Bacterial ice nucleation activity in rainfall and on crop residues may be increasing frost damage in WA cropping systems

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Abstract
Stubble retention and rainfall prior to frost events increases frost damage in the WA cropping system, and ice nucleating bacteria may be responsible. Pseudomonas syringae were found to be present in spring rainfall and on crop residues which were able to produce ice nucleating proteins which cause freezing at warmer subzero temperatures. In-field thermography found canopies freeze from the ground up with ice nucleation caused by ice nucleating proteins produced by P. syringae on stubble, before moving to older senesced leaves and up the plant to the head. Some spring rainfall collected from frost prone regions of the WA production zone contain P. syringae and their ice nucleation activity was highest (-5.5°C) in <5mm events which fell in the early evening. Commercially derived ice nucleating proteins from P. syringae, when applied as simulated rainfall prior to frost events, increased frost damage several fold in a dose response manner. Hence it's likely that rainfall prior to frost events and retained stubble increase frost damage due to greater bacterial ice nucleation activity and the ice nucleating proteins they produce. Ongoing frost research needs to address biological ice nucleating activity in the western cropping system.

Keywords
Frost, wheat, Pseudomonas syringae, thermography, stubble, proteins.

Introduction
Stubble retention and rainfall prior to frost events increases frost damage in the WA cropping system, and ice nucleating bacteria may be responsible. Pseudomonas syringae were found to be present in spring rainfall and on crop residues which were able to produce ice nucleating proteins which cause freezing at warmer subzero temperatures. In-field thermography found canopies freeze from the ground up with ice nucleation caused by ice nucleating proteins produced by P. syringae on stubble, before moving to older senesced leaves and up the plant to the head. Some spring rainfall collected from frost prone regions of the WA production zone contain P. syringae and their ice nucleation activity was highest (-5.5°C) in <5mm events which fell in the early evening. Commercially derived ice nucleating proteins from P. syringae, when applied as simulated rainfall prior to frost events, increased frost damage several fold in a dose response manner. Hence it's likely that rainfall prior to frost events and retained stubble increase frost damage due to greater bacterial ice nucleation activity and the ice nucleating proteins they produce. Ongoing frost research needs to address biological ice nucleating activity in the western cropping system.

Methods
Infield thermography
To determine where and when frost damage occurs as a result of ice nucleation, in-field thermography was carried out during the night, with forecast frost events, in the spring of 2019 and 2020. A thermal camera (FLIR-T62101 in 2019 and FLIRAX5 in 2020) was set up to observe the stubble/crop residue in the inter-row, as well as ~30 stems and spikes. Plants were targeted for the most frost susceptible stages from early head emergence to early grain set (~Z49-71). Manual interpolation of the thermal images was used to determine the source of ice nucleation as well as movement of the freezing process.

Rainwater ice nucleation activity
Spring rainfall was collected throughout the frost prone areas of the Great Southern and south coast, in 2018 to 2020, from manual rain gauges (~07:00-09:00am), and samples were immediately frozen/stored at -20°C. The ice nucleating temperature (INT50) was determined in Dec 2020 by droplet freezing assay, described by Vali and Stansbury (1965), with minor modifications. In brief, an aliquot of 20 droplets (10 µl) of rainfall was placed on Parafilm, floating on a temperature-controlled cooling bath, pre-chilled to 0°C. The temperature of the cooling bath was lowered by 1°C every five min and thermography (FLIR-T62101) was used to capture the freezing events of the droplets. The temperature at which 50% of the droplets (INT50) freezes was determined.

Dose response to ice nucleating proteins
Trials were sown into a flat, frost-susceptible part of a paddock, which had a history of multiple frost events. Scepter wheat was sown using local agronomic practices. The trials consisted of 1.8x5m plots with 6 sowing dates at ~250GDD intervals ranging from ~ 10th April to 25th May, in order to expose plants to reproductive frost damage throughout the frost window between August and September of 2019 and 2020. Prior to a forecast frost event (minimum 2°C night temperature), the sowing dates between ear peep and early grain set (Z49-71) were sprayed in the early evening, with 0, 2, 4,6,8 g^{-1}L 2019 or 0, 0.004, 0.04, 0.4 and 4 g^{-1}L 2020 of ice nucleating protein until the canopy was at dripping point. (~0.5mm rainfall). There are two controls a dry canopy control of no water and a wet canopy control of sterile water (0.0g^{-1}L. From Zadok (Z) 40 (flag-leaf sheath extending) onwards, plots were assessed weekly for crop developmental stage. At Z85 (late dough) 30 heads were collected from three locations near temperature sensors for floret sterility (FS) assessments, irrespective of whether frosts had occurred or not. Biomass cuts were collected at Z89 (hard dough) for harvest index, 1000-grain weight, hectolitre weight and screenings. At maturity, final grain yield was determined with a plot-header. Grain yield and its components were analysed by ANOVA and LSD0.05 used to compare the means of the 4 reps. Values presented are the predicted means plus and minus standard errors. ANOVA indicated where treatment effects were not significant (NS) at $P<0.05$.

Results

Infield thermography
Infield thermography during frost events in 2019 and 2020, after the emergence of the head, indicates the canopies freeze from the ground up. An example is displayed in Image 1. Ice nucleation seen as a rise in temperature associated with the latent heat of freezing (seen as white plant parts indicated with arrows) started first on the stubble and crop residue in the inter-row, before moving to the older senesced leaves and up the plant leaf sheath, stems and spikes (Image 1). The freezing process was also observed to be a gradual process, taking ~20-30min for a plant to freeze from the ground up to the spike. A machine learning based approach to optimise and merge digital and thermal images and identify heat exotherms/freezing is ongoing. Our lab results on ice nucleation activity of various tissues confirm the likely order of freezing (Bekuma et al 2021) and indicate that stubble and/or older leaves are the primary sites of ice nucleation (-4.7°C to -5.7°C) which increase the risk of frost damage during the most susceptible stages. The higher INA on the stubble and older leaves is likely to be caused by ice-nucleating bacteria (INB) which were plated out on selective media and are undergoing sequencing.

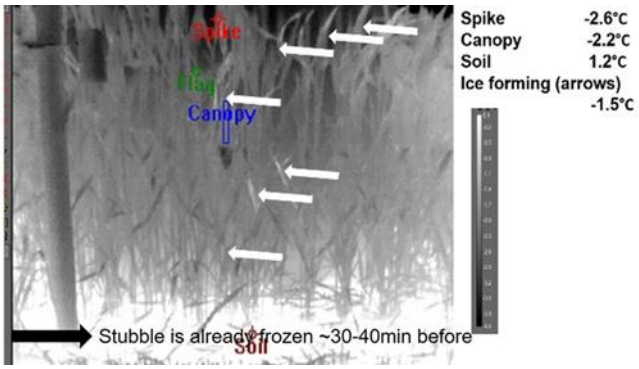

Image 1. Infra-red thermography image from the infected stubble treatment at Dale Research Station on 6th September 2019 at around 5:30 am. Arrows indicate a heat exotherm associated with the ice nucleation and freezing of the plant tissue.

Dose response to ice nucleating proteins
There was a clear increase in visual symptoms of frost damage from ice nucleating protein treatments, during 2019 and 2020, in a dose response manner. The frost damage symptoms on stem, head, flower and grain were consistent with the ice nucleating protein treatments and simulating rain on the crop in the late afternoon/early evening before a frost event but was not observed to the same extent in controls.
In 2019, when there was 10-60% floret sterility in the no water control, the ice nucleating proteins increased frost damage in 17th April to 17th May sowings (Figure 1a). There was no frost damage observed in the no water control of 10th April 2019 sowing, while frost damage occurred (>60%) in the no water control of 24th April 2019 sowing, however, was not statistically different. The lower harvest index and lower grain yield was associated with the increase in floret sterility, occurred from ice nucleating protein treatments (Figure

1a), however, biomass and 1000 grain weight remained unaffected (data not shown). Application of sterile water alone (0.0g/L) did not show any significant increase in frost damage over the no water control. This suggests an ice nucleator must be present to increase frost damage, not just wetting the canopy.

In 2020, the Dale site experienced fewer frosts, and not all TOS had the ice nucleating protein applied. The first sowing of 15th April was not exposed to frost and not sprayed; hence the data is not presented. In the sowings of 29th April and 5th May, there were several minor frost events which caused ~10% sterility in the controls, however the ice nucleating protein was applied to them. From the minor frost conditions, and by the application of the ice nucleating protein, increased floral sterility due to frost damage occurred in a logarithmic manner from ~10% in the dry and no water controls to ~45% with $4g^{-1}L$ ice nucleating protein in the 29th April and 35% in 5th May sowing. The increase in floret sterility was also associated with a reduction in harvest index and grain yield (Figure 1b). The application of sterile water alone ($0.0g^{-1}L$) on 29th April and 5th May did not show any significant increase in frost damage over the control, suggesting that an ice nucleator presence is must for the increased frost damage. As there was no frost forecast (<2°C) during the canopy susceptible window (Z45-71), the ice nucleating protein was not applied to the following sowing date, therefore, no data was presented. However, the forecast temperature threshold was relaxed to an overnight minimum of <4°C for the sowing dates of 19th and 26th May to capture a frost. Several applications were made on this forecast, but none eventuated into a frost, therefore, these sowings were not frosted (floret sterility <10% in all treatments). Without frost, there was no effects of the ice nucleating proteins on sterility, harvest index or grain yield indicating that the proteins or sterile water alone does not cause any damage to the crop.

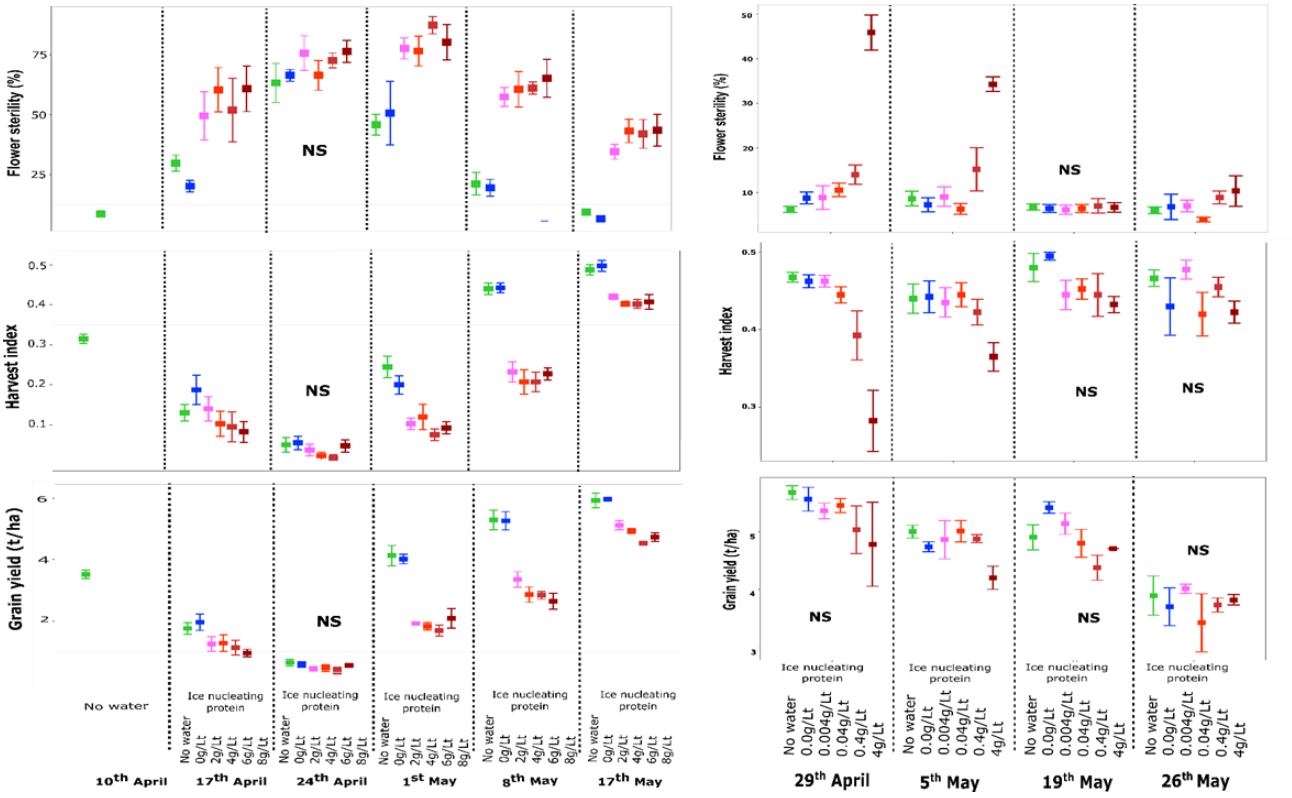

Figure 1. Floret Sterility (a), harvest index (b) and grain yield (c) of Scepter wheat at Dale 2019 (a) and (b) 2020 as affected by application of sterile water and dose response of ice nucleating protein across different times of sowing. Values are the mean of 4 reps +/- SE.

Ice nucleation activity of spring rainfall

Spring rainfall collected across 15 sites in the frost-prone areas of the Great Southern and South Coast wheat belt were assayed for ice nucleation activity. Of the 175 samples assayed, ice nucleating activity was detected in 30 samples, 16 of which were from rainfall events of <5mm. Of the 16 ice nucleated rainfall samples the highest activity (INT50 >-8°C) was observed from the rainfall events that fell in the late afternoon or early evening between late September (26-27th Sept) and early October (8th Oct) 2020. All these rainfall events occurred in 4 sample locations in the western and upper Great Southern (4 sites within 150km of each other) at Wickepin, Wagin Qualeup and Kojonup. The areas with 5 to 10 mm rainfall events

also detected some ice nucleation activity (5 out of 30 samples) (INT50 >-12°C) but the activity was less at greater rainfall events >10mm (2 out of 30 samples). There was limited activity in the 2018 and 2019 samples The longer storage time (24-12 months) compared to the 2020 samples may have contributed to this since the storability of rainwater and ice nucleation activity is unknown (data not shown).

Conclusion

Rainfall prior to frost events in combination with retained stubble appears to increase frost damage due to greater ice nucleation activity. Infield thermography after head emergence indicates canopies freeze from the ground up. Ice nucleation starts first on the stubble and crop residue in the inter-row, before moving to the older senesced leaves and up the plant leaf sheath, stems and reproductive tissue of wheat and this is confirmed by the INT50 of samples which peak at flowering. Increasing concentrations of ice nucleating protein applied prior to frost events increased frost damage several fold in a dose response manner, but a wet canopy by itself did not increase frost damage. Spring rainfall has P. syringae ice nucleation activity and this is highest in <5mm rainfall events that fall late in the afternoon/early evening which is likely to be responsible for increased frost damage in commercial crops. Further research needs to study the management practices in order to develop techniques to control ice nucleating bacteria and the associated frost damage other than stubble removal.

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