after transfer of single shoots to rooting medium. The analysis of data revealed non significant differences for rooting frequency from shoots originally derived from callus cultures initiated at different levels of 2,4-D. Hence, unlike regeneration frequency, this auxin did not interfere with rooting potential of shoots of LLR-16. Of all the shoots transferred to rooting medium, overall, a good rooting response was observed ranging from 70 to 76% at 8 mg/L and the rest of 2,4-D levels, respectively. Although the initial 2,4-D doses showed marked influence on callus induction, callus morphology and regeneration frequency of callus cultures, however, on root formation this effect seemed to be negligible (Table 5)

LLR-15

The average rooting frequency was observed between 56.76 to 70.00, from shoot cultures originally derived from calli induced at 2 and 8 mg/L of 2,4-D (Table 5). The higher 2,4-D concentrations did not show prolonged detrimental effect on rooting of callus-derived shoot cultures.

LLR-13

The rooting frequency data has a mixed trend as far as the carried over effect of 2,4-D concentrations is concerned, setting a range of 76.67 to 86.67, respectively for mean rooting frequency (Table 5). Comparison of the three genotypes revealed that LLR-13 produced the best rooted shoots (67.34), followed by LLR-16 (60) and LLR-15 (46).

Acclimatization of *in vitro*-derived plants

The rooted plantlets of LLR16 were transferred to polythene bags filled with soil mixture for adaptation in the incubation room and were subjected to the same culture conditions as described earlier for callus to plant regeneration. The surviving plants were shifted to pots in the glasshouse. A total of nine regenerants were obtained at the end from immature embryo-derived calli of LLR-16; five regenerants of LLR-15 and seventeen of LLR13 survived. These were due to be tested for leaf rust resistance. However, future studies on the evaluation of these somaclones at the molecular level would be of great importance.

DISCUSSION

This research employed immature embryos as explants. Earlier studies also confirmed these as the most widely-used explants being highly regenerative under *in vitro* conditions (Benkirane et al., 2000; Li et al., 2003; Haliloglu et al., 2005). Different attempts in the past to replace immature embryos with other explant sources have shown that all except immature embryos had a lower regeneration potential of calli derived from alternative explants than those obtained from immature embryos (Dodig et al., 2010). Callus induction and regeneration in this study have proved to be genotype–dependent and strongly influenced by the components of the medium used. Other reports are also in agreement with our findings (Yasmin et al., 2009). Immature embryos produced varying degree of callus and with different texture on varying concentrations of 2,4-D. The choice of 2,4-D for callus induction in this study was based on the earlier investigations in wheat which mostly used this auxin for somatic embryogenesis. Although, we

have observed that higher 2,4-D concentrations improved callusing ability of immature embryos, they adversely affected the regeneration of the callus cultures originally induced on higher amounts of 2,4-D, and this is in agreement with other reports (Purnhauser et al*.,* 1987). Hence, the regeneration media are mostly devoid of auxin or have a reduced concentration thereof (Delporte et al*.,* 2001). Contrary to our studies, Nasircilar et al. (2006) observed regeneration in wheat callus cultures on MS + 2,4-D or MS + 1-napthaleneacetic acid (NAA) media.

This present research is the pioneer attempt on the assessment of *in vitro* response of wheat land races of Pakistan. All the three land races were responsive and regenerative under axenic conditions. It opens up the possibilities of exploiting these land races in various biotechniques especially somatic hybridization as a donor parent for transferring their disease resistant trait to the important wheat varieties which are susceptible to leaf rust.

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