

Callus growth and proline accumulation in response to sorbitol and sucrose-induced osmotic stress in rice

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Abstract

This study investigated the influence of osmotic stress, induced by sorbitol and sucrose combinations, on growth and proline accumulation in callus cultures of rice (*Oryza sativa* L.). Dehusked mature seeds, cv. Hassawi, were induced to callus on MS medium supplemented with 4.52 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.32 μ M 6-furfurylaminopurine (kinetin). The medium also contained 29.2, 58.4, 87.6, and 116.8 mM sucrose combined with 0, 54.9, 109.8, and 164.7 mM sorbitol. Callus formation was observed in about 35 % of the cultured seeds irrespective of the sugar treatment. An increase in callus mass was observed as sucrose concentration increased reaching a maximum growth at 87.6 mM. Callus growth was enhanced in response to 54.9 mM sorbitol but at higher concentration it was inhibitory. Best callus growth was obtained on a medium containing 54.9 mM sorbitol combined with 87.6 mM sucrose. Increasing osmotic stress, as a consequence of increasing sucrose and sorbitol concentrations, induced proline accumulation and the highest concentration of proline, 5.8 μ mol g⁻¹(f.m.), was obtained on 164.7 mM sorbitol combined with 116.8 mM sucrose.

Additional key words: carbon source, *Oryza sativa*, sugar, tissue culture.

Sucrose is generally used as the major source of carbon and energy in tissue culture media of rice (Rueb *et al.* 1994, Al-Khayri *et al.* 1996, Al-Khayri and Al-Bahrany 2000, Duong *et al.* 2000). Other sugars including mannitol, maltose, and sorbitol also have been used, often in combination with sucrose (Kishor and Reddy 1986, Swedlund and Locy 1993, Okamoto *et al.* 1996, Laxmi and Reddy 1997). The addition of sorbitol has been observed to enhance *in vitro* culture growth and morphogenesis in certain rice genotypes (Yoshida *et al.* 1994, Al-Khayri *et al.* 1996, Huang and Huang 1999).

In addition for being major carbon sources for *in vitro* growth, sorbitol and sucrose act as osmotic agents that may introduce osmotic stress above certain concentrations. Studies on the mechanism of osmotic adjustment in plants are limited by the fact that whole plants contain mostly non-growing cells which makes characterizing biochemical processes in growing cells in response to osmotic changes difficult (Turner and Jones 1980). The use of cultured plant cells provides a means to overcome this difficulty since it allows careful measurements of growth in response to

various osmotic changes in the environment (Bressan *et al.* 1982).

Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of the possible means for overcoming osmotic stress caused by the loss of water (Al-Bahrany 1994, Shankhadhar *et al.* 2000). This study investigated the influence of osmotic stress, induced by sorbitol and sucrose combinations supplied to the culture medium at various concentrations, on callus growth and proline accumulation in rice callus cultures.

Mature rice (*Oryza sativa* L. cv. Hassawi) seeds which is tolerant to high salinity and drought were obtained from Hofuf Regional Agricultural Research Center, Ministry of Agriculture and Water, Kingdom of Saudi Arabia. The seeds were dehusked manually and surface sterilized for 1 min in 70 % ethanol followed by 30 min shaking in 2.6 % m/v sodium hypochlorite containing 3 drops *Tween* 20 per 100 cm³ solution. The seeds were rinsed three times in sterile distilled water and cultured on callus induction medium.

Received 12 July 2001, accepted 22 November 2001.

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Callus induction and growth medium consisted of MS salts (Murashige and Skoog 1962) supplemented with 1 mg dm⁻³ thiamine-HCl, 1 mg dm⁻³ pyridoxine-HCl, 1 mg dm⁻³ nicotinic acid, 2 mg dm⁻³ glycine, 100 mg dm⁻³ myo-inositol, 4.52 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.32 μ M 6-furfurylaminopurine (kinetin). To examine the response to carbon source and sugar concentration, the medium was also augmented with 29.2, 58.4, 87.6, and 116.8 mM sucrose combined with 0, 54.9, 109.8, and 164.7 mM sorbitol. The medium was adjusted to pH 5.8 with 1 M KOH and solidified with 8 g dm⁻³ agar (Agar-agar or Gum agar; Sigma, St. Louis, MO). Culture medium was dispensed in 150 \times 25-mm culture tubes (15 cm³ medium per tube), and autoclaved at 121 °C and 10⁵ Pa for 15 min.

Seeds were placed horizontally on the surface of the medium (one seed per culture tube) and incubated at 24 ± 2 °C under a 16-h photoperiod of cool-white fluorescent light (irradiance of 40 μ mol m⁻² s⁻¹). After 4 weeks, the number of seeds that induced callus per treatment was noted and calli were separated from the seed explants and transferred to fresh identical media to encourage further proliferation. Callus cultures were maintained for an additional 4 weeks after which individual calli were weighed to determine the effect of osmotic stress on callus growth expressed in fresh callus mass. This experiment was setup as a 4 \times 4-factorial designed in which the main factors were sucrose and sorbitol concentrations at four levels each. Thirty seeds were cultured per sucrose and sorbitol combination, and 10 randomly selected calli per treatment were weighed. Callus data were subjected to analysis of variance and the means were compared, where appropriate, using a least significant difference (LSD) at 5% significance. The experiment was repeated twice with similar results, thus the data presented pertain to a single experiment.

Extraction and estimation of proline was conducted according to the procedures described by Bates *et al.* (1973). Fresh callus, 500 mg per sample, was homogenized in 10 cm³ of 3% (m/v) aqueous sulphosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. In a test tube 2 cm³ of the filtrate was mixed with 2 cm³ acid ninhydrin and 2 cm³ glacial acetic acid and incubated in 100 °C water bath for 1 h. The reaction mixture was terminated by placing in ice bath, extracted with 4 cm³ toluene, and the chromophore phase was aspirated from the aqueous phase. The absorbance was read at 520 nm using LKB Novaspec Model 4049 spectrophotometer (LKB Biochrom, Cambridge, England). The effect of sucrose and sorbitol on the accumulation of proline was assessed based on 3 replications per treatment. Data were subjected to analysis of variance and the means were compared, where appropriate, using LSD at 5% significance.

Seed germination proceeded callus formation that emerged from the scutellum region of the seed.

Germinating seeds produced only small shoots and failed to form roots in response to the presence of 2,4-D, which stimulated callus proliferation in about 35% of the cultured seeds. Although callusing percentage was irrelevant to sugar treatment, maximum callus formation, 47%, occurred on a combination consisting of 109.8 mM sorbitol and 29.2 mM sucrose, and the minimum, 19%, was obtained on a medium containing 164.7 mM sorbitol and 58.4 mM sucrose.

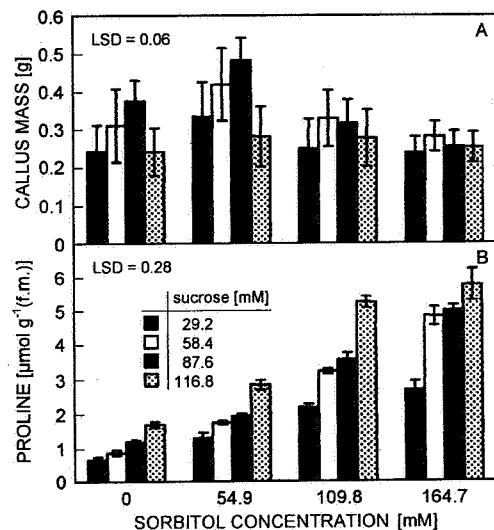


Fig. 1. Effect of sorbitol and sucrose concentration in Hassawi rice callus culture medium on callus mass (A), and proline accumulation (B).

In the absence of sorbitol, as the concentration of sucrose increased callus growth improved reaching maximum growth on 87.6 mM sucrose beyond which callus growth was suppressed (Fig. 1A). When 54.9 mM sorbitol, the lowest concentration tested, was added callus growth was significantly promoted with each sucrose concentration. The degree of growth enhancement, however, was related to the specified sucrose level in which 87.6 mM sucrose supported highest callus mass (Fig. 1A). With 54.9 mM sorbitol, percentage increases in callus mass, compared to the corresponding non-sorbitol treatments, were 37, 35, 29, and 17%, in response to 29.2, 58.4, 87.6, and 116.8 mM sucrose, respectively. However, additional increase in sorbitol concentration did not further improve callus growth. Sorbitol at 109.8 mM either unchanged, in combination with 116.8 mM sucrose, or suppressed, in combination with 29.2 to 87.6 mM sucrose, callus proliferation. At 164.7 mM sorbitol, further inhibition of callus growth was observed regardless of sucrose level, as compared to 54.9 mM sorbitol. It appears that with high concentrations of sorbitol, above 54.9 mM, the effect of sucrose concentration on callus proliferation gradually diminished.

Generally as the concentration of sorbitol increased, proline accumulation in rice callus increased, regardless of

the sucrose concentration (Fig. 1B). However, the amount of increase varied depending on sucrose concentration. At 29.2 mM sucrose, increasing sorbitol concentration to 54.9, 109.8, and 164.7 mM resulted in 1.9, 3.3, and 4-folds increase in proline accumulation compared to no sorbitol. Whereas, with 58.4 mM sucrose, increasing sorbitol concentration to 54.9, 109.8, and 164.7 mM resulted in 2, 3.7, and 5.6 folds increase in proline accumulation. At 87.6 mM sucrose, increasing sorbitol concentration to 54.9, 109.8, and 164.7 mM resulted in 1.6, 3, and 4.2 folds increase in proline accumulation. Similarly, with 116.8 mM sucrose, increasing sorbitol concentration to 54.9, 109.8, and 164.7 mM resulted in 1.7, 3.1, and 3.4 folds increase in proline accumulation in comparison to the corresponding sucrose treatments with no sorbitol.

With any given sorbitol concentration, increasing sucrose concentration further enhanced the accumulation of proline in response for the resultant higher osmotic stress. This suggests that both sorbitol and sucrose contributed to the osmotic stress, and in turns the enhanced

accumulation of proline, in an additive manner. The highest content of proline, 5.8 $\mu\text{mol g}^{-1}$ (f.m.), was obtained from callus cultured on 164.7 mM sorbitol combined with 116.8 mM sucrose, the highest concentrations tested. The lowest proline concentration, 0.7 $\mu\text{mol g}^{-1}$ (f.m.), was found in cultures, subjected to the least osmotic stress, grown on 29.2 mM sucrose alone.

In conclusion, this paper compared the *in vitro* responses of Hassawi rice callus with respect to growth and proline accumulation as influenced by osmotic stress of sorbitol and sucrose carbon sources. Although the lowest concentration of sorbitol, 54.9 mM, enhanced callus growth, it is clear that callus growth was inhibited in response to increasing sorbitol to higher levels. Under these conditions, the resultant increase in osmotic stress enhanced proline accumulation that continued to rise reaching its maximum on a treatment representing the greatest osmotic stress induced by the highest sugar concentrations.

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