Effect of water stress and heavy metals on induction of somatic embryogenesis in wheat leaf base cultures

Debasis Patnaik, A Mahalakshmi & Paramjit Khurana*
Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, Dhaula Kuan, New Delhi 110 021, India

Received 14 March 2005; revised 5 May 2005

In vitro cultures of plant tissues are known to mimic the response of field-grown plants when subjected to stress treatments. This investigation on *Triticum aestivum* explores the effect of drought stress on somatic embryogenesis and endogenous proline content. Leaf bases were cultured on MS medium supplemented with 2,4-D (10 μM) and different concentrations of PEG (2.5, 5, 7.5%) or mannitol (0.25 and 0.5 M) and also subjected to different periods of aerial drying in the laminar flow for one-day and subsequently transferred to MS basal medium. PEG treatment induced a high percentage (up to 50%) of embryoid formation. However, with mannitol and aerial drying, percentage of embryoid formation decreased with increasing concentrations and duration. After ten days, the endogenous proline content of explants treated with different concentrations of PEG, mannitol and different durations of aerial drying increased with increasing concentration and increasing duration of the treatment, thus, corroborating the role of proline as an osmolyte during stress conditions. Similarly, addition of metals such as cadmium and cobalt caused a reduction in percentage explants depicting embryogenesis. However, when cadmium was employed alone, 22% explants displayed somatic embryogenesis as compared to 54% in 2,4-D treated cultures.

Keywords: Cadmium, Heavy metals, Somatic embryogenesis, Water stress, Wheat

Leaf base explants of wheat provide a model system for the understanding of somatic embryogenesis by short-term auxin treatment. This work was initiated to study correlation, if any, simulated stress treatment and induction of somatic embryogenesis. Leaf bases when treated with the plant growth regulator 2,4-D for durations as short as one day undergo somatic embryogenesis after transfer to an auxin free basal medium and subsequently regenerate into plantlets. Although the importance of a mild osmotic stress in cell growth and plant regeneration in vitro is well-documented, the underlying mechanism remains obscure. Several studies involving late embryogenesis proteins also confirm how a normal physiological process such as embryo desiccation and stress can overlap during normal embryo development. Recent evidences indicate proline may act as an osmoprotectant by interacting with crucial macromolecules of the cell and modulating their biological activity. This study was, therefore, undertaken to analyze the effect of different treatments mimicking water stress, namely, mannitol and polyethylene glycol on the induction of somatic embryogenesis in wheat leaf base cultures, earlier optimized for differentiation of somatic embryogenesis. This study indicated a positive correlation between different stress treatments with accumulation of endogenous proline as well as induction of somatic embryogenesis.

Since induction of somatic embryogenesis by 2,4-D has been extrapolated to be similar to a stress response, and various metal ions are reported to be effective modulators of somatic embryogenesis, in the present study, we also investigated the induction of somatic embryogenesis by heavy metals in the presence as well as absence of 2,4-D.

Materials and Methods

Seeds of *Triticum aestivum* cv. HD2329 were obtained from The National Seeds Corporation, Pusa, New Delhi. These were inoculated on MS basal medium after surface sterilization and raised in the culture room with the light/dark cycle of 16/8h at 26°±2°C. After removal of the second and third leaf (from outside, excluding the coleoptile), basal leaf bases of 5-7 mm size were excised aseptically and inoculated on MS medium in Petri dishes containing the plant growth regulator 2,4-D at a concentration of 10 μM and incubated for one day. The explants were then transferred to MS basal medium and kept in dark
for 10 days¹. Thirty to forty explants were inoculated per treatment and repeated at least thrice. Observations were recorded on the tenth day from the day of inoculation using a stereo microscope (SMZU, Nikon).

Treatments of mannitol and polyethylene glycol (PEG 8000) were given in the 2,4-D containing induction medium for one day. Half of the explants were transferred to basal medium without 2,4-D and for the other half the stress treatments were continued in the basal medium for ten days. The explants for aerial drying were exposed for drying on the induction medium for varying periods in the laminar flow. Explants not exposed to the stress treatments on the MS basal and on the 2,4-D supplemented media served as controls. The polyol mannitol, was included in the induction medium at a concentration of 0.25 and 0.5 M. Explants were transferred to MS basal and MS medium supplemented with the same concentrations of mannitol in the induction medium after 24 hr. Heavy metals were added to the MS medium prior to autoclaving, either alone or in presence of 2,4-D, at a concentration of 10 μM or as specified. All experiments were repeated 3-4 times and the mean data are presented.

Estimation of endogenous proline content — The endogenous proline content was estimated following the modified protocol of Bates¹⁹. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

Results and Discussion

Stress-induced somatic embryogenesis has been reported in many plants. In carrot, 2,4-D functions as an auxin and a stress inducer²⁰. Of the various stress conditions conducive for somatic embryogenesis, osmotic stress, dehydration stress, heat shock stress and heavy metal ion stress are the most potent stressors reported¹⁶,¹⁷,²¹-²⁵. The wheat leaf bases were subjected to various abiotic stress treatments and evaluated after ten days of transfer to basal medium with 2,4-D (10μM) for embryogenic response. The requirement of 2,4-D in this system¹ has been optimized for one day (induction medium) and thereafter embry development takes place on a basal medium (differentiation medium). Endogenous proline levels were also determined with increasing durations of various treatments.

Simulated water stress — Polyethylene glycol, a non-penetrating osmotic agent that lowers the water potential of the medium, has been used extensively to simulate drought stress in plants²⁶. In the present work, when the leaf base explants were subjected to polyethylene glycol treatment in the induction medium, one of the significant results was the increase in percentage of embryo formation at 2.5% concentration of PEG (Fig. 1A). This response was also observed when PEG was present in the basal medium during the embryo differentiation phase. There was a significant rise in embryo formation per explant also at 2.5% PEG in the medium (Fig. 1A). However, with increasing concentration of PEG, percentage embryo formation declined. From the results it is inferred that PEG acts synergistically with 2,4-D for embryo induction. The endogenous proline levels were found to increase with increasing concentrations of PEG treatment both after 1 day as well as 10 day (Fig. 2A). Upto 2-3 fold increase in endogenous proline levels were observed when PEG treatment was given for one day. Endogenous proline levels were found to increase when PEG treatment was continued in the basal medium for 10 days (increase upto 2-3.5 fold as compared to the control). Such a PEG enhanced regeneration in vitro has been reported earlier in rice³.

Mannitol was selected as an osmoregulatory agent since it neither supports in vitro tissue growth nor is it metabolized by higher plants. When mannitol was present in the induction medium at concentrations of 0.25 to 0.5 M for 1 day, percentage embryoid formation declined after 10 days with increasing concentrations and reduced up to 50% as compared with the control (Fig. 1B). However, when the mannitol treatment was continued at 0.5 M on the basal medium for 10 days no somatic embryogenic was found. The endogenous proline level increased with increasing concentrations and durations of the treatment. Up to 3-5 fold increase in proline levels were observed when the explants were treated with mannitol for 1 day as compared with both the controls on the basal and on 2,4-D (10 μM) containing medium (Fig. 2B). When this treatment was continued in the basal medium for 10 days the increase was much higher (up to 9-10 fold higher) than the controls (Fig. 2B), but had no effect on somatic embryogenesis.

Aerial drying treatment — When leaf base explants were aerial dried in the laminar flow for durations ranging from 1 to 4 hr, a significant reduction in somatic embryogenesis was observed (Fig. 1C). This
decline in embryogenic response continued when explants were subjected to 2 and 3 hr of aerial drying. The embryogenic response of explants subjected to 4 hr of aerial drying was comparable to that observed with explants subjected to 1 hr of aerial drying (Fig. 1C). The endogenous levels of proline in explants was also higher when subjected to aerial drying on MS+10 mM, 2,4-D (Fig. 2C). Nonetheless, the role of endogenous hormones in modulation of the final response cannot be overruled.

![Graphs showing effect of simulated abiotic stress treatment on induction of somatic embryogenesis in T. aestivum leaf bases. C= leaf bases without corresponding stress treatment, (A) — Polyethylene glycol treatment; (B) — Mannitol treatment; and (C) — Aerial drying.]
The above mentioned results indicate that simulated water stress can result in induction of somatic embryogenesis and even enhance regeneration efficiency without the obligate need for exogenous hormones. Water stress in the form of drought or salt stress has been found to be promotory for regeneration by other workers as well in different systems.

Non-fatal osmotic stress affects the growth and differentiation by probably altering endogenous phytohormone content, therefore, it is understandable that exogenous proline results in increased somatic embryogenesis and regeneration frequency. Refreshing the idea that stress or stress effects are important regulators of regeneration. In fact, it has been proposed that at low concentrations, osmotic stress may cause a disruption of plasmodesmatal interconnections between pre-embryonic cells, allowing cells to be physiologically isolated, thus aiding in a greater number of cells to differentiate.

**Effect of heavy metals** — Since induction of somatic embryogenesis by 2,4-D was extrapolated to be similar to a stress response, and various metal ions are reported to be effective, with cadmium being the most potent, the present study also investigated the involvement of metal ions on the induction of somatic embryogenesis in the wheat leaf base system. When the heavy metal ions cadmium, copper, zinc, nickel and silver were provided at 300 \( \mu M \) levels either alone or in combination with 2,4-D (10 \( \mu M \)) in the presence of 2,4-D, all of them displayed a general inhibition of somatic embryogenesis (Fig. 3A). The order of their inhibition was \( \text{Zn}^{++} \), \( \text{Cd}^{++} \), \( \text{Co}^{++} \), \( \text{Ni}^{++} \) and \( \text{Ag}^{+} \). When these metals were provided without 2,4-D treatment, surprisingly, only cadmium displayed induction of somatic embryogenesis *albeit* to a lesser extent than 2,4-D. The fact that cadmium alone could cause induction of somatic embryogenesis was quite intriguing. Cadmium was subsequently tried over a concentration range and 500 \( \mu M \) (in absence of 2,4-D) and was found to be effective for induction of somatic embryogenesis though much less than the control (Fig. 3B). Moreover, a time course study revealed that cadmium treatment for 3 days elicit the maximum response and further incubation is in fact inhibitory (Fig. 3C). However, in none of the experiments, the frequency of the cadmium-induced somatic embryogenesis was higher than that induced by 2,4-D. When 2,4-D was supplemented with metal ions, a decline in somatic embryos was observed, in contrast to the observation of Roustan *et al.* where a stimulation of somatic embryogenesis occurred by cobalt and nickel in the presence of auxin, which was attributed to the inhibition of

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**Fig. 2** — Effect of simulated abiotic stress treatments on endogenous proline content of *T. aestivum* 10-day-old leaf base cultures. C0 = leaf bases without 2,4-D, C = leaf bases + 2,4-D. [(A) — Polyethylene glycol; (B) — Mannitol treatment; and (C) — Aerial drying]
ethylene production. However, regeneration from these embryoids on MS basal medium was not observed which is contrary to the observations made by Harada et al.\(^1\), wherein a better regeneration frequency was obtained by stress than by 2,4-D alone.

The result indicated the possibility of overlapping functions of two gene expression programs, one leading to the embryogenic state and the other in response to simulated drought stress. Recent evidence strongly suggests the alteration of the gene expression by both drought stress and auxin treatment leading to embryogenic pathway\(^9\). Overproduction of proline results in an increased tolerance to osmotic stress of transgenic tobacco plants. Over the past five years, a number of transgenic plants have been produced in which over accumulation of osmolytes leads to increased tolerance to osmotic stress\(^2\).

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Fig. 3 — Effect of metal ions on the induction of somatic embryogenesis in 10-day old leaf base cultures of T. aestivum. C= leaf bases+ 2,4-D. [(A) — Effect of various metals at 300 μM in the presence of 2,4-D (10 μM); (B) — Effect of cadmium in the absence of 2,4-D; and (C) — Effect of cadmium (300 μM) for varying durations]


