Stubble and senesced leaves are the primary sites of ice nucleation activity in wheat

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Abstract
Economic loss to frost damage is increasing in WA wheatbelt due to the increased sensitivity of cereal plants to frost. This study investigated upper and lower leaf canopy, stubble and soil as a potential site of ice nucleation activity (INA) and tracked the changes in INA during the plant development. We found that older leaves of wheat are the primary sites of ice nucleation (-4.7°C) followed by stubble (-5.7°C) which increase the risk of frost damage during the most susceptible stages (heading and flowering). However, healthy and green upper canopy leaves (flag and flag-2) and the soil have lower INA (<-11°C) during these stages. We anticipate the higher INA on the stubble and older leaves was caused by ice-nucleating bacteria (INB), known to cause frost injury to sensitive plants at -5°C. Stubble retained or applied during the growing season further exacerbate additional frost risk by potentially increasing the INB load.

Keywords:
Frost, activity (INA), wheat, stubble, leaves

Introduction
Agronomic, genetic and climatic research indicates a very weak correlation between air temperature and frost damage. Previously, trials and treatments that experience similar temperatures often showed different levels of frost damage and these inconsistent results regularly occur within a season at different sites and from year to year at the same site. One possibility that has not been explored within the WA cropping system is whether ice nucleation bacteria (INB), either present in rainfall before frost events associated with the weak frontal systems or in situ in crop residue and dispersed by rainfall and/or actively growing on the plant canopy, could be responsible for the increased sensitivity of cereal plants to frost. Two bacterial genera, *Pseudomonas syringae* and *Pantoea agglomerans* (formerly *Erwinia herbicola*), found positive for ice nucleating activity (Kozloff et al. 1983; Newton & Heyward 1986; Mazarei & Kerr 1987) are present in the WA cropping landscape. The implication within a cropping environment is that in the presence of these bacteria, water and plant tissue can freeze at temperatures well above what it would normally freeze at, causing cell damage, desiccation and death, resulting in significant yield loss. Without these ice nucleating agents, a plant may remain supercooled with no damage to the reproductive and vegetative parts. Even frost-sensitive plants grown under a glasshouse environment can supercool to -8°C to -10°C with mild injury (Lindow 1983).

Past frost and stubble management trials have found differences in frost severity, duration and damage (Jenkinson et al, 2014; Smith et al, 2017 and Biddulph et al, 2019). However, the cause-effect relationships have not been established. For instance, stubble retention even with low stubble loads (1 t/ha) can increase frost damage in wheat (Biddulph et al, 2019). However, it is not known whether stubble increases the frost damage, which increases the frost severity and duration, or whether the stubble increases the frost severity and duration, thereby increasing the damage. The mechanism by which stubble increases frost risk in wheat crops remains to be investigated. The work presented here investigates the temporal variability in the ice-nucleating ability of leaf, stubble and soil samples collected from wheat plots managed under stubble retention and blanket burnt from head emergence to grain filling stages.

Methods
Leaf, stubble and soil samples were collected from replicated plots of wheat (*Triticum aestivum* cultivar Scepter), planted on 17th May 2020 and grown under "infected stubble" (crop residue from frosted wheat in the previous season) and removed stubble management (burnt) approach at DPIRD Dale frost research site, WA. Starting from 20th August to 26th October 2020, samples were collected on six different dates at approximately two-week intervals. The plants were at awn emergence stage (Z49) when sampling started and sampling ended at late ripening (Z87). Stubble samples were collected from plots applied with "infected stubble", while soil samples were collected from plots with no stubble. Leaf samples were collected from

three different leaf positions: flag leaf; flag minus 2; flag minus 4 and older senesced leaves from both plots with “infected stubble” and no stubble.
The INA of samples was determined by droplet freezing assay as described by Vali and Stansbury 1965) but with minor modifications. Flame-sterilized scissors were used to cut 1 g of leaf and stubble samples into 2-3mm long pieces, which were added to 5 ml sterile distilled water and mixed on a wrist shaker for 2 minutes. Soil suspensions (20% w/v) were prepared by vigorous mixing with sterile distilled water. A total of 20 droplets (10 µl each) of plant homogenate or soil suspension were aliquoted on a parafilm floating on a temperature-controlled cooling bath pre-chilled at 0°C. The temperature of the cooling bath was lowered by 1°C every five minutes and thermographic camera (FLIR-T62101) was used to capture the freezing events of the droplets. Data collection was terminated when the sterile distilled water control droplet started to freeze. Since the same water was used in sample preparation, any nucleation at lower temperatures after this could not be attributed to the sample. The average temperature required to freeze 50% of the droplets (INT50) was then determined.

Results

Older leaves and stubble are the site of high INA

The present study was undertaken to identify the location of ice nucleation and to understand its temporal variation from head emergence to grain filling in wheat grown under contrasting stubble management (stubble retained and burnt). Results indicated that flag-4 leaves (plus older senescing leaves) had the highest INA (INT50 between

-4.7 and -6.3oC) followed by stubble (-5.7 to -6.7°C) of all the samples (Figure 1) suggesting older leaves and stubble are the site of highest INA that trigger freezing in wheat during natural frost events. The high INT50 values (-4 oC and -6oC) also suggest the presence of biologically active ice-nucleating bacteria (INB), known to cause frost injury to sensitive plants at -5oC (Lindow et al, 1982). Older leaves on lower parts of the plant and stubble, both protected from direct sunlight, seems to be preferred by INB consistent with previous findings that identified decaying leaf litter as a source of biological ice nucleating microorganisms (Leory et al, 1974; Schnell and Vali 1972; Schnell and Vali 1976; Vali et al, 1976).

Highest INA coincides with the most sensitive stage

INA of stubble and flag-4 samples increased from initial INT50 values of between -5.7oC to -6.7oC at Z49 to peak values during heading (Z55) and flowering (Z65) (between 20th Aug and 17th Sept). The high INA (~ -4.7oC) was maintained through to the grain milk stage (Z73) before starting to decline as the grain entered the dough development stage (Figure 1). The peak ice nucleation
activity period coincided with the most frost susceptible stages (heading and flowering). Interestingly, stubble removal by blanket burning did not keep flag-4 and older leaves free from high ice active nuclei but rather only appeared to delay peak activity (Figure 1). Higher INA was detected at bottom of the plant, consistent with infield thermography studies that have shown wheat plants freeze from the ground-up (Biddulph et al, 2021; Livingston et al, 2017).

Frost risk from the soil and upper canopy leaves is negligible

Leaf samples derived from the upper and mid-canopy of the wheat plants (flag and flag minus 2) as well as from soil froze at low temperatures, as indicated by the INT50 values (Figure 1). However, as the season progressed and these leaves started to senesce naturally, their activity briefly increased and then declined again. This difference between young and senescing leaves could be explained based on the numbers of INB bacteria present, as has been shown in Lindow and Upper (1978). The peak INA activity of these leaves (INT50 ~ -6oC) was not high enough to pose a frost risk and was also reached during a stage of growth when wheat is least susceptible to frost damage. Consequently, the role of the upper canopy leaves in frost risk is negligible. On the other hand, the soil has weak INA which remained below -9oC at all sampling times (Figure 1), consistent with the poor survival of INB such as P. syringae in a soil (Moor 1988; Hollaway and Bretag 1997).

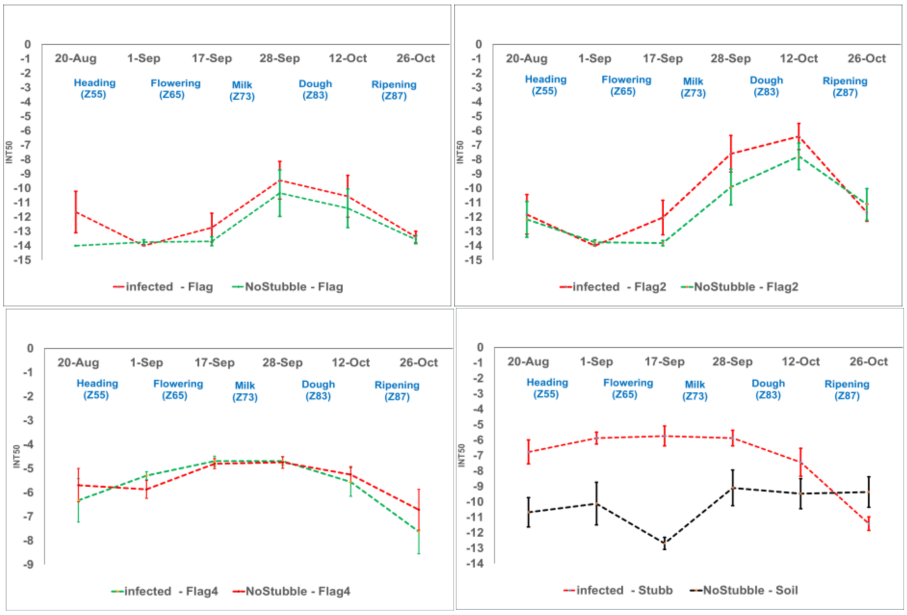

Figure 1. Ice nucleation temperature required for freezing 50% of the droplets (INT50) of soil, stubble, flag, flag-2 and flag-4 and above leaf samples collected from wheat plots grown on infected stubble and no stubble treatments. Sampling was carried out six times starting from heading to ripening stage. Values are the mean of 8 reps +/- SE.

Stubble retention is likely to increase the risk of frost damage

Even with the low rate of stubble residue (2 t/ha) used on our experimental plots, there seems to an increased risk for frost damage in the infected stubble plots compared to the blanket burnt plots (Figure 1). This is probably because the frosted stubble is serving as an inoculum source of overwintering-INB that migrate to the leaves and show increased activity when the conditions permit.

Our data also indicated that stubble removal doesn't guarantee frost protection but might offer a slight advantage in delaying the frost injury at flowering (Figure 1 and 2) by delaying the onset of high INA activity until after flowering (the most frost susceptible stage). Further multi-location and multi-season sampling and diagnostic studies are required for a conclusive answer. In the meantime, growers are advised to continue managing stubble loads in frost prone landscapes as per the recommendation by DPIRD (Smith et al, 2017).

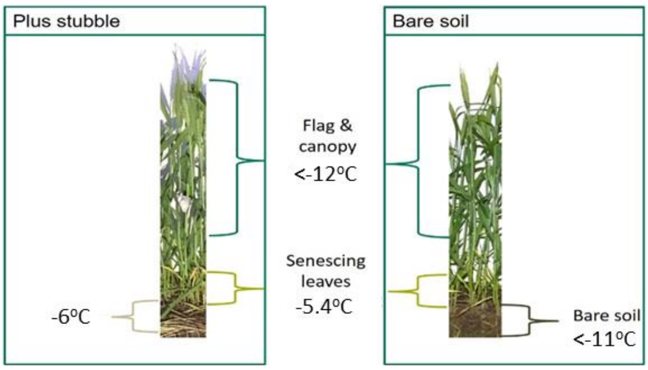

Figure 2. An illustration of the ice nucleation activity on wheat canopy, bare soil and stubble as measured by the INT50 temperature of samples collected during frost-sensitive stage (heading to flowering), values are the mean of 48 reps, LSD0.05= 2°C.

Conclusion

The senesced and senescing leaves of wheat crop at the soil-plant interface are the primary points of ice nucleation that freeze plants at warmer sub-zero temperatures. Stubbles retained or applied during the growing season further exacerbate additional frost risk by potentially increasing the INB load. Unfortunately, with stubble retention, peak INA overlaps with the most frost susceptible stage of wheat (heading and flowering) which also coincides with a wetter and colder time of the growing season. Currently, the evidence is mounting to implicate the role of INB behind the increased INA, and our increased understanding offers new management options that target INB on stubble and older leaves. The origin of INB is the subject of an ongoing investigation but likely sources in WA cropping systems include rainwater and seed.

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