HOKKAIDO UNIVERSITY

Title	Anti-ice nucleation activity in xylem extracts from trees that contain deep supercooling xylem parenchyma cells
Author(s)	Kasuga, Jun; Mizuno, Kaoru; Arakawa, Keita et al.
Citation	Cryobiology, 55(3), 305-314 https://doi.org/10.1016/j.cryobiol.2007.08.010
Issue Date	2007-12
Doc URL	https://hdl.handle.net/2115/32347
Type	journal article
File Information	Manuscript.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Anti-ice nucleation activity in xylem extracts from trees that contain deep supercooling xylem parenchyma cells

Jun Kasuga, Kaoru Mizuno, Keita Arakawa, Seizo Fujikawa*

Laboratory of Woody Plant Biology, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
*Corresponding author. Address: Laboratory of Woody Plant Biology, Graduate School of Agriculture, Hokkaido University, Kita-9 Nishi-9, Kita-ku, Sapporo 060-8589, Japan. Fax: +81 011 706 3859. E-mail address: sfuji@for.agr.hokudai.ac.jp (S. Fujikawa)

This study was supported by a grant from the Japan Society for the Promotion of Science (17•9014 to J.K.) and a grant-in-aid for scientific research from the Ministry of Education, Sports, Culture, Science and Technology of Japan (17380101 to S.F.).

Abstract

Boreal hardwood species, including Japanese white birch (*Betula platyphylla* Sukat. var. *japonica* Hara), Japanese chestnut (*Castanea crenata* Sieb. et Zucc.), katsura tree (*Cercidiphyllum japonicum* Sieb. et Zucc.), Siebold's beech (*Fagus crenata* Blume), mulberry (*Morus bombycis* Koidz.) and Japanese rowan (*Sorbus commixta* Hedl.), had xylem parenchyma cells (XPCs) that adapt to subfreezing temperatures by deep supercooling. Crude extracts from xylem in all these trees were found to have anti-ice nucleation activity that promoted supercooling capability of water as measured by a droplet freezing assay. The magnitude of increase in supercooling capability of water droplets in the presence of ice-nucleation bacteria, *Erwinia ananas*, was higher in the ranges from 0.1 to 1.7°C on addition of crude xylem extracts than freezing temperature of water droplets on addition of glucose in the same concentration (100 mosmol/kg). Crude xylem extracts from *C. japonicum* provided the highest supercooling capability of water droplets. Our additional examination showed that crude xylem extracts from *C. japonicum* exhibited anti-ice nucleation activity toward water droplets containing a variety of heterogeneous ice nucleators, including ice-nucleation bacteria, not only *E. ananas* but also *Pseudomonas syringae* (NBRC3310) or *Xanthomonas campestris*, silver iodide or airborne impurities. However, crude xylem extracts from *C. japonicum* did not affect homogeneous ice nucleation temperature as analyzed by emulsified micro-water droplets. The possible role of such anti-ice nucleation activity in crude xylem extracts in deep supercooling of XPCs is discussed.

Key words: Supercooling; Cold acclimation; Boreal hardwood species; Anti-ice nucleation substance; Heterogeneous ice nucleator

Introduction

Most trees have xylem parenchyma cells (XPCs) that adapt to subfreezing temperatures by deep supercooling [3, 14, 15, 31, 32, 36, 41]. Water in XPCs that have adapted to subfreezing temperatures by deep supercooling can be maintained in a liquid state at very low temperatures for long periods. XPCs can survive in such a supercooling state, but lethal intracellular freezing occurs if the temperature falls below the limit of supercooling [14, 15]. Damage to XPCs caused by intracellular freezing

may lead to death of entire trees [10, 35, 37]. Since the freezing resistance of XPCs that adapt by deep supercooling is weaker than that of other tissue cells that adapt by extracellular freezing [36, 37], the geographic distribution of trees in cold areas is determined by the temperature limits of supercooling in XPCs [5, 7, 14, 16, 18].

The supercooling capability of XPCs changes greatly depending on the environmental temperature. The temperature limits of supercooling in XPCs gradually decrease from warm areas toward cold areas in parallel with latitudinal temperature reduction, possibly reflecting evolutional cold acclimation [14, 18]. The temperature limits of supercooling in XPCs also change depending on seasonal environmental temperature as a result of seasonal cold acclimation and deacclimation [14, 25, 37, 46]. Furthermore, supercooling capability of XPCs is changed by artificial cold acclimation and deacclimation of twigs in trees [22, 23]. Thus, trees adapt to environmental temperature by changing the supercooling capability in their XPCs.

However, the mechanisms of deep supercooling in XPCs, especially the mechanisms of the fluctuation of supercooling capability, are unclear. The mechanism of deep supercooling in XPCs has been explained solely by the physical state of protoplasts as an isolated water droplet [2, 17]. However, it is difficult to explain the cause of the change in supercooling capability of XPCs only by such a physical state of water. On the basis of a previous hypothesis, it has been suggested that the temperature limit of supercooling corresponds to the temperature at which cell walls lose their barrier property against penetration of extracellular ice. Our previous study, however, indicated that walls of a wide variety of plant cells, even those of extracellular freezing cells in trees, can inhibit penetration of extracellular ice [48].

Our recent studies have indicated that supercooling capability of XPCs is significantly changed by release of intracellular substances and suggested that a variety of intracellular substances might have important roles in the supercooling capability of XPCs [26, 27]. We have hypothesized that there are intracellular substances in XPCs that promote the supercooling capability of water. In the present study, therefore, we tested this possibility. We prepared crude extracts from the xylem of several boreal hardwood species that have deep supercooling XPCs and examined their effects on supercooling of water droplets. The results indicated that these crude xylem extracts had anti-ice nucleation activity which might be related to deep supercooling in XPCs.

Materials and methods

Plant materials

Four to six-year-old twigs of Japanese white birch (*Betula platyphylla* Sukat. var. *japonica* Hara), Japanese chestnut (*Castanea crenata* Sieb. et Zucc.), katsura tree (*Cercidiphyllum japonicum* Sieb. et Zucc.), Siebold's beech (*Fagus crenata* Blume), mulberry (*Morus bombycis* Koidz.) and Japanese rowan (*Sorbus commixta* Hedl.) were harvested during winter (December to February) from adult trees growing in the campus of Hokkaido University. The twigs were immediately placed on crushed ice and transported to the laboratory. Xylem tissues, after the barks and piths had been removed, were used as experimental materials.

Differential thermal analysis (DTA) for determination of supercooling capability in XPCs

A block of fresh xylem tissue (300 mg) was connected to a copper-constantan thermocouple (AUG #36) and wrapped with parafilm. Samples were placed in a deep freezer (MDF-192, Sanyo Co., Ltd., Tokyo, Japan) equipped with a programmable digital temperature controller (ES-100P, Tajiri Co., Ltd., Sapporo, Japan). The samples were kept at 4°C for 60 min and then cooled at a rate of 0.2°C/min to –60°C, and the differences between thermal responses of fresh samples and those of oven-dried reference samples were recorded. All measurements were replicated three times.

Preparation of crude xylem extracts

Xylem tissues were cut into small pieces with a razor blade, frozen with liquid nitrogen and ground into fine pieces with a mortar and pestle. Approximately 1 g of xylem powder was extracted with 5 ml of 80% (v/v) ethanol overnight at room temperature in the dark. The tissue debris was removed by centrifugation at 14,000 x*g* for 10 min at 4°C. From the supernatants, ethanol and water were eliminated by evaporation and the remnants were lyophilized. The lyophilized remnants were redissolved in 200 µl of distilled water. The resultant suspensions were again centrifuged at 14,000 x*g* for 10 min at 4°C and the supernatants were used as crude xylem extracts. The crude xylem extracts were stored at –80°C until subsequent examinations. The osmolarity of sample solutions was determined by using a vapor

pressure osmometer (Model 5520, Wescor Inc., Utah, USA).

Preparation of ice nucleators

UV-sterilized and lyophilized ice-nucleation bacteria, *Erwinia ananas*, and lyophilized cell debris of ice-nucleation bacteria, *Xanthomonas campestris*, were purchased (Wako Pure Chemical Industries, Ltd., Osaka, Japan). These were used as ice nucleators without further treatment.

Ice-nucleation bacteria, *Pseudomonas syringae* (NBRC3310), were kindly provided by NITE Biological Resource Center in Japan. *P. syringae* was grown on a liquid medium composed of 1% (w/v) bacto tryptone (BD, Sparks, USA), 0.2% (w/v) yeast extract (BD, Sparks, USA) and 0.1% (w/v) $MgSO_4 \cdot 7H_2O$ for 2 days at 30°C. The bacterial cells were collected by centrifugation at 500 x*g* for 10 min at 20°C. The pellet composed of bacterial cells was rinsed three times with deionized water and immediately lyophilized. The lyophilized *P. syringae* were used as an ice nucleator.

Silver iodide (Nacalai Tesque Ltd., Kyoto, Japan) was used for another kind of ice nucleator as a suspending solution after it had been ground to a fine powder by a mortar and pestle.

Measurements of ice nucleation temperatures by a droplet freezing assay

Ice nucleation (freezing) temperatures of solutions were analyzed by a droplet freezing assay described by Vali [43] with some modifications. The solutions consisted of diluted phosphate buffer (50 mM potassium phosphate, pH 7.0) containing one of the ice nucleators and either crude extract or glucose, depending on the purpose. When ice nucleators were added, their concentrations were 2 mg/ml in *E. ananas*, 1 mg/ml in *P. syringae*, 1 mg/ml in *X. campestris*, and 10 mM in silver iodide.

Two µl of droplet solution was placed on the surface of a copper plate coated with mineral oil (Nacalai Tesque Ltd., Kyoto, Japan). The copper plate was set in an alcohol bath (F26, Julabo Labortechnik GmbH, Seelback, Germany) maintained at 0°C and immediately cooled at a rate of 0.2°C/min to –30°C. Freezing of droplets was judged by the naked eye. The percentage of cumulative number of frozen droplets with 0.5°C temperature decrements was plotted as the cumulative ice nucleation spectrum. In each treatment, 120 droplets in total from 3 separated examinations were counted [43]. The temperature required for freezing of 50% of the droplets was indicated as INT_{50} (ice

nucleation temperature of 50% of the droplets).

Measurements of ice nucleation temperatures by an emulsion freezing method

Emulsions of diluted buffer solutions (50 mM potassium phosphate, pH 7.0) were prepared in silicone oil (TSF451-10, GE Toshiba Silicones, Tokyo, Japan) containing 5% (w/w) of SPAN 65 (Wako Pure Chemical, Osaka, Japan) as an emulsifier. Diluted buffer solutions contained either crude extract or glucose, depending on the purpose. Diluted buffer solutions also contained ice nucleators, depending on the purpose. An aqueous solution in silicone oil (distilled water / silicone oil = 1 / 2) was emulsified by sonication with an ultrasonic cell disruptor (XL2000, Misonix Inc., NY, USA). Droplets of less than 10 μm in diameter were prepared in emulsions with confirmation of droplet size by light microscopic observation (BH-2, Olympus, Tokyo, Japan). Thirty microliters of an emulsified sample was put into a micro test tube, and a copper-constantan thermocouple (AUG #36) was inserted in each sample solution. To determine the temperature of freezing of each sample by DTA, the differences between thermal responses of emulsified samples and silicone oil were recorded during cooling from 4°C to –50°C at a rate of 0.2°C/min. All measurements were replicated five times.

Results

Freezing behavior of XPCs in 6 boreal hardwood species

The freezing behavior of XPCs in 6 boreal hardwood species was examined by DTA. In xylem tissues of *C. crenata*, *C. japonicum*, *F. crenata, M. bombycis* and *S. commixta*, the DTA profiles produced two clear exothermal peaks, i.e., high temperature exotherm (HTE) and low temperature exotherm (LTE), under cooling. It is believed that while an HTE is produced by the freezing of apoplast water, an LTE is produced by intracellular freezing of XPCs when the temperature exceeds the limit of supercooling as evidence of supercooling adaptation of XPCs [4, 17, 19, 21, 24, 37, 38]. The peak temperatures of the LTE in these winter samples were –31.9 ± 0.9°C in *C. crenata*, –40.0 ± 0.3°C in *C. japonicum*, –39.2 ± 0.5°C in *F. crenata*, –28.8 ± 0.6°C in *M. bombycis* and –40.1 ± 0.1°C in *S. commixta* (Fig.1).

In contrast, xylem tissues of *B. platyphylla* var. *japonica* showed an HTE but did not produce an LTE (Fig. 1). However, our previous study showed that although DTA profiles of *B. platyphylla* var. *japonica* lacked an LTE, cryo-scanning electron

microscopic observations confirmed supercooling of XPCs with the limit to –60°C during winter [31]. Thus, XPCs in all boreal hardwood species used in this study adapt to subfreezing temperatures by deep supercooling.

Effects of addition of crude xylem extracts from 6 boreal hardwood species on supercooling in water droplets

The effects of crude xylem extracts from 6 boreal hardwood species on supercooling in water droplets containing ice-nucleation bacteria, *E. ananas*, were examined by a droplet freezing assay (Fig. 2). In these samples, INT_{50} was –5.7°C without addition of glucose or crude xylem extracts. In the presence of glucose at concentrations of 10, 50 and 100 mosmol/kg, the INT_{50} was reduced in a concentration-dependent manner, although the reduction rate was very slight. At 100 mosmol/kg glucose, INT_{50} was reduced to –5.9°C (a reduction of 0.2°C in comparison to that without addition of glucose).

On the other hand, the presence of crude xylem extracts from 6 boreal hardwood species more significantly reduced INT_{50} compared with the effect of glucose (Fig. 2). The degree of reduction in INT_{50} caused by the addition of crude xylem extracts varied among species and essentially did not show a linear relation with the concentration, although INT_{50} was generally more greatly reduced by a higher concentration. The crude xylem extracts from *C. japonicum* showed the highest supercooling (anti-ice nucleation) activity from –5.7°C without addition of extracts to –7.6°C (reduction of 1.9°C) at a concentration of 100 mosmol/kg. The magnitudes of reduction in INT_{50} caused by the addition of 100 mosmol/kg of crude xylem extracts were 1.0°C (from –5.7°C to –6.7°C) in *B. platyphylla* var. *japonica*, 1.2°C (from –5.7°C to –6.9°C) in *C. crenata*, 1.3°C (from –5.7°C to –7.0°C) in *F. crenata* and 1.0°C (from –5.7°C to –6.7°C) in *S. commixta*. The lowest activity was observed in crude xylem extracts from *M. bombycis* at 100 mosmol/kg with reduction in INT_{50} of 0.3°C (from –5.7°C to –6.0°C).

Effects of addition of crude xylem extracts from C. japonicum including different ice nucleators on supercooling in water droplets

The effects of crude xylem extracts from *C. japonicum* on supercooling in water droplets containing different kinds of ice nucleators, including not only *E. ananas* bacteria (Fig. 2) but also other ice-nucleation bacteria, *P. syringae* or *X. campestris*, or

silver iodide, were examined. The *C. japonicum* crude xylem extracts promoted supercooling capability of water droplets containing all of these ice nucleators, although the magnitude of reduction in INT_{50} differed depending on the ice nucleator (Fig. 3A-C). The INT_{50} values of water droplets without addition of crude xylem extracts or glucose were –2.9, –7.0 and –8.8°C in the presence of *P. syringae* (Fig. 3A), *X. campestris* (Fig. 3B) and silver iodide (Fig. 3C), respectively. By addition of 100 mosmol/kg glucose, INT_{50} was reduced to –3.2°C (reduction of 0.3°C) in *P. syringae* (Fig. 3A), to –7.3°C (reduction of 0.3°C) in *X. campestris* (Fig. 3B) and to –9.4°C (reduction of 0.6°C) in silver iodide (Fig. 3C). On the other hand, by addition of 100 mosmol/kg crude xylem extracts from *C. japonicum*, INT_{50} values of water droplets were reduced to –3.6°C (reduction of 0.7°C) in *P. syringae* (Fig. 3A), to –8.3°C (reduction of 1.3°C) in *X. campestris* (Fig. 3B), and to –10.6°C (reduction of 1.8°C) in silver iodide (Fig. 3C). These results showed that crude xylem extracts from *C. japonicum* facilitated supercooling of water droplets including a variety of heterogeneous ice nucleators and that such facilitation of supercooling was greater than that by glucose.

Furthermore, crude xylem extracts from *C. japonicum* also reduced ice nucleation temperature of water droplets without intentional addition of ice nucleators (Fig. 3D). The INT_{50} of water droplets without addition of crude xylem extracts or glucose was –11.3°C. By addition of 100 mosmol/kg glucose, INT_{50} value was reduced to –11.6°C (reduction of 0.3°C). On the other hand, by addition of 100 mosmol/kg crude xylem extracts from *C. japonicum*, INT_{50} value was reduced to –13.0°C (reduction of 1.7°C). Thus, INT_{50} value was more reduced (1.4°C) by addition of 100 mosmol/kg of crude xylem extracts than that by addition of the same concentration of glucose. However, this effect cannot be attributed to the fact that crude xylem extracts from *C. japonicum* also affect homogeneous ice nucleation. INT_{50} of water droplets without addition of ice nucleators was –11.3°C (Fig. 3D), much higher than the homogeneous ice nucleation temperature of approximately –34°C with 2 µl water droplets, according to formula by Mason [33]. It is suggested that water droplets are contaminated with airborne ice nucleators that exist ubiquitously in the external environment. A previous study showed the difficulty of removing active heterogeneous ice nucleators from water droplets at microlitter order [20, 44]. Crude xylem extracts from *C. japonicum* also functioned to reduce INT_{50} against such airborne ice nucleators.

Effects of crude xylem extracts from C. japonicum on homogeneous ice nucleation

In order to determine whether or not crude xylem extracts from *C. japonicum* affect homogeneous ice nucleation temperature, emulsified micro-water droplets smaller than 10 μm in diameter were prepared and the ice nucleation temperatures were examined by DTA (Fig. 4). Angell [1] showed that water droplets that were smaller than 10 μm in diameter tended to keep supercooling near the homogeneous nucleation temperature.

A large exothermal peak by freezing of micro-water droplets in control medium (buffer solution without addition of crude extracts or glucose) was produced at –37.6 ± 0.6°C (Fig. 4A). Exothermal peaks of micro-water droplets containing 100 mosmol/kg glucose and 100 mosmol/kg crude xylem extract were produced at –38.0 ± 0.6°C (Fig. 4B) and –37.8 ± 0.3°C (Fig. 4C), respectively. Thus, there is no significant difference in the freezing temperatures among them, and the freezing temperatures corresponded to homogeneous ice nucleation temperatures [39]. These results indicate that crude extracts from xylem tissues of *C. japonicum* do not affect homogeneous ice nucleation.

In addition, a single exothermal peak or a few small exothermal peaks were produced frequently at higher temperatures around –15°C by cooling of emulsified micro-water droplets (Fig. 4A and C). It is thought that these small peaks were produced by heterogeneous nucleation by the presence of airborne ice nucleators, in small numbers of micro-water droplets. The addition of ice-nucleation bacteria, *E. ananas,* to micro-water droplets resulted in loss of the large peak corresponding to homogeneous ice nucleation and instead produced a number of distinct peaks at higher temperatures above –20°C by heterogeneous ice nucleation (Fig. 4D).

Discussion

XPCs in 6 boreal hardwood species harvested in winter exhibited high supercooling capability in the range from –28 to –60°C as revealed by DTA (Fig. 1) and the results of a previous cryo-scanning electron microscopic study [31]. The crude xylem extracts from these 6 hardwood species, which contained deep supercooling XPCs, exhibited greater effects to facilitate supercooling of water droplets than the effect by glucose at the same concentration (Fig. 2).

Increasing the osmotic concentration of general solutes depresses the equilibrium melting point of an aqueous solution to 1.86°C per mole solute per kg water by the

so-called colligative effect [42]. Thus, 100 mosmol/kg of general solute, a concentration used frequently in this study, depresses the melting point to –0.186°C. Furthermore, general solutes depress the ice nucleation (freezing) temperature of an aqueous solution by about two-fold more than the level of depression of equilibrium melting point [39]. Another study has been shown that general solutes depress homogeneous nucleation temperature by three-fold and depress heterogeneous nucleation temperature by about two-fold [9]. Although the values changed depending on the heterogeneous ice nucleator used in this study, glucose at 100 mosmol/kg resulted in reduction of ice nucleation temperature by 0.2 to 0.6°C (Figs. 2 and 3). Thus, glucose behaved as a general solute. On the other hand, crude xylem extracts, at the same concentration as that of glucose, reduced INT_{50} of water droplets including *E. ananas* as ice nucleators by 1.0°C in *B. platyphylla* var. *japonica*, 1.2°C in *C. crenata*, 1.9°C in *C. japonicum*, 1.3°C in *F. crenata*, 0.3°C in *M. bombycis* and 1.0°C in *S. commixta* (Fig. 2). In the case of *M. bombycis*, although the magnitude of reduction of nucleation temperature caused by crude xylem extracts was very small (0.3°C), it was still 0.1°C higher than that in glucose solution including *E. ananas* (0.2°C). These results indicate that in all hardwood species examined, crude extracts from xylem including deep supercooling XPCs have specific anti-ice nucleation activity.

There are some previous reports about anti-ice nucleation substances that enhance supercooling of water as listed in Table 1. Antifreeze proteins from insects [11], antifreeze proteins and antifreeze glycoproteins from fish [20, 34, 45], anti-nucleating proteins from bacteria [29], and polysaccharides from bacteria [49] exhibit anti-ice nucleation activity toward water droplets. As substances originating from plants, hinokitiol from the leaves of Taiwan yellow cypress [28] and eugenol from clove reduce ice nucleation activity of water [30]. Crude extracts from seeds of woody plants and supernatant liquid from germinating legume seeds exhibit very high anti-ice nucleation activity toward water droplets, although causative substances for supercooling in these plant extracts were not identified [8]. As chemical substances, polyvinyl alcohol and polyglycerol enhance supercooling of an aqueous solution [20, 47]. However, this study is the first study to show anti-ice nucleation activity in crude extracts from xylem of trees that contain deep supercooling XPCs.

Since the supercooling capability of water changes greatly depending on experimental conditions, such as cooling rate, volume of samples, concentration of

added anti-ice nucleation substances and kinds of ice nucleators used, it is difficult to compare simply anti-ice nucleation activities of different substances. In previous studies, the anti-ice nucleation activity was investigated using only one or two kinds of ice nucleators, except for the study using a 130-kDa polysaccharide [49] and polyvinyl alcohol [47]. Thus, it is not clear whether these anti-ice nucleation substances are effective or not for a wide variety of ice nucleators. On the other hand, the present study showed that crude extracts from xylem tissues of *C. japonicum* had extensive anti-ice nucleation activity toward a variety of heterogeneous ice nucleators, including 3 kinds of ice-nucleation bacteria, silver iodide and airborne ice nucleators (Figs. 2 and 3). The 130-kDa polysaccharide did not function as an anti-ice nucleation substance toward airborne ice nucleators [49].

On the other hand, we confirmed that crude xylem extracts of *C. japonicum* did not affect to homogeneous ice nucleation (Fig. 4). We confirmed this by using emulsified micro-water droplets with diameters less than 10 μm [1]. Antifreeze glycoproteins from fish also have the same effects as those of crude xylem extracts, showing the presence of anti-ice nucleation activity toward heterogeneous ice nucleators [34, 45] but absence of activity toward homogeneous ice nucleation [13, 45].

The current understanding of deep supercooling in XPCs neglects the presence of heterogeneous ice nucleators in the protoplasts of XPCs [6]. If this is true, anti-ice nucleation activity in crude xylem extracts, which show activity toward only heterogeneous ice nucleators, should not influence the supercooling capability of XPCs. However, no direct evidence showing the absence of heterogeneous ice nucleators in deep supercooling XPCs has been obtained. Rather, George and Burke [17] showed that ice nucleation activity in supercooling XPCs of shagbark hickory (*Carya ovata* L.) was intermediate between homogeneous ice nucleation for pure water [12] and the weak heterogeneous ice nucleation in yeast cells [40]. This finding indicates that although the activity level is very low, some heterogeneous ice nucleators might be present in XPCs. Thus, it is thought that any anti-ice nucleation substances in crude xylem extracts that inhibit a wide variety of heterogeneous ice nucleators (Figs. 2 and 3) may serve to inhibit intracellular freezing due to heterogeneous ice nucleators in protoplasts of XPCs and may enhance supercooling of XPCs.

While it is thought that the presence of anti-ice nucleation substances in crude xylem extracts from trees that contain deep supercooling XPCs may play a role in enhancing

or stabilizing supercooling of XPCs, the exact role of such substances in the deep supercooling of XPCs is unclear at present. The anti-ice nucleation activities of crude xylem extracts (Fig. 2, Table 1) did not correlate with supercooling capability of XPCs in the 6 hardwood species (Fig. 1). For example, while XPCs in *M. bombycis* exhibited high supercooling of –40.1°C (Fig. 1), the magnitude of reduction of nucleation temperature by crude xylem extracts was only 0.1°C higher than that by glucose (Fig. 2). However, crude xylem extracts might be contaminated with ice nucleators included in the xylem apoplast. In our preliminary examination, anti-ice nucleation activity was significantly increased after partial purification of crude xylem extracts from *C. japonicum* (data not shown). In order to compare the activity of anti-ice nucleation substances with supercooling capability of XPCs, purification and identification of anti-ice nucleation substances are undoubtedly necessary. We are currently trying to purify and identify anti-ice nucleation substances as well as confirm their expression in XPCs in relation to the fluctuation of supercooling capability in XPCs.

References

[1] C.A. Angell, Supercooled water, in: F. Franks (Eds.), Water: A Comprehensive Treatise Vol. 7, Plenum Press, New York, 1982, pp. 1-17.

[2] E.N. Ashworth, F.B. Abeles, Freezing behavior of water in small pores and the possible role in the freezing of plant tissues, Plant Physiol. 76 (1984) 201-204.

[3] E.N. Ashworth, P. Echlin, R.S. Pearce, T.L. Hayes, Ice formation and tissue response in apple twigs, Plant Cell Environ. 11 (1988) 703-710.

[4] E.N. Ashworth, D.J. Rowse, L.A. Billmyer, The freezing of water in woody tissues of apricot and peach and the relationship to freezing injury, J. Am. Soc. Hortic. Sci. 108 (1983) 299-303.

[5] M.R. Becwar, C. Rajashekar, K.J.H. Bristow, M.J. Burke, Deep undercooling of tissue water and winter hardiness limitations in timberline flora, Plant Physiol. 68 (1981) 111-114.

[6] M.J. Burke, L.V. Gusta, H.A. Quamme, C.J. Weiser, P.H. Li, Freezing and injury in plants, Annu. Rev. Plant Physiol. 27 (1976) 507-528.

[7] M.J. Burke, C. Stushnoff, Frost hardiness: a discussion of possible molecular causes of injury with particular reference to deep supercooling of water, in: H. Musell, R.C. Staples (Eds.), Stress Physiology in Crop Plants, Wiley, New York, 1979, pp. 197-225.

[8] G. Caple, R.G. Layton, S.N. McCurdy, C. Dunn, L. Culbertson, Biogenic effects in heterogeneous ice nucleation, CryoLetters 4 (1983) 59-64.

[9] S. Charoenrein, D.S. Reid, The use of DSC to study the kinetics of heterogeneous and homogeneous nucleation of ice in aqueous systems, Thermochim. Acta 156 (1989) 373-381.

[10] J.W. Daniell, F.L. Crosby, Occlusion of xylem elements in peach trees resulting from cold injury, Proc. Am. Soc. Hortic. Sci. 93 (1968) 128-134.

[11] J.G. Duman, The inhibition of ice nucleators by insect antifreeze proteins is enhanced by glycerol and citrate, J. Comp. Physiol. B 172 (2002) 163-168.

[12] N.H. Fletcher, The Chemical Physics of Ice, Cambridge University Press, London, 1970, pp. 73-103.

[13] F. Franks, J. Darlington, T. Schenz, S.F. Mathias, L. Slade, H. Levine, Antifreeze activity of Antarctic fish glycoprotein and a synthetic polymer, Nature 325 (1987) 146-147.

[14] S. Fujikawa, K. Kuroda, Cryo-scanning electron microscopic study on freezing

behavior of xylem ray parenchyma cells in hardwood species, Micron 31 (2000) 669-686.
[15] S. Fujikawa, K. Kuroda, K. Fukazawa, Ultrastructural study of deep supercooling of xylem ray parenchyma cells from *Styrax obassia*. Micron 25 (1994) 241-252.
[16] M.F. George, M.R. Becwar, M.J. Burke, Freezing avoidance by deep undercooling of tissue water in winter-hardy plants, Cryobiology 19 (1982) 628-639.
[17] M.F. George, M.J. Burke, Cold hardiness and deep supercooling in xylem of shagbark hickory, Plant Physiol. 59 (1977) 319-325.
[18] M.F. George, M.J. Burke, H.M. Pellet, A.G. Johnson, Low temperature exotherms and woody plant distribution, HortScience 9 (1974) 519-522.
[19] L.V. Gusta, N.J. Tyler, T.H.H. Chen, Deep undercooling in woody taxa growing north of the –40°C isotherm, Plant Physiol. 72 (1983) 122-128.
[20] C.B. Holt, The effect of antifreeze proteins and poly(vinyl alcohol) on the nucleation of ice: a preliminary study, CryoLetters 24 (2003) 323-330.
[21] S.G. Hong, E. Sucoff, Units of freezing of deep supercooled water in woody xylem, Plant Physiol. 66 (1980) 40-45.
[22] S.G. Hong, E. Sucoff, Rapid increase in deep supercooling of xylem parenchyma, Plant Physiol. 69 (1982) 697-700.
[23] S.G. Hong, E. Sucoff, Temperature effects on acclimation and deacclimation of supercooling in apple xylem, in: P.H. Li, A. Sakai (Eds.), Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implication, Academic press, New York, 1982, pp. 341-356.
[24] S.G. Hong, E. Sucoff, O.Y. Lee-Stadelmann, Effect of freezing deep supercooled water on the viability of ray cells, Bot. Gaz. 141 (1980) 464-468.
[25] S. Kaku, M. Iwaya, Low temperature exotherms in xylems of evergreen and deciduous broad-leaved trees in Japan with reference to freezing resistance and distribution range, in: P.H. Li and A. Sakai (Eds.), Plant Cold Hardiness and Freezing Stress. Academic Press, New York, 1978, pp. 227-239.
[26] J. Kasuga, K. Arakawa, S. Fujikawa, High accumulation of soluble sugars in deep supercooling Japanese white birch xylem parenchyma cells, New Phytol. 174(2007) 569-579.
[27] J. Kasuga, K. Mizuno, N. Miyaji, K. Arakawa, S. Fujikawa, Role of intracellular contents to facilitate supercooling capability in beech (*Fagus crenata*) xylem

parenchyma cells, CryoLetters 27 (2006) 305-310.
[28] H. Kawahara, K. Masuda, H. Obata, Identification of a compound in *Chamaecyparis taiwanensis* inhibiting the ice-nucleating activity of *Pseudomonas fluorescens* KUIN-1, Biosci. Biotechnol. Biochem. 64 (2000) 2651-2656.
[29] H. Kawahara, I. Nagae, H. Obata, Purification and characterization of a new anti-nucleating protein isolated from *Acinetobacter calcoaceticus* KINI-1, Biocontrol Sci. 1 (1996) 11-17.
[30] H. Kawahara, H. Obata, Identification of a compound in spices inhibiting the ice-nucleating activity of *Erwinia uredovora* KUIN-3, J. Antibact. Antifungal Agents Jpn 24 (1996) 95-100.
[31] K. Kuroda, J. Kasuga, K. Arakawa, S. Fujikawa, Xylem ray parenchyma cells in boreal hardwood species respond to subfreezing temperatures by deep supercooling that is accompanied by incomplete desiccation, Plant Physiol. 131 (2003) 736-744.
[32] S.R. Malone, E.N. Ashworth, Freezing stress response in woody tissues observed using low-temperature scanning electron microscopy and freeze substitution techniques, Plant Physiol. 95 (1991) 871-881.
[33] B.J. Mason, The supercooloing and nucleation of water, Adv. Phys. 7 (1958) 221-234.
[34] A. Parody-Morreale, K.P. Murphy, E. Di Cera, R. Fall, A.L. DeVries, S.J. Gill, Inhibition of bacterial ice nucleators by fish antifreeze glycoproteins, Nature 333 (1988) 782-783.
[35] G.F. Potter, Low temperature effects on woody plants, Proc. Am. Soc. Hortic. Sci. 36 (1938) 185-195.
[36] H.A. Quamme, P.M. Chen, L.V. Gusta, Relationship of deep supercooling and dehydration resistance to freezing injury in dormant stem tissues of 'Starkrimson Delicious' apple and 'Siberian C' peach, J. Am. Soc. Hortic. Sci. 107 (1982) 299-304.
[37] H. Quamme, C. Stushnoff, C.J. Weiser, The relationship of exotherms to cold injury in apple stem tissues, J. Am. Soc. Hortic. Sci. 97 (1972) 608-613.
[38] H. Quamme, C.J. Weiser, C. Stushnoff, The mechanism of freezing injury in xylem of winter apple twigs, Plant Physiol. 51 (1973) 273-277.
[39] D.H. Rasmussen, A.P. MacKenzie, Effect of solute on ice-solution interfacial free energy; calculation from measured homogeneous nucleation temperatures, in: H.H.G. Jellinek (Eds.), Water Structure at the Water-polymer Interface, Plenum Press, New

York, 1972, pp. 126-145.
[40] D.H. Rasmussen, A.P. MacKenzie, Supercooling and nucleation of ice in single cells, Cryobiology 12 (1975) 328-339.
[41] A. Sakai, W. Larcher, Frost Survival of Plants: Response and Adaptation to Freezing Stress, Springer, Berlin, 1987.
[42] T.E. Sweeney, C.A. Beuchat, Limitations of methods of osmometry: measuring the osmolality of biological fluids, Am. J. Physiol. Regul. Integr. Comp. Physiol. 264 (1993) R469-R480.
[43] G. Vali, Quantitative evaluation of experimental results on heterogeneous freezing nucleation of supercooled liquids, J. Atmos. Sci. 28 (1971) 402-409.
[44] P.W. Wilson, A.F. Heneghan, A.D.J. Haymet, Ice nucleation in nature: supercooling point (SCP) measurements and the role of heterogeneous nucleation, Cryobiology 46 (2003) 88-98.
[45] P.W. Wilson, J.P. Leader, Stabilization of supercooled fluids by thermal hysteresis proteins, Biophys. J. 68 (1995) 2098-2107.
[46] M. Wisniewski, E.N. Ashoworth, A comparison of seasonal ultrastructural changes in stem tissue of peach (*Prunus persica*) that exhibit contrasting mechanisms of cold hardiness, Bot. Gaz. 147 (1986) 407- 417.
[47] B. Wowk, G.M. Fahy, Inhibition of bacterial ice nucleation by polyglycerol polymers, Cryobiology 44 (2002) 14-23.
[48] T. Yamada, K. Kuroda, Y. Jitsuyama, D. Takezawa, K. Arakawa, S. Fujikawa. Roles of the plasma membrane and the cell wall in the responses of plant cells to freezing, Planta 215 (2002) 770-778.
[49] Y. Yamashita, H. Kawahara, H. Obata. Identification of a novel anti-ice-nucleating polysaccharide from *Bacillus thuringiensis* YY529, Biosci. Biotechnol. Biochem. 66 (2002) 948-954.

Figure legends

Fig. 1. Typical DTA profiles during cooling of xylem tissues in 6 boreal hardwood species harvested in winter (January). Each xylem tissue was cooled at a rate of 0.2°C/min from 4°C to –60°C. Arrowheads show peaks of low temperature exotherm (LTE).

Fig. 2. Effects of crude xylem extracts from 6 boreal hardwood species harvested in winter (December to February) on ice nucleation temperatures of water droplets. Water droplets consisted of diluted phosphate buffer solution including 2 mg/ml of ice-nucleation bacteria *Erwinia ananas* and different concentrations of crude xylem extracts or glucose. Freezing spectrums were obtained by a droplet freezing assay using 2 µl water droplets cooled at a rate of 0.2°C/min. ○: Control, without crude extracts or glucose; ▲:10 mosmol/kg of crude extracts or glucose; ■: 50 mosmol/kg of crude extracts or glucose; ♦: 100 mosmol/kg of crude extracts or glucose. In each treatment, 120 droplets in total from 3 separated examinations were used.

Fig. 3. Effects of crude xylem extracts from *C. japonicum* on ice nucleation temperatures of water droplets containing different ice nucleators. Water droplets consisted of diluted buffer solution including 1 mg/ml of *P. syringae* (A), 1 mg/ml of *X. campestris* (B), 10 mM silver iodide (C) or without addition of ice nucleators (D), and crude xylem extracts from *C. japonicum* at 100 mosmol/kg (■), glucose at 100 mosmol/kg (▲) or without crude extract or glucose (○). Freezing spectrums were obtained by a droplet freezing assay using 2 µl water droplets cooled at a rate of 0.2°C/min. In each treatment, 120 droplets in total from 3 separated examinations were used.

Fig. 4. Typical DTA profiles showing effects of crude xylem extracts from *C. japonicum* on ice nucleation temperature in emulsified micro-water droplets with diameters of less than 10 µm. Micro-water droplets consisted of diluted phosphate buffer solution alone (A), diluted buffer solution including 100 mosmol/kg of glucose (B), diluted buffer solution including 100 mosmol/kg of crude xylem extracts (C), and diluted buffer solution including 2 mg/ml of *E. ananas* (D). Thermographs were obtained during cooling of emulsified samples at a rate of 0.2°C/min.

Table 1. A list of anti-ice nucleation substances.

[a] Anti-ice nucleation activity is expressed by the difference between INT_{50} of water droplets containing anti-ice nucleation substances and that without anti-ice nucleation substances. No compensation of INT_{50} with INT_{50} of water droplets containing same concentration of general solutes was done.

[b] No ice nucleators were intentionally added. However, it is suggested that these water droplets contained airborne ice nucleators.

[c] The analysis was done by an emulsion freezing method, suggesting effects on homogeneous ice nucleation.

[d] It is shown that significant activity was not detected.

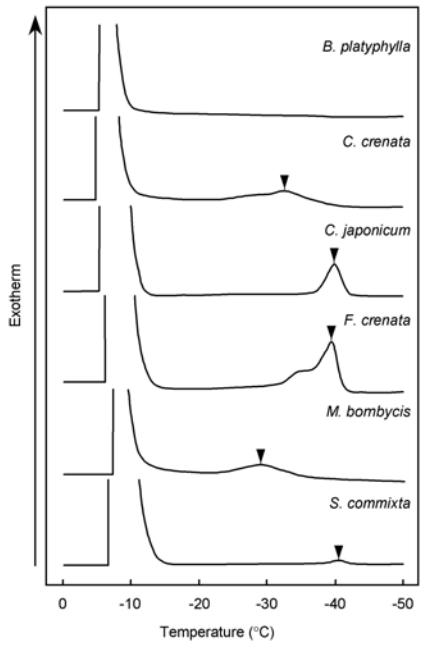
B. platyphylla
C. crenata
C. japonicum
F. crenata
M. bombycis
S. commixta
Exotherm
0
-10
-20
-30
-40
-50
Temperature (°C)

Fig. 1

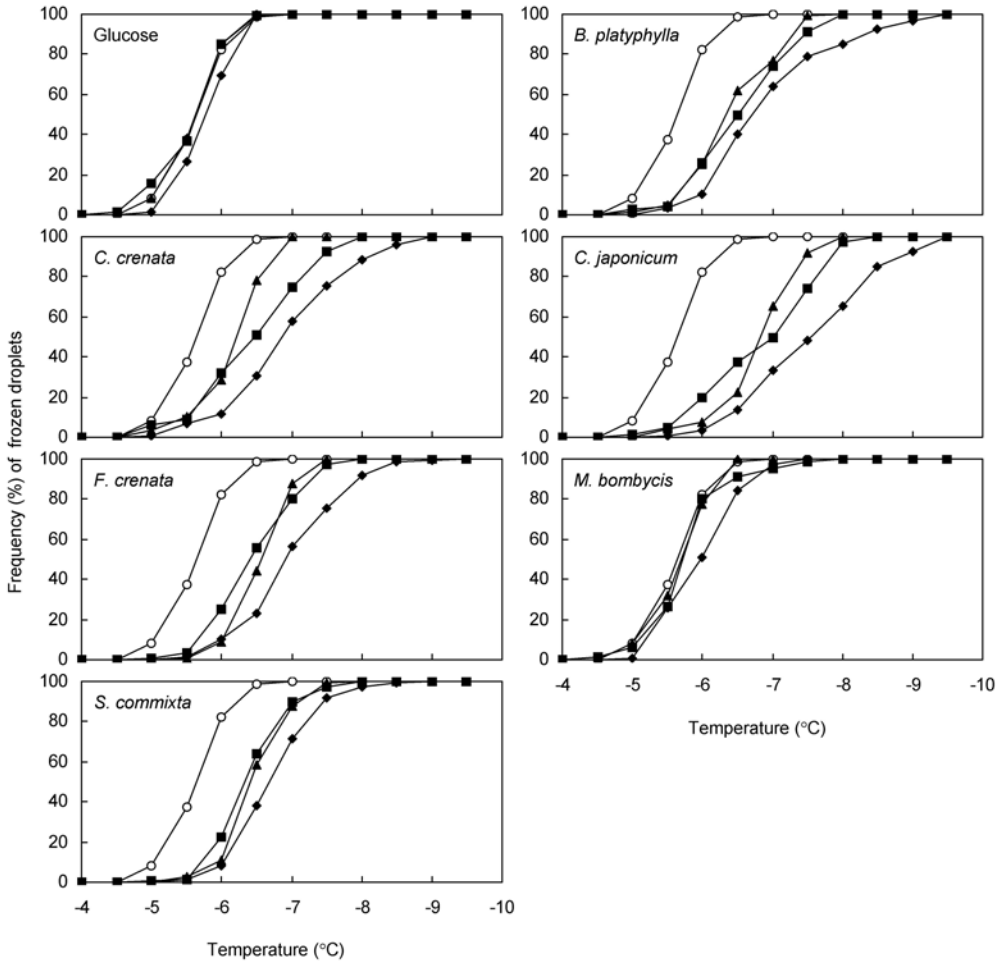
Glucose
B. platyphylla
C. crenata
C. japonicum
F. crenata
M. bombycis
S. commixta
Frequency (%) of frozen droplets
Temperature (°C)
Temperature (°C)
100
80
60
40
20
0
-4
-5
-6
-7
-8
-9
-10

Fig. 2

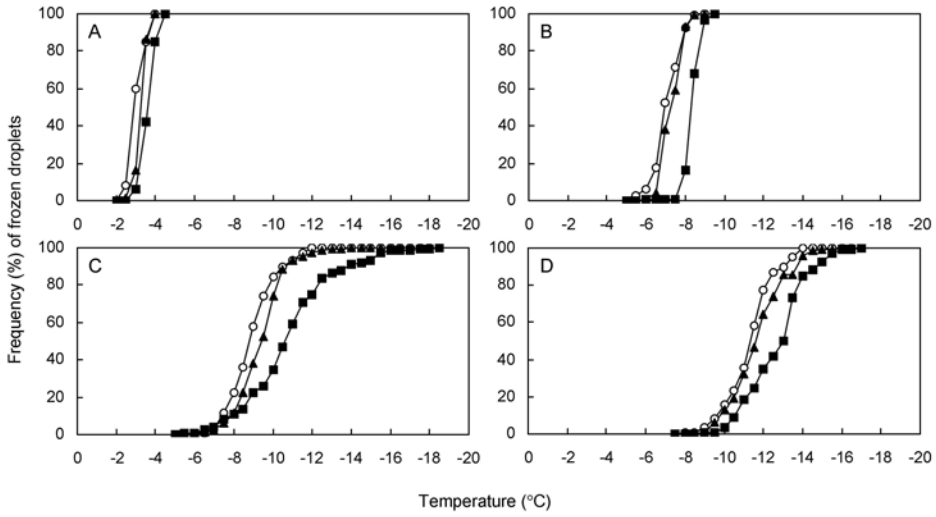
A
B
C
D
Frequency (%) of frozen droplets
Temperature (°C)
100
80
60
40
20
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20

Fig. 3

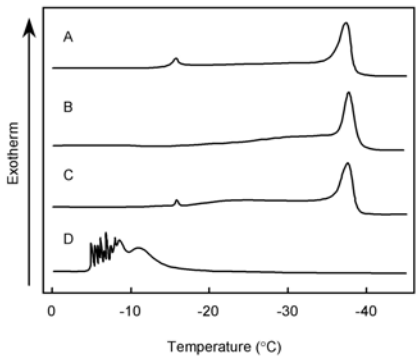

Fig. 4

Table 1

Substance	Experimental condition				Anti-ice nucleation activity (°C) [a]	Reference
	Concentration	Ice nucleator	Volume of droplets (μl)	Cooling rate (°C/min)		
Xylem extract						
Betula platyphylla var. *japonicum*	100 mosmol/kg	*Erwinia ananas*	2	0.2	1.0	Present paper
Castanea crenata	100 mosmol/kg	*E. ananas*	2	0.2	1.2	
Fagus crenata	100 mosmol/kg	*E. ananas*	2	0.2	1.3	
Morus bombycis	100 mosmol/kg	*E. ananas*	2	0.2	0.3	
Sorbus commixta	100 mosmol/kg	*E. ananas*	2	0.2	1.0	
Cercidiphyllum japonicum	100 mosmol/kg	*E. ananas*	2	0.2	1.9	
	100 mosmol/kg	*Pseudomonas syringae* NBRC3310	2	0.2	0.7	
	100 mosmol/kg	*Xanthomonas campestris*	2	0.2	1.3	
	100 mosmol/kg	Silver iodide	2	0.2	1.8	
	100 mosmol/kg	None (airborne ice nucleators) [b]	2	0.2	1.7	
	100 mosmol/kg	None (homogeneous ice nucleation) [c]	$< 5 \times 10^{-7}$	0.2	0.2 [d]	
Antifreeze protein						
Dendroides canadensis	Not shown	Protein ice nucleator	1	1.0	1.7	[11]
	Not shown	*P. syringae*	1	1.0	1.2	
Dissostichus mawsoni	0.1 ~ 1% (w/w)	None (airborne ice nucleators) [b]	100	0.6	0.3 ~ 4.9	[20]
Macrozoarces americanus	0.1 ~ 0.5% (w/w)	None (airborne ice nucleators) [b]	100	0.6	0.7 ~ 1.5	
Antifreeze glycoprotein						
Dissostichus mawsoni	1 mg/ml	*Erwinia herbicola*	1	Not shown	1.0	[34]
	10 mg/ml	*Hemideina maori* hemolymph	3	Not shown	2.5	[45]
Notothenia neglecta	1% (w/w)	None (homogeneous ice nucleation) [c]	2×10^{-8}	1.25	0.1 [d]	[13]
55-kDa protein (*Acinetobacter calcoaceticus* KINI-1)	10 μg/ml	*Erwinia uredovora* KUIN-3	10	1.0	2.2	[29]
130-kDa polysaccharide (*Bacillus thuringiensis* YY529)	50 μg/ml	*Pantoea ananas* KUIN-3	10	1.0	2.3	[49]
	50 μg/ml	*Xanthomonas Translucens* IFO 13558	10	1.0	1.0	
	50 μg/ml	*Pseudomonas fluorescens* KUIN-1	10	1.0	0.3	
	50 μg/ml	Silver iodide	10	1.0	4.2	
	50 μg/ml	Metaldehyde	10	1.0	0.1	
	50 μg/ml	Fluoren-9-one	10	1.0	1.1	
	50 μg/ml	Phenazine	10	1.0	1.0	
	50 μg/ml	None (airborne ice nucleators) [b]	10	1.0	0	
Hinokitiol	10 mM	*P. fluorescens* KUIN-1	10	1.0	2.1	[28]
Hinokitin	10 mM	*P. fluorescens* KUIN-1	10	1.0	1.1	
α-Pinene	10 mM	*P. fluorescens* KUIN-1	10	1.0	1.1	
α-Terpinene	10 mM	*P. fluorescens* KUIN-1	10	1.0	0.4	
Limonene	10 mM	*P. fluorescens* KUIN-1	10	1.0	1.6	
Eugenol	1 mg/ml	*E. uredovora* KUIN-3	10	1.0	1.9	[30]
α-Methoxyphenol	1 mg/ml	*E. uredovora* KUIN-3	10	1.0	0.2	
2-Allylphenol	1 mg/ml	*E. uredovora* KUIN-3	10	1.0	2.5	
4-Allylanisole	1 mg/ml	*E. uredovora* KUIN-3	10	1.0	1.2	
Seed extract						
Simmondsida chinesis	Not shown	Silver iodide	10	1.0	4.0	[8]
Prunus persica	Not shown	Silver iodide	10	1.0	2.6	
Prunus america	Not shown	Silver iodide	10	1.0	4.2	
Supernatant liquid surrounding germinating pea (*Pisum sativum*) seeds	Not shown	Silver iodide	10	1.0	8.1	
Polyvinyl alcohol	0.5 ~ 1% (w/w)	*P. syringae*	100	0.6	1.4	[20]
	0.1 ~ 1% (w/w)	None (airborne ice nucleators) [b]	100	0.6	2.3 ~ 4.6	
	0.1% (w/w)	*P. syringae*	1	2.0	0.8	[47]
Polyglycerol	0.001 ~ 1% (w/w)	*P. syringae*	1	2.0	1.2 ~ 6.6	