Salicylic acid-induced freezing tolerance and the effect of short-term versus prolonged freezing on freeze-thaw injury in spinach (Spinacia oleracea L.): Understanding the cellular mechanism through metabolite profiling

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Salicylic acid-induced freezing tolerance and the effect of short-term versus prolonged freezing on freeze-thaw injury in spinach (*Spinacia oleracea* L.): Understanding the cellular mechanism through metabolite profiling

by

Kyungwon Min

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

This dissertation constitutes two main research projects: 1) salicylic acid (SA)-induced freezing tolerance, and 2) the effect of short versus prolonged freezing on freeze-thaw injury. Both studies used spinach (Spinacia oleracea L.) as the model. Regarding the SA study, freezing tolerance and metabolome changes were investigated for spinach seedlings sub-fertigated with 0.5 mM SA. Experiments included seedlings sub-fertigated with SA at both the ambient (non-acclimation; NA) and cold acclimation (CA) temperatures, i.e. NASA and CASA, respectively. SA-fed plants exhibited, in general, similar growth performance as non-treated controls. SA application improved freezing tolerance of spinach at both warm- and cold temperatures; CASA-plants were the most freezing tolerant followed by CA-, NASA-, and NA-plants. Metabolite profiling revealed that SA-feeding differentially alters plant metabolism at warm or cold. SA-induced freezing tolerance may be due to improved tolerance to oxidative stress and freeze-desiccation, as indicated by accumulation of antioxidants, compatible solutes, and osmolytes. Additionally, SA-induced FT appears to be mediated cooperatively by NO and H₂O₂ signaling since removal of H₂O₂ or NO reduced beneficial effect of SA on freezing tolerance. This study proposed that exogenous SA application could be an important strategy to improve plant freezing tolerance. In the second study, we conducted metabolite profiling to gain metabolic insight into the differential response by spinach leaves to a short (0.5 and 3.0-h freezing) versus prolonged freezing (5.5 and 10.5-h freezing) at a sub-lethal temperature (-4.5 °C) causing a reversible versus irreversible injury, respectively. Some of the key findings from this study are that: 1) SA may induce tolerance/recovery response in leaves exposed to short-term freezing whereas trigger programmed cell death in irreversibly injured leaves following prolonged freezing, 2) GABA accumulation in freeze-thaw stressed tissues serves as a ‘pH-stat’ against cytoplasmic
acidification in leaves exposed to short-term freezing, 3) chloroplast functions may be less sensitive to prolonged freezing than mitochondria, 4) increased accumulation of fatty acids and policosanols with increasing freezing duration indicates incremental injury to membrane lipids and epicuticular waxes, respectively, and 5) ascorbic acid and α–tocopherol accumulation during short-term freezing may facilitate recovery by removal of free radicals. This study provided insight into several cellular events/alterations potentially associated with reversible versus irreversible injury following a short-term or prolonged freezing, respectively, and thereby advance our fundamental understanding of plant response to freezing.
CHAPTER 1: GENERAL INTRODUCTION

Literature review

Freezing stress and freeze-thaw process

Freeze-thaw stress is one of the major abiotic stresses impacting crop production and distribution of plant species. Resistance to freeze-thaw stress may either take the form of avoidance (i.e. preventing any ice-formation in tissues or preventing freeze-desiccation despite the presence of ice in the tissue) or tolerance (minimizing the injurious effects of extracellular freezing) (Levitt, 1980).

Under a natural frost episode, ice-crystal forms around extracellular spaces at relatively mild temperatures (i.e. -0.5 to -3.0 °C) and relatively slow cooling rates (1-3 °C/h) since extracellular fluid has a higher freezing point due to a lower solute concentration than the intracellular fluid (Guy, 1990; Thomashow, 1999; Arora, 2018). Slow cooling allows the diffusion of cellular water from cytoplasm to extracellular ice as fast as the temperature drops, permitting increase in solute concentration and thus an equilibrium between the chemical potential of cytoplasm and extracellular ice (Levitt, 1980; Arora and Palta, 1991; Arora, 2018). As temperature increases, plant cells experience thaw-rehydration and both freeze-desiccation and thaw-rehydration (i.e. contraction and expansion of membranes, respectively) are considered as stressful events to plant cells (Fig. 1) (Levitt, 1980; Arora, 2018). Extracellular freezing can be tolerated by plant tissues depending upon their threshold tolerance for freeze-desiccation (Arora, 2018).
**Freeze-thaw injury**

Two cellular lesions have been widely understood based on the mechanistic model of freeze-thaw injury of plants: 1) structural and functional perturbation in cell membrane and 2) free radical accumulation resulting in oxidative stress (Arora, 2018).

Due to freeze-desiccation, cell membrane experiences mechanical stress and therefore, is considered as a primary locus of freezing injury (Palta and Li, 1980; Steponkus, 1984; Arora and Palta, 1991; Uemura et al., 2006). Freeze-desiccation is thought to cause not only the structural perturbation but also altered cellular milieu that may be favorable condition to generate excessive accumulation of free radicals (e.g. OH-, O2-, and H2O2) (Fig. 1) (Kendall and McKersie, 1989; Uemura et al., 2006; Chen and Arora, 2014). Indeed, freeze-thaw stress causes accumulation of free radicals and thereby oxidative damage to various cellular components including cell membrane (Kendall and McKersie, 1989; Mittler, 2002; Chen and Arora, 2014; Min et al., 2014; Shin et al., 2018). Moreover, these structural perturbations are known to cause alteration of membrane transport function leading to enhanced ion-leakage; such ion-leakage often coincides with water-soaking due to water efflux to extracellular space during freezing (Palta et al., 1977; Arora and Palta, 1991; Min et al., 2014; Arora, 2018).

![Fig. 1. An illustration of freeze-thaw process/stress at the plant cell level. +/- and Ø represent ion-leakage and water soaking, respectively. CM, cell membrane, CW, cell wall.](image-url)
Plant cold acclimation

Many plants from temperate regions have an ability to improve their FT when exposed to low temperatures (and other inductive conditions), an adaptive process known as cold acclimation (Levitt, 1990; Thomashow, 1999; Xin and Browse, 2000). Cold acclimation is a multi-factorial, complex response involving many physiological, biochemical and molecular changes that are associated with induction of FT (Xin and Browse, 2000); these changes, for instance, include reduced growth, membrane modification, accumulation of compatible solutes (osmolytes) and antioxidants, changes in hormone levels, and alterations in gene expression (Fig. 2) (Guy, 1990; Arora et al., 1996; Thomashow, 1999; Xin and Browse, 2000).

Fig. 2. Various examples of cellular changes induced by plant cold acclimation.
Free radicals and antioxidants

Free radicals are partially reduced forms of atmospheric oxygen. They typically result from the excitation of $O_2$ to form singlet oxygen ($^1O_2$) or from the transfer of one, two or three electrons to $O_2$ to generate, respectively, a superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) or a hydroxyl radical ($HO^-$). It has been well known that plants accumulate free radicals in response to abiotic stresses including freeze-thaw stress (Kendall and McKersie, 1989; Chen and Arora, 2014; Min et al., 2014; Shin et al., 2018). Excessive free radicals are very toxic molecules that can cause oxidative damage to proteins, DNA, and lipid if not properly scavenged (Foyer et al., 1994; Mittler, 2002; Rossini et al., 2006).

Plants have evolved antioxidant mechanism to counteract the toxic effect of free radicals via antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), and antioxidant metabolites (e.g. ascorbic acid, tocopherol, and glutathione) (Guta et al., 2018). SOD catalyze the conversion of superoxide ($O_2^-$) into $H_2O_2$ whereas CAT and APX are responsible for detoxifying $H_2O_2$ into $H_2O$ (Guta et al., 2018). Ascorbic acid (vitamin C) essentially serves as a substrate in glutathione-ascorbate cycle, catalyzed by APX to detoxify $H_2O_2$ (Smirnoff, 2000). Tocopherol (vitamin E) are also potent antioxidants that scavenge lipid peroxyl radical generated during lipid peroxidation (Munné-Bosch and Peñuelas, 2003; Yusuf et al., 2010).

Compatible solutes

In response to cellular desiccation, many plants accumulate compatible solutes, irrespective of whether the desiccation is caused by drought, freezing or osmotic shock (Hoekstra et al., 2001). Compatible solutes are small organic molecules that accumulate under stress conditions and considered to stabilize proteins and membranes by preferential
exclusion and contribute to balancing the cell osmotic pressure (Hoekstra et al., 2001; Kaplan et al., 2004; Chen et al., 2007; Wani et al., 2013). Examples include betaines, amino acids (e.g. proline), and the sugars (e.g. trehalose, and sucrose) (Hoekstra et al., 2001; Wani et al., 2013). Evidence has been provided for a close relationship between elevated concentration of these compatible solutes and improved stress tolerance including freezing (Hoekstra et al., 2001; Kaplan et al., 2004; Lee et al., 2012).

**Cold damage under climate change**

Although mean global temperature is annually rising due to global warming, frequency of temperature extremes/sudden swings is also increasing, which can induce even greater risk of frost damage to plants (Arora, 2018). Such injurious frosts can cause tremendous economic loss to important fruit/vegetable crops (Gu et al., 2008). Unusually late spring or early fall frosts could cause injury to tender annuals. Unseasonal warm temperature during winter season or early spring can cause premature de-acclimation wherein plant tissues are sensitive to unseasonal frost; also, a warmer than normal fall temperature can delay cold acclimation or hinder attainment of maximum cold hardiness in which plant tissues are vulnerable to severe winter (Arora, 2018). Therefore, it is imperative to develop efficient strategies for improving FT in order to reduce potential frost damage from such unseasonal/erratic frost episode.

**Improving freezing tolerance via chemical priming**

Breeding is conventionally selected as a strategy to improve stress tolerance against various abiotic stresses including freezing. However, this method is somewhat time-
consuming mainly due to the long generation time of plants as breeding needs frequent crossing and self-fertilization. As an alternative, plants can be transiently ‘prepared’ in short time against future abiotic stress via ‘chemical priming’ (i.e. pre-exposure of plants to natural or synthetic chemical compounds) (Savvides et al., 2016). Chemical priming is known to activate ‘protection response’ much faster, stronger or both when a stress pressure is encountered. Thus, chemical priming would be fascinating method for farmers to protect or transiently enhance freezing tolerance of their crop against upcoming or unseasonal freezing stress. Indeed, various chemical compounds (e.g. melatonin, ABA, proline, etc.) has been used to improve cold tolerance (Abromeit et al., 1992; Fu et al., 2017; Mohammadrezakhani et al., 2019).

**Spinach (Spinacia oleracea L.)**

Spinach is a cool season vegetable crop and has become an increasingly important economic crop worldwide. The world production of spinach was 27.9 million tons in 2017 (FAO, 2019). The United State is the second largest producer of spinach (estimated to be 8.48 million tons in 2018) following China, and the value of the crop totaled $423 million in 2018 U.S (NASS, 2019). During the last 15 years, spinach industry in the US has observed a substantial increase in fresh market demand, which motivated to increase spinach production (Shi et al., 2017). Given that spinach planting is made during spring or fall season, it may be essential to prepare methods for protecting spinach against unseasonal frost. Chemical priming could be employed as one of the strategies to achieve this objective.
Salicylic acid

Salicylic acid (SA) is one of the phenolic compounds synthesized in plants. The name of SA is derived from the word *Salix*, the scientific name of the willow tree (*Salix alba* L.) (Vlot et al., 2009; Hara et al., 2012; Miura and Tada, 2014). SA is the oxidized form of salicyl alcohol which constitutes salicin (i.e. isolated from the willow bark) with glucose (Mahdi et al., 2006). The plants containing SA were frequently used for therapeutic purpose throughout the ancient world and its acetylated derivate (i.e. known as aspirin) is one of the most widely used as a drug in the world (Dempsey and Klessig, 2017).

SA is typically present in plants in quantities of few μg/g fresh weight or less in a free state or in the form of methylated, glycosylated, or amino acid conjugates (Raskin et al., 1990; Lee et al., 1995). Two pathways have been recognized to synthesize SA in plants. One is the phenylalanine ammonia-lyase (PAL) pathway and the other is the iso-chorismate synthase (ICS) pathway, of which ICS pathways is known as the major pathways for SA synthesis in *Arabidopsis*, *Nicotiana benthamiana*, and tomato (Hara et al., 2012).

SA is known as one of the phytohormones involved in biotic stress response (Dempsey et al., 2011). Plants accumulate endogenous SA in the necrotic lesion following pathogen attack and induces hypersensitive response (HR) as well as the expression of pathogenesis-related genes by which the spread of infection is prevented (Enyedi et al., 1992; Vlot et al., 2009). It has been also noted that SA is required for systemic acquired resistance (SAR) which is a slower systemic (whole plant) response occurring following HR (Gaffney et al., 1993). In addition to plant defense, SA is also known to be involved in the response to abiotic stresses. Several studies have been reported that exogenous SA enhanced plant tolerance against various abiotic stresses including drought, salt, ozone, UV radiation and heavy metals (Yalpani et al., 1994; Sharma et al., 1996; Miura and Tada, 2014; Hara et al.,
2012; references in Khan et al., 2015). However, the effect of exogenous SA on FT in plant tissues has not been well investigated.

Nitric oxide (NO) and hydrogen peroxide (H$_2$O$_2$) along with SA are well-known signaling molecules. It has been reported that SA induces the production of H$_2$O$_2$, and H$_2$O$_2$ also modulates the SA biosynthesis in response to various biotic and abiotic stresses (Jayakannan et al., 2015). On the other hand, H$_2$O$_2$ induces NO synthesis and NO is also known to modulate H$_2$O$_2$ levels (Zhang et al., 2007; Neill et al., 2008). Therefore, a crosstalk has been suggested between these signalling molecules (Fig. 3) (Esim and Atici, 2014; Miura and Tada, 2014; Qiao et al., 2014; Mostofa et al., 2015). Several studies reported that exogenous SA, H$_2$O$_2$, and NO improved salt stress tolerance by alleviating oxidative stress in various plant species (Li et al., 2011; Khan et al., 2014). However, a potential crosstalk between SA, NO and H$_2$O$_2$ remains to be elucidated in relation to FT.

![A crosstalk between SA, NO, and H$_2$O$_2$ under biotic- and abiotic-stresses.]

**Fig. 3.** A crosstalk between SA, NO, and H$_2$O$_2$ under biotic- and abiotic-stresses.
Plant metabolomics

The main goal of plant metabolomics is to provide a non-biased characterization of the total metabolite pool of a plant tissue (Fiehn, 2002; Kumar et al., 2017; Jorge and António, 2018). The metabolites can be defined as the end-products of gene expression or enzymatic activity that can be altered depending upon environmental conditions and therefore, metabolomics has been proposed as a useful tool for studying the molecular and biochemical mechanism underlying plant responses to abiotic stresses (Jorge and António, 2018). The size of metabolome differs between different organisms and the plant kingdom has been estimated that more than 200,000 different primary and secondary metabolites present over a large dynamic range in concentrations that can vary from femtomolar to millimolar (Fernie, 2003; Dunn and Ellis, 2005). Therefore, it is impossible to analyze all metabolites in a single analysis with current analytical equipment. In order to address this problem, different analytical approaches (e.g. extraction method, type of chromatography and column, etc.) have been required to answer specific biological questions (Fiehn, 2002).

Mass spectrometry (MS)-based analytical tools are the most widely employed in plant metabolomics. Among them, powerful chromatographic techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) have been frequently used to gain comprehensive pictures of the plant metabolome across various plant species (Obata and Fernie, 2012; Jorge et al., 2016).

The first large-scale plant metabolomics was made by Roessner et al. (2000, 2001) on potato tubers (Solanum tuberosum L.) and Arabidopsis thaliana leaf extract (Fiehn et al. 2000) using GC-MS. These two metabolomics studies motivated other researchers to address different biological questions including the changes in the metabolome during cold acclimation (Cook et al., 2004; Kaplan et al., 2004) and the metabolite profile in spinach
treated with various chemical compounds including nitrate and CeO$_2$ nanoparticles (Okazaki et al., 2008; Liu et al., 2016; Zhang et al., 2019).

**Freeze-thaw injury under prolonged freezing**

Several factors associated with freeze-thaw process affect the degree of injury in plant tissues; these are, for example, the extent of freezing temperature, the rate of cooling, whether or not tissues were ice-nucleated, the rate of thawing and the duration of freezing (Gusta and Wisniewski, 2013; Arora, 2018). Although the first three of them are relatively well understood, the last two are not understood yet (Arora, 2018).

Temperature-controlled, laboratory-based freeze-thaw assays are routinely employed to determine FT of excised tissues across various plant species. Typically, in these tests, tissues are subjected to various test temperatures for 30 to 60 min following ice-nucleation. After thawing at 0 to 4 °C, tissues are used to estimate injury using an electrolyte-leakage based assay and the FT is determined as LT$_{50}$, a lethal temperature causing 50 % injury (Lim et al., 1998). While widely used successfully for estimating relative FT of plant tissues, these tests do not consider the effect of duration of freezing on the estimated LT$_{50}$ since tissues are typically held at test temperatures for a relatively short duration (Min et al., 2014).

That short-term versus prolonged freezing can cause significantly different injury was first reported by Pomeroy et al. (1975) in winter wheat. Later work noted that prolonged freezing test is more useful in selecting more hardy genotypes, which otherwise possessed similar LT$_{50}$ as the less hardy ones when evaluated by a conventional freeze-test (Gusta et al., 1997; Waalen et al., 2011). Nagao et al. (2008) demonstrated that ultrastructure changes in plasma membrane caused by prolonged freezing were more severe than those exposed to
short-term freezing. Also, we have previously reported that a freezing temperature regarded as sub-lethal based on the LT_{50} could indeed be lethal to spinach leaves depending upon the duration of freezing. Moreover, the ability of recovery from freeze-thaw injury was also significantly influenced by the duration of freezing at a given freezing temperature (Min et al., 2014). Given that tissues exposed to a fixed sub-freezing temperature under slow/equilibrium freezing protocol would undergo a fixed level of freeze-dehydration, it is curious as to why or how plants undergo substantially greater (or irreversible) injury following prolonged freezing than those subjected to shorter-freezing experiencing only moderate (or recoverable) injury (Fig. 4). Thus, in-depth investigation is needed to discern the cellular/molecular mechanism of injury due to shorter versus prolonged freezing.

**Fig. 4.** An illustration of substantially different levels of freeze-thaw injury when tissues are stressed for shorter versus prolonged freezing at a constant freeze-desiccation. Small versus large size of the symbols, +/− and  represent lower versus higher ion-leakage and water soaking, respectively. Similarly, small versus big font-size of ‘cellular dysfunction’ represents the relative level of injury under two scenarios. CM, cell membrane; CW, cell wall.
Research objectives and hypotheses

The overall general objectives of this dissertation are to study:

(1) the effect of exogenous salicylic acid (SA) on freezing tolerance (FT) in spinach (Spinacia oleracea L.) at the cellular and biochemical levels; and

(2) possible cellular mechanism for differential response by spinach leaves to a short *versus* prolonged freezing at a sub-lethal temperature causing a reversible *versus* irreversible injury, respectively.

Specific hypotheses of this research are as follows:

- **Hypothesis:** exogenous SA-application induces FT in excised spinach leaves, and the two signaling molecules NO and/or H$_2$O$_2$ are involved in SA-induced response (*Chapter 2*). Nutrient solution, with or without SA and other molecules, was administered to excised petiolate leaves in a ‘microfuge’ system (details under chapter 2).

- **Hypothesis:** exogenous SA improves FT of spinach seedlings at the whole-plant level, and is associated with altered leaf metabolome (*Chapter 3*). SA was supplied to seedlings via sub-fertigation (details under chapter 3).

  Additionally, the effect of SA feeding on leaf metabolite profiles and freezing tolerance of non-acclimated (NA) *versus* cold acclimated (CA) seedlings was compared to gain insight into the cellular basis for differential response under two temperature regimes, as follows:
a) FT of SA-fed plants grown and exposed to ambient temperatures, i.e. NA condition (NASA) versus those that were SA-fed and grown at non-acclimated conditions but were also cold acclimated (CASA);

b) comparison of leaf metabolite profiles among NA, NASA, CA, and CASA using gas chromatography-mass spectrometry (GC-MS).

- **Hypothesis:** metabolite profiles of thawed leaves following a short-term (0.5- and 3.0-h) vs. prolonged freezing (5.5- and 10.5-h) at a sub-lethal temperature is different and associated with reversible or irreversible injury, respectively (Chapter 4).
Thesis Organization

This dissertation includes 5 chapters.

- **Chapter 1** is General Introduction/Literature review

- **Chapter 2** is ‘Exogenous salicylic acid improves freezing tolerance of spinach (Spinacia oleracea L.)’; published in Cryobiology (2018) 81, 192-200.

- **Chapter 3** is ‘Salicylic acid-induced freezing tolerance in spinach (Spinacia oleracea L.) leaves explored through metabolite profiling’; published in Experimental and Environmental Botany (2018) 156, 214-227.


The contents (i.e. text, tables, and figures) of chapter 2, 3, & 4 are identical to that of the published versions.

- **Chapter 5** is General Conclusions.
References


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CHAPTER 2. EXOGENOUS SALICYLIC ACID IMPROVES FREEZING TOLERANCE OF SPINACH (Spinacia oleracea L.)


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ABSTRACT

Salicylic acid (SA)-treatment has been reported to improve plant tolerance to various abiotic stresses. However, its effect on freezing tolerance has not been well investigated. We investigated the effect of exogenous SA on freezing tolerance of spinach (*Spinacia oleracea* L.) leaves. We also explored if nitric oxide (NO) and/or hydrogen peroxide (H$_2$O$_2$)-mediation was involved in this response, since these are known as primary signaling molecules involved in many physiological processes. A micro-centrifuge tube-based system used to apply SA to petiolate spinach leaves (0.5 mM over 4-d) was effective, as evident by SA content of leaf tissues. SA-treatment did not hamper leaf growth (fresh and dry weight; equatorial and longitudinal length) and was also not significantly different from 25% Hoagland controls vis-à-vis growth. SA application significantly improved freezing tolerance as evidenced by reduced ion-leakage and alleviated oxidative stress (lower accumulation of O$_2^-$ and H$_2$O$_2$) following freeze-thaw stress treatments (-6.5, -7.5, and -8.5 °C). Improved freezing tolerance of SA-treated leaves was paralleled by increased proline and ascorbic acid (AsA) accumulation. A 9-d cold acclimation treatment also improved leaf freezing tolerance (compared to non-acclimated control) and was accompanied by accumulation of SA and proline. Our results indicate that increased FST may be associated with accumulation of compatible solutes (proline) and antioxidants (AsA). Notably, the beneficial effect of SA on freezing tolerance was abolished when either H$_2$O$_2$- or NO-scavenger (1 µM N-acetylneuraminic acid, NANA or 100 µM hemoglobin, HB, respectively) was added to SA as pretreatment. Our data suggest that SA-induced freezing tolerance in spinach may be mediated by NO and H$_2$O$_2$ signaling.
1. Introduction

Freeze-thaw is one of the major environmental stressors impacting crop yield and distribution of plant species. Long enough exposure to temperatures cooler than the freezing tolerance threshold of plant tissues can result in irreversible injuries. By far, the primary cause of injury due to an equilibrium freezing episode is cellular dysfunction resulting from dehydration and contraction (during freezing) followed by rehydration and expansion (during thaw) [4]. Thus far, two cellular loci have been predominantly implicated to explain the mechanism of freeze-thaw injury: 1) structural and functional perturbations in cell membranes, evident in solute leakage [1, 64], and 2) excessive production of reactive oxygen species (ROS) [3, 30], which, if not adequately removed through antioxidants, damage cellular components including membranes, proteins, and nucleic acids [43].

Salicylic acid (SA), a hormone-like plant phenolic, has been widely implicated as signal molecule mediating defense mechanisms against pathogens, such as hypersensitive response and development of systemic acquired resistance [8]. Evidence is also accumulating for SA-application enhancing tolerance against abiotic stresses including heat, chilling, drought, and salt stress [31, 42, 55]. However, the effect of exogenous SA on freezing tolerance in plant tissues has not been well investigated.

Nitric oxide (NO) and hydrogen peroxide (H$_2$O$_2$) are well known key signaling molecules in plants, regulating biological processes and mediating diverse protective mechanisms against abiotic stresses [51]. Accordingly, to gain mechanistic insights into SA’s protective role, several laboratories have explored potential cross-talk among SA, H$_2$O$_2$ and
Research also suggests that improved abiotic stress tolerance or stress-alleviation in the presence of exogenous SA may be mediated via: 1) bolstered pool and/or activity of enzymatic / non-enzymatic antioxidants, and/or 2) accumulation of compatible solute, proline, both of which have been widely correlated with acquisition of stress tolerance [2, 43, 59, 61, 67]. Indeed, pretreatment of SA improves chilling tolerance of maize seedlings via increase in \( \text{H}_2\text{O}_2 \) concentration which in turn induced an increase in antioxidant enzymes activities [25]. Moreover, combined application of NO and SA to wheat seedlings represses more efficiently the accumulation of malondialdehyde (MDA) and ROS and enhances activities of antioxidant enzymes than NO and SA alone [9]. However, a potential cross-talk among SA, NO, and \( \text{H}_2\text{O}_2 \) remains unknown in relation to freezing tolerance.

Spinach (\textit{Spinacia oleracea} L.), an important horticultural crop, is vulnerable to sudden and unseasonal spring frost often resulting in frost-damage and economic losses. Frequency of such occurrences is predicted to increase in future due to vagaries of climate change. Development of strategies or cultural practices to improve freezing tolerance would, therefore, be beneficial to horticulture industry. The major objectives of the present study were to determine whether exogenous SA-application could induce freezing tolerance in spinach leaves, and whether NO and / or \( \text{H}_2\text{O}_2 \) are involved in SA-induced response. In order to gain insight into the mode of action for SA-treatment, experiments were also conducted to monitor leaf growth and tissue content for SA, proline and a non-enzymatic antioxidant, ascorbic acid (AsA). A micro-centrifuge tube-based system
2. Materials and Methods

2.1. Plant Material and Growing Conditions

Seeds of *Spinacia oleracea* L. ‘Reflect’, an F₁ hybrid cultivar (Johnny’s selected seeds, Inc., Winslow, ME) were sown in plug flats using Sunshine LC-1 mix (Seba Beach, Alberta, Canada) and placed in growth chamber at 15/15 °C (D/N) under average photosynthetically active radiation (PAR) of ~300 μmol m⁻² s⁻¹ and 12-h photoperiod provided by incandescent and fluorescent lights. Seedlings were watered as needed (~ 4-d interval). Two weeks later, the growth temperature was elevated to 20/18 °C (D/N) and 300 ppm EXCEL nutrition solution (Scotts Sierra Horticultural Products Company, Marysville, OH) was fed to seedlings through sub-irrigation. About 17-day-old seedlings were harvested and used for a SA and other treatments in a micro-centrifuge tube system.

2.2. Micro-Centrifuge Tube System and Treatments

First two true petiolate leaves were excised in deionized water to prevent embolism of xylem and inserted in a 1.5 mL micro-centrifuge tubes (containing treatment solutions) through an adequate sized hole drilled in the lids of the tubes; these tubes are hereafter referred to as ‘micro-tubes’ (Fig. 1 inset). Control (25% Hoagland nutrition solution; [20]) and all the treatments, also made with 25% Hoagland as solvent, (all at ~ pH 6.5± 0.4) are as follows: (1) HG-control [25% Hoagland nutrition solution (HG)], (2) 0.5 mM or 1 mM SA, (3) 1 μM N-acetylneuraminic acid (NANA, a H₂O₂ scavenger), (4) 0.5 mM SA + 1 μM NANA, (5) 100 μM hemoglobin (HB, a NO scavenger), (6) 0.5 mM SA + 100 μM HB, and (7) 0.5 mM SA+ 1 μM NANA+ 100 μM HB. Tubes held in micro-tube racks were placed in a growth chamber at 20/18 °C (D/N), ~300 μmol m⁻² s⁻¹, and 12-h photoperiod. To avoid any dehydration, especially in the early stages of sample transfer to the growth chamber, micro-
tube racks were covered with a perforated transparent plastic dome with moist paper towels placed under it. The tubes were examined every 24 h and refilled with the spent solution as needed. After a 4-d treatment (precisely 96 h), samples were used for freeze-tolerance assays and other experimentation described below.

2.3. Leaf Growth Measurement

Leaf-growth was estimated by measuring length (longitudinal/equatorial), fresh weight, and dry weight. Leaf length and fresh weight were measured before and after the 4-d treatment in each solution. Dry weight was measured only after the 4-d treatment by oven-drying leaves at 75 ± 1 °C for 72 h. Data were obtained from 2 independent experiments with total of 32 technical replicates (16 technical replicate/experiment sampled from 8 individual plants; one leaf/technical replicate).

2.4. Freezing Tolerance Measurement

Leaf freezing tolerance was determined using the ion-leakage-based laboratory freeze-thaw protocol, as detailed by Chen and Arora [3]; Min et al. [41]. Essentially, a pair of petiolate leaves (dip-rinsed four times with deionized water and blotted) was placed in a 2.5 × 20 cm test tube with petioles standing in 150 µL deionized water and slowly cooled (−0.5 °C/30 min) in a glycol bath (Isotemp 3028; Fisher Scientific, Pittsburgh, PA) to various freezing treatment temperatures following ice-nucleation at −1 °C. Tissues were held for 30 min at each temperature and thawed on ice overnight. Unfrozen control (UFC) leaves were maintained at 0 °C throughout the freeze-thaw cycle.

For selecting the freezing treatment temperatures, first a LT50 curve was generated for micro-tube control (HG-control) samples by freezing the leaves at −3 to −12 °C. Tubes were
removed from the glycol bath at –1 °C intervals. This experiment was repeated thrice, each with 5 technical replicates/temperature. Percent injury at individual treatment temperatures from these experiments were pooled and used to generate a sigmoid curve fitting the Gompertz function [39]; LT$_{50}$ (°C), mid-point between the minimum and maximum injury, was defined as the leaf freezing tolerance. Guided by this sigmoid curve, 3 treatment temperatures, –6.5, –7.5, and –8.5 °C, were selected for comparing freezing tolerance of HG-control and 0.5 mM or 1.0 mM SA-treated leaves. For freezing tolerance experiments involving all 7 treatment solutions (i.e., HG, SA, NANA, HB, SA+NANA, SA + HB, and SA+NANA+HB), leaves were exposed to only two freezing treatments, -6.5 and -7.5 °C.

Samples were removed from the glycol bath following freezing at selected temperatures and thawed as explained above. Freezing tolerance tests were independently repeated five times for HG-control, 0.5 mM and 1.0 mM SA comparison, and thrice for the 7 treatments experiment, respectively, each including 4 to 8 technical replications per treatment temperature (two leaves/technical replicate). Percent injury data from these independent experiments were pooled to obtain treatment means.

2.5. Staining of ROS (Superoxide and Hydrogen Peroxide)

Histochemical detection of superoxide (O$_2^•$) and hydrogen peroxide (H$_2$O$_2$) was performed using the procedures previously used in our laboratory for spinach leaves [3, 41]. Staining intensities were visually evaluated for HG-control and 0.5 mM SA-treated leaves following exposure to freeze-thaw at -6.5 and -7.5 °C. Staining experiments were independently repeated twice, each with two replications (two leaves/replicate) per temperature per treatment solution. A representative picture of that data is presented here.
2.6. Quantification of SA, Pro, and AsA in Leaf Tissues

SA, proline, and AsA were quantified in HG-control and 0.5mM SA-treated leaves using gas chromatography-mass spectrometry (GC-MS). Protocols used to extract SA, proline, and AsA were as described by Noutsos et al. [49], with some modifications. Two-hundred mg of liquid N2-ground leaf powder was mixed with internal standard (25 μg of ribitol) and homogenized with 0.35 mL hot methanol (60°C) for 10 min followed by 10 min sonication. Chloroform (0.35 mL) and 0.3 mL of HPLC grade water were then added to the mixture and vortexed for 1 min followed by centrifugation (10,000g) for 10 min. Upper, polar phase (200 μl), was removed and transferred into GC-MS vial (2 mL) and dried in a SpeedVac for 10-h. Extracts were methoximated using methoxyamine hydrochloride (50 μl of 20 mg/mL) and incubated for 90 min at 30 °C. Samples were silylated [by adding 70 μl of bis-trimethyl silyl trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS)] for 30 min at 37 °C, and subjected to GC-MS (7890A GC, 5975C MSD, and separation column HP5MS; Agilent Technologies). Three target molecular species were identified using the method of Sumner et al. [60] by comparing the mass spectral to NIST08 Library and using Retention Indices followed by quantification based on internal standards.

Measurements consisted of three biological replications, each with two technical replicates (each technical replicate was a 200 mg powder sub-sampled from the bulk generated by grinding 4-5 leaves).

2.7. Non- and Cold-Acclimation Treatments

These treatments were administered on whole seedlings grown in the greenhouse media (not the micro-tube-treated ones). Plants grown for ~21-d in growth chamber (same conditions as described above) were considered as non-acclimated (NA). Nineteen-day old
seedlings, grown same as NA plants, were transferred to a cold room (4 °C and 12-h photoperiod at ~75 µmol m⁻² s⁻¹) for 9-d, and used as cold-acclimated (CA) plants. LT₅₀ of NA and CA plants was evaluated as described under ‘freezing tolerance Measurements’. Experiments consisted five biological replications, each including 5-8 (for NA) and 4-5 (for CA) technical replicates.

2.8. Statistical Analysis

Statistical differences were assessed using analysis of variance (ANOVA) employing R (version 3.2.2, The R Foundation for Statistical Computing, ISBN 3-900051-07-0). Mean differences were analyzed by Duncan’s multiple range test or the Student’s t-test.

3. Results

3.1. Growth Measurements in SA-Treated and HG-Control Leaves

Leaves treated with 0.5 mM SA in micro-tubes for 4-d and their corresponding HG-controls continued to expand, as evident by increase in longitudinal and equatorial dimensions. While no significant difference for the two sets in equatorial growth was observed (relative to respective 0-d dimensions), SA-treated leaves grew relatively less longitudinally, though only by ~3% (Table 1). SA-treated leaves showed slightly lower gain in FW over 4-d treatment compared to HG-control but FW/DW ratios for the two sets were essentially same (Table 2).

3.2. LT₅₀ of Spinach Leaves Maintained in Micro-Tube System

Freeze-thaw response curve (-3 to -12 °C) for ‘Reflect’ spinach leaves treated in micro-tubes with 25% HG for 4-d is presented in Fig. 1. Minimum and maximum injury
percentages were 0.2 % and 81.9 %, respectively, with -6.9 °C corresponding to the mid-point of percent injury (41.1%), hence was considered as LT50 or freezing tolerance. A picture of the micro-tube system is presented as an inset.

3.3. Effect of Exogenous SA on Freezing Tolerance and SA Content

Three test temperatures, -6.5, -7.5 and -8.5 °C, were selected to compare freezing tolerance of HG-control and SA-treated leaves. In preliminary experiments, four SA concentrations, i.e. 0.25, 0.5, 1.0 and 2.0 mM, were tested as leaf treatments. No significant difference was observed for freezing tolerance at 0.25 mM SA compared to HG control, while 2 mM SA treatment resulted in somewhat stunted growth and wilted appearance of leaves during the 4-d exposure (data not shown). Hence these two SA concentrations were dropped for subsequent experiments.

Leaves treated with 0.5 mM SA were significantly more freeze-tolerant than HG-controls as evidenced by a reduction in freeze-thaw injury by 64.9, 43.9, and 14.2 % at -6.5, -7.5, and -8.5 °C, respectively (Fig. 2A). Likewise, percent injury for 1.0 mM SA treated tissues frozen at the same three temperatures was significantly reduced by 60.8, 33.1, and 12.2 %, respectively. Since no significant difference in percent injury was detected between 0.5 mM- and 1.0 mM SA-treatment, only 0.5 mM SA was selected for subsequent freezing tolerance and other experiments. Also, since the improvement in freezing tolerance by SA-treatment was more pronounced at -6.5 and -7.5 °C stress level, the third treatment temperature (-8.5°C) was not included in subsequent experiments.

SA content of leaves treated with 0.5 mM and 1.0 mM SA was ~6.7 and 270-fold of HG-Control (Fig. 2B), indicating that micro-tube system was effective in SA-uptake by petiolate spinach leaves.
3.4. ROS (O$_2^-$ and H$_2$O$_2$) Staining in SA-Treated and HG-Control Leaves

Distribution of superoxide (O$_2^-$) (blue stain) and hydrogen peroxide (H$_2$O$_2$) (brown stain) showed similar pattern across two independent experiments; Fig. 3 presents a representative set of images. Although stain intensity was not digitally quantified, a higher accumulation of O$_2^-$ and H$_2$O$_2$ was apparent in injured tissues compared to unfrozen controls (UFCs) wherein the two ROS were essentially undetectable. Moreover, ROS accumulation increased with severity in freezing stress (-6.5 versus -7.5 °C). Data also indicate that ROS staining intensity in SA-treated leaves was lower than HG-control at both stress levels, indicating reduced ROS accumulation (compare Fig. 3A, C with Fig. 3B, D).

3.5. Freezing Tolerance and SA Content of NA and CA Leaf Tissues

Freezing tolerance (LT$_{50}$) of NA and CA leaves were -5.8 and -9.9°C, respectively (Fig. 4A). SA content of NA and CA tissues was 0.14 and 0.65 pmol/mg FW, respectively, indicating ~5-fold accumulation during CA (Fig. 4B).

3.6. Proline and AsA Content of SA-Treated, NA and CA Leaf Tissues

Proline and AsA contents of 0.5 mM SA-treated tissues were significantly higher (~1.5- and 1.75-fold, respectively) than those of HG-Control (Fig. 5A and B). A 9-d CA treatment (4°C) of spinach seedlings also resulted in significant increase in proline content (~28-fold) compared to NA tissues (Fig. 5C) while no marked change in AsA content was observed after CA (Fig. 5D).
3.7. Effect of Exogenous SA (with or without \( \text{H}_2\text{O}_2 \) or NO-Scavenger) on Freezing Tolerance

To explore if SA-induced freeze-tolerance potentially involves \( \text{H}_2\text{O}_2 \) or NO mediation, experiments were conducted to evaluate freezing tolerance of leaves (at -6.5 and -7.5 °C stress treatments) that were pre-treated with ‘SA-alone’ or in the presence of \( \text{H}_2\text{O}_2 \) or NO-scavenger (NANA and HB, respectively); NANA-alone, HB-alone, SA+NANA+HB, and HG-control were included as controls. Freezing tolerance experiment was repeated thrice, each with 4 to 5 technical replicates / temperature / treatment (two leaves/replicate). Means with standard errors from the pooled data across these experiments are presented in Fig. 6.

Leaves treated with 0.5 mM SA suffered ~59% and 40% less injury at -6.5 and -7.5 °C, respectively, compared to HG-control, and thus provided the highest protection from freezing stress among six treatments. Notably, the beneficial effect of SA on freezing tolerance at both stress temperatures was completely offset when \( \text{H}_2\text{O}_2 \) scavenger (NANA) was added to SA as pretreatment; this treatment essentially resulted in similar freezing tolerance as HG-control. ‘NANA-alone’ pretreatment too was ineffective at eliciting any significant increase in freezing tolerance compared to HG-control but also did not adversely affect freezing tolerance. ‘SA+NO-scavenger’ (SA+HB) appeared slightly effective at improving freezing tolerance but only at -7.5 °C. Finally, ‘HB-alone’ and ‘SA+HB+NANA’ were not only ineffective but the only treatments that appeared somewhat deleterious, both causing ~ 30% increase in freeze-injury at -6.5 °C compared to controls while >2.5-fold injury compared to ‘SA-alone’.
4. Discussion

Evidence is accumulating for the beneficial effects of SA application on plant tolerance to abiotic as well as biotic stresses [24, 31]. However, research on SA’s effect on freezing tolerance remains scarce. Investigations also show that SA’s effect might involve NO and/or H$_2$O$_2$ signaling and that improved performance by tissues may result, in part, from increased tolerance to oxidative stress. Accordingly, we set out to explore a hypothesis that SA application increases freezing tolerance of Spinacia oleracea L. ‘Reflect’ leaves and that this effect involves NO and/or H$_2$O$_2$ mediation.

To treat leaves with SA without potential confounding effect on the whole plant, we devised a micro-tube system. Our data on the SA contents of 4-d treated leaves indicates that this system allowed SA uptake through leaf petioles. Solution uptake was also evident by the need to having to refill the micro-tubes over time to replace spent solutions ensuring the submergence of petiole bases under liquids throughout. Leaf growth parameters and visual observations (leaf turgor or any indication of chlorosis) recorded over 4-d treatment period indicate that SA treatment used in this study was not detrimental. Based on the literature survey of SA concentrations used across diverse species for effecting tolerance against drought, chilling, salt, heat, or cold [29, 45, 47, 55], we first tested 4 levels of SA application, 0.25, 0.5, 1.0, and 2.0 mM, of which, only 0.5 mM was selected for most of the work for reasons discussed below and elsewhere.

4.1. SA Treatment Increases Freezing Tolerance

Based on the freeze-response curve of HG-control leaves from micro-tube system (Fig. 1), we selected three stress temperatures, -6.5, -7.5, and -8.5 °C, to compare freezing tolerance of SA-treated and control tissues; these represented physiologically relevant injury
levels, namely, substantial yet relatively mild (~25%), approx. LT_{50} level (~50%), and a level more severe than LT_{50} (~70%) but milder than maximum injury.

Leaves treated with 0.5 mM and 1.0 mM SA were significantly less injured (ion-leakage assay) compared to controls at all stress levels, and both treatments were statistically equally effective at improving freezing tolerance (Fig. 2A). Since the leaves treated with 1.0 mM SA showed slight wilting, though only during the initial 24-h of treatment (data not shown), and accumulated intriguingly/abnormally high SA (~270-fold of control; Fig. 2B), this treatment too was dropped from subsequent physiological/biochemical investigations. Our selection of 0.5 mM SA for this study is in accordance with Hara et al. [15] who noted that optimal SA treatment for most plants’ enhancement of abiotic stress tolerance ranged between 0.1 – 0.5 mM.

Though several studies have explored SA’s role in conferring chilling tolerance (references in [53]), we are aware of only three reports to have shown increase in freezing tolerance by SA application [45, 62, 66]; one dealt with in vitro cultured potato plantlets while others employed SA ‘spray’ on winter-wheat leaves and grape leaves (unlike petiole-uptake in our study); moreover, neither study provided any data on SA uptake by tissues. Another important distinction of our study from these three is that we evaluated freezing tolerance using a temperature-controlled freeze-thaw protocol, regulating gradual and realistic cooling and thawing, and ensuring extracellular ice formation (ice-nucleation). In a recent study with WT and mutant Arabidopsis plants carrying the sid2-1 mutation, a loss of function of ICS1 with primary role in SA biosynthesis, Kim et al. [33] concluded that SA biosynthesis did not contribute to freeze-tolerance, an observation seemingly in contrast to our finding of SA-induced freezing tolerance in spinach leaves. Interestingly, however, a close examination of freeze-thaw response curves for WT and sid2-1 mutant genotypes in
their study reveals that despite similar LT$_{50}$ (approximately -4°C) for both genotypes, $sid2$-$I$ mutants were significantly more injured (by ~20%) compared to WT control at ~-3°C, a stress slightly milder than LT$_{50}$, supporting our results with spinach leaves (Fig. 2A). This revelation is significant and seems to caution that comparing freezing tolerances across treatments based solely on LT$_{50}$ may not always reveal real and significant freezing tolerance differences particularly in the sub-lethal range which may be more meaningful, physiologically, than injuries too severe to be remedied.

Excess accumulation of ROS (O$_2$· and H$_2$O$_2$) during freeze-thaw and resultant oxidative damage by ROS have been widely proposed to explain freezing injury [2, 30]. Our data are consistent with this notion since freeze-thaw stressed leaves accumulated greater amount of O$_2$· and H$_2$O$_2$ (visual intensity) compared to unfrozen controls (Fig. 3); similar observations were made in our previous studies with ‘Bloomsdale’ spinach [3, 41]. ROS accumulation, however, was relatively higher in control than SA-treated leaves at both stress levels, supporting the SA-induced freezing tolerance evident from the ion-leakage assay (Fig. 2A). Several studies have shown that exogenous SA application protected plants against abiotic stresses by alleviating the accumulation of O$_2$· and/or H$_2$O$_2$ (see references [15, 31, 44]).

4.2. Freezing Tolerance vis-à-vis SA content of NA and CA leaf tissues

A 9-d CA of whole plants resulted in gain of 4.1°C in freezing tolerance (Fig. 4A). NA and CA LT$_{50}$ values obtained in our study are in accordance with those previously reported in [3, 14, 41]. Notably, the freezing tolerance (LT$_{50}$) of micro-tube-treated leaves (-6.9°C) was 1.1 °C colder (more negative) than for those sampled from whole-plants (see NA-
LT_{50}), despite the use of identically controlled freeze-thaw protocol (compare Fig. 1 and Fig. 4A). This small yet significant discrepancy in freezing tolerance from the two scenarios highlights the rationale for first determining freeze-response curve for micro-tube treated leaves so as to select physiologically relevant freeze-stress treatments for this study.

CA leaves had higher SA content than NA controls; ~5-fold accumulation during CA (9-d at 4°C) in our study is supported by a similar 4.1 to 6.3-fold accumulation in cold-exposed (12-d at 5 °C) Arabidopsis leaves [53] and SA accumulation in cold acclimating wheat [35]. Increased freezing tolerance paralleled by SA accumulation during CA lends support to a similar outcome for leaves fed with SA in micro-tubes. However, gain in freezing tolerance vis-a-vis SA accumulation in CA tissues was substantially higher than that by SA-feeding (compare Fig. 2A, B with Fig. 4A, B). This is not surprising since CA is a multi-factorial, complex response involving myriad of cellular, molecular and whole plant level adjustments, and SA accumulation maybe one of the contributing factor to this additive response.

4.3. Why are SA-Treated Leaves More Freezing Tolerant?

In order to explore probable physiological/biochemical explanation for SA-induced freezing tolerance, we studied the leaf-growth dynamic and tissue levels of two molecules (proline and AsA) known to be associated with acquisition of freezing tolerance.

4.3.1. Leaf growth and increased Freezing Tolerance

Data showed only a slight reduction (~3%) in longitudinal growth (none equatorially) in SA-fed leaves over the treatment duration; these leaves also gained slightly less FW. Though unable to discount completely, we are inclined to assume a negligible effect
of leaf growth differences on freezing tolerance. Others have also noted a growth-retardation effect of exogenously applied SA (0.1 mM), e.g. in stem of potato microplants [40] or tobacco seedlings [7].

4.3.2. Proline accumulation and increased Freezing Tolerance

Proline, a compatible solute, has been widely reported to accumulate at higher level in CA tissues compared to NA ones [22, 36, 50, 67] and is recognized as one of the components of abiotic stress tolerance mechanism [61]. Due to its hydrophilic nature, proline is believed to protect macromolecules and membranes against freeze-induced desiccation [21]. Proline accumulation under stress is also implicated in detoxification of ROS and cellular osmotic adjustment [18]. In the present study, more freeze-tolerant SA-treated spinach leaves also accumulated ~45% higher proline compared to HG-controls. Similarly, CA spinach leaves accumulated higher proline content compared to NA. Moreover, much greater proline accumulation in CA leaves relative to SA-fed ones is also paralleled by a substantially greater gain in freezing tolerance by the former (compare Fig 5A vs. Fig. 5C with Fig. 2A vs. Fig. 4A). In support of our results, others have also noted increased proline accumulation and concomitant amelioration of abiotic stresses (heat or salt) in 0.5 mM SA-treated wheat [32], Torreya grandis [37], and lentil [42]. No experiments were conducted in our study to investigate why SA-treatment results in proline accumulation. However, Misra and Saxena [42] and Khan et al. [32] noted increased activities of proline biosynthetic enzyme (γ-glutamyl kinase, a component of 1-pyrroline-5-carboxylic acid (P5C) synthetase complex) in SA-treated tissues; both groups also noted decreased activity of proline catabolizing enzyme (proline oxidase) in SA-treated tissues, an outcome also expected to favor proline accumulation.
4.3.3. AsA accumulation and increased Freezing Tolerance

Ascorbic acid (AsA) or ascorbate is one of the important non-enzymatic antioxidants, widely implicated in protecting plants against oxidative stress emanating from various abiotic stresses including freeze-thaw [2, 57] and references therein. AsA essentially serves as a substrate in glutathione-ascorbate (GSH-AsA) cycle, catalyzed by ascorbate peroxidase (APX) to detoxify H$_2$O$_2$ [59]. In our study, SA treatment markedly improved freezing tolerance as well as caused lower O$_2$•– and H$_2$O$_2$ accumulation following freeze-thaw, suggesting improved ROS scavenging by SA-fed tissues. These tissues also accumulated higher level of AsA (~1.75-fold) compared to HG-control (Fig. 5B), which could be one of the components of bolstered ROS scavenging. Others have also noted AsA accumulation by exogenous SA application [38, 46].

Understanding why SA-feeding increases AsA accumulation is beyond the scope of this study. However, literature survey leads us to offer an explanation for potential ‘indirect inducement’. Research shows that exogenous SA reduces activity of catalase [5, 23, 46], another antioxidant enzyme responsible for detoxifying H$_2$O$_2$. Therefore, it may be argued that, if not scavenged, this could result in excess H$_2$O$_2$ accumulation under stress conditions (such as freeze-thaw). And, upregulation of APX activity will, conceivably, be needed to compensate for diminished catalase activity, thereby requiring even greater amount of AsA (substrate). Indeed, increased APX activity or transcripts has been reported in response to SA-application [7, 29, 62].

Upregulation of enzymatic and non-enzymatic antioxidants, including AsA, during CA has been reported [6, 26, 28]. To determine if CA-induced freezing tolerance was also accompanied by AsA accumulation, we compared NA and CA tissues but were intrigued to find no significant change (Fig. 5D). Speculatively, AsA may not constitute a major
component of scaled-up antioxidant capacity in cold acclimated ‘Reflect’ spinach leaves, a proposal warranting further investigation.

4.4. Does SA-Induced Freezing Tolerance Require H$_2$O$_2$ or NO Mediation?

SA, NO, and H$_2$O$_2$ are regarded as key signaling molecules regulating various biological processes and mediating diverse stress protective mechanisms [44, 48, 51]. A ‘self-amplifying feedback loop’ concept suggests a ‘duplex signal transduction’ whereby SA modulates H$_2$O$_2$ accumulation and vice-versa [13, 16, 27, 54]. Evidence also exists for SA-induced NO production [69] or enhancement of NO-regulated antioxidant enzymes [34], and for NO modulating SA and H$_2$O$_2$ levels [13, 52]. Using an NO scavenger (hemoglobin, HB), Mostofa et al. [46] demonstrated that NO mediates SA-induced salt tolerance in rice. Despite investigations of potential ‘cross-talk’ among SA, NO, and/or H$_2$O$_2$ in mediating abiotic stress tolerances, no such information exists for freezing tolerance.

Our results showed while 0.5 mM SA-treatment induced significant freezing tolerance in spinach leaves, adding 1μM NANA, a H$_2$O$_2$-scavenger, to SA (SA+NANA) essentially abolished the beneficial effect of SA (Fig. 6). This suggests that SA exerts its effect in H$_2$O$_2$-dependent manner, a notion reinforced by reports that H$_2$O$_2$ signaling acts downstream of SA [68]. Accumulation of H$_2$O$_2$ at exceedingly low levels has been implicated as a second messenger for activating various stress responses [43, 48]. Harfouche et al. [16] noted that exogenous application of SA, in a range of 0.1 – 0.5 mM, gave rise to low ROS accumulation and activation of antioxidant enzymes [19]. Research also shows that exogenous H$_2$O$_2$ enhances tolerance to salt [12], heat [65], and chilling stresses [17], and in most cases, the protective mechanism included enhanced antioxidant enzymes activity. Although antioxidant enzymes activities were not measured in the present study, our data on
reduced ROS accumulation in freeze-stressed leaves pretreated with SA, coupled with their higher AsA accumulation compared with HG-control, lend support to such notion. A similar response for ‘SA+NANA’, ‘NANA only’ and ‘HG-control’ treatments suggests that 1μM NANA used in our study was sufficient to remove most of H₂O₂, endogenous as well as any putatively induced by SA, and was also not detrimental to unstressed or freeze-stressed spinach leaves.

Our data indicates that scavenging of NO using ‘SA+HB’ pretreatment negated the beneficial effect of ‘SA alone’ on freezing tolerance, especially at -6.5°C stress. Moreover, ‘HB-alone’ treatment resulted in slightly higher injury than HG-control at -6.5°C, a treatment which evoked similar response as ‘SA+NANA+HB’ pretreatment. This suggests a role for NO signaling and for its mediation in SA-induced freezing tolerance in spinach leaves. However, explanation for slightly lower injury at -7.5°C in ‘SA+HB’ pretreated leaves compared to HG-control, ‘HB alone’ or ‘SA+NANA+HB’ is intriguing. Simaei et al. [58] noted that ‘SA+NO-donor’ pretreatment improved salt stress tolerance of soybean seedlings via increased antioxidant enzymes activity. Reports also exist for improved tolerance by NO-donor-application against chilling [10], salt/heat [63] and drought [11]. Also, NO has been shown to protect plants from oxidative stress by promoting detoxification of O₂⁻ via enhancing H₂O₂-scavenging enzymes [56]. Fan et al. [10] reported a reduced level of electrolyte leakage in NO-donor-treated plants after chilling stress, paralleled by enhanced antioxidant enzymes activity in response. Mostofa et al. [46] provided evidence for cross-talk between SA, H₂O₂ and NO in enhancing salt stress tolerance in rice seedlings.

In conclusion, the present study provides evidence that a 4-d treatment of 0.5 mM exogenous SA improved freezing tolerance (reduced membrane leakage and lower ROS accumulation) of spinach leaves without causing any detrimental effect on leaf health. The
micro-tube system used here allowed efficient absorption of SA by the leaves. Our results indicate that SA-induced increased freezing tolerance may be associated with greater accumulation of compatible solute, proline and antioxidant AsA, and may involve NO and H$_2$O$_2$ mediation (Fig. 7). Future studies should test the effect of SA-feeding on freezing tolerance at whole plant level. Moreover, further mechanistic elucidation of SA-induced freezing tolerance of spinach leaves is warranted, potentially involving the investigation of antioxidant enzyme activity, gene expression associated with proline biosynthesis, and monitoring of other factors contributing to freeze-tolerance.

Acknowledgments

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[38] G. Li, X. Peng, L. Wei, G. Kang, Salicylic acid increases the contents of glutathione and ascorbate and temporally regulates the related gene expression in salt-stressed wheat seedlings, Gene 529 (2013) 321–325.


Table 1. Leaf length and ratio of leaf length of 25% Hoagland solution (HG-Control)- vs. 0.5 mM SA-treated spinach (*Spinacia oleracea* L. cv. Reflect) leaves

<table>
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<th>Ratio of leaf length (4-d/0-d)</th>
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<td>Longitudinal</td>
<td>Equatorial</td>
<td>Longitudinal</td>
<td>Equatorial</td>
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<tr>
<td>HG-Control</td>
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<td>3.55 ± 0.05</td>
<td>2.14 ± 0.04</td>
<td>1.05</td>
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<td>0.5 mM SA</td>
<td>3.37 ± 0.04</td>
<td>2.06 ± 0.03</td>
<td>3.47 ± 0.05</td>
<td>2.10 ± 0.02</td>
<td>1.02(^y)</td>
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Data are the means of 32 technical replicates from two independent experiments (16 technical replicates each) with standard errors analyzed with Student’s *t*-test

\(^y\) *p* ≤ 0.01

\(^z\) non-significant
Table 2. Leaf weight and ratio of leaf weight of Hoagland’s solution (HG-Control)- vs. 0.5 mM SA-treated spinach (*Spinacia oleracea* L. cv. Reflect) leaves

<table>
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<th>Treatment</th>
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<th>Ratio of leaf weight</th>
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<td>4-d</td>
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</tr>
<tr>
<td>HG-control</td>
<td>0.135 ± 0.005</td>
<td>0.156 ± 0.007</td>
<td>0.025 ± 0.001</td>
</tr>
<tr>
<td>0.5 mM SA</td>
<td>0.131 ± 0.004</td>
<td>0.143 ± 0.004</td>
<td>0.023 ± 0.001</td>
</tr>
</tbody>
</table>

Data are the means of 32 technical replicates from two independent experiments (16 technical replicates each) with standard errors analyzed with Student’s *t*-test

<sup>y</sup> *p* ≤ 0.01

<sup>z</sup> non-significant
Fig. 1. Freeze-thaw injury response of micro-tube-based leaves of 3-week old spinach (Spinacia oleracea L. cv. Reflect) seedlings; spinach seedlings were grown in greenhouse media for 17-d (see methods) and petiolate leaves were transferred to micro-centrifuge tubes containing 25% Hoagland solution for 4-d before administering the freezing test. The sigmoid curve was generated from percent injury at individual treatment temperatures using Gompertz function; LT$_{50}$ (°C), the mid-point (41.1% corresponding to -6.9°C) between the minimum (0.2%) and maximum (81.9%) injury was defined as the temperature causing 50% injury. Inset: Illustration of micro-centrifuge tube system used in this study (see Methods).
Fig. 2. (A) Effect of exogenous SA on leaf freezing tolerance of 3-week old spinach (*Spinacia oleracea* L. cv. Reflect) seedlings; plants were grown in green house media for 17-d and petiolate leaves were incubated for 4-d in three different treatments (as follows) before exposure to freezing treatments: (1) 25% Hoagland solution (HG-Control), (2) 0.5mM SA, and (3) 1.0 mM SA using micro-tube system as shown in Fig. 1 inset. Unfrozen leaves corresponding to each treatment were used as control (UFC). Different letters indicate significant differences between treatments at \( p \leq 0.05 \), according to Duncan’s multiple range test. (B) SA concentration in leaf tissues incubated with three solutions as explained above.
Fig. 3. Distribution of superoxide (O$_2^-$) (A and B) and hydrogen peroxide (H$_2$O$_2$) (C and D) in unfrozen controls (UFC) and freeze-thaw injured spinach (*Spinacia oleracea* L. cv. Reflect) leaves, that were pretreated with either 0.5 mM SA or with 25% Hoagland solution (HG-Control) before exposure to two freezing treatments, -6.5 and -7.5 °C. Superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) were visualized by histochemical staining.
Fig. 4. (A) Freeze-thaw injury response in non-acclimated (NA) and cold-acclimated (CA) spinach (Spinacia oleracea L. cv. Reflect) leaves. The sigmoid curves were generated from percent freeze-injury using Gompertz function; LT$_{50}$ (°C) of NA leaves (-5.8°C) corresponded to mid-point injury of 47.7%, between the minimum (0.2%) and maximum (95.02%) injury, and that of CA leaves (-9.9 °C) corresponded to mid-point injury of 45% between the minimum (0.2%) and maximum (88.7%) injury. (B) SA content in NA- and CA-spinach leaves. *, p ≤ 0.05, analyzed by Student’s t-test
Fig. 5. Proline and ascorbic acid (AsA) content in Hoagland solution (HG-control) vs. 0.5 mM SA-treated spinach (*Spinacia oleracea* L. cv. Reflect) leaves (A and B), and non-acclimated (NA) vs. cold-acclimated (CA) leaves (C and D). Plants used for A and B were grown in greenhouse media for 17-d and petiolate leaves were incubated for 4-d in HG-control or SA. See methods for details on NA and CA treatments. NS, no significant difference: *, p ≤ 0.05; **, p ≤ 0.01, analyzed by Student’s *t*-test.

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*Proline concentration (mmol g⁻¹ fresh weight)*

**A**

HG-Control | 0.5 mM SA

**B**

HG-Control | 0.5 mM SA

**C**

NA | CA

**D**

NA | CA
Fig. 6. Effects of SA with or without \( \text{H}_2\text{O}_2 \)- and NO-scavengers (NANA and HB, respectively) on freezing tolerance of \((\text{Spinacia oleracea} \ L. \ \text{cv. Reflect})\) leaves; plants were grown in green house media for 17-d and petiolate leaves were incubated in seven different treatments (as follows) for 4-d using micro-tube system before exposure to two freezing treatments (i.e., -6.5 and -7.5 °C): (1) 25% Hoagland solution (HG-Control), (2) 0.5 mM SA (SA), (3) 0.5 mM SA + 1 μM NANA (SA+NANA), (4) 0.5 mM SA + 100 μM HB (SA+HB), (5) 0.5 mM SA+ 1 μM NANA + 100 μM HB (SA+NANA+HB), (6) 1 μM N-acetylneuraminic acid (NANA), and (7) 100 μM hemoglobin (HB). Different letters indicate significant differences between treatments at \( p \leq 0.05 \), according to Duncan’s multiple range test.
Fig. 7. Schematic illustration of the effect of SA-pretreatment on freezing tolerance of spinach (*Spinacia oleracea* L. cv. Reflect) leaves. SA-accumulating leaves suffer lower oxidative stress and reduced membrane injury (ion-leakage) following a freeze-thaw cycle. Proposed mechanism involves accumulation of proline and ascorbic acid in SA-fed leaves in a H$_2$O$_2$- and NO-dependent manner. SA, salicylic acid; NO, nitric oxide; H$_2$O$_2$, hydrogen peroxide.
CHAPTER 3. SALICYLIC ACID-INDUCED FREEZING TOLERANCE IN SPINACH

(Spinacia oleracea L.) LEAVES EXPLORED THROUGH METABOLITE PROFILING

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Abstract

Freezing tolerance and metabolome changes were investigated for spinach (*Spinacia oleracea* L. ‘Reflect’) seedlings treated with 0.5 mM salicylic acid by sub-fertigation. Experiments included seedlings treated with salicylic acid at both the ambient (non-acclimation; NA) and cold acclimation (CA) temperatures, i.e. NASA and CASA. Except for slight reduction in the leaf size, salicylic acid-fed plants exhibited, in general, similar growth performance as non-treated controls based on the dry weight/fresh weight ratio, percent water content, and dry weight/leaf area. Temperature-controlled in situ freeze-thaw of seedlings revealed NASA plants were more freeze-tolerant (visual estimates and ion-leakage test) and had higher salicylic acid content than NA control. Metabolite profiling revealed NASA had higher trehalose, ascorbic acid, γ-tocopherol, proline, and leucine, whereas lower mannose and aconitic acid than NA tissues. Excised leaf freeze-thaw tests revealed CASA leaves to be the most freeze-tolerant of the four conditions followed, respectively, by CA, NASA, and NA. Principal component analysis distinctly separated metabolic phenotypes for NA, NASA, CA, and CASA, indicating salicylic acid differentially affected metabolism at warm vs. cold. CASA leaves had higher compatible solutes (osmolytes), antioxidants, and salicylic acid than CA control. Our data suggests that altered accumulation of trehalose, ascorbic acid, and aconitic acid was a salicylic acid-specific response. Additionally, 7 metabolites (5-oxoproline, fructose, glucose, maltose, proline, sucrose, and tartaric acid) were quantitatively associated with the freezing tolerance levels across four conditions.

**Key words.** Ascorbic acid, freezing tolerance, metabolomics, proline, tocopherol, trehalose
1. Introduction

Salicylic acid (SA) is a well-known signaling molecule that regulates various physiological processes and mediates defense mechanisms against pathogens in plants (Raskin, 1992; Horváth et al., 2007; Vincent and Plasencia, 2011; Khan et al., 2015). Numerous studies have also reported that exogenous SA improved plant tolerance to various abiotic stresses, such as ozone and UV-exposure, heat, chilling, drought, and salt (Yalpani et al., 1994; Sharma et al., 1996; Miura and Tada, 2014; references in Khan et al., 2015). However, little is known about SA’s effect on freezing tolerance (FT) in plants.

Freeze-thaw stress is one of the major environmental constraints on crop performance and yield. We have recently demonstrated improvement in FT of excised leaves of spinach \( (Spinacia oleracea \text{ L.}) \) when supplied with exogenous SA (Shin et al., 2018). Although, few other studies have noted beneficial effect of SA treatment on FT, such as in winter-wheat leaves (Tasgín et al., 2003), \textit{in vitro} cultured potato plantlets (Mora-Herrera et al., 2005), and grape leaves (Wang and Li, 2006), ours was the first such study to have ensured direct uptake of SA by the leaves and used FT assessment protocols that included precisely controlled cooling and warming rates, and ice-nucleation. Results from our study had also showed that increased FT in SA-treated leaves was accompanied by greater tolerance to reactive oxygen species (ROS) and accumulation of an antioxidant (ascorbic acid) and a compatible solute (proline) (Shin et al., 2018). Cold acclimation (CA), a process where FT is induced in certain plants under inductive environmental conditions, typically involves similar metabolic changes, along with other cellular and molecular reprogramming (Thomashow, 1999; Xin and Browse, 2000). Cold exposure / CA has also been shown to result in accumulation of SA in some plant tissues. For example, SA concentration of \textit{Arabidopsis} leaves increased during CA (Scott et al., 2004; Kaplan et al., 2004; Kim et al.,...
2013), and our previous work with spinach showed >4-fold accumulation of SA in ∼9d-cold acclimated leaves which were also substantially more freeze-tolerant than non-acclimated (NA) controls (Shin et al., 2018).

Above observations collectively provide preliminary mechanistic insight into the SA-induced FT. However, a comprehensive analysis of metabolome changes underlying SA-induced FT could further clarify the cellular mechanism for such a response. Also, comparative study of FT acquisition as well as metabolome reprogramming in SA-alone vs. SA+CA treated tissues will provide insights into the similarity or uniqueness of metabolic changes elicited specifically by SA or cold as related with improved FT. Furthermore, in order for SA-induced FT to have potential application on a practical scale, it is imperative that its promotive effect on FT be examined in situ at a ‘whole plant’ level. Such studies may result in the identification of beneficial metabolites associated with higher FT while also advancing fundamental understanding of the in vivo role of SA in improving FT.

With these questions in mind, the main goals of the present study were to: (1) determine the effect of SA-feeding on FT of spinach seedlings at the whole plant level, (2) compare and contrast: a) FT of SA-fed plants at warm (or in non-acclimated (NA) condition; NASA) vs. cold (CA plus SA; CASA), and b) changes in the leaf metabolome among NA, NASA, CA, CASA using gas chromatography-mass spectrometry (GC-MS). Suitability of spinach leaves for FT acquisition experiments has been previously established by us and others through a series of experiments (Guy et al., 1987; Chen and Arora, 2014; Min et al., 2014; Shin et al., 2018).
2. Materials and methods

2.1. Plant material

Seeds of spinach (*Spinacia oleracea* L. cv. Reflect), a F₁ hybrid cultivar (Johnny’s selected seeds, Inc., Winslow, ME, USA), were sown in plug flats containing Sunshine LC-1 mix (Seba Beach, Alberta, Canada) and transferred to growth chambers at 15/15 °C (D/N) with 12-h photoperiod under average photosynthetically active radiation of ~300 µmol m⁻² s⁻¹ at plant height provided by incandescent and fluorescent lamps (Shin et al., 2018). Seedlings were watered as needed through sub-irrigation (approximately, 5-d interval). Two weeks from the sowing, chamber temperature was elevated to 20/18 °C (D/N), and seedlings were sub-irrigated with either 300 ppm EXCEL (Scotts Sierra Horticultural Products Company, Marysville, OH, USA) nutrient solution (i.e., NA controls) or with 0.5 mM SA dissolved in 300 ppm EXCEL (SA-feeding; NASA); this means that plants were fertilized only once and SA treatment was for 8 to 10 days. SA concentration used in this study has previously been adjudged to improve FT in excised spinach leaves (Shin et al., 2018). About 22-24-day-old spinach seedlings were used for studies as described below.

2.2. Leaf Growth Measurement

Leaf fresh weight (FW), dry weight (DW), and area were measured to compare leaf-growth between control vs. SA-fed seedlings. Briefly, twelve pairs of leaves (total 24) per treatment were used to measure leaf area using LI-3100 Area Meter (LI-COR, Inc., Lincoln, NE, USA), quickly followed by the measurement of FW on the same leaves. Dry weight was measured after oven-drying leaves at 75 ± 1 °C for 72-h. Data of leaf-growth from five biological replications (24 leaves per biological replicates) were pooled to calculate the representative treatment means with standard errors.
2.3. Determination of Freezing Tolerance

2.3.1. Whole plant (in situ) freezing test

This test was conducted only with fertigated controls (NA) and SA-fed (NASA) plants. Two test temperatures, -5.5 and -6.5 °C were used to compare FT of NA and NASA seedlings. A pair of plug flats — one of fertigated control and the other with SA-fed plants (50-60 seedlings/flat) — was transferred to a freezing chamber (Percival, E-41L1LT, Percival Scientific, Inc., Perry, IA, USA) maintained at 0 °C; another such pair of flats was transferred to another identical freezing chamber. The two chambers, respectively, were used for freezing up to -5.5 or -6.5 °C and subsequent thawing following the identical freezing protocol (Fig. 1). After 2-h at 0 °C, temperature was lowered at 1°C/h to -2 °C. Thereafter, plants were quickly misted with pre-chilled (0 °C) de-ionized water to promote ice-nucleation and plants were held at this temperature for an hour. Both chambers were then cooled at 0.5°C/30min to reach -5.5 or -6.5 °C. Plants were held at the target temperatures for 30 min. Thawing consisted raising the temperature to 0 °C and allowing plants to thaw overnight (~13-h). Gradual thaw continued by exposing plants to 5 °C for 2-h. Entire freezing and thawing was conducted in dark and the temperature of leaf surface, air, and growing media (~2.5 cm deep) was monitored using copper-constantan thermocouples (Omega DP 465, Omega Engineering, Inc., Stamford, CT, USA) (Fig. 1). Unfrozen control plants were exposed to 0 °C in dark throughout this time in another but identical chamber. All the plants (freeze-stressed and controls) were then transferred from chambers to the lab bench (~20 °C) under dim-light (~15 μmol m^-2 s^-1, cool white fluorescent) for ~12 h. During this time, to avoid unintended drying, plants were periodically but lightly misted with de-ionized water. Freeze-thaw injury to seedlings was then assessed visually and photographed. Whole-plant freeze tests were independently repeated thrice.
Additional estimation of freeze-injury / tolerance of NA and NASA plants was made by measuring ion-leakage on leaves excised from plants that were subjected to whole-plant freezing. These measurements were also repeated thrice, each including 12 to 19 plants per temperature per treatment (two leaves/plant replicate). Injury percent data from three biological replications were pooled to calculate the representative treatment means with standard errors. Mean differences were analyzed by LSD (Least Significant Difference) test.

2.3.2. Excised-leaf freezing test or ‘Bath freezing’

Excised-leaf freezing test was used to assess LT$_{50}$, (temperature at which 50% injury occurs and defined as FT) of the four treatments, i.e. NA and NASA plants, and those that were cold acclimated after being fed with just the fertilizer (CA) or with SA (CASA). Briefly, excised leaves from these treatments were exposed to a temperature-controlled freeze-thaw protocol in a glycol bath (Isotemp 3028; Fisher Scientific, Pittsburgh, PA, USA) (hereon referred as ‘bath freezing’). Leaves were slowly frozen to various temperatures, ensuring the ice-nucleation, followed by ion-leakage-based freeze-injury estimation as per our previous studies (Chen and Arora, 2014; Shin et al., 2018). LT$_{50}$ curves for NA and NASA leaves were generated based on -3 to -11 °C freezing treatments. For CA and CASA treatments, 19-d old seedlings were transferred to a cold acclimation regimen comprising 9-d exposure to 4°C at ~75 μmol m$^{-2}$ s$^{-1}$ (12/12h; D/N) (Shin et al., 2018). LT$_{50}$ was estimated after replicated samples of excised leaves were exposed to freezing temperatures ranging from -7 to -17 °C (Min et al., 2014; Shin et al., 2018). ‘Bath freezing’ tests were independently repeated thrice, each with 5 technical replicates per temperature per treatment (two leaves/technical replicate). Injury percent data from three independent experiments were pooled to calculate the representative treatment means with standard errors.
2.4. Metabolite profiling

2.4.1. Sample preparation

Leaf samples, previously stored at -80 °C, from four treatments, i.e., NA, NASA, CA, and CASA, were used for metabolite profiling using GC-MS. Each treatment was composed of two biological replications, each with 2 technical replications. Leaves were ground with LN$_2$ to powder. The metabolites from each treatment were then extracted using the method described by Noutsos et al. (2015), with slight modifications. Briefly, aliquots of ~30 mg of frozen powder were mixed with internal standard, 25 μl (1 mg/ml stock) of ribitol for polar compounds and 20 μl (1 mg/ml stock) of nonadecanoic acid for non-polar compounds and homogenized with 0.35 mL of 80 % (v/v) hot methanol (60 °C) for 10 min followed by 10 min of sonication. After centrifugation (10,000 g) for 10 min, supernatant was transferred to GC-MS vial followed by drying in speed-vac for 10-h. For methoximation, methoxyamine hydrochloride in dry pyridine (50 μL of 20 mg/mL stock) was added to dried sample and incubated at 30 °C for 1.5 h with continuous shaking. Samples were then silylated by mixing with 70 μL of bis-trimethyl silyltrifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1% TMCS) for 30 min at 37 °C. Derivatized samples were analyzed using an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with HP5MS separation column. The mass range was 40-800 m/z.

2.4.2. Metabolite identification and quantification

Identification of the compounds, i.e. specific resolved peaks in the mass spectra, was determined by conducting database searches based on compounds’ chromatographic retention time indices following deconvolution of raw GC-MS chromatograms using AMDIS software; the database used was a NIST14 mass spectra library. Each compound was selected as a
metabolite if identified with a match factor > 650 on a scale of 0 to 1,000 and retention time index deviation ± 50. Each identified metabolite was quantified based on internal standards; missing data were replaced by a number (i.e. the smallest peak area /2) for further statistical analysis. (Xia et al., 2009). Using a detection limit for missing values is standard for GC/LC data. Because the systems cannot give “0” as a value, there must be some minimum threshold “area” value before the system can detect a compound as a “peak”. So, to compare values of detected compounds to undetected compounds a detection limit value must be used in place of “0”. In this study, we found that only one metabolite, i.e., trehalose was undetectable in NA and CA controls but was present in SA-fed leaves (NASA and CASA). Therefore, for the calculations of fold-change in trehalose concentration (e.g. CASA vs. CA or NASA vs. NA) half of the smallest peak area value found in the dataset was quantified to be 0.000432698 pmols mg⁻¹ FW (Table S1) and used as the denominator. Using this protocol, trehalose in NASA leaves, for example, was adjudged to be 135-fold of NA [0.058416524 (NASA)/0.000432698 (NA)] (Table S1).

2.4.3. Statistical analysis of metabolite profiling data

Statistical differences were assessed by analysis of variance (ANOVA) using R (version 3.2.2, The R Foundation for Statistical Computing, ISBN 3-900051-07-0). For principal component analysis (PCA) and visualization, R was used on log₁₀-transformed relative responses.

Pair-wise comparison of metabolic changes between treatments was performed using R and then volcano plots were generated using log₂-scaled mean metabolite concentrations and log₁₀-transformed p-values adjusting the false discovery rate using the Benjamini-Hochberg correction (padj). A two-fold criterion was used to assess the magnitude of change
in metabolite contents among treatments. Only those metabolites were name-labeled on the volcano plots that either met both thresholds, i.e. significantly different (padj < 0.05) as well as fold change in abundance of >|2|, or met only one of these two conditions. The two vertical lines in volcano plots indicate a two-fold cut off for either increase or decrease in abundance whereas the horizontal line represents a threshold of \(-\log_{10} \text{padj} = 0.05\).

3. Results

3.1. Effect of exogenous SA on plant growth parameters under ambient and cold acclimation conditions

Leaf area of NASA plants was ~ 29% smaller than the NA whereas it was reduced by only ~9% in CASA relative to CA (Table 1). However, no significant difference was observed for % water content, DW/leaf area, DW/FW ratio, as well as the ‘actual’ tissue DW used for metabolite analyses when comparing ‘with’ or ‘without SA’ treatments under ambient (NA vs. NASA) or cold acclimation (CA vs. CASA) conditions. Also no significant difference in DW/FW ratio was noted between NASA and CA treatments. Finally, no significant difference in ‘actual’ DW used for metabolite analyses was observed for all three pair-wise comparisons (used for volcano plots), i.e. NA vs. NASA, NASA vs. CA, and CA vs. CASA.

3.2. Freezing tolerance and SA content of NA vs. NASA tissues

As a reminder, NA and NASA mentioned under this heading are same as the fertilizer control and SA-fed seedlings maintained under warm (ambient) conditions. Two test temperatures, -5.5 and -6.5 °C, were selected for the ‘whole-plant freezing test’. A representative image of plants subjected to freeze-thaw stress is shown in Fig. 2A where SA-
fed (NASA) plants are visually more freeze-tolerant than the fertilizer controls (NA) at both stress levels. Moreover, the beneficial effect of SA seemed more pronounced at the milder stress level. FT-improvement by SA was also evident from ion-leakage measurements of the leaves taken from seedlings that had undergone whole-plant freezing (Fig. 2B). Accordingly, NASA plants suffered ~70% and ~33% less injury at -5.5 and -6.5 °C, respectively, as compared to the NA control, which corroborated visual estimates of injury. Bath freezing test with excised leaves from NA and NASA plants also confirmed the beneficial effect of SA on FT improvement (Fig. 2C) with LT50 of NASA leaves being more negative than NA controls by -0.7 °C (-6.5 °C vs. -5.8 °C) (Fig. 2C). Here too, respective injury percentages for NA and NASA samples at -5.5 and -6.5 °C were similar to those observed in Fig. 2B. Taken together Fig. 2 A, B, & C show that SA-fed spinach seedlings/leaves are more freeze-tolerant than the untreated controls, and that beneficial effect declines with the severity of freezing stress.

Data on SA-content shows NASA leaves accumulated 60% higher SA than NA controls (Fig. 2D).

3.3. Metabolite profiles of NASA vs. NA tissues

Metabolite contents for two treatments maintained at ambient growing conditions, i.e., NA and NASA, were analyzed by GC-MS. In total, 58 metabolites were identified and clustered into 5 groups (i.e., amino acid, carbohydrate, lipid component, TCA intermediates, and others). It consisted of 16 amino acids, 12 carbohydrates, 9 lipid components, 5 TCA intermediates, and 16 others (Table S1).

Mean concentrations of metabolites from NASA and NA were pair-wise compared, i.e. NASA/NA, using log2-scaled fold change and -log10 scaled p-values adjusted with Benjamini-Hochberg correction (Padj). Only those metabolites were name-identified on a
volcano plot that were significantly different in abundance (padj < 0.05) as well as changed by (>2) or met only one of these two thresholds (Fig. 3). Data indicate that 3 metabolites (blue dots) showed major changes meeting both the criteria: two of these, trehalose and ascorbic acid were more abundant in NASA tissues while aconitic acid was less abundant compared to NA controls; trehalose could not be detected in NA samples in the present study and the relative fold change (NASA vs. NA) was calculated using the procedure described under ‘Methods- Metabolite Profiling’ section. Five other metabolites also showed changes in abundance between NA and NASA but met only one of the two thresholds for the magnitude of change (red or green dots). Of these, proline and γ-tocopherol increased whereas mannose decreased in abundance in NASA compared to NA tissues meeting >2 criterion, while SA and leucine, were more abundant in NASA vs. NA tissues based on padj < 0.05 threshold (Fig. 3).

3.4. Freezing tolerance and SA content of CA vs. CASA tissues

SA feeding improved FT of cold acclimated leaves with LT50 of CASA leaves being more negative than CA by -1.4 °C (-9.9 vs. -11.3 °C) (Fig. 4A). SA content of CASA-leaves was ~3.8-fold of that in CA-leaves (Fig. 4B); this was in contrast with NASA plants (SA-fed under warm) which had ~1.6-fold SA relative to their NA counterparts (compare Fig. 4B and Fig. 2D).
3.5. Principal component analysis (PCA) of metabolite phenotypes for NA, CA, NASA, and CASA treatments

PCA was conducted to distinguish metabolite phenotypes among NA, NASA, CA, and CASA, and to determine which metabolites contributed the most for such differences. PCA of 58 metabolites revealed clear differentiation among the four treatments wherein mainly the two components accounted for 81% of the total variance (Fig. 5). The first principal component (PC1) explained 54% of the total variance of data set (Fig. 5), indicating a different response to warm (NA) and cold (CA) at the metabolic level. In order to further investigate which metabolites contributed to such difference between the treatments, the metabolite loading values in PC1 were ranked (Table S2). The 5 most positive loading values (i.e. warm-temperature related), in descending order, were for aconitic acid, succinic acid, oxalic acid, putrescine, and ferulic acid whereas the 5 most negative loading values (i.e. cold-temperature related), in descending order, correspond to proline, galactinol, mannose, isoleucine, and maltose. The second principal component (PC2) accounted for 27% of the total variance of data set (Fig. 5) indicating a SA-feeding effect. The 5 most positive loading values (i.e., SA-treated) were for trehalose, putrescine, glycine, fumaric acid, and mannose, while the 5 most negative (i.e., non-SA treated) for aconitic acid, leucine, asparagine, tyrosine, and methionine (Table S2).

3.6. Metabolite profiles of CA- vs. NASA-tissues

Mean concentrations of total 58 identified metabolites were pair-wise compared, i.e., CA/NASA, using log2-scaled fold change and -log_{10} scaled p-values adjusted with Benjamini-Hochberg correction (Padj). Only those metabolites were name-identified on a
volcano plot that were quantitatively different fulfilling either both thresholds (padj < 0.05 and >|2|) or only one of these two criteria (Fig. 6). Data indicates that 22 metabolites (blue dots) showed major changes meeting both the criteria: eleven of these, proline, methionine, tyrosine, asparagine, glutamine, galactinol, myo-inositol, talose, maltose, aconitic acid, and citric acid were more abundant in CA tissues while trehalose, glycine, fumaric acid, succinic acid, ferulic acid, glyceric acid, cis-coutaric acid, oxalic acid, threonic acid, p-coumaric acid, and putrescine were less abundant relative to NASA tissues. Again, trehalose could not be detected in CA samples in the present study and the relative fold change (CA vs. NASA) was calculated using the procedure described under ‘Methods- Metabolite Profiling’ section. Eleven other metabolites also exhibited changes in abundance between CA and NASA but met only one of two thresholds for the magnitude of change (red or green dots). Of these, isoleucine, leucine, valine, phenylalanine, fructose, and mannose increased whereas serine decreased in abundance in CA compared to NASA tissues meeting >|2| criterion, while SA, benzoic acid, lactic acid, and ascorbic acid were more abundant in NASA than CA tissues based on padj < 0.05 threshold (Fig. 6).

3.7. Metabolite profiles of CASA- vs. CA-tissues

Mean concentrations of total 58-identified metabolites were pair-wise compared, i.e., CASA/CA, using log₂-scaled fold change and -log₁₀ scaled p-values adjusted with Benjamini-Hochberg correction (Padj). Only those metabolites were name-identified on a volcano plot that were quantitatively different fulfilling either both thresholds (padj < 0.05 and >|2|) or only one of these two criteria (Fig. 7). Data indicate that 21 metabolites (blue dots) showed major changes meeting both the criteria: twenty of these, trehalose, mannose, xylose, threitol, fructose, maltose, proline, threonine, glycine, malic acid, tartaric acid, cis-coutaric acid,
phosphoric acid, glyceric acid, glycolic acid, lactic acid, putrescine, SA, ascorbic acid, and \( \alpha \)-tocopherol were more abundant in CASA tissues while aconitic acid was less abundant compared to CA tissues. Since trehalose could not be detected in CA samples (mentioned before), the relative fold change (CASA vs. CA) was calculated using the procedure described under ‘Methods- Metabolite Profiling’ section. Fifteen other metabolites also exhibited changes in abundance between CASA and CA but met only one of two thresholds for the magnitude of change (red or green dots). Of these, fumaric acid, oxalic acid, gluconic acid, threonic acid, stigmasterol, and \( \gamma \)-tocopherol increased whereas leucine decreased in abundance in CASA tissues compared to CA tissues meeting >|2| criterion. Glutamic acid, 5-oxoproline, sucrose, glucose, myo-inositol, benzoic acid, and p-coumaric acid were more abundant in CASA tissues whereas tyrosine was less abundant compared to CA tissues meeting \( \text{padj} < 0.05 \) threshold (Fig. 7).

3.8. Seven metabolites vs. FT differences across NA, NASA, CA, and CASA

Based on the LT\(_{50}\) values, CASA was the most freeze-tolerant (most negative LT\(_{50}\), -11.3 °C), followed by CA (-9.9 °C), NASA (-6.5 °C), and NA being the least freeze-tolerant (-5.8 °C) (Fig. 8; also Fig. 2C and 4A). To determine which metabolites’ abundance most mirrors these LT\(_{50}\) differences among four treatments, levels of 58 metabolites (increasing order) were screened against the increasing FT of 4 treatments. Since, ‘actual’ tissue DW used for metabolite analysis across 4 treatments was slightly but significantly different (Table 1), these seven metabolites were quantified on DW basis (Fig. 8). Analysis revealed that levels of 7 leaf metabolites progressively increased in parallel with relative increase in FT, i.e. from least freeze-tolerant (NA) to most freeze-tolerant (CASA) treatment. Tissue contents (log\(_2\) scale) of these metabolites (5-oxoproline, fructose, glucose, maltose, proline, sucrose,
and tartaric acid) as a function of LT$_{50}$ are shown in Fig. 8. Except for sucrose, all 6 metabolites were significantly higher in CASA leaves compared to other three treatments, whereas CA leaves had significantly higher levels of four metabolites (i.e., 5-oxoproline, fructose, maltose, and proline) compared to NA and NASA samples. Although the absolute levels of all 7 metabolites were higher in more freeze-tolerant NASA than NA leaves, the increase was not significant (Fig. 8).

### 4. Discussion

Exogenous application of beneficial chemicals has previously been employed successfully as a promising strategy to improve plant stress tolerance against various abiotic stresses (Savvides et al., 2016). We have previously reported that SA-application improved the FT of excised spinach leaves (Shin et al., 2018). Few studies, including ours, noting an improvement in plant FT by SA application have suggested diverse explanations for this response, ranging from altered ice-nucleation activity in apoplastic proteins (Taşgın et al., 2003), changes in antioxidant enzymes’ activity (Mora-Herrera et al., 2005; Wang and Li, 2007), to the accumulation of compatible solutes and antioxidant metabolites (Shin et al., 2018). To complement the limitation of ‘excised-leaf based’ results and gain more comprehensive understanding at the metabolic level of SA-mediated increase in FT, here, we have evaluated the effect of SA-fertigation on FT at the ‘whole-plant level’ and performed metabolite profiling of leaves before and after SA-treatment. Moreover, experiments were conducted to compare and contrast the effect of SA application on the FT and metabolome reprogramming at warm vs. cold temperatures.
4.1. SA application and its effect on plant growth

Effect of SA on plant growth and physiology is dependent upon the concentration and mode of application (Miura and Tada, 2014). Our previous study, where excised, petiolated leaves of ‘Reflect’ spinach were fed with a range of SA concentrations (0.25, 0.5, 1.0 and 2.0 mM), showed 0.5 mM to be most optimal in terms of FT-induction as well as non-interference with leaf growth. (Shin et al., 2018). Hara et al. (2012) also noted that the optimal concentration of SA application for most plants in biotic/abiotic stress tolerance experiments ranged between 0.1 and 0.5 mM. Accordingly, in the present study, 0.5 mM SA was applied to whole plants/seedlings via sub-fertigation. Higher leaf SA content of SA-fed plants (~1.6-fold) than the fertilizer-controls (Fig. 2D) indicates that seedlings effectively absorbed and assimilated SA. While plants did not show visual signs of any detrimental effect of SA-feeding, a closer examination showed some reduction in the leaf-area of SA-fed seedlings at both ambient (29% reduction; NA vs. NASA) as well as cold acclimation (~9% reduction; CA vs. CASA) conditions (Table 1). However, no significant change in DW/FW ratio, % water content, DW/leaf area or actual tissue DW used for metabolite analyses was observed for these two pair-wise comparisons (Table 1). Notably, no reduction in the leaf-area was observed when excised leaves were fed with SA in our earlier study (Shin et al., 2018). The slight discrepancy between the two studies, in terms of SA’s effect on leaf growth, may be attributed to differences in the method and duration of SA application – excised leaves used in Shin et al. (2018) were nearly fully expanded when treated with SA for 4-d whereas in the present study leaves of the seedlings were in a relatively earlier phase of leaf expansion when first fed with SA and evaluated after 8-10 days of SA treatment. There is some evidence in literature for SA application to result in slower plant growth. Nazar et al. (2011) reported retarded growth in mung bean treated with 1.0 mM SA and Scott et al. (2004)
noted wild-type *Arabidopsis* plants to have somewhat smaller leaf-area than the mutants (*NahG*) that are deficient for SA accumulation. SA has been also shown to affect photosynthetic processes including stomatal closure (Izumi et al., 2001; Khokon et al., 2011). Presumably, SA-induced retarded leaf-growth in the present study may have been linked to stomatal closure but it cannot be confirmed at this time. Also, SA-induced leaf area reduction could involve GA regulation since there is some evidence for cross talk between SA and GA (Navarro et al., 2008).

4.2. *Exogenous SA improves freezing tolerance of spinach leaves (NASA vs. NA)*

Temperature-controlled, laboratory-based freeze-thaw protocol is conventionally and routinely used to estimate FT of ‘excised’ plant tissues, e.g. stem, bud, or leaf. While such tests provide valuable data on the relative FT of tissues / treatments in question, ‘whole plant freezing tests’ can be more informative for evaluating and comparing ‘real-world’ hardiness of plants. Here, we tested the effect of SA on FT of seedlings through an *in situ* ‘realistic’ freeze-thaw protocol, i.e. exposing whole plants anchored in growing media to a gradual cooling-warming cycle and also ensuring the ice formation in tissues, as evidenced by an exothermic event at ~ -2 °C (Fig. 1). Selection of two stress levels in the FT tests, -5.5 and -6.5 °C, was based on our previous determinations of leaf freezing response curve and LT<sub>50</sub> (temperature at which 50% injury occurs) for this cultivar (Shin et al., 2018) which showed that these temperatures bracketed the LT<sub>50</sub> of ~ -6°C. Thus, -5.5 and -6.5 °C were selected to represent ‘physiologically relevant’ levels of stress, one moderate and other relatively severe (but not too severe), respectively. Two methods of FT evaluations — one visual observation of freeze-thaw-injured seedlings and the other based on ion-leakage measurements on leaves taken from the whole-plants after the freeze-thaw cycle — show that SA-feeding increased FT
of spinach seedlings (Fig. 2A, B). Moreover, a ‘bath-freezing’ assay routinely used in our laboratory with excised spinach leaves (Chen and Arora, 2014; Min et al., 2014; Shin et al., 2018) showed that LT$_{50}$ of SA-fed leaves (NASA) was more negative, thus they were more freeze-tolerant, relative to NA controls (Fig. 2C).

4.3. Metabolite profiling vis-à-vis FT in NASA vs. NA leaves

Our analysis revealed 3 metabolites to be changing in SA-specific manner: increase in trehalose and ascorbic acid and decrease in aconitic acid (Fig. 3). Besides, four other metabolites showed increase (proline, γ-tocopherol, leucine) or decrease (mannose) but only by one of two criteria for quantitative change (Fig. 3). These metabolites, except for SA which was expectedly higher in NASA leaves due to exogenous supply, are discussed under the following sub-headings:

4.3.1. Compatible solutes (trehalose and proline)

Trehalose, a non-reducing disaccharide, has unique physical properties in comparison with other sugars, including high hydrophilicity and chemically stable structure (López-Gómez and Lluch, 2012) and hence, is also regarded as a compatible solute (Lunn et al., 2014). In vitro studies have demonstrated that trehalose replaces water via hydrophobic interactions with membranes and/or macromolecules during dehydration and freezing (Crowe, 2007). Few studies have also reported trehalose accumulation in response to chilling (Kaplan et al., 2004; Pramanik and Imai, 2005). Trehalose in NASA samples was adjudged to be ~130-fold of NA leaves (Fig. 3, Table S1); indeed, trehalose concentrations in NA samples were below the GC-MS detection limit as explained under ‘Methods’. To our
knowledge, this is the first report of trehalose accumulation induced by SA application in plant tissues. And even more significant is the concomitant increase in FT by SA-fed, trehalose accumulating tissues. NASA leaves accumulated proline at ~3.0-fold as compared to NA tissues (Fig. 3, Table S1). Several studies have noted proline accumulation in SA-fed tissues with improved tolerance against heat/salt stress (Misra and Saxena, 2009; Khan et al., 2013; Li et al., 2014). Our previous study with excised leaves also showed that more freeze-tolerant, SA-treated spinach leaves accumulated more proline compared to less-hardy, controls (Shin et al., 2018). Proline, a compatible solute, is believed to stabilize macromolecules against freeze-desiccation due, primarily, to its hydrophilic nature (Hoekstra et al., 2001) and can detoxify free radicals (Hayat et al., 2012).

4.3.2. Antioxidants (ascorbic acid and tocopherols)

One of the consequences of freeze-thaw injury is elevation in free radicals (O$_2^-$, H$_2$O$_2$, OH) and resultant oxidative injury to membranes and enzymes (Kendall and McKersie, 1989; Baek and Skinner, 2003; Chen and Arora, 2014; Min et al., 2014). Numerous studies have reported upregulation of antioxidant capacity of tissues during induction of FT (McKersie et al., 1993; Janda et al., 2003; Akram et al., 2017). Our data showed that NASA leaves had ascorbic acid at ~2-fold compared to NA (Fig. 3, Table S1). Ascorbic acid (vitamin C) is one of the non-enzymatic antioxidants involved in glutathione-ascorbate cycle (as a substrate for ascorbate peroxidase; APX) responsible for converting H$_2$O$_2$ into H$_2$O (Smirnoff, 2000). Why do SA-fed leaves accumulate higher ascorbic acid? One possible explanation may be related to the activity of catalase (CAT), an enzyme responsible for converting H$_2$O$_2$ into water. SA has been reported to inhibit CAT activity, which can potentially result in accumulation of H$_2$O$_2$ (Chen et al., 1993; Horváth et al., 2002).
If not properly scavenged, excess H$_2$O$_2$ can be damaging to cells. Therefore, upregulation of APX activity may be needed to compensate for diminished CAT activity, thus requiring higher amount of ascorbic acid. Evidence exists for exogenous SA to enhance the activity of enzymes involved in glutathione-ascorbate cycle or increased ascorbate and glutathione in plant tissues, and concomitantly improve salt and drought tolerance (Li et al., 2013; Nazar et al., 2015).

Tocopherols (vitamin E) are also potent antioxidants that scavenge lipid peroxyl radical generated during lipid peroxidation (Yusuf et al., 2010). Also, it has been known to play a pivotal role in protecting the photosynthetic membranes against oxidative stress (Munné-Bosch and Alegre, 2002; Munné-Bosch and Peñuelas, 2003). Increase in isoprenoids, including tocopherol, has been reported in tissues exposed to certain abiotic stresses (Fukuzawa et al., 1982; Peñuelas and Munné-Bosch, 2005) and both SA and α-tocopherol were found to accumulate under drought stress (Munné-Bosch and Peñuelas, 2003). Our results show that both α- and γ-tocopherol were higher in NASA leaves than NA (Table S1), however, increase in the latter was more pronounced (~3.5 fold of NA) (Fig. 3, Table S1). This is the first report, to our knowledge, on exogenous SA-induced tocopherol accumulation in non-stressed plant tissues accompanied by increased FT.

4.3.3. Aconitic acid and Mannose

Aconitic acid level in NASA was significantly reduced (~1/4$^\text{th}$ concentration) as compared to NA-leaves (Fig. 3, Table S1). Aconitic acid is a TCA cycle intermediate produced during the conversion of citric acid to isocitrate (Krebs and Holzach, 1952). It has been reported that SA specifically binds to iron-containing enzymes including aconitase,
thereby resulting in inhibition of their activity (Rüffer et al., 1995); aconitase catalyzes conversion of citric acid into cis-aconitic acid. Therefore, the reduction of aconitic acid in NASA could be a SA-specific response but it is not clear as to how this reduction may be associated with increased FT. However, Carrari et al. (2003) reported an Aco1 tomato mutant to have higher photosynthetic sucrose synthesis; Nunes-Nesi et al. (2005) later noted that this Aco1 mutant had substantially higher ascorbic acid levels although no such data were shown in their study. Interestingly, our data showed higher sucrose levels in NASA (~1.5 fold) than NA (Table S1). Speculatively, SA treatment could inhibit aconitase activity leading to higher sucrose and ascorbic acid (by mechanism unclear to us), which, in turn, could contribute to improved FT. NASA leaves had about ½ the mannose concentration as that in NA samples (Table S1). Reason for this is not clear at this time. However, Ishikawa et al. (2006) suggested mannose to be a primary precursor for ascorbic acid biosynthesis. Supposedly, decrease in mannose in NASA leaves in our study could be a reflection of its increased consumption for ascorbic acid biosynthesis.

4.4. CASA tissues are more freezing tolerant than NASA or CA

It is well known that plants adapted to colder climates are more freezing tolerant when cold-acclimated than their NA counterparts, and this FT-induction is accompanied by a myriad of adjustments in cellular physiology/biochemistry (Xin and Browse, 2000; Cook et al., 2004; Kaplan et al., 2004; Kim et al., 2013), including recently reported accumulation of SA by cold-acclimated spinach leaves (Shin et al., 2018). Our results show that SA-treatment increased FT of both excised (Shin et al., 2018) as well as whole plants (this study). We also tested if the combination of SA-application and CA treatment (CASA) resulted in higher FT
compared to NASA or CA alone. Our data indicates this to be true and that LT$_{50}$ of CASA was 4.8°C and 1.4°C more negative than that of NASA or CA, respectively (compare Figs. 2C and 4A). Moreover, a closer comparison of freezing injury percentages at -9 to -11°C range from LT$_{50}$ response curves for CASA and CA (Fig. 4A) indicates CASA to be $\geq2$-fold as cold-hardy, an improvement in FT not readily apparent from just the LT$_{50}$ comparisons. Thus, SA treatment along with CA results in higher leaf FT in spinach than CA alone.

CASA leaves had substantially higher SA concentration (~ 3.8-fold or ~ 3-fold) compared to CA or NASA leaves, respectively (compare Figs. 2D and 4B). But CA leaves which were more freeze-tolerant than NASA (LT$_{50}$ of -9.9 vs. -6.5 °C; compare Figs. 2C and 4A) did not accumulate higher SA than NASA. These results suggest that while SA levels may not be a strict quantitative indicator of relative FT, SA accumulation especially under cold temperatures could be one of the important contributors to a multi-factorial response of enhanced FT.

4.5. Distinct metabolic responses of exogenous SA under warm vs. cold

PCA revealed specific metabolic phenotypes for four treatments (i.e. NA, NASA, CA, and CASA); PC1 and PC 2 clearly separated treatments depending upon the temperature (warm vs. cold) and SA application (treated vs. non-treated), respectively (Fig. 5). In other words, metabolism differentially responded to SA feeding at warm vs. cold. Discussion below under various sections further highlights this interpretation.
4.6. Metabolite profiling vis-à-vis FT in CA vs. NASA leaves

Comparative abundance of metabolites in more freeze-tolerant CA vs. less freeze-tolerant NASA are presented in Fig. 6 and discussed under following sub-headings:

4.6.1. Compatible solutes (osmolytes) and ascorbic acid

CA leaves accumulated relatively higher levels of compatible solutes (osmolytes), including galactinol, myo-inositol, maltose and talose. Fructose and mannose were also more abundant in CA leaves although the magnitude of increase only met one of the two criteria, i.e. >|2| (Fig. 6). Other than talose, these metabolites are known to accumulate in cold-acclimated tissues, supposedly to serve as compatible solutes (osmolytes) (Wanner and Junntila, 1999; Cook et al., 2004; Kaplan et al., 2004; Lee et al., 2012). Galactinol and myo-inositol, especially, are known to be involved in raffinose family oligosaccharides (RFOs) biosynthesis whereby myo-inositol is one of the components for galactinol synthesis and galactinol, a precursor for RFO biosynthesis (Kannan et al., 2016). RFOs have been shown to accumulate during cold acclimation and function as compatible solute (Bachmann and Keller, 1994; Castonguay et al., 1995; Zuther et al., 2004; Kaplan et al., 2004; Bocian et al., 2015). Indeed, raffinose stabilizes isolated chloroplast thylakoid membranes during freeze-thaw cycle (Hincha, 1990). Although no tri-saccharides, such as raffinose, could be detected in the present study due to technical limitations of the GC-MS instrument used, it is tempting to speculate that higher levels of myo-inositol/galactinol in CA leaves could potentially reflect greater accumulation of raffinose in these tissues conferring higher FT. Talose, a monosaccharide, has been reported to accumulate in plant tissues when exposed to abiotic stresses such as cadmium (Xie et al., 2014) and salt (Shen et al., 2016); the former group
suggested accumulation of talose and other sugars under Cd stress to be due to their reduced utilization. However, to our knowledge, this is the first report of talose accumulation associated with increase in FT (~4.8-fold accumulation in CA vs. NA and 5.9-fold in CA vs. NASA) and maybe a spinach-specific response.

Trehalose was not detected in CA (or NA) leaves in the present study but its presence was noted in NASA (and CASA) leaves. It is not clear if the lack of trehalose detection in CA (and NA) tissues was strictly related to the detection limitation of GC-MS protocol used or to other unknown reason. However, our data seems to indicate trehalose accumulation may be a SA-specific response at least in ‘Reflect’ spinach leaves (see further discussion under ‘CASA vs. CA’ comparison below). Indeed, Suzuki et al. (2008) have reported an increase in trehalose concentration following SA accumulation in Arabidopsis under heat stress.

Ascorbic acid in NASA leaves was ~1.6-fold of CA samples, indicating a possible linkage between SA and ascorbic acid biosynthesis. This notion is further reinforced by a significantly lower level of aconitic acid in NASA relative to CA leaves (Fig. 6). As discussed before, SA can reduce aconitase activity (Rüffer et al., 1995) resulting in a decreased aconitic acid which, in turn, may lead to increased ascorbic acid content as reported by Nunes-Nesi et al. (2005). Curiously, two of the organic acids generated from ascorbic acid catabolism, i.e. oxalic acid and threonic acid (Green and Fry, 2005; Ishikawa et al., 2006), were also found to be at higher levels in NASA tissues in the present study (Fig. 6).

4.6.2. Amino acids

CA leaves had significantly higher levels of several amino acids compared to NASA tissues based on both the thresholds of magnitude change (Fig. 6); these included proline,
methionine, tyrosine, asparagine, and glutamine. Other amino acids (isoleucine, leucine, phenylalanine, and valine) also were more abundant in CA leaves but based only on >2 criterion (Fig. 6). Much greater accumulation of proline, a compatible solute, in more freeze-tolerant CA leaves indicates that cold exposure induces greater proline accumulation than the SA application at warm temperature (Table S1). This observation is further supported by a relatively higher abundance of glutamine and glutamic acid in CA leaves (2.4- and 1.5-fold, respectively, of NASA leaves) (Fig. 6, Table S1). Glutamine can be converted into glutamic acid using glutamate synthase and glutamic acid is known as a primary precursor for proline biosynthesis (Hare and Cress, 1997; Forde and Lea, 2007; Hayat et al., 2012).

Increase in methionine has been reported in cold-acclimated Arabidopsis leaves (Kaplan et al., 2007). Also, upregulation of methionine synthase has been noted in crowns of velvet bentgrass during CA process (Espevig et al., 2012). Methionine, a sulfur-containing amino acid, serves as a building block for protein synthesis. Several studies have reported synthesis of various proteins during CA process, such as dehydrins and heat shock proteins (Neven et al., 1992; Wisniewski et al., 1996; Ukaji et al., 1999), cold-regulated (COR) proteins (Thomashow, 1999), apoplastic antifreeze proteins (Antikainen and Griffith, 1997). Therefore, higher methionine in CA leaves (3.8-fold of NASA leaves) (Fig. 6, Table S1) may be associated with higher demand of this amino acid for protein synthesis required for FT induction.

Valine, leucine, and isoleucine in CA leaves were 2.4-, 5.1- and 11.3-fold, respectively, of NASA leaves (Table S1). Increase in these branched amino acids was also observed in Arabidopsis during cold acclimation (Kaplan et al., 2004). Also, phenylalanine and tyrosine, the aromatic amino acids, accumulated at higher levels in CA leaves (6.2- and 3.3-fold, respectively, of NASA leaves) (Table S1) similar to observations in cold-acclimated
Arabidopsis (Kaplan et al., 2004). Branched and aromatic amino acids have been known to serve as precursors for various secondary metabolites involved in plant defense mechanism against pathogens (Bennett and Wallsgrove, 1994; Vetter, 2000; Dixon, 2001). Expression of secondary metabolism genes has been well correlated with the induction FT (Hannah et al., 2006). For example, reduction of flavonoid compounds caused an impairment of FT, whereas the accumulation of quercetin (a polyphenol from flavonoid group) and anthocyanin improved FT (Schulz et al., 2016).

Asparagine in CA was about 5.0-fold of that in NASA leaves (Table S1). Similar asparagin accumulation during cold acclimation has been observed in Arabidopsis (Kaplan et al., 2007) while no specific role was ascribed to it. Our data also showed that glycine and serine were relatively less in CA compared to NASA tissues (Table S1). Glycine and serine are interconvertible amino acids (Mouillon et al., 1999). Since glycine is one of the components of glutathione biosynthesis pathway (Lu, 2013), conceivably, its higher amount in NASA leaves could lead to potentially higher accumulation of glutathione (needed for glutathione-ascorbate cycle) (Li et al., 2013; Nazar et al., 2015). Indeed, NASA tissues accumulated higher ascorbic acid compared to CA leaves as has been mentioned earlier.

4.6.3. TCA intermediates and other metabolites

The level of metabolites involved in TCA cycle was significantly different between CA- and NASA-leaves; aconitic acid and citric acid were more abundant in CA compared to NASA leaves (Fig. 6). As discussed before, SA can inhibit conversion of citric acid into cis-aconitic acid via inhibition of aconitase. Therefore, it is reasonable that NASA have reduced aconitic acid levels compared to CA leaves. Higher citric and aconitic acid in CA leaves
could potentially lead to higher $\alpha$-ketoglutaric acid, a TCA intermediate also involved for glutamic acid synthesis (van den Heuvel et al., 2004; Vuoristo et al., 2016); our data showed CA tissues to contain higher glutamic acid than NASA, as has been previously discussed. This hypothesis could also explain why CA leaves had relatively lower levels of succinic acid and fumaric acid, the two metabolites that follow $\alpha$-ketoglutaric acid in TCA cycle.

Putrescine, one of the polyamines, can serve as a precursor for the biosynthesis of other polyamines, i.e., spermidine and spermine (Cuevas et al., 2008). Accumulation of these polyamines associated with increased FT has been reported for various plant species (Cuevas et al., 2008; Alcázar et al., 2011). Intriguingly, however, our data indicated less freeze-tolerant NA leaves to contain relatively higher putrescine than NASA and CA tissues (Fig. 6, Table S1); no other polyamines could be detected in spinach leaves in our study. Why/how more freeze-tolerant tissues had relatively lower putrescine content as found in our study is not clear. Similarly, the rationale for higher accumulation of benzoic acid, lactic acid, p-coumaric acid, ferulic acid, cis-coutaric acid and glyceric acid in NASA leaves compared to CA tissues deserves further investigation.

4.7. Metabolite profiling vis-à-vis FT in CASA vs. CA leaves

CASA-leaves were more freezing tolerant than CA-leaves by ~ 1.4 °C based on the LT$_{50}$ values (Fig. 4A). Moreover, injury percent in CASA leaves was significantly less than that in CA leaves, especially between -9 to -11 °C (Fig. 4A), suggesting exogenous SA along with cold acclimation treatment induces higher FT than cold acclimation alone. In order to gain insight into this phenomenon, CASA vs. CA metabolites profiles were compared (Fig. 7).
4.7.1. Sugars/osmolytes and non-enzymatic antioxidants

CASA leaves accumulated significantly higher levels of compatible solutes (osmolytes) than CA tissues which included trehalose, mannose, maltose, fructose, sucrose, glucose, myo-inositol, xylose, and threitol. Once again, trehalose was detected in CASA but not in CA tissues, and its fold change (~173-fold of CA leaves) (Table S1) was calculated as described under “Methods”. An apparent high accumulation of this compatible sugar in CASA leaves may be one of the contributors to their higher FT, and as mentioned above, accumulation of trehalose in CASA as well as NASA leaves may be a SA-specific response in spinach. Moreover, SA treatment together with cold exposure (CASA) appear to result in higher (~1.3 fold) trehalose accumulation than SA at warm temperatures (NASA) (Table S1).

Mannose, maltose, fructose, sucrose, glucose, and myo-inositol in CASA leaves were 18.8-, 2.0-, 2.1-, 1.4-, 1.3-, and 1.5-fold, respectively, of CA leaves (Table S1). As discussed before, these metabolites could serve as compatible solutes or osmolytes and, thereby, allow greater tolerance to freeze-desiccation by CASA leaves. Threitol and xylose in CASA leaves were 2.3- and 3.0-fold, respectively, of CA leaves (Table S1). Threitol, a four-carbon sugar alcohol, is believed to serve as a biocompatible solute in freeze-tolerant and freeze-avoiding insects (Walters et al., 2009). Our data indicate accumulation of threitol may not be associated with cold acclimation since its level was similar in NA and CA leaves. This suggests that high accumulation of threitol in CASA leaves might have been a response to ‘SA + cold-acclimation’ treatment and associated with their higher FT. The role of threitol or its accumulation in relation to plant FT have not yet been reported, and this is the first such proposal to the best of our knowledge. Xylose, a monosaccharide, has been reported to accumulate in Arabidopsis (Kaplan et al., 2004) and sugar maple (Wong et al., 2003) during cold acclimation. Xylose is associated with hemicellulose component of the cell wall (Prade,
1996). Cell wall augmentation is known to be a component of increased FT (Huner et al., 1981; Rajashekar and Lafta, 1996; Stefanowska et al., 1999). Xylose is also reported as a precursor for threitol biosynthesis (Walters et al., 2009) and its level in most freeze-tolerant, CASA leaves was higher than CA, NASA or NA (Table S1).

Non-enzymatic antioxidants (ascorbic acid and tocopherol) were also more abundant in CASA compared to CA leaves (Fig. 7). Ascorbic acid in CASA was ~4.1-fold of CA leaves (Table S1); in comparison, it was ~2.6-fold of NASA leaves. This indicates that ‘SA + cold’ induced higher ascorbic acid accumulation than either cold alone or SA at ambient temperatures. Interestingly, aconitic acid in CA leaves was 6.3-fold of CASA leaves, supporting our earlier discussion about lower aconitic acid levels potentially leading to a higher ascorbic acid accumulation. Lipophilic antioxidants, α- and γ -tocopherol, in CASA leaves were 2.5- and 2.1-fold, respectively, of CA leaves (Table S1). Taken together, our data suggests that a higher FT in CASA leaves may be due to their higher protection capacity from oxidative stress and freeze-desiccation.

4.7.2. Amino acids

Several amino acids in CASA were more abundant than in CA leaves, including proline, glycine, threonine, glutamic acid, and 5-oxoproline (Fig. 7). Proline in CASA was 3.4-fold of CA leaves, indicating that ‘SA + cold temperature’ cause higher proline accumulation than cold-temperature alone; the presence of significantly higher glutamic acid (a precursor for proline biosynthesis) in CASA leaves (1.9-fold of CA leaves) supports this observation (Table S1). A relatively higher level of 5-oxoproline in CASA tissues (1.2-fold of
CA leaves) (Table S1) may possibly be another explanation for higher proline accumulation, since glutamic acid can be produced from 5-oxoproline (Mazelis and Pratt, 1976).

Glycine in CASA was 7.0-fold of CA leaves (Table S1). Glycine is one of components involved in biosynthesis of glutathione, part of antioxidant system (Lu, 2013) and is also known as a metabolite with compatible solute property (Kaplan et al., 2004). Tyrosine and leucine in CA leaves was 1.7- and 3.9-fold, respectively, of the CASA leaves (Table S1). It appears that while being potentially important for increased FT during cold acclimation as discussed earlier, these branched and aromatic amino acids do not seem to be a factor for additional FT of CASA leaves.

4.7.3. TCA intermediates and other metabolites

Malic acid and fumaric acid in CASA were more abundant than CA leaves (Fig. 7). Indeed, malic acid in CASA leaves was the highest across four treatments (~3.5-, ~3.8-, and 5.4-fold of NA, NASA, and CA leaves, respectively) (Table S1). It is tempting to suggest that such malic acid accumulation in spinach leaves maybe a specific response to ‘SA + cold’ treatment. Since malic acid can serve as a compatible solute (Kaplan et al., 2004), its higher amount in CASA leaves may contribute to greater FT.

Putrescine in CASA leaves was 11.4-fold of CA leaves (Table S1), indicating ‘SA + cold’ induced greater accumulation than cold alone. Indeed, Németh et al. (2002) noted that maize leaves subjected to 0.5 mM SA + chilling (5 °C) had higher putrescine than those exposed only to chilling. Oxalic, threonic and tartaric acids, the three metabolites that generate from ascorbic acid catabolism (Ishikawa et al., 2006), were also more abundant in CASA leaves which also had the highest ascorbic acid content across all treatments. Other
metabolites including cis-coutaric acid, phosphoric acid, glyceric acid, lactic acid, glycolic acid, p-coumaric acid, stigmasterol, and gluconic acid were more abundant in CASA than CA tissues but no explanation is available at this time for their role, if any, in higher FT.

4.8. Seven specific metabolites vis-à-vis FT

In order to explore which metabolites (out of the total of 58) are predominantly associated with improved FT, their abundance was screened against the LT50 values across 4 treatments (NA, NASA, CA, and CASA). Resultantly, 7 metabolites were found to incrementally accumulate with increase in FT; these were 5-oxoproline, fructose, glucose, maltose, sucrose, proline and tartaric acid (Fig. 8). As discussed above, carbohydrates (e.g. fructose, glucose, maltose, and sucrose) and proline are well-known compatible solutes and osmolytes potentially useful for increased tolerance to freeze-desiccation while 5-oxoproline can be used for biosynthesis of glutamic acid, a precursor for proline synthesis. Also, conversion of 5-oxoproline to glutamic acid is required for the regeneration of glutathione involved in glutathione-ascorbate cycle which detoxifies hydrogen peroxide (H2O2) (Yu et al., 2012). No report exists, to date, on specific role of tartaric acid for increased FT, if any, but curiously it is one of the catabolism products of ascorbic acid which accumulated at higher levels in more freeze-tolerant leaves.

5. Conclusion

In the present study, we examined the effect of exogenous SA feeding on ‘whole-plant’ FT of spinach at physiological and biochemical level. Salicylic acid application differentially induced FT at warm vs. cold; CASA plants were the most freezing tolerant,
followed by CA-, NASA- and NA-plants. Taken together the comparative metabolite analyzes of NASA vs. NA and CASA vs. CA revealed that SA treatment influenced plant metabolism differentially at warm- vs. cold-temperature (Fig. 9). Spinach seedlings grown under ambient condition with 0.5 mM SA feeding (NASA) accumulated more trehalose, proline, tocopherol, and ascorbic acid than non-SA treated leaves (NA). Cold-acclimated plus 0.5 mM SA-fed leaves (CASA) accumulated higher amount of compatible solutes (osmolytes) and antioxidants than the CA alone. Through comparative analysis of three metabolite pairs, each comprising a more vs. less freeze-tolerant treatment, respectively, i.e., NASA vs. NA, CA vs. NASA, and CASA vs. CA, we inferred that NASA- and CASA-leaves (i.e. SA-feeding as common denominator), specifically reduced aconitic acid level. Lowering of aconitic acid has been linked to accumulation of ascorbic acid (Nunes-Nesi et al., 2005). Accordingly, we hypothesize that SA-feeding of spinach seedlings results in high accumulation of ascorbic; how does lower aconitic acid result in ascorbic acid accumulation remains unclear at this time. Another unique finding of this study was the presence of trehalose in SA-fed treatments only suggesting a SA-specific response. Finally, the level of 7 metabolites (5-oxoproline, fructose, glucose, maltose, sucrose, proline and tartaric acid) was quantitatively associated with the magnitude of FT across CASA, CA, NASA, and NA treatments (Fig. 8).

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References


Table 1. Various plant growth parameters for NA (control), NASA (0.5 mM SA), CA (cold-acclimated control), and CASA (0.5 mM SA + cold acclimation) spinach (*Spinacia oleracea* L. cv. Reflect) leaves. FW, fresh weight; DW, dry weight.

<table>
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<tr>
<th>Growth parameters</th>
<th>NA</th>
<th>NASA</th>
<th>CA</th>
<th>CASA</th>
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<td>Water content (%) on FW basis</td>
<td>87.8 ± 0.3 ab</td>
<td>88.4 ± 0.4 a</td>
<td>86.7 ± 0.1 bc</td>
<td>86.1 ± 0.5 c</td>
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<tr>
<td>DW/FW (g)</td>
<td>0.12 ± 0.05 b</td>
<td>0.12 ± 0.07 b</td>
<td>0.13 ± 0.07 ab</td>
<td>0.14 ± 0.05 a</td>
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<td>Leaf area (cm²)</td>
<td>4.54 ± 0.03 a</td>
<td>3.25 ± 0.12 c</td>
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<td>DW/leaf area (mg/cm²)</td>
<td>2.64 ± 0.02 b</td>
<td>2.81 ± 0.05 b</td>
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<td>3.29 ± 0.08 a</td>
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<td>DW used for metabolomics (mg)</td>
<td>4.1 ± 0.1 c</td>
<td>4.2 ± 0.1 bc</td>
<td>4.5 ± 0.06 ab</td>
<td>4.7 ± 0.1 a</td>
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*Pooled means ± SE from five biological replications, each including 10-12 plants. Two leaves/ plant were used resulting in a total of 20-24 leaves/biological replication. Values with the same letter within the same row are not different (LSD test, *p* < 0.05)*

*Fresh weight (average of 3 to 4 replicates) used for metabolite profiling experiments was 33.5, 36.3, 34.5, and 34.0 mg for NA, NASA, CA, and CASA, respectively. ‘DW in a given amount of FW used for metabolomics’ ± SE for each of the four treatments was extrapolated using the percent water content indicated in this Table; values with the same letter within the same row are not different (LSD test, *p* < 0.05)*
Fig. 1. Diagrammatic representation of *in situ* freeze-thaw protocol used in this study. Air (closed square), soil (open circle), and leaf (open triangle) temperatures were monitored using copper-constantan thermocouples. Ice-nucleation was initiated by spraying pre-chilled (0°C) ddH$_2$O onto spinach (*Spinacia oleracea* L. cv. Reflect) leaves and is indicated as an exothermic event.
Fig. 2. Responses to in situ freezing (-5.5 and -6.5 °C) and leaf SA content in spinach (Spinacia oleracea L. cv. Reflect) seedlings sub-fertigated with either fertilizer alone (NA control) or with fertilizer + 0.5 mM salicylic acid (NASA). (A) visual record of freeze-thaw injury; UFC, unfrozen control (B) injury assessed by ion-leakage from leaves excised from the seedlings subjected to in situ freeze-thaw cycle; different letters indicate significant differences between treatments at P < 0.05 as per LSD test; (C) freezing tolerance (LT_{50}, a lethal temperature causing 50 % injury) of NA- and NASA-leaves as determined by excised-leaf freeze-thaw test; the sigmoid curves were generated from percent freeze-injury (means ± S.E.) using Gompertz function; LT_{50} (°C) of NA leaves (-5.8°C) corresponded to mid-point injury of ~47.7%, between the minimum (~0.2%) and maximum (~95.2%) injury, and that of NASA leaves (-6.5 °C) corresponded to mid-point injury of ~47.3% between the minimum (~0.3%) and maximum (~94.2%) injury. (D) SA content (means ± S.E.) in NA- vs. NASA-leaves.
Fig. 3. Volcano plot of comparative abundance of metabolites for NASA vs. NA biological conditions in spinach (Spinacia oleracea L. cv. Reflect) leaves; NASA, non-acclimated with 0.5 mM SA-feeding; NA, non-acclimated without SA feeding. Each dot represents a metabolite with the -log_{10} of the Benjamini-Hochberg adjusted P value (padj) as a function of abundance difference between two biological conditions (log2 on the abscissa). Only those metabolites were name-labeled that either met both thresholds, i.e. significantly different (padj < 0.05) as well as the fold change in abundance of >|2| or met only one of these two conditions. The two vertical lines in volcano plots indicate a two-fold cut off for either increase or decrease in abundance whereas the horizontal line represents a threshold of -log_{10} (padj) = 0.05. Blue dot denotes metabolites that met both thresholds for significant change; red dot denotes metabolites that changed in abundance based on padj < 0.05; green dot denotes metabolites that changed based on (>|2|); black dot denotes metabolites that do not meet either threshold.
Fig. 4. (A) Freezing tolerance (LT$_{50}$, lethal temperature causing 50% injury) of cold acclimated (CA) and ‘cold acclimated + 0.5 mM SA-fed’ (CASA) leaves of spinach (Spinacia oleracea L. cv. Reflect). The sigmoid curves were generated from percent freeze-injury (means ± S.E.) using Gompertz function; LT$_{50}$ ($^\circ$C) of CA leaves (-9.9$^\circ$C) corresponded to mid-point injury of ~45.7%, between the minimum (~0.1%) and maximum (~91.2%) injury, and that of CASA leaves (-11.3 $^\circ$C) corresponded to mid-point injury of ~42.5% between the minimum (~0.1%) and maximum (~84.8%) injury. (B) SA concentration (means ± S.E.) in CA- vs. CASA-leaves.
Fig. 5. Metabolic phenotype clustering via principal component analysis (PCA) of log₁₀-scaled 58 metabolite data for 14 samples originating from four treatments (i.e. NA, NASA, CA, and CASA). Principal component 1 (PC1) indicates differential response to warm- and cold-temperature. Principal component 2 (PC2) indicates differential response to SA application. NA, non-acclimated (yellow), NASA, non-acclimated with 0.5 mM SA-feeding (red), CA, cold-acclimated (black), and CASA, cold-acclimated with 0.5 mM SA-feeding (green) are shown in 2D plot.
**Fig. 6.** Volcano plot of comparative abundance of metabolites for CA vs. NASA biological conditions in spinach (*Spinacia oleracea* L. cv. Reflect) leaves; CA, cold acclimated; NASA, non-acclimated with 0.5 mM SA-feeding. Each dot represents a metabolite with the $-\log_{10}$ of the Benjamini-Hochberg adjusted $P$ value ($\text{padj}$) as a function of abundance difference between two biological conditions ($\log_2$ on the abscissa). Only those metabolites were name-labeled that either met both thresholds, i.e. significantly different ($\text{padj} < 0.05$) as well as the fold change in abundance of $>2$ or met only one of these two conditions. The two vertical lines in volcano plots indicate a two-fold cut off for either increase or decrease in abundance whereas the horizontal line represents a threshold of $-\log_{10}(\text{padj}) = 0.05$. Blue dot denotes metabolites that met both thresholds for significant change; red dot denotes metabolites that changed in abundance based on $\text{padj} < 0.05$; green dot denotes metabolites that changed based on ($>2$); black dot denotes metabolites that do not meet either threshold.
Fig. 6. Volcano plot of comparative abundance of metabolites for CA vs. NASA biological conditions in spinach (Spinacia oleracea L. cv. Reflect) leaves; CA, cold acclimated; NASA, non-acclimated with 0.5 mM SA-feeding. Each dot represents a metabolite with the $-\log_{10}$ of the Benjamini-Hochberg adjusted $P$ value ($\text{padj}$) as a function of abundance difference between two biological conditions ($\log_2$ on the abscissa). Only those metabolites were name-labeled that either met both thresholds, i.e. significantly different ($\text{padj} < 0.05$) as well as the fold change in abundance of $>|2|$ or met only one of these two conditions. The two vertical lines in volcano plots indicate a two-fold cut off for either increase or decrease in abundance whereas the horizontal line represents a threshold of $-\log_{10}(\text{padj}) = 0.05$. Blue dot denotes metabolites that met both thresholds for significant change; red dot denotes metabolites that changed in abundance based on $\text{padj} < 0.05$; green dot denotes metabolites that changed based on ($>|2|$); black dot denotes metabolites that do not meet either threshold.
Fig. 7. Volcano plot of comparative abundance of metabolites for CASA vs. CA biological conditions in spinach (Spinacia oleracea L. cv. Reflect) leaves; CASA, cold-acclimated with 0.5 mM SA-feeding; CA, cold acclimated. Each dot represents a metabolite with the $-\log_{10}$ of the Benjamini-Hochberg adjusted $P$ value (padj) as a function of abundance difference between two biological conditions ($\log_{2}$ on the abscissa). Only those metabolites were name-labeled that either met both thresholds, i.e. significantly different (padj $<$ 0.05) as well as the fold change in abundance of $>$|2| or met only one of these two conditions. The two vertical lines in volcano plots indicate a two-fold cut off for either increase or decrease in abundance whereas the horizontal line represents a threshold of $-\log_{10}$ (padj) = 0.05. Blue dot denotes metabolites that met both thresholds for significant change; red dot denotes metabolites that changed in abundance based on padj $<$ 0.05; green dot denotes metabolites that changed based on ($>2$); black dot denotes metabolites that do not meet either threshold.
Fig. 8. Quantitative relation between seven specific metabolites and freezing tolerance (LT$_{50}$) across four treatments, i.e., NA, NASA, CA, and CASA. Mean concentration of each metabolite was transformed as log$_2$-scale. Different letters within each rectangle indicate significant differences in each metabolite between treatments at $p < 0.05$ according to LSD test. NA, non-acclimated; NASA, non-acclimated with 0.5 mM SA-feeding; CA, cold-acclimated; CASA, cold-acclimated with 0.5 mM SA-feeding.
**Fig. 9.** Schematics of effect of SA-pretreatment on plant metabolism at warm- and cold-temperatures vis-à-vis improved freezing tolerance. (▲) or (▼) indicate increased or decreased metabolite level. One or two arrows indicate the relative magnitude of change in abundance. SA binding inhibits aconitase resulting in lowered aconitic acid. Ascorbic acid level is elevated at lower aconitic acid condition, but its mechanism is unclear. In the two circles, three metabolites common under two conditions (warm and cold) are highlighted in bold. NA, non-acclimated; NASA, non-acclimated with 0.5 mM SA-feeding; CA, cold-acclimated; CASA, cold-acclimated with 0.5 mM SA-feeding
Table S1. Concentrations (p.mols mg$^{-1}$ FW) of 58 metabolites across 4 biological conditions (NA, NASA, CA, and CASA) as detected by GC-MS. Values are averages of 3 to 4 replications per biological condition. Metabolites are grouped under 5 categories. NA, non-acclimated; NASA, non-acclimated with 0.5 mM SA-feeding; CA, cold-acclimated; CASA, cold-acclimated with 0.5 mM SA-feeding.

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### TCA intermediates (n=5)

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Table S2. Rankings and loading values of 58 metabolites as analyzed by principle component analysis (PCA) on log10-transformed relative response across 4 biological conditions (NA, NASA, CA, and CASA). PC1 accounts for 54 % of total variance of data set, whereas PC2 account for 27 % of the total variance of data set. NA, non-acclimated; NASA, non-acclimated with 0.5 mM SA-feeding; CA, cold-acclimated; CASA, cold-acclimated with 0.5 mM SA-feeding.

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CHAPTER 4. SHORT VERSUS PROLONGED FREEZING DIFFERENTIALLY IMPACTS FREEZE-THAW INJURY IN SPINACH LEAVES: MECHANISTIC INSIGHTS THROUGH METABOLITE PROFILING


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Abstract

Plant tissues subjected to short or prolonged freezing to a fixed sub-freezing temperature are expected to undergo similar freeze-desiccation but former causes substantially less injury than the latter. To gain metabolic insight into this differential response, metabolome changes in spinach (*Spinacia oleracea* L.) leaves were determined following short-term (0.5 and 3.0 h) vs prolonged freezing (5.5 and 10.5 h) at -4.5°C resulting in reversible or irreversible injury, respectively. LD_{50}, the freezing duration causing 50% injury, was estimated to be ~3.1 h, and defined as the threshold beyond which tissues were irreversibly injured. From 39 identified metabolites, 19 were selected and clustered into 3 groups: 1) signaling-related (salicylic acid, aliphatic and aromatic amino acids), 2) injury-related (GABA, lactic acid, maltose, fatty acids, policosanols, TCA intermediates), and 3) recovery-related (ascorbic acid, α-tocopherol). Initial accumulation of salicylic acid during short-term freezing followed by a decline may be involved in triggering tolerance mechanisms in moderately injured tissues, while its resurgence during prolonged freezing may signal programmed cell death. GABA accumulated with increasing freezing duration, possibly to serve as a ‘pH-stat’ against cytoplasmic acidification resulting from lactic acid accumulation. Mitochondria seems to be more sensitive to prolonged freezing than chloroplast since TCA intermediates decreased after LD_{50} while salicylic acid and maltose, produced in chloroplast, accumulate even at 10.5-h freezing. Fatty acids and policosanols accumulation with increasing freezing duration indicates greater injury to membrane lipids and epicuticular waxes. Ascorbic acid and α-tocopherol accumulated after short-term freezing, supposedly facilitating recovery while their levels decreased in irreversibly injured tissues.
**Abbreviations** — FT, freezing tolerance; GAD, glutamate decarboxylase; Osm, osmolal; PCA, principal component analysis; PCD, programmed cell death; SA, salicylic acid; UFC, unfrozen control

**Introduction**

Several factors related to the freeze-thaw process can impact the magnitude of injury to plant tissues. Some of these are: extent of freezing temperature, rate of cooling especially in sub-freezing range, whether or not tissues were ice-nucleated, rate of thaw, and the duration of freezing (Gusta and Wisniewski 2013, Arora 2018). While the first three factors have been well investigated, research on the ‘rate of thaw’ and the ‘effect of, and cellular response to, varying duration of freezing’ remains sparse (Arora 2018). Temperature-controlled, laboratory-based freeze-thaw tests are conventionally employed to determine freezing tolerance (FT) of excised tissues (e.g., leaves, stems, buds, etc.) of diverse plant species. Generally, in these tests, tissues are exposed to various temperatures for 30 to 60 min. After thaw at 0-4°C for 12-20 h, tissues are used to estimate injury, typically via ion-leakage test, and FT is determined as LT$_{50}$, a lethal temperature causing 50 % injury (Lim et al. 1998). Though widely used successfully for estimating relative FT of plant tissues, these tests do not consider the effect of ‘duration of freezing’ on the estimated LT$_{50}$ since tissues are generally held at test temperatures for a relatively short duration (Min et al. 2014).

Few studies have been reported on the effect of duration of freezing on FT of plants. Pomeroy et al. (1975) first noted significantly different injuries in winter wheat subjected to shorter vs longer freezing at an identical freezing temperature. Later work noted that prolonged freezing test are more useful in selecting more hardy genotypes, which otherwise
possessed similar LT$_{50}$ than the less hardy ones, when evaluated by a conventional freeze-test (Gusta et al. 1997, Waalen et al. 2011). Using Arabidopsis leaves, Nagao et al. (2008) demonstrated that ultrastructural changes in plasma membrane induced by long-term freezing were more severe than those exposed to short-term freezing and attributed it to the prolonged exposure of cells to concentrated solutions of intracellular chaotropic phytotoxic ions. Also, we have previously demonstrated that a freezing temperature interpreted to be sub-lethal based on the LT$_{50}$ could indeed be lethal to spinach leaves depending upon the duration of freezing; moreover, an ability of recovery from freeze-thaw injury was also significantly influenced by the duration of freezing at a given freezing temperature (Min et al. 2014). Given that tissues subjected to a fixed sub-freezing temperature under slow/equilibrium freezing protocol would experience a fixed level of vapor-pressure gradient or freeze-dehydration (i.e. the decline of water potential of ice at -1.16 MPa per ºC) (Hansen and Beck, 1988, Arora 2018), it is curious as to why or how (at the cellular level) plants experience substantially greater (or irreversible) injury after a prolonged freezing than those exposed to shorter freezing-duration experiencing only moderate (recoverable) injury.

To address this curiosity, we conducted a study using spinach leaves, a previously used system in our laboratory (Chen et al. 2013, Min et al. 2014, Shin et al. 2018), to explore the tissue metabolome changes, using gas chromatography-mass spectrometry (GC-MS), after short-term versus prolonged freezing at a fixed sub-lethal temperature. Since longer freezing duration is known to result in greater injury relative to short-term freezing, we hypothesized that metabolome of thawed tissues in these two scenarios would also be different. We categorized the metabolites in 3 major groups: ‘signaling-related’, ‘injury-related’, and ‘recovery-related’. These categories were assigned based on a two-fold criteria: 1) well-defined roles of these metabolites (in respective categories) in abiotic/biotic stress
tolerance as described in literature, and 2) the pattern of their appearance or dissipation along the ‘duration of freezing’ time-course. For example, ‘signaling-related metabolites’ were expected to increase in shorter freezing duration followed by decrease before the ‘threshold duration’ separating reversible from irreversible injury. Similarly, recoverable tissues were expected to exhibit a reduction after the threshold duration in metabolites known to have roles in ‘recovery’ from abiotic/biotic stress. Finally, tissues injured at progressively longer freezing durations were expected to incrementally accumulate ‘injury-related’ metabolites.

Materials and methods

Plant material

Spinach (Spinacia oleracea L. cv. Bloomsdale) seeds (Stokes Seeds, Inc.) were sown in plug flats filled with Sunshine LC-1 mix (Seba Beach) and placed in a growth chamber at 15/15°C (day/night) with 12-h photoperiod under average photosynthetically active radiation at plant height (~250 µmol m⁻² s⁻¹) provided by incandescent and fluorescent lamps (Chen and Arora 2014, Min et al. 2014). Seedlings were watered as needed through sub-irrigation (about 5-d interval). Two weeks from the sowing, the growth chamber temperature was increased to 20/18°C (day/night) and seedlings were sub-fertigated with 300 ppm Excel nutrient solution (Scotts Sierra Horticultural Products Company) at weekly interval. Four-week-old leaves excised from seedlings were used for assays described below.
Freeze-thaw protocol

A fixed sub-lethal temperature (-4.5°C) and four different durations of freezing (i.e. 0.5, 3.0, 5.5, and 10.5 h) were selected based on our previous study (Min et al. 2014) which showed the LT$_{50}$ of ‘Bloomsdale’ spinach to be $\sim$ -5.5°C and that these freezing durations resulted in a range of injury from minimal to moderate to severe. Essentially, a pair of petiolated leaves was placed, standing up, in a 2.5 cm x 20 cm test tube containing 150 μl of distilled-deionized water (ddH$_2$O) and transferred to 0°C in a glycol bath (Isotemp 3028; Fisher Scientific). After 1 h at 0°C, samples were slowly cooled down to -1°C (ice-nucleation) and then to -4.5°C at a rate of -1°C h$^{-1}$ (Chen and Arora 2014, Min et al. 2014). Samples held at -4.5°C were removed from the glycol bath following a desired duration of freezing and subsequently thawed on ice overnight. Unfrozen control (UFC) leaves were kept at 0°C throughout the freeze-thaw cycle. The next morning, thawed samples were held at 4°C for 1 h followed by 1 h at room temperature (~20°C) before measuring ion-leakage. Twenty ml of ddH$_2$O was then added and percent ion-leakage from injured tissues was measured as described by Chen and Arora (2014). Percent injury was calculated from percent ion-leakage data as described by Lim et al. (1998).

LD$_{50}$, the freezing duration at which tissues reach their 50% of maximum injury, was calculated from injury vs duration using an asymmetric sigmoid function (Lim et al. 1998). The scatter plot for this analysis included 15 data points for injury at each of the four durations.
**Metabolite profiling**

Leaf metabolites were extracted from UFC and the thawed tissues after four freezing durations, i.e., 0.5, 3.0, 5.5 and 10.5-h freezing, using the method described by Noutsos et al. (2015), with slight modification. Briefly, leaves from four duration treatments were ground in liquid nitrogen using a mortar and pestle. Aliquots of ~25 mg of frozen powder were mixed with two internal standards, 20 \( \mu \)l (1mg ml\(^{-1}\) stock) of ribitol for polar compounds and 20 \( \mu \)l (1mg ml\(^{-1}\) stock) of nonadecanoic acid for non-polar compounds, and homogenized with 350 \( \mu \)l of hot MeOH (60°C) followed by incubation for 10 min. Samples were then sonicated using Branson 2510 ultrasonic cleaner (Marshall Scientific LLC) for 10 min at 40 kHz. After sonication, samples were mixed with 350 \( \mu \)l of chloroform, vortexed for 10 sec, and then mixed with 300 \( \mu \)l of water and vortexed for 10 sec. After centrifugation (10 000 g), 200 \( \mu \)l of upper layer (polar phase) and lower layer (non-polar phase) were transferred to separate GC-MS vials. The vials containing polar or non-polar extraction were placed in a Savant speed-vac concentrator 100H (Savant Instrument INC.) for 10 h. Samples were derivatized by the addition of 50 \( \mu \)l of 20 mg ml\(^{-1}\) methoxyamine hydrochloride in dry pyridine to dried polar extraction followed by incubation at 30°C for 1.5 h with continuous shaking. Subsequently, trimethylsilylation was conducted for polar and non-polar samples by the addition of 70 \( \mu \)l of bis-trimethyl silyltrifluoroacetamide with 1% trimethylchlorosilane for 30 min at 37°C. Derivatized samples were analyzed using an Agilent 7890A gas chromatograph (Agilent Technologies) equipped with HP5MS separation column. The mass range was 40-800 \textit{m/z}. 
**Metabolite identification and quantification**

Metabolites were identified by performing database searches following deconvolution of raw GC-MS chromatograms using AMDIS software (Min et al. 2018); the database used was a NIST 14 mass spectra library. Each compound was determined as a metabolite if identified with a match factor $>650$ on a scale of 0 to 1000 and retention time index deviation $\pm 50$. Each identified metabolite was quantified based on fresh weight and internal standards. Undetectable metabolites were allocated a number equal to the smallest peak area /2 for statistical analysis purposes (Xia et al. 2009).

**Statistical analysis**

Freezing tests (for four durations) were independently repeated thrice with five replications per duration per biological replicate. Percent injuries from three experiments, namely 15 individual points, were pooled to acquire means and standard errors. Mean differences were analyzed by HSD (Honest Significant Difference) test using R (version 3.2.2, The R Foundation for Statistical Computing). Metabolite analysis for each duration treatment consisted of three technical replications from one biological replication.

Principal component analysis (PCA) was conducted with R after normalization and log-transformation of the metabolite concentration data. The pair-wise comparison of abundance of each metabolite across five treatments, i.e. 0 (UFC), 0.5-, 3.0-, 5.5-, and 10.5-h freezing, were performed by HSD test with R (Table S1).

Statistical significance of the contribution of metabolites to PC1 or PC2 was assessed non-parametrically using permutation tests as described by Linting et al. (2011). The importance of each metabolite (c) relative to the sample segregation at one principal
component was estimated as: \( c = l^2 \times v \), wherein \( l^2 \) is the squared loading value of a metabolite at one PC, and \( v \) is the percentage of data variation explained by this PC. The corresponding p-value was calculated through a permutation test described in Linting et al. (2011). The R script used for permutation test and calculating p-values is detailed in Supplementary Method file.

**Results**

**Freeze-thaw injury in response to four freezing durations**

Based on our previous study (Min et al. 2014), -4.5°C freezing stress, a temperature warmer than the LT50 (~ -5.5°C), was selected as a sub-lethal stress treatment for comparing percent injury in response to increasing duration of freezing. Our data indicated tissues were progressively more injured with increasing duration of freezing (Fig. 1A), and the injury ranged from relatively mild (~16%) by 0.5 h freezing to severe (~ 68%) following 10.5 h freezing. The levels of injury sustained at different freezing durations in this study are similar to our previous study with spinach leaves (Min et al. 2014) which, based on a post-thaw recovery observation over a 6-d period, had also demonstrated that relatively shorter freezing (0.5 and 3.0 h) caused injury that was reversible whereas the longer freezing (5.5 and 10.5 h) induced irreversible injury.

Injury percentages from all 15 data points across three biological replications each with five technical replications were used to develop a scatter plot. An asymmetric sigmoid Gompertz function was fitted to this data to generate injury percent vs freezing duration response curve. This was used to estimate LD50, the freezing duration causing 50% injury, which was defined as the ‘threshold duration’ beyond which tissues are irreversibly injured.
Our data indicate LD_{50} to be ~ 3.1 h (Fig. 1B).

**Principal component analysis (PCA) of metabolites**

Metabolite contents for five treatments, i.e. 0 (UFC), 0.5, 3.0, 5.5, and 10.5-h freezing, were analyzed by GC-MS. In total, 39 metabolites were identified and clustered into 5 groups: 14 amino acids, 7 carbohydrates, 7 lipid components, 4 TCA intermediates, and 7 others (Table S1).

Principal component analysis (PCA) was performed to distinguish metabolite phenotypes between the unfrozen control (0 h, UFC) and the four freezing durations to determine which metabolites were affected the most by the treatment differences, if any. PCA showed differentiation across five treatments in which the two components explained 78.6% of the total variance (Fig. 2). The first principal component (PC1) accounted for 56.5% of the total variance of data set, indicating a different response at the metabolic level by uninjured (0 h, UFC) vs freeze-thaw injured-tissues. In order to explore which metabolites contributed the most for such difference, the metabolite loading values in PC1 were ranked (Table S2). The 5 most positive loading values (i.e. freeze-thaw stress related), in descending order, were for GABA, maltose, lactic acid, glutamine and glyceric acid, whereas the 5 most negative loading values (i.e. no-freezing), in increasing order, correspond to fructose, ascorbic acid, α-ketoglutaric acid, glucose, and valine. The second principal component (PC2) explained 22.1% of the total variance of data set, separating recoverable/short-term freezing (0.5 and 3.0 h) vs irrecoverable/prolonged freezing (5.5 and 10.5 h); the 5 most positive loading values (i.e. short-term freezing related) in PC2, in descending order, were for glutamic acid, aspartic acid, SA, glutamine, and ascorbic acid whereas the 5 most negative values (i.e. prolonged freezing related), in increasing order, were for glucose, α-linolenic acid, phytol, fructose, and maltose.
The statistical significance of the contribution of 39 metabolites to PC1 or PC2 was non-parametrically assessed using permutation tests (Table 1). Other than α-linolenic acid, monoglyceride, 1-tetracosanol, and palmitic acid, all significantly contributed to separation between treatments on PC 1, while all but SA, 1-tetracosanol, glucose, sucrose, and monoglyceride, did so on PC 2.

**Metabolite changes vis-à-vis durations of freezing**

Nineteen of the total 39 metabolites were clustered into 3 groups as described earlier in the introduction: (1) signaling-related (Fig. 3A, B), (2) injury-related (Fig. 4A-D), and (3) recovery-related metabolites (Fig. 5). Signaling-related metabolites included SA, leucine, isoleucine, valine, and phenylalanine. As per our data SA levels showed a bi-phasic response, i.e., increasing at 0.5 h, maintaining its level up to 3.0 h, decreasing after 3.0 h up to 5.5 h, and again increasing until 10.5 h freezing duration. Aliphatic and aromatic amino acids levels increased up to 3.0 h of freezing and then decreased, except for phenylalanine which increased at 10.5-h freezing (Fig. 3B).

Injury-related metabolites consist of GABA, lactic acid, maltose (Fig. 4A), TCA intermediates (citric acid, α-ketoglutaric acid, succinic acid, and malic acid; Fig. 4B), fatty acids (palmitic acid, α-linolenic acid, and stearic acid; Fig. 4C); and policosanols (1-tetracosanol and 1-hexacosanol; Fig. 4D). These metabolites progressively increased with increasing duration of freezing except for the TCA intermediates. As per our data, citric acid, succinic acid, and malic acid increased up to 3.0 h of freezing followed by a decrease up to 5.5 h after which no change was noted until 10.5-h freezing. Whereas, α-ketoglutaric acid stayed at relatively lower level than UFC at all freezing durations (Fig. 4B).
Again, based on the criteria defined earlier in the introduction, ascorbic acid and \( \alpha \)-tocopherol were grouped as recovery-related metabolites. Levels of both peaked at 3.0 h followed by continued decline until 10.5-h freezing (Fig. 5).

**Discussion**

We have previously reported that short-term (minutes) vs prolonged (hours) duration of freezing caused significantly different injury to plant tissues as estimated by ion-leakage (Min et al. 2014). For instance, ‘Bloomsdale’ spinach leaves exposed to 3.0 h at -4.5°C, i.e., a sub-lethal temperature determined by ion-leakage based LT\(_{50}\) of -5.5°C, resulted in ~30% injury while additional freezing for 2.5 h at the same temperature caused ~70% injury (Min et al. 2014). Data from the present study (Fig. 1A) confirms these observations and reinforces the notion that a freezing temperature interpreted to be sub-lethal based on the LT\(_{50}\) curve could indeed be lethal depending upon the duration of freezing. Additionally, leaves frozen for shorter durations (0.5 and 3.0 h) were recoverable from injury during post-thaw whereas those frozen for 5.5 and 10.5 h were irreversibly injured. Excised leaves in this study were exposed to temperature-controlled ‘equilibrium freezing’, i.e. holding the tissues for about 1.5 h following ice-nucleation at -1°C followed by slow cooling of 0.5°C /30 min until the target temperature of -4.5°C. Such freezing protocol is expected to exert similar degree of cellular desiccation (efflux of osmotically active water from the cell) regardless of the duration of freezing (Arora 2018). For ‘Bloomsdale’ spinach leaves, the cell sap concentration at -4.5°C would be ~ 2.4 Osm (efflux of ~88% of freezable water) given their initial osmotic pressure at the unfrozen state to be ~0.3 Osm (Min et al. 2014, Arora 2018). This observation raises the question: why/how leaves get more or irreversibly injured following a longer freezing duration despite being equivalently freeze-desiccated as from a
relatively shorter freezing duration? To gain insight into this phenomenon at the cellular level, we performed metabolite profiling of thawed tissues exposed to four durations (0.5, 3.0, 5.5, and 10.5 h) of freezing at -4.5°C.

PCA revealed metabolism differentially responded to various durations of freezing (Fig. 2). Ensuing discussion explores potential connection of the 19 metabolites and their changes with the different consequences following shorter vs longer freezing duration.

**Signaling-related metabolites – SA, and aliphatic and aromatic amino acids**

In this study, five metabolites, including SA, leucine, isoleucine, valine (aliphatic amino acids) and phenylalanine (aromatic amino acid) fit the two-pronged criteria for ‘signaling-related metabolites’ as defined under Introduction. These metabolites were also statistically significant on PC1 separating the uninjured vs injured tissues (Table 1 and Fig. 2).

SA is a well-known signaling molecule regulating defense mechanisms against plant pathogens (Raskin 1992, Horváth et al. 2007, Vincent and Plasencia 2011, Miura and Tada 2014, Khan et al. 2015). SA accumulation in response to abiotic stresses (e.g. cold, heat, and drought) and its role in stress-tolerance mechanisms have also been reported lately (Munné-Bosch and Peñuelas 2003, Kaplan et al. 2004, Suzuki et al. 2008, Shin et al. 2018, Min et al. 2018). Hence, SA accumulation could indicate initiation of defense/tolerance processes after 0.5- or 3.0-h freezing stress that causes moderate, ~16-33% injury (Fig. 1A and 3A).

Accumulation of aliphatic and aromatic amino acids (i.e. valine, leucine, isoleucine and phenylalanine) in these tissues (Fig. 3B) could facilitate SA-induced defense system since these are precursors for various secondary metabolites involved in plant defense mechanism, especially pathogens (Bennett and Wallsgrove 1994, Vetter 2000, Dixon 2001, Dučaiová et al.
2013, Hildebrandt et al. 2015). Plant tissues injured by freeze-thaw can become an infection court for pathogens during the post-thaw, and therefore, activation of such defense could be beneficial to plant survival, especially when only moderately injured. All five metabolites also follow the accumulation dynamic criterion defined for signaling-related metabolites, i.e. increase during short duration followed by a decrease before the ‘threshold duration’.

Why/how SA levels started to decrease around LD$_{50}$ and again increased at 10.5 h is not clear but SA level is associated with the degree of injury. Tissues frozen for >3.0 h are severely or irreversibly injured (~60%; Fig. 1A), and possibly, are not able to establish defense systems. Corresponding decrease in aliphatic and aromatic amino acids around LD$_{50}$ (Fig. 3B) supports this assumption. In contrast, an increase in SA at 10.5 h, when tissues are even more severely injured, may be linked to programmed cell death (PCD), shown to be regulated by SA (Radojičić et al. 2018). Corresponding rise in phenylalanine at 10.5 h (Fig. 3B) supports its role as a substrate for SA biosynthesis via phenylalanine ammonia lyase pathway (Chen et al. 2009). Since, chloroplasts are the major site of SA synthesis (Nomura et al. 2012, Serrano et al. 2013), our data suggests that while membranes transport functions are acutely injured (high ion-leakage) after 10.5-h freezing, chloroplasts might not have suffered complete dysfunction. Such differential tolerance to freeze-thaw stress by various cell organelles (plasma membrane, chloroplast, mitochondria etc.) has previously been reported (Singh et al. 1977, Steffen et al. 1989). Alternatively, SA accumulation may occur due to its decreased consumption in severely injured tissues.
Injury-related metabolites (1) – GABA, lactic acid, maltose and TCA intermediates

Those metabolites that progressively increased with incremental injury, i.e. increasing duration of freezing, were defined as ‘injury-related metabolites’. GABA, lactic acid, and maltose followed this pattern (Fig. 4A) while TCA intermediates (citric acid, α-ketoglutaric acid, succinic acid, and malic acid) did not (Fig. 4B). But since they are linked to GABA metabolism, a major injury-related metabolite in our study, we grouped TCA intermediates also under this category. These metabolites were statistically significant on PC2 separating the duration treatments between relatively less injured (i.e. 0.5 and 3.0 h) vs severely injured (5.5 and 10.5 h) tissues (Table 1 and Fig. 2).

GABA is a four-carbon non-proteinogenic amino acid which is typically maintained at low levels in plant cells ranging from 0.03 to 2.00 μmol g⁻¹ fresh weight (Shelp et al. 1999). However, GABA level could increase several-fold in response to abiotic stresses such as drought, hypoxia, cold and heat shock (Shelp et al. 1999). Mazzucotelli et al. (2006) reported that exposure of barley leaves to -3°C for 16 h resulted in 16-fold increase in GABA. In accordance with this report our data indicate that GABA levels progressively increased with increasing duration of freezing (increasing injury; Fig. 4A); however, an important distinction between our study and experiments with barley is that tissues were indeed frozen in our study by ensuring ice-nucleation.

GABA shunt, conversion of glutamic acid to succinic acid via GABA, involves two enzymes — glutamate decarboxylase (GAD) and GABA transaminase (GABA-T). The former enzyme converts glutamic acid into GABA in cytoplasm (Tsushida and Murai 1987, Narayan and Nair 1990), whereas the latter catalyzes reversible conversion of GABA to succinic aldehyde, which can be further metabolized to succinic acid through succinic semialdehyde dehydrogenase in mitochondrion (Shelp et al. 1999, Michaeli and Fromm 2015). Our data
indicate levels of glutamic acid continuously decreased with concomitant increase in GABA from 0.5- to 10.5-h freezing (Table S1), suggesting increase in GAD activity with the increasing duration of freezing. How/why GABA progressively accumulated with increased freezing duration is not clear. However, it may be associated with cytoplasmic acidification, one of the potential consequences of freeze-thaw injury (Arora 2018). Lowering of cytosolic pH in freeze-thaw injured tissues can occur due to inhibition of plasma membrane H⁺-ATPase (Arora and Palta 1991) and/or demixing of vacuolar contents due to injured tonoplast (Yoshida et al. 1979a, 1979b). Increase in cytosolic H⁺ has been reported to precede GABA accumulation and has been explained by GAD’s H⁺-consuming property (Carroll et al. 1994, Crawford et al. 1994).

Alternatively, cytosolic acidification may occur via accumulation of lactic acid (Davies et al. 1974, Perata and Alpi 1993). As per our data, lactic acid progressively accumulated with increasing duration of freezing (Fig. 4A), an indirect indication of tissues experiencing anaerobic conditions. Freeze-thaw results in water-soaking of tissues, a condition promotive of hypoxia/anoxia. Our earlier study noted water-soaking of spinach leaves to progressively increase with freezing-duration (Min et al. 2014). Data in the present study indicated levels of TCA intermediates (citric acid, succinic acid, and malic acid) peaked at 3.0 h, followed by decrease at 5.5 h and 10.5 h (Fig. 4B). Decrease in succinic acid around LD50 along with continued GABA accumulation points to possible mitochondrial dysfunction, reinforcing anaerobic respiration scenario. This is so because GABA is catabolized into succinic acid via GABA-T in mitochondria. Supposed inhibition of GABA-T is further indicated by decreasing levels (after 3 h) of alanine (Table S1), a byproduct of GABA-T activity (Shelp et al. 1999, Michaeli and Fromm 2015). Hypoxic condition in severely water-soaked tissues (after prolonged freezing) and mitochondrial dysfunction
would also result in lack of ATP needed for H\(^+\)-ATPases at both plasma membrane and tonoplast. Anoxia has been shown to cause severe alterations in mitochondrial ultrastructure (Vartapetian and Andreeva 1986).

Above discussion leads to our hypothesis that tissues frozen for shorter duration (0.5 and 3.0 h) are able to perform aerobic respiration during post-thaw whereas those severely injured after prolonged freezing (5.5 and 10.5 h) predominantly undergo anaerobic respiration. Furthermore, accumulation of GABA with increasing freezing duration, i.e., increasing injury, may be a component of cellular pH-stat against cytoplasmic acidification, a condition detrimental to cellular health (Perata and Alpi 1993). And since tissues exposed to \(\geq 5.5\) h of freezing were irreversible injured, remedial role of GABA accumulation against cytoplasmic acidification has certain limit.

The only TCA intermediate with continuous lower abundance in injured tissues relative to UFC was \(\alpha\)-ketoglutaric acid (Fig. 4B). This may be an evidence for its continuous consumption for synthesis of glutamic acid (Forde and Lea 2007), a substrate for GABA, which continues to accumulate with increasing injury/prolonged freezing.

Maltose accumulates in chloroplast when plant (Arabidopsis) tissues are exposed to cold and has been suggested to protect stromal proteins against cold stress (Kaplan and Guy 2004). In the present study, maltose progressively accumulated with increasing duration of freezing whereby irreversibly injured tissues (5.5- and 10.5-h freezing) had higher maltose than those reversibly injured (0.5- and 3.0-h freezing) (Fig. 4A). Its continued accumulation in severely injured tissues can be attributed to one of two possibilities: 1) that starch breakdown in chloroplast is higher in irreversibly injured cells, or 2) that maltose is increasingly less consumed with increasing injury, and thus accumulates.
### Injury-related metabolites (2) – *Fatty acids, polycosanols*

Three fatty acids (Fig. 4C) and two types of polycosanols (Fig. 4D) were also selected as ‘injury-related’ metabolites. They progressively increased with incremental injury, i.e. increasing duration of freezing, except for alpha-linolenic acid which transiently decreased at 0.5-h freezing duration but continually increased thereafter. Other than 1-tetracosanol, these selected metabolites were statistically significant on PC2 separating the duration treatments between relatively less injured (i.e. 0.5 and 3.0 h) vs severely injured (5.5 and 10.5 h) tissues (Table 1 and Fig. 2).

Three fatty acids (i.e. palmitic acid, stearic acid, and α-linolenic acid) continuously increased in response to prolonged freezing, suggesting incremental lipid degradation with increasing freezing duration although α-linolenic acid transiently declined at 0.5-h freezing (Fig. 4C). Freeze-thaw stress causes accumulation of free radical species (Kendall and McKersie 1989, Chen and Arora 2014, Min et al. 2014) that can be injurious to cellular components, including membrane lipids (Mittler 2002). Indeed, Kendall et al. (1985) noted a 13-fold increase in the free fatty acid: phospholipid ratio in the membranes of wheat crowns after freeze-thaw. We had previously showed prolonged freezing to cause accumulation of reactive oxygen species (ROS) with concomitant increase in membrane injury (ion-leakage) and lipid peroxidation (Min et al. 2014).

Two types of polycosanol, i.e., a mixture of long-chain (C<sub>20</sub>-C<sub>30</sub>) aliphatic primary alcohol, were identified in the present study including 1-tetracosanol (C<sub>24</sub>) and 1-hexacosanol (C<sub>26</sub>), and their levels progressively increased with increasing duration of freezing (Fig. 4D). Polycosanol is a component of waxes in spinach, rice, sugar cane, and beeswax (Irmak et al. 2006, Asikin et al. 2012, Ishaka et al. 2014), suggesting an increased degradation of epicuticular wax with increasing freezing duration. While epicuticular waxes can protect
from frost damage by impeding ice propagation into subtending plant tissues (Jenks and Ashworth 1999), no direct evidence exists, to the best of our knowledge, for whether or how freeze-thaw causes degradation of epicuticular wax.

**Recovery-related metabolites - Non-enzymatic antioxidants (ascorbic acid, α-tocopherol)**

Metabolites known to have a role in remediation of injury responses, such as scavenging of free radicals, as well as accumulating after short-term freezing but decrease after the LD$_{50}$ were categorized as ‘recovery-related metabolites’. Two metabolites, ascorbic acid and α-tocopherol (Fig. 5), fit the first criteria. However, while they both increase after short-term freezing duration as expected, they begin to decrease just around the LD$_{50}$, instead of after LD$_{50}$ in accordance with the second criterion. It is important to note, however, that we do not have any duration treatments between 3.0 and 5.5 h in this study. Therefore, it is difficult to ascertain if these metabolites decreased indeed right around or possibly after LD$_{50}$. Higher resolution of freezing durations, especially between 3.0 and 4.0 h in future studies, could resolve this question.

These two metabolites were statistically significant on PC2 separating duration treatments between recoverable (i.e. 0.5 and 3.0 h) vs irrecoverable (5.5 and 10.5 h) tissues (Fig. 2 and Table 1) from freeze-thaw injury.

As a reminder, tissues frozen for 0.5 and 3.0 h were recoverable from freeze-thaw stress whereas those frozen for 5.5 and 10.5 h were irreversibly injured. It has been reported that post-thaw recovery in spinach leaves is marked by a bolstered antioxidant system and was evidenced by upregulation of antioxidant enzymes’ activity and scavenging of free radicals that had accumulated right after freeze-thaw (Chen and Arora 2014). Data from the
present study indicates accumulation of non-enzymatic antioxidants, i.e., ascorbic acid and α-tocopherol, in tissues frozen up to 3.0 h, which could contribute to the recovery in these moderately injured tissues. In contrast, these antioxidants’ abundance decreased in tissues frozen for ≥5.5 h that were unable to recover (Fig. 5).

Conclusions

Based on our previous discussion, a hypothetical model is suggested to illustrate cellular events in spinach leaves that are exposed to a short versus prolonged freezing during a freeze-thaw cycle (Fig. 6).

- SA signaling. SA as a signaling molecule may be involved in inducing tolerance/defense mechanisms against cellular lesions in recoverable tissues (0.5- and 3.0-h freezing); one such defense can be against potential pathogen-infection during post-thaw. Whereas in irrecoverable tissues, especially frozen for 10.5 h, SA accumulation could be involved in PCD.

- GABA metabolism. Increasing duration of freezing may cause cytoplasmic acidification due to: 1) inhibition of plasma membrane H⁺-ATPase; 2) demixing of vacuolar contents due to tonoplast injury; 3) accumulation of lactic acid due to excessive water-soaked tissues experiencing anaerobic conditions; the latter leading into compromised TCA cycle and lack of ATP.

- Differential sensitivity of mitochondria vs chloroplast to a prolonged freezing. TCA intermediates, and ascorbic acid, whose synthesis requires a mitochondrial enzyme (Bartoli et al. 2000), decrease right around LD₅₀ while SA and maltose, produced in chloroplast, accumulate even up to 10.5 h. This seems to suggest mitochondria to be more sensitive to a
prolonged freezing than chloroplast whereby an altered milieu of severely injured cells is relatively more injurious to mitochondria. This notion warrants further in-depth testing since SA and maltose accumulation could also arise from their decreased consumption.

- **Lipid breakdown.** Free fatty acids and policosanols accumulate with an increasing duration of freezing, indicating progressively greater injury to membrane lipids and epicuticular waxes.

- **Antioxidants.** Ascorbic acid and α–tocopherol accumulate in tissues subjected to shorter freezing duration (0.5 and 3.0 h), supposedly to scavenge free radicals and facilitate recovery. Their levels decline in tissues exposed to longer freezing (5.5 and 10.5 h) that are irreversibly injured.

**Author Contributions**

R.A. and K.M. jointly conceived the idea and designed experiments. K.M. performed the experiments and analyzed the data with K.C. assistance. K.M. and R.A. jointly wrote the paper. R.A. provided all financial support for this research.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Pair-wise comparisons of differences in mean concentration of thirty-nine metabolite across five different ‘duration of freezing’ treatments, i.e. 0 (UFC), 0.5, 3.0, 5.5, and 10.5 h. Metabolites are clustered into 5 groups (i.e. amino acids, carbohydrates, lipid components, TCA intermediates, and others).

Table S2. Rankings and loading values of 39 metabolites as analyzed by principal component analysis (PCA).

Supplementary Method. The R script (converted to text file) used for permutation tests and p-values to determine the significance of metabolite contribution to PC1 and PC2.
Fig. 1. (A) Percent injury in freeze-thaw stressed leaves of spinach (*Spinacia oleracea* L. Bloomsdale) exposed to four different duration of freezing (0.5, 3.0, 5.5, and 10.5 h) at -4.5°C; 0 h represents unfrozen control (UFC). Tissues frozen for 0.5 and 3.0 h were ‘recoverable’ from freeze-thaw stress after 6-d post-thaw recovery period, whereas those frozen for 5.5 and 10.5 h were ‘irrecoverable’; the interpretation for “recoverable” or “irrecoverable” injury was derived from our previous study (Min et al. 2014). Means and standard errors were obtained from the pooled percent injury data across three independent ‘duration’ tests, each with five replications per duration treatment. Different letters indicate significant differences between treatments at p < 0.05, according to honest significant different (HSD) test. (B) Response curve (Gompertz function) for freeze-thaw injury as a function of ‘freezing durations’; LD₅₀ (i.e. ~3.1 h) corresponds to the mid-point injury (~36%), between the minimum (~1.2%) and maximum (~68.3%) injury, and is defined as the duration causing 50% injury at -4.5°C freezing. Each dot represents 15 individual points, i.e. three biological replications, each with five technical replications.
Fig. 2. Metabolic phenotype clustering via principal component analysis (PCA) of log_{10}-scaled 39 metabolite data for 15 samples originating from 0 h/UFC and four freezing durations, i.e. 0.5, 3.0, 5.5, and 10.5 h; each treatment with 3 replications. Principal component 1 (PC1) differentiates UFC vs freeze-thaw stressed. Principal component 2 (PC2) differentiates responses to 4 durations of freezing, i.e. 0.5 and 3.0 h (reversible injury) vs 5.5 and 10.5 h (irreversible injury).
Fig. 3. Log₂-mean concentrations and standard errors of ‘signaling-related metabolites’ in response to four durations of freezing (0.5, 3.0, 5.5, and 10.5 h) at -4.5°C; 0 h represents unfrozen control (UFC). A dashed vertical line represents LD₅₀, the ‘duration threshold’ (~3.1 h) that separates reversible from irreversible injury. SA, salicylic acid. Some standard errors are too small to be visible.
Fig. 4. Log$_2$-mean concentrations and standard errors of ‘injury-related metabolites’ in response to four durations of freezing (0.5-, 3.0-, 5.5-, and 10.5-h) at -4.5 °C; 0-h represents unfrozen control (UFC). A dashed vertical line represents LD$_{50}$, the ‘duration threshold’ (~3.1-h) that separates reversible from irreversible injury. Some standard errors are too small to be visible.
Fig. 5. Log$_2$-mean concentrations and standard errors of ‘recovery-related metabolites’ in response to four durations of freezing (0.5, 3.0, 5.5, and 10.5 h) at -4.5°C; 0 h represents unfrozen control (UFC). A dashed vertical line represents LD$_{50}$, the ‘duration threshold’ (~3.1 h) that separates reversible from irreversible injury. Some standard errors are too small to visible.
Fig. 6. A proposed model illustrating cellular events in freeze-thaw injured tissues after a short (0.5 h to 3.0 h) vs prolonged freezing (5.5 h to 10.5 h) at an identical temperature (−4.5°C). Small vs large size of the symbols, +/- and represent lower vs higher ion-leakage and water soaking, respectively. LD₅₀ is the freezing duration threshold causing 50% injury beyond which tissues are irreversibly injured. PM, plasma membrane; CW, cell wall; SA, salicylic acid; ROS, reactive oxygen species; FAs, fatty acids; PCs, policosanol; PCD, programmed cell death; GAD, glutamate decarboxylase.
Table S1. Pair-wise comparisons of differences in mean concentration of thirty-nine metabolites across five different ‘duration of freezing’ treatments, i.e. 0 (UFC), 0.5, 3.0, 5.5, and 10.5-h. Metabolites are clustered into 5 groups (i.e. amino acids, carbohydrates, lipid components, TCA intermediates, and others).

<table>
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<th>Metabolite</th>
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<th>5.5-h</th>
<th>10.5-h</th>
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Different letters indicate significant differences between treatments at p < 0.05, according to honest significant differences (HSD) test.
Table S2. Rankings and loading values of 39 metabolites as analyzed by principal component analysis (PCA).

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<th>PC1 (56.5 %)</th>
<th>Metabolites (n=39)</th>
<th>PC2 (22.1 %)</th>
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CHAPTER 5. GENERAL CONCLUSIONS

Chapters 2, 3 and 4 represent published work. In this chapter, I summarize main findings from the entire dissertation.

In the first study, effect of exogenous SA on FT was determined using micro-centrifuge tube-based excised leaf system to supply SA to spinach leaves (Chapter 2).

- This SA-application method was effective since treated leaves had higher SA content than controls. Four-day application of SA did not affect leaf growth.
- SA treatment significantly improved leaf-FT.
- SA-induced FT is associated with accumulation of compatible solutes (proline) and antioxidants (ascorbic acid).
- SA-induced FT appears to be mediated cooperatively by NO and H$_2$O$_2$ signaling.

Next, experiments were designed to study the effect of SA-feeding (via sub-fertigation) on FT of ‘whole-plant’/seedlings along with comparative analysis of leaf metabolite profiles of SA-fed and control tissues. Experiments also included seedlings treated with SA at both the ambient (non-acclimated; NA) and cold acclimated (CA) conditions, i.e. NASA and CASA, respectively (Chapter 3).

- Sub-fertigation was effective to supply SA since treated seedlings had higher SA content than controls.
- CASA-leaves were most freeze-tolerant followed by CA-, NASA-, and NA-leaves, whereas CASA-leaves had the highest SA content followed by NASA-, CA-, and
NA-leaves. This suggests that 1) SA and cold have an additive effect on FT, and 2) tissue SA content may not be a strict quantitative indicator of relative FT.

- Principal component analysis (PCA) revealed four distinct metabolite phenotypes for NA, NASA, CA, and CASA treatments.

- Greater FT of NASA and CASA than NA and CA controls, respectively, was paralleled by a relatively higher amounts of leaf compatible solutes (trehalose and proline) and antioxidants (ascorbic acid and tocopherol). Moreover, CASA leaves had higher abundance of several osmolytes, i.e. sucrose, fructose, xylose, mannose, glucose, talose, threitol, maltose, myo-inositol.

- SA-fed tissues had reduced aconitic acid level, presumably facilitating the accumulation of antioxidant ascorbic acid.

Based on the data obtained in chapter 2 and 3, it is proposed that exogenous SA application could be an important strategy to improve plant FT. SA-feeding, presumably, primes a plant to trigger protection responses when a freezing stress is encountered. Our research suggests enhanced antioxidant systems and accumulation of compatible solutes (osmolytes) as being two of these protection responses.

Plant tissues exposed to short or prolonged freezing to a given sub-freezing temperature are expected to experience similar freeze-desiccation. However, plants undergo substantially greater (or irreversible) injury following a prolonged freezing (5.5 and 10.5 h) compared to shorter freezing-duration (0.5 and 3.0 h) that results in moderate (recoverable) injury (Chapter 4). Some of the key findings from this study are:
\begin{itemize}
  \item The LD$_{50}$, the freezing duration causing 50 \% injury, was estimated to be $\sim$3.1 h and defined as the threshold beyond which spinach leaves are irreversibly injured.
  \item PCA clearly separated duration treatments depending upon uninjured- \textit{versus} injured and recoverable- \textit{versus} irrecoverable-tissues.
  \item SA may induce tolerance/recovery response in tissues exposed to short-term freezing whereas trigger programmed cell death in irreversibly injured tissues after prolonged freezing.
  \item GABA accumulation in freeze-thaw stressed tissues serves as a ‘pH-stat’ against cytoplasmic acidification in tissues exposed to short-term freezing.
  \item Chloroplast functions may be less sensitive to prolonged freezing than mitochondria.
  \item Increased accumulation of fatty acids and policosanols with increasing freezing duration indicates incremental injury to membrane lipids and epicuticular waxes, respectively.
  \item Ascorbic acid and $\alpha$–tocopherol accumulation during short-term freezing may facilitate recovery by removal of free radicals.
\end{itemize}

Metabolite data from \textbf{chapter 4} provided insight into several cellular events/alterations potentially associated with reversible \textit{versus} irreversible injury following a short-term or prolonged freezing, respectively, and thereby advance our fundamental understanding of plant response to freezing. This study also has two other impacts: (1) discrepancies among laboratories for reported LT$_{50}$ or \% injury for the same species/tissue could be attributable, in part, to the differences in ‘freezing durations’ used in freezing tests. Moreover, the extent of injury as well as an ability of post-thaw recovery of freeze-injured
tissue could also be influenced by the duration of freezing; 2) on the practical level, this information may be useful to predict what to expect or when to protect plants under a given frost forecast. For instance, a tissue with an LT50 value of $-T^\circ C$ may conventionally be expected to escape / suffer less injury if the coldest temperature overnight was no colder than $-T^\circ C$. However, this tissue may in fact get severely damaged, even irreversibly, if the duration of freezing was longer than LD50.
APPENDIX A. SPINACH SOWING

1. Seeds of spinach (*Spinacia oleracea* L.) were sown in a plug tray containing Sunshine LC-1 mix (Seba Beach, Alberta, Canada).

2. Mix was sufficiently watered using a watering can before seeds were sown.

3. Two seeds per cell were placed on the top of mix using a pincer.

4. Spread a thin layer of mix to cover the seeds.

5. Plug tray were then transferred to a growth chamber at 15/15°C for day and night with 12-h photoperiod (PAR ~300 µmol m⁻² s⁻¹ at plant height).

*Note: seeds were generally germinated 5 or 6 days after sowing.

6. Two weeks after sowing, seedlings were sub-fertigated using 300 ppm EXCEL nutrient solution. While sub-fertigated, extra seedling was thinned out, allowing one seedlings to be grown per cell (you planted 2 seeds on each cell).

7. Chamber temperature was elevated to 20/18 °C for day and night with 12-h photoperiod (PAR ~300 µmol m⁻² s⁻¹ at plant height).

8. About 22-24-day old spinach seedlings were used for research.

*300 ppm nutrient composition

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<tr>
<th>Concentration (ppm)</th>
<th>Mg</th>
<th>Ca</th>
<th>Inject Ratio</th>
<th>Electrical Conductivity (EC)**</th>
<th>mS/cm/cm</th>
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<tr>
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<td>2.25</td>
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*Approximate Gallons Required to Dissolve One 25 lb. Bag of 15-5-15

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APPENDIX B. EXOGENEOUS APPLICATION OF SALICYLIC ACID
(SUB-FERTIGATION)

Materials:

- 0.5 mM SA: SA (0.06906g / L) was completely dissolved in 300 ppm EXCEL nutrient solution.

Procedures:

1. Seeds of spinach (*Spinacia oleracea* L.) were sown in a plug tray containing Sunshine LC-1 mix (Seba Beach, Alberta, Canada).

2. Mix was sufficiently watered using a watering can before seeds were sown.

3. Two seeds per cell were placed on the top of mix using a pincer.

4. Spread a thin layer of mix to cover the seeds.

5. Plug tray were then transferred to a growth chamber at 15/15°C for day and night with 12-h photoperiod (PAR ~300 µmol m⁻² s⁻¹ at plant height).

6. Two weeks from sowing, temperature in a chamber was elevated to 20/18 °C for day and night.

7. A plug tray containing seedlings was sub-fertigated with 300 ppm EXCEL nutrient solution – Fertilizer control, and another same plug tray was sub-fertigated with 0.5 mM SA – SA treatment; each treatment was applied for ~15 min.

8. While sub-fertigated, extra seedling was thinned out, allowing one seedlings to be grown per cell (you planted 2 seeds on each cell)

9. About 22-24-day old spinach seedlings were used for research.
APPENDIX C. WHOLE-PLANT FREEZING PROTOCOL
(A CASE STUDY IN SPINACH PLANT)

Materials:

- Freezing chamber (Percival, E-41L1LT, Percival Scientific, Inc., Perry, IA, USA)
- Copper-constantan thermocouple (Omega DP 465, Omega Engineering, Inc., Stamford, CT, USA)
- Pre-chilled (0 °C) distilled de-ionized water (ddH₂O)

Procedures:

1. Temperature of freezing chamber was preset to 0 °C (1-h before experiment).
2. Plug trays containing plants were placed in a freezing chamber (0 °C) for 2-h.
3. After 2-h, temperature of freezing chamber is lowered to -2 °C (-1 °C h⁻¹).
4. After 1-h at -2 °C, plants were ice-nucleated by quickly spraying the pre-chilled ddH₂O (0 °C) onto leaves, and kept at -2 °C for another 1-h.
5. After 1-h at -2 °C, the temperature of freezing chamber was lowered 0.5 °C/30 min to a desired temperature.
6. After freezing at a desired temperature for 30 min, plants were allowed to be thaw overnight by increasing temperature of freezing chamber to 0 °C.
7. Next morning, temperature of freezing chamber was increased to 5 °C and kept at this temperature for 2-h.

*Note: Entire freezing and thawing was performed in dark and the temperature of leaf surface, air, and growing media (~2.5 cm deep) was monitored using copper-constantan thermocouple (Fig. 1).
8. After 2-h at 5 °C, plants were transferred to room temperatures (~ 20 °C) under dim-light (~15 μmol m⁻² s⁻¹, cool white fluorescent) for 1-d. During this time, plants were
periodically but lightly misted with deionized water to avoid unintended drying.

9. Unfrozen control (UFC) plants were kept at 0 °C in dark throughout this time in another but identical chamber.

10. After 1-d at room temperature, freeze-thaw injury to seedlings was then assessed visually and photographed. Additional estimation of freeze-injury / tolerance of plants was measured via ion-leakage assay on leaves excised from plants that were subjected to whole-plant freezing.

**Fig. 1.** Representative picture of whole-plant freezing test as conducted in a freezing chamber. Air, soil, and leaf temperatures were monitored during freeze-thaw cycle using a thermocouple.
APPENDIX D. DETERMINE FREEZING TOLERANCE (i.e. LT₅₀) IN SPINACH TRUE LEAVES

Materials:

1. Deionized distilled water (ddH₂O)
2. Glycol bath (Isotemp 3028: Fisher Scientific, Pittsburgh, PA)
3. Conductivity meter (model 3100; YSI Inc., Yellow Spring, OH)

Procedures:

Day 1

1. A pair of petiolated spinach leaves was placed, standing up, in a 2.5 x 20 cm test tube containing 150 μl of ddH₂O. Each temperature treatment contained 5 technical replications (one seedling per tube).
2. Cover all tubes except the unfrozen control (UFC) with metal bolts. The UFC was covered with plastic caps.
3. Transfer all tubes (other than UFC) into the temperature-controlled glycol bath at 0 °C. The UFC was kept at 0 °C throughout the freeze-thaw cycle.
4. Equilibrate the tissue temperature (~20 °C) with bath temperature (0 °C) for 1-h.
5. Change the bath temperature to -1 °C.
6. Equilibrate the tissue temperature (0 °C) with bath temperature (-1 °C) for 1-h.
7. Initiate ice-nucleation by dropping ice crystals in each test tube at -1 °C.
8. Equilibrate the tissue temperature with bath temperature at -1 °C for another 1-h.
9. Cool the tissues to various sub-freezing treatment temperatures at slow cooling rates (0.5 °C / 30 min down to -6 °C, and 1°C / 30 min from -6 to -10 °C).
10. Take out the tubes from glycol bath at various test temperatures, and let the tissues thaw in ice overnight.

**Day 2**

1. Take out all tubes (including UFC) from the ice.

2. Gradual thaw continued by incubating samples at 4 °C for 1-h, followed by 1-h incubation at room temperature (~20 °C).

3. Add 20 ml ddH₂O to each tube.

4. Vacuum-infiltreate the samples for 3 times (3 min each) at ~100 kPa.

5. Shake the samples at 250 rpm for 1.5-h.

6. Determine the initial ion-leakage for each sample with a conductivity meter.

7. Autoclave the samples at 120 °C for 20 min.

8. Cool the samples to the room temperature (~20 °C).

9. Determine the final ion-leakage.

**Data analysis**

- Process the raw data

  Following formulas were employed to calculate the adjusted percent injury

  - Ratio (R) (%) = initial leakage/Final leakage x 100
  - \( R_{ave,cont} \) = the average of R. control
  - Percent Injury (PI) (%) = (R - \( R_{ave,cont} \)) / (100 - \( R_{ave,cont} \)) x 100
  - \( PI_{ave,max} \) (%) the average of PI at the lowest temperature.
  - Adjusted percent injury (API) = PI / \( PI_{ave,max} \) x 100
- Define API to be 100 % when they are ≥ 100 %
- Define API to be 0 % when they are ≤ 0 %

- Calculate the average and standard error for API under each temperature treatment.
- Calculate LT₅₀ with SigmaPlot 10 (Systat Software Inc., San Jose, CA, USA)
  - Open SigmaPlot 10 > select ‘New’ > import the APIs as well as standard errors under individual temperatures, as shown in table below.

<table>
<thead>
<tr>
<th>T (0°C)</th>
<th>API (%)</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1481</td>
<td>0.0834</td>
</tr>
<tr>
<td>-1</td>
<td>1.6130</td>
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<td>5.5742</td>
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<td>-6</td>
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<tr>
<td>-10</td>
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<td>0.2080</td>
</tr>
</tbody>
</table>

- Highlight the table > ‘Create Graph’ tab > select ‘Scatter’ chart > choose ‘Create a simple vertical bar chart with error bars’. A chart would be created as shown below:
Click the X-axis to change the scale > define the start value to be 1, and end value to be -10. The bar chart would be changed accordingly as shown below:

Right-click the bars > select ‘Curve fit’ > select ‘Gompertz, 4 parameter’ from the drop-down menu of ‘Equation name’ > click ‘Finish’. A 4-parameter Gompertz curve would be created to fit the data, as shown below:
- Go to ‘Tool’ tab > click ‘Draw line’ button > Draw a first line starting from ‘50’ in Y-axis and parallel to X-axis, and draw a second line parallel to Y-axis and starting from the crossing point between the first line and Compertz curve > calculate the LT$_{50}$. As shown in the figure below, the LT$_{50}$ for the example dataset is -5.8 °C.
APPENDIX E. EXTRACTION FOR NON-TARGETED METABOLITE PROFILING

*Note: it is very important to have a black extraction along with the real extraction.

**Reagents:**

- Ribitol (1 mg/ml)
- Non-adecanoic acid (1 mg/ml)
- Methanol
- Chloroform
- Methoxyamine hydrochloride in dry pyridine (20 mg/ml)
- Bis-trimethyl silyl trifluoroacetamide with 1% trimethylchlorosilane

**Procedures:**

*Day 1*

1. Take 20-40 mg plant samples (i.e. ground with LN₂ to powder) into 1.5 ml of Eppendorf tube and add following internal standards: ribitol (25 μl) and non-adecanoic acid (20 μl).

2. Add 0.35 ml of hot methanol (60 °C) and immediately incubated at 60 °C for 10 min followed by vortex for 10 sec.

3. Put samples into a sonication water bath for 10 min at 40 kHz.

4. Add 0.35 ml of chloroform and vortex for 30 sec., and add 0.3 ml of HPLC water, followed by vortex for 30 sec.

5. After centrifugation (10,000 g) for 10 min, take out 200 μl of upper layer (polar fraction) and 200 μl of lower layer (non-polar fraction) and add them to GC-MS vials.

6. Dry samples in a Savant speed-vac concentrator (Savant instrument Inc.) for 10-h.
Day 2

1. For methoximation (to protect carbonyl groups in sugars), add 50 μl of 20 mg/ml methoxyamine hydrochloride in dry pyridine to dried polar extract and incubate at 30 °C for 1.5-h with continuous shaking.

2. Trimethylsilylation (TMS) is conducted by the addition of 70 μl of bis-trimethyl silyl trifluoroacetamide with 1% Trimethylchlorosilane (BSTFA+1% TMCS) for 30 min at 37 °C.

*Note: TMS needs to perform on both non-polar and polar fraction.

3. Samples were analyzed using an Agilent 7890A gas chromatography (Agilent Technologies) equipped with HP5MS separation column.
APPENDIX F. AMDIS GC-MS DATA ANALYSIS WORKFLOW

Materials:

- GC-MS data files
- Automated Mass Spectral Deconvolution & Identification System (AMDIS) software
- National Institute of Standards and Technology (NIST) or other GC-MS libraries
- Hydrocarbon standards library and blank library file
- Align3 macro 2003 excel sheet

General introduction:

Open AMDIS

Open Data in Window:

- Select File (Experimental sample)
- Select open
- Find sample

For 2 different samples in same window:

- After first sample is open (see above)
- Select File (control sample)
- Select open in ➔ Active window
- Find Sample

Open data in a new window:

- Select File
- Select Open in New window
- Find Sample
If wanted peaks are not detected:

- Go to “analyze -> settings -> Deconv.”
- For narrower peaks use a lower component width (wider higher)
- Adjacent peak subtraction can be 1 or 2 depending on the number of overlapping peaks observed
- Higher resolution and sensitivity will allow for more and smaller peaks to be detected
- Lower shape requirements will allow for more and smaller peaks to be detected

View spectra of a specific peak:

- After running an analysis of a data file click the triangles above the peaks
  To search the NIST library
- right click in the spectra window -> NIST -> go to NIST MS program

Find one fragment in the data file:

- Select Options
- Select m/z
- Fill in the mass of the desired compound
- Creating a retention index calibration file:
- Load the hydrocarbon data file

**Data Analysis:**

- Select Analyze menu
- Select Analyze GC-MS Data…
- Set Analysis type to **RI calibration/performance**
- Load the a hydrocarbon .csl as the Calib./Std. Lib.
  - Click select new find new .csl file
- Load name a new .cal file in the RI Calib. Data
  - Click “select new” and find the new .cal file
- Hit “Save”
- Hit “run”
- A warning will appear to check .csl and .cal. Verify they are correct and click “Ok”
- Adjust settings and or the library until all standard peaks are targeted with a T shown above and are in order from as low as 1000 up to 4000 by 200’s, your sample may not have them all.
  - To adjust the library go to the library menu and select “File”—>
    “build one library” and load the .csl you are using for your retention index calibration
    - You have to change the file type from .msl to .csl
  - click on the triangles above the peaks you want to use and search the NIST library to confirm their identity
  - With the triangle of a known peak selected, hit **add** in the “build library” window. Edit the compound information to have the correct retention index and name for that hydrocarbon peak. Finally, Click “save”. A box will appear titled RI Calibration, ensure that “Use for RI calibration” is checked.

- Continue until all the hydrocarbon peaks in your file are identified when you click Run
• View of analysis settings for generating a .cal file:

![Image of analysis settings](image1)

![Image of recommended settings](image2)

**Data Analysis**

- Go to “Analyze” and click analyze GC/MS Data
- Select Analyze type “use retention index” and load the correct .cal and the empty .msl file:
• Open an experimental data file (pick a good one)
  o Adjust the Decanv.
• Settings until a reasonable number of peaks are detected

**Building a custom library:**

• Target
  o If it is in the library a T will show up above the peak’s triangle.
• If not in the library
  o Use NIST library to identify and help add to own Library
    ▪ Can be used for known and unknown compounds
    ▪ Go to “Analyze” -> “search NIST library”
  o Best to add to Library one by one.
  o Use these settings and click “Analyze”: 
- The NIST results will show up as Ts above the peaks
- To add a good NIST library match to a new library: Got to “Library” > build one library (and with the name you want selected) click add. Select the extracted spectra to add to the library.
- Only add a compound to the library if it is a good spectral match and has a reasonable retention index value compared to the NIST (less than 100 units off, check probability, and compare chromatograms)
- Once the library has at least the top 5 compounds add to it, load a new data file and hit run. Make sure the peaks are identified correctly in the second sample.

**Generate report:**
- Select an example data file
- Make sure your settings are detecting and identifying the peaks
- Click run, then got to file generate report
  - Find the desired file
    - Save as a new file “batch1”
    - Select the next file
    - Click run each time a new file is loaded followed by file->generate report (this saves the analysis). Repeat until all wanted data files are saved in a single batch file.
• Will be saved as a text file
  ▪ Need to open in Excel
  ▪ Find and replace all question marks in the data
  ▪ Save as 97-2003 worksheet
  ▪ Open align3 sheet and run alignment on the batch file.
  ▪ Done.