# Supercooling, ice nucleation and crystal growth: a systematic study in plant samples

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## ARTICLE INFO

## ABSTRACT

This paper presents an innovative technological platform which is based on infrared video recording and is used for monitoring multiple ice nucleation events and their interactions, as they happen in 96 well microplates. Thousands of freezing curves were obtained during this study and the following freezing parameters were measured: cooling rate, nucleation point, freezing point, solidus point, degree of supercooling, duration of dendritic phase and duration of crystal growth. We demonstrate the use of this platform in the detection of ice nuclei in plant samples. Future applications of this platform may include breeding for frost tolerance, cryopreservation, frozen food technology and atmospheric sciences.

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## 1. Introduction

Plants are susceptible to temperature variations therefore their temperature follows air temperature changes [14]. When air temperature drops below 0 °C, conditions start to become intolerant for some plants, such as tomatoes [16], whereas others, like Boreal conifers, survive temperatures down to −50 °C [18]. The response of plants to subzero temperatures is manifested mainly by two competing phenomena; supercooling and ice nucleation. Supercooling is a metastable state of water in which it remains liquid below its freezing point. It is a freeze avoidance mechanism, which in some cases, is supported by the presence of antifreeze proteins [5] and antifreeze agents. Supercooling is disrupted by freezing, which by itself is initiated by ice nucleation. The latter can be either homogeneous or heterogeneous [19]. In nature freezing is heterogeneous [20] and is initiated by extrinsic or intrinsic ice nucleators. Extrinsic ice nucleators are produced by a source other than the organism in question, f.x. in plants extrinsic ice nucleators are produced by bacteria, such as *Pseudomonas*, *Xanthomonas* and *Erwinia*, or fungi such as *Fusarium*, which often reside in the phyllosphere [21]. A plethora of extrinsic ice nucleators have been studied, some of them have been identified as proteins and their corresponding genes have been cloned [13]. Apart from the extrinsic ice nucleators, there is evidence that plants can produce intrinsic ice nucleators [2,4,11]. Regardless of whether the nucleator in a sample is intrinsic or extrinsic, the longer the sample remains at a certain subzero temperature, the more probable it is to freeze at this temperature. This is due to the stochasticity underlying ice nucleations.

In nature, during a frost event, temperature decreases slowly usually by less than 2 °C/h [17]. Therefore plants have enough time to withdraw a substantial amount of cellular water to the extracellular space. As a result, the concentration of osmolytes inside the cell increases causing a freezing point depression [3]. Although freezing tolerant plants can survive extracellular freezing they cannot do the same with intracellular freezing. Cold hardy plants, after a period of cold acclimation, produce various osmolytes, such as sugars, amino acids, organic acids, lipids and polyamines [7]. Due to the presence of osmolytes not only the freezing point but also the nucleation point is decreased [20].

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In order to detect ice nucleators in plant samples based on their ice nucleation activity we developed a high throughput screening platform involving microplates. Previous work on microplate freezing assays proved the existence of temperature variability among and within microplates and the need to use blocked experimental designs and single well plots to reduce the experimental error [23]. In this study, we further improved the accuracy of microplate freezing assays by incorporating an infrared camera in the screening platform, in order to facilitate the continuous monitoring and data recording of multiple freezing processes and their interactions. We demonstrate the usefulness of the developed platform by comparing the ice nucleation activity of *Hippophae rhamnoides*, a freezing tolerant plant that can survive temperatures as low as −40 °C [9], with grape plants that are significantly less tolerant. *Hippophae rhamnoides* was used as a positive control in platform validation studies, as it produces the best studied plant ice nucleator [10,11].

## 2. Materials and methods

### 2.1. Experimental material

*Hippophae rhamnoides* plants were cultivated in the field of the TEI of Thessaly, Greece, (Latitude: 39.627227, Longitude: 22.386257). *Vitis vinifera* plants, varieties Merlot, Syrah, Malagouzia, Cabernet Savignon and Pausanias, were cultivated at the Vine Institute (Likovrisi, Athens, Greece) (Latitude: 38.069869, Longitude: 23.77582). All plants received only watering, when necessary. Leaves were collected during the Fall − Spring 2014. Leaves were washed three times in tap water. Leaf homogenates were then obtained from 1 g of leaf in 20 mL of water, using a homogenizer (Wise Tis HG-15D). Each microplate well was filled with 150 μL of experimental material. Water with no traces of experimental material in it, served as the negative control (Table 1). Water was rejected if ice nucleators were present in it, in the freezing range of the experimental material. Sample sizes (n) were 8, 16 or 24 wells.

### 2.2. Platform set up

The platform consists of three parts. A circulating cooling water alcohol bath (Lauda RE120), a thermal camera (FLIR A310) calibrated at the factory, which is mounted on top of a circulating cooling bath and a computer connected to the camera. Up to eight microplates could be accommodated on the surface of the cooling bath. All experiments were performed in 96 well microplates (Nunc Cat No. 167008), which were partially submersed in the cooling liquid. The microplates without lids were kept in place with screws. The temperature of the bath was lowered at a constant cooling rate of about −0.2 °C/min, from 2 °C to −25 °C, with continuous mild agitation.

**Table 1**
Nucleation temperatures of ten *H. rhamnoides* clones and water obtained from 8 observations per sample.

| *H. rhamnoides* clone | Nucleation temperature | SD |
|---|---|---|
| 14 | −2.21 | 0.37 |
| 11 | −2.31 | 0.34 |
| 15 | −2.59 | 0.23 |
| 116 | −2.62 | 0.20 |
| 17 | −2.65 | 0.30 |
| 26 | −2.75 | 0.21 |
| 212 | −2.84 | 0.23 |
| 210 | −2.88 | 0.24 |
| 27 | −2.94 | 0.15 |
| 216 | −3.01 | 0.20 |
| **Water** | −17.26 | 0.92 |

### 2.3. Software development

The μThermalyzer software program was developed for the specific needs of our experimental set up. The μThermalyzer software uses a special grid of 96 points, which can be adjusted to fit the dimensions of each plate under various perspective angles. The μThermalyzer can measure the temperature of any point in the microplate; in our case, it measured the temperature in the center of each well. The software exports the continuous thermal data of each well in MS Excel format.

### 2.4. Data acquisition

The platform comprises a thermal video camera (FLIR A310) which is recording the temperature of the microplates at a rate of 1 frame/sec. During each freezing trial, thermal responses were video recorded by the infrared camera. All necessary precautions were taken to avoid disturbances (e.g. shaking) of the experimental set up, during freezing and particularly during data recording. The size of the thermal image was $240 \times 320$ pixels, which is large enough to accommodate up to 8 microplates (768 wells) simultaneously.

### 2.5. Data analysis

The freezing curves were analyzed and the following freezing parameters were measured for each microplate well: cooling rate, nucleation point (np), freezing point (fp), solidus point (sp), degree of supercooling ($\Delta$Tsc), duration of dendritic phase and duration of crystal growth. The average and standard deviation were calculated where appropriate. Differential Thermal Analysis (DTA) was used in all freezing curves for the calculation of (np), (fp) and (sp). The temperature differences referred to temperature of the same points 80 frames apart.

## 3. Results and discussion

### 3.1. Platform development and validation

In the described experimental setting hundreds of temperature measurements per frame could be obtained (Fig. 1A), as each pixel of the camera constituted a thermometer. More than 7000 temperature data per well were used to generate a freezing profile using the μThermalyzer software, a program developed in house (Fig. 1A and B, Video S1). Three characteristic points in any freezing profile, the (np), (fp) and (sp) were obtained. These three points delimitated the two phases of crystallization, the nucleation and the crystal growth. Leaf homogenates of *Hippophae rhamnoides* which produces a plethora of ice nuclei, were used as positive controls for platform validation (Table 1). The average nucleation temperature was −2.88 °C and characterized by small standard deviations of 0.24. According to our results, non-isothermal conditions in microplate freezing assays can be attributed to two sources a) the microplate format, which generates systematic temperature differences among wells and b) the release of latent heat of freezing (Lf) from samples, which generates nonsystematic temperature variations (Fig. 1B). Due to the release of Lf, non-isothermal conditions are common to all freezing assays involving samples closely spaced and therefore should be taken into account in the design of these assays.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.cryobiol.2016.03.012.

### 3.2. Qualitative and quantitative analysis of ice nucleators

Although there are plants such as rye which express intrinsic ice