Freezing Injury and Colonization of ‘Redwing’ Red Raspberries with- Ice-nucleation-active Bacteria

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Abstract. In 1993, ice-nucleation-active (INA) bacteria were isolated from ‘Redwing’ red raspberries (Rubus idaeus L. var. idaeus) at five pigmentation stages. Fruit were also subjected to thermal analysis to determine the ice nucleation temperatures. INA bacteria were recovered from nearly all fruit samples, and the bacterial populations tended to decrease with greater red color development (i.e., fruit maturation). However, the ice nucleation temperature was not affected by the stage of fruit pigmentation. In 1994, INA bacterial densities were similar among fruit at the three pigmentation stages sampled. INA bacteria were recovered more often from the calyx rather than the drupe surface of these fruit. INA bacteria also were detected on pistils of some fruit. Red and pink fruit, which were nucleated with ice, had greater receptacle injury than mottled, yellow, or green fruit, but INA bacterial densities apparently were not related to injury. Thus, the injury response of fruit at different pigmentation (or development) stages indicated that nonbacterial ice nuclei may be involved in freezing injury of developing raspberries.

One of the primary factors that limits production of red raspberries is low-temperature injury (Daubeny and Fear, 1992; Nonnecke and Luby, 1992; Swartz et al., 1992). In regions where low temperatures frequently occur, primocane-fruited raspberries are often grown rather than floricanes-fruited types to avoid freezing injury during midwinter (Nonnecke and Luby, 1992). However, low-temperature injury can occur in the fall before all of the fruit is harvested, limiting yields of primocane-fruited plants (Nonnecke et al., 1993; Warmund and Nonnecke, 1993).

Low-temperature injury may be exacerbated by the occurrence of bacterial species on plant surfaces that incite ice formation at subfreezing temperatures (Lindow et al., 1982a; Maki et al., 1974). These ice-nucleation-active (INA) species are a component of the complex array of bacteria that commonly colonize plants. Bacterial colonization of plant surfaces varies in relation to plant characteristics and microenvironmental conditions. For example, morphological features of surfaces, such as cuticular wax (O’Brien and Lindow, 1989) trichomes, and venous and intercellular depressions (Leben, 1981), influence the spatial patterns of bacterial colonization of leaves. Additionally, populations generally increase immediately after rainfall but decline under drying conditions from solar radiation and wind.

While INA bacteria have been isolated from the foliage of many plant species, including Rubus idaeus (Lindow et al., 1978), the incidence of these bacteria on the fruit surfaces of primocane-fruited raspberries and their relationship to low-temperature injury have not been investigated. Raspberry morphology is complex, consisting of multiple druplets, the receptacle, and sepals. The sizes and surface characteristics of these tissues vary with stage of fruit maturity and, thus, may influence of INA bacterial colonization patterns. However, the distribution of these microorganisms has not been characterized on reproductive organs of any small fruit species. Therefore, the objectives of these studies were to 1) determine if INA bacteria reside on the fruit of primocane-fruited raspberry; 2) identify the site(s) on developing fruit where these bacteria may reside; and 3) determine if there is a relationship between fruit development stage and freezing injury of fruit naturally colonized with INA bacteria.

Materials and Methods

Fruit were collected from a 3-year-old planting of ‘Redwing’ red raspberries growing at the Horticultural Research Station at New Franklin, Mo. Fruit at five pigmentation stages [green, yellow, mottled (a mixture of yellow and pink druplets), pink, and red] were sampled to represent different stages of development (Perkins-Weazie and Nonnecke, 1992). Fruit samples (with the calyx and pedicel attached) were collected at 60 ± 15 cm above the soil surface, sealed in polyethylene bags, and immediately transported to the laboratory.

Thermal analysis. Fruit at each pigmentation stage were subjected to thermal analysis to determine their ice nucleation temperatures. Because of equipment limitations, one fruit of each pigmentation stage was sampled on 4, 5, 6, 7, 8, and 11 Oct. 1993 for thermal analysis. Each fruit was misted with about 0.5 ml deionized water, wrapped in aluminum foil, and placed on a 3 × 3-cm thermoelectric module (Melcor Materials Electronic Products Corp., Trenton, N.J.) inside an aluminum block in a programmable freezer (Tenney Engineering, Union, N.J.). The temperature in the freezer was lowered rapidly to 2 C, held at this temperature for 3 h, and then lowered 1C/h to −15 C. Fruit surface temperatures were monitored with a 5-µm-diameter copper constantan thermocouple (RdF Corp., Hudson, N.H.) attached to each module and recorded at 1-min intervals with a ADALAB-PC data acquisition system (Interactive Microwave, State College, Pa.) interfaced with a microcomputer. Data from each sample were then plotted and the temperature at which there was a sharp right angle deflection from the baseline of the thermal analysis curve was considered to be the ice nucleation temperature (Anderson and Ashworth, 1985). Temperature data were subjected to analysis of variance (ANOVA) and means were separated by LSD.

Isolation of bacteria from fruit. INA bacteria were isolated and quantified from fruit after thermal analysis. Six additional fruit at each pigmentation stage were collected from the field on 4, 6, and 7 Oct. 1993 to enumerate the natural INA bacterial population from a larger sample size than that used for thermal analysis. These
samples were not exposed to low temperatures before bacterial populations were quantified.

From both collections, individual fruit were placed in test tubes with 0.1 M phosphate buffer (pH 7.0) and vortexed for 1 min. Each fruit was removed and the buffer suspension was centrifuged at 3,000×g for 10 min. To quantify bacteria, the pellet was resuspended in the phosphate buffer, diluted serially, and plated on nutrient agar (Difco, Detroit) supplemented with 2.5% glycerol (v/v) and 100 ppm cycloheximide (NAGC medium) (Lindow et al., 1978). After plates were incubated at 25°C for 48 h, bacterial colonies were counted directly from NAGC plates. The INA fraction of these colonies was estimated by the plate-harvesting technique (Anderson and Ashworth, 1985). To harvest the bacteria, each petri dish was flooded with 5 ml sterile water. Each bacterial suspension was then placed in individual test tubes that were submerged in a refrigerated bath at -3°C. After 30 min at -3°C, the bath temperature was adjusted to -5°C and test tubes were held at this temperature for 30 min. Bacterial suspensions that froze at either of the test temperatures were considered to contain INA bacteria. The highest dilution at which a suspension froze was used to provide an order-of-magnitude estimate of INA bacterial density (Anderson and Ashworth, 1985; Anderson et al., 1987). This method was used because of its capacity to detect low densities of INA bacteria (Anderson and Ashworth, 1985; Anderson and Whitworth, 1993).

Bacterial population data from the thermal analysis experiment and from the additional unfrozen samples were subjected to a square-root transformation, and data for each date were analyzed separately by ANOVA. However, since the bacterial populations did not differ by collection date or exposure to freezing temperatures, data were pooled for statistical analysis by ANOVA and means were separated by LSD.

Isolation of INA bacteria from drupes, pistils, and sepals. On 6 Sept. 1994, fruit at green, mottled, and red pigmentation stages were collected to characterize further INA bacterial colonization on various parts of the fruit and the calyx. Sepals were removed from each of six fruit at each pigmentation stage, with the receptacle and drupe remaining intact. INA bacteria were then isolated from each separate fruit and calyx sample as previously described. Additionally, five pistils were removed from each of ten fruit at each of the three pigmentation stages. All pistils were plated directly on NAGC medium to detect the presence of INA bacteria. The density of INA bacteria on the sepals was greater (6.4 × 10^3 CFU/g fresh weight, respectively. Of these bacteria, INA forms occurred sporadically at detectable levels. For example, INA bacteria were not detected on any green drupe surfaces, and they were isolated from only 33% of mottled or red drupe surfaces. In contrast, INA bacteria were detected on the sepals of 83%, 100%, and 67% of green, mottled, and red fruit, respectively. The average density of INA bacteria on the sepals was greater (6.4 × 10^3 CFU/g fresh weight).

Enumeration of pistil density. Ten fruit each at the green, mottled, and red pigmentation stages were sampled on 1 Sept. 1994. The number of pistils present on two sides of a fruit in a 40x field of view of a dissecting microscope was recorded.

Low-temperature injury of fruit. On 6 Sept. 1994, fruit at each of the five pigmentation stages were collected to assess fruit injury after exposure to low temperatures. Ten fruit at each pigmentation stage were placed in moist cheesecloth and wrapped in aluminum foil for each of the seven test temperatures estimated to cause fruit injury. A 0.5-mm-diameter (24-gauge) copper constantan thermocouple was placed in contact with the drupe surface of one of the ten fruit contained in the foil packets to monitor tissue temperature during exposure to the low temperatures. Thermocouple output was read with a digital thermometer (HH-25T, Omega Engineering, Stamford, Conn.). Fruit samples were placed in a programmable freezer, (Tenney Jr. ; Tenney Engineering, Union, N.J.). After samples were held at 2°C for 3 h, they were cooled at 1°C/h and removed from the freezer during nucleation (as the first exotherm was observed) and after nucleation at –2.5, –3.0, –3.5, –4.0, 4.5, and –5.0°C. Samples were then thawed for 24 h in a refrigerator at 2°C before the fruit injury was assessed using a dissecting microscope.

Results

Thermal analysis. The first abrupt exotherm detected by thermal analysis represented ice nucleation (Fig. 1). The mean ice nucleation temperature for fruit at all pigmentation stages was –3.0°C ± 0.6. The nucleation temperature was not affected by fruit pigmentation stage (data not shown). A second broad exotherm was also detected from each sample subjected to thermal analysis at –3.4 ± 0.6°C. In most fruit tested, this broad exotherm was followed by multiple, abrupt low-temperature exotherms between –4.7 and –9.2°C.

Isolation of INA bacteria from fruit in 1993. Neither exposure to low temperatures nor collection date affected the total density of bacteria from fruit that grew on NAGC medium or INA bacteria (data not shown). However, bacterial populations generally declined as the fruit progressed in development from green to red (Table 1).

Of the sampled fruit, 97% was colonized with INA bacteria. INA bacteria, which represented <10% of the total bacteria recovered from the NAGC medium, were positively correlated with total bacteria (r = 0.61). The populations of INA bacteria recovered from individual fruit ranged from 8.4 × 10^3 to 1.5 × 10^4 colony-forming units (CFU)/g fresh weight.

Bacterial colonization of the sepals, drupes, and pistils in 1994. Total densities of bacteria (that grew on NAGC medium) on sepals and drupes from all samples averaged 3.7 × 10^4 and 6.4 × 10^4 CFU/g fresh weight, respectively. Of these bacteria, INA forms occurred sporadically at detectable levels. For example, INA bacteria were not detected on any green drupe surfaces, and they were isolated from only 33% of mottled or red drupe surfaces. In contrast, INA bacteria were detected on the sepals of 83%, 100%, and 67% of green, mottled, and red fruit, respectively. The average density of INA bacteria on the sepals was greater (6.4 × 10^3 CFU/g fresh weight).
g fresh weight) than that on drupes (5.4 × 10^7 CFU/g fresh weight). However, densities of INA bacteria (the sum of bacteria on sepals and drupe surfaces) were not affected by the pigmentation stage of samples collected in 1994.

Only 5 of 30 fruit had detectable levels of INA bacteria on pistils. These bacteria were recovered from a single pistil on a green and mottled fruit and two red fruit. On another red fruit, INA bacteria were recovered from three of the five pistils tested.

**Pistil density.** Green, mottled, and red fruit averaged 19, 6, and 4 pistils in the 40× field of view of the microscope. While pistils on the green and mottled fruit appeared intact, some of those observed on the red fruit were broken.

**Fruit injury after exposure to low temperatures.** The cheesecloth surrounding the red and pink fruit was stained with pigment, indicating that fruit were injured by low temperatures. However, staining was not evident when mottled, yellow, and green fruit were cooled to −6.5°C. Regardless of the staining response, when the fruit subjected to subfreezing temperatures were bisected, the receptacle tissue appeared water-soaked and oxidative browning was evident. In contrast, browning was not observed in the receptacle tissue of unfrozen controls. Thus, oxidative browning in the receptacle tissue was selected as the criterion by which injury was assessed. Extracellular voids also were observed in the receptacle tissue of some samples that exhibited oxidative browning. These voids were most likely a result of extracellular ice formation (Ashworth et al., 1989; Warmund et al., 1992).

**Discussion**

In 1993, INA bacteria were recovered from nearly all of the fruit (with attached sepals). The following year, INA bacteria were also recovered from most fruit, however, more bacteria were recovered from the sepals than from the drupe surfaces. INA bacteria were detected from the surface of only 22% of all drupes tested. In contrast, 83% of the calyxes tested were colonized with INA bacteria. These results indicate that the calyx is an important structure in the INA bacterial colonization of raspberries. The reason that green fruit were more heavily colonized with INA bacteria than red fruit in 1993 may be that the calyx encloses much of the developing fruit. Thus, moisture may be trapped or retained on developing fruit enveloped by a calyx, providing a favorable environment for bacterial growth. As the fruit enlarges, the calyx encloses a smaller proportion of the fruit and eventually the calyx is reflexed, resulting in a less-protected environment for bacterial multiplication.

Pistil density may also be a factor in the capacity of bacteria to colonize the fruit. Since INA bacteria were also found in association with individual pistils, these structures may also be sites for INA bacterial colonization on the fruit. The greater pistil density on green than on red fruit indicates that these organs may enhance moisture retention or enhance the relative humidity near the drupe surface. Thus, pistil density may also contribute to the differences in the INA bacterial populations isolated from the fruit at different pigmentation stages in 1993.

Leben (1981) reported that bacteria reside on protected sites when environmental conditions do not favor bacterial growth. *Pseudomonas glycinea* (Coerper) Stapp has been observed within soybean buds and trichomes on foliar surfaces (Leben et al., 1968). On soybean leaves, bacteria in water enter hollow trichomes and may survive in the lumen after the foliage has dried. These bacteria then resume growth under favorable environmental conditions.

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**Table 1. Natural populations of bacteria isolated from ‘Redwing’ red raspberries collected at five stages of pigmentation in 1993.’**

<table>
<thead>
<tr>
<th>Fruit pigmentation stage</th>
<th>Total bacteria (log CFU/g fresh wt)</th>
<th>INA bacteria (log CFU/g fresh wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>5.23</td>
<td>3.72</td>
</tr>
<tr>
<td>Yellow</td>
<td>4.45</td>
<td>3.50</td>
</tr>
<tr>
<td>Mottled</td>
<td>4.84</td>
<td>3.39</td>
</tr>
<tr>
<td>Pink</td>
<td>4.47</td>
<td>3.33</td>
</tr>
<tr>
<td>Red</td>
<td>4.18</td>
<td>3.02</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>0.42</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Values represent mean bacterial densities isolated from fruit that were unexposed and exposed to low temperatures in thermal analysis before bacterial isolations. Mean separation by LSD, *P* = 0.05.

*Mean densities (colony-forming units/g fresh weight) of all bacteria recovered from fruit that grew on NAGC medium.

*Mean densities of INA bacteria as determined by the plate harvesting method.

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**Table 2. Number of ‘Redwing’ raspberries that exhibited oxidative browning in the receptacle tissue at selected drupe surface temperatures.**

<table>
<thead>
<tr>
<th>Fruit pigmentation stage</th>
<th>Tissue temp (°C)</th>
<th>0.0°C</th>
<th>2.5°C</th>
<th>4.0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td></td>
<td>0 a</td>
<td>7 a</td>
<td>10 a</td>
</tr>
<tr>
<td>Yellow</td>
<td></td>
<td>1 a</td>
<td>7 a</td>
<td>8 a</td>
</tr>
<tr>
<td>Mottled</td>
<td></td>
<td>1 a</td>
<td>8 a</td>
<td>10 a</td>
</tr>
<tr>
<td>Pink</td>
<td></td>
<td>7 b</td>
<td>10 a</td>
<td>10 a</td>
</tr>
<tr>
<td>Red</td>
<td></td>
<td>10 b</td>
<td>10 a</td>
<td>10 a</td>
</tr>
</tbody>
</table>

*Means within the same column that are followed by the same letter do not differ significantly as determined by a chi-square test, *P* = 0.05, n = 10.

*Samples were removed from the freezer as the first exotherm was observed at a drupe surface temperature of 0°C.

*Samples were removed from the freezer after ice nucleation and additional cooling at 1°C/h.

During the viability test, the surface temperature of most fruit in contact with a thermocouple dropped to about −3°C before an exothermic response was observed. One foil packet, containing fruit at each stage of maturity, was removed during the exothermic response when the drupe surface temperature increased to 0°C. More pink and red fruit removed from the freezer at this time exhibited oxidative browning than green, yellow, or mottled fruit, as determined by chi-square analysis (χ² = 28, *P* = 0.05) (Table 2). Nearly all fruit removed from the freezer after the first exothermic response was observed were injured at or below −2.5°C.
O’Brien and Lindow (1989) reported that cells of *Pseudomonas syringae* van Hall (an INA bacterium) were able to survive on foliar surfaces when incubated at high light intensity and low humidity (40% RH) for 72 h. However, in their study, plant leaves with waxy cuticles generally had lower epiphytic populations than those with rough trichomatic surfaces. Since prickles and dense pubescence are prominent on the surface of raspberry sepal, these may be protected sites for INA bacteria.

Although this study presents evidence that the calyx is an important site of INA bacterial colonization near the fruit, its contribution to freezing injury is unknown. There may be other favorable microsites for INA bacterial colonization (e.g., interdruplet depressions) that influence ice formation and fruit injury. Bacterial populations have typically been estimated as CFU/g fresh weight or cells/g fresh weight (Anderson and Ashworth, 1985; Anderson et al., 1987; Andrews et al., 1986; Gross et al., 1984; Lindow et al., 1978). However, this unit of measurement may not be appropriate for comparing bacterial colonization among microsites on tissues that vary in size and surface morphology. In the future, it may be appropriate to express bacterial density relative to unit surface area of tissue rather than biomass.

INA bacterial densities were only a small proportion of the total bacterial densities detected on NAGC medium (Table 1). The reason for the lower INA densities is that not every bacterial cell or CFU in a population serves as an ice nucleus (Hirano et al., 1985; Lindow et al., 1982b). The calculation of total bacteria is based on the total number of CFU counted directly from the NAGC plates multiplied by the dilution factor. In contrast, the plate-harvesting technique used to quantify INA bacteria is based on the assumption that only one bacterial colony occurs at the lowest dilution at which the bacterial suspension froze (Anderson and Ashworth, 1985; Anderson et al., 1987; J.A. Anderson, personal communication).

Results from thermal analyses indicated that there were multiple freezing events that occurred during low-temperature exposure (Fig. 1). The first abrupt exotherm represented the initiation of ice formation (i.e., nucleation) (Anderson and Ashworth, 1985). The second broad exotherm most likely represented extracellular ice formation (Graham and Mullin, 1976; Quamme, 1978). In most fruit tested, this exotherm was followed by multiple, abrupt low-temperature exotherms, which may represent freezing of isolated fractions of water in druplets or in other organs (Warmund et al., 1988).

Pigmentation stage did not affect the ice nucleation temperature of fruit sampled for thermal analyses, even though INA bacterial populations differed among stages in 1993 (Table 1). In the following year, densities of INA bacteria were similar among fruit at all pigmentation stages tested. However, bacterial densities recovered from the fruit were low in this collection. Others have suggested that a threshold level of INA bacteria is necessary to increase the number of ice nuclei on plant tissues significantly (Anderson et al., 1987; Hirano et al., 1985; Maki et al., 1974). Thus, bacterial densities may have been too low to affect the ice nucleation temperature. In both years of this study, no precipitation was recorded in the 4 days preceding the collection of fruit. Although dew, relative humidity, and solar radiation were not measured, they may have influenced the bacterial densities on the fruit (Leben, 1981; O’Brien and Lindow, 1989).

The relationship between the INA bacterial colonization and freezing injury of raspberries is unclear. The range of temperatures at which receptacle injury was observed was similar to that for ice nucleation. Although the ice nucleation temperature was not affected by pigmentation stage in the thermal analyses, results from the viability test indicate that freezing injury is affected by pigmentation stage or, more likely, fruit developmental stage. Andrews et al. (1983) reported that less injury was associated with higher nucleation temperatures in *Prunus* floral buds inoculated with INA bacteria, just before petal tip emergence. However, immature *Prunus* fruit were injured whenever the tissue froze. Proebsting et al. (1982) suggested that ice crystals formed within the vascular system of the stem attached to developing *Prunus* fruit and then spread progressively into the fruit. In our study, the differential injury response of fruit at different pigmentation (or development) stages suggests that there may be other factors, such as intrinsic ice nucleators or ice nuclei of nonbacterial origin, involved in freezing injury of developing raspberries (Andrews et al., 1983; Anderson et al., 1987). To elucidate the relationship between INA bacterial colonization and freezing injury, future work should include raspberries that are free of INA bacteria.

**Literature Cited**


