Strategies for Frost Protection in Cereal Crops: Assessing the Efficacy of Ice Nucleation Inhibiting Bactericides and Cryoprotectants

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Abstract

Winter-grown cereal crops are sensitive to frost damage at the reproductive stage which can negatively impact yield. Ice Nucleating Bacteria (INB) are naturally present in cropping systems and increase the risk of frost damage. These bacteria possess an Ice Nucleation Protein (INP), which can catalyse freezing at temperatures as warm as -2°C. Previous studies focusing on INB and in-field thermography have identified older leaves and stubble as primary sites of ice formation, leading to subsequent frost damage in the plant's reproductive parts. In this study we have evaluated two bactericidal products, Cu+Zn and K-Soap, as well as two cryoprotectants (Cryo-E, and Cryo-F). In the 2023 season, we have measured the effects of these chemical products to inhibit ice nucleation activity of senesced leaf samples, and to limit yield loss from frost damage of field grown wheat and barley. Senesced leaf tissue samples were collected fortnightly to determine their freezing point, which was used as a proxy for the potential frost susceptibility of the plants. Floret sterility and grain yield data indicated that mild to moderate frost events in 2023 didn't allow discrimination among treatments for frost damage. However, the observed reduction in the freezing temperature of older leaves following direct application of some of the products might impact floret sterility and yield if a discriminative level of frost occurs during the reproductive stages of the plant. Future research will focus on optimising product application and establishing correlation between frost damage and ice nucleation activity in older leaves of wheat and barley.

Keywords

Frost management, broadacre cereal crops, Ice Nucleating Bacteria, *Pseudomonas fluorescens*.

Introduction

In Western Australia (WA), winter grown cereal crops are susceptible to frost damage at the reproductive stage, resulting in yield loss and reduced quality of grain. On average economic losses from frost damage are $400 million per annum, however, this can vary widely depending on the severity of frost. One factor that increases the risk of frost damage is the presence of Ice Nucleating Bacteria (INB). INB are ubiquitous in the environment, typically inhabiting decaying leaf matter (Lindow et al., 1978). In WA cropping systems these bacteria live on senesced leaves and increase the risk of frost damage to cereal crops (Bekuma et al., 2021; Biddulph et al., 2021). INB include *Pseudomonas* species such as *P. syringae* and *P. fluorescens* (Maki et al., 1974). These bacteria produce an Ice Nucleating Protein (INP) which catalyses ice formation in water at temperatures as warm as -2ºC (Lukas et al., 2022). The INP can remain active, even after the bacterial cell has died, therefore to reduce the risk of ice nucleation through chemical means, the protein must be degraded (Lindow, 2023).

There has been some previous work to test various bactericides to inhibit INB *in vitro* (Zagory et al., 1983), and *in planta* (Menkissoglu and Lindow, 1991). However, bactericides to inhibit nucleation activity of INB have not been tested in cereal crops in a field environment. Cryoprotectants are substances that increase freezing tolerance and some testing has been done to use them to increase the frost tolerance of wheat (Todorova et al., 2012). In the current study, two bactericidal products, a Cuprous Oxide and Zinc Oxide (Cu+Zn) mixture and a Potassium Soap with Silicate (K-Soap), and two cryoprotectants (Cryo-E and Cryo-F) have been tested to see whether they can reduce the ice nucleation activity of INB on senesced leaves, and whether they can protect the crops from yield loss due to frost damage.

Methods

Description of field trial site

A field experiment was conducted in 2023 to determine the efficacy of some bactericide and cryoprotectant products in protecting crops from yield loss due to frost damage. The trial was conducted at the Department of Primary Industries and Regional Development's (DPIRD) research site in Dale, Western Australia (-32.203395, 116.757311), which has a history of being frost prone (Leske and Biddulph, 2022; Stutsel et al., 2020).

Field trial to assess frost protection products

Wheat (*Triticum aestivum* cv. Sceptre) and barley (*Hordeum vulgare* cv. Spartacus) were planted at Dale, across 4 different times of sowing (TOS 1 April 13th, TOS 2 April 28th, TOS 3 May 5th, TOS 4 May 11th), to increase chances of the frost occurrence at the reproductive stage of the crop. Two bactericides and two cryoprotectants were tested in the trial, along with INB treated plots inoculated with *P. fluorescens* strain RW117 and a control with no treatments applied (Table 1). Stubble from the previous year was not burnt, and it served as the source of natural inoculum of INB for the rest of the trial. The treatments were then applied throughout the season, as described in Table 1. A frost event was defined as the forecast temperature dropping below 2°C to determine the threshold for bactericide application. Plots were sprayed with the bactericides at canopy level until mid-August. After mid-August, the spraying shifted to the interrow to target the lower canopy leaves. Cryoprotectants were applied to the upper canopy throughout the season as per recommndation.

Table 1. Description of frost protection treatments and application details used in the 2023 field trial at Dale, WA

Treatment	**Abbreviation**	**Classification**	**Application**
Cuprous Oxide and Zinc Oxide	Cu+Zn	Bactericide	Applied ~12 hours prior to frost event
Potassium Soap with Silicate	K-Soap	Bactericide	Applied ~12 hours prior to frost event
Cryoprotectant-E	Cryo-E	Cryoprotectant	One off application at Z37
Cryoprotectant-F	Cryo-F	Cryoprotectant	Applied at Z37, then fortnightly after
Ice Nucleating Bacteria	INB	Control	One off application from Z35-40
Control		Control	N/A

Sampling field trial

To assess the ice nucleation activity of the plants, senesced leaf tissue samples were collected from each plot fortnightly, once the plants had reached Z50. To avoid transfer of bacteria between samples, hands were sterilised with 70% ethanol between plots with different treatments. Leaf tissue samples were stored in a freezer at -20ºC until ready for assessment. To assess the extent of frost damage to the crop 30 random heads were picked from each plot after the plants had reached Z80. The heads were stored in a freezer at -20ºC until ready for assessment.

Ice nucleation activity assessment of leaf tissue samples

Leaf tissue samples were prepared by chopping them into small pieces using flame sterilised scissors. Chopped leaf was added to sterile distilled water in a 1:5 ratio and thoroughly mixed. To assess the ice nucleation activity, or freezing temperature, of the leaf tissue solutions, four-inch wide Parafilm sheets were floated in the reservoir of a programable refrigerated circulator cooling bath, which was set at 0°C. Ten 15 µl droplets of the solutions were aliquoted on to the Parafilm. The temperature of the cooling bath was reduced at a rate of 1°C every 5 minutes while an infrared thermography camera, positioned above, captured a timelapse of images every 15 second. As freezing is a thermographic reaction, heat is released as the droplets freeze, which is captured by the infrared camera. To assess the ice nucleating temperature at which 50% of the droplets had frozen (INT50°C), the images were analysed by counting the number of droplets that turned orange (freezing initiated) in each image.

Floret sterility of wheat and barley heads

The floret sterility of the wheat and barley heads from the trial was measured to assess the extent of frost damage to the crop. The sterility of the two outer florets of each spikelet was measured. The floret sterility is expressed as the % of sterile florets in a head.

Results

Ice nucleation activity of leaf tissue samples

The INT50 (°C) is a measure of the freezing temperature of the leaf tissue samples, giving an indication to the presence of INB and the increased risk of frost damage they may pose. When the freezing temperature of the sample is lower, the frost risk is decreased, as the leaf tissues require much lower temperatures for freezing to occur. When senesced leaf tissue samples were taken within two days after the bactericides were applied on the lower canopy, there was a reduction in freezing temperature of some treated leaf tissues (Fig. 1). In early sown wheat (Fig. 1, plot a), there were significant differences ($p>0.001$) in the freezing point of leaf samples from plots with different chemical treatments. Samples from Cu+Zn treated plots exhibited a freezing temperature of -5.3°C, which was -0.8°C lower than samples from INB inoculated plots. Similarly, in early sown barley (Fig. 1, plot b), there were also significant differences ($p=0.003$) in the freezing temperature of leaf tissue samples. The Cu+Zn and K-Soap treated samples had significantly lower freezing points (-4.7°C and -4.6°C respectively), than the INB samples which froze at -4.3°C. It was expected that the cryoprotectants would not significantly impact the ice nucleation activity of the senesced leaves, as they are not antimicrobial, therefore will not affect the INB.

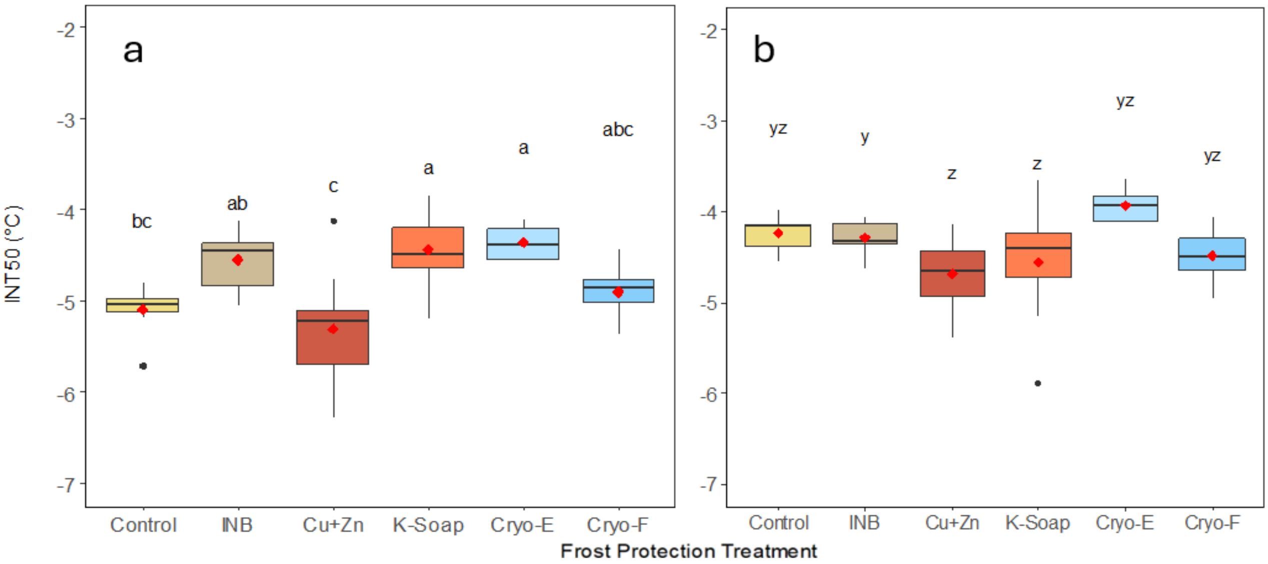

Figure 1. The freezing point, INT50 (^OC), of senesced leaf tissue samples of wheat (a) and barley (b) plants from TOS 1 of the field trial in Dale, WA. Leaf samples were taken within two days of the bactericides being applied to the lower canopy. Red dots indicate mean INT50 (^OC) values. Same letters indicate that mean INT50 values are not significantly different at (α=0.05) following Tukey's HSD post-hoc test.

The Cu+Zn product contains Cuprous Oxide and Zinc Oxide, which are known antimicrobials. Copper products have previously been show to be effective in reducing populations of INB in fruit tree crops (Menkissoglu and Lindow, 1991). The K-Soap product is a soap, which are known to solubilise bacterial membranes through surfactant action (Falk, 2019). Scanning Electron Microscopy of cells of an ice nucleating strain *P. fluorescens* treated with the Cu+Zn and K-Soap products show that the bacterial membranes are damaged, which may degrade the INP and explain the reduction in ice nucleation activity of senesced leaves (Cooper et al., 2024).

Floret sterility of wheat and barley heads

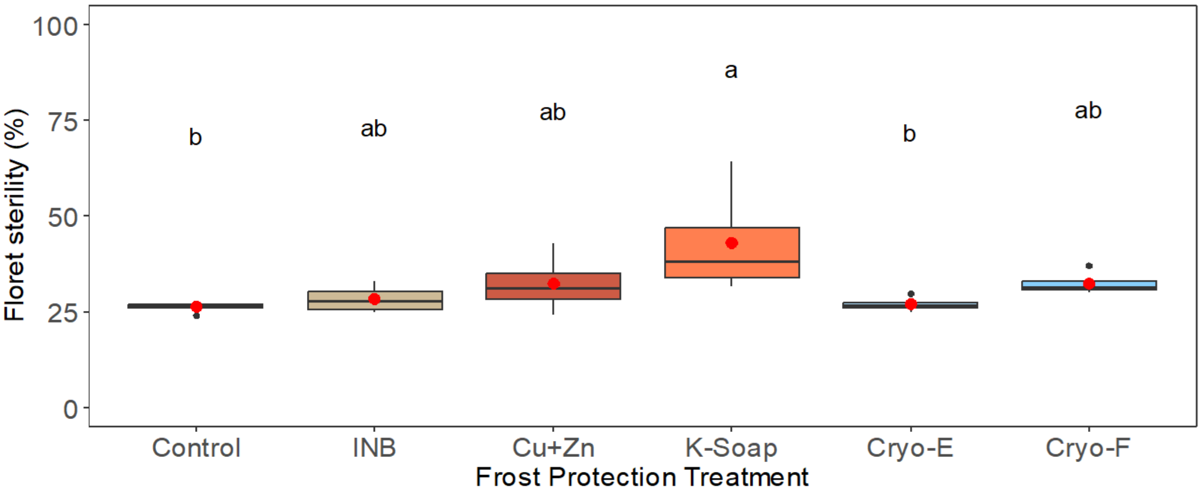

Figure 2. The floret sterility (%) of wheat from TOS 1 from the field trial at Dale. Different chemical treatments have been applied to the plots prior to frost events. Red dots indicate mean floret sterility (%) values. Same letters indicate that mean floret sterility values are not significantly different at (α=0.05) following Tukey's HSD post-hoc test.

The floret sterility is a measure of the number of florets that did not form grain, which gives an indication to the potential yield loss from frost damage. In early sown wheat, there were significant differences in the floret sterility of the heads from plots treated with different frost protection products. There was greater sterility (p=0.033) in heads from plots treated with the K-Soap bactericide (43%), than there were in the control plots (26%). In the early sown barley, there were no significant differences in the floret sterility between treatments (data not presented). The increased sterility in K-Soap treated plots may be related to the surfactant nature of the product, which may increase the spread of water on the surface of the plant, aiding in the spread of ice from nucleation events. The mild to moderate frost events in 2023 season didn't allow discrimination among treatments for the level of floret sterility from frost damage.

Conclusion

In this field trial, we evaluated bactericides and cryoprotectants for their efficacy in reducing ice nucleation activity in senesced leaf tissues and protecting wheat and barley from frost related yield loss. The Cu+Zn product was the most effective at reducing ice nucleation activity of the senesced leaves, however, these results are from one year of field testing, so further years of this field trial are required to confirm its efficacy. Cu+Zn shows potential for inhibiting ice nucleation activity of INB in cereal crops, although further research is needed to determine whether this product is effective at mitigating frost related yield loss. Given that a 1°C increase in frost tolerance is equivalent to an 8% increase in yield (Zheng et al., 2015), the results of this study show that a further research on these chemical products may provide significant benefits to growers in the future.

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