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IN VITRO SCREENING AND REGENERATION OF WATER STRESS TOLERANT CULTURE OF TOMATO

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SUMMARY

Callus of tomato (*Lycopersicon esculentum* Mill.) was initiated from hypocotyls on MS medium supplemented with 3 mg l^{-1} BAP + 0.50 mg l^{-1} NAA. For proliferation of callus the hormone concentrations were reduced to half. Cell clumps of about 1mm diameter were exposed to increasing concentrations of polyethylene glycol (PEG-6000) ranging from 10 g l^{-1} to 100 g l^{-1} for water stress tolerance. Upon incubation for 40 days the cells, which could tolerate this concentrations of PEG, grew to form calli. Selected calli were further subcultured on the selective medium for proliferation. The selected calli when transferred from the normal to the selective medium, were capable of growing on it. The shoot regeneration from the *in vitro* selected water stress tolerant calli was obtained in MS medium supplemented with 100 g l^{-1} PEG + 2 mg l^{-1} BAP+ 1 mg l^{-1} IAA. The *in vitro* regenerated shoots were transferred on MS medium supplemented with 100 g l^{-1} PEG + 1.25 mg l^{-1} BAP for shoot elongation. High percentage of root regeneration from *in vitro* regenerated shoots was obtained in MS medium supplemented with 100 g l^{-1} PEG + 0.1 mg l^{-1} NAA.

Key worlds: **Callus culture, regeneration, tomato, water stress.**

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable crop grown all over the world. It is an annual vegetable crop of wide spread culture and popularity. In India, tomato has become an important vegetable during the past half a century and presently occupies an area of 5, 20,000 ha with production of 85, 00,000 MT (Ananymous 2003). Deleterious effects of water deficit can be envisaged at any stage of plant growth and development. The physiological responses of the plant to drought and the many characters implicated in drought tolerance are extremely complex and vary with the type of plant as well as the degree and the time of exposure to drought. Considerable progress has been made in the understanding of physiological and metabolic changes influenced by developing water deficit (Hsiao 1973), and the effect of these physiological changes on growth and yield. There is an increasing awareness of the potential and limitation of the technique of tissue and cell culture for the production of novel genotypes with valuable attributes for agriculture particularly in relation to water tolerance (Nabors *et al*. 1980, Kumar and Sharma 1989, Siddeswar and Kavi Kishore, 1989). However, water stress tolerance cell culture also represents an ideal system for assessing the physiological effects of water stress at the cellular level (Handa *et a*l. 1983). The potential of *in vitro* cell selection for the production of novel genotype with valuable attributes for agriculture has attracted considerable attention and resulted in a number of reports on the selection of abiotic and biotic stress tolerance cell lines/plants (Nabors *et al.*

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1980, Handa *et al.* 1982, McHughan and Swartz 1984, Hasegawa *et al.* 1986, Pua and Thorpe 1986, McCoy 1987, Kumar and Sharma 1989, Siddeswae and Kavi Kishore 1989). The present study deals with *in vitro* screening and regeneration of water stress tolerant callus of tomato using polyethylene glycol (PEG) in the medium. The selected cells have been characterized for growth behaviors and shoot regeneration.

MATERIALS AND METHODS

Establishment of callus culture: Callus cultures of tomato (*Lycopersicon esculentum* Mill) cv. Solan vajar were initiated from the hypocotyls segments of aseptically grown seedlings. Hypocotyl explants (0.5-1.0 cm long) were cultured on the MS medium (Murashige and Skoog, 1962) supplemented with BAP (3 mg l^{-1}) and NAA (1 mg l^{-1}) (Fig. 1 A&B). All the cultures were incubated under 5000 lux at 25± 2 °C with 16 h photoperiod. The MS medium supplemented with 3 % sucrose, 100 mg l^{-1} inositol gelled with 0.8 % agar and pH adjusted to 5.8 used as the basal medium. For sub-culturing of callus, concentration of the hormones was reduced to half. The calli were sub-cultured at an interval of 4 weeks.

Selection of water stress tolerant cell lines: Cell clumps of about 1mm diameter were exposed to different concentration of polyethylene glycol (PEG-6000) ranging from 10 g l^{-1} to 100 g l^{-1}. The cells were sub-cultured on the same selective medium for 6 weeks to check their stability and then transferred on to normal MS medium (1.5 mg l^{-1} BAP + 0.25 mg l^{-1} NAA) without PEG for further proliferation. Selected and non-selected callus were characterized for their growth behavior.

Measurement of growth: The growth of *in vitro* selected and non-selected cell lines (control) were studied on normal MS medium supplemented with BAP 1.5 mg l^{-1} + NAA 0.25 mg l^{-1} and selective MS medium containing 100 g l^{-1} PEG in addition to BAP 1.5 mg l^{-1} + NAA 0.25 mg l^{-1}. The callus was cut into small pieces. Three pieces of callus each weighing approximately 159±0.2 mg was cultured in each flask. Subsequently at 10 days interval the callus from the flask was removed and its fresh weight was determined. It was then oven dried at 80 °C for 48 h till constant dry weight. This procedure was repeated till 40 days after inoculation.

Regeneration of tomato plants: Selected calli were excised from 40-45 days old water stress tolerant calli. The selected calli were cut into small pieces of 0.1 to 1.0 cm^2 in size. These small pieces were cultured on the MS medium supplemented with various combinations and concentration of BAP, kinetin, IAA, and NAA. Frequency of shoot bud regeneration varied with hormonal combinations and concentrations in the medium with earlier reports (Venkatachalam *et al.* 2000). The maximum numbers of shoots per explant were found in MS medium supplemented with PEG 100 g l^{-1} + BAP 2 mg l^{-1} + IAA 1 mg l^{-1}. Elongated shoot (about 2-3 cm in length) obtained from the *in vitro* selected water stress tolerant calli were excised and cultured on MS medium supplemented with various concentration of different auxins i.e. IAA, NAA and IBA for rooting. The periods for induction of roots were variable among various concentrations of different auxins but it was generally after 10-12 days in culture.

RESULTS AND DISCUSSION

Selection of PEG tolerant cell line: Callus tissue of higher plants have been used to select numerous variant cell lines (Crroughan *et al.* 1978), the variant cells are usually selected from a population of cells by imposing a particular stress on the population. It may be possible to select mutations from increased drought tolerance at the cellular level using tissue culture. In the present investigation, PEG-6000 was used as osmotic agent because it stimulates water stress by acting as a non-penetrating osmotic agent which lowers the water potential of the medium. Thus cells resistant to water stress might be selected from a population by using PEG as a result of which water stress occurred in the selective medium containing 100 gl^{-1} PEG and viable clones (only 2-3 clones) were recovered from the cultures containing 100 gl^{-1} PEG (Fig. 1C). Selected clones were sub-cultured on the selective medium for 6 weeks and transferred to the normal MS medium (Fig. 1D&E). To check the stability of tolerance at cellular level selected calli were transferred from the normal to the selective medium (with the same concentration of PEG) (Fig. 1F). The selected clones were capable of growing on it. Selection of cell lines resistant to water stress was done by exposing callus to a medium containing (PEG) the cells grew much better than the

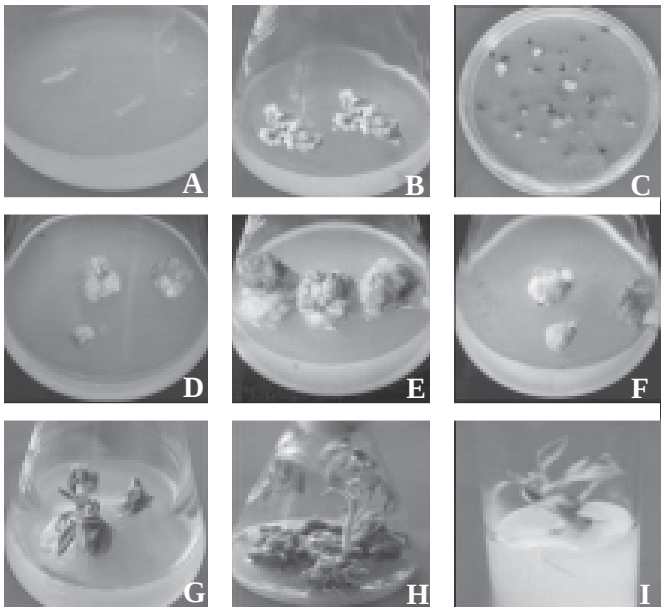

Fig. 1. A. Callus initiation from hypocotyl explants on MS medium containing BAP (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}), B. Growth of callus from hypocotyl explants after 30 days in culture (MS + BAP 3 mg l^{-1} + NAA 0.5 mg l^{-1}), C. Growth of calli on the selective medium (MS + 100 g l^{-1} PEG + 1.5 mg l^{-1} BAP + 0.25 mg l^{-1} NAA) after 3 weeks interval, D. Selected calli growing on fresh selective medium (MS + 100 g l^{-1} PEG + 1.5 mg l^{-1} BAP + 0.25 mg l^{-1} NAA) after 6 weeks, E. Growth of non-selected calli on selective medium (MS + 100 g l^{-1} PEG + 1.5 mg l^{-1} BAP + 0.25 mg l^{-1} NAA) after 6 weeks, F. Growth of selected calli on normal medium (MS + 1.5 mg l^{-1} BAP + 0.25 mg l^{-1} NAA) after 6 weeks interval, G. Shoot bud regeneration from water stress tolerant calli on MS medium supplemented with BAP (2 mg l^{-1}) and IAA (1 mg l^{-1}) with (100 g l^{-1}) PEG, H. Shoot bud elongation on MS medium supplemented with BAP (1.2 mg l^{-1}), + (100 g l^{-1}) PEG, I. Root induction from the elongated shoot on MS medium containing NAA (0.1 mg l^{-1}) and (100 g l^{-1}) PEG

controlled unselected cells on medium containing non-penetrating osmoticum recorded by Bressan *et a*l. (1981). When studying the growth pattern of the selected and non-selected cell lines on both normal and selective medium, there was a steady increase in dry weight with the increase in fresh weight. The non-selected callus showed a higher growth rate on normal medium than the selected callus. This may be attributed to the fact that the selected callus was under stress conditions and so when it was transferred into normal medium, the growth reduced as result of time taken for acclimatization to the normal medium. However, selected callus grew better on selective medium than non-selected callus which showed a continuous decrease in fresh weight. This may be due to stress condition of selected callus which enabled it to adopt itself better than the non-selected callus in the selective medium. The cells were selected at 100 g l^{-1} PEG upon incubation for 30 days, most of the callus died but only the few cells which could tolerate this concentration of PEG grew to form cell clones. Selected clones were successfully sub-cultured on the selective medium 100 g l^{-1} PEG for 8 weeks and then transferred to the normal MS medium has also been reported in tomato by Srivastava *et al*. (1995). The selected calli when transferred from the normal to the selective medium, were capable of growing on it. It has also been reported in bell pepper by Nath *et al. (*2005). The use of *in vitro* cell selection for the isolation of water stress tolerant cell line has also been reported in several crops like carrot (Fallon and Phillips, 1989), chilli pepper (Santos Diaz *et al.* 1994), wheat (Islam *et al.* 1998),

Table 1. Simple correlation between fresh and dry weight of normal callus and selected callus on normal and selective MS medium (BAP 1.5 mgl^{-1} + NAA 0.25 mgl^{-1} + PEG 100 gl^{-1})

Variables	Values of r* (Correlation value)
Fresh and dry weight of selected callus on selective MS medium	+0.999
Fresh and dry weight of selected callus on normal MS medium	+0.991
Fresh and dry weight of non-selected callus on selective medium	+0.996
Fresh and dry weight of non-selected callus on normal MS medium	+0.950
Dry weight of selected callus and dry weight of normal callus on selective MS medium	-0.901
Dry weight of non-selected callus and dry weight of selected callus on normal MS medium	+0.967
Fresh weight of selected callus and fresh weight of non-selected callus on selective MS medium	-0.859
Fresh weight of non-selected callus and fresh weight of selected callus on normal MS medium	+0.980

*Significant at 5% level of significance

ground nut (Purushotham *et al.* 1998) and tomato (Cheng *et al.* 2002). When the growth data were expressed as ratio of the final weight for the two cell lines, it was noted that the ratio remained fairly constant (Table 1), thereby indicated that both the cell lines grew well on the normal MS medium. A significant positive correlation was observed during the growth experiment between fresh and dry weight of non-selected cell lines (0.996), fresh and dry weight of water stress tolerant cell lines (0.999), dry weight of non-selected and water stress tolerant cell lines (0.967) and fresh weight of non-selected and water stress tolerant cell lines (0.980).

Shoot regeneration from water stress tolerant calli: Regeneration ability of water stress tolerant calli was tested on MS medium containing different combinations and concentrations of BAP+IAA+100g l^{-1}PEG and Kn+ IAA+ 100g l^{-1}PEG. Out of eight combinations of BAP and NAA in selected MS medium tried maximum number of shoots per explant were found in I-AP8 (0.98) with 38.51 percent of explants showed regeneration, which was superior to all other combinations under study (Table 2 and Fig. 1G). High frequency of plant regeneration from hypocotyls on MS medium supplemented with different concentrations and combinations of various auxins and cytokinins, BAP was the most effective plant growth regulator, which has also been shown earlier in tomato by Venkatachalam *et al.* (2000). Shoot elongation occurred when regenerated shoots were transferred to the medium with lower cytokinin concentration and best result was observed with MS+1.25mg l^{-1} BAP (Table 3 and Fig. H).

Table 2. Effect of various combinations and concentrations of BAP+IAA and kinetin +IAA on shoot regeneration from *in vitro* selected calli of tomato

Treatment/ medium	Growth hormones	Average number of shoots formed per calli	Percent callus showing regeneration
I-AP1	MS+PEG 100 g l^{-1}+BAP 1 mg l^{-1}+IAA 0.2 mg l^{-1}	0.17	7.17 (15.54)
I-AP2	MS+PEG 100 g l^{-1}+BAP 2 mg l^{-1}+IAA 0.2 mg l^{-1}	0.32	16.70 (24.12)
I-AP3	MS+PEG 100 g l^{-1}+BAP 2.5 mg l^{-1}+IAA 0.2 mg l^{-1}	0.11	3.04 (10.05)
I-AP4	MS+PEG 100 g l^{-1}+BAP 1 mg l^{-1}+IAA 0.5 mg l^{-1}	0.20	8.12 (16.56)
I-AP5	MS+PEG 100 g l^{-1}+BAP 2 mg l^{-1}+IAA 0.5 mg l^{-1}	0.46	16.95 (24.32)
I-AP6	MS+PEG 100 g l^{-1}+BAP 2.5 mg l^{-1}+IAA 0.5 mg l^{-1}	0.98	36.58 (37.22)
I-AP7	MS+PEG 100 g l^{-1}+BAP 1 mg l^{-1}+IAA 1 mg l^{-1}	0.16	22.09 (28.03)
I-AP8	MS+PEG 100 g l^{-1}+BAP 2 mg l^{-1}+IAA 1 mg l^{-1}	0.98	38.51 (38.36)
III-AP1	MS+PEG 100 g l^{-1}+Kn 1 mg l^{-1}+IAA 0.2 mg l^{-1}	0.04	3.65 (10.91)
III-AP2	MS+PEG 100 g l^{-1}+Kn 2 mg l^{-1}+IAA 0.2 mg l^{-1}	0.16	8.60 (17.05)
III-AP3	MS+PEG 100 g l^{-1}+Kn 2.5 mg l^{-1}+IAA 0.2 mg l^{-1}	0.20	19.11 (25.97)
III-AP4	MS+PEG 100 g l^{-1}+Kn 1 mg l^{-1}+IAA 0.5 mg l^{-1}	0.12	9.28 (17.73)
III-AP5	MS+PEG 100 g l^{-1}+Kn 2 mg l^{-1}+IAA 0.5 mg l^{-1}	0.13	16.00 (23.58)
III-AP6	MS+PEG 100 g l^{-1}+Kn 2.5 mg l^{-1}+IAA 0.5 mg l^{-1}	0.15	14.70 (22.54)
III-AP7	MS+PEG 100 g l^{-1}+Kn 1 mg l^{-1}+IAA 0.1 mg l^{-1}	0.30	24.07 (29.38)
III-AP8	MS+PEG 100 g l^{-1}+Kn 2 mg l^{-1}+IAA 1 mg l^{-1}	0.23	17.82 (24.97)
CD at P=0.05		0.059	0.580 (0.415)

Transformation value of per cent shoot regeneration given in parenthesis

Table 3. Effect of cytokinins on shoot elongation of *in vitro* raised shoots

Medium no.	Medium	Relative shoot elongation
PP1	MS+100 g l^{-1} PEG+1 mg l^{-1} Kn+0.1 mg l^{-1} IAA	+
PP2	MS+100 g l^{-1} PEG+1 mg l^{-1} Kn	++
PP3	MS+100g l^{-1} PEG+2 mg l^{-1} BAP	+
PP4	MS+100 g l^{-1} PEG+1.25 mg l^{-1} BAP	+++

+++, ++ and +: Representing relatively fast, medium and slow shoot elongation respectively

Rooting of in vitro shoots: Elongated shoots (about 2-3 cm in length) obtained from the *in vitro* selected water stress tolerance calli were excised and cultured on selected MS medium supplemented with various concentrations of different of auxins i.e. IAA, NAA, and IBA. The period for induction of roots was variable among various concentrations of different auxins but it was generally after 10-12 days in culture. The percentage of rooted shoots reached above 45 percent within 20 days in culture. All the treatments were statistically significant from one another. It is clear from the data that MS medium supplemented with NAA resulted in highest percentage of root regeneration (48.85 %). Whereas, MS medium supplemented with IBA, resulted in lowest percentage (14.74 %) of root regeneration (Table 4 and Fig. 1I).

Present investigation shows that MS medium supplemented with different concentrations of PEG resulted in the selection of water stress tolerant calli. High efficiency of water stress tolerant calli could be obtained on the medium containing 100 g l^{-1} PEG under four weeks of incubation period. PEG improves water stress tolerance, which is an important attribute under drought conditions. During the entire study high frequency shoot regeneration could not be obtained. Still more work is required to standardize the method for high frequency shoot regeneration from water stress tolerant calli in the cultivar 'Solan vajar' of tomato.

Table 4. Effect of various combinations & concentrations of IAA, NAA and IBA on percent root regeneration from *in vitro* raised shoot

Treatment/ medium	Medium	Percent root regeneration
I-RM1	MS+100 g l^{-1}PEG+0.5 mg l^{-1}IAA	36.4 (37.08)
I-RM2	MS+100 g l^{-1}PEG+0.10 mg l^{-1}IAA	34.74 (36.11)
II-RM3	MS+100 g l^{-1}PEG+0.20 mg l^{-1}NAA	45.50 (42.34)
II-RM1	MS+100 g l^{-1}PEG+0.5 mg l^{-1}IAA	43.17 (41.07)
II-RM2	MS+100 g l^{-1}PEG+0.10 mg l^{-1}NAA	48.85 (44.34)
II-RM3	MS+100 g l^{-1}PEG+0.20 mg l^{-1}NAA	32.89 (35.00)
III-RM1	MS+100 g l^{-1}PEG+0.5 mg l^{-1}IBA	20.70 (27.06)
III-RM2	MS+100 g l^{-1}PEG+0.10 mg l^{-1}IBA	16.30 (23.81)
III-RM3	MS+100 g l^{-1}PEG+0.20 mg l^{-1}IBA	14.74 (22.57)

Transformation value of per cent shoot regeneration given in parenthesis

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