A robust and rapid pollen viability test using impedance flow cytometry for high throughput screening of heat tolerant wheat (*Triticum aestivum*) germplasm

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Abstract
Pollen viability is an important physiological character for the screening of heat tolerant wheat germplasm. A robust and rapid analysis is essential to determine pollen thermotolerance. Our pre-breeding program assessed the Ampha Z32 impedance flow cytometer from Amphasys as a tool for rapid determination of pollen viability. The Ampha Z32 detects viable pollen based on the electric properties of cells which react to an applied alternating current, simultaneously generating information on cell size, membrane integrity and cytoplasmic conductivity. This study tested the hypothesis that pollen viability assessment using the conventional method by staining and counting under a microscope is comparable with the Ampha Z32 for rapid analysis of wheat pollen viability under high temperature stress. Pollen viability of three wheat genotypes was examined after heat stress during meiosis including an untreated control, using both the Ampha Z32 and conventional Lugol's solution (KI)-stain-count. The cytometer provided comparable data and positive correlations with the conventional staining method.. The cytometer-generated data can enable much larger populations to be screened at lower cost, thus enhancing selection of wheat pollen for heat tolerance.

Key words
Wheat breeding, heat tolerance, pollen viability, meiosis, impedance flow cytometry

Introduction
Genetic variation in floral development and pollen viability is essential for the development of stress tolerant wheat genotypes (Mesihovic et. al., 2016; Baninasab et al 2017). Pollen development has been identified as the most heat-sensitive stage in the plant reproduction process, with both meiosis and gametogenesis being highly thermosensitive (Bokszczanin and Fragkostefanakis 2013). Grain crops like wheat are critically dependent on successful reproductive development. Pollen viability is determined by various methods, such as staining with chemical solutions or *in vitro* pollen germination; however, each method has limitations in its reliability and time requirementwhich varies with plant species. Impedance flow cytometry (IFC) can analyse pollen condition and viability in a high-throughput and species independent manner, which also predicts pollen germination (Heidmann et al 2016). IFC is based on the electric properties of cells that react to an applied alternating current. By varying the frequency (2 or 12 MHz) of the alternating current, cell size, membrane integrity and cytoplasmic conductivity are simultaneously obtained. IFC has been used in microbiology and human cell cultures to characterise cell types (Crocetti et al 2013) and culture conditions (Pierzchalski et al 2012) but has not yet been widely used for plant cells (Heidmann et al 2016). This study tested the hypothesis that pollen viability assessment using a conventional staining method and visual counting under microscope is comparable with the Amphasys impedance flow cytometer (Ampha Z32) for rapid analysis of wheat pollen.

Methods
Pollen of three wheat genotypes; one heat tolerant line (Line 1), one heat susceptible line (Line 2) and the variety Crusader were studied for their viability after heat stress at meiosis. The plants were grown in a microclimate room at the Plant Breeding Institute of the University of Sydney at Cobbitty. Heat stress was imposed for 3 days at 35/22□C day/night temperature during meiosis and the control plants continuously grown at 22 /15□C day/night conditions.

Collection and preparation of pollen sample
Pollen viability test was determined on mature pollen collected at anthesis. Four pollen samples were collected from different flowers of similar maturity in each spike (two anthers were selected from the lower and two from the upper part of the spike), for analysis using the Ampha Z32 cytometer and staining with

Lugol's solution (1% I_2 in 2% KI) (Weerasinghe et al, 2016; Dwivedi et al 2017). The anthers of each pair of samples were divided and placed into two different Eppendorf tubes; one for cytometer analysis and the other for KI-staining.

Staining with Lugol's solution

An aliquot of 300 µl Lugol's solution (KI) was added to the Eppendorf tube with anthers and mixed with a sample crusher to disperse pollen. From the mixed pollen suspension, 70 µl was dispensed to the Sedgwick Rafter plate to count pollen. Counting of viable and non-viable pollen was performed under a light microscope and four grids were counted randomly for each sample. All viable pollen were fully stained with dark brown or black colouration while the non-viable pollen were partially stained or light coloured (Fig 1A).

Ampha Z32 cytometer

In an Eppendorf tube, 1 ml of Ampha buffer #6 was added to previously collected anthers and mixed with a sample crusher to disperse the pollen. The buffer with the pollen was filtered through a 70 µm sieving filter to a 5 ml sample holding tube. Further 1 ml buffer was added to the Eppendorf tube and the filter rinsed for the collection of remaining pollen to the sample holding tube for analysis through the cytometer. Specific populations like debris, non-viable and viable pollen are displayed with their counts in a marked template (Fig 1B).

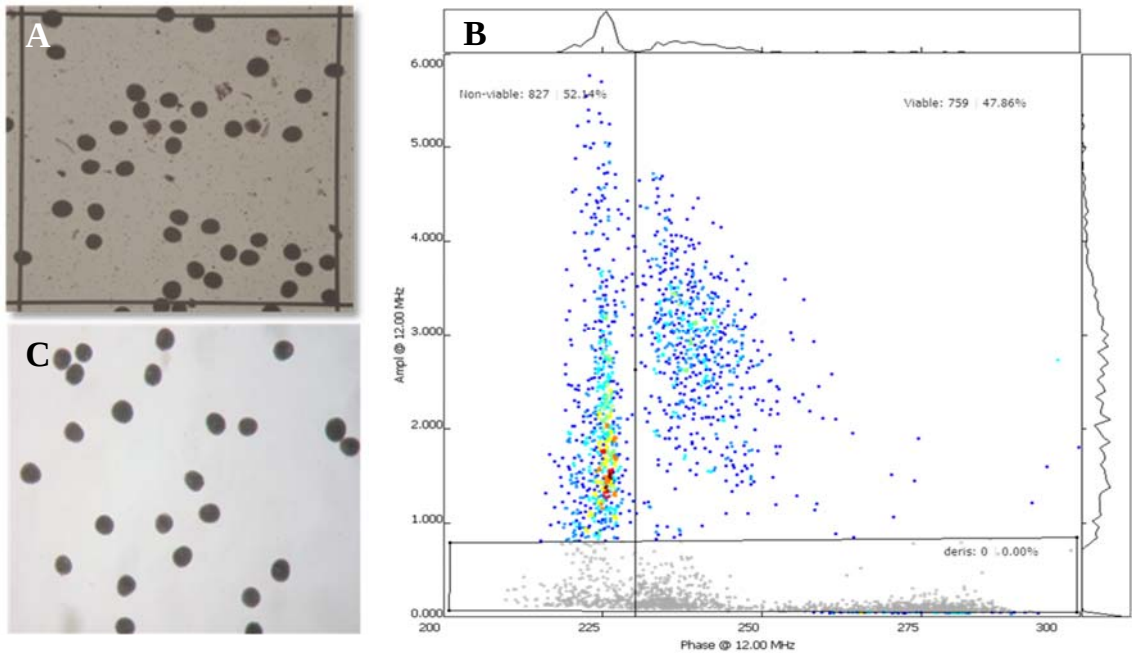

Figure 1. Pollen count by staining with Lugol's solution dispensed to Sedgwick Rafter plate in a 1 cm^2 grids (A) and Ampha cytometer chart at 12.00 MHz for different categories of cell count (B) that generates a separate data table. Pollen suspension in the Ampha solution showing intact pollen ready for analysis in the cytometer (C).

Results and Discussion

Percent viable pollen

The pollen viability of the wheat genotypes in both treatment conditions was similar (see Figure 2). However, the percentage of viable pollen in the control treatments was higher using KI staining than the Ampha Z32 method, while the reverse was observed for heat treated samples. KI stains pollen based on starch only (Dwivedi et al 2017); however, Ampha Z32 detects viable pollen based on the electric properties of cells such as cell size, membrane integrity and cytoplasmic conductivity that react to an applied alternating current at varying frequency (2 or 12 MHz). Thus cell size, membrane integrity and cytoplasmic conductivity are simultaneously obtained with the cytometer. Smaller pollen may not have been detected as viable by the Ampha Z32 due to insufficient induction. On the other hand, most of the non-viable deformed

pollen from the heat-treated florets were discarded as debris by the Ampha cytometer, resulting in a lower number for the non-viable pollen-cells. As a consequence, the value of the percent viable pollen increased.

Correlation between the Ampha Z32 and KI assays

There was a strong relationship between the Ampha Z32 and KI-stain pollen counts ($r^2 = 0.67$, $p<0.001$, Figure 3). Some higher estimates of viable pollen in the KI-stain-count were balanced lower corresponding values in the Ampha Z32 analysis. This may be because KI-stain counts all the living cells irrespective of size as viable pollen whereas, Ampha Z32 may have discarded undersized and/or disintegrated membrane-cells due to low conductivity, lowering the viable pollen count. On the other hand, most of the Ampha Z32 data for viable pollen had comparable values with corresponding KI analysis. This suggests that the analysis of pollen viability using the Ampha Z32 is robust for wheat pollen. KI-stain-counts may have some bias whereas the Ampha Z32 seems to be more reliable for objective determination of viable pollen. Despite some variation, the strong correlation suggests that both methods are comparable and equally applicable for assessing pollen viability. However, the Ampha Z32 can be more conveniently and rapidly used for effective fast throughput pollen analysis, which is useful for high throughput phenotyping by wheat breeders.

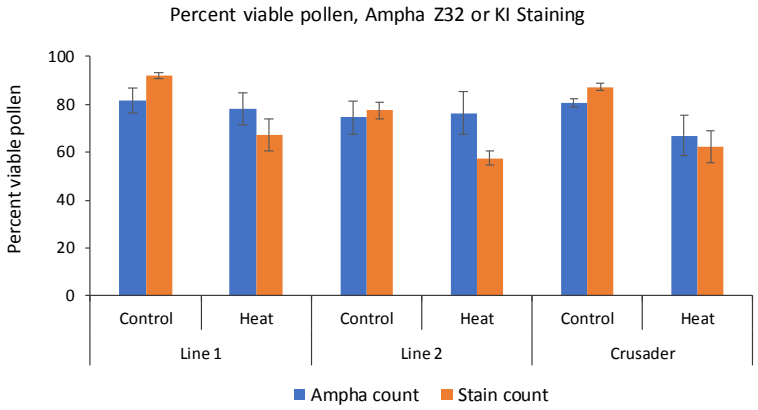

Figure 2. Percent viable pollen of wheat breeding lines/variety from the control or heat-treated florets using the Ampha Z32 cytometer or KI staining counts. The bars representing SE of the mean.

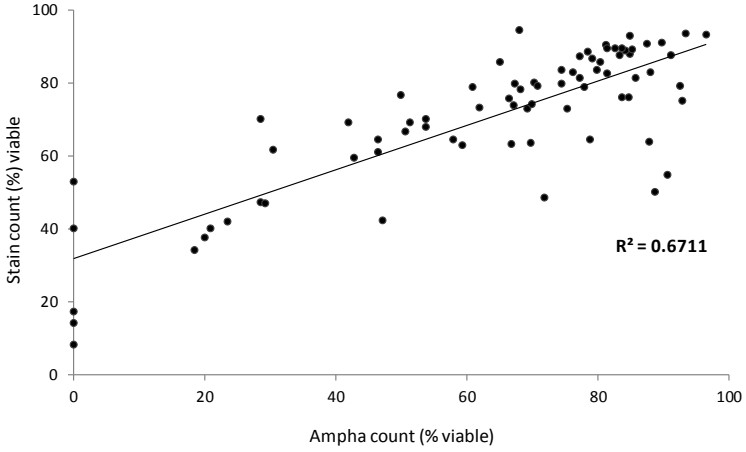

Figure 3. Correlation in pollen count of viable pollen between the Ampha Z32 cytometer and KI staining counts. A total of 86 corresponding pollen samples were counted from the anthers of wheat florets of various treatment combinations (n=86).

Conclusions

The Ampha Z32 cytometer is comparable to conventional KI-staining methods for the detection of viable pollen. Despite some variation in the estimation of viable pollen, the Ampha Z32 showed a consistent and strong correlation with the KI-staining method irrespective of wheat genotype at high meiosis temperatures.

However, both methods have limitations and advantages. KI-staining is cumbersome, time consuming and laborious. However, stained-pollen can be preserved for a few days for subsequent analyses. In contrast, the Ampha Z32 analysis is at least 10 times faster and an immediate report is generated. However, samples have to be analysed immediately after preparation in the buffer. The Ampha Z32 could potentially be a powerful tool for high throughput pollen viability phenotyping and may be useful for screening pollen sensitivity to high temperatures.

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