Sample size is critical when exploring the grain set in wheat cultivars grown under frost-prone field conditions in Western Australia

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Abstract
Quantifying the grain set and floret sterility (FS) of wheat cultivars is crucial to benchmarking their susceptibility to frost and exploring cultivar differences so that genetic improvement can be made. Ensuring that the measurement of grain set and FS is accurate and adequately captures the variation within and between spikes and cultivars, is vital to making progress in this space. A field trial with eight times of sowing blocks (from mid-April to early-June) was established at Dale, Western Australia to evaluate the susceptibility to frost of 15 wheat cultivars, of which three are reported on in this paper. Variance component analysis was used to determine the optimal sample size from a sub-set of three wheat cultivars with known differences in their susceptibility to frost damage (Impose CL Plus, Kunjin and Magenta). The optimal sample size was determined to be 15 to 20 spikes per plot, when grain positions per spike ranged from 25 to 35.. Future phenotyping experiments exploring stresses or traits related to spike fertility would benefit from undertaking a variance component analysis to ensure efficient use of their resources.

Key Words
Triticum aestivum L, floret sterility, phenotyping, grain mapping

Introduction
The genetic improvement of wheat to reduce its susceptibility to frost is a key objective to lift the yield potential of wheat and reduce risk to growers in frost-prone areas of Australia. Accurate phenotyping methods are required to screen current cultivars. Current field phenotyping methods for frost susceptibility developed by Reinheimer *et al.* (2004) have been used to screen and rank commercial cultivars (Biddulph *et al.* 2015; GRDC National Frost Initiative 2019). This method measures floret sterility (FS) on the proximal florets, grain positions G1 and G2 using the nomenclature of Feng *et al.* (2018). Post heading frost causes damage both to anthers and ovules, resulting in floret sterility. Previous studies have de-grained wheat spikes, mapped the grains, recorded their weights and used sample sizes of 9, 10 and 20 spikes respectively; to understand the pattern of grain filling, competition between florets along the spike and evaluate mechanisms that could be contributing to final grain weight (Rawson and Evans 1970; Bremner and Rawson 1978; Calderini and Reynolds 2000). To study the effect of nitrogen on durum wheat, a random selection of 10 spikes was used to map the grain set under different treatments (Ferrante *et al.* 2013). Ferrante *et al.* (2017) previously reported on grain set in frosted wheat spikes using grain mapping and related this to yield and its components using a sample size of five spikes. Another study compared five winter wheat cultivars in China using a sample size of 30 spikes (Feng *et al.* 2018). While all these studies have used different sample sizes of wheat spikes to map grain set, there was no justification stated for the sample size to be used. Grain mapping is a labour intensive measurement and sample size is often constrained by the availability of resources: labour, infrastructure/limitations of experimental design and time. In this study we set out to determine a standard grain mapping sample size, which adequately captures the variation of grain set within the subset of three wheat cultivars, when grown in a frost prone landscape. Previous work by Ferrante *et al.* (2017) mapped grain from frosted wheat spikes, this paper builds on that work by quantify an appropriate sample size to do this.

Methods
Trial design
A randomised block design trial with eight times of sowing (TOS) blocks was established at one site in Western Australia at Dale; 20 km south-west of Beverley (-32.20°, 116.75°) in 2017. The site was located in a frost prone area of the landscape; stubble was burnt prior to sowing. To ensure germination soon after sowing, one 25 mm application of irrigation was applied (via a lateral irrigator) two weeks prior to seeding and a further 25 mm was applied the day before seeding, for all sowing dates. No further irrigation was applied after sowing. Sowing dates were selected based on a predicted equidistant thermal time of 250 growing degree days from April 12 to June 8. This was done to ensure that wheat would flower from early August to early October, the typical frost window for the area. The plant density of each plot was 150 plants

m^{-2}. Fourteen commercial wheat cultivars and one synthetic derived line (AUS30323) were sown; these were chosen based on contrasting reputations in their susceptibility to low temperature. Of these cultivars, detailed results of 3 cultivars (Impose CL Plus, Kunjin and Magenta) are reported in this paper. Each cultivar had three replicates per TOS block and were randomised in two directions in the same manner to (Leske *et al.* 2017).

Measurements

The canopy temperature and screen temperature were measured as described in (Leske *et al.* 2017). Loggers were moved up the pole as the crop grew in height, to maintain the logger at canopy height. Plant development stage in each plot was scored weekly (from Z45-70) according to the Zadok scale (Zadoks *et al.* 1974). Canopy heading (Z55) and flowering dates (Z65) of the cultivars were estimated from these scores (Zheng *et al.* 2013). Floret Sterility (FS) is the reduction in grain number per head expressed as a percentage of the total number of possible grains that could have formed in the proximal florets. When the spikes reached grain filling stage (Z85), 30 main tillers were collected and the FS of the two proximal florets were determined (discarding the top and bottom florets). Harvest index cuts and their yield components were determined as per Leske *et al.* (2017). Grain yield was obtained from the whole plot (1.65 x 3 m) using a small plot harvester specifically set up to retain the small frost affected grains.

Grain mapping and analyses

From the harvest index cuts, five main tillers were sub-sampled per plot from 11 cultivars. Three cultivars (Kunjin, Magenta and Impose CL Plus) had 30 tillers sub-sampled to explore the within sub-sample variation in grain set, so that an optimal sample size could be determined. Grain mapping was carried out on these samples as described by (Feng *et al.* 2018), with the variation that undeveloped spikelets at the base of the spike were discarded and spikelet position one began at the first fully formed spikelet. Grain set was mapped on one side of the spike (Ferrante *et al.* 2017). A variance component analysis was used to determine the optimal sample size (Snedecor and Cochran 1956). A linear mixed model was used to analyse grain set within spikes; ID (grain and spike position information) as a fixed term and replicate as a random term (Genstat - VSN-International Ltd. 2018).

Frost events

The timing of frost events (i.e. sub-zero canopy air temperatures) was during the susceptible stages of flowering (Figure 1). The coldest being when Impose CL Plus was heading (-3.4°C) and others when Scout (-2.7°C and -2.4°C) and AUS3023 (-1.2°C and -0.9°C) were flowering.

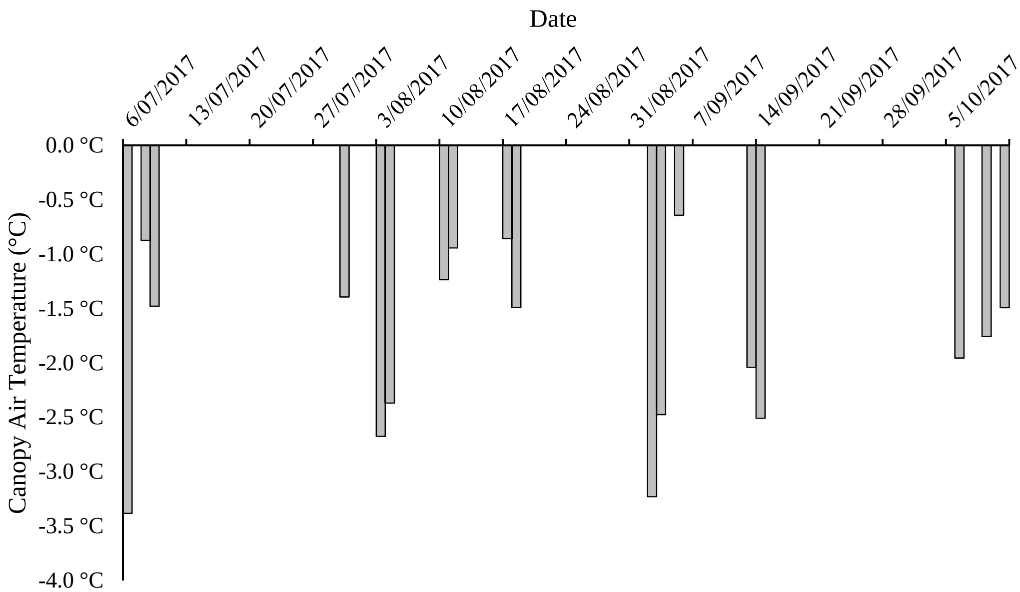

Figure 1. Minimum canopy air temperatures on dates with sub-zero recordings at Dale frost nursery from July to October 2017.

Results and Discussion

The timing, intensity and severity of frost events is highly variable. The results and our variance component analysis showed that the largest sample size of 30 spikes per plot, provided the best estimate of grain set and floret sterility when compared to smaller sample sizes (Table 1). The variance component analysis of the three cultivars showed 15 or 20 spikes per plot offered an improved accuracy, as compared to five spikes which had been used in the past. However, it should be noted that the study by Ferrante *et al.* (2017) had three plot replicates, so pooled sample size would have been adequate. The optimal sample size remained the same even when the number of grain positions, that might be present in a spike was varied from 25 and 30. When the number of grain positions within a spike increased, the variance of the mean number of grains and the error were reduced. This means that in cultivars with longer spikes, more spikelets and those that set grains from G1 to G4 and G5, smaller sample sizes can be used, without comprising accuracy. Smaller sample sizes would also be acceptable, below an increase of five percent in the standard error and variance of the mean grain number threshold. For example, if the average number of grain positions is 25, then 15 to 20 spikes could be mapped. If the average number of grain positions is 35, then a sample size of 10 to 15 spikes could be mapped. An increase of four and five percent in the standard error and variance of the mean would result, with these reduced sample sizes (Table 1).

Table 1. Variance components from REML model of 30 spikes (6 sets) per cultivar (3 cultivars) mapped for grain set. Bold text highlights the threshold of five percent change in the mean variance and standard error to give the minimum sample size required for a given average number of grain positions in the spikes.

Number of spikes mapped for grain set	Number of grain positions	Mean variance	Standard error of the mean	Ratio of SE	Ratio of mean variance
5	25	0.12	0.35	1.16	1.35
10	25	0.11	0.32	1.07	1.14
15	25	0.10	0.31	**1.04**	1.08
20	25	0.10	0.31	1.02	**1.04**
25	25	0.09	0.31	1.01	1.02
30	25	0.09	0.30	1.00	1.01
5	30	0.12	0.34	1.14	1.30
10	30	0.10	0.32	1.06	1.12
15	30	0.10	0.31	**1.03**	1.06
20	30	0.09	0.31	1.01	**1.03**
25	30	0.09	0.30	1.01	1.01
30	30	0.09	0.30	1.00	1.00
5	35	0.12	0.34	1.12	1.26
10	35	0.10	0.32	**1.05**	1.10
15	35	0.10	0.31	1.02	**1.05**
20	35	0.09	0.31	1.01	1.02
25	35	0.09	0.30	1.00	1.00
30	35	0.09	0.30	1.00	0.99

Variance of cultivar = 0.259, variance of five spike sets mapped = 0.027, variance of grain positions = 2.13, mean grain number per position = 2.894, standard error of the mean = 0.304

Conclusion

The results from this study have shown that when measuring grain set in wheat under frost conditions, a relatively large sample size of 30 spikes or greater is ideal. However, due to the cost and time constraints of grain set and FS measurements, 15 to 20 spikes offers an adequate compromise. It is recommended that FS is measured first, to determine the extent of frost damage, and then also used to determine an appropriate sample size for mapping grain set. Grain mapping is conducted afterwards, as measuring FS can cause breakage of the spike. The results of this study showed, that the current phenotyping approach used to rank wheat cultivars for their ability to maintain grain number under frost, had a suitable standard sample size.

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