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Short Communication

In vitro hardening — the role of supra-optimal sucrose on acclimation stress in *Kniphofia leucocephala*

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Supra-optimal sucrose concentrations (6% and 9%) *in vitro* resulted in morphological and ultrastructural differences between control and stressed plantlets of *Kniphofia leucocephala*. Shoot length, in particular, was significantly reduced by increasing sucrose concentrations. Supra-optimal sucrose concentrations did not confer significant benefits on the stressed plantlets after transplanting, indicated by the similar maximum photochemical efficiency of photosystem A. However, environmental stresses *ex vitro* may not have been sufficiently great to elicit different responses from the control and stressed plantlets.

Abbreviations: CIP = culture induced phenotype; F_o, F_m and F_v = minimum, maximum and variable fluorescence, respectively; F_v/F_m = maximum efficiency of photosystem A

In tissue culture media, sucrose is both a respirable carbohydrate reserve and an osmoticum, which may participate in adaptation to water stress. These factors have been identified as the main causes for losses during acclimation. For example, in *Spathiphyllum*, although the shoots were photosynthetically competent, the determining factor for survival appeared to be the quantity of respirable reserves, particularly sucrose (Van Huylenbroeck and Debergh 1996). However, in asparagus, poor survival was attributed to uncontrolled excessive transpiration (Yue *et al*. 1992). Furthermore, conditions *in vitro* may result in culture-induced phenotypes (CIPs) (Korban and Donnelly 1994). These CIPs often exhibit abnormal anatomy, morphology and physiology, which complicate the acclimation of plantlets to *ex vitro* conditions (Premkumar *et al*. 2001). Researchers have therefore stressed plantlets by adding supra-optimal concentrations of sucrose *in vitro*, in an attempt to harden plantlets against damage during transplantation, and thus improve survival rates *ex vitro*. This has been shown to improve the acclimation in certain species, although the precise mechanism/s may differ between species. The aim of this study was to determine the effect of supra-optimal sucrose concentrations *in vitro* on the stress incurred *ex vitro* during the acclimation of *Kniphofia leucocephala* Baijnath. The conservation of this species is of major significance as only a few plants remain in the wild.

A continuous culture system for *Kniphofia leucocephala* (McCartan and Van Staden 2003) was used as a source of explants for these experiments. The shoots were trimmed to approximately 20mm, and transferred to media supplemented with 3%, 6% or 9% sucrose for 28 days. The 3% sucrose medium acted as a control, and the 6% and 9% sucrose media provided supra-optimal levels of sucrose and acted as stress-inducing media.

As reported in other studies (Short *et al.* 1987, Smith *et al.* 1990), *in vitro* hardening caused morphological differences between control and stressed plantlets. After 28 days *in vitro*, shoot length decreased significantly with increasing sucrose concentration (Figure 1). Plantlets grown on 9% sucrose had robust, erect leaves. Similar results have been found in shoots of other species stressed *in vitro* by the addition of an osmoticum to the medium (Dami and Hughes 1997). This suggests that reduction in shoot length is in response to water stress. Furthermore, where the vapour pressure of the water in the vessel was reduced by using ventilated culture vessels, similar results have been obtained (Short *et al.* 1987, Smith *et al.* 1990, McCartan *et al.* 2004).

The shoots produced in the media containing 6% and 9% sucrose were darker green compared with the control. Surprisingly, chlorophyll content (estimated by extraction in *N,N*-dimethylformamide as described by Inskeep and Bloom 1985) was significantly reduced by sucrose (Figure 2).

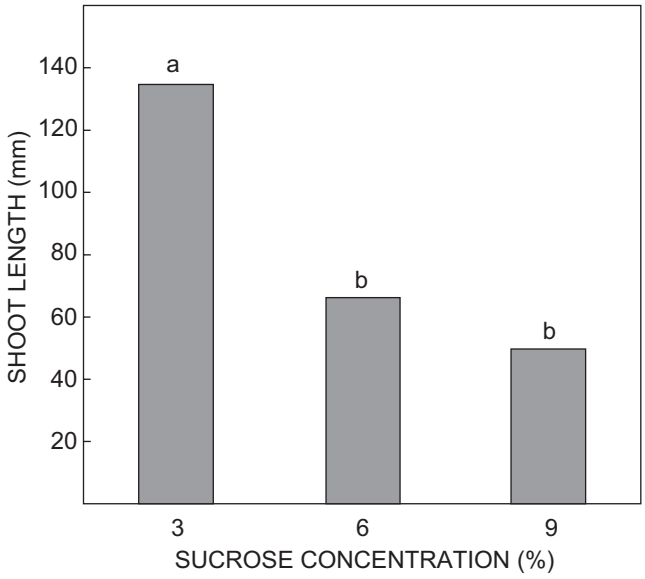

Figure 1: The effect of sucrose concentration on the mean shoot length of *K. leucocephala* plantlets after 28 days *in vitro*. Columns with the same letter do not differ significantly (P > 0.05)

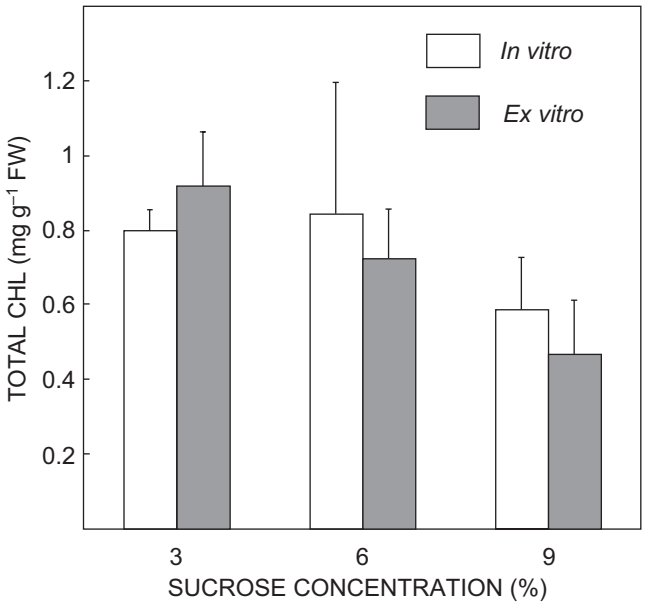

Figure 2: The effect of sucrose concentration on the chlorophyll content of *K. leucocephala* plantlets after 28 days *in vitro* and seven days *ex vitro*. Two way analysis of variance showed that sucrose (P = 0.005) but not culture conditions (P = 0.569) affected chlorophyll content

Observations of electron micrographs of leaf sections of plants grown in 6% and 9% sucrose revealed approximately twice as many starch grains as those grown on 3% sucrose (data not shown). Similar results have been obtained with other plants (Cappelades *et al.* 1991, Van Huylenbroeck and Debergh 1996).

After 28 days *in vitro*, the shoots were transplanted into vermiculite and transferred to the mist house. After transplantation, the control and stressed plantlets retained their respective habits for the following seven days. No new leaves developed and the chlorophyll concentration decreased slightly but not significantly (Figure 2). In contrast, in *Spathiphyllum* a significant increase in chlorophyll was seen within a few days of transplantation (Van Huylenbroeck and Debergh 1996). In tissue culture, in general, this is often attributed to the change from shade- to sun-leaves, and the associated increase in the cell density and thickness of the palisade layer (Marin and Gella 1988).

The photosynthetic capability or fitness of the plantlets was also determined, as previously described in detail (McCartan *et al.* 2004). The maximum photochemical efficiency of PSII (F_v/F_m) was measured using a Hansatech FMS2 fluorometer (King's Lyn, UK). The actinic light (22µmol m^{-2} s^{-1}) was switched on and the maximum fluorescence was recorded; thereafter, the actinic light was switched off and after 5min, by which time a steady state had been reached, the minimum fluorescence was recorded (McCartan *et al*. 2004). Similar F_v/F_m values of c. 0.8 were observed in plantlets *in vitro* and following transplantation, although F_v/F_m increased significantly with increasing sucrose concentration (P = 0.049) and slightly but not significantly following transplantation (P = 0.085). These results suggest that the addition of sucrose did not confer any significant benefit to the plantlet, in terms of photochemical efficiency. Reports on other studies have provided conflicting results and as a consequence micropropagated plantlets have been grouped into two categories based on their photosynthetic capability to adapt to conditions *ex vitro* (Grout 1988). One group includes species such as potato and chrysanthemum, in which the foliage formed *in vitro* persists after transplanting. In the second group represented by strawberry, cauliflower and African violet, the leaves senesce after transplanting and the plantlet requires the development of new foliage to survive. *Kniphofia* belongs to the first group. It has also been observed that in rose (Cappelades *et al*. 1991) and *Spathiphyllum* (Van Huylenbroeck and Debergh 1996) the photosynthetic capability was reduced for plantlets on high sucrose concentrations. This was not found in *Kniphofia*. However, plants such as cauliflower have lower ribulose biphosphate carboxylase activity despite having light-stimulated electron transport systems that are comparable to those of plants *in vivo* (Grout and Donkin 1987).

In conclusion, both control and stressed plantlets were successfully acclimatised in the misthouse. Despite some morphological and ultrastructural differences, *in vitro* hardening by osmotic stress conferred no apparent benefits to the photosynthetic capacity of the plantlets, as indicated by the similar F_v/F_m. However, it is possible that the environmental stresses *ex vitro* were not severe enough to elicit different responses from the control and stressed plantlets. Thus, more severe environmental stresses may be required to evaluate the potential of *in vitro* hardening by osmotic stress.

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