Cold temperature under aerobic conditions increases spikelet sterility in rice (*Oryza sativa* L.)

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Abstract
Aerobic rice production (well-watered, non-flooded) has been proposed to improve water productivity. However, little research has been conducted on the effect aerobic conditions have on cold induced spikelet sterility. Two glasshouse experiments were conducted to examine the interaction between genotypes and water availability under cold temperatures. In each experiment, four genotypes were grown under aerobic and flooded conditions and half of all plants were exposed to cold temperatures (15/21°C) for a minimum of 14 days during the late booting stage. Water use was measured weekly until harvest and spikelet sterility was determined on the main stem panicle. Pollen number, anther size and stigma size were quantified. Under warm conditions, reductions in water use in aerobic conditions ranged from 58 to 85% compared to flooded (26L plant-1). When plants were exposed to cold temperatures, flooded conditions (34-48%) resulted in a significantly lower sterility than aerobic (70-80%). The genotypic effect in the cold treatment was significant in both experiments and sterility ranged between 36-78%. The lack of a significant interaction in both experiments indicates the mechanism for cold tolerance may be similar for flooded and aerobic conditions. Within the cold treatment, spikelet sterility was negatively correlated with the % viable pollen (r=-0.51*) and mean area of viable pollen (r=-0.60*) which reaffirms that the failure of the pollen grains is the leading cause for cold induced spikelet sterility at the late booting stage.

Key words
Aerobic, cold, booting stage, spikelet sterility, floral architecture

Introduction
Traditionally, rice has been grown under flooded conditions; however, increasing population and decreasing water availability has been applying pressure to improve water productivity. Recently aerobic production (well-watered, non-flooded) has been proposed to improved water productivity with field trials demonstrating an increase of 27-89% in water productivity (kg m^{-3}) in comparison to flooded production with no to small reductions in yield (Peng et al., 2006, Kato et al., 2009). A limitation to the adoption of aerobic production in temperate regions, such as the NSW Riverina, is cold induced sterility at the microspore stage where flooding with a 25cm 'water blanket' is recommended to act as a temperature buffer to protect the developing floral structures (Farrell et al., 2006). There is a current industry push to improve genotypic cold tolerance to reduce the depth of the water blanket with the eventual aim to introduce the opportunity to produce rice aerobically. These two glasshouse experiments examined the interaction of genotypes and water availability on intrinsic water use, cold induced sterility and its underlying physiological mechanisms.

Methods
Two temperature controlled glasshouse experiments were conducted at the University of Queensland, Brisbane, Queensland.

Experiment 1
Experiment 1 was conducted between March and August 2012. Reiziq (Australian cold susceptible lowland), Sherpa (Australian cold tolerant lowland), WAB 38 (African cold susceptible landrace) and Yunlu 29 (Chinese moderately cold tolerant upland) were grown in a completely randomised design with four levels of replication under aerobic and flooded conditions under control temperatures (21/28°C). At the late-booting stage, half were exposed to cold temperatures (15/21°C night/day) for 14 days and then returned to the control temperatures.

On 16 of March 2012, six seeds of were sown into four litre ANOVA® pots filled with 4.2kg of a Gatton black vertosol with 33.6g of Osmocote Exact 3-4M (16-9-12+ 2MgO + Trace elements). Pots designated

for the aerobic treatment had a 10cm petri dish placed approximately two-thirds of the way down to reduce root escape from the central hole in the ANOVA® pot. At 14 days after sowing (DAS), plants were thinned to two plants per pot and the water treatment imposed. A constant water table -22cm and +2cm from the soil surface was maintained for the aerobic and flooded treatments respectively via a valve. The valve for the aerobic treatment was within a 'pot in bucket system' placed below the pot and water was supplied to the pot via a capillary mat as described by Hunter et al. (2012) . The supply of water for each pot was from an individual five litre graduated bottle which enabled the measurement of the plant's water use (WU) from 14DAS to harvest. In the flooded treatment, the valve was placed on the soil surface. Pots were thinned to one plant at 42DAS and plant water use before then was assumed to be half of the measurement from the graduated bottle. In both treatments, the top and sides of the pots were covered with insulation to prevent excessive heating and evaporation. When the auricle of the flag leaf and penultimate leaf of the main stem intersected (AD = 0±2cm), half of all plants were transferred into the cold room (15/21°C) for 14 days before being returned to the warm room. WU was measured weekly. The number of filled and unfilled spikelets on the main panicle was determined at physiological maturity for determination of spikelet sterility.

Experiment 2

Experiment 2 was completed over 2012-2013. Lijiangheigu (Chinese cold tolerant landrace), M205 (USA lowland), and Australian varieties Reiziq and Sherpa were grown and exposed to cold temperature treatments as described for experiment 1. On the 7th of August 2012, six seeds of were sown into four litre ANOVA pots filled with 2100g ± 50g of composted pine bark with 20g of basacote 3M (16-8-12+ 2Mg+ 5S + Trace elements) and grown as experiment 1 with the media's moisture content in aerobic conditions maintained around field capacity. Water use and spikelet sterility were measured as per experiment 1.

The original settings for the cold temperature glasshouse (15/21°C night/day) based on Experiment 1 led to an elevated day temperature of 25°C due to increasing ambient temperature. To compensate the settings were adjusted 12 days after the initial transfer of the first replicates which led to cold glasshouse temperatures of 11/21°C. The exposure to the cold temperatures was also extended from 14 to 18 days to ensure all replicates received a minimum of five days in the lower temperatures.

Sampling for pollen characteristics occurred on the main stem one to two days after heading (only the cold treatment reported). Spikelets were sampled from the third, fourth and fifth spikelets on the first, second and third lower primary branches (Gunawardena et al., 2003). If the panicle was not fully extruded, the sheath was peeled back to permit sampling. The spikelets were stored in a 50% ethanol solution at 4°C for up to 12 weeks before processing. Three spikelets were dissected to expose the anther and stigma and an image was captured for later image analysis (Olympus SZX10®; Lumenera's Infinity1-1M®). Two anthers from each of three spikelets were then stained with an Iodine Potassium Iodide solution (IKI; 5g I2 and 20g KI in 1L of water). The anthers were macerated individually using a splinter probe and the pollen distributed on a slide for image capture under a stereo microscope. Analysis of anthers, pollen and stigma style was undertaken with freeware program ImageJ®. Anther length was measured from the apex of the thecea to the base of the long locule, width was measured at the widest point and area was determined via the number of pixels. Stigma and style traits were calculated on the same basis of the anthers measurements with length being from the base of the style to the apex of the longest stigma, the widest point across the stigmas and area being calculated with pixels. Pollen which contained carbohydrate stained black with IKI is a proxy for pollen viability (Figure 1). Pollen which lacked carbohydrates appeared as transparent yellow and were classed as unviable pollen. Pollen with partial staining was also present, which was clearly different from viable pollen, was also classed as unviable pollen. Analysis with ImageJ® allowed for the quantification of total pollen number per anther, viable pollen number (stained black) per anther, % viable pollen (viable pollen number / total pollen number) and mean area of the viable pollen. The latter was examined as it has been identified as a key determinant in pollen fertility (r=0.89**) in *Mimulus guttatus* with the diameter of unviable pollen being approximately 13µm smaller than viable pollen (Kelly et al., 2002).

Data Analysis

Analysis of Variance (ANOVA) was completed using Genstat 17®. Two-way factorial analysis was partaken separately between cold and warm temperatures as the assumption of homogenous variance was not valid using three-way ANOVA. The variable temperature in the cold treatment in Experiment 2, led to the use

of analysis of covariance using the mean minimum temperature of time in the cold room as the covariate (Farrell et al., 2006). The effect of the temperature was compared using a student's t-test of non-homogenous variance.

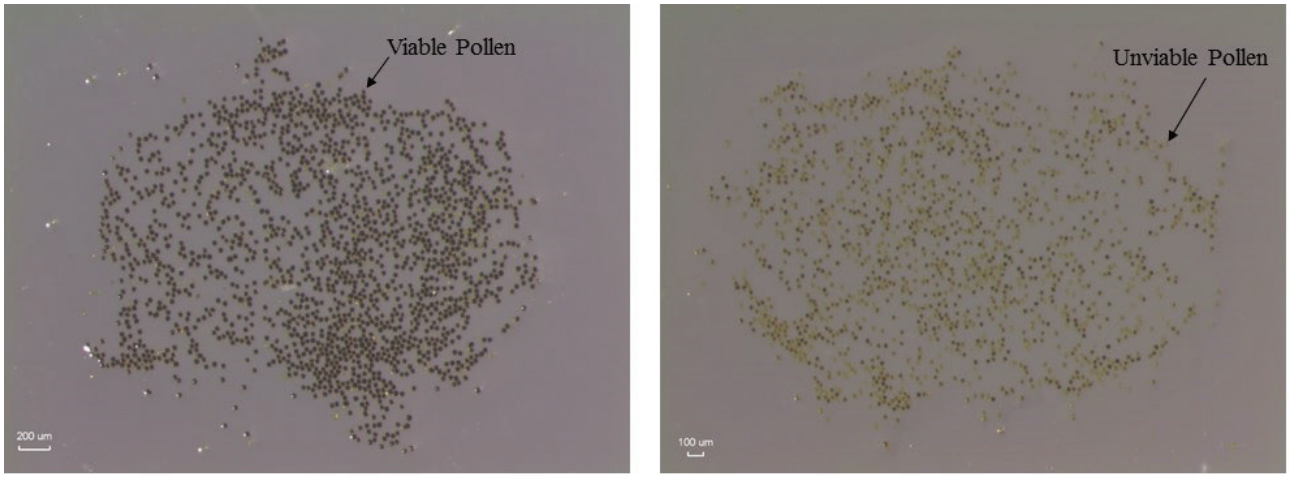

Figure 1: An example of M205 pollen development under warm (a) and cold (b) temperatures under aerobic conditions.

Results and discussion

A highly significant ($p<0.01$) effect of the water regime (WR) existed for cumulative water use under warm temperatures in both experiments with the flooded (24.5-25.7L) utilising more than aerobic conditions (3.8-10.4L). Aerobic conditions in experiment 1 (3.8 L) had a lower level of water utilisation than experiment 2 (10.4L), which is indicative of a lower water availability in the black soil.

In experiment 1, spikelet sterility under aerobic (45%) conditions was significantly ($p<0.01$) higher than the flooded (10%) under warm temperatures (Table 1). A highly significant ($p<0.01$) WR by genotype interaction effect existed, with genotypes not being significantly different from each other (5-15%) in flooded conditions. Under aerobic conditions, Sherpa (9%) had a significantly ($p<0.05$) lower sterility than WAB38 (80) and Reiziq (65%) in experiment 1. However, in experiment 2 there was no significant genotype effect, WR or genotype by WR interaction in the warm treatment (Aerobic (A) 16; Flooded (F) 15%). A highly significant ($p<0.01$) temperature effect occurred in both experiments with warm temperatures (15-27%) having a lower spikelet sterility than the cold (57-59%). Within the cold temperature, spikelet sterility was significantly ($p<0.01$) higher in aerobic conditions (70-80%) than the flooded (34-48%). In both experiments, a significant ($p<0.05$) genotypic effect occurred with Sherpa (36%) having a lower spikelet sterility than Reiziq (58%) and WAB38 (78%) in experiment 1. In experiment 2, Lijiangheigu (46%) had a significantly ($p<0.05$) lower spikelet sterility than M205 (71%). The lack of an interaction suggests that there is a net impact of a reduction in water availability on spikelet sterility which increases with reducing availability. In experiment 2, there was a significant ($p<0.05$) effect of the temperature covariate.

Within the cold treatment (experiment 2), there was a significant ($p<0.05$) effect of the water regime on total pollen number (A 1379; F 1154 pollen grains per anther), viable pollen number (A 700; F 995 viable pollen grains per anther), % viable pollen (A 49; F 86%) and mean area of viable pollen (A 576; F 856 um^2). With the exception of total pollen number, aerobic conditions had a negative impact on the pollen characteristics in comparison to the flooded. No significant effects occurred in anther or stigma style dimensions. Spikelet sterility was significantly ($p<0.05$) and negatively correlated with the % viable pollen ($r=-0.51*$) and mean area of viable pollen per anther ($r=-0.60*$) which reaffirms that failure of the pollen grains are the leading cause of cold induced sterility (Farrell et al., 2006, Gunawardena et al., 2003, etc). It also suggests that the ability for the available pollen to remain viable (% viable pollen and mean area) is important for cold tolerance. Spikelet sterility is commonly reported to have a negative correlation with viable pollen number per anther and anther length (Gunawardena et al., 2003, Farrell et al., 2006), this did not occur in this study nor was there any relationship with dimensions of the style and stigma. Farrell et al. (2006) also reported that the correlation was not significant in all experiments that confirms the conclusion that there are various mechanisms leading to spikelet sterility.

Table 1: Spikelet sterility (%) under warm and cold conditions in Experiment 1 and 2 (Experiment 2 adjusted for the temperature covariate).

Experiment 1							Experiment 2		
	Warm			Cold				Warm	Cold
	Aerobic	Flooded	Mean	Aerobic	Flooded	Mean			
Reiziq	65	5	35	87	31	58	Lijiangheigu	18	46
Sherpa	9	12	11	57	16	36	M205	19	71
WAB38	80	15	47	99	57	78	Reiziq	18	59
Yunlu 29	24	8	16	80	31	55	Sherpa	6	59
WR Mean	45	10		80	34		Aerobic	16	70
							Flooded	15	48
Temperature Mean	27			57			Mean	15	59
LSD 5%							LSD 5%		
Genotype			22**			18**		ns	11*
WR			16**			13**		ns	14**
Genotype x WR			31**			ns		ns	ns
Temperature			11**					13**	

Conclusion

Aerobic conditions reduced the water used by rice plants by 58-85% compared to flooded conditions. When water availability was maintained at field capacity, there was no detrimental impact on spikelet sterility under warm temperatures. However, in cold temperatures the reduction in water availability led to a net increase in spikelet sterility (97-140%). The lack of interaction for spikelet sterility in experiment 1 suggest that genotypes with adaption to lower water availability does not decrease the level of sterility in comparison to lowland genotypes when exposed to cold temperatures. To confirm the hypothesis, an experiment should be conducted with a larger range of genotypes with adaption to low water availability. Experiment 2 reaffirmed that failure of the pollen grains is the leading cause for spikelet sterility, however, the correlation with % viable pollen and mean viable pollen diameter rather than viable pollen per anther indicates that there are multiple pathways in which spikelet sterility occurs. Further elucidation of the underlying physiological mechanisms is required to improve cold tolerance under flooded and aerobic conditions.

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