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The Use of High-Resolution Infrared Thermography (HRIT) for the Study of Ice Nucleation and Ice Propagation in Plants

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Abstract:	Freezing events that occur when plants are actively growing can be a lethal event, particularly if the plant has no freezing tolerance. Such frost events often have devastating effects on agricultural production and can also play an important role in shaping community structure in natural populations of plants, especially in alpine, sub-arctic, and arctic ecosystems. Therefore, a better understanding of the freezing process in plants can play an important role in the development of methods of frost protection and understanding mechanisms of freeze avoidance. Here, we describe a protocol to visualize the freezing process in plants using high-resolution infrared thermography (HRIT). The use of this technology allows one to determine the primary sites of ice formation in plants, how ice propagates, and the presence of ice barriers. Furthermore, it allows one to examine the role of extrinsic and intrinsic nucleators in determining the temperature at which plants freeze and evaluate the ability of various compounds to either affect the freezing process or increase freezing tolerance. The use of HRIT allows one to visualize the many adaptations that have evolved in plants, which directly or indirectly impact the freezing process and ultimately enables plants to survive frost events.
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Dear Dr. Prather,

I would like to take this opportunity to submit the second revision of our manuscript entitled, "**The Use of High-Resolution Infrared Thermography (HRIT) for the Study of Ice Nucleation and Ice Propagation in Plants."** I have addressed all the comments and concerns indicated by the reviewers. The changes are visible in the revised document as all changes were made using "Track Changes." I have also uploaded a response to each comment.

I trust that you will find everything in suitable order. Thank you for the invitation to publish this protocol.

Sincerely,

Michael Wisniewski

TITLE:
The Use of High-Resolution Infrared Thermography (HRIT) for the Study of Ice Nucleation and Ice Propagation in Plants

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KEYWORDS:
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SHORT ABSTRACT:
Here we present a protocol that allows one to visualize sites of ice formation and avenues of ice propagation in plants utilizing high resolution infrared thermography (HRIT).

LONG ABSTRACT:
Freezing events that occur when plants are actively growing can be a lethal event, particularly if the plant has no freezing tolerance. Such frost events often have devastating effects on agricultural production and can also play an important role in shaping community structure in natural populations of plants, especially in alpine, sub-arctic, and arctic ecosystems. Therefore, a better understanding of the freezing process in plants can play an important role in the development of methods of frost protection and understanding mechanisms of freeze avoidance. Here, we describe a protocol to visualize the freezing process in plants using high-resolution infrared thermography (HRIT). The use of this technology allows one to determine the primary sites of ice formation in plants, how ice propagates, and the presence of ice barriers. Furthermore, it allows one to examine the role of extrinsic and intrinsic nucleators in determining the temperature at which plants freeze and evaluate the ability of various

compounds to either affect the freezing process or increase freezing tolerance. The use of HRIT allows one to visualize the many adaptations that have evolved in plants, which directly or indirectly impact the freezing process and ultimately enables plants to survive frost events.

INTRODUCTION:

Freezing temperatures that occur when plants are actively growing can be lethal, particularly if the plant has little or no freezing tolerance. Such frost events often have devastating effects on agricultural production and can also play an important role in shaping community structure in natural populations of plants, especially in alpine, sub-arctic and arctic ecosystems [1-6]. Episodes of severe spring frosts have had major impacts on fruit production in the USA and South America in recent years [7-9] and have been exacerbated by the early onset of warm weather followed by more typical mean low temperatures. The early warm weather induces buds to break, activating the growth of new shoots, leaves, and flowers all of which have very little to no frost tolerance [1, 3, 10-12]. Such erratic weather patterns have been reported to be a direct reflection of ongoing climate change and are expected to be a common weather pattern for the foreseeable future [13]. Efforts to provide economical, effective, and environmentally-friendly management techniques or agrochemicals that can provide increased frost tolerance have had limited success for a host of reasons but this can be partly attributed to the complex nature of freezing tolerance and freezing avoidance mechanisms in plants. [14]

The adaptive mechanisms associated with frost survival in plants have traditionally been divided into two categories, freezing tolerance and freezing avoidance. The former category is associated with biochemical mechanisms regulated by a specific set of genes that allow plants to tolerate the stresses associated with the presence and the dehydrative effect of ice in its tissues. While the latter category is typically, but not solely, associated with structural aspects of a plant that determine if, when, and where ice forms in a plant [14]. Despite the prevalence of freeze avoidance as an adaptive mechanism, little research has been devoted in recent times to understanding the underlying mechanisms and regulation of freeze avoidance. The reader is referred to a recent review [15] for greater details on this subject.

While the formation of ice at low temperatures may seem like a simple process, many factors contribute to determining the temperature at which ice nucleates in plant tissues and how it spreads within the plant. Parameters such as the presence of extrinsic and intrinsic ice nucleators, heterogeneous vs. homogeneous nucleation events, thermal-hysteresis (antifreeze) proteins, the presence of specific sugars and other osmolytes, and a host of structural aspects of the plant can all play a significant role in the freezing process in plants. Collectively, these parameters influence the temperature at which a plant freezes, where ice is initiated and how it grows. They can also affect the morphology of the resulting ice crystals. Various methods have been used to study the freezing process in plants under laboratory conditions, including nuclear magnetic resonance spectroscopy (NMR) [16], magnetic resonance imaging (MRI) [17], cryo-microscopy [18-19], and low-temperature scanning electron microscopy (LTSEM). [20] Freezing of whole plants in laboratory and field settings, however, has mainly been monitored with thermocouples. The use of thermocouples to study freezing is based on the liberation of heat (enthalpy of fusion) when water undergoes a phase transition from a liquid to a solid. Freezing is then recorded as an exothermic event. [21-23] Even though thermocouples are the typical method of choice in studying freezing in plants, their use has many limitations that limit the amount of information obtained

during a freezing event. For instance, with thermocouples it is difficult to nearly impossible to determine where ice is initiated in plants, how it propagates, if it propagates at an even rate, and if some tissues remain free of ice.

Advances in high-resolution infrared thermography (HRIT) [24-27], however, have significantly increased the ability to obtain information about the freezing process in whole plants, especially when used in a differential imaging mode. [28-33] In the present report, we describe the use of this technology to study various aspects of the freezing process and various parameters that affect where and and at what temperature ice is initiated in plants. A protocol will be presented that will demonstrate the ability of the ice-nucleation-active (INA) bacterium, *Pseudomonas syringae* (Cit-7) to act as an extrinsic nucleator initiating freezing in a herbaceous plant at a high, subzero temperature.

High-Resolution Infrared Camera

The protocol and examples documented in this report utilize a high resolution infrared video radiometer. The radiometer (**Figure 1)** supplies a combination of infrared and visible spectrum images and temperature data. The spectral response of the camera is in the range of 7.5 to 13.5 µm and provides 640 X 480 pixel resolution. Visible spectrum images generated by the built-in camera can be fused with IR-images in real time, which facilitates the interpretation of complex, thermal images. A range of lenses for the camera can be used to make close-up and microscopic observations. The camera can be used in a stand-alone mode, or interfaced and controlled with a laptop using propietary software. The software can be used to obtain a variety of thermal data embedded in the recorded videos. It is important to note that a wide variety of infrared radiometers are commercially available. Therefore, it is essential that the researcher discuss their intended application with a knowledgeable product engineer and that the researcher test the ability of any specific radiometer to provide the information needed. The imaging radiometer used in the described protocol is placed in an acrylic box (**Figure 2)** insulated with styrofoam in order to deter exposure to condensation during the warming and cooling protocols. This protection is not needed for all cameras or applications.

PROTOCOL:

1. **Preparation of Plant Materials**

1.1. Use either leaves or whole plants of subject plant material (*Hosta* spp. or *Phaseolus vulgaris*).

2. **Preparation of Water Solutions Containing Ice Nucleation Active (INA) Bacteria**

2.1. Culture the INA bacterium, *Pseudomonas syringae* (Strain Cit-7) in petri dishes at 25 ºC on Pseudomonas Agar F prepared with 10 g/L of 100% glycerol per the manufacturer's direction.

2.2. After cultures have grown sufficiently, place at 4 ºC until needed but keep at 4 ºC for two days prior to ensure a high level of ice nucleation activity.

2.3. Scrape bacteria from a single plate from the surface of the agar with a plastic, disposable or re-usable metal spatula at the time of use and place in 10 – 15 ml of deionized water in a 25 ml disposable cuvette. The concentration should be in the range of 1×10^7 to $1 \times 10^9 \cdot ml^{-1}$. The solution will appear cloudy. There is no need to confirm the concentration using a hemocytometer or spectrophotometer, as concentration need only be approximate.

2.4. Vortex the cuvette for a minimum of 10 seconds to distribute the bacteria.

Note: The specific concentration of the resulting INA mixture is not important and the protocol described will provide more than an adequate level of ice nucleation activity. This mixture of INA bacteria and water will be used later in the nucleation experiments.

3. **Setting Up A Freezing Experiment**

3.1. Place the high resolution infrared camera (SC-660) inside the protective acrylic box so that the lens projects through the opening in the front of the box and the wires connecting the camera to a laptop or recording device exit through the rear opening of the box. Secure the lid of the box and place the box inside the environmental chamber or freezer in a location that will allow the subject plant material to be seen.

3.1.1. Provide a dark background around the plant material by lining the walls of the chamber with black construction paper to prevent interference from reflected infrared energy.

3.1.2. Fit the chamber with LED lighting to minimize heating from the light source when recording images in visible wavelengths is required. Only a minimum of lighting, such as a battery-operated closet light or other small LED device, is required for the plants to be visible by the camera.

3.1.2.1. Once visible images of the subject plant material are taken, turn off the LED lighting. Distribute all external wired connections (firewire connection to computer, power cord, etc.) to the camera via a port or other opening in the chamber.

3.1.3. Fill any extra space in the port or opening with insulating foam material to avoid or reduce temperature gradients within the chamber. Set the initial temperature of the chamber at 1 °C.

3.2. Align plants or plant parts so that the plant material is in the field-of-view of the camera and the plant material is visible on the remote viewing screen or within the chosen software.

3.3. Allow plants to equilibrate at 1 °C for 30 minutes to 1 h, depending on the size of the plant material, prior to initiating a controlled freezing experiment. This ensures that the temperature of the plant will not lag behind air temperature by many degrees once the freezing experiment is initiated. Equilibration is achieved when the temperature of the plant material is within 0.5 °C of air temperature.

3.3.1. Place a layer of Styrofoam insulation on top of the soil of potted plants if potted plants are used. Once the plants have equilibrated, commence cooling of the chamber.

Note: The layer of insulation on the soil surface of the pot reduces the amount of continued heat loss from the pot to the air surrounding the plant, and prevents the roots from freezing, as this would not typically occur during a frost event in nature due to the massive reservoir of residual heat present in the soil.

3.4. Set the desired camera parameters (color palette, temperature range, specific areas of interest, etc.), as discussed in 3.4.1 – 3.4.4.

3.4.1. Select the rainbow palette to display the temperature variations while viewing the live image.

3.4.2. Set the temperature span to 5 °C by adjusting the temperature bar located just below the image in the software.

3.4.3. Choose the linear scale (algorithm) for converting the infrared data into the false color image as defined by the selected palette (rainbow) and set the range of temperature to 5 °C and to track automatically based on the image. Alternatively, adjust the set range manually while conducting the experiment.

3.4.3.1. Use the temperature of a specific point or an average temperature within the defined area of interest provided by the software. Retrieve the temperature data of all pixels from the recorded video sequence or from the information embedded in the image file. **Figure 3** shows a typical screenshot from within ResearchIR software.

3.4.4. Place a cursor on a location on the plant tissue that represents a specific point of interest. Define the area of interest as points (1 -3 pixels in size), boxes, lines, ellipses, or circles. Multiple combinations of points or shapes can be located over the image.

3.5. Recording a video sequence

3.5.1. Set the camera to record at ten frames per second and for the recording to be stopped manually.

3.5.2. Indicate the location on the computer or external drive where the recorded video file will be placed.

3.5.3. Commence recording.

Note: Recording to an external hard drive is highly recommended since large video files will be generated. Recorded video files can be later edited to contain only the portion containing the necessary information. This will greatly reduce the file size.

3.5.4. Lower the temperature of the chamber incrementally by 0.5 – 1.0 °C. Wait until the plant temperature equilibrates with air temperature and then lower the temperature again by 0.5 – 1.0 °C. Depending on the mass of the plant tissue being observed and its morphology, equilibration can take 10 to 15 minutes. Thus, giving a cooling rate of about 4 °C/hr.

3.5.5. Continue in this manner until the plant freezes and observations are completed. End the recording when the freezing process has been completed.

Note: The plant tissue has equilibrated with air temperature when the plant material and background are the same color since they are at the same temperature. Since the background temperature and the temperature of the plant tissue are the same, it may be difficult to visualize the plant material until you again lower the temperature and there is temperature differential between the plant tissue and air temperature.

REPRESENTATIVE RESULTS:

Ice-nucleating activity of the Ice+ bacterium, *Pseudomonas syringae* (strain Cit-7).

A 10 µl drop of water and 10 µl of water containing *P. syringae* (Cit-7) were placed on the abaxial surface of a Hosta leaf (*Hosta* spp.) (**Figure 4)**. As illustrated, the drop of water containing the INA bacteria froze first and was responsible for inducing the leaf to freeze while the drop of water on the leaf surface remained unfrozen.

Freezing and Ice Propagation in a Woody Plant

Figure 5 illustrates both ice initiation and ice propagation in a stem of oak (*Quercus robur*). Ice formation was initiated in the vascular cambium\phloem region of the stem and propagated circumferentially around the stem. The rate of ice propagation in woody plant stems is much greater in a longitudinal direction than in a lateral and circumferential direction.[31]

Rates of Ice Propagation and Barriers to Ice Propagation

Ice was initiated in the stem of a bean plant (*P. vulgaris*) at the site where INA bacteria had been placed (**Figure 6A - arrow)**. Following the initial freezing event, ice propagated up and down the stem (**Figure 6B-C)**. Using the video sequence, which has a time stamp, and measuring distance on the stem, enables one to calculate the rate of ice propagation over a given distance. The graph in **Figure 6** presents the rate of ice propagation up the stem of the bean plant from the point of initial freezing and illustrates a decreased rate of ice propagation as ice passes through the nodal region of the plant. Using infrared thermography also allows one to determine the presence of any physical barriers that prevent the propagation of ice into specific tissues. **Figure 7** illustrates freezing in an alpine species, *Loiseleuria procumbens*, where the vegetative part (stem and leaves) of the plant has frozen but the terminal flower buds remain unfrozen. Ice formation did not occur until 126 -164 minutes after freezing of the stem and leaves had occurred and the resulting exothermic response had dissipated. As reproductive shoots of alpine woody species are freezing sensitive [3, 33], freezing avoidance is crucially important to reproductive success.

Ability of Hydrophobic Barriers to Block Extrinsic Ice Nucleation Induced Freezing

A tomato plant (*Solanum lycopersicum*) was coated with a hydrophobic kaolin-based material (**Figure 8A**) in order to determine if the hydrophobic barrier could block extrinsic ice nucleation

induced freezing. The degree of contact of droplets of liquid with the leaf surface was much greater in uncoated leaves (**Figure 8B**) compared to coated leaves (**Figure 8C**). As illustrated in **Figure 8D**, uncoated plants (right) exhibited an exothermic event typical of a freezing event, while coated plants (left) remained unfrozen and supercooled to approximately -6.0 °C. Details of these experiments can be found in Wisniewski et al. [34] A trend of greater hydrophobicity in the leaf structure of native plant species along an altitudinal gradient has been noted by Aryal and Neuner. [35]

Figure 1. High-resolution Infrared Radiometer. The model illustrated is a FLIR SC-660 Infrared Video Camera.

Figure 2. Protective enclosure for the infrared camera. An acrylic box is used to house the camera and prevent condensation from forming on the infrared camera during freezing and thawing experiments. **A.** Box with top removed. **B**. Camera inserted in the acrylic box and lid closed.

Figure 3. Viewing and Analyzing Infrared Images and Remote Camera Control. Screen shot from the ResearchIR software. The software is used to view the live image, change camera settings, record single images, make video recordings, and analyze temperature data in the images. Insert on right shows options for changing camera settings while insert on lower left shows a temperature histogram of the live image.

Figure 4. Extrinsic nucleation induced freezing of Hosta leaf (*Hosta* spp.). Unfrozen droplets of water and INA bacteria, *Pseudomonas syringae* (strain Cit-7), are present on the abaxial surface of the leaf (A). INA droplet freezes first (B) and initiates freezing of the leaf (C). Ice spreads throughout the leaf (D) and despite the freezing of the leaf, the water droplet remains unfrozen (E). Droplet of water on surface of leaf freezes after the entire leaf has frozen and is beginning to cool at its edges (F).

Figure 5. Ice initiation and propagation in the stem of a woody plant (*Quercus robur*). Left Panel: Cross-section of a woody stem of oak. A – H. Initiation of freezing event in the region of the phloem and vascular cambium (A) and progression of ice formation around the stem (B – H).

Figure 6. Rate of ice propagation in a bean plant (*Phaseolus vulgaris)* calculated using high-resolution infrared thermography. A. Ice initiated in stem (arrow). B – C. Ice propagation up and down the stem. Graph at top of figure displays the rate of ice propagation presented as the distance ice traveled over time as it moved up the stem from the original site of freezing. A delay in ice propagation occurred as ice moved through the nodal portion of the plant stem. This figure was modified from Wisniewski *et al.*[24]

Figure 7. Barrier to ice formation in the alpine woody plant, *Loiseleuria procumbens* (alpine azalea). A. Visible light image of the stem of alpine azalea showing central stem, attached leaves, and terminal buds. B – C. Freezing is initiated in the stem and ice propagates out into leaves. Terminal buds remain unfrozen. D – E. Terminal buds freeze independently 126 - 164 minutes after initial freezing of stem and leaves. During this time the heat of enthalpy produced by the freezing of the stem and leaves has already dissipated.

Figure 8. Hydrophobic barriers block extrinsic nucleation induced freezing of tomato (*Solanum lycopersicon*). A. Hydrophobic kaolin-based material applied to tomato plants. B – C. Reduced level of contact between the leaf surface and drops of liquid containing INA bacteria on coated (B) vs. uncoated (C) leaves. D. Uncoated plant (right) undergoes an exothermic response associated with freezing of the plant while coated plant remains supercooled, and unfrozen at approximately -6 °C. This figure has been modified from Wisniewski et al.[34]

DISCUSSION:

Water has the ability to supercool to temperatures well below 0 °C and the temperature at which water will freeze can be quite variable.[36] The temperature limit for supercooling of pure water is about – 40 °C and is defined as the homogeneous nucleation point. When water freezes at temperatures warmer than – 40 °C it is brought about by the presence of heterogenous nucleators that enable small ice embryos to form which then serve as a catalyst for ice formation and growth.[37] There are a multitude of molecules in nature that act as very efficient ice nucleating agents, thus most freezing of water in nature occurs at temperatures just below 0 °C. The ability to regulate or influence the activity of heterogenous nucleating agents has significant potential as a novel approach to providing frost protection to plants. Understanding how ice forms and propagates in freezing sensitive and freezing-tolerant plants is essential to achieving this objective.

As indicated in the Introduction, various methods have been used to study the freezing process in plants under laboratory conditions, however, freezing of plants in nature has mainly been monitored with the use of thermocouples. High-resolution infrared thermography (HRIT) [24-28, 34], offers several distinct advantages as a method for studying the freezing process in plants. HRIT allows one to observe the initial site of ice formation, the number of freezing events needed to freeze an entire plant, actually observe how ice is propagated in a plant and if any barriers to ice propagation are present, and determine if any portions of a plant remain ice free. Most importantly, it allows one to observe the freezing process in whole plants rather than small, isolated portions of a plant that have been removed from the parent plant.

The present report outlines the application of HRIT to the study of freezing in intact plants or plant parts, and provides several examples of how this technology can be used to examine several parameters that can influence how and when ice forms in plants, and how ice is propagated. Critical aspects of conducting these studies involve the sensitivity and accuracy of the infrared camera, the parameters used in the setup of the camera and the recording of video sequences, the cooling rate, the structural/morphological complexity of the subject being viewed, and knowledge about infrared science. These items will be addressed individually.

Sensitivity and accuracy of the infrared camera (radiometer)

The exothermic events during freezing of plant tissues that are being visualized are very small, ranging from <0.1 to about 0.5 °C. Therefore the infrared camera must be sensitive enough to easily differentiate small changes in temperature. Temperature accuracy is also an important aspect and requires that the camera is calibrated on a regular basis (at least once a year). While this can be done by the user, it requires the use of several black bodies covering a wide range of temperatures. Therefore, it is best to have the camera factory-calibrated. If a high level of

temperature accuracy is absolutely essential, it is highly recommended that a thermocouple be used in conjuction with the infrared camera. This can be mounted near the object being studied to give an accurate estimate of air temperature.

Camera parameters

A host of parameters can be adjusted on advanced, high-end infrared cameras. In using the camera to view and/or record freezing events, it is important that image averaging be used in order to reduce a noisy image, thus making it easier to visualize plant parts and freezing events. Image averaging occurs when a high quality image is selected in the camera settings. Since minor freezing exotherms are expected, it is also important when viewing the freezing process to set the temperture span of the camera to cover a small temperature range (2 – 5 °C). This is needed because the software will distribute the selected color pallette over the full span set for the camera. Therefore, if there are 10 colors in the pallette and one has the span set to 100 °C, their would only be a change in color if there was a 10 °C change in temperature. A high capture rate (ten frames per second) should be used so that small exothermic events, which dissipate quickly, are not missed. Different color palettes and grey scales can be selected from a drop down menu. Selection of the most appropriate palette should be based on whether or not it provides the best option for visualizing the thermal events of interest. Advanced cameras also offer several options for recording a video sequence and/or capturing single images. A specific number of frames over a set time duration can be selected. This is best for recording sequences of short duration (minutes) rather than hours. Alternatively, the number of frames per second can be indicated and the camera set to stop recording manually or after a specific number of frames. Advanced cameras also offer the option for recordings to commence or end based on pre-defined triggers (temperature or time).

Cooling rate

It is important that the temperature of plant material being viewed does not differ dramatically from the air temperature during cooling. If the temperature is lowered too quickly, plants will supercool and freeze at a lower air temperature than they would under natural cooling rates. Most studies recommend a cooling rate of 1-2 °C h^{-1}, especially at temperatures above -5 °C, which provides ample time for plants to come to equilibrium with the air temperature. In actuality, the plant material may come into equilibrium much faster. This can be determined by comparing the temperature of the plant material with the temperature of the background around the plant. If the plant is in equilibrium, it will be difficult to discern the plant from its background in the infrared image as it will be at the same temperature as the background and the image will appear to be nearly homogeneous in color.

Structural\morphological complexity of the object being viewed

Since the images being viewed represent images of temperature, objects that overlap will appear as contiguous objects rather than discrete objects. This can make discerning where freezing events are occuring very difficult and also increase the difficulty in determining how ice is being propagated in the plant. The best way to deal with this problem is to first work with simple objects (individual leaves, stems, etc.) and then build up to more complex objects. Experience in working with specific material has great value in dealing with this problem. Additionally, the ability to overlay the infrared image on top of a digital, visible-light image can also greatly assist in analyzing and understanding the infrared data.

Knowledge of infrared science
Although it would be advantageous to be able to simply point the camera at an object and know that the temperature data received is 100% accurate, understanding how infrared energy interacts with its environment can greatly increase ones understanding of how best to use the research-grade infrared cameras and interpret the data. One should become somewhat familiar with the terms emissivity, reflectance, and absorbance. For the most part, the camera can be used without worrying about these parameters, however, they can help to explain the nature of the image being displayed and its overall quality and accuracy. Briefly, when infrared energy hits an object it can be either reflected or absorbed and then emitted. The nature of the object being studied, therefore, can affect the accuracy of the data being received. If an object has a high reflectance, one will receive an image more representative of the surrounding objects that are emitting infrared energy than the object itself. Absorbance of infrared energy without emitting the infrared energy can also lead to obtaining false temperature data from the object being studied. The camera sensors detect emitted infrared energy, therefore, the most accurate temperatures are obtained from objects that have a high level of emissivity. Fortunately, plants do have a high level of emissivity allowing accurate temperature measurements. Lower levels of emissivity can be compensated for by adjusting this parameter in the camera settings which will then use an algorithm to make an appropriate adjustment in the temperature readout.

The ability to accurately determine how and when plants freeze is essential to understanding the evolution of freeze-avoidance mechanism and the role of plant structure in the freezing process. Freezing, despite its apparent simplicity, is a complex process and plants have evolved a host of structural adaptations to avoid freezing, compartmentalize ice formation, and prevent the propagation of ice. High resolution infrared thermography is a novel and powerful tool that can be used to study the complexity of the freezing process in plants and lead to the development of effective new methods of frost protection. A better understanding of freeze-avoidance can also help us to understand how these adaptive mechanisms have evolved, and the role they play in the biology and survival of different plant species.

DISCLOSURES:
The authors have no competing financial interests or conflicts of interest.

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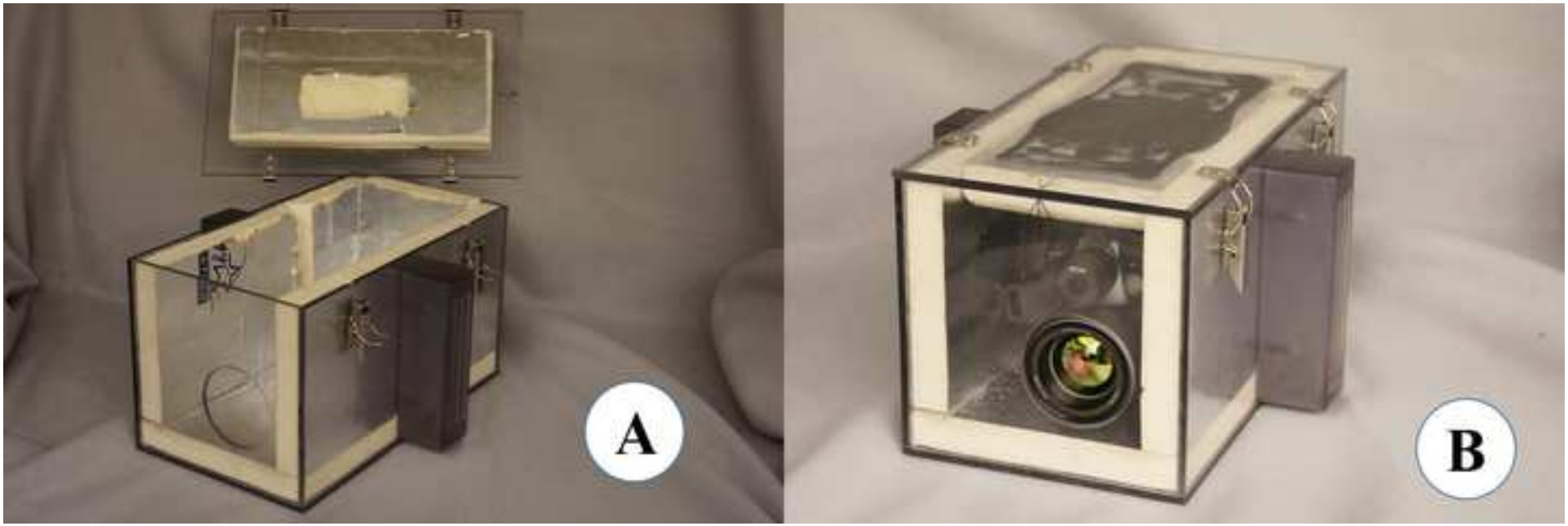

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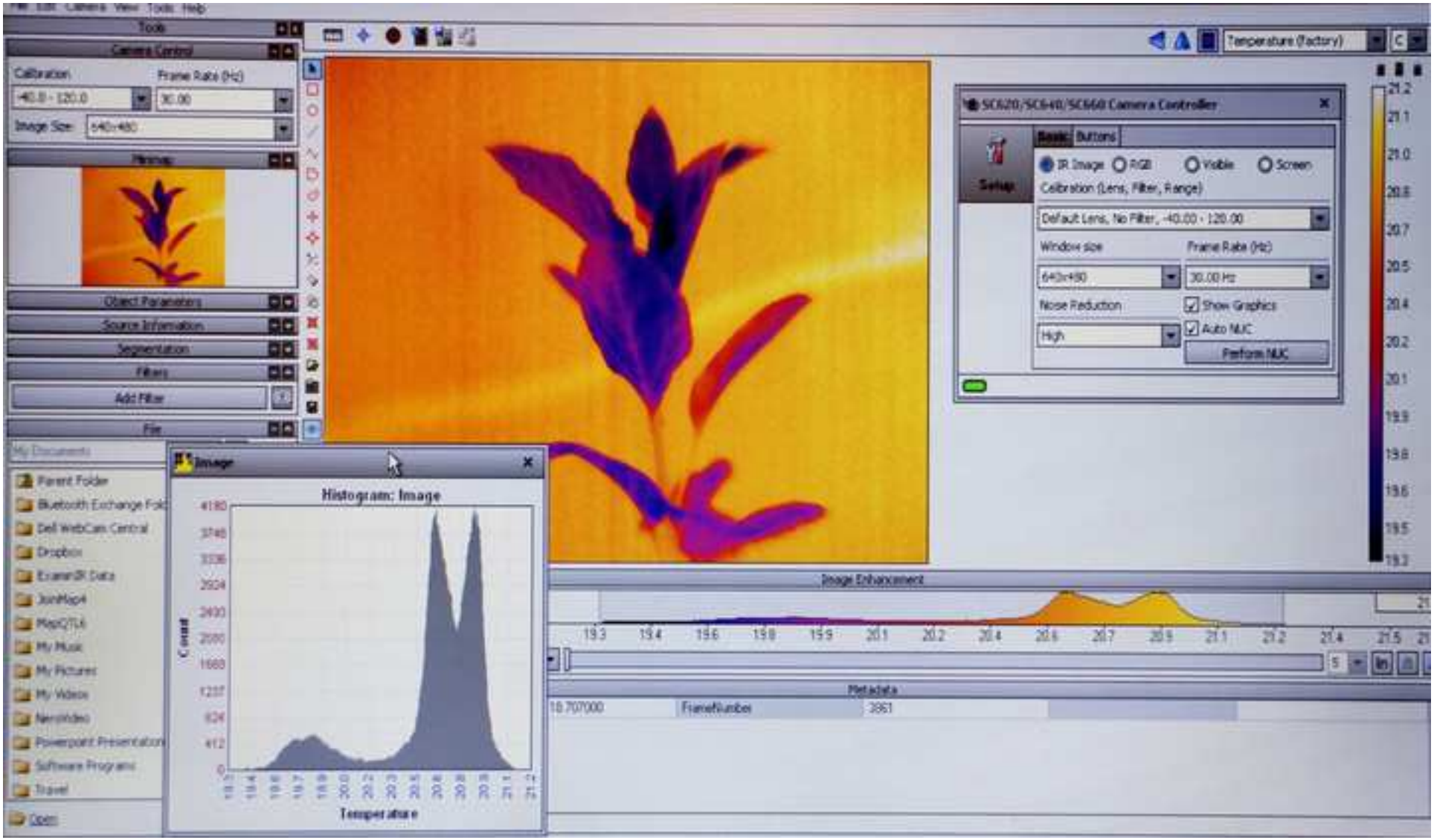

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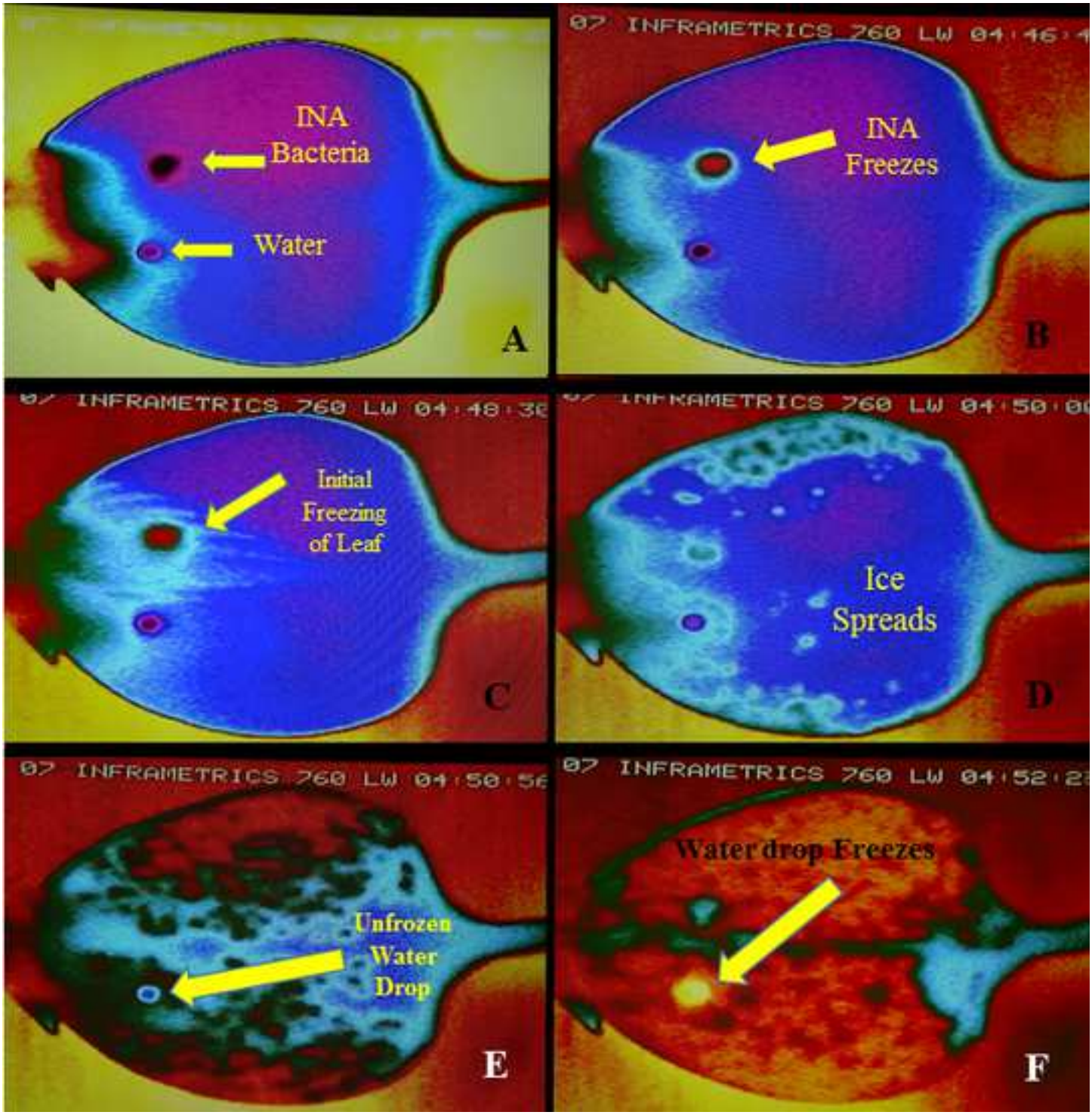

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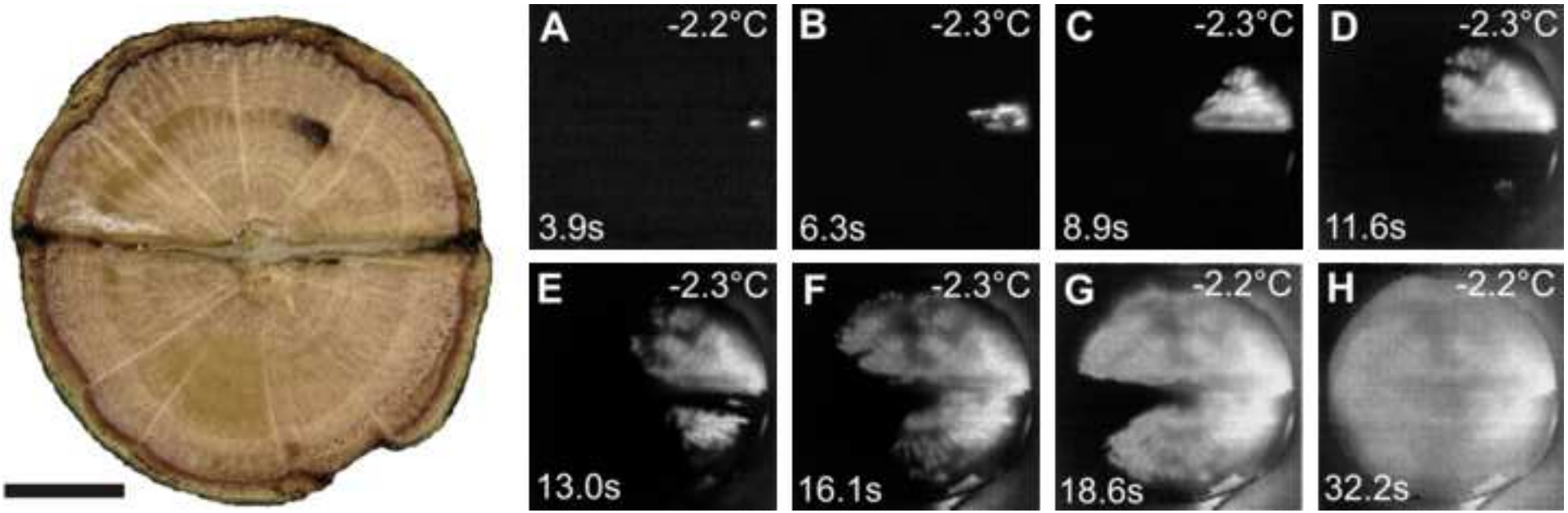

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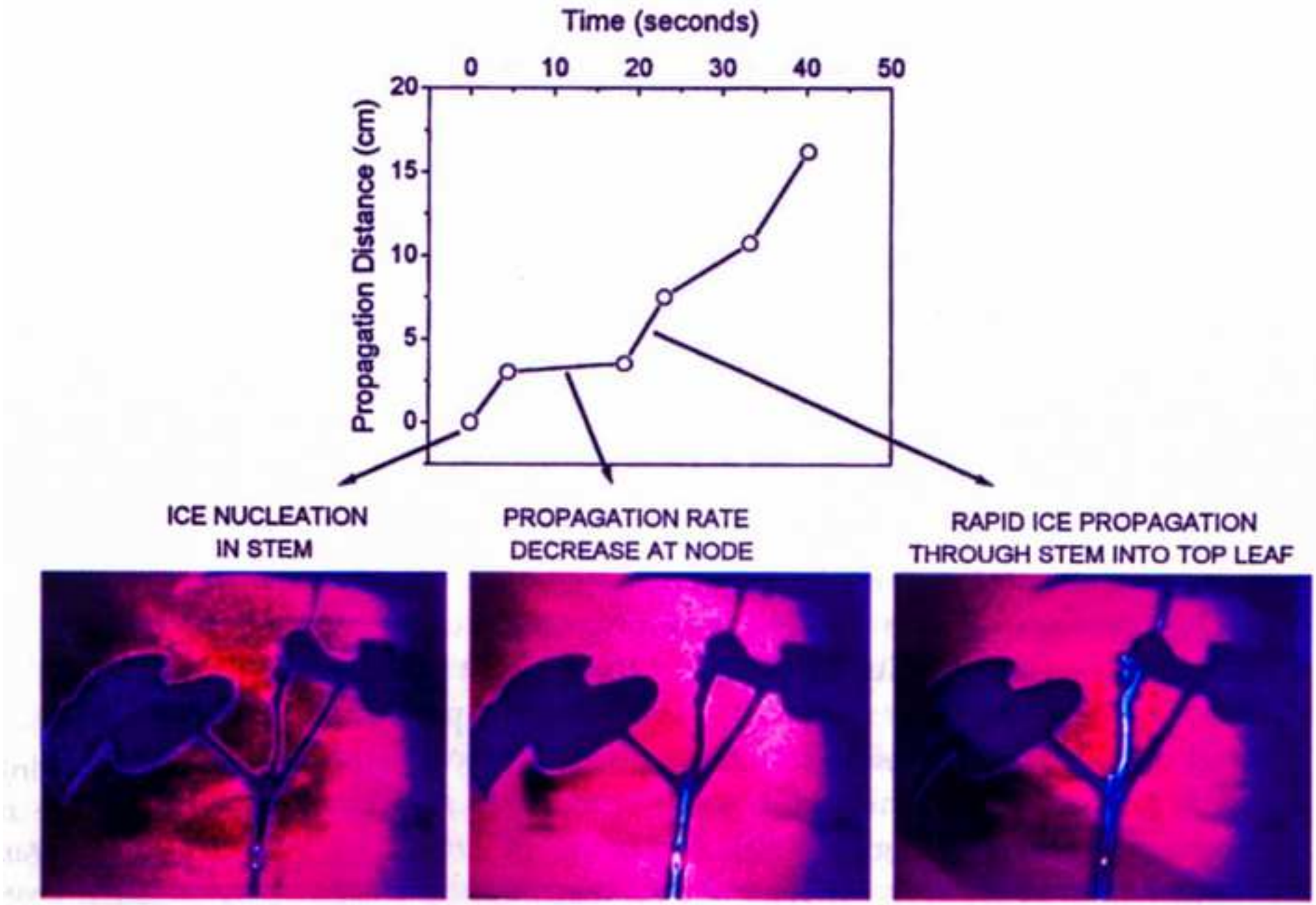

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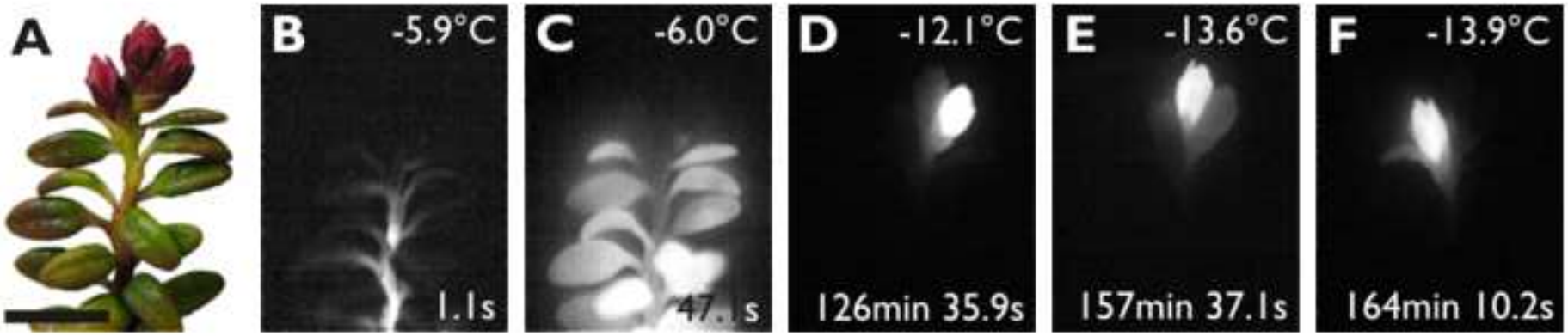

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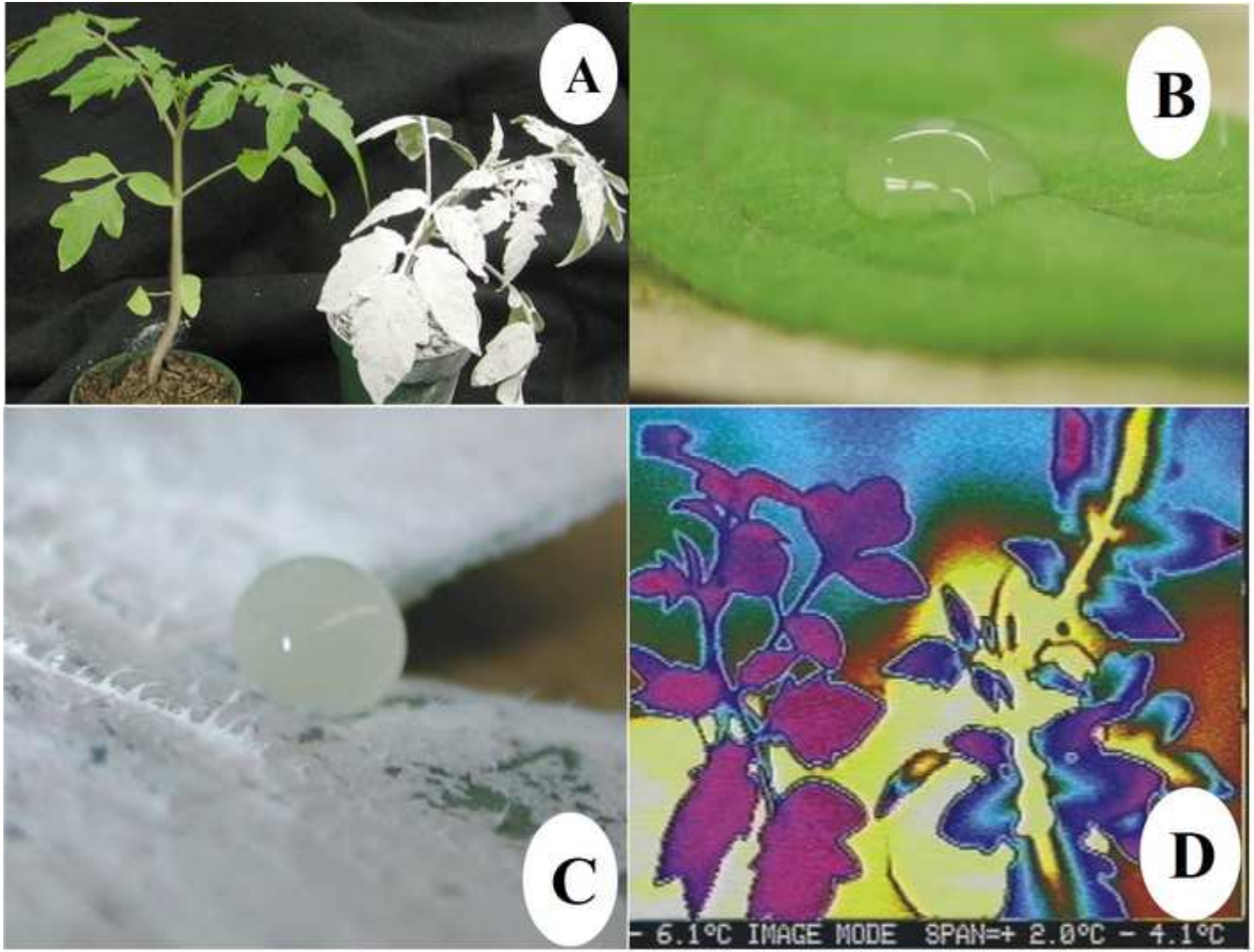

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Name of Material Or Equipment	Company	Model Number	Comments/Description
Infrared Camera	FLIR	SC-660	Many models available depending on application
Infrared Analytical Software	FLIR	ResearchIR 4.10.2.5	$3,500
Pseudomonas syringae (strain Cit-7)			Kindly provided by Dr. Steven Lindow, University of California Berkeley icelab@berkeley.edu
Pseudomonas Agar F	Fisher Scientific	DF0448-17-1	

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Author(s): Michael Wisniewski, Gilbert Neuner, Lawrence Gusta

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CORRESPONDING AUTHOR:

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Department: Appalachian Fruit Research Station

Institution: USDA-ARS

Article Title: The Use of High Resolution Infrared Thermography for the Study of Ice Nucleation in Plants

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Changes made by the Science Editor:

1. There have been edits made to the manuscript.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please provide step by step detail for step 3.4.3 and 3.4.3 (pull info from note below).

Reviewers' comments:

Reviewer #1:
Excellent description of an important and relatively new technique to study freezing in plants

Major Concerns:
None

Minor Concerns:
P3L103. Suggest using "high" in place of "warm" its hard to think in terms of "subzero" and "warm" The term high has been substituted for warm. In many prior manuscripts, people have equated the term high with greater, so readers have interpreted the term high to mean a greater sub-zero temperature. It is a problem I have had for over 30 years writing on the subject. That is why I used the term warm, sub-zero temperature.

P3L110. I know high resolution and high definition are not synonymous terms but the adjective "high" has certain connotations in the imaging world. I am not sure I would call 640X480 high resolution at this time. 640X480 is considered Standard Definition while High Definition is 1280X720. This is certainly not a major objection and I would not require the authors to change their terminology. It is just something they should consider. The reviewer is correct. My use of high resolution refers to not only the specific resolution but also as a comparison of what is generally available on low-cost infrared imagers, and also the temperature resolution. This is the description that I have used in many other published manuscripts (as have others), so I would prefer to keep the present terminology.

P6L252,253. Suggest rewording this sentence to: The direction of ice propagation in woody plant stems is more longitudinal than [NOT "then"] lateral or circumferential. This error has been

corrected.

Reviewer #2:
Major Concerns:
N/A

Minor Concerns:
The paper of Wisniewski et al. is an important contribution to the literature related to the ice nucleation and propagation in plants. It fits perfectly to the scope of Journal of Visualized Experiments, and I can recommend the MS for publication with minor revisions below.

l. 55: Why not also for Sub-Arctic and Arctic ecosystems? e.g.
-Bokhorst et al. (2010) Physiol Plantarum 140: 128-140;
-Taulavuori et al. (2013) Env Exp Bot 87: 191-196;
-Taulavuori K (2013) Vegetation at northern high latitudes under global warming (In) I Dincer, C.O. Colpan and F Kadioglu (eds) Causes, Impacts and solutions to global warming, Springer, 3-16. – First two references were added.

l.59. Couple of more suggested citations:
-Skre et al (2008) Env Exp Bot 62 (3), pp. 254-266;
-Hänninen & Tanino (2011) Trends in Plant Science 16 (8), pp. 412-416 Both references were addes.

l. 67-75: This paragraph may need a sentence or couple to refer that there are ecological adaptations for freezing avoidance, i.e. overwintering below snow (Raunkier's chamaephytes), and some reference, e.g.
-Taulavuori et al. (2011) Env Exp Bot 72 (3), pp. 397-403
There might be also good to define and separate intracellular freezing and extracellular freezing (e.g. in the second paragraph of Introduction). I agree with the reviewer that there are ecological adaptations associated with freezing avoidance but since the focus of this manuscript is the technique, I feel that the general references provided are sufficient for readers to begin to explore the topic in more detail. The manuscript is not meant to be an extensive review of the topic of freeze avoidance.

l. 104: remove the other dot .. The extra dot was removed.

l. 141: Please explain the concentration range. The concentration provided is just an estimate. As indicated in the subsequent sentence, the concentration is not really that important. The bacterial solution is only being used to demonstrate that there are biological-based nucleating agents present in the environment that can play a role (as demonstrated in the illustrated experiment) in inducing plants to freeze.

l. 229-230: Please mention the freezing rate. The freezing rate was avoided on purpose as it can vary depending on the nature of the freezing experiment, the tissue mass, etc. The main thing is the temperature of the plant tissue should not lag several degrees behind the air temperature as

this will give a false impression of the ability of the plant tissue to supercool. In general, it takes about 10 minutes for the size plants we use to equilibrate with the air temperature inside the temperature. A general rate has been added.

l. 264: Loiseleuria. procumbens (remove the dot). The extra dot has been removed.

l. 395: Citations for the freezing rate recommendations. I think the range is wider than 1-2°C h-1. The range has been clarified, it mainly pertains to temperatures from 0 to -5 C, below that temperature a faster rate can generally be used. The main thing, as mentioned in the paragraph, is that the plant tissue is in equilibrium with the air temperature. This can be easily visualized with the infrared camera.

Reviewer #3:

Manuscript Summary:

In this manuscript, the authors are showing a protocol using HRI video radiometer to identify sites of ice formation and how it propagates throughout the plant. It is very clear and comprehensive. Strengths and weaknesses are also clearly exposed, and the illustrations are well-chosen.

There are only minor remarks:

Minor Concerns:

*Fig.6 might be of better quality- better figure has been substituted and will also try to produce a completely new figure prior to publication.

*Thre are a couple of mispellings in the manuscript: line 107, there is a 'a' that has to be removed, - Removed line 113, shouldn't it be stand-alone? – Corrected line 163 wavelengths, - Corrected line 264 remove the dot after Loiseleuria, Corrected line 272, should be lycopersicum Corrected

*More information should be given on the material in the text, at least the brand of the camera, of the brand and name of the software (3.4.2)- This was specifically avoided at the request of the journal editor as it is the journal policy to not mention brand names as much as possible and I was asked to remove the details. This information is provided in the Materials List.

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