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EMPLOYMENT OF IMMATURE EMBRYO CULTURE FOR *IN VITRO* SELECTION OF DROUGHT TOLERANT SOMACLONES OF WHEAT

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Abstract

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Drought, a serious threat to world agriculture, demands neo-breeding approaches. Tissue culture being a mutagenic process induces somaclonal variations, which can be manipulated for improving drought tolerance of commercial cultivars of wheat. Present study was conducted to explore potential of somaclonal variation to produce drought tolerant somaclones of wheat. PEG-6000 tolerant calli induced from immature embryos of wheat cv. GA-2002 were screened and regenerated to R_0 somaclones. The R_0 somaclones were selfed to produce R_1 seeds. The progeny of R_1 seeds (R_1 generation) and their donor parent cv. GA-2002 were raised in pots and compared for drought tolerance by withholding water for 2, 4, 6, and 8 days along with control. Water stress led to reduction of relative water content (RWC), excise leaf water loss (ELWL), leaf succulence and specific leaf weight (SLW), while an increase in ABA content of both R_1 somaclones and their parent cv. GA-2002. The R_1 somaclones showed significantly greater tendency to conserve RWC, leaf succulence and less ELWL in response to higher regimes of water stress imposed for six or eight days. Similarly, significantly higher ABA contents were accumulated by R_1 somaclones than parent cv. GA-2002 in response to water stress of 4, 6 and 8 days. Results from physico-chemical bases of drought tolerance indicated that R_1 somaclones had higher drought tolerance than their parent cv. GA-2002 and suggested possibility of *in vitro* selection of drought tolerant plants of wheat using immature embryo culture.

Key words: somaclonal variations, callus, tissue culture, water stress, wheat

Abbreviations: ABA (abscisic acid), RWC (relative water content), ELWL (excise leaf water loss), SLW (specific leaf weight), cv (cultivar)

Introduction

Drought is a scourge to crop productivity worldwide, limiting yield than any other factor of rain-fed agriculture. Wheat is predominantly grown in arid or semi-arid regions of the world under rain-fed conditions facing erratic drought spells. The productivity of rain-fed wheat can considerably be enhanced by selecting/breeding wheat genotypes better adapted to drought prone areas. Conventional plant breeding for drought tolerance is time consuming and problematic due to restricted gene pool availability, species barrier and other biological limitations. The role of transgenics to offer ecosystem friendly drought tolerant genotypes with no potential risks to human health is also questionable and yet experimental in nature, although a considerable progress had been made (Kasukabe et al., 2004). However, exploitation of tissue culture induced somaclonal variations in the presence of suitable selection agent may be an alternative, inexpensive and ecosystem friendly approach for breeding drought tolerant plants for developing countries with limited resources. Callus culture is a novel approach addressing cultured cells as selection units independent of whole plant. Natural varia-

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tions for drought tolerance existing among cell lines can be exploited *in vitro* in the presence of suitable concentration of osmoticum and stress duration (Rai et al., 2011; Mahmood et al., 2012a). In addition, improvements in tissue culture techniques had widened the genetic variability in crops and are clearly a mutagenic procedure (Afrasiab and Iqbal, 2012).

Generally, tissue culture regenerants appeared normal but a significant proportion of plants may have altered expressions/characteristics termed as somaclonal variation (Larkin and Scowcroft, 1981) and are generally attributed to pre-existing genetic variation in somatic cells (Walbot, 1985). Single gene mutation, aneuploidy, transposable elements, cytogenetic changes and DNA methylation are considered some of the possible causes of somaclonal variations (Rego and Faria, 2001; Obute et al., 2007). The extent of variation among the somaclones depends on type of explants, genotype, age of the explant donor plant and type and concentration of growth regulators used in the culture medium (Peredo et al., 2006).

In vitro screening of tolerant somaclones possessing tissue culture induced genetic variability can curtail conventional selection cycles by three to four generations (Lapitan et al., 2004) and had been exploited effectively for improvement many crops (Rai et al., 2011) including wheat (Hasissou and Bouharmont, 1994), sugarcane (Taghian, 2002), maize (El-Aref, 2002) and alfalfa (Dragiiska et al., 1996) against various biotic and a biotic stresses. The approach is based on induced or spontaneous mutation during *in vitro* culture (Afrasiab and Iqbal, 2012) and involves subjecting a population of de-differentiated mass of cells (callus) to a suitable selection pressure, recovering small fraction of variants presumptively acclimated to new conditions by physiological changes or spontaneous mutation and then regenerating plants from selected mutants (Ehsanpour and Razavizadeh, 2005). The tolerance operating at the unorganized cellular level is also effective at the whole plant level as well and the tolerance of genetic basis can be transmitted to the next progeny (Kumar and Kumar, 2000).

PEG-6000 or PEG of higher molecular weight has long been used in the culture medium to induce osmotic stress in order to exploit tissue culture induced somaclonal variation for drought tolerance in maize (El-Aref, 2002), wheat (Hasissou and Bouharmont, 1994; Mahmood et al., 2012c), alfalfa (Dragiiska et al., 1996) and rice (Biswas et al., 2002). Once the PEG tolerant callus lines are regenerated into intact plants, they are to be compared with their explant donor parent in vivo, in order to confirm in vitro acquired or improved drought tolerance. The drought tolerant and susceptible genotypes/plants can be characterized on the bases of various morhpho-physiochemical indices like relative water content (Khan et al., 2010; Raziuddin et al., 2010), leaf succulence (Gong et al., 2005; Bagatta et al., 2008), excised leaf water loss (Dhanda and Sethi, 2002, Ali et al., 2009), specific leaf weight (Brown and Byrd, 1997; Thumma et al., 1998), accumulation of ABA (Chandrasekar et al., 2000; Guóth et al., 2010) and many more.

The present study was therefore carried out to explore potential of immature embryo culture in the prescience of osmoticum (PEG-6000) for improving drought tolerance level of wheat. Callus induced from immature embryos were cultured for four weeks on MS based media supplemented with sub-lethal level of PEG-6000 induced osmotic stress as selection agent with subsequent another selection cycle. The tolerant calli were regenerated (R0) and progeny of selected somaclones R1 was then set out in the green house/plant shed along with explants donor parent to compare their level of drought tolerance based on physico-chemical drought indices.

Materials and Methods

Plant material

Preliminary studies were conducted to find out wheat cultivar possessing maximum regeneration potential under prevailing conditions. Wheat cultivar GA-2002 was selected based on its maximum regeneration potential, among seven cultivars currently under cultivation in the farmers' fields (Mahmood et al., 2012b) to explore potential of immature embryo culture for improving level of drought tolerance of wheat.

Tissue culture procedure

Immature caryopses of cv. GA-2002 were collected 2 to 3 weeks post-anthesis and surface sterilized with 90% ethanol for five minutes followed by rinsing three times with sterile distilled water. Caryopses were again sterilized with 6.5% sodium hypochlorite containing 0.1% Tween-20 for thirty minutes, followed by rinsing with four changes of sterile distilled water. Immature embryos were removed aseptically and placed on MS (Murashige and Skoog, 1962) based previously standardized callus induction media (4.0 mg/l 2,4-D, 30 g/l sucrose and 6 g/l agar) (Mahmood et al., 2012b), keeping scutella side upward. The culture was incubated in total darkness at 25±1 0C for three weeks. The calli were proliferated for another period of three weeks on callus proliferation medium (MS+30 g/l sucrose+6g/l agar+2mg/l of 2,4-D). The media was refreshed after every 14-21 days.

Selection of osmotic stress tolerant calli

Proliferated calli were divided into micro-clumps of 100±10 mg and were shifted onto previously standardized callus selection medium (MS + 2 mg/l 2,4-D + 30 g/l sucrose + 6 g/l agar + PEG-6000 induced osmotic stress of -0.9MPa)

for period of four weeks (Mahmood et al., 2012a). The tolerant surviving calli were selected and proliferated for two weeks on callus proliferation medium devoid of PEG-6000. These calli were again divided into micro-clumps and passed onto another selection cycle of four weeks in order to minimize chances of escaping non-tolerant calli.

Regeneration of osmotic stress tolerant calli

The surviving calli (PEG-6000 tolerant) were regenerated on previously standardized regeneration medium for cv. GA-2002 (MS + 0.2 mg/l IAA + 0.5mg/l kinetin + 0.5 mg/l of BAP + 30 g/l sucrose + 6 g/l agar) (Mahmood et al., 2012b). The regenerated plants (R0 generation) were selfed to produce R1 seed.

Comparison of R_1 selected somaclones with their explant donor parent cv. GA-2002 for drought tolerance

The R1 seeds were raised to R1 generation along with original explant donor parent cv. GA-2002 in earthen pots. The R1 somaclones and plants of parent cv. GA-2002 were kept in control shed and compared for drought tolerance by withholding water in the pots prior to booting for period of 2, 4, 6 and 8days along with control. Following drought tolerance adopted traits were studied:

Relative water content (RWC) (%)

Fully developed leaves were collected and fresh weight (F_W) was immediately taken. Leaves were then submerged for 4 hour in distilled water at room temperature under a constant light. After soaking for 4 hours turgid weight (T_W) of leaves was taken. The leaves were then dried for 36 hours at 70 °C for dry weight (D_W). RWC were computed as proposed by Barrs and Weatherley (1962).

$$RWC\ (\%) = \frac{Fw - Dw}{Tw - Dw} \times 100$$

Excised leaf water loss (ELWL) (%).

Data for excised leaf water loss was recorded from flag leaf and two fully expanded preceding leaves as described by Dhanda and Sethi (2002). To circumvent any complications, predawn sampling was exercised. Fresh weight of the leaves was recorded immediately after sampling. The leaves were then dried at 50% RH and 28°C in incubator for period of 6 hours, followed by drying in an oven for period of 24 h at

$$ELWL\ (\%) = \frac{Fresh\ weight - Weight\ after\ 6\ h}{Fresh\ weight - Dry\ weight} \times 100$$

Succulence (mg of H_2O/cm^2 of leaf segment)

Randomly taken leaves were weighted for their fresh weight followed by measurements of their leaf area. Then the leaves were dried until constant weight at 70°C. Succulence was calculated by following formula:

$$Succulence = \frac{Fresh\ weight - Dry\ weight}{Leaf\ area}$$

Specific leaf weight (mg/cm^2)

Specific leaf weight (SLW) was executed as proposed by Yang et al. (2003). Leaf discs were sliced, area measured and dried at 70°C till constant dry weight. SLW was calculated by using following formula:

$$Specific\ leaf\ weight = \frac{Dry\ wt.\ of\ leaf\ disc}{Area\ of\ leaf\ disc}$$

Leaf abscisic acid contents (µg/g dry wt.)

Leaf abscisic acid (ABA) contents were worked out using Zeevart (1980) method reported by Chandrasekar et al. (2000). Two-gram leaf samples were frozen in liquid nitrogen. Samples were crushed and stored at -20°C. Extraction of frozen samples was carried out thrice with 80% acetone (10 ml v/v) (1.0 ml glacial acetic acid, 100.0 mg of 2, 6 di-tert-butyl 4-methylphenol plus 80 ml of acetone in total volume of 100ml). The extract was collected in volumetric flask of 100 ml. The tissue residues were then again homogenized with 80% acetone (v/v) using pestle and mortar and filtered with whatman no.1 filter. To evaporate acetone, the filtrate together with the extracts prepared earlier was shifted to boiling flask of the rotary flash vacuum evaporator. Liquid soluble fraction was left on walls of boiling flask. The collected liquid soluble fraction was dissolved in 1.0% acetic acid solution. Solution with resultant amber color was transferred into 10 ml vials. Prior to sample injection into HPLC, samples were filtered using 2.5ml syringe and 0.45 mm millipore filter. The flow rate was adjusted to 2.5 ml/min and wave length at 265 nm. 20 µl of standard ABA (10ppm) was used to identify the peaks belonging to ABA. The ABA concentration of the sample was worked out by comparing with area under ABA standards.

Data analysis

The data so collected was analyzed using MSTAT-C software (Freed and Eisensmith, 1986) and treatment means were compared by Least Significant Difference (LSD) test (α=0.05).

Results

Comparative performance of *in vitro* selected R_1 somaclones and their parent cv. GA-2002 in response to simulated water stress

The interaction of genetic material under investigation and level of water stress had significant effect on RWC (Figure 1), ELWL (Figure 2), leaf succulence (Figure 3) and ABA

(Figure 4). The RWC, ELWL, leaf succulence and specific leaf weigh of both R_1 selected somaclones and their parent cv. GA-2002 declined significantly with increasing duration of water stress. In contrast, ABA contents were increased with rising water stress in pots (Figure 4). Contrary, specific leaf weight did not differ significantly among R_1 somaclones and donor parent cv. GA-2002 with non-significant interactive effect of water stress and genetic material (P<0.05) (Table 1).

The selected R_1 somaclones maintained higher RWC than their parent cv. GA-2002 in response to all treatments of water stresses. Significantly, higher RWC of 69.4% and 51.87% were maintained by the selected somaclones in response to

Table 1
Specific leaf weight (mg/cm²) of R1 selected somaclones and their parent wheat cv. GA-2002 in response to water stress

	Water stress					
	control	2 days	4 days	6 days	8 days	Mean
Somaclones (R_1)	6.04	6.03	5.81	5.49	5.37	**5.75**[a]
cv. GA-2002(Parent)	6.28	5.85	5.66	5.28	5.03	**5.62**[a]
Mean	6.16[a]	5.94[ab]	5.73[b]	5.38[c]	5.20[c]	
LSD values = [NS]Genetic material= 0.146, *Stress = 0.232, [NS]Stress × Genetic material = 0.327 [NS]Non-significant; *Significant						

Entries sharing similar letters do not differ significantly at 5% probability level

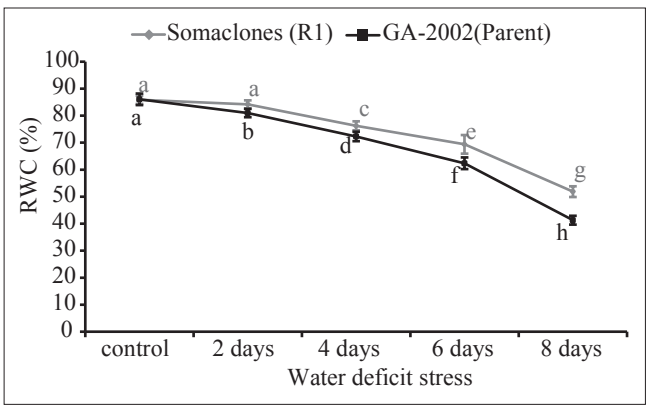

Fig. 1. RWC (%)

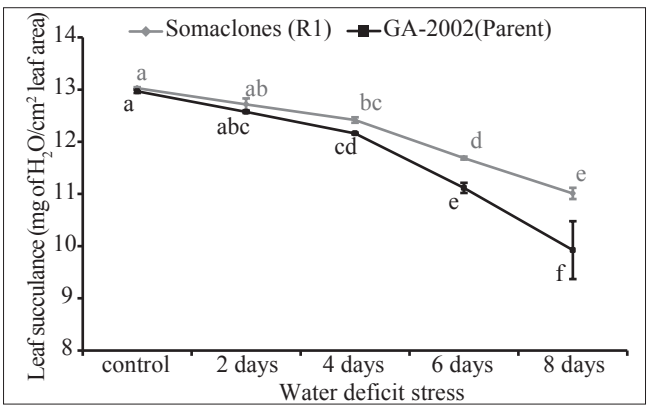

Fig. 3. leaf succulance (mg of H_2O/cm² of leaf area)

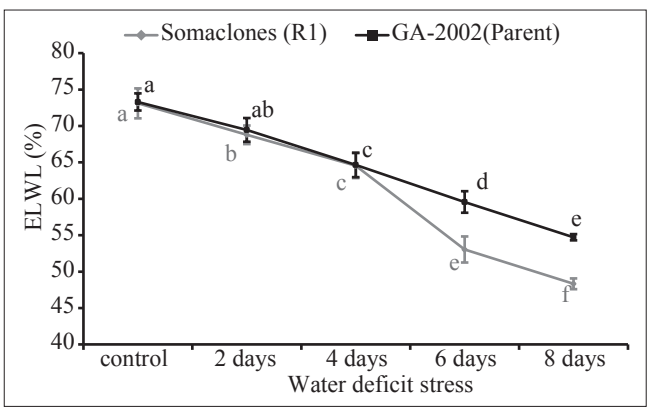

Fig. 2. ELWL (%)

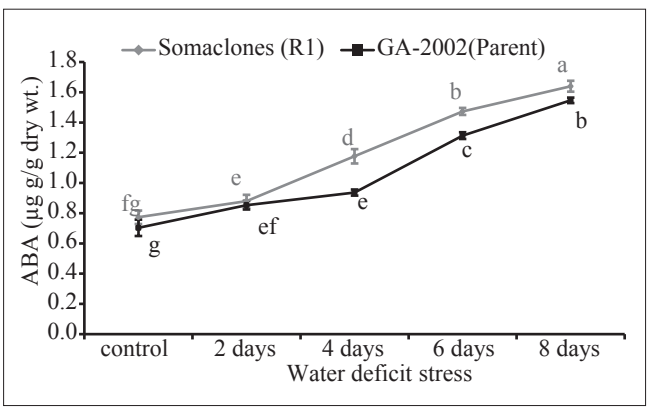

Fig. 4. ABA contents (µg/g dry wt.)

Physico-chemical response of R_1 selected somaclones and their parent wheat cv. GA-2002 in response to water stress. Vertical bars represent standard errors. Bars sharing similar letters do not differ significantly (P<0.05).

6 and 8days of water stress, respectively, against parent cv. GA-2002 with 62.37% and 41.30% in response to 6 and 8days of water stress, respectively (Figure 1). The R_1 somaclones also differed significantly from their parent cv. GA-2002 in term of ELWL under extreme water stress of 6 or 8 days. The selected R_1 somaclones demonstrated significantly less values of ELWL compared with their donor parent cv. GA-2002 (Figure 2) and exhibited ELWL of 53.04% against 59.56% for parent cv. GA-2002, when water was withheld for 6 days. Similarly, ELWL of 48.36% and 54.72% was witnessed for R_1 somaclones and parent cv. GA-2002 respectively, in response to water stress of 8 days. Also, the R_1 selected somaclones maintained significantly higher leaf succulence of 11.69 vs. 11.12 mg/cm^2 in response to 6 days of water stress and 11.01 vs 9.92 mg/cm^2 when water was withheld for 8 days, compared with parent cv. GA-2002 (Figure 3). Similarly, the R_1 somaclones accumulated significantly higher ABA (µg/g dry wt.) in response to water stress of 4, 6 or 8days, viz., 1.18, 1.47 and 1.64 µg/g dry weight, respectively, against parent cv. GA-2002 with respective ABA content of 0.94, 1.31 and 1.55 µg/g dry wt. for 4, 6 and 8 days of water stress (Figure 4).

Discussion

Drought stress disrupts water balance of plants, which results in decreased leaf water relations (RWC, ELWL and succulence). Leaf water relations are closely associated with cell volume and it may possibly elucidate balance between leaf water supply and transpiration rate. The ability of plants to maintain higher RWC indicate their ability to maintain higher tissue water contents and possibly be a more precise marker of tissue hydrous status under limited supply of water. Accordingly, the trait is exploited to investigate drought tolerance of plants (Dhanda and Sethi, 2002). A declining trend in RWC was observed for both R1 somaclones and their explant donor cv. GA-2002 in response to increasing water stress (Figure 1). The observations are supported by many other investigators who also documented decrease in RWC with increasing simulated drought stress, for example in wheat (Raziuddin et al., 2010) and brassica (Khan et al., 2010). Comparative study of RWC had enabled the researchers to select superior drought tolerant landraces for water deficient environments (Dhanda and Sethi, 2002), since, drought tolerant genotypes maintain higher RWC than drought susceptible ones (Khan et al., 2010).

Similarly, leaf succulence index is often exploited to evaluate ability of the plants for osmotic adjustment. Leaf succulence of both R_1 somaclones and their parent cv. GA-2002 declined with increasing water stress (Figure 3) and is in line as reported by Gong et al. (2005) and Bagatta et al. (2008). Drought tolerant genotypes always maintain higher water content regardless of soil moisture stress (Dedio, 1975). The R_1 somaclones regenerated from PEG-6000 adapted callus lines maintained significantly higher leaf succulence compared with their explant donor parent cv. GA-2002 in response to water stress of 6 and 8 days (Figure 3) and can be designated more drought tolerant based on leaf succulence under water stress conditions. The observation are also supported by previous studies, suggesting that *in vitro* PEG adapted cell lines maintained higher water contents than non adapted cell lines (Heyser and Nabors, 1981).

Excised leaf water loss (ELWL) or excised leaf water retention (ELWR) is another excellent indicator of water status of the leaves. Most of the evaporation after leaf excision is from the epidermis and is presumably an indirect measure of cuticular thickness and cuticular transpiration (Clarke and McCaig, 1982). The genotypes with reduced ELWL are less affected by evapotranspiration water losses (Ali et al., 2009) and are recognized as more drought tolerant due to conserved water contents. Therefore, ELWL which presumably is the measure of cuticular transpiration or indirectly epicuticular wax load (Clarke and McCaig, 1982) has been proposed as a screening criterion to select for drought tolerant genotypes of wheat adapted to dry areas (Ali *et al.,* 2009). Accordingly, the genotypes with reduced ELWL are considered more drought tolerant (Dhanda and Sethi, 2002). Comparatively lower ELWL was exhibited by R_1 somaclones than parent cv. GA-2002 in response to moisture deficit stress (Figure 2) and it can be designated better adopted to drought prone areas based on ELWL.

The ability of R_1 regenerated somaclones to maintain higher RWC, ELWL and leaf succulence may be ascribed to their pre-existing or *in vitro* acquired ability of the callus lines for osmotic adjustment by active accumulation of solutes within symplast in response to water stress; since, osmotic adjustment and leaf water relations are closely related due to single locus on chromosome (Lilley et al., 1996). In wheat, heritability of leaf water relations like ELWL (Chandra and Islam, 2003) and especially of RWC is much higher than yield or yield components (Bayoumi et al., 2008). Tentatively, it can be revealed that genetically inherited or acquired drought tolerance of R_1 somaclones (in terms of RWC and ELWL) from PEG-6000 tolerant callus lines can be transmitted to next the progenies as well (R_2) (Afrasiab and Iqbal, 2012) and the selected somaclones may potentially be incorporated in plant breeding program. The results that immature embryo culture induces somaclonal variation for ELWL/waxiness in R_1 generation of wheat are consistent with those reported by Larkin et al. (1984) in regenerated somaclones of wheat, who also suggested that induced variations are heritable and stable (Afrasiab and Iqbal, 2012).

Specific leaf weight (SLW) is the ratio of leaf mass to leaf area (Thumma et al., 1998). Plants with higher SLW had increased cell wall constituents, non-structural carbohydrates and often some specific proteins (Brown and Byrd, 1997). The trait is reported to be associated with drought tolerance of plants and is suggested as a useful selection criterion for plants breed for low rainfall targeted areas (Thumma et al., 1998). Cultivars with more SLW had thick leaves (reduced surface area to volume ratio) and exhibit improved water use efficiency (Brown and Byrd, 1997; Thumma et al., 1998). The genotypes differ for SLW at most of their growth stages (Thumma et al., 1998). Contrary, R_1 selected somaclones and their parent cv. GA-2002 did not differ significantly for SLW. Non-significant differences between SLW of R_1 somaclones and their donor parent cv.GA-2002 can be attributed to the fact that both regenerated R_1 somaclones and their parent cv. GA-2002 had same genetic origin. Theoretically, it can be revealed that *in vitro* tissue culture induced somaclonal variation may not contribute significantly to improve SLW of wheat.

Concentration of abscisic acid (ABA) increases many fold in droughted plants than well watered plants (Chandrasekar et al., 2000; Raziuddin et al., 2010). The accumulated ABA plays a pivotal role in long distance signaling process in plants in response to drought stress (Davies et al., 2002) and is major cause of stomatal closure. Drought tolerant genotypes accumulate more ABA than drought sensitive ones under conditions of water deprivation (Chandrasekar et al., 2000; Guóth et al., 2010), suggesting ABA content as a suitable physiological trait to evaluate drought tolerant and susceptible cultivars (Guóth et al., 2010). The results elucidated that water stress stimulated synthesis of endogenous ABA more in R_1 somaclones than their parent cv. GA-2002 (Figure 4), which in turn induced stomatal closure resulting in higher RWC and succulence of R_1 somaclones and is evident from results on RWC and leaf succulence reported here . Several genes are induced by drought stress (Shinozaki and Shinozaki, 1996) and ABA is synthesized as a result of nuclear gene expression, translation and transcription (Guerrero and Mullet, 1986). The ABA associated gene of R_1 somaclones might be induced during *in vitro* callus adaption phase with repeated selection cycles under PEG induced osmotic stress and the ability was transmitted to the regenerated plants (R_0) and to R_1 progeny thereafter. Based on ABA contents, the somaclones can be regarded as better adapted to drought compared with their parent cv. GA-2002.

The source of enhancement of drought tolerance of R_1 somaclones as is evident from our results may also be attributed to pre-existing variability among cultured cell lines, in addition to somaclonal variations induced during callus culture (Rego and Faria, 2001; Taghian, 2002). Somaclonal variations may be aggravated by the presence of PEG-6000 used as stressing agent *in vitro*. Stressing agents used *in vitro* also act as mutagenic agent for the cultured cell lines (Rego and Faria, 2001; Bressan *et al.,* 1985) and may resulted in methylation alteration in genes, ploidy change or translocation of genes associated with drought adopted traits (Obute et al., 2007). Previous studies and reviews (Rai et al., 2011) show that tissue culture induces somaclonal variations and these variations were exploited to regenerate drought tolerant plants of various crops including wheat (Hasissou and Bouharmont, 1994; Mahmood et al., 2012c), rice (Biswas et al., 2002) and maize (El-Aref, 2002).

Conclusion

Based on physio-chemical drought adopted traits of R_1 selected somaclones and their parent cv-GA-2002, it is inferred that the R_1 selected somaclones regenerated from PEG-6000 tolerant calli are more drought tolerant than their explant donor parent cv. GA-2002. Immature embryo culture in the presence of suitable selection agent can be employed for developing drought tolerant R_1 somaclones of wheat. It is suggested that to confirm whether acquired drought tolerance of R_1 somaclones is of genetic or epigenetic nature, the stability of drought tolerance of somaclones should also be tested in R_2 and R_3 generations.

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