Validity of *in vitro* Viability Tests for Predicting Response of Different Vine Cacti in the Field to High and Low Temperatures♦

Dan Pelah[1], Ram-Avtar Kaushik[1], Avinoam Nerd[1], Yosef Mizrahi[1,2*]

[1]The Institutes for Applied Research and [2]Department of Life Sciences,
Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
*corresponding author Telephone 972 –7-6461966 or 6461930

Fax: 972-7-6472969 or 6472984

Email: mizrahi@bgumail.bgu.ac.il

ABSTRACT

Several species of hemiepiphytic vine cacti have recently been introduced to Israel as fruit crops. As part of the domestication process, these species must be evaluated for their response to environmental conditions. Differences in tolerance to low and high field temperatures were noticed between species. *In vitro* viability tests followed by field observations were conducted in an effort to find a rapid and reliable method for predicting response to high and low temperatures. Neutral-red and 2,3,5-triphenyltetrazolium chloride (TTC) tests were found to be appropriate for monitoring high temperature stress, but proved inadequate for evaluating response to low temperatures.

Keywords: cacti, temperature, viability, heat stress, cold stress

1. INTRODUCTION

Several species of hemiepiphytic vine cacti originating in tropical regions of the Americas have recently been introduced as fruit crops to regions characterized by a subtropical/tropical climate, including Israel, California, Vietnam, and Taiwan (Mizrahi and Nerd, 1999; Mizrahi et al., 1997). The attractive scaly fruits known as pitahaya in Latin America command high prices in markets. The most popular species are *Hylocereus undatus*, a pitahaya with a red skin and white flesh, and *Selenicereus megalanthus*, also white fleshed but with a yellow skin and spines on the tubercles. With the aim of developing the pitahaya industry in Israel, a number of clones representing different species of vine cacti were introduced. This material is being utilized for the selection and breeding of appropriate cultivars. Unlike in the tropics, in Israel vine pitahayas grown outdoors must be shaded to prevent bleaching (Raveh et al., 1998). Furthermore, plants may suffer damage when site ambient temperatures rise above 40°C or decline to zero.

In vitro viability tests are considered a credible tool for the characterization and quantification of cell death in plant tissue exposed to stress conditions (Porter et al., 1994). Some of these methods are based on cellular absorption of stains, such as Evans blue (Swain and De, 1994), trypan blue (Hou and Lin, 1996) or neutral red (Swain and De, 1994), and others on enzymatic activity, e.g., the 2,3,5-triphenyltetrazolium chloride (TTC) test (Steponkus and Lanphear, 1967). When evaluating diversified plant material, such

♦ Received 6 November 2002

viability tests can save considerable time, effort, and money for purposes of selection for tolerance to extreme environmental conditions. In the present study, several *in vitro* viability tests were examined in order to select a reliable method for assessing temperature tolerance in vine pitahaya. The validity of the selected test was then checked against field observations.

2. MATERIALS AND METHODS

2.1 Plant Material and Orchard Conditions

The plant material used for the study consisted of four accessions of vine pitahaya, *Hylocereus undatus* (#89024), *H. costaricensis* (#89023), *H. polyrhizus* (#89028), and *Selenicereus megalanthus* (#90003) (Weiss et al., 1994). Plant performance in the field was monitored in orchards located at three sites (Qetura, Gilgal, and Besor) differing in their environments. All the stem tissue for the *in vitro* test was obtained from Besor. At all the sites plants were grown in net-houses (40% to 60% shade) and were trained on a 1.6-m-high trellis system for support. Irrigation with a nutrient solution containing 200 mg l^{-1} fertilizer (23 parts N, 7 parts P_2O_5, 23 parts K_2O, plus trace elements, Deshanim, Israel) was applied the year round in amounts calculated to maintain the moisture content at close to field capacity to a 40-cm depth, having water potential above –0.15 Mpa (Raveh et al., 1998). Irrigation water was brackish at Qetura (EC = 3.8 to 4 dS m^{-1}) and of good quality at the other sites (EC = 0.8 to 1.0 dS m^{-1}).

2.2 Field Observations

Orchards were visited once a month during the period 1997–1999. As for monitoring high- and low-temperature damage, samples were taken during the summer (June through September) and winter (December through February), respectively. The average extreme temperatures during the summer were between 42°C and 17°C in the inner valleys of Qetura and Gilgal, while reaching 35°C to 12°C at Besor. During winter, average extreme temperatures were 28°C to 2°C at all sites. Stems (2 meters in length) were taken from all sites after extreme high or low temperatures and damage was evaluated visually for stem browning (Table 1).

2.3. *In vitro* Studies

2.3.1 Temperature Treatments

Samples of one-year-old stems were taken from June to September (heat stress) and December to February (cold stress) 1999 from three plants of each of the investigated species. For the high temperature treatment, rib discs 4 mm in diameter were taken with a cork borer and placed in 1-ml PCR tubes containing 300 µl of 0.3 M sorbitol. The tubes were then incubated for 1 h at either 20°C (control), 45°C, or 55°C using a Hybaid Omni Gene PCR machine (Franklin, Mass., USA). For the low-temperature treatments, mature 20-cm-long stem segments were exposed to –1°C for 14 h in a freezer. Stems left at room temperature (20°C) were used as controls. Each experiment was repeated three times during the season with nine replications for each treatment at each date. During all experiments, a thermocouple was used to monitor internal tissue temperature.

2.3.2 Measurements

Discs taken from the high-temperature treatments or excised from the stem segments of the cold treatments (1, 7, and 14 d after the treatment) were used for the TTC and neutral-red tests.

2.3.2.1 TTC test

Discs were placed in glass vials containing 8 ml of 0.6% TTC solution in 0.05 M potassium phosphate buffer, pH 7.4, plus a few drops of Triton X 100. Samples were vacuum infiltrated for 2 minutes and incubated for 16 h at 31°C in the dark. Then, the discs (~0.3-mm diameter) were macerated in 95% ethanol and the formazan concentration of the extract was determined by measuring the optical density at

485 nm (Chandler et al., 1997). Absorbance values from the boiled samples were subtracted from the measured values to remove any interference from nonenzymatic TTC reduction (Orzech and Burke, 1988). All viability results were expressed in terms of TTC reduction as percent of the nonstressed control.

2.3.2.2 Neutral-Red Test

Discs were kept for 15 min in a petri dish containing 0.1% neutral-red (Sigma) dissolved in 0.1 M potassium phosphate buffer, pH 7.4. Then, the tissue was washed with the buffer solution and the number of nonstained and stained cells was determined in slices under the microscope (Axioskop 2, Carl Zeiss, Gottingen, Germany); there were three fields containing 50 cells each in every sample. Viability was expressed as the ratio of stained cells to total cell number (Nobel et al., 1998).

3. RESULTS AND DISCUSSION

3.1 Monitoring Heat And Cold Stress Response In The Field

High temperatures, above 42°C, measured in separate events in summer in the inner valleys Qetura and Gilgal were correlated with browning and liquidation of stem segments, indicating tissue collapse. *H. undatus* was the most sensitive specie to high temperature (about 50% of the canopy was injured) followed by other *Hylocereus* spp., while *S. megalanthus* was the least sensitive. Hence, cultivation of *H. undatus* in extremely hot areas is not recommended.

S. megalanthus was found to be the most sensitive to low temperatures, showing stem bleaching and dieback. Visible damage was around 42% at Besor and around 26% and 25% at Qetura and Gilgal. The three *Hylocereus* spp showed no significant damage at all sites (Table 1).

Table 1. Visible damage (length of brown tissue vs total length) due to high and low temperatures of *Hylocereus* species and *S. megalanthus* at different growing sites in Israel.

(Shoot segments (2 meters in length) were taken from *H. costaricensis*, *H. undatus*, *H. polyrhizus* species and *S. megalanthus* grown in orchards in the Gilgal, Qetura and Besor regions and evaluated visually for stem browning. Values are means ±SE (n=3). For temperature details, see Materials and Methods.)

Species	**% Visible Damage (± SE) (High Temperature)**		
	Location		
	Qetura	**Gilgal**	**Besor**
H. undatus	50 ± 5.5	48.3 ± 3.4	no damage
H. polyrhizus	30 ± 2.2	28.1 ± 4.8	no damage
H. costraricensis	34.8 ± 4.4	30.2 ± 3.6	no damage
S. megalanthus	6 ± 1.1	5.3 ± 2	no damage

Species	**% Visible Damage (± SE) (Low Temperature)**		
	Location		
	Qetura	**Gilgal**	**Besor**
H. undatus	4.2 ± 0.2	8 ± 0.5	10.1 ± 1
H. polyrhizus	no damage	no damage	4.8 ± 0.7
H. costraricensis	no damage	no damage	4.5 ± 0.8
S. megalanthus	26.3 ± 4	25 ± 2.2	42 ± 5.2

3.2 Monitoring Heat and Cold Stress Response *in vitro*

When samples were incubated at 45°C for 1 h, TTC viability reduction testing indicated that the viability of *H. costaricensis, H. undatus*, and *S. megalanthus* had not been significantly impaired, while that of *H. polyrhizus* was appreciably reduced. After incubation of the discs for 1 h at 55°C, the results showed complete destruction of *H. undatus* and moderate damage to the rest of the clones (Figure 1A). Clakins and Swanson (1990), in a critical review on viability assays following stress, concluded that no one test was reliable and that, therefore, several viability tests should be correlated for a particular tissue. Furthermore, because stress response is a multifunctional event that involves both functional components (monitored by the TTC viability test) and structural components, it was important to check whether the pattern of response of different species to heat stress was similar while checking a different parameter (membrane integrity) using neutral red as a vital stain.

Staining slices with neutral-red (NR) vital stain (Figure 1B) showed a pattern of response similar to that indicated by the TTC-reduction test: All the study species maintained high viability when incubated for 1 h at 45°C, but upon incubation for 1 h at 55°C, *H. undatus* lost all viability, while that of the others demonstrated a decline to values ranging from 22% to 55%.

For the low-temperature studies, isolated discs and whole stems taken from the four species were subjected to freezing temperatures for up to 14 h. The sample failed to exhibit any visible damage or decline in viability (as measured by the two *in vitro* methods, data not shown), presumably because low-temperature damage is a slow process. When whole stems subjected to the freezing treatment as above were subsequently restored to room temperature, tissue damage became apparent after 7 days, then increased sharply in severity up to 14 days poststress. At the end of the experiment, *S. megalanthus* exhibited complete collapse of tissue (up to 100%), *H. polyrhizus* and *H. undatus* showed an intermediate response (30% to 50%), while *H. costaricensis* showed only minor damage (up to 5%). When the viability tests were repeated seven days after the cold-stress application, i.e., on appearance of tissue damage, an increase in TTC viability values compared to the control was recorded for all four species (Figure 2). Surprisingly, *S. megalanthus,* the most severely affected species in relation to tissue damage, showed the highest increment in viable activity. Increase in TTC viability values has also been shown in previous studies. Keith and McKersie (1986) and Ishikawa et al. (1995), working on callus cultures of *Lotus corniculatus* and bromegrass, respectively, reported a similar phenomenon during ABA application and freezing stress. As a tentative explanation for these findings, we suggest that could be the greater availability of electron donors for reduction of TTC in the mixture of viable and injured cells and/or (as in our case) the invasion of damaged tissue by pathogens may have been responsible for the higher viability score. Furthermore, when TTC tests were conducted on hybrids of the test species, which had a different response to low temperature, no correlation was found between low-temperature damage and reduction in viability (data not shown). As in the case of *S. megalanthus*, the hybrids, which suffered the most also, showed higher values of TTC reduction. Viability results 14 days after stress were close to the control value.

Thus, our attempt to verify viability by using neutral-red staining encountered a major difficulty. Up to seven days poststress, stressed tissue showed the same degree of staining as control cells. Beyond seven days the tissues deteriorated rapidly, and sampling proved feasible only for *H. costaricensis* (data not shown).

In vitro tests for viability appears to be an adequate tool for rapid screening of species with sensitivity to high temperatures. Thus, in the case of *H. undatus*, which exhibited severe damage in the field after exposure to heat-stress conditions, the laboratory tests established a corresponding decline in viability. However, in regard to low temperatures, no correlation could be established between the laboratory results used in this study and visible damage to the tissue. Further testing is needed to determine the nature of the relationship between viability results and low-temperature stress.

REFERENCES

Calkins, J.B., Swanson, B.T., 1990. The distinction between living and dead plant tissue-viability tests in cold hardiness research. Cryobiology 27:194-211.

Candler, J.W., Abrams, S.R., Bartels, D., 1997. The effect of ABA analogs on callus viability and gene expression in *Craterostigma plantagineum*. Physiol. Plant 99:465-469.

Hou, B.H., Lin, C.G., 1996. Rapid optimization of electroporation conditions for soybean and tomato suspension cultured cells. Plant Physiol. 111:[Suppl] 2, 166.

Ishikawa, M., Robertson, A.J., Gusta, L.V., 1995. Comparison of viability test for assessing cross-adaptation to freezing, heat and salt stresses induced by abscissic acid in bromegrass (*Bromus inermis* Leyess) suspension cultured cells. Plant Sci. 107:83-93.

Keith, C.N., McKersie, B.D., 1986. The effect of abscisic acid on the freezing tolerance of callus cultures of *Lotus corniculatus* L. Plant Physiol. 80:766-770.

Mizrahi, Y., Nerd, A., Nobel, P.S., 1997. Cacti as crops. Hort. Rev. 18:291-320.

Mizrahi, Y., Nerd, A., 1999. Climbing and columnar cacti: new arid land fruit crops. In: Janick, J. (ed.). Perspectives on new crops and new uses, ASHS, Alexandria, VA. p. 358-366.

Nobel, P.A., Castaneda, M., North, G., Pimienta-Barrios, E., Ruiz, A., 1998. Temperature influences on leaf CO_2 exchange, cell viability and cultivation range for *Agave tequilana*. J. of Arid Environ. 39:1-9.

Orzech, K.A., Burke, J.J., 1988. Heat shock and the protection against metal toxicity in wheat leaves. Plant Cell Environ. 11:711-714.

Porter, D.R., Nguyen, H.T., Burke, J.J., 1994. Quantifying acquired thermal tolerance in winter wheat. Crop Sci. 34:1686-1689.

Raveh, E., Nerd A., Mizrahi, Y., 1998. Responses of two hemiepiphytic fruit crop cacti to different degrees of shade. Sci. Hort. 73:151-164.

Steponkus, P.L., Lanphear, F.O., 1967, Refinment of triphenyl-tetrazolium chloride method of determining cold injury. Plant physiol. 42:1423-1426

Swain, D., De, D.N., 1994. Vital staining, a technique for rapid screening of plant protoplast viability. Indian J Exp Biol 32:501-506.

Weiss, J., Nerd A., Mizrahi, Y., 1994. Flowering behavior and pollination requirements in climbing cacti with fruit crop potential. HortScience. 29:1487-1492.

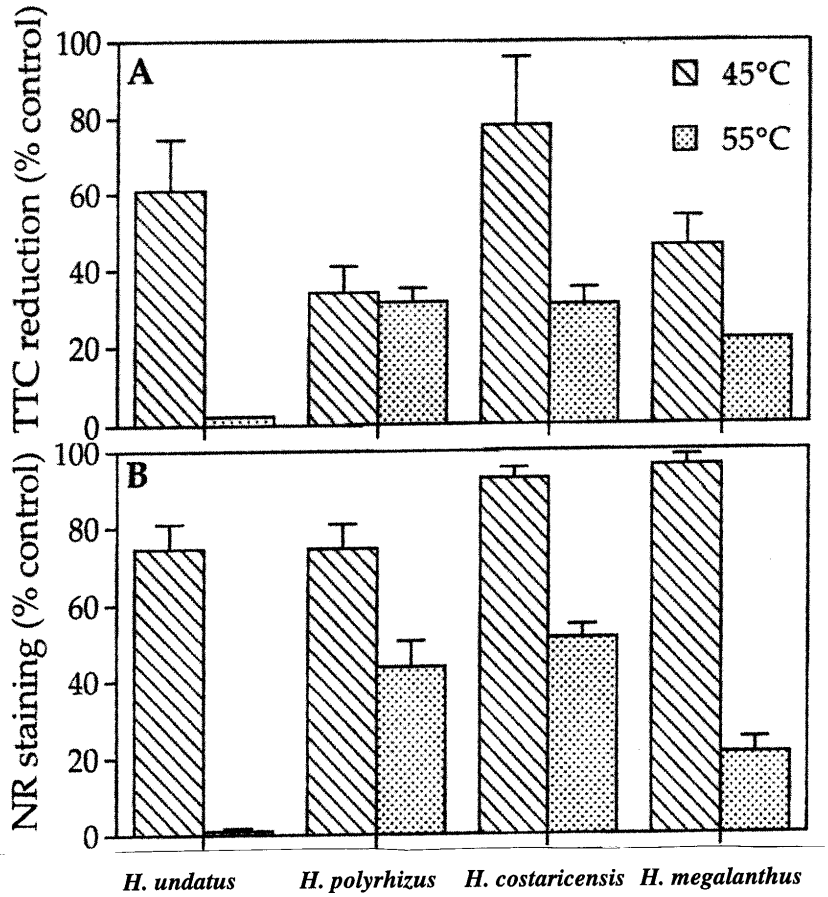

Figure 1. Effect of High-Temperature Stress on Isolated Discs of Three *Hylocereus* species and *Selenicereus megalanthus* as Determined by TTC reduction (A) and NR Staining (B).
Discs were subjected to heat stress at 45°C or 55°C for 1 h. TTC tests and NR staining were conducted immediately after each treatment. Values have been normalized so that control values (discs incubated at 20°C for 1 h) represent 100% viability. Values are means ±SE (n=3).

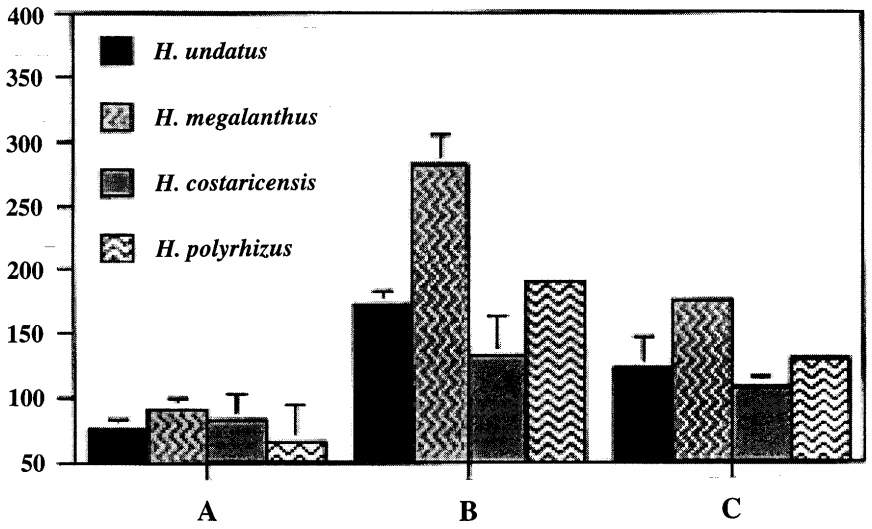

Figure 2. Effect of Low-Temperature Stress on Discs Isolated from Whole Stems of Three *Hylocereus* Species and *Selenicereus megalanthus*, as Determined by TTC.
Whole stems were subjected to low-temperature stress at −1°C up to 14-h (A), then held at room temperature for 7 d (B) and 14 d (C). TTC tests were conducted immediately after each treatment. Values have been normalized so that the control (discs incubated at room temperature up to 14 d) represent 100% viability. Values are means ±SE (n=3).