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Freezing Resistance in Tomato (*Lycopersicum esculentum* Mill.) Using Potential Cryoprotectors

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Summary

Tomato (*Lycopersicum esculentum* Mill.) plants 'Manitú F_1' were sprayed with several plant cryoprotection treatments at the phenological stage with plants bearing four true leaves. The active ingredients of the potential cryoprotectors used were: Me_2SO, proline, polyvinylpyrrolidone (PVP), a preparation of free amino acids plus nitrogen and a preparation of glycerol plus vitamin E. The effectiveness of the cryoprotector was assessed 24 hours after spraying using differential scanning calorimetry (DSC) on leaf tissue and whole plant freezing simulations. The treatment composed of an aqueous solution of 5 % glycerin and 0.35 % vitamin E was found to be the most effective cryoprotector for reduction of freeze damage on tomato leaf tissue.

Key words. PVP – glycerol – Me_2SO – proline – tomato seedlings – freeze damage – DSC

Introduction

Low temperatures limit the distribution of crops throughout the world (Parker 1963) and (Pearce 2001). Frost usually occurs outside of tropical areas, where freeze damage to horticultural crops is a problem of major economic importance (Snyder and Melo-Abreu 2005).

Spain is the second producer of tomatoes in Europe after Italy, with a production of 4749200 tons (Fao 2009). In Spain, tomato plants are transplanted to open fields in late winter for production to serve the early market in Europe. In some cases, these young plants cannot survive and are affected by low temperature or frost producing damage. Tomato is a warm-season plant and is chill-sensitive (Lyons and Raison 1970; Wang 1990). Chill-sensitive plants are injured when exposed to temperatures below 10 °C (Levitt 1980). For this reason, cold acclimation is not advisable for these plants; however, there are studies involving short-term cold acclimation by increasing the frost hardiness of tomato leaves (Shen and Li 1983).

Freezing or chilling injury depends on stage of development of the plant (Rodrigo 2000) intensity and duration of low temperature, rates of temperature decreasing or increasing (Burke et al. 1976; Strang et al. 1980), and previous and current climatic conditions in relation to the low temperature (Westwood 1993; Perry 1998). According to Snyder and Melo-Abreu (2005), the range of critical damage temperature for tomato plants is between 0 and −1 °C. Wisniewski and Glenn (2002) showed that young 'Rutgers' tomato plants (*Lycorpersicum esculemtum* Mill.) froze at −1.5 to −2.5 °C. Perry et al. (1992) observed frost injury in young tomato plants (*Lycopersicon esculenum* Mill. 'Pik Red') at temperatures below −1 °C, Anderson et al. (1982, 1984) observed frost injury at −4 °C for young tomato plants (*Lycopersicum esculemtum* Mill. 'Walter'). The freezing temperature of leaves is −2.8 °C if the leaves have ice nucleation active (INA) bacteria, while the freezing temperature of leaves without INA bacteria can reach −12 °C (Wisniewski and Glenn 2002). INA bacteria (nucleation active) are bacteria commonly found on leave surfaces as epiphytes that show to be active catalysts for ice formation (Lindow 1983). Shen and Li (1983) reported temperatures of −1.5 °C will kill tomato leaves (*Lycopersicum esculentum* Mill. 'Early Cascade').

Several methods provide important information on damage to plants by low temperatures and frost. The methods include differential thermal analysis (DTA), nuclear magnetic resonance (NMR), magnetic resonance imaging (MRI), infrared video thermography (IRVT), scanning electron microscopy (SEM), low-temperature scanning electron microscopy (LTSEM) (Pearce 2001) and differential scanning calorimetry (DSC) (Anglea et al. 1993).

Cryoprotectors have been widely studied in recent years as a tool for cryonics, i.e., the science that attempts to prolong human life by freezing and for long term storage programs in gene banks. The physical-chemical principles that occur in animal and plant cells are similar, so strong developments in the field of human cells and tissue also have repercussions for plants. Some examples are glycerol, ethylenglycol, and dimethyl sulfoxide (Me_2SO).

Active materials such as polyvinylpyrrolidone (PVP) and vinyl acetate (Coulther and Jackson 1962), proline and hydroxyproline (Prasad and Potluri 1996), glycerol and ethy-

lenglycol combined with α-tocopherol (Wölfel and Noga 1998; Albrecht et al. 2004), alcohol derivatives such as propylene glycol combined with polyoxyethylene (Perry et al. 1992), *Pseudomonas syringae* plus spectinomycin (Anderson et al. 1984), DMSO (Dhawan et al. 1986) and hydrophobic particle film (Wisniewski and Glenn 2002; Fuller et al. 2003) have been used as a plant sprays for freeze protection. Today many agrochemical companies have products to minimize the risk of occasional freeze damage, which are composed of fan amino acid and nutrient base.

The objective of this experiment was to study the effect of five potential cryoprotectors used in 23 treatments plus control on tomato leaves by means of differential scanning calorimetry (DSC) parameters, and on young tomato plants by means of simulation of frost in a chamber for freeze protection of 'Manitú F_1' tomato plants (*Lycopersicum esculentum* Mill.).

Material and Methods

Plant material and cryoprotector application

Tomato plants 'Manitú F_1' were grown in a greenhouse with daytime temperatures of 26.0±2.0 °C and nighttime temperatures of 19.0±2.0 °C without supplemental lighting. The plants were bearing four true leaves, as prescribed for stage 104 under the BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemical Industry) classification of the phenological stages in the development of solanaceous plants.

Table 1. Cryoprotectors treatments and mean values 24 h after spraying tomato (*Lycopersicum esculentum* Mill. cv. 'Manitú F_1'). Critical Biological Temperature (CBT, °C), Critical Physical-Chemical Temperature (CPT, °C), Transformed Water (TW, %) and Energy Absorbed in the Second Transition (STE, J g^{-1}) for the analysis in leaf tissues and survival percentage for frost simulation in chamber in whole plants.

Active Ingredient	Treatment	Concentration	DSC Analysis				Chamber Analysis
			CBT	CPT	TW	STE	Survival (%)
Control	0	–	−2.33 a+	−2.37 a	68.40 a	230.33 a	17 ab
Dimethyl sulfoxide (DMSO) (ppm)	1	25	−2.87 b–f	−2.37 a	66.26 a–c	219.33 a–d	20 ab
	2	50	−2.83 b–e	−2.57 ab	67.90 ab	229.33 ab	13 a
	3	100	−2.67 b	−2.67 a–c	61.73 c–f	204.00 d–h	23 ab
	4	200	−2.83 b–e	−3.13 d–g	66.39 a–c	227.67 a–c	27 ab
	5	300	−2.73 bc	−2.97 b–f	66.29 a–c	219.00 a–d	23 ab
Proline ($g \cdot L^{-1}$)	6	1.25	−2.83 b–e	−2.93 b–f	62.43 b–f	209.33 a–h	17 ab
	7	2.50	−3.03 d–g	−3.90 ij	64.26 a–e	214.33 a–g	13 a
	8	5.00	−3.07 e–g	−3.47 g–i	61.97 c–f	208.00 b–h	17 ab
	9	7.50	−2.97 c–g	−3.97 j	64.44 a–e	213.00 a–g	20 ab
Polyvinylpyrrolidone (PVP) (‰)	10	1.25	−2.97 c–g	−2.87 b–f	65.15 a–d	216.33 a–f	13 a
	11	2.50	−2.87 b–f	−2.70 a–d	57.30 f–h	193.00 g–i	17 ab
	12	5.00	−2.97 c–g	−3.17 e–g	65.44 a–d	216.67 a–f	20 ab
	13	10.00	−3.20 g	−2.67 a–c	58.74 e–h	195.33 f–i	20 ab
	14	20.00	−2.93 b–g	−2.63 a–c	65.20 a–d	217.00 a–e	20 ab
Aminoacid (24 %) +Nitrogen (8 %) ($mL\ L^{-1}$)	15	1.0	−2.73 bc	−3.03 c–g	61.56 c–f	206.33 c–h	17 ab
	16	1.5	−2.90 b–f	−3.27 f–h	65.42 a–d	218.33 a–d	13 a
	17	2.0	−2.87 b–f	−2.83 b–f	62.76 a–f	211.00 a–h	17 ab
	18	2.5	−3.00 c–g	−3.07 c–g	64.50 a–e	213.33 a–g	17 ab
	19	5.0	−2.77 b–d	−2.77 a–e	59.46 d–g	201.00 d–h	13 a
Glycerol (50 %) +Vitamin E (3.5 %) (%)	20	5	−2.93 b–g	−3.07 c–g	61.09 c–f	204.00 d–h	37 b
	21	10	−3.03 d–g	−3.63 h–j	53.09 h	176.33 i	83 c
	22	15	−2.97 c–g	−3.47 g–i	54.81 gh	195.67 e–i	40 b
	23	20	−3.13 fg	−2.97 b–f	58.10 f–h	190.67 hi	33 ab
Significance			***	***	***	***	**

***, **: Significant at P<0.001, or P<0.01, respectively. *Values followed by a common letter in a column for each treatment are not significantly different at P<0.05.

A selection of potential active cryoprotector materials in several different concentrations were used to determine 23 treatments plus a control group (Table 1). The active materials were: (1) dimethyl sulfoxide (Me_2SO), (2) proline, (3) polyvinylpyrrolidone (PVP, 40000 g mol^{-1}), (4) a preparation of total nitrogen (8 %) and free amino acids (24 %), these amino acids were composed of Aspartic Acid (1.3 %), Glutamic Acid (2.7 %), Serine (0.7 %), Histidine (0.1 %), Glycine (8.2 %), Threonine (0.2 %), Alanine (3.0 %), Arginine (0.7 %), Tyrosine (0.7 %), Valine (0.3 %), Methionine (0.1 %), Phenylalanine (0.6 %), Isoleucine (0.6 %), Lysine (0.6 %), Hidroxiproline (1.5 %) and Proline (2.4 %) and (5) a preparation of watery solution composed of 50 ml water + 50 ml glycerol + 3.5 g Vitamin E, called Glycerol (50 %) * Vitamin E (3.5 %).

The various active materials (Table 1) were diluted in water and 10 mL and was sprayed with a developer-plate atomizer using a Richard bulb onto each seedling, making sure that the leaves were completely coated. The seedlings were kept in a Phytotron with 14 h of light at 25 °C and 10 h of darkness at 15 °C and humidity relative of 70 % during 24 h. Thirty-three plants were sprayed for each treatment. Three sprayed plants were used for DSC analysis and thirty for simulation of natural frost in controlled atmosphere chamber. The control group plants were not sprayed but were kept in the Phytotron with the same weather conditions as the other treatments.

Differential scanning calorimetry in leaves tomato

Calorimetric analysis tests were done with a Perkin-Elmer differential scanning calorimeter (mod. DSC6). The calorimeter had a built-in Julabo Ultratemp ethanol cooler (mod. FP35). The instruments were calibrated using indium and n-hexane, and an empty aluminium pan was used as reference. Version 1.01 of the pertinent Perkin-Elmer software was used in a personal computer connected to the calorimeter to control and manage the DSC6. Working temperatures for the calorimeter range from −120 to 450 °C, with ±0.1 °C accuracy. Samples were always taken from the terminal leaflet of the first true leaf; avoiding the main vein. A sample was made up of two leaf tissue discs of 5 mm in diameter placed inside one small, sealed aluminium capsule. The weight of the two leaf disks in a capsule ranged from 14 to 16 mg. We analyzed three plants for each treatment. The calorimetric method consisted of a first stage, where temperatures decreased from +15 to −20 °C at a gradient of −1 °C min^{-1}, and a second stage from −20 to +15 °C with an ascending gradient of +2 °C min^{-1}.

The following variables in the leaf tissue samples were determined with the calorimeter: FTE (First Transition Energy), which is energy per unit mass (J g^{-1}) released in the first transition (liquid to solid). STE (Second Transition Energy), which is energy per unit mass (J g^{-1}) absorbed in the second transition (melting). TW (Transformed Water), which is the percentage of water involved in crystallization and melting processes. FTT (First Transition Temperature), which is the temperature (°C) at which first transition occurs. CPT (Critical Physical-Chemical Temperature), which is the temperature at which tissue freezes. CBT (Critical Biological Temperature), which is the critical biological temperature (°C) for 20 % liquid water in tissue.

All variable data were obtained directly by means software of DSC6 except CPT which was calculated as the intersection of the tangent lines between the baseline and the curve described by the sample in the second stage (melting), which is the inflection point associated with slope change (Rascón-Chú et al. 2000).

The most effective cryoprotector should have the lowest values for CBT, CPT, TW, and STE, and these criteria were judged in the selection process. CBT and CPT values indicate that the leaf tissue generates ice crystals at lower temperatures. Low TW and STE values indicate that less water is used in the phase changes, and therefore the leaf tissues are more resistant to ice formation (Moratiel 2006). Singer and Baugerod (1986) observed that there was a relationship between the water content of the tomato leaves and the supercooling temperature. The wet leaves supercooled to about −7 °C while dry leaves supercooled to −12 °C.

Simulation a frost in a chamber

This experiment was conducted with whole 'Manitu F_1' tomato plants as previously described. The controlled atmosphere chamber used was Selecta mod. Hot- Cold UL 4000695 that was modified by means of a controller of temperature and processor OMRON (mod. ES100). The processor was connected to a computer that controlled the chamber using ES100 software version 1.01. Thirty treatment plants were moved from the Phytotron to chamber and remained at temperature of 10 °C during 2 h without light. The chamber was cooled at rate of −3 °C·h^{-1} to −4 °C and held one hour. The increase of temperature was 6 °C per hour and the plant remained at 10 °C for 2 hours. These rates of temperature increase and decrease are common during late winter and spring in radiation frost in Spain. After exposure to frost the plants were moved to a Phytotron, and they were evaluated five days later. The evaluation identified the number of dead and living plants. Dead plants showed evidence of turgor loss, colour change, and inability to continue growth and development. (Fig. 1)

Statistics

For DSC, the effects exerted by the cryoprotectors on the study variables were statistically evaluated using an ANOVA (one−way). When significance was detected, *a posteriori* comparisons were tested by Fisher's least square difference (LSD). The whole plant data were subjected to analysis using Z-test for level of significance $P \leq 0.05$. The analysis was done using the program Systac 12 (Cranes Software International, Chicago, IL).

Results

Differential scanning calorimetry in tomato leaves

Highly significant differences were observed ($p \leq 0.001$) between the various cryoprotection treatments for CBT, CPT, TW, and STE while FTT showed no significant effects. For the tissue to freeze, an ice-nucleating precursor is generally required. The FTT show the temperature of ice formation,

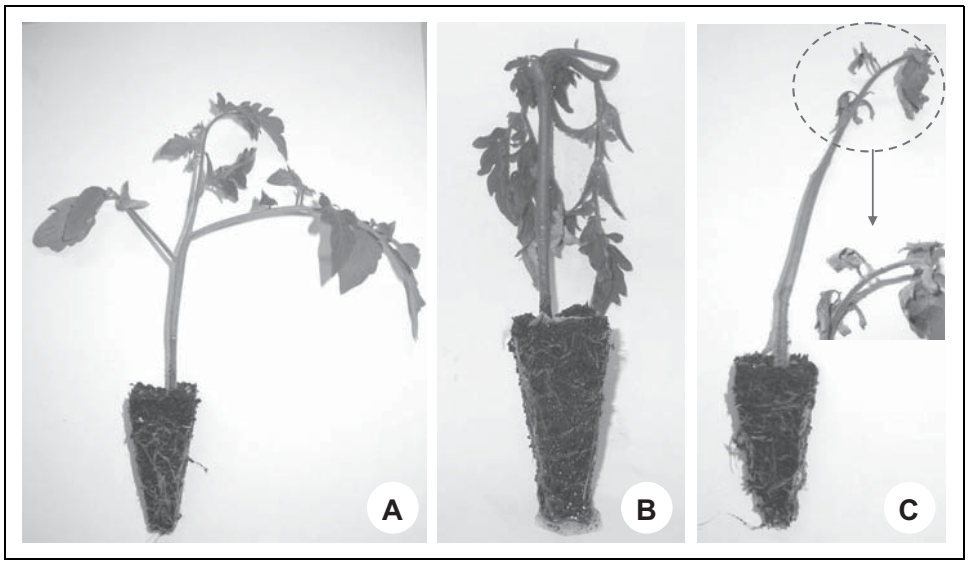


Fig. 1. Young tomato plants after being subjected to freezing in the simulation chamber: A, unharmed; B, plant dead (6 h after freezing) and C, plant dead (5 days after freezing).

which is dependent on several factor such as volume of the sample (Franks 1985) or heterogeneous nucleators, including INA bacteria, biological or molecular structures, or organic and inorganic debris (Pearce 2001). This means that the temperature was changeable and erratic. The variable FTE was not analyzed because it is equivalent to the energy absorbed in the second transition STE±5 %. All analysis and interpretation of STE can be applied to FTE.

Based on the group of cryoprotectors, the one with active ingredient DMSO performed the worst while cryoprotectors with active ingredients glycerol and vitamin E preformed the best. The treatment with the lowest values for CBT, CPT, TW and STE was treatment 21.

A good relationship was observed between variables STE and TW because, when the tissues have more water to transform (TW) to ice, the change releases more energy (STE). The linear relationship is: STE = 1.3*TW +13 (R^2=0.94). The variable CPT is comparable to the nucleation temperature or the first exothermic, in hardened tissues. The crystallization takes place outside the cells and does not cause freeze injury (Sakai and Larcher 1987), but tomato plant tissue dies at this temperature (CPT).

Simulation a frost in a chamber

Table 1 shows the average values of survival of whole tomato plants subjected to the freeze simulation in the chamber. A temperature of −4 °C maintained for one hour with decreasing and increasing rates of −3 °C h^{-1} and +6 °C h^{-1}, respectively, caused a death rate of 83 % on the control tomato plants. Only in treatments 20, 21 and 22 were there differences from the control. Furthermore, the treatment which best protected plants from low temperatures was treatment 21, in which the survival rate increased 66 % compared to the control. This treatment also preformed the best faced with the DSC.

Discussion

The results show that treatment 21, composed of 5 % Glycerol and 0.35 % Vitamin E performed the best in both experiments, the DSC technique and the simulation chamber. DSC or DTA techniques used to determine frost resistance in species such as *Rhododendron* (Iwaya-Inoue and Kaku 1986; Swiderski et al. 2006), Prunus sp. (Quamme et al. 1982; Mathers 2004), *Malus* sp. (Ketchie and Kammereck 1987), *Nothofagus menziessi* (Neumer et al. 1997), and *Physalis ixocarpa* (Rascón-Chú et al. 2000). This technique is compared with laboratory analysis, such as conductometric electrolyte study (Ketchie and Kammereck 1987; Rascón-Chú et al. 2000; Swiderski et al. 2006), freezing simulation chambers (Neumer et al. 1997), or visual browning (Mathers 2004). Normally, the DSC or DTA technique is used to study tissues in which cells adapt to subfreezing temperatures by supercooling, for example xylem ray parenchyma and winter buds of tree (George and Burke 1977), but in our case we have observed that DSC is a useful reference for screening frost-resistant sensitive species such as tomato. Authors such as Rascón-Chú et al. (2000) have shown the utility of this technique for examining husk tomato.

Little information exists on frost hardiness in tomatoes, and less still for tomato leaves. In these studies, temperature was the most analyzed variable for the assessment of damage from low and freezing temperatures. Shen and Li (1983) reported that the leaf tissue of the 'Early Cascade' tomato could be cold-acclimated to tolerate freezing for 0.5 h at −3 °C, whereas non-treated plants were killed at −1.5 °C with rate drops of 1 °C per hour. In the best treatments of the cryoprotectors (7, 9, and 21) relative to DSC analysis and especially with the CBT and CPT variables, the temperature was about −3 °C. The results were close to those obtained by Shen and Li (1983) after subjecting their tomato plants to a cold-acclimation. Studies like Anderson et al. (1984) on cv. 'Walter' tomato plants with a height of 10–15 cm, showed a mortality rate of 5 % at temperatures of −3 °C for one hour. This mortality was reduced to 0 % when they pulverized watery mixtures of 0.5 ml per plant composed of *Pseudomonas syringae* (4×10^8 cells ml^{-1}) plus spectinomycin (250 ppm). The leaf ice nucleation temperatures above −4 °C are a function of the population levels of INA bacteria (Anderson et al. 1982). According

to LINDOW (1983) tomato plants can survive temperatures of −5 °C or lower with application of bacterial ice-nucleation inhibitors. In our case, without having simulated temperatures lower than −4 °C, we can expect that nucleation will be reduced by the cryoprotectors that performed the best. Different treatments done on tomato leaves to control the INA bacteria population are capable of reducing the damage 33 % at a temperature of −4 °C (HIRANO et al. 1985) while in our experiments with treatment 21 whole plant damage was reduced 66 % at the same temperature. Tomato plants without INA bacteria were supercooled and survived to −8 °C (ANDERSON et al. 1982). According to WISNIEWSKI and GLENN (2002), use of a hydrophobic particle film as a barrier to extrinsic ice nucleation reduces the percentage of cv. 'Rutgers' killed when held for 15 min at −3 °C by about 50 %.

In our study, the best treatment was 21 [10 % concentration of Glycerol (50 %) +Vitamin E (3.5 %)]. The results were similar to those reported by other authors studying the control of population of INA bacteria on plant tissue (ANDERSON et al. 1982; HIRANO et al. 1985), investigating cold acclimation at which tomatoes are damaged by 1.5 °C (SHEN and LI 1983), and the use of hydrophobic particle film WISNIEWSKI and GLENN (2002).

WÖLFEL and NOGA (1998) and ALBRECHT et al. (2004) used α-tocopherol/glycerol, and reduced freeze damage in apple branches by 0.25 %/5 % w/v. The cryoportector glycerol decrease the freezing point in treated plant tissue. Glycerol produces an accumulation of the compound sn-glycerol-3-P and O-phosphohomoserine in the cytoplasmic compartment and decreases the rate of respiration (AUBERT et al. 1994). This osmotic effect is shown in the variable TW (Table 1) with a reduction of transformed water. Moreover, we can expect to increase of cryoprotection with increasing of concentration of Glycerol (treatments 22 and 23) but the high concentration produces osmotic stress that tends to cancel the cryoprotective effect. Vitamin E plays a part in reducing reactive oxygen species (mainly singlet oxygen and hydroxyl radicals) in photosynthetic membranes and limiting the extent of lipid peroxidation by reducing the lipid peroxyl radicals to the corresponding hydroperoxides (MUNNÉ-BOSH 2005). No studies have been done on freeze protection for tomato plants with active materials of glycerin (glycerol) and vitamin E. PERRY et al. (1992) tested a cryoprotector composed of an alcohol base on tomato plants. During the frost and freeze occurrences in the field, the cryoprotector was ineffective for temperatures below −3.5 °C for more than three hours, but the authors mentioned that it could be tested for temperatures in the range of −1.0 to −3.0 °C. WISNIEWSKI and GLENN (2002) observed that the major factors limiting supercooling in herbaceus plants are the present of moisture and epiphytic ice nucleating agents on the plant surface, and they used a hydrophobic material to delay or block the effect of moisture or ice-nucleations agents with good result.

In summary, our studies demonstrated that DSC is a useful reference technique for screening sensitive chilling species. Moreover, the treatment composed of Glycerol and vitamin E at 10 % concentration gave the best performance as a cryoprotector. This result is comparable with other techniques such as cold acclimation or INA bacteria population control for protection of young tomato plants.

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