IN VITRO SELECTION OF TISSUE CULTURE INDUCED SOMACLONAL VARIANTS OF WHEAT FOR DROUGHT TOLERANCE

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ABSTRACT

This study was carried out in the Department of Agronomy, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan, during the year 2008-10. The objective was to explore potential of somaclonal variation for improving drought tolerance in wheat. The proliferated calli from immature embryos of wheat (cv. GA-2002) were cultured on MS based medium supplemented with PEG-6000 (polyethylene glycol) induced osmotic stress of -9.0 bars for four weeks. PEG-6000 tolerant calli were selected and regenerated (R_0). The somaclones (R_0 regenerants) were grown to produce R_1-seeds. The progeny of selected plants (R_1-generation) and their donor parent were tested in pots for drought tolerance under simulated water stress. The results revealed that selected somaclones were less affected by water stress than their donor parent. Leaf rolling was observed in both R_1 and their parent with increasing severity of stress but symptoms of leaf rolling were rather earlier and clearly noticeable in parent than R_1 selected somaclones. The results further showed that R_1 somaclones maintained higher leaf water potential than donor parent in response to all treatments of water stress. Maximum leaf water potential of -1.23, -1.63 and -2.21 MPa was noted in 4, 6 and 8 days of water stress, respectively compared with parent cv. GA-2002 (-1.37, -1.80 and -2.50 MPa). Compared to parental control, significant higher drought tolerance of selected R_1 somaclones was evident based on symptoms of leaf rolling and leaf water relation. Somaclonal variations are potential source of genetic variability and can effectively be employed to develop drought tolerant plants *in vitro*. The protocol can effectively be used for *in vitro* screening and improvement of wheat for drought tolerance.

KEYWORDS: ***Triticum aestivum;*** **embryo culture; drought resistance; Pakistan.**

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INTRODUCTION

Drought is the most serious threat to world agriculture; limiting plant growth, development and productivity (1, 40). The yield increment in world cereals, particularly wheat, is declining due to increasing environmental production constraints like drought (17) and does not keep pace with increasing world population. The situation demands crop breeding for drought prone areas which does not seem feasible with classical plant breeding strategies, due to slow process, restricted gene pool availability, species barrier and other biological limitations (32).

Tissue culture is undoubtedly a mutagenic process. Tissue culture induced somaclonal variations are novel source of genetic variability for crop improvement against biotic and abiotic stresses.

In vitro tissue culture based screening of tolerant cell lines with pre-existing natural tolerance has elucidated the dilemma which is based on spontaneous or induced mutation *in vitro* (31). Tissue culture generates a wide range of genetic variability in plants. This variability in regenerants is termed as somaclonal variation (24) and can be effectively used by plant breeders for crop improvement. Somaclonal variations called as tissue culture induced somaclonal variation, could be the result of mutation caused by genetic and epigenetic changes during tissue culture processes (10). Exploitation of somaclonal variation for crop improvement is a relatively simple and easy technique, practicable where other methods are not possible, or where genes of interest are not available for crop improvement (26).

Drought tolerant plants can be obtained by supplementing polyethylene glycol (PEG) or mannitol as selection agents in culture media containing explants. Only explants competent to tolerate selective agent survive in the long run and are selected. Cells resistant to selective agent are selected and subsequently regenerated into plantlets due to their 'totipotency'. The selected somaclones for desired characters may be genetically stable and helpful for crop improvement (31).

Significant improvement in drought tolerance was made in crops like maize (9, 11), sugarcane (41), rice (25), wheat (19) and mungbean (14) by exploiting somaclonal variation. PEG was used to simulate *in vitro* osmotic stress in the culture media and drought tolerant somaclones were regenerated (11).

The plants can be categorized as drought tolerant or drought susceptible on the basis of symptoms of leaf rolling (22, 33), wilting (34), leaf water potential (LWP) (4, 44) and many more.

Conceptual models suggested that leaf rolling could be used as visual criteria to select plants for habitats with fluctuated or limited water supply (33) and may be a genotypic specific character. The trait may effectively be used as selection criteria when parent and their progenies are to be compared for their index or degree of dehydration avoidance. Leaf rolling is associated with increased stomatal resistance due to altered leaf anatomy under water deficit stress and helps reduce transpirational water losses (7).

Leaf rolling and wilting is reported to be an important adaptive response to drought stress (38, 42). Leaf rolling causes an increase in stomatal resistance to save water, protect photosynthetic machinery from desiccation injury and contributes towards yield under drought stress (7, 30). LWP declines with increasing drought stress (5, 8, 12, 15, 16, 18, 20, 21, 23, 37, 39) and was also successfully exploited to evaluate drought tolerance of wheat cultivars and other crop species (2, 3, 28, 36).

The present study was conducted to explore potential of somaclonal variation in wheat for improving drought tolerance.

MATERIALS AND METHODS

This study was carried out in the Department of Agronomy, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan, during the year 2008-10. Wheat cultivar GA-2002 was used for improvement of its level of drought tolerance using immatured embryos as explant source collected approximately 2 to 3 weeks post-anthesis. Immatured caryopses were removed and surface sterilized with 90 percent ethanol for five minutes followed by rinsing three times with sterile distilled water. Caryopses were sterilized again for 30 minutes in 6.5 percent sodium hypochlorite containing 0.1 percent Tween-20, followed by thoroughly washing with four changes of sterile distilled water. Immatured embryos were removed from immatured seeds aseptically using forceps. Callus were induced by placing immatured embryos on MS based (27) callus induction medium (MS based medium+30 g/l sucrose+6 g/l agar and 4 mg/l 2,4-D) already standardized for GA-2002 in laboratory keeping scutella side upward. The explants were incubated in total darkness at 25±1°C for three weeks. The calli were proliferated for another period of three weeks on previously standardized callus proliferation medium

(MS +30 g/l sucrose+6 g/l agar+2mg/l of 2,4-D). The media was refreshed after every 14-21 days. The proliferated calli were divided into small clumps of 100±10 mg and were shifted onto already standardized callus selection medium (MS based medium+30 g/l sucrose+6 g/l agar+2 mg/l 2,4-D + PEG induced osmotic stress of -0.9MPa) for a period of four weeks. Highly globular, white to yellowish in colour, nodular and friable calli were selected and proliferated for two weeks on callus proliferation medium devoid of PEG. After this, calli were passed onto another selection cycle of four weeks on callus selection medium to minimize chances of escaping non-tolerant calli. The tolerant calli were then shifted onto already standardized regeneration medium for cv. GA-2002 (MS based medium + 30 g/l sucrose + 6 g/l agar + 0.2 mg/l IAA + 0.5mg/l kinetin + 0.5 mg/l of BAP) for shooting and were incubated at 26^{o}C temperature with 16 hour light and 8 hour dark photoperiod (42). For rooting the regenerated shoots were shifted onto half strength MS (35). The regenerated plants (R_0 generation) were selfed to produce R_1 seed. R_1 seeds of selected somaclones alongwith their parent cv. GA-2002 were surface sterilized and placed in petri-plates for germination. Established seedlings were shifted into plastic boxes measuring 18 × 14 × 4 cm^3 filled with sterilized sand for further growth. Then the seedlings were transferred into earthen pots filled with sandy loam soil and shifted in green house. Four pots per treatment and five plants per pot were maintained. R_1 generation of somaclones and parent were compared for drought tolerance by withholding water in pots for 2, 4, 6 and 8 days alongwith control based on symptoms of leaf wilting, rolling and leaf water potential.

Visual observations on leaf rolling and leaf wilting were made on daily basis. LWP was evaluated from pressure chamber technique (43), as described by Bhutta *et al.* (6). The leaves were collected in the morning hours when they were fully turgid. Petiole of detached leaf was then quickly placed through the chamber lid and secured tightly, with the cut edge of petiole facing outside and leaf blade inside the chamber. Pressure from the tank of compressed nitrogen was applied and edge of the petiole was observed for appearance of a drop of water (sap). As soon as the drop appeared, corresponding pressure from the chamber gauge was recorded. This pressure value reflected the LW P read in negative MPa. Differences for temperature were observed near the walls and centa of greenhouse during day time. Therefore, the experiment was laid out in RCBD with factorial arrangement replicated thrice to minimize effects of differences of temperature. Ten samples per treatment were inspected and averaged. The data were analyzed using MSTAT-C software (13) and treatment means were compared by least significant difference (LSD) test (α=0.05).

RESULTS AND DISCUSSION

Leaf wilting and leaf rolling were observed in both R_1 somaclones and parent cv. GA-2002 with increasing severity of stress. As water stress progressed, the symptoms of leaf rolling and wilting were rather earlier in parent than R_1 somaclones. Slight symptoms of leaf rolling were noted in both R_1 and donor cv. GA-2002 at water stress of 4 or 6 days but it was difficult to discriminate level of drought tolerance based on extent of leaf rolling among R_1 somaclones and their parent at this level of water stress. However, symptoms of leaf rolling were clearly noticeable in both genetic materials under investigation in response to water stress of 8 days being higher in parent. It indicated that R_1 selected somaclones had better capability of osmotic adjustment than parent at same level of stress. Previously, wheat genotypes with delayed leaf rolling were characterized as more drought tolerant (22). Leaf wilting is reported to be delayed in drought tolerant genotypes more than drought susceptible ones in response to increasing water stress treatment. Similarly, Ritchie *et al*. (34) reported wilting of plants of drought susceptible genotype 'Sturdy' on day 9 of water stress due to reduced relative water content whereas, plants of drought tolerant genotype 'TAM W-101' did not show symptoms of wilting and maintained higher relative water content above this level of water stress treatment. It seemed that osmotic adjustment delayed leaf wilting and rolling in selected somaclones (R_1) and may be an important morpho-physilogical response to tolerate drought stress (38).

The rolling of leaves in plants happens under water deficit conditions resulting in reduced leaf area. The frequency of leaf rolling was observed to increase with severity of water stress (30) and had been reported to play an important role in preventing photosynthetic machinery from desiccation injury. In *Ctenanthe setosa* the carotenoid and chlorophyll as well as chlorophyll stability index were shown to be declined rapidly in the start of drought stress period but was little later on, as leaf rolling increased in response to drought stress (42).

Visual observations indicate that R_1 selected somaclones and their parent GA-2002 coped with water stress by different adaptive leaf rolling characters and R_1 somaclones were more tolerant to water stress than parent GA-2002.

Leaf water potential (-MPa)

LWP shows the hydrous status of plant tissues. A small decrease in soil water potential results in a significant decline of leaf water potential (3) and the trait could be instrumental for assessment of species' susceptibility or

tolerance to drought (2, 28). In this study, statistically significant differences were witnessed for LWP of plants subjected to variable level of water stress (Table-1). Maximum LWP (0.76MPa) was observed when the plants were well provided with water (control), which declined, with increasing osmotic stress to -0.88, -1.30, -1.72 and -2.35MPa in response to water stress imposed for 2, 4, 6 and 8 days, respectively. Water stress was developed slowly in unirrigated pots and maximum change (decrease) in predawn leaf water potential was witnessed between 6 and 8 days of water stress. The least percent change (15.78%) in LWP was observed after two days of water stress. It indicated that mild water stress (2 days) resulted in small change in leaf water status; and progressively more and significant decline happened in response to higher water stress.

Table 1. Leaf water potential (MPa) of R_1 selected somaclones and their parent cv. GA-2002 response to water stress.

Genetic material	Drought stress (days)					
	Control	2	4	6	8	Mean
Somaclones (R_1)	-0.72h	-0.86g	-1.23f	-163d	-2.21b	-1.33b
GA-2002 (parent)	-0.80gh	-0.89g	-1.37c	-1.80c	-2.50a	-1.47a
Mean	-0.76e	-0.88d	-1.30c	-1.72b	-2.35a	

LSD values: **Genetic material = 0.0510; **Stress = 0.0806; **R_1 x Parent = 0.1140
Any two means sharing same letter did not differ significantly @ = 0.05 (LSD). **Significant.

The above cited results are supported by Jafar *et al*. (23) who reported leaf water potential of -0.88MPa in sorghum in response to 3 days of water stress which declined to -3.43MPa, in 12 days prolonged water stress. Siddique *et al*. (37) reported LWP of -0.63MPa in plants provided with ample supply of water and -2.0 MPa in water stressed plants. Similarly, upto 81 percent decrease in leaf water potential of *Phaseolus vulgaris* was reported by Stoyanov (39) under water stress conditions compared with control (unstressed). Many other researchers had also documented decrease in LWP with increasing soil moisture deficit in wheat (18, 21) and sunflower (20).

Significant difference in total LWP of R_1 somaclones and donor GA-2002 was observed under simulated water stress (Table1). The highest leaf water potential (-1.33MPa) was witnessed in R_1 somaclones against the lowest (-1.47MPa) for donor cv. GA-2002 subjected to various levels of water stress. The R_1 somaclones maintained 10.53 percent higher leaf water potential compared with their parent under drought conditions.

The interaction of genetic material under investigation and levels of water stress was also significant (α=0.05) (Table-1). LWP declined simultaneously

for both R_1 and donor with increasing stress (Fig). Non-significant difference for LWP of R_1 somaclones (-0.86MPa) and their parent (-0.89MPa) was witnessed at drought stress imposed for 2 days. Significant differences for LWP were noted in prolonged water stress of 4, 6 or 8 days. The least LWP (-2.50 MPa) was recorded for parent cv. GA-2002 in response to water stress of 8 days preceded by R_1 somaclones (-2.21MPa) at same level of water stress. It indicated that R_1 somaclones are more tolerant and better adapted to water stress in terms of LWP as plants maintain higher leaf water potential than sensitive ones (44). The findings are in line with those of Adjei and Kirkham (3) for drought resistant and sensitive cultivars of wheat. They observed lower leaf water potential (-25.5 bars) of drought sensitive cultivar of hard red winter wheat 'Centurk' than that of cultivar 'Concho' (-18.8 bars), under water deprivation conditions. Similar observations were also reported by Sassi *et al*. (36) in *Phaselous vulgaris* who reported that mannitol induced osmotic stress for 15 days caused 25 percent decrease in LWP in drought tolerant and 80 percent in drought susceptible genotypes compared with unstressed plants.

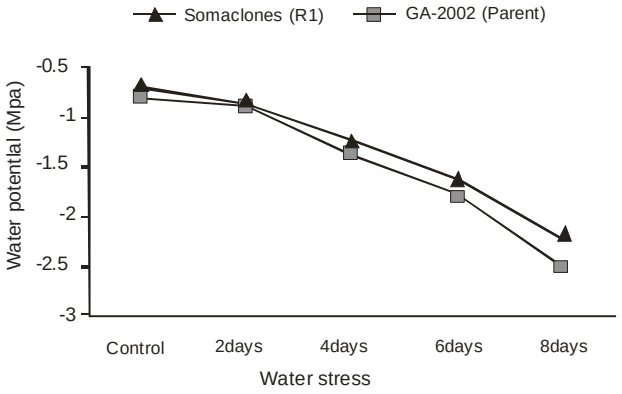

Fig. Leaf water potential of R1 somaclones and their parent GA-2002 in response to water stress.

The plants of parent GA-2002 were characterized by more negative values of LWP under all drought treatments (Fig.), while R_1 somaclones reached these values later suggesting that the parent experienced a faster water loss than R_1, possibly due to delayed activation of drought stress responsive genes. These observations are supported by Forgóné (12) who reported that leaf water status of drought susceptible genotypes declined earlier and more significantly under osmotic stress as well as under well irrigated conditions while tolerant genotypes did not show significant change until final senescence period. The results revealed that better ability of R_1 somaclones to maintain higher tissue water potential during early, mild and elevated

phases of drought episodes could be ascribed to their better drought tolerance. Similar results were reported by Alves and Setter (4) in cassava.

The decline in LWP is mainly associated with soil moisture deficit, while maintenance of higher LWP may partially be associated with active accumulation of compatible solutes like proline (28) and inorganic ions like K^+ (29). The less decrease in LWP of R_1 somaclones at higher levels of water stress might be attributed to better active accumulation of proline, K^+ and reduced stomatal conductance. Theoretically, the stomatal conductance may decrease more and earlier in R_1 somaclones than parent due to rapid and significant accumulation of ABA in somaclones in response to increasing water stress, which ultimately led to reduced transpiration to conserve water as described by Guóth *et al*. (16).

The results further showed that mild dehydration stress can predominantly induce stress response mechanisms equally in both R_1 somaclones and their parent with equal evapo-transpiration water loss. It suggested that under conditions of normal (control) and mild water stress it is difficult to discriminate drought tolerance level of various germlines based on LWP; as has been stated by Aprile *et al*. (5). Both drought tolerant and sensitive genotypes of wheat exhibited slight decline in water relations in response to mild water stress (8). Non-significant differences in LWP of both drought tolerant and sensitive genotypes of mustard and canola have also been reported earlier (15) under well irrigated conditions, until the plants experienced severe water stress (15).

It is further observed that duration of withholding water should be enough to impose water stress in root zone to elucidate physiological responses to be used as selection criteria. It is supported by Nabizadeh *et al*. (28), who studied water relations of eight doubled haploid and two wheat commercial lines (Azar2 and Zarrin) at different water regimes (25, 50, 75 or 100% of soil field capacity) and concluded that LWP of commercial varieties and double haploid was non-significant at 100 and 75 percent of field capacity. However, they found significant difference as water deficit increased to 25 and 50 percent of field capacity. As low as LWP of -2.4MPa was recorded in doubled haploid line DH14 and as high as -1.4MPa in cultivar Azar2 at soil moisture of 25 percent of field capacity.

The ability of R_1 regenerated somaclones to maintain higher LWP with increasing water stress may be ascribed to pre-existing or *in vitro* acquired ability of callus lines for osmotic adjustment. The manifestation of drought resistance in progeny of regenerated plants (R_1 somaclones) points to their

genetic nature (9). After successive cycles of *in vitro* selection in presence of selective agent, the cellular component of stress resistance might be transmitted to the progenies (25). The increased tolerance of R_1 somaclones may be the result of mutation caused by genetic (permanent and heritable) changes during tissue culture processes (10). Earlier, tissue culture induced somaclonal variations were successfully exploited to improve drought tolerance of wheat (19), rice (25), maize (9, 11), sugarcane (41) and mungbean (14). Similarly, wheat plants with improved disease resistance were also obtained by exploiting tissue culture induced somaclonal variation (26).

CONCLUSION

The study concludes that R_1 somaclones are more drought tolerant than parent GA-2002 and tissue culture induced somaclonal variation is potential source of genetic variability and can potentially be employed to develop drought tolerant plants *in vitro*.

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