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# Freezing tolerance and cold acclimation in guava (Psidium guajava L.)

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# Freezing tolerance and cold acclimation in guava (*Psidium guajava* L.)

by

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## ABSTRACT

Guava (*Psidium guajava* L.) is a tropical, woody perennial with nutritious fruit. It exceeds most tropical and subtropical fruit plants in adaptability and productivity. This thesis focused on the physiology of freezing tolerance and cold acclimation in guava. Freezing tolerance tests, growth and leaf relative water content measurements, anthocyanin content analyses, and protein analyses were performed on nonacclimated and cold-acclimated leaves of guava cultivars Lucknow-49 and Ruby × Supreme. The freezing tolerance of nonacclimated leaves were -2.3 ºC and 2.7 ºC, and enhanced to -4.2 ºC and -4.6 ºC after cold acclimation for 'Lucknow-49' and 'Ruby × Supreme', respectively. Growth and leaf relative water content reduced, while anthocyanins accumulated in guava during cold acclimation. Protein analyses which were performed after cold acclimation and drought stress by electrophoresis and immunoblots revealed that three proteins (69, 23.5, and 17.4 kDa) accumulated in response to low temperatures, and three proteins (78, 17.4, and 16 kDa) accumulated in response to drought stress. One common 17.4 kDa dehydrin accumulated in response to cold and drought stresses. In summary, we indicated that guava possesses some freezing tolerance, and also is able to cold acclimated. Diverse mechanisms are used by guava to tolerate freezing stress. Cold acclimation in guava appears to involve complex physiological and biochemical changes, and also overlapping responses with drought stress.

# CHAPTER 1. GENERAL INTRODUCTION

## Introduction

*Freezing injury and tolerance in plant tissue*

Growth, productivity, and distribution of crops, including horticultural plants, are affected by environmental stresses which can be defined as any environmental factor potentially unfavorable to living organism (Levitt, 1980; Thomashow, 1999). Cold and drought stresses are acknowledged to be the two most dominant factors (Weiser, 1970; Palva et al., 2002). There are two types of cold stress: chilling stress, which extends down to 0 °C, and freezing stress, which is below 0 °C (Levitt, 1980). Freezing stress induces ice crystallization, which determines the course and location of freezing injury (Levitt, 1980; Guy, 1990; Palva et al., 2002).

Freezing injury may happen in all plants. It may appear as tissue, organ, or plant death, or it may induce specific disease symptoms, such as frost cankers and burls in conifers (Zalasky, 1975a, b; Levitt, 1980). Freezing injury may occur directly due to intracellular freezing or indirectly as extracellular freezing (Levitt, 1980). Ice formation can occur in two markedly distinct locations within the tissues of most plants depending on the rate of tissue cooling. Intracellular freezing, which means ice crystallizes within the cells, may be caused by rapid freezing stress, usually $\geq 3$ °C / min for plant cells (Levitt, 1980; Guy, 1990). Intracellular freezing is always fatal (Levitt, 1980). In nature, the air temperature rarely decreases more than a few degrees per hour (Weiser, 1970; Steffen et al., 1989). Under most

circumstances, ice nucleation is generally initiated in the intercellular spaces and on the surface of cell walls as temperature reaches sub-zero. This is partly due to the extracellular fluid having a higher freezing point because of a lower solute concentration than the intracellular fluid (Jeffree et al., 1987; Guy, 1990; Thomashow, 1999; Xin and Browse, 2000). Ice will remain in the external region of cells only if the plasma membrane remains intact and the cooling rate is slow (Guy, 1990). The formation of extracellular ice creates a sudden decline in water potential outside the cell because the water potential of ice is much lower than liquid water at a given temperature. Consequently, this water potential gradient leads the cellular water moving from the cytoplasm to the intercellular spaces, resulting in cellular dehydration (Levitt, 1980; Guy, 1990; Arora and Palta, 1991; Ristic and Ashworth, 1997; Thomashow, 1999; Xin and Browse, 2000). Cellular dehydration increases with decreasing temperature until dehydration and/or ice formation is enough to kill the cells (Breton et al., 2000).

The plasma membrane of the cell has been considered the primary site of freezing injury in plants (Levitt, 1980; Steponkus, 1984; Arora and Palta, 1991; Thomashow, 1999; Mahajan and Tuteja, 2005). It is well known that freeze-induced membrane damage, including expansion-induced-lysis, lamellar-to-hexagonal-II, and fracture jump lesions, are, primarily, due to the severe cellular dehydration associated with freezing (Steponkus, 1984; Steponkus et al., 1993; Uemura et al., 1995; Thomashow, 1999; Mahajan and Tuteja, 2005). Freezing-induced cellular dehydration also links freezing with other stresses, such as drought and high salinity (Palva, 1994; Xin and Browse, 2000; Palva et al., 2002). Therefore, plants may exhibit, at least partly, overlapping responses to these stresses (Close, 1997; Li et al.,

2004). Besides cellular dehydration, certain additional factors may also contribute to membrane damage during freezing stress, such as freezing-stress-induced reactive oxygen species and protein denaturation occurring at low temperatures (McKersie and Bowley, 1997; Thomashow, 1999; Palva et al, 2002; Mahajan and Tuteja, 2005).

Resistance to freezing stress may take the form of either avoidance, to prevent any ice formation within tissues, or tolerance, to minimize the injurious effects of extracellular freezing (Levitt, 1980). In plants, freezing tolerance is the major mechanism of freezing resistance since plants are essentially unable to avoid the freezing stress (environmental freezing temperatures) (Levitt, 1980). Freezing tolerance varies greatly among species, genotypes, and the developmental age of the plant (Bray et al., 2000; Kozlowski, 2002). The development of freezing tolerance in plants is generally initiated by nonfreezing low temperatures, moderate drought, exogenous abscisic acid (ABA), short photoperiod, or combinations of these (Guy, 1990; Arora et al., 1992; Kozlowski, 2002; Palva et al., 2002; Li et al., 2004).

*Cold acclimation and role of protein metabolism*

Gradual increasing stress might initiate physiological and biochemical adjustment that protects plants from injury when environmental stresses abruptly occur (Kozlowski, 2002). Cold acclimation is a phenomenon that occurs when the freezing tolerance of plants increases after exposure to low, nonfreezing temperatures (Levitt, 1980; Guy, 1990; Arora et al., 1992). Almost all temperate perennials, and many annual and biennial plants can alter their tissue and cellular freezing tolerances when exposed to low, nonfreezing temperatures

(Levitt, 1980; Guy, 1990). In woody plants, cold acclimation usually follows a two-stage pattern (Arora et al., 1992). In the first stage, an increase in cold hardiness is initiated by cooler temperatures or shorter photoperiods. It involves the cessation of growth and a myriad of metabolic changes that affects the response of plants exposed to subzero temperatures. In the second stage, a prolonged exposure of already moderately cold-hardy plants to non-lethal, subzero temperatures induces further hardening (Weiser, 1970; Levitt, 1980; Guy, 1990; Arora et al., 1992; Beck et al., 2004). In both annual, and other herbaceous plants, cold acclimation and induction of freezing tolerance require, mainly, low temperatures exposure (Fennell and Li, 1985; Guy, 1990; Palva et al., 2002). Many studies of freezing tolerance induced by cold acclimation revealed that the rate of acclimation is highly temperature and species dependent (Chen et al., 1979; Fennell and Li, 1985; Guy, 1990). Woody plants may require longer low-temperature exposure than herbaceous species (Palva et al., 2002).

Cold acclimation is a complex process associated with physiological and biochemical changes in plants, including modifications in membrane lipid composition, increases in soluble sugars, amino acids, and organic acids, synthesis and accumulation of antioxidants and protective proteins, changes in hormone levels, and alterations in gene expression (Steponkus, 1984; Guy, 1990; Palva, 1994; Arora et al., 1996; Hughes and Dunn, 1996; Palva and Heino, 1997; Tao et al., 1998; Thomashow, 1999; Palva et al., 2002; Li et al., 2004; Wei et al., 2006; 2005). The key function of cold acclimation is to protect and stabilize the integrity of cellular membranes against freezing injury, which may be achieved by the changes in membrane lipid composition and accumulation of sucrose and other simple sugars during cold acclimation (Steponkus et al., 1993; Uemura and Steponkus, 1997; Thomashow,

1999). Numerous electrophoretic studies have reported both quantitative and qualitative differences in protein content between nonacclimated and cold-acclimated tissues and new sets of proteins synthesized upon exposure to low temperatures (Craker et al., 1969; Brown and Bixby, 1975; Rosas et al., 1986; Guy and Haskell, 1988; Guy, 1990; Arora and Wisniewski, 1994; Arora et al., 1992; 1996). Although various genetic studies of cold acclimation isolated and characterized some cold-induced genes, there are still many cold-induced genes with unknown function (Palva, 1994; Hughes and Dunn, 1996; Thmahsow, 1998; Palva et al., 2002). However, proteins induced by low temperatures are conserved due to related genes being reported in different plants (Palva et al., 2002).

Dehydrins (*dehydr*ation *in*duced), also known as the *Lea* (*l*ate *e*mbryogenesis *a*bundant) D II family of proteins, are encoded by one of the most common categories of genes induced by low temperatures (Close, 1996; 1997; Arora et al., 1996; Kozlowski, 2002; Palva et al., 2002; Svensson et al., 2002; Marian et al., 2004). They are immunologically distinct family of proteins in plants that accumulate during the late stages of embryogenesis or in response to low temperatures or any environmental stress leading to dehydration, such as drought and high salinity (Close, 1997). There is evidence that dehydrins exist in all photosynthetic organisms and could be induced by cold acclimation (Arora and Wisniewski, 1994; Arora et al., 1996; Close, 1997, Palva et al., 2002; Marian et al., 2004; Kalberer et al., 2007). This family of proteins is hydrophilic and remains soluble after boiling (Close, 1996; Palva et al., 2002). They contain one or more copies of a putative amphipathic α-helix-forming K-segment with a highly-conserved, 15 residue consensus sequence EKKGIMDKIKEKLPG, a phosphorylatable S-segment, and an N-terminal Y-segment

(Vilardell et al., 1990; Close, 1996; Close, 1997). The biochemical properties and accumulation pattern imply that dehydrins may stabilize cellular structure during dehydration stress (Close, 1997; Rinne et al., 1998; 1999; Thomashow, 1999; Nylander et al., 2001; Palva et al., 2002; Peng et al., 2008).

*Anthocyanins*

Anthocyanins are a group of water-soluble flavonoids, which are the most widespread class of phenolic components (Terasawa et al. 2007). Anthocyanins are mainly responsible for colors ranging from scarlet to blue in flowers, fruits, leaves, stems and storage organs (Oren-Shamir and Nissim-Levi 1999; Terasawa et al., 2007). They are concentrated in cellular vacuoles and absorb visible light in the range 475-560 nm (Harborne, 1976; Hopkins, 1995). Anthocyanins also function as free-radical scavengers (antioxidants) to inhibit or delay the oxidation of other molecules. This results in the prevention of ascorbic acid oxidation and activity inhibition against oxidative enzymes (Rice-Evans et al., 1997; Pennycooke et al., 2005; Terasawa et al., 2007). Moreover, anthocyanins have positive therapeutic properties, for example, risk reduction of coronary heart disease and some chronic diseases (Waterhouse, 1995; Bridle and Timberlake, 1997).

Previous studies indicate that anthocyanin accumulation in plant leaves is mainly induced by environmental stresses, such as low temperatures and high light intensities (Oren-Shamir and Nissim-Levi, 1999; Nissim-Levi, 2003). In plant cells, freeze-induced reactive oxygen species (ROS), which destroy the oxygen-scavenging system and are highly toxic, contribute to plasma membrane damage during freezing (McKersie and Bowley, 1997;

Pennycooke et al., 2005). Antioxidant metabolites, such as anthocyanins, help to maintain ROS at low, steady-state levels (Asada 1992; Pennycooke et al., 2005). Anthocyanin synthesis has been induced by low temperatures in cranberry leaves (Hall and Stark, 1972), winter oilseed rape leaves (Solecka et al., 1999), the leaves of some maize genotypes (Pietrini et al., 2002), and petunia leaves (Pennycooke et al., 2005). In nonacclimated maize seedlings, freezing injury is partly caused by induction of ROS, and the freezing tolerance of cold-acclimated seedlings is partly due to an increased antioxidant system which prevents ROS accumulation (Anderson et al., 1995; Pennycooke et al., 2005).

*Guava (Psidium guajava* L.*)*

The plant chosen for this thesis is tropical Guava. Guava belongs to the order *Myrtales*, family *Myrtaceae*, and the genus *Psidium* L.. *Psidium* includes 150 species, most of which are fruit-bearing trees native to tropical and subtropical America (Yadava, 1996b). Guava is a small evergreen tree (Menzel and Paxton, 1986; Yadava, 1996a) native to tropical America and tolerates a wide range of frost-free environments (Samson, 1986; Yadava, 1996a). It flourishes in both humid and dry climates at elevations between sea level and 2,100 m (Yadava, 1996a). It can withstand temporary waterlogging and very high temperatures. Guava exceeds most of the tropical and subtropical fruit trees in adaptability and productivity due to its chilling, drought, and salinity tolerance (Samson, 1986; Yadava, 1996b). Guava is prolific, and regular-bearing, and flowers and fruits continuously throughout the year in climates suitable for production (Rathore, 1976; Thonte and Chakrawar, 1982; Yadava, 1996a).

Guava fruit is either eaten fresh or consumed in processed foods. It is very nutritious that is rich in vitamin C, dietary fiber (from 5 to 7%), vitamin A, and several minerals important for human health (Wilson, 1980; Yadava, 1996a). It is a staple food of some countries and is widely used in many dietetic and medical products (Menzel, 1985). Guava jelly, guava juice, guava cheese, guava cakes, and guava jams are popular around the world (Rathore, 1976; Menzel, 1985). In tropical countries, the roots, bark, leaves, and green fruit are used in medicine for gastroenteritis, diarrhea, and dysentery (Rathore, 1976; Yadava, 1996a).

The guava plant is cultivated in many countries all around the world, including India, Brazil, South Africa, Venezuela, Cuba, the Philippines, and New Zealand (Yadava, 1996a). It is important in international trade and the domestic economy of more than 50 tropical and subtropical countries (Menzel, 1985; Yadava, 1996a).

The majority of the guava plants in the United States are cultivated only in a few favorable locations of California, Florida, and Hawaii (Hawaii Agricultural Statistics Service (HASS), 1999). In recent years, the American market demand for exotic fruits, like guava, has been increasing, mainly due to increased immigration from Asia, Latin America, and other warm countries (Yadava, 1996b). With a long harvest period, guava can be a potential, alternative high-value cash crop in the U.S. (Thonte and Chakrawar, 1982; Yadava, 1996a,b). The major limitation with expanding guava cultivation to further north in the U.S. is its low cold tolerance (Yadava, 1996a). Therefore, guava is a good subject for the study of freezing injury, freezing tolerance, and cold acclimation.

## Objectives and Significance

The main objective of this thesis is to understand the physiology of freezing tolerance and cold acclimation in guava. I determined the leaf freezing tolerance of two cultivars of guava, and developed a cold-acclimation protocol for these cultivars in controlled environments. I also investigated the physiological changes associated with cold acclimation, such as changes in stress proteins and anthocyanins. Additionally, experiments were also conducted to evaluate drought tolerance of the two cultivars and understand the physiology of drought tolerance in guava.

The information gained will be valuable to further studies on molecular and genetic mechanisms of freezing tolerance and cold acclimation of guava. Moreover, results from this study may provide the potential for new strategies to develop freezing-tolerant guava cultivars. The project may also trigger interest in the response of other tropical and subtropical plants to cold stress.

## Thesis Organization

The format for this dissertation consists of one journal-styled manuscript preceded by a general introduction and followed by a general conclusion. The manuscript will be submitted to a peer-reviewed horticulture journal. The content of Chapter 2 is identical to the initial submission of the manuscript except for necessary formatting changes to meet the

standards of the graduate college for theses. Wei Hao was the primary investigator for this work under the supervision of Dr. Rajeev Arora and is the first author of the manuscript.

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# CHAPTER 2. FREEZING TOLERANCE AND COLD ACCLIMATION IN GUAVA

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## Abstract

Guava (*Psidium guajava* L.) is a tropical, evergreen tree tolerating a wide range of frost-free environments. In recent years, the American market demand for exotic and nutritious fruits, like guava, has been increasing, and, with a long harvest period, guava can be a potential alternative, high-value cash crop in the United States. However, the main problem with commercializing guava cultivation in the US is its low cold tolerance. In this paper, we studied the physiology of freezing tolerance and cold acclimation in guava. Freezing tolerance tests, growth and leaf relative water content measurements, anthocyanin content analyses, and protein analyses were performed on nonacclimated and cold-acclimated leaves of guava cultivars Lucknow-49 and Ruby × Supreme. The freezing

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tolerance ($LT_{50}$) of nonacclimated leaves were -2.3 ºC and 2.7 ºC, and enhanced to -4.2 ºC and -4.6 ºC after an environmentally controlled cold acclimation regime for 'Lucknow-49' and 'Ruby × Supreme', respectively. Growth and leaf relative water content reduced, while anthocyanins accumulated in guava during cold acclimation. Protein analyses which were performed after cold acclimation and drought stress by electrophoresis and immunoblots revealed that three proteins (69, 23.5, and 17.4 kDa) accumulated in response to low temperatures, and three proteins (78, 17.4, and 16 kDa) accumulated in response to drought stress. One common 17.4 kDa dehydrin accumulated in response to cold and drought stresses. Our data indicate that guava possesses some freezing tolerance, and also exhibits cold acclimation ability. Leaf freezing tolerance at individual freezing treatment temperatures varied significantly between 'Lucknow-49' and 'Ruby × Supreme' cultivars at nonacclimated and cold-acclimated states. Such variability among guava genotypes may be exploited to develop cold tolerant cultivars in the future. Cold acclimation in guava appears to be a multifactorial process involving complex physiological and biochemical changes, and also, overlapping responses with drought stress.

## Introduction

Cold and drought stresses are acknowledged to be the two most dominant environmental stresses that affect the growth, productivity, and distribution of crops and horticultural plants (Weiser, 1970; Levitt, 1980; Thomashow, 1999; Palva et al., 2002). The plasma membrane of the cell has been considered the primary site of freezing injury in plants

(Levitt, 1980; Steponkus, 1984; Arora and Patal, 1991; Thomashow, 1999; Mahajan and Tuteja, 2005). Freeze-induced damages of the membrane are primarily due to severe cellular dehydration associated with freezing (Steponkus, 1984; Steponkus et al., 1993; Thomashow, 1999; Mahajan and Tuteja, 2005), which also links freezing with other stresses, such as drought and high salinity (Palva, 1994; Xin and Browse, 2000; Palva et al., 2002). Therefore, plants might exhibit at least partly overlapping responses to these stresses (Close, 1997; Li et al., 2004).

In plants, the major mechanism of freezing resistance is tolerance rather than avoidance since plants are essentially unable to avoid environmental freezing temperatures (Levitt, 1980). Gradual increasing stress might initiate physiological and biochemical adjustment that protect plants from injury when environmental stresses abruptly occur (Kozlowski and Pallardy, 2002). Cold acclimation is a phenomenon that occurs when the freezing tolerance of plants increases after exposure to low, nonfreezing temperatures (Levitt, 1980; Guy, 1990). Almost all temperate perennials, and many annual and biennial plants can alter their tissue and cellular freezing tolerance when exposed to low, nonfreezing temperature (Levitt, 1980; Guy, 1990). Cold acclimation is a complex process associated with physiological and biochemical changes in the plants, including modifications in membrane lipid composition, increases in soluble sugars, amino acids, and organic acids, synthesis and accumulation of antioxidants and protective proteins, changes in hormone levels, and alterations in gene expression (Steponkus, 1984; Guy, 1990; Palva, 1994; Arora et al., 1996; Hughes and Dunn, 1996; Palva and Heino, 1997; Tao et al., 1998; Thomashow, 1999; Palva et al., 2002; Li et al., 2004; Wei et al., 2005a,b).

Numerous electrophoretic studies have reported both quantitative and qualitative differences in protein content between nonacclimated and cold-acclimated tissues, and new sets of proteins synthesized upon exposure to low temperatures (Craker et al., 1969; Brown and Bixby, 1975; Rosas et al., 1986; Guy and Haskell, 1988; Guy, 1990; Arora et al., 1996). Dehydrins (*Lea* D II family), which are encoded by one of the most common categories of genes induced by low temperatures, accumulate during the late stages of embryogenesis or in response to low temperatures or any environmental stress that lead to dehydration, such as drought and high salinity (Close, 1997; Kozlowski and Pallardy, 2002; Palva et al., 2002). Previous studies imply that the dehydrins may stabilize cellular structure during dehydration stress (Close, 1997; Rinne et al., 1998; 1999; Thomashow, 1999; Nylander et al., 2001; Palva et al., 2002; Peng et al., 2008).

Guava (*Psidium guajava* L.), which belongs to the *Myrtaceae* family, is a small evergreen tree (Menzel and Paxton, 1986; Yadava, 1996a) tolerating a wide range of frost-free environments and flourishing in both humid and dry climates at elevations between sea level and 2,100 m (Samsom, 1986; Yadava, 1996a). Guava exceeds most of the tropical and subtropical fruit trees in adaptability and productivity due to its chilling, drought, and salinity tolerance (Samson, 1986; Yadava, 1996b). Guava is prolific and regular-bearing, and can produce continuously throughout the year in climates suitable for production (Rathore, 1976; Thonte and Chakrawar, 1982; Yadava, 1996a).

In recent years, the American market demand for exotic fruits, like guava, has been increasing, mainly because of increased immigration from Asia, Latin America, and other warm-climate countries (Yadava, 1996b). With a long harvest period, guava can be a

potential alternative, high-value cash crop in the United States (Thonte and Chakrawar, 1982; Yadava, 1996a,b). Guava plants are cultivated in many countries including India, Brazil, South Africa, Venezuela, Cuba, the Philippines, and New Zealand (Hawaii Agricultural Statistics Service, 1999). However, the majority of guava plants in the U.S. are cultivated only in a few favorable locations of California, Florida, and Hawaii (Yadava, 1996a). The main problem with expanding guava cultivation to further north in the US is its low cold tolerance (Yadava, 1996a). However, freezing tolerance and cold acclimation of guava have received little attention by researchers. A physiological and biochemical understanding of freezing tolerance and cold acclimation in guava will be valuable to further studies on its molecular and genetic mechanisms. It may provide the potential for new strategies to develop freezing-tolerant guava cultivars, and may also trigger interests in the responses of other tropical and subtropical plants to cold stress.

We used two high-productive guava cultivars Lucknow-49 and Ruby × Supreme in this study to examine the physiology of freezing tolerance in guava. The major objectives of this study were (1) to determine whether guava, which is native to the tropics, possess any freezing tolerance, (2) to determine whether freezing tolerance of guava can be enhanced by an environmentally controlled cold acclimation regime, and (3) to investigate the physiological changes, such as growth, leaf water content, anthocyanins, and proteins, associated with cold acclimation.

## Materials and Methods

*Plant materials and growth conditions*

Seeds by of 'Lucknow-49' and 'Ruby × Supreme' from Fort Valley State University (Fort Valley, GA) were germinated in December 2006 in plug trays (52.5 × 26 × 6 cm; 50 plugs) with Peat-Lite mix (Sunshine LC1 Mix, Sun Gro Horticulture Ltd., Bellevue, WA) in a temperature-controlled greenhouse. Three-month-old guava seedlings were transplanted to 21 × 20 cm (diameter × height) plastic pots and maintained in a greenhouse at 24 ± 2 °C with a 16-h photoperiod with irradiance 70 ± 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The plants were fertilized and irrigated as needed.

*Cold acclimation treatment*

Three uniform one-year-old plants (nonacclimated) of each cultivar were chosen randomly and transferred to a growth chamber for four weeks with a 15-h photoperiod under cool-white fluorescent lamps that provided PAR of 60 ± 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants were exposed to a step-wise lowering temperatures in the growth chamber as follows: 18 °C / 12 °C (day / night) for one week, then 15 °C / 9 °C (day / night) for one week, then 10 °C / 5 °C (day / night) for one week, and finally 7 °C / 3 °C (day / night) for one week. Another three uniform nonacclimated plants of each cultivar were maintained at 24 ± 2 °C in the greenhouse for four weeks as a control for growth analysis. This four-week, cold-acclimation treatment was repeated three times in the same growth chamber for three independent experiments. Here, we present data from one such representative experiment.

*Growth analysis*

Growth parameters were measured for nonacclimated (control) and cold-acclimated plants before and during three repeated cold-acclimation experiments. In each experiment, fresh weight, saturated-fresh weight, and dry weight of leaves were determined for each cultivar. After measuring the fresh weight of freshly harvested leaves, they were placed into containers with distilled, deionized water for 24 to 30 h to a constant weight, and saturated-fresh weight was measured for each sample. Dry weight was obtained after drying fresh leaves at 70 °C in an oven for 48 to 72 h to a constant weight. In all measurements, fully expanded leaves of a similar age were utilized. Leaf relative water content was calculated as (leaf fresh weight – leaf dry weight) / (leaf saturated-fresh weight – leaf dry weight) * 100 (Barrs and Weatherley, 1962).

Shoot elongation was determined by measuring the distance between a reference mark and the shoot tip before and during cold-acclimation treatments. The reference was made between the shoot tip and the first node with a piece of red thread before cold acclimation treatment. Measurements were made on three plants per cultivar for nonacclimated and cold-acclimated plants.

*Leaf freezing tolerance*

Freezing tolerance of leaf tissue was determined by measuring changes in electrical conductivity (Dexter et al., 1932; Palta et al., 1977; Arora and Palta, 1991). The freezing tolerance of nonacclimated leaves was performed on the same day before the plants were transferred to the growth chamber for cold acclimation while cold-acclimated leaves were

evaluated for leaf freezing tolerance at the end of the cold-acclimation treatment. Leaves of similar age were sampled from three plants from each cultivar and cut into discs of 1.9 cm diameter. Three discs for each treatment temperature of each cultivar were placed separately into test tubes (200 × 25 mm, 70 mL) containing 100 µl distilled, deionized water. Test tubes with leaf discs were placed in a glycol bath (Model 3028, Fisher Scientific Inc., Pittsburgh, PA) for freezing treatment. Treatment temperatures were -0.5, -1, -1.5, -2, -2.5, -3, -3.5, and -4 °C for nonacclimated plants, and -0.5, -1, -1.5, -2, -2.5, -3, -3.5, -4, -5, -6, -7, and -8 °C for cold-acclimated plants. Three unfrozen discs of each cultivar were placed at 0 °C on ice as the control. Samples in the glycol freezing bath were ice nucleated with an ice chip to initiate freezing at -0.5 °C. Thereafter, samples were equilibrated for 1 h, then cooled at the rate of 0.5 °C every 30 min to -4 °C (nonacclimated plants) and subsequently at the rate of 1 °C every 40 min to -8 °C (cold-acclimated plants). Tubes were removed from the glycol bath at each treatment temperature, first placed in ice for overnight thaw, and then transferred to 4 °C for 1 h. Samples were then incubated for 1 h at room temperature. Subsequently, 20 mL of distilled deionized water was added to each tube and samples were vacuum infiltrated at about 100 kPa for 3 min, and then shaken at 250 rpm on a platform shaker (Model Innova 2300, New Brunswick Scientific Co. Inc., Edison, NJ) for 1.5 h. Electrical conductivity of the leachate was measured with a conductivity instrument (Model 3100, YSI Inc., Yellow Springs, OH) at room temperature before (initial electrical conductivity [IEC]) and after (final electrical conductivity [FEC]) leaf discs were autoclaved at 121 °C for 20 min with a slow exhaust cycle of 7.56 $g \cdot s^{-1}$. Percentage ion leakage (% IL) was calculated as the ratio of IEC to FEC, and percentage injury (% injury) was calculated as follows: $\%\ injury_t = (\%\ IL_t - \%$

$IL_c$) / (100 - % $IL_c$) * 100. Here, % $IL_t$ and % $IL_c$ represent percentage ion leakage of each treatment temperature and control, respectively. Percentage adjusted injury (% adjusted injury) was obtained by adjusting percentage injury to the maximum freezing injury taken as 100% as follows: % adjusted $injury_t$ = (% $injury_t$ / % $injury_{max}$) * 100 (Lim et al., 1998). $LT_{50,}$ the temperature at which 50% injury occurred was estimated by fitted sigmoidal curves with Gompertz function (Lim et al., 1998; Peng et al., 2007).

*Anthocyanin contents analysis*

Relative anthocyanin contents were determined followed a modified protocol described in Neff and Chory (1998) and Kleine et al. (2007). In brief, 0.1 g leaf tissue powder was incubated overnight in 300 µl acidified methanol (1% HCl). The next day, anthocyanin were separated from chlorophyll by adding 200 µl distilled deionized water and 500 µl chloroform. Absorbance at 530 nm and 657 nm of the aqueous phase was determined using a spectrophotometer (Model DU-640, Beckman Coulter, Inc., Fullerton, CA). The relative anthocyanin contents per fresh weight were calculated as: $A_{657}$ - 0.25×$A_{530}$ by compensating the contribution of chlorophyll and its degradation products to the absorbance of anthocyanins. 1% HCl methanol was used as the blank absorbance.

*Drought stress treatment and water potential measurement*

Four uniform, well-watered, eighteen-month-old plants were chosen from each cultivar. They were subjected to drought stress by withholding watering until wilting was observed, and then allowing them to recover by resuming irrigation. The fully-expanded

youngest leaf with petiole was cut from each plant before the drought stress treatment (Control), every two or three days during the water deficit, and during the recovery at 9:00 am immediately after leaf collection leaf water potential ($\Psi_{leaf}$, MPa) was measured using a pressure bomb (Model 670, P.M.S. Instrument Company, Albany, OR) (Scholander et al., 1964). Control and drought-stressed leaves were freeze dried by liquid nitrogen and stored at -80 °C for subsequent protein analysis.

*Leaf protein extraction and measurement*

Leaf proteins were extracted by the method described by Gaspar et al. (1997) with minor modifications. Leaves from nonacclimated and cold-acclimated plants, well-watered, drought-stressed (20 and 18 d of water deficit for 'Lucknow-49' and 'Ruby × Supreme', respectively), and drought-stress-recovered (5 d of recovery) plants, were collected, lyophilized, and ground to fine powder. 0.3 g powder of each tissue sample was washed at 4 °C with 20 mL solution (methanol: acetic acid: water = 10: 1: 9), and spun at 20,000 × g for 30 min. The pellet was then washed with 15 mL hexane and 15 mL acetone consecutively at 4 °C. The supernatant was discarded and the pellet was dried for a few hours at room temperature. Dry pellet was then treated with 1.5 mL extraction buffer (80 mM Tris-HCl buffer (pH 6.8), 0.1 M β-mercaptoethanol, 2 % (w/v) SDS, and 15 % (v/v) glycerol) and immediately heated at 100 ºC for 10 min. The samples were stored at 4 ºC overnight after being cooled to room temperature. The next day, samples were centrifuged at 15,800 × g for 30 min, yielding supernatant as the source of leaf tissue protein for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Protein concentration was determined by a modification of the Esen protein assay (Esen, 1978). Equal aliquots (5 µl; triplicates) from various extractions and BSA standards (0 – 4 mg / ml; triplicates) were spotted on Whatman No. 1 chromatography paper and stained with 0.1 % Coomassie brilliant blue dye R-250. After rinsing briefly with water and drying, the stained spots were eluted with 1% SDS, and absorbance of the dry-protein complex was measured at 600 nm.

*SDS-PAGE and immunoblotting*

Samples with equal amounts of protein (12 µg) were subjected to SDS-PAGE and then visualized by Coomassie stain followed a slight modified protocol described in Arora et al. (1992). In brief, discontinuous SDS-PAGE was performed with a PROTEAN II electrophoresis unit (Bio-Rad) using 4% stacking gel and a 12.5% separating gel. Gels were stained by Brilliant blue G - Colloidal (Sigma-Aldrich Co., St. Louis, MO).

For immunoblots, separated proteins (4 µg) from an unstained gel were transferred to nitrocellulose membranes as described in Arora and Wisniewski (1994). Membranes were probed at 1:500 dilution with antibody (kindly provided by Dr. Timothy Close) directed against a synthetic peptide of a 15 residue consensus sequence (EKKGIMDKIKEKLPG) which is highly conserved at the C-terminus of dehydrin proteins (Close et al., 1993). Immunoreactive bands were detected by alkaline phosphatase assay using ProtoBlot Western Blot AP Kit (Promega Corp., Madison, WI).

*Statistical analysis*

Results were expressed as mean values ± standard error. Comparisons between cultivars and treatment means, using a significance level of $p < 0.05$, were performed using the two-way analysis of variance (ANOVA) of the Statistical Analysis System (SAS) software (SAS Institute Inc., 2002).

## Results

*Effect of cold acclimation on growth and anthocyanins*

Shoot elongation of 'Lucknow-49' and 'Ruby × Supreme' was reduced by the cold acclimation treatment (Fig. 1A). Daily elongation rates (final shoot elongation / days) of cold-acclimated plants were less than that of nonacclimated plants, and shoots of 'Lucknow-49' elongated more than shoots of 'Ruby × Supreme' for both nonacclimated and cold-acclimated plants. Mean elongation rates of nonacclimated plants were 374 and 172 $nm \cdot d^{-1}$ in 'Lucknow-49' and 'Ruby × Supreme', respectively, whereas for cold-acclimated plants 152 and 81 $nm \cdot d^{-1}$, respectively (data not shown). The leaf relative water content of the two cultivars was reduced during cold acclimation treatment. 'Ruby × Supreme' had relatively higher levels of leaf relative water content before and during cold acclimation (Fig. 1B).

After subjecting plants to cold acclimation for four weeks, a color change, from green (nonacclimated leaves) to mostly red in cold-acclimated leaves, was visually observed in both cultivars (Fig. 2A). Subsequently, the anthocyanin contents were determined on nonacclimated and cold-acclimated plants. Cold-acclimated leaves accumulated anthocyanin

in both ‘Lucknow-49’ and ‘Ruby × Supreme’ (Fig. 2B). Relative anthocyanin contents increased by four fold and three fold in ‘Lucknow-49’ and ‘Ruby × Supreme’, respectively, compared to their nonacclimated levels. ‘Lucknow-49’ had a relatively higher level of anthocyanins than ‘Ruby × Supreme’ in both nonacclimated and cold-acclimated plants.

*Leaf freezing tolerance of nonacclimated and cold-acclimated plants*

Freezing tolerance of leaf tissue from nonacclimated and cold-acclimated cultivars varied with treatment temperatures (Fig. 3). Nonacclimated leaf tissue did not suffer injury when frozen at -0.5 °C, had minimum injury at -1 °C, and suffered incremental injury with decreasing treatment temperatures. ‘Lucknow-49’ suffered more freezing injury than ‘Ruby × Supreme’ at all treatment temperatures. At -2.5, -3, and -3.5 °C, ‘Lucknow-49’ suffered greater freezing injury (by ≥ 29%) than ‘Ruby × Supreme’. Maximum injuries recorded for nonacclimated leaf tissue were 91% for ‘Lucknow-49’ and 70% for ‘Ruby × Supreme’ at -4 °C. Freezing injury increased gradually with decreasing temperature in both cultivars until -2.5 °C, after which the injury increased abruptly in both cultivars. However, the trend reached a plateau at -3.5 and -4 °C in ‘Lucknow-49’, whereas this response was not seen in ‘Ruby × Supreme’ (Fig. 3A).

Both cultivars exhibited cold-acclimation capacity when exposed to cold acclimation treatment. Cold-acclimated leaf tissue showed minimum injury at -2 °C while maximum injury recorded was 88% and 79% at -8 °C for ‘Lucknow-49’ and ‘Ruby × Supreme’, respectively. A gradual increase in freezing injury was also seen in both cultivars in the cold-acclimated plants; however, the injury increased abruptly from -4 to -5 °C, and reached a

plateau at -7 and -8 °C in both cold-acclimated cultivars. ‘Lucknow-49’ exhibited more injury than ‘Ruby × Supreme’ at all treatment temperatures, but the cultivar difference was smaller compared with the nonacclimated plants of both cultivars (Fig. 3B). Both nonacclimated and cold-acclimated ‘Ruby × Supreme’ showed less freezing injury than ‘Lucknow-49’ at every treatment temperature and therefore was more freezing tolerant.

$LT_{50,}$ as a measurement of freezing tolerance was derived for nonacclimated and cold-acclimated cultivars by determining the temperature at which 50% tissue is injured (Fig. 4). For nonacclimated plants, leaf $LT_{50}$ were -2.3 and -2.7 °C for ‘Lucknow-49’ and ‘Ruby × Supreme’, respectively. After cold acclimation, leaf tissue $LT_{50}$ decreased in both cultivars, to -4.2 and -4.6 °C for ‘Lucknow-49’ and ‘Ruby × Supreme’, respectively (Table 1). Freezing tolerance of both cultivars (assessed as $LT_{50}$) increased after cold acclimation by at least 1.9 °C which was obtained by the end of 10 °C / 5 °C (day / night) step of the cold acclimation regime with no further gain in freezing tolerance achieved after 7 °C / 3 °C (day / night) exposure for an additional week (data not shown).

*Protein alterations during cold acclimation*

Coomassie-stained SDS-PAGE profiles of total proteins and their anti-dehydrin immunoblots for ‘Lucknow-49’ and ‘Ruby × Supreme’ in both nonacclimation and cold-acclimation are presented in Fig. 5A, B. SDS-PAGE data (Fig. 5A) indicated that three of these proteins, with molecular weights of 69, 23.5, and 17.4 kDa, accumulated in cold-acclimated leaf tissue from both cultivars. The extent of accumulation of these proteins differed among cultivars. Except for the 23.5 kDa protein, the accumulation levels of the

other two were higher in ‘Lucknow-49’ than ‘Ruby × Supreme’. The levels of three proteins, with molecular weights of 65, 59 and 22 kDa, reduced after cold acclimation in both cultivars, with more reduction in ‘Lucknow-49’. Only the level of 48-kDa protein seemed to increase in ‘Lucknow-49’ but slightly decrease in ‘Ruby × Supreme’.

Anti-dehydrin immunoblots of the leaf proteins revealed differing levels of accumulation of two dehydrins in nonacclimated and cold-acclimated plants (Fig. 5B). A 17.4 kDa dehydrin was present in the cold-acclimated plants while undetected in the nonacclimated samples for both cultivars. Levels of the 17.4 kDa dehydrin in cold-acclimated leaves from ‘Lucknow-49’ appeared to be higher than from ‘Ruby × Supreme’ from visual observation. Another low accumulation level dehydrin (48 kDa) was detected in both nonacclimated and cold-acclimated leaves from the two guava cultivars, with a relatively higher level in ‘Lucknow-49’ (Fig. 5B). None of these dehydrins was detected when parallel samples were probed with pre-immune serum (data not shown). None of the bands detected by anti-dehydrin immunoblotting corresponded to major bands on the SDS-PAGE protein gels. The relatively abundant 17.4 kDa dehydrin on immunoblots appeared as a faint band on the corresponding protein gel (Fig. 5B). The reason for the apparent lower dehydrin intensities on SDS-PAGE is not clear. However, other reports showed similar low intensities of leaf dehydrins (on the SDS-PGAE profiles) from woody plants (Arora et al., 1996; Artlip and Wisniewski, 1997).

*Effects of drought stress on leaf water potential, total protein contents in leaf tissue*

Plants experienced reduced water potential, from -0.44 MPa (Control) to -1.4 and -1.55 MPa (fully-stressed) for 'Lucknow-49' and 'Ruby × Supreme', respectively, when continually exposed to drought stress. 'Lucknow-49' exhibited more drought resistance than 'Ruby × Supreme' as it required more time to become drought-stressed as indicated by loss in turgor and leaf water potential -- 20 and 18 d of water deficit for 'Lucknow-49' and 'Ruby × Supreme', respectively. When watering was resumed to control levels, the leaf water potential of stressed plants fully recovered in both cultivars (Fig. 6).

Coomassie-stained SDS-PAGE profiles of total proteins and their anti-dehydrin immunoblots for 'Lucknow-49' and 'Ruby × Supreme' in controlled, drought-stressed, and recovered plants are presented in Fig. 7A, B. Various protein bands were observed to undergo quantitative changes after drought stress, but reverted to control level after recovery (Fig. 7A). Three of these proteins, with molecular weights of 78, 17.4, and 16 kDa, accumulated in leaf tissue of drought-stressed plants in both cultivars. The level of the 34-kDa protein reduced after drought stress, and then reversed to a slightly lower intensity than the control in recovered plants in both cultivars.

Anti-dehydrin immunoblotting of these two guava cultivars revealed a dehydrin differing in molecular weight and level of accumulation among control, fully drought-stressed, and recovered plants (Fig. 7B). A 17.4 kDa dehydrin was present in the fully drought-stressed plants, while absent in both control and recovered plants for both cultivars. Level of the 17.4 kDa dehydrin was higher in 'Ruby × Supreme' than 'Lucknow-49'. The 17.4 kDa dehydrin on immunoblots appeared as a faint band on the corresponding protein gel.

## Discussion

The results presented here provide information on the physiological responses of guava to freezing stress and cold acclimation. Our study emphasized the ability of a sensitive plant organ, the guava leaves, to tolerate freezing stress and exhibit cold acclimation, highlighted the related cold-induced changes in total protein contents and anthocyanin contents, and also integrated the overlapping responses to cold and drought stresses.

We found that guava leaves accrued freezing tolerance to a maximum of -2.7 °C at nonacclimated state, and freezing tolerance among the two cultivars varied (Table 1; Fig. 3A). Moreover, both cultivars exhibited significant cold acclimation ability with at least 1.9 °C gain in the leaf freezing tolerance (Table 1; Fig. 3B). This is the first laboratory study on freezing tolerance and cold acclimation of guava since prior reports have mainly been based on visual observations (Rathore, 1976; Morton, 1987; Yadava, 1996a).

Comparison of structural components or metabolites from nonacclimated and cold-acclimated plants, or various genotypes is a common way to investigate the biochemical basis of freezing tolerance (Xin and Browse, 2000). Guava growth was largely reduced during cold acclimation (Fig. 1A). This may be considered as a mechanism for the plant to cope with stresses. Cessation of elongation growth is usually associated with cold acclimation (Pellet, 1998; Renaut et al., 2005). It may be due to low root absorption and transportation of water and nutrients during low temperatures or related to the onset of cold acclimation for woody plants (Bassirirad et al., 1991; Pella, 1998; Ensminger et al., 2006). Cold acclimation is associated with the synthesis and accumulation of metabolites, such as

pigments, carbohydrates, and compatible solutes (Larher et al., 2003; Kaplan et al., 2004; Renaut et al., 2005). Anthocyanin accumulation was observed in leaves of guava after cold acclimation (Fig. 2). Previous studies indicate that anthocyanin accumulation in plant leaves is mainly induced by environmental stresses, such as low temperatures and high light intensity (Oren-Shamir and Nissim-Levi, 1999; Nissim-Levi, 2003). In plant cells, anthocyanins function as antioxidants, helping maintain high toxic reactive oxygen species (ROS) at low steady-state levels (Asada 1992; Pennycooke et al., 2005). It is conceivable that enhanced freezing tolerance of cold-acclimated guava leaves may partly be associated with increased antioxidant system from induced anthocyanins.

The water status of a plant tissue usually reflects the degree of freezing injury of the plant (Kacperska, 1993; Li et al., 2004). The freeze-induced injury is primarily due to the cellular dehydration associated with freezing (Steponkus, 1984; Steponkus et al., 1993; Thomashow, 1999; Mahajan and Tuteja, 2005). Therefore, tissues with a great amount of free water are highly susceptible to freezing injury (Li et al., 2004). The leaf relative water content of guava decreased after cold acclimation (Fig. 1B), which is consistent with prior reports showing the reduction in tissue water content during cold acclimation in woody plants (Li et al., 2003). 'Ruby × Supreme' showed relative higher levels of leaf relative water content than 'Lucknow-49' before and during cold acclimation, suggesting that 'Ruby × Supreme' may be more sensitive to freezing stress than 'Lucknow-49'.

$LT_{50}$, estimated by electrolyte leakage, usually represents freezing tolerance of a plant species. Guava cultivars Lucknow-49 and Ruby × Supreme did not show a significant difference in $LT_{50}$ (Table 1). However, by comparing percent freezing injury of the two

cultivars at various sub-freezing treatment temperatures, ‘Ruby × Supreme’ exhibited much less injury than ‘Lucknow-49’ at all temperatures in both nonacclimation and cold acclimation regimes (Fig. 3A, B). Nonacclimated ‘Lucknow-49’ had almost twice the percentage injury at -3.5 °C and 1.5 times at -4 °C compared with ‘Ruby × Supreme’ (Fig. 3A). This contrast was maintained even after cold acclimation since, at -4 and -5 °C, ‘Ruby × Supreme’ suffered only about 75% injury of that in ‘Lucknow-49’ (Fig. 3B). ‘Ruby × Supreme’ thereby showed more freezing tolerance than ‘Lucknow-49’, suggesting that $LT_{50}$ may not be a comprehensive parameter to discern small but significant difference in freezing tolerance among genotypes. Furthermore, concerning the opposite conclusion from the study of leaf water contents showing that ‘Ruby × Supreme’ may be more sensitive to freezing stress than ‘Lucknow-49’, it appears that cold acclimation response of guava genotypes involves complex physiological changes.

Gradual increasing stresses may induce physiological adjustment that protects plants from growth inhibition and/or injury that follow when environmental stresses are abruptly imposed (Kozlowski and Pallardy, 2002). It has been suggested that to obtain a proper freezing tolerance and reach the maximum tolerance, temperatures may need to be changed in sequence (Weiser, 1970). Optimal growth temperature for guava is about 25 °C (Yadava, 1996b). The final cold acclimation temperatures were approached gradually in this study. If a plant cannot adjust cellular processes for proper function during long-term exposure to low, nonfreezing temperatures, it probably has reached its maximal freezing tolerance (Kozlowski and Pallardy, 2002). In this study, freezing tolerance of guava leaf tissue did not further increase after exposure to 7 °C / 3 °C (day / night) for additional week compared with the

level attained after 10 °C / 5 °C (day / night) exposure (data not shown). This study provides, for the first time, an environmentally-controlled cold-acclimation protocol for guava.

Cold acclimation of plants also involves the synthesis and accumulation of protective proteins (Thomashow, 1999; Li et al., 2004). The study of proteins expressed in the leaves of guava during cold acclimation by SDS-PAGE and immunoblotting showed the cold-related accumulation of proteins. Three proteins (69, 23.5, and 17.4 kDa) were found to accumulate in response to cold acclimation (Fig. 5A), which may be due to increased protein synthesis and / or decreased turnover (Wake and Fennell, 2000; Renaut et al., 2004). Two bands (48 and 17.4 kDa) responsive to anti-dehydrin antibodies were detectable after cold acclimation in both cultivars. It appears that the more abundant 17.4 kDa dehydrin in cold-acclimated leaves was not constitutively expressed in nonacclimated leaves. However, the 48 kDa dehydrin was present in nonacclimated leaves and accumulated in cold-acclimated leaves, suggesting the 17.4 kDa dehydrin may be mainly cold-induced, but 48 kDa dehydrin may be inactive under favorable conditions but become functional in response to low temperatures (Renaut et al., 2005). However, the level of dehydrins accumulation did not coincide with the tolerance to cold stress among guava genotypes. The more freezing tolerant 'Ruby × Supreme' (Fig. 3A, B), accumulated less dehydrins than 'Lucknow-49' during cold acclimation (Fig. 5B), indicating the multifactorial character of cold hardiness at the molecular level.

Plants may exhibit partly overlapping responses to freezing and drought stress since freeze-induced injury in plants is mainly due to cellular dehydration (Close, 1997; Xin and Browse, 2000; Li et al., 2004). By comparing proteins expressed in the leaves during cold

acclimation and drought stress by SDS-PAGE and immunoblotting, one common dehydrin (17.4 kDa) was found in both cultivars. This result may imply that the accumulation of non constitutive 17.4 kDa dehydrin is triggered by dehydration in response to drought or low temperatures, which is consistent with previous research (Close, 1997).

In summary, the results of this study indicate that guava is a chilling-tolerant species that possesses some freezing tolerance, and is also able to be cold acclimated. Leaf freezing tolerance at nonacclimated and cold-acclimated states varied significantly between 'Lucknow-49' and 'Ruby × Supreme' cultivars. It appears that diverse mechanisms, such as growth and leaf water content reductions, anthocyanins accumulation, and stress protein turnover (specifically dehydrins), are used by guava to tolerate freezing stress. Cold acclimation in guava appears to be a multifactorial process involving complex physiological and biochemical changes, and also, overlapping responses with drought stress.

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Table 1. Leaf tissue freezing tolerance ($LT_{50}$)[z] of *Psidium guajava* L. 'Luckonw-49' and 'Ruby × Supreme' in nonacclimation (NA) and cold acclimation (CA) regimes.

| Cultivar | **$LT_{50}$ (°C)** | |
|---|---|---|
| | NA | CA |
| Lucknow-49 | -2.3 a[x] | -4.2 b |
| Ruby × Supreme | -2.7 a | -4.7 b |

[z] $LT_{50}$ represents temperature at which 50% tissue is injured.

[x] Means within each row followed by different letters are significantly difference at $P \leq 0.05$ according to the two-way analysis of variance (ANOVA).

Fig. 1. Growth analysis of *Psidium guajava* L. 'Lucknow-49' and 'Ruby × Supreme' on (A) shoot elongation in nonacclimation (NA) and cold-acclimation (CA) regimes, and (B) changes of relative leaf water content from nonacclimation regime (0 d) to 28-d cold acclimation regime. Values are means ± SE of three replicates.

Fig. 2. (A) Changes in leaf color, and (B) leaf relative anthocyanin content of *Psidium guajava* L. 'Lucknow-49' and 'Ruby × Supreme' in nonacclimation (NA) and cold-acclimation (CA) regimes. Values are means ± SE of three replicates.

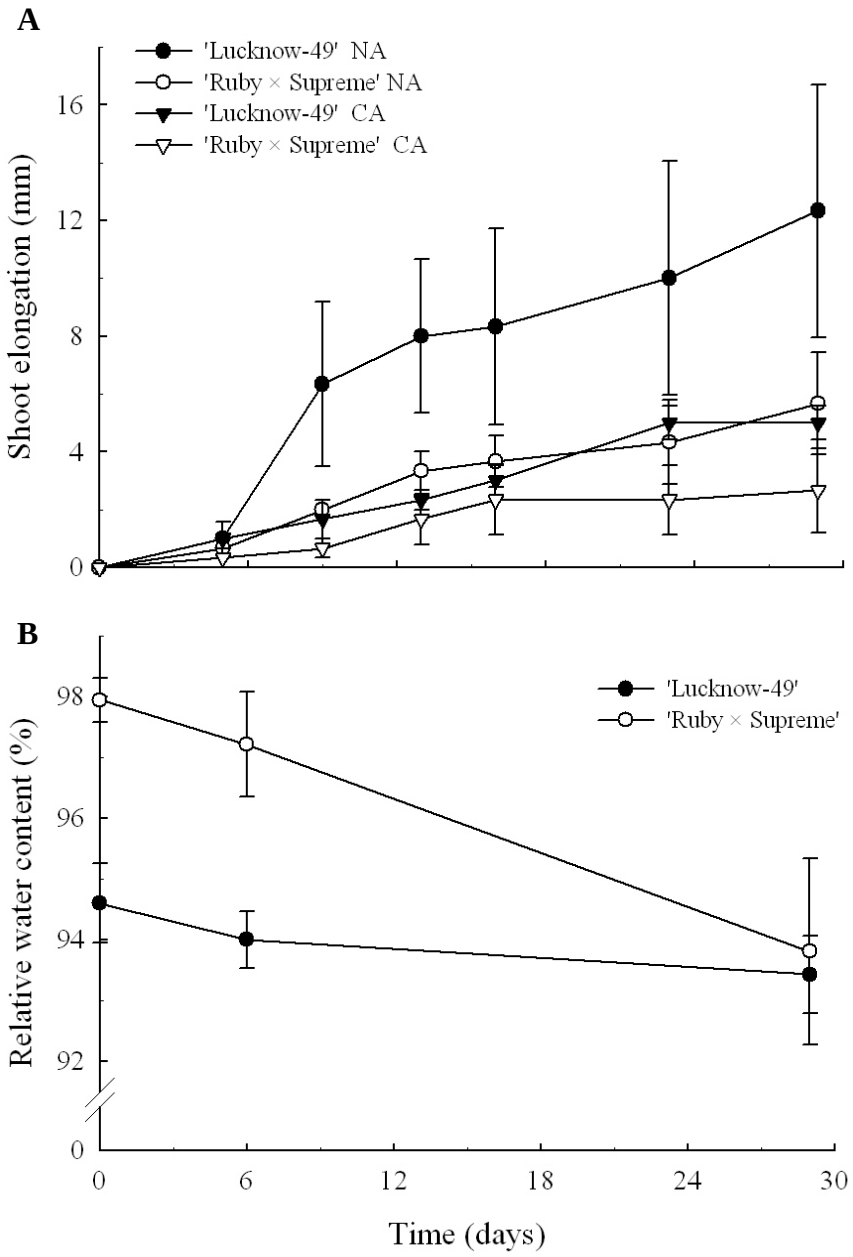
Fig. 3. Leaf percentage freezing injury measured at different freezing treatment temperatures in *Psidium guajava* L. 'Lucknow-49' and 'Ruby × Supreme' in (A) nonacclimation regime, and (B) cold-acclimation regime. Vertical bars represent means ± SE of three samples.

Fig. 4. Leaf freezing tolerance ($LT_{50}$) of *Psidium guajava* L. 'Lucknow-49' and 'Ruby × Supreme' in a nonacclimation regime. Arrows show the $LT_{50}$ values. Values are means ± SE of three replicates.

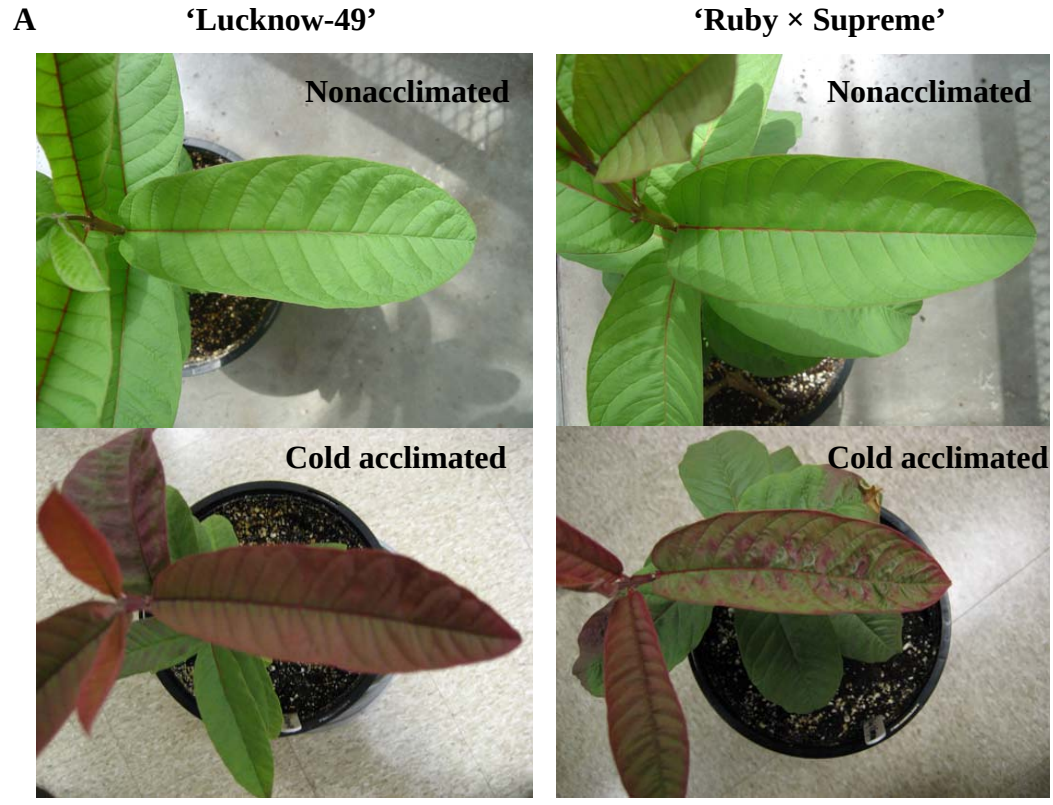
Fig. 5. (A) SDS-PAGE profiles (12 μg protein per lane), and (B) Anti-dehydrin immunoblotting (4 μg protein per lane) of total soluble proteins from leaves of *Psidium guajava* L. 'Lucknow-49' and 'Ruby × Supreme' in nonacclimation (NA) and cold-acclimation (CA) regimes. * indicates the 48 kDa band, and ** indicates the 17.4 kDa band.

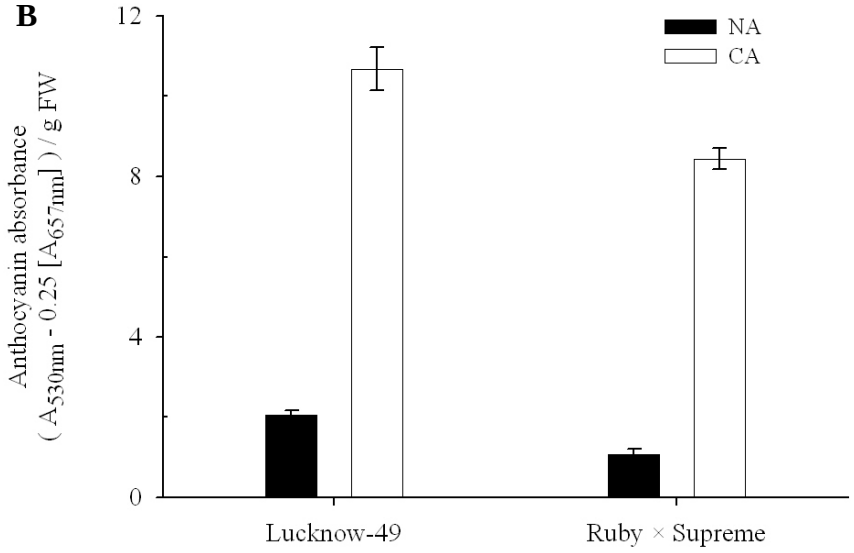
Fig. 6. Leaf water potential ($\Psi_{leaf}$, MPa) of *Psidium guajava* L. 'Lucknow-49' and 'Ruby × Supreme' under 20-d and 18-d drought stress treatment, respectively, and during recovery. Recovery period started from 20$^{th}$ day of drought stress and lasted for 5 d for both cultivars. Dotted line (for 'Ruby × Supreme') shows the period between the last measurements made on 18$^{th}$ day of drought treatment and the resumption of watering on 20$^{th}$ day. Values are means ± SE of four replicates.

Fig. 7. (A) SDS-PAGE profiles (15 µg protein per lane), and (B) Anti-dehydrin immunoblotting (6 µg protein per lane) of total soluble proteins from leaves of *Psidium guajava* L. 'Lucknow-49' and 'Ruby × Supreme' from control (C), drought-stressed (DS), and recovered (R) plants. Control leaves were collected before plants were subjected to drought stress treatment. Drought-stressed plants were subjected to water deficit for 20 and 18 d for 'Lucknow-49' and 'Ruby × Supreme', respectively. Recovered plants were watered well for 5 d after the 20-d drought stress for both cultivars. * indicates the 17.4 kDa band.


A
'Lucknow-49' NA
'Ruby × Supreme' NA
'Lucknow-49' CA
'Ruby × Supreme' CA
Shoot elongation (mm)
0
4
8
12
16
B
'Lucknow-49'
'Ruby × Supreme'
Relative water content (%)
92
94
96
98
0
0
6
12
18
24
30
Time (days)

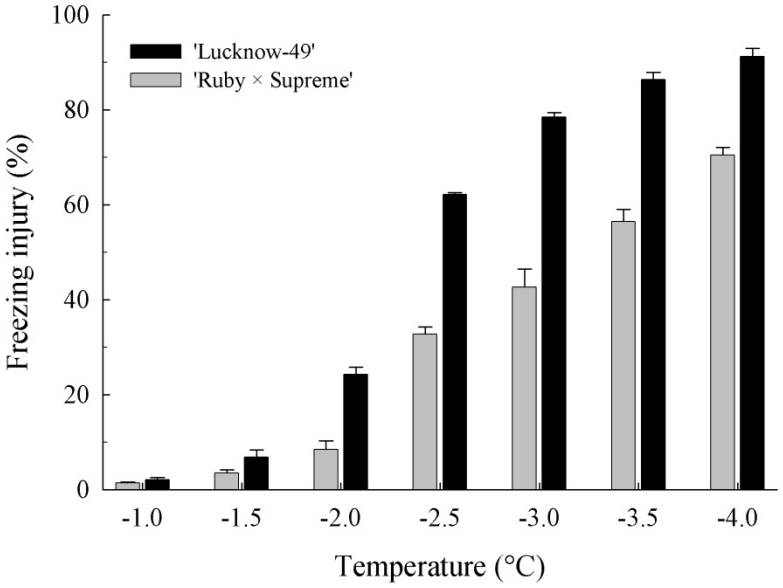
(Fig. 1)


A
'Lucknow-49'
'Ruby × Supreme'
Nonacclimated
Nonacclimated
Cold acclimated
Cold acclimated

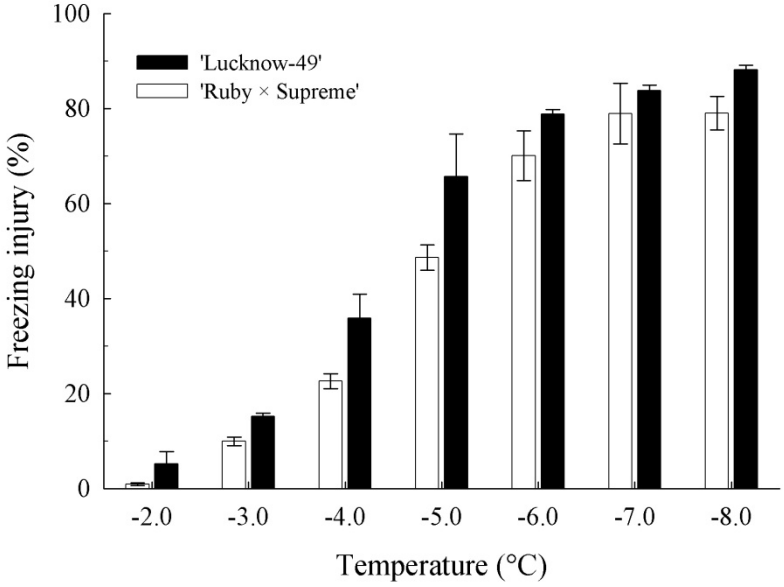

B
Anthocyanin absorbance
( A530nm - 0.25 [A657nm] ) / g FW
12
8
4
0
NA
CA
Lucknow-49
Ruby × Supreme

(Fig. 2)

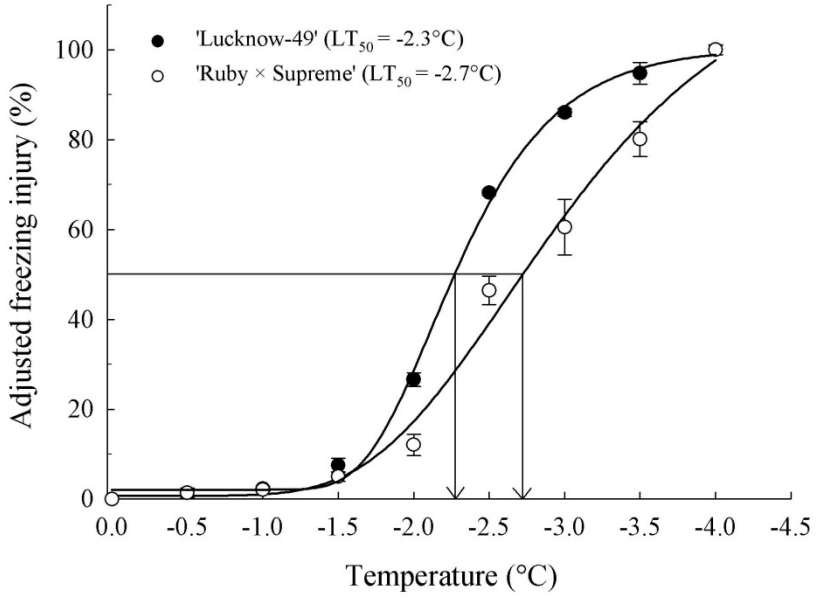
**A Nonacclimated**



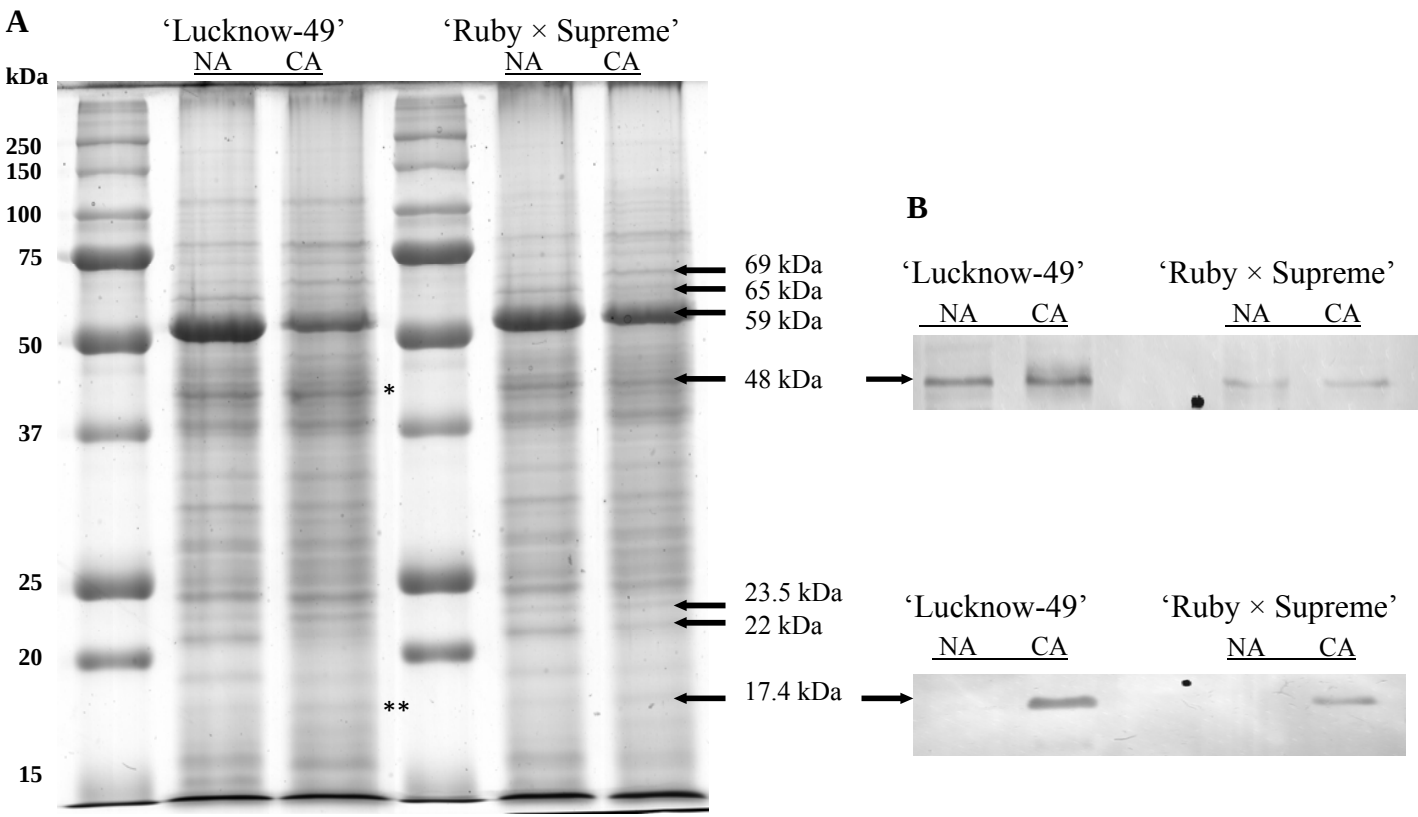

**B Cold-acclimated**



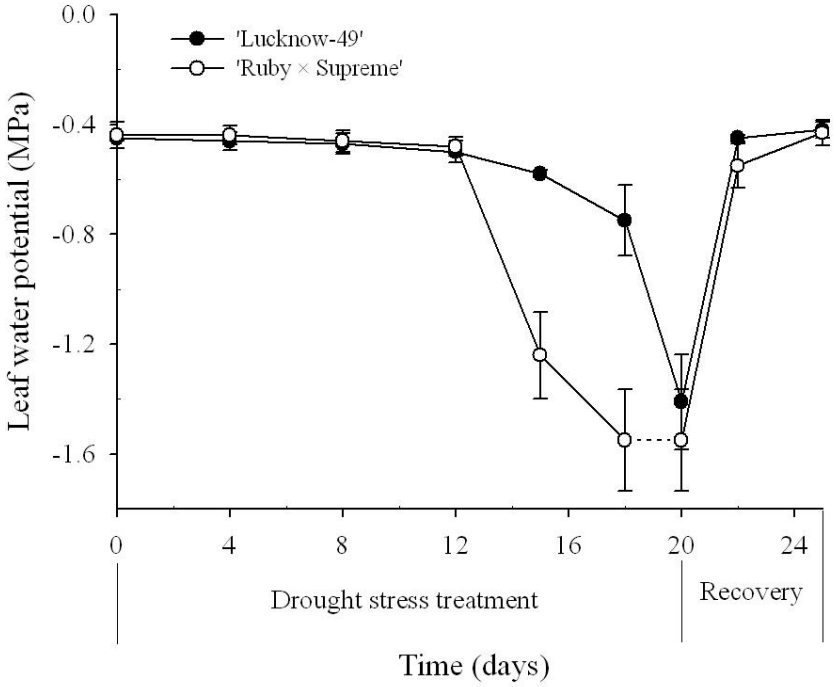

(Fig. 3)


'Lucknow-49' (LT50 = -2.3°C)
'Ruby × Supreme' (LT50 = -2.7°C)
Adjusted freezing injury (%)
100
80
60
40
20
0
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
Temperature (°C)

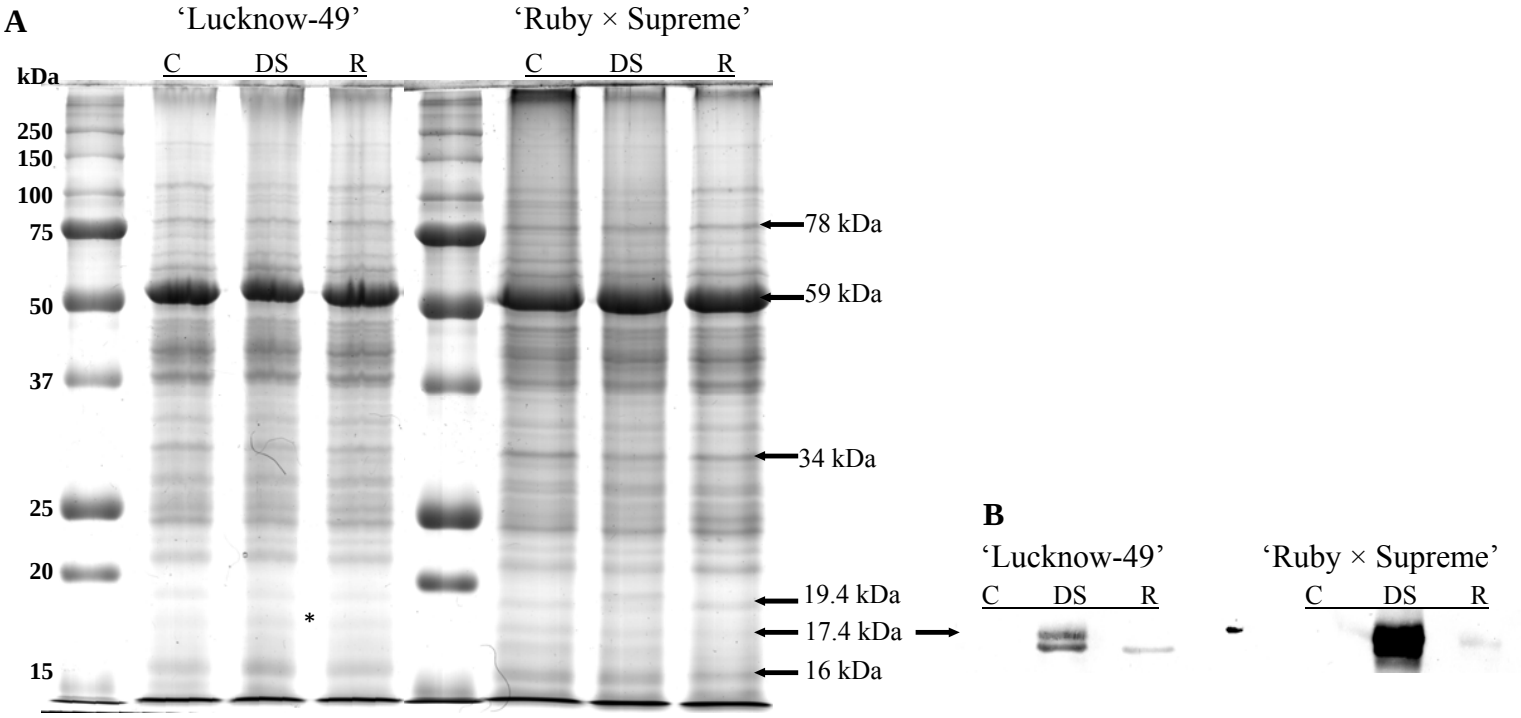
(Fig. 4)


A
‘Lucknow-49’
‘Ruby × Supreme’
NA
CA
NA
CA
kDa
250
150
100
75
50
37
25
20
15
*
**
69 kDa
65 kDa
59 kDa
48 kDa
23.5 kDa
22 kDa
17.4 kDa
B
‘Lucknow-49’
‘Ruby × Supreme’
NA
CA
NA
CA
‘Lucknow-49’
‘Ruby × Supreme’
NA
CA
NA
CA

(Fig. 5)


0.0
-0.4
-0.8
-1.2
-1.6
Leaf water potential (MPa)
'Lucknow-49'
'Ruby × Supreme'
0
4
8
12
16
20
24
Drought stress treatment
Recovery
Time (days)

(Fig. 6)


A
‘Lucknow-49’
‘Ruby × Supreme’
C
DS
R
C
DS
R
kDa
250
150
100
75
50
37
25
20
15
*
78 kDa
59 kDa
34 kDa
19.4 kDa
17.4 kDa
16 kDa
B
‘Lucknow-49’
‘Ruby × Supreme’
C
DS
R
C
DS
R

(Fig. 7)

# CHAPTER 3. GENERAL CONCLUSIONS

In this project, we studied the physiological and biochemical basis of freezing tolerance and cold acclimation of guava. Guava cvs. Lucknow-49 and Ruby × Supreme were used for this project. We performed freezing tolerance tests on leaves, the sensitive tissue, of guava. The results indicated that the $LT_{50}$ were -2.3 ºC and 2.7 ºC for nonacclimated plants, and the leaf freezing tolerance could be enhanced to -4.2 ºC and -4.6 ºC after cold acclimation for 'Lucknow-49' and 'Ruby × Supreme', respectively, when exposed to a step-wise, environmentally controlled cold acclimation treatment. In conclusion, guava is able to withstand some freezing stress, and also be cold acclimated. Specifically, we developed a step-wise, cold-acclimation protocol for guava as: cold exposure at 18 °C / 12 °C (day / night) for 7 d, then 15 °C / 9 °C (day / night) for 7 d, then 10 °C / 5 °C (day / night) for 7 d with a 15-h photoperiod under cool-white fluorescent lamps that provided PAR of about 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Although 'Lucknow-49' and 'Ruby × Supreme' did not show a significant difference in $LT_{50,}$ data on actual freezing injury at sub-freezing test temperatures, indicated that 'Ruby × Supreme' was more freezing tolerant than 'Lucknow-49', suggesting that $LT_{50}$ alone may not be a sufficient parameter to distinguish small yet significant differences in freezing tolerance among genotypes.

Growth measurements, leaf relative water contents and anthocyanins analyses were performed on nonacclimated and cold-acclimated leaves of 'Lucknow-49' and 'Ruby × Supreme.' Growth and leaf water content reduced while anthocyanins accumulated during

cold acclimation in both cultivars. We concluded that the freezing tolerance of cold-acclimated guava leaves may be due, in part, to an increased antioxidant and the decrease of free water in leaf cells. However, 'Ruby × Supreme' showed much greater reduction of leaf water content than 'Lucknow-49' during cold acclimation, suggesting that 'Ruby × Supreme' may be more sensitive to freezing stress than 'Lucknow-49'.

Protein analyses performed after cold acclimation and drought stress by electrophoresis and immunoblotting revealed that three proteins with molecular weights of 69, 23.5, and 17.4 kDa accumulated in response to low temperatures, and three proteins with molecular weights of 78, 17.4, and 16 kDa accumulated in response to drought stress. This suggests increased protein synthesis and / or decreased turnover when exposed to environmental stresses (Wake and Fennell, 2000; Renaut et al., 2004). One common dehydrin (17.4 kDa) accumulated in response to cold and drought stresses. Plants may exhibit partly overlapping responses to freezing and drought stresses since freeze-induced injury in plants is mainly due to cellular dehydration (Close, 1997; Xin and Browse, 2000; Li et al., 2004). This result may imply that the accumulation of the non constitutive 17.4 kDa dehydrin is triggered by dehydration in response to drought or low temperatures.

In summary, we have gained important insights into the freezing tolerance and cold acclimation of guava, however, our overall understanding remains far from complete. In the future, further study of the molecular and genetic mechanisms of freezing tolerance and cold acclimation in guava should be performed to fully understand the diverse mechanisms of guava related to cold stress response.

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