African Journal of Biotechnology Vol. 11(17), pp. 4000-4006, 28 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2610
ISSN 1684ï 5315 © 2012 Academic Journals

Full Length Research Paper

Interaction of callus selection media and stress duration for *in vitro* selection of drought tolerant callus of wheat

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Accepted 16 February, 2012

Callus culture is a novel approach addressing cultured cells as selection units independent of whole plant. Natural variations for drought tolerance existing among cell lines can be exploited *in vitro* in the presence of suitable concentration of osmoticum and stress duration. The study was aimed to standardized callus selection media and culture duration to select drought tolerant cell line (callus) of wheat. Calli were induced from immature embryos on Murashige and Skoog (MS) based medium supplemented with 4 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D). Proliferated calli were cultured on various callus selection medias (MS based media + 30 g/L sucrose + 6 g/L agar + 4 mg/L 2,4-D + various levels of polyethylene glycol-600 (PEG-6000) induced osmotic stress including -0.3, -0.6, -0.9 or -1.2 MPa along with control, that is, 0.0 MPa) for two, three or four weeks. Callus health, callus growth rate, callus survival (%) and regeneration (%) declined significantly on media supplemented with higher levels of PEG-6000 induced osmotic stress and with increasing stress duration. Not a single callus could survive nor regenerate on selection media comprising PEG-6000 induced osmotic stress of -1.2 MPa imposed for four weeks. The highest callus growth rate (CGR) was recorded on PEG-6000 free media when calli were cultured for four weeks (-7.32%) on the media supplemented with PEG-6000 induced osmotic stress of -1.2 MPa. Callus selection media comprising PEG-6000 which induced osmotic stress of -0.9 MPa for four weeks was found selective and sub-lethal for wheat calli with 8.63% CGR, 26.62% callus survival and 21.50% regeneration; and seemed ideal for screening drought tolerant somaclonal cell lines of wheat.

Key words: Interaction, polyethylene glycol-600 (PEG-6000), culture duration, sub-lethal, regeneration, somaclonal variation.

INTRODUCTION

Drought, the most serious threat to world agriculture (Kulkarni and Deshpande, 2007; Aazami et al., 2010) demands breeding for drought prone areas. Classical plant breeding for stressful environment is time consuming and inefficient because of multi-genic tolerance mechanism of plants, lack of well defined selection criteria (Ehsanpour and Jones, 2001) and undesirable genes incorporated during classical breeding (Richards, 1996).

One of the most supportive and promising breeding approaches to achieve stable drought tolerant wheat genotypes is to exploit natural diversity of the gene pool carrying desired genes for drought tolerance. When plant tissues are subjected to tissue culture processes comprising callus phase, these natural variation are amplified and are termed as somaclonal variations (Nhut

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et al., 2000). Somaclonal variation for drought tolerance existing among cell lines can be explored *in vitro* in the presence of suitable osmoticum.

Callus culture is a novel approach dealing with cultured cells as independent selection units rather than the whole plant. This approach involves subjecting a population of de-differentiated mass of cells (callus) to a suitable selection pressure/osmoticum, selecting tolerant cell lines and then to regenerate tolerant somaclones (Matheka et al., 2008). The tolerance operating at unorganized cellular level (callus) can act to some level of effectiveness at the whole plant level as well (Kumar and Kumar, 2000). The approach can be employed for genetic improvement of pre-existing wheat genotypes against biotic and abiotic stresses (Sharma et al., 2010). However, it is obligatory to give consideration to the screening and selection criteria for tolerance. Morp-physiological changes (e.g. callus morphology, callus growth rate, callus survival and callus regeneration) taking place in callus by increasing osmotic stress (Wani et al., 2010; Aazami et al., 2010) can be used as selection criteria for screening drought tolerant cell lines. However, a suitable protocol for selection of tolerant cell lines with reasonable regeneration of surviving calli must be available.

In general, polyethylene glycol (PEG), induced osmotic stress is used for *in vitro* selection of drought tolerant cell/callus of wheat (Abdel Ghany et al., 2004). Too high osmotic stress kills the tolerant cells while suboptimal level of osmoticum results in survival of non tolerant cell lines. In addition, embryogenic callus induction and regeneration of plantlets from selected calli is inversely proportional to increasing concentrations of PEG used in selection media (Matheka et al., 2008). Prolonged callusing period decreases morphogenesis potential, increases frequency of calli with only roots and seedlings with albino shoots (Wen et al., 1991). Therefore, selection media with PEG induced osmotic stress and stress duration is crucial for obtaining drought tolerant plants.

Nonetheless, limited information is available on the interaction of callus selection media and stress duration for *in vitro* selection of tolerant calli. Therefore, the present work was conducted to determine the efficient callus selection media and stress duration for *in vitro* selection of drought tolerant calli of wheat.

MATERIALS AND METHODS

Plant material

Wheat (*Triticum aestivum* L.) variety GA-2002 was selected due to its maximum regeneration potential based on preliminary experimentations conduced in our lab (data not presented).

Evaluation procedure for callus selection media and stress duration

Immature caryopses of cv. GA-2002 were removed about two weeks post-anthesis. The caryopses were surface sterilized for 5 min in 90% ethanol and rinsed three times in sterile distilled water. Caryopses were sterilized again for thirty minutes in 6.5% sodium hypochlorite with 0.1% Tween 20, followed by rinsing with four changes of sterile distilled water. Immature embryos were removed aseptically using forceps and placed on MS based (Murashige and Skoog, 1962) callus induction media (MS + 30 g/L sucrose + 6 g/L agar + 4 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D)) keeping the scutella side upward. pH of the medium was adjusted to 5.7 prior to autoclaving at 121°C for 20 min. The explants were incubated in total darkness at 25±1°C temperature for four weeks. Afterward, induced calli were shifted to callus multiplication media (MS media + 30 g/L sucrose + 6 g/L agar+ 2 mg/L of 2,4-D) for another period of four weeks. The media were refreshed after every 14 to 18 days. Highly globular, white to yellowish in colour, nodular and friable calli were chosen, divided into micro-clumps of equal size and were transferred on to various callus selection media described subsequently for two, three or four weeks. Villela et al. (1991) table was used for relationship between osmotic potential and concentration of PEG-6000 solution; negating osmotic potential of MS based medium and gelling agent which was found to be -0.3 MPa. The calli were sub-cultured on fresh selection media after every week. The experiment was laid out according to completely randomized design (CRD) with factorial arrangement replicated four times.

Treatments

Callus selection media:

M1 = MS + 30 g/L sucrose + 6 g/L agar + 4 mg/L 2,4-D (control),
M2 = MS + 30 g/L sucrose + 6 g/L agar + 4 mg/L 2,4-D + PEG-6000 induced osmotic stress of -0.3 MPa,
M3 = MS + 30 g/L sucrose + 6 g/L agar + 4 mg/L 2,4-D+ PEG-6000 induced osmotic stress of -0.6 MPa,
M4 = MS + 30 g/L sucrose + 6 g/L agar + 4 mg/L 2,4-D+ PEG-6000 induced osmotic stress of -0.9 MPa,
M5 = MS + 30 g/L sucrose + 6 g/L agar + 4 mg/L 2,4-D+ PEG-6000 induced osmotic stress of -1.2 MPa.
Stress level and culture duration at which 25 to 30% calli survived with reasonable regeneration was considered sub-lethal and most suitable to screen drought tolerant calli of cv. GA-2002.

Visual characterization of callus

The calli were frequently observed and characterized as follow: A, No browning (excellent); B, callus with slightly brown surface (very good); C, whole callus tissue brown with optimum growth (good); D, entire callus tissue deeply brown with suboptimal growth (moderate); E, whole callus tissue deeply brown with no growth at all (poor).

Callus growth rate (CGR) %

The callus growth rate in stress (M_2, M_3, M_4 and M_5) and unstressed medium (M_1) was measured in terms of percent increase in fresh weight. Micro clumps of calli approximately of almost equal size with known weight (100 ± 10 mg) were incubated for two, three or four weeks on selection media. At the end of stress period, random samples of calli (n = 5 to 15) were weighted aseptically, averaged and callus growth rate was determined using following formula:

$$CGR = \left(\frac{Final\ weight - Initial\ weight}{Initial\ weight} \right) \times 100$$

Figure 1. (A) Callus culture on M_1 for two weeks; (B) callus culture on M_4 for four weeks.

Callus survival (%) after stress

The calli cultured on various callus selection medias for two, three or four weeks were shifted onto recovery media devoid of PEG-6000 (MS + 30 g/L sucrose + 6 g/L agar + 4 mg/L 2,4-D) for a period of three weeks in total darkness for recovery and proliferation of putative drought tolerant cell lines. The calli which survived and continued to proliferate were counted and callus survival percentage was computed as:

$$Callus\ survival\ (\%) = \left(\frac{No.\ of\ calli\ survived}{Total\ no.\ of\ calli\ cultured} \right) \times 100$$

Regeneration (%) of calli after stress

The calli were cultured on various callus selection media for two, three or four weeks and then shifted to regeneration medium (MS + 30 g/L sucrose + 6 g/L agar + 0.2 mg/L indole-3-acetic acid (IAA) + 0.5 mg/L kinetin + 0.5 mg/L of 6-benzylaminopurine (BAP)), previously optimized for cv. GA-2002 in our Lab. The calli were incubated at 26°C temperature with 16 h light and 8 h dark photoperiod. The regeneration medium was refreshed after every 12 TO 15 days. Regeneration (%) of calli was computed as:

$$Callus\ regeneration\ (\%) = \left(\frac{No.\ of\ calli\ regenerated}{Total\ no.\ of\ calli\ cultured} \right) \times 100$$

Statistical analysis

Analysis of variance (ANOVA) was executed and treatment means were compared by least significant difference (LSD) test at 5% probability level using MSTATC software (Freed and Eisensmith, 1986).

RESULTS AND DISCUSSION

Visual observations (callus health)

In general, increase in callus browning was recorded with increasing level and duration of stress. Calli cultured on stress free medium (M_1) were found to be healthier with unrestricted growth (excellent) than those on the stressed media. Calli subjected to M_5 (-1.2 MPa) for four weeks exhibited very poor health. Callus culture for two weeks on M_1 or M_2 did not affect callus health and callus looked light yellow to whitish in color with no browning (Figure 1). Whole callus tissues were brown when calli were cultured for two weeks on M_5 or for three weeks on M_4 or for four weeks on M_3 with optimum callus growth (Figure 1). The calli exhibited good to moderate health when cultured on medium with osmotic stress of -0.9 MPa (M_4) for three or four weeks, respectively (Table 1).

Callus browning rate was found to be good indicator of callus sensitivity to PEG-6000 induced osmotic stress. It seemed that tolerant calli had lower browning rates at higher levels of osmotic stress and survived. At higher level of osmotic stress, toxicity in addition to water paucity may deter cell growth. Exposure of calli for four weeks to M_5 (-1.2 MPa) spoiled them with evident necrosis. Necrosis was more on the surface of the callus facing medium. These observations are in line with those of Hassan et al. (2004). They reported that *in vitro* osmotic stress of -1.0 MPa for eight months of culture onto PEG-6000 containing media is lethal for sunflower calli with evident necrotic tissues on callus surface.

It can be revealed that callus culture of wheat for three or four weeks on MS based media containing PEG-6000 induced osmotic stress of -0.9 MPa (M_4) had sub-lethal effect on callus health and is selective for calli of cv. GA-2002, allowing drought tolerant calli to survive.

Growth kinetics (Callus growth rate) %

Interaction of various callus selection media and stress duration showed significant effect on callus growth rate (Table 2). Progressive increase in CGR was observed when calli were incubated on M_1 or M_2 for two, three or four weeks. Highest CGR of 277.86% was witnessed when calli were cultured for four weeks on M_1 (no stress).

Table 1. Effect of various callus selection medium and stress duration on callus health of what cv. GA-2002 (visual observations).

Stress duration	Callus selection media				
	M_1	M_2	M_3	M_4	M_5
2 weeks	A (excellent)	A (excellent)	B (very good)	B (very good)	C (good)
3 weeks	A (excellent)	B (very good)	B (very good)	C (good)	D (moderate)
4 weeks	A (excellent)	B (very good)	C (good)	D (moderate)	E (poor).

A, No browning (excellent); B, callus with slightly brown surface (very good); whole callus tissue brown with optimum growth (good); C, entire callus tissue deeply brown with suboptimal growth (moderate); D, whole callus tissue deeply brown with no growth at all (poor).

Table 2. Effect of PEG-6000 induced osmotic stress and stress duration on callus growth rate (%).

Stress duration	Callus selection media					
	M_1	M_2	M_3	M_4	M_5	Mean
2 weeks	203.09^{c}	112.10^{f}	46.89^{h}	33.51^{i}	15.78^{jk}	82.28^{b}
3 Weeks	266.61^{b}	128.38^{e}	59.41^{g}	19.38^{j}	3.74^{l}	95.50^{a}
4 weeks	277.86^{a}	142.88^{d}	39.21^{hi}	8.63^{kl}	-7.32^{m}	92.25^{a}
Means	249.19^{a}	127.78^{b}	48.50^{c}	20.51^{d}	4.07^{e}	

Entries sharing similar letters do not differ significantly at 5% probability level. LSD values: **, weeks = 4.776; **, Osmotic stress = 6.165; **, weeks × osmotic stress = 10.678; *, non-significant; **significant.

CGR declined from M_1 to M_5 with increasing osmotic stress irrespective of stress duration. It was noticeable that on M_3 (-0.6 MPa) initially CGR increased from 46.89 to 59.41% up to three weeks and then regress to 39.21% when stress duration was increased to four weeks. CGR progressively declined on M_4 and M_5 with increasing stress duration. CGR of 8.63% was recorded on M_4 when calli were incubated for four weeks. The least CGR of -7.32% (negative growth rate) was witnessed on M_5 when stress duration was increased to four weeks preceded by CGR of 3.74% on M_5 with three week culture period.

Reduced cell growth is the most immediate and sensitive response of the plants to osmotic stress (Levitt, 1980). The growth of the calli is significantly restricted by increasing and continuous presence of PEG in the media (Hsissou and Bouharmont, 1994; Dragiiska et al., 1996) over time. Decline in CGR% on media containing higher PEG-6000 induced osmotic stress might be due to reduced cell division, shrinking cytoplasmic volume and loss of cell turgor, nutritional imbalance due to reduced up take of water, an increase in electrolyte leakage and decrease in cell water contents with increasing stress (Lokhande et al., 2010). Earlier, similar results were reported in wheat (Barakat and Abdel-Latif, 1995), sunflower (Hassan et al., 2004), and rice (Wani et al., 2010).

The results show that callus growth rate anticipated on fresh weight basis exhibited negative values (-7.32%) on M_5 (-1.2 MPa) with stress duration of four weeks (Table 2). It indicates that no growth was evident at this level of stress, indicating dehydration induced death of cell lines and loss of water contents from dead cells. It is worth accentuated that callus growth on M_4 (-0.9 MPa) for four weeks culture period was very small but not lethal, while incubation of calli on M_5 for three or four weeks seemed lethal. Incubation of calli on M_4 for four weeks is critical inhibitory and sub-lethal and can be used for selecting drought tolerant somaclonal cell lines of wheat. However, the critical inhibitory/sub-lethal levels of PEG-6000 induced osmotic stress in the selection media may vary depending upon molecular weight of PEG and species under investigation. For example, for sunflower, -0.8 MPa stress from PEG-6000 for three months culture period (Hassan et al., 2004) and for *Triticum durum,* 25% of PEG-10000 for six months (Hsissou AND Bouharmont, 1994) are reported as critical inhibitory for callus growth.

Survival (%) of stressed calli

Callus survival percentage was significantly affected with culture time, type of selection media and their interaction (Table 3). Callus survival percentage decreased with increasing PEG-6000 induced osmotic stress in culture media and stress duration. Significant effect of interaction helped to identify selection media and stress duration to be imposed for selecting drought tolerant cell lines of wheat. Callus culture on M_1 either for two, three or four weeks did not show significant effect on callus survival. However, callus survival percentage declined significantly on all other selection media with increasing incubation period of calli (Table 3). Most of the calli which were incubated on M_2, M_3, M_4, and M_5 for period of two weeks recovered with recovery percentage of 91.75, 85.00, 72.12 and 54.50%, respectively. Callus culture on M_4 selection media (-0.9 MPa) for four weeks resulted in

Table 3. Effect of PEG-6000 induced osmotic stress and stress duration on callus survival (%) of what cv. GA-2002.

Stress duration	Callus selection media					
	M_1	M_2	M_3	M_4	M_5	Mean
2 weeks	97.62[a]	91.75[b]	85.00[c]	72.12[e]	54.50 [f]	80.20 [a]
3 weeks	96.25[a]	86.75[c]	76.50[d]	35.12[h]	14.62 [j]	61.85 [b]
4 weeks	94.50[ab]	78.12[d]	44.12[g]	26.62[i]	00.00 [k]	48.68 [c]
Means	96.12[a]	85.54[b]	68.54[c]	44.62[d]	23.042 [e]	

LSD values: **, weeks=1.407, **, Osmotic stress = 1.816, **, weeks × osmotic stress=3.146; *, non-significant; **, significant.

Table 4. Effect of PEG-6000 induced osmotic stress and stress duration on callus regeneration (%) of what cv. GA-2002.

Stress duration	Callus selection media					
	M_1	M_2	M_3	M_4	M_5	Mean
2 weeks	60.50[a]	51.25[bc]	46.25[cd]	35.50[fg]	26.75[h]	44.05[a]
3 weeks	57.75[a]	48.50[c]	38.75[ef]	31.00[gh]	12.75[j]	37.75[b]
4 weeks	56.000[ab]	41.75[de]	31.25[gh]	21.50[i]	00.00[k]	30.10[c]
Means	58.08[a]	47.17[b]	38.75[c]	29.33[d]	13.17[e]	

LSD values: **, weeks = 2.290; **, Osmotic stress = 2.957; **, weeks × osmotic stress = 5.122; *, non-significant; **, significant.

survival of 26.62% of the callus lines. Majority of the calli cultured for three weeks on M_5 were unable to recover and remained necrotic with recovery percentage of 14.62%. Incubation of calli for a period of four weeks on M_5 (-1.2 MPa) was found highly lethal and not a single callus was able to tolerate this extent of stress (Table 3).

The declining trend in survival rate of calli with increasing osmotic stress in selection media is in accordance with those reported by AbdElGhany et al. (2004) and Hassanein (2010). Calli cultured for four weeks on M_5 were unable to recover and remained necrotic. Necrosis intensity was even increased after culturing onto recovery medium indicating that cells were predominantly permanently wilted during stress and were unable to regain turgor and died. These findings are in complement with those reported by Matheka et al. (2008) in maize, Biswas et al. (2002) in rice and by Abdel Ghany et al. (2004) in wheat. Mohamed et al. (2000) had also reported almost similar observation for shoot clumps of *Tagetes minuta* cultured on media comprising above sub-optimal level of mannitol (80 mM) as osmoticum.

The results show that selection medium containing sub-optimal levels of osmotic stress (M_2) facilitated the cell lines to acclimate to unfavorable conditions over time, reducing the possibility of sorting out genetically drought tolerant callus/cell lines. Callus culture on medium containing intermediate level of osmotic stress (M_3) for three or four weeks seemed to be selective; while, callus culture on medium with higher level of osmotic stress (M_4) for four week was proved to be more selective (survival 26.62%). Culturing calli on M_5 for three weeks is extremely selective and may even kill potentially tolerant calli (survival 14.62%). The survival of only small fraction of cell lines incubated for four weeks on M_4 or for three weeks on M_5 indicated their inbuilt tolerance at cellular level or possible induction of *de novo* synthesized drought tolerance during *in vitro* culture (Biswas et al., 2002). Exposure of callus on M_5 for four weeks was found lethal and none of the calli survived and which may be associated with increased activities of RNase and DNase (Yupsanis et al., 2001) or injury to cells by over production of reactive oxygen species (ROS) under severe water deficiency (Abdellaoui et al., 2010).

It can be concluded that callus culture on M_4 selection media (osmotic stress -0.9 MPa) for four weeks is sub-lethal and selective with 26.62% of callus survival. The surviving calli at these treatments grew into compact, bright white and friable callus with globular structure when cultured on recovery media and could be designated drought tolerant. The observations are supported by Hassan et al. (2004) who found that callus culture on media containing osmotic stress of -0.8 MPa for three months is sub-lethal and ideal to select for drought tolerant cell lines of sunflower.

Regeneration (%) of stressed calli

The regeneration potential of wheat calli of cv. GA-2002 after exposure to PEG-6000 induced osmotic stress is presented in Table 4. The interaction of callus selection

media and stress duration was significant. The regeneration potential of calli decreased significantly with increasing stress duration and osmotic stress in culture media (Table 4). The highest regeneration was witnessed (60.50%) when calli were exposed for a period of two weeks on M_1, that is, media devoid of PEG-6000. Regeneration of calli incubated on M_1 for 2, 3 or 4 weeks was not influenced significantly. Incubation of calli on M_4 for four weeks resulted in 21.50% regeneration. The least regeneration of 12.75% was recorded for calli cultured on M_5 for three weeks. Contrary, not a single plant was regenerated from calli which were incubated for four weeks on M_5 (Table 4).

Addition of PEG-6000 in solid media lowers water potential of the medium that adversely affect cell division leading to reduced callus growth and consequently influences regeneration (Ehsanpour and Razavizadeh, 2005; Sakthivelu et al., 2008). Also, regeneration ability of explants is usually decreased by repeated subculture over time in many plants (Dragiiska et al., 1996; Mohamed et al., 2000). A parallel decrease in plantlet regeneration with increasing *in vitro* osmotic stress was reported in rice (Biswas et al., 2002; Wani et al., 2010), wheat (Barakat and Abdel-Latif, 1995) and tomato (Aazami et al., 2010).

Drought stress causes profuse mutation in cellular metabolism including protein functioning and alteration in amount of proteins (Plomion et al., 1999). The decrease in regeneration frequency on media with higher osmotic stress may be due to altered gene expression (Visser, 1994) controlling this trait or the genes may express themselves but the resultant proteins may be denatured due to increased stress. The motives behind regenerative potential loss are not clearly legitimated and may be consequence of malfunctioning or loss of substances supporting regeneration of tissues, epigenetic changes or somaclonal variations (George, 1993).

On the bases of results of regeneration on various callus selection media and stress duration it seemed that incubation of wheat calli on M_4 for four week is optimum to regenerate potentially drought tolerant somaclones with increased mutation.

Conclusion

Interactive effect of callus selection media comprising PEG-6000 induced osmotic stress and stress duration had significant effect on callus morphology, callus growth rate, survival and regeneration and should be given consideration for screening drought tolerant callus lines of wheat. Incubation of wheat calli derived from immature embryos on callus selection media supplemented with PEG-6000 induced osmotic stress of -0.9 MPa for four weeks seemed sub-lethal and can be expected to kill non-tolerant calli and allow only tolerant ones to survive with reasonable regeneration potential.

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