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# Effect of high temperature and water stress on pollen germination and spikelet fertility in rice

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ABSTRACT

In future climates, rice could more frequently be subjected to simultaneous high temperature and water stress during sensitive developmental stages such as flowering. In this study, five rice genotypes were exposed to high temperature, water stress and combined high temperature and water stress during flowering to quantify their response through spikelet fertility. Microscopic analyses revealed significant differences in anther dehiscence between treatments and genotypes, with a moderately high association with the number of germinated pollen grains on the stigma. There was a strong relationship between spikelet fertility and the number of germinated pollen on stigmas. Although, all three stress treatments resulted in spikelet sterility, high-temperature stress caused the highest sterility in all five genotypes. A cumulative linear decline in spikelet fertility with increasing duration of independent high-temperature stress and in combination with water stress was quantified. Better anther dehiscence, higher *in vivo* pollen germination, and higher spikelet fertility were observed in both the N22 accessions compared with IR64, Apo and Moroberekan under high temperature, water stress and combined stress, indicating its ability to tolerate multiple abiotic stresses.



## 1. Introduction

Rice is a major staple cereal grown in irrigated cropping systems of South and Southeast Asia, with maximum day temperatures either close to or higher than the critical threshold ranging between 33 °C (Nakagawa et al., 2002) and 35 °C (Yoshida, 1981). Recent global climate models predict an increase in mean temperature by 2–4.5 °C and the rice area affected by water stress to double by the end of this century (IPCC, 2007). Recently, hot spots for combined high temperature and water stress occurring at the sensitive flowering and grain-filling stage of rice were identified using data from the rice almanac and spatial analysis using geographical information system (Wassmann et al., 2009). Hence, overcoming the effects of high temperature and water stress on rice production is essential for food security in the future.

High-temperature stress is defined as the rise in temperature beyond a critical threshold for a period of time sufficient to cause irreversible damage to plant growth and development (Wahid et al., 2007). Rice responses to high temperature differ according to the developmental stage, with the highest sensitivity recorded at the reproductive stage. Temperatures >35 °C at anthesis and lasting for more than 1 h can lead to high sterility in rice (Jagadish et al., 2007). A high-temperature stress-induced increase in spikelet sterility was attributed to abnormal anther dehiscence (Matsui and Omasa, 2002), impaired pollination (Matsui et al., 2005), and pollen germination (Jagadish et al., 2010). Moreover, high temperature of 39 °C given a day before flowering resulted in poor anther dehiscence during subsequent anthesis (Matsui and Omasa, 2002).

Water limited condition (also referred to as drought), affecting 23 m ha of rice regularly (Pandey et al., 2007) is a condition related to insufficient soil moisture available to support average crop production. The response of plants to water stress depends on the duration and severity of the stress (Araus et al., 2002; Bartels and Souer, 2004) and the developmental stage (Zhu et al., 2005). Rice is sensitive to drought stress particularly during flowering stage, resulting in severe yield losses (Liu et al., 2006). The physiological processes during the sensitive flowering stage, negatively affecting spikelet fertility under water stress [anther dehiscence (Ekanayake et al., 1989, 1990; Liu et al., 2006); pollen germination (Saini and Westgate, 2000)] were similar to high-temperature stress (Yoshida, 1981; Jagadish et al., 2010). Additionally, panicle exsertion (O'Toole and Namuco, 1983), and peduncle length (He et al., 2009) were partly responsible for increased sterility under water stress.

The simultaneous occurrence of multiple abiotic stresses rather than one particular stress is commonly noticed under field conditions (Mittler, 2006). The combination of high temperature and

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water stress represents an excellent example of multiple abiotic stresses occurring concomitantly in the field. It was found that combined stress had a significantly greater detrimental effect on growth and productivity than exposure to a single stress in *Hordeum vulgare* (Savin and Nicolas, 1996) and *Poa pratensis* (Wang and Huang, 2004). Effects of this combination in *Arabidopsis thaliana* (Rizhsky et al., 2004), *Triticum aestivum* (Shah and Paulsen, 2003) and *Nicotiana tabacum* (Rizhsky et al., 2002) have been documented. However, relatively little information is available for rice in response to combined high temperature and water stress in general and at the most sensitive flowering stage in particular. Experiments were therefore carried out with the following objectives to (1) study the effect of high temperature (HT), water stress (WS) and combined high temperature and water stress (HT + WS) on the flowering period, peduncle elongation and panicle exsertion in rice; (2) record the impact on anther dehiscence, pollen count and germination on the stigma and spikelet fertility when exposed to the above mentioned stresses at flowering; and (3) test the hypothesis that "high temperature a day before flowering affects fertility of spikelets opening on the subsequent day".

## 2. Materials and methods

### 2.1. Crop husbandry

Five rice (*Oryza sativa* L.) genotypes differing in their response to either HT or WS at flowering were used for this study (Table 1). Seeds were sown in seeding trays with clay loam soil after breaking dormancy (2 d, 50 °C). Fourteen-day-old seedlings were transplanted into plastic pots with two holes at the bottom sealed with stoppers to facilitate water control. Each pot was filled with 6.0 kg of the same clay loam soil with 2.0 g $(NH_4)_2SO_4$ (urea), 1.0 g muriate of potash (KCl) and 1.0 g single super phosphate (SSP). An additional 2.5 g of $(NH_4)_2SO_4$ was top dressed, 25–30 d after transplanting. Cypermethrin (Cymbush) 0.42 g $L^{-1}$ was sprayed 30 d after transplanting to control white flies (*Bemisia* spp.). There were no other pest or disease problems.

### 2.2. Greenhouse

Plants were grown in a temperature-controlled greenhouse maintained at 29/21 °C day/night temperature [actual: 28.8 °C (SD {standard deviation} = 0.84)/20.9 °C (SD = 0.27)] and day/night relative humidity (RH) of 75–85% [actual 75.2% (SD = 0.11)/86.7% (SD = 0.07)] under natural sunlight conditions at the International Rice Research Institute (IRRI), Philippines. Plants were placed on a bench spaced at 30-cm to avoid shading effects. Ambient air temperature and RH were measured using thermocouples (Chessell 392, USA) every 10 s and averaged over 10 min.

### 2.3. Growth chamber

Indoor growth chambers (Thermoline, Australia) were used with temperatures automated to gradually increase from 29 °C to 38 °C starting from 0730 to 0830 (2.5 h after dawn) and maintained at 38 °C (SD = 1.23) until 1430, with an RH of 75% (SD = 3.88) during both Experiments 1 and 2. Both temperature ($P > 0.97$) and RH ($P > 0.73$) were maintained consistently between the experiments to avoid any chamber effects on plant observations recorded. Plants were spaced at approximately 15–20 cm to avoid crowding. A thermocouple placed above the canopy in the growth chamber measured the ambient air temperature and RH every 10 s and averaged them over 10 min (Chessell 392, USA). Photosynthetic photon flux density was maintained at 640 μmol $m^{-2}$ $s^{-1}$. $CO_2$ concentration was not measured.

### 2.4. Stress treatments

Two experiments were carried out with slightly different conditions. Five and seven replicate plants were used for each of the four treatments (control, HT, WS and HT + WS) in Experiment 1 and 2, respectively. Plants of all five genotypes were grown in temperature-controlled greenhouse conditions at 29/21 °C and used as absolute controls for the experiments.

For Experiment 1, plants were exposed to HT for 6 h (0830–1430), on the first day of anthesis (i.e. the appearance of anthers) and then moved back to the control conditions (29/21 °C). Similarly, transfer between the control and HT conditions were continued for five consecutive flowering days. For WS, main tillers at 5 d before heading (DBH) were selected and tagged. Stoppers at the bottom of these pots were unplugged for overnight draining to reach maximum water holding capacity by the following morning. The main-tiller flag leaf in all five genotypes began to roll after 5 successive non-watering days before heading. Following main-tiller flag-leaf rolling and based on the average water lost (pot weight at leaf rolling-weight at subsequent weighing), across genotypes, a constant volume (500 mL) of water was added back daily until the main-tiller completed anthesis followed by complete flooding. A different set of plants as identified at 5 DBH for HT + WS treatment and exposed to WS as described earlier. These selected plants were exposed to both WS and two days of high-temperature (38 °C) starting on the first day of anthesis.

For Experiment 2, high-temperature stress was imposed as described in Experiment 1 for four consecutive flowering days. Similarly, for WS, main tillers at 5 DBH were identified as described in Experiment 1 and WS was initiated. WS was continuously monitored by recording flag-leaf relative water content (RWC) using the following formula of RWC (%) = $[(W - DW)/(TW - DW)] \times 100$ (Liu et al., 2006), where *W*: fresh weight, TW: turgid weight, and DW: dry weight. Unlike the first experiment, the volume of water added back was determined based on individual pot weight. The

**Table 1**
Five rice genotypes having differential response to either HT or WS at flowering. Numbers in parenthesis are IRGCIS (International Rice Genebank Collection Information System) accessions.

| Cultivar | Origin | Species | Expected stress tolerance | References |
|---|---|---|---|---|
| N22 (03911) | India | *O. sativa* aus | Heat and drought | Prasad et al., 2006; Jagadish et al., 2008, 2010; Selote and Chopra, 2004 |
| N22 (19379) | India | *O. sativa* aus | Heat and drought | Prasad et al., 2006; Jagadish et al., 2008, 2010; Selote and Chopra, 2004 |
| Apo (115128) | Philippines | *O. sativa* indica | Drought | Kumar et al., 2008 |
| IR64 (116793) | Philippines | *O. sativa* indica | Moderately heat and drought | Jagadish et al., 2008; Liu et al., 2006 |
| Moroberekan (117272) | Guinea | *O. sativa* japonica | Heat sensitive and drought tolerant | Jagadish et al., 2008, 2010; Liu et al., 2006 |

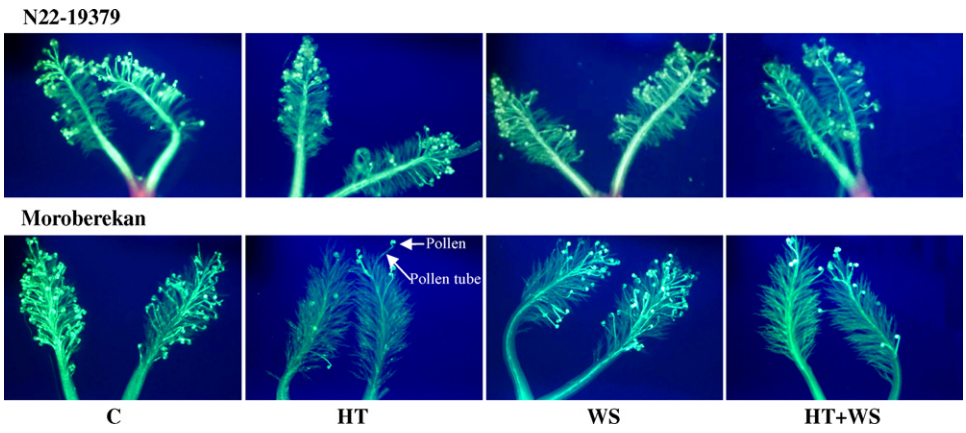


**Fig. 1.** Pictorial illustration of high temperature (HT), water stress (WS) and combined stress (HT + WS) affecting pollen count and pollen germination in the heat and drought tolerant N22 and the drought tolerant and heat sensitive Moroberekan.

HT + WS was imposed as in Experiment 1 except that the HT was given for four consecutive flowering days starting from the first day of anthesis.

### 2.5. Observations

#### 2.5.1. Peduncle length and panicle exsertion

Peduncle length (PeL) starting from the panicle node to the immediately preceding node was measured. Since panicles were not completely exserted because of WS or HT + WS, portions of the panicle outside (exserted) and inside (trapped) were measured with the flag-leaf collar as the reference. Peduncle and panicle length (PaL) were measured in both the experiments.

#### 2.5.2. Microscopic analysis

Fifteen to 20 spikelets were randomly sampled between 1030 and 1200, from the four treatments and five genotypes. For this, spikelets about to flower on the main tiller were marked using acrylic paint, and, after pollination (around 30 min after the spikelet closed) marked spikelets were collected into vials filled with FAA (50% absolute ethanol, 5% acetic acid, 27% formaldehyde and 18% sterilized water) fixative following the protocol by Jagadish et al. (2010). Anthers separated from the fixed spikelets were used to record percent anther dehiscence. Spikelets were washed in de-ionized water before dissecting under a stereo-microscope (Olympus SZX7, Olympus Corp., Japan). Isolated stigmas were cleared in 8 N NaOH for 3–5 h at room temperature and stained with aniline blue dissolved in 0.1 M $K_2HPO_4$ for 5–10 min and number and germinated pollen on the stigma were recorded. Images were taken with a DP70 digital camera attached to an Axioplane 2 microscope (Carl Zeiss, Germany) at 100× (Fig. 1).

#### 2.5.3. Flowering period and anther dehiscence

The number of days taken by the main tiller to complete flowering was recorded as flowering period (FP) from four treatments and all five genotypes. Anthers from the spikelets collected to record the pollen count and germination with either basal and/or apical pore open were recorded as dehisced and the remaining as un-dehisced using a stereo-microscope (Olympus SZX7, Olympus Corp., Japan). Anther dehiscence was calculated as the ratio of the number of dehisced anthers to the total number of anthers (dehisced + un-dehisced).

#### 2.5.4. Spikelet fertility

Spikelet fertility (SF) from both experiments was estimated from the main-tiller panicle using the procedures of Prasad et al. (2006) and Mohammed and Tarpley (2009). Fifteen to twenty days after the completion of anthesis, spikelet fertility was estimated by pressing the spikelet between the thumb and forefinger to determine whether it was filled or not. Both partially and fully filled spikelets were categorized as filled spikelets. Spikelet fertility was calculated as the ratio of filled spikelets to total number of spikelets. Additionally, in the second experiment, spikelets opening on four consecutive flowering days were identified using different-colored acrylic paint following the protocol of Jagadish et al. (2007, 2008). Tillers used for collecting spikelets for microscopic analysis were not including for fertility analysis.

### 2.6. Statistical analysis

Data on flowering period, peduncle length, panicle length, panicle exsertion (PE), anther dehiscence, and spikelet fertility were analyzed as a two way completely randomized design using SPSS 13.0 (Version 13, LEAD Technologies Inc.) with 5 and 7 replications in Experiment 1 and 2, respectively. Tukey's least significant difference (LSD) at a probability level of 5% and 1% was used to compare the differences between treatments and genotypes.

## 3. Results

### 3.1. Phenology at flowering

Managed WS resulted in flag-leaf RWC being maintained around 50–60% throughout the stress period, which coincided with flag-leaf rolling in all five genotypes, and this provided evidence that plants in Experiment 1 were at similar water content as in Experiment 2 (Fig. 2). In all five genotypes, flowering period was significantly extended ($P < 0.05$) when exposed to HT, WS and HT + WS in both the experiments compared with control plants (Table 2). The effect between WS and HT + WS treatment was non-significant ($P > 0.05$). Across treatments, the FP between genotypes varied significantly, ranging between 5.5 and 7.6 d, with Moroberekan recording the longest FP in both Experiment 1 and 2. Similarly, Peduncle length was significantly ($P < 0.05$) reduced by HT (8%), WS (24%) and HT + WS (27%) in all genotypes and in both experiments compared with the control (Table 2). The genotypes responded consistently to PeL under stress, with Moroberekan having the longest peduncle while both the N22 accessions having the shortest peduncles. Although, there was no difference in panicle length under HT, WS and a HT + WS in the five genotypes (data not show), panicle exsertion showed significant differences with WS related treatments. Under WS and HT + WS, PE was significantly hindered ($P < 0.05$) compared with the control, whereas HT stress had no effect ($P > 0.05$) (Table 2). IR64 and Moroberekan has significantly

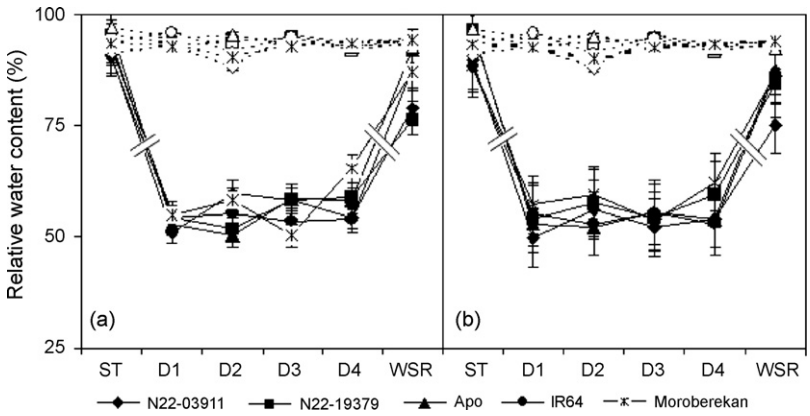


**Fig. 2.** Flag-leaf relative water content (RWC) under WS (a) and HT + WS (b) (solid lines) compared to controls (dotted lines). 50–60% RWC was used as a criterion for imposing WS and HT + WS starting from the initiation of flowering till the main tiller completed anthesis. ST indicates start of treatment; D1, D2, D3 and D4 the first, second, third and fourth day of stress; WSR (water stress recovery) three days after re-watering. The duration to reach D1 was different among genotypes and the 3 d water stress recovery phases are indicated by two parallel line breaks. Bars indicate ±SD.

**Table 2**
Effect of control, HT, WS and HT + WS on flowering period (days), peduncle length (cm) and panicle exsertion (%) in five rice genotypes.

| Experiment 1<br>Trait | Genotypes | Control | HT | WS | HT + WS | Mean | 5% LSD |
|---|---|---|---|---|---|---|---|
| Flowering period | N22-03911 | 5.4 | 5.8 | 7.8 | 8.0 | 6.8 | *0.5* |
| | N22-19379 | 5.2 | 5.2 | 6.4 | 6.6 | 5.9 | |
| | Apo | 3.4 | 5.8 | 6.4 | 6.4 | 5.5 | |
| | IR64 | 3.6 | 5.6 | 6.4 | 7.0 | 5.7 | |
| | Moroberekan | 4.0 | 5.6 | 7.6 | 7.8 | 6.3 | |
| | Mean | 4.3 | 5.6 | 6.9 | 7.2 | | |
| | 5% LSD | *0.45* | | | | | |
| Peduncle length | N22-03911 | 38.0 | 34.7 | 26.9 | 23.8 | 30.9 | *2.1* |
| | N22-19379 | 39.0 | 33.7 | 25.1 | 26.4 | 31.1 | |
| | Apo | 44.1 | 42.2 | 30.8 | 29.7 | 36.7 | |
| | IR64 | 36.7 | 34.1 | 33.3 | 32.3 | 34.1 | |
| | Moroberekan | 47.1 | 42.2 | 38.2 | 34.6 | 40.5 | |
| | Mean | 41.0 | 37.4 | 30.9 | 29.4 | | |
| | 5% LSD | *1.9* | | | | | |
| Panicle exsertion | N22-03911 | 97.1 | 88.5 | 36.1 | 24.4 | 61.5 | *10.6* |
| | N22-19379 | 87.0 | 93.3 | 40.5 | 36.0 | 64.2 | |
| | Apo | 94.2 | 93.2 | 56.9 | 48.4 | 73.2 | |
| | IR64 | 100 | 100 | 89.1 | 84.2 | 93.3 | |
| | Moroberekan | 100 | 98.9 | 76.8 | 66.2 | 85.5 | |
| | Mean | 95.7 | 94.8 | 59.9 | 51.8 | | |
| | 5% LSD | *9.5* | | | | | |
| Experiment 2 | | | | | | | |
| Flowering period | N22-03911 | 5.1 | 5.4 | 8.1 | 8.0 | 6.7 | *0.32* |
| | N22-19379 | 5.0 | 6.1 | 7.9 | 8.3 | 6.8 | |
| | Apo | 5.3 | 6.3 | 8.3 | 8.5 | 7.1 | |
| | IR64 | 4.9 | 5.6 | 8.1 | 8.3 | 6.7 | |
| | Moroberekan | 5.4 | 6.6 | 9.3 | 9.1 | 7.6 | |
| | Mean | 5.1 | 6.0 | 8.3 | 8.4 | | |
| | 5% LSD | *0.29* | | | | | |
| Peduncle length | N22-03911 | 33.8 | 31.3 | 24.9 | 23.3 | 28.3 | *2.0* |
| | N22-19379 | 32.9 | 31.8 | 24.4 | 22.7 | 28.0 | |
| | Apo | 40.1 | 36.1 | 25.1 | 25.4 | 31.7 | |
| | IR64 | 30.5 | 28.5 | 31.1 | 29.6 | 29.9 | |
| | Moroberekan | 38.8 | 37.4 | 31.1 | 29.6 | 34.2 | |
| | Mean | 35.2 | 33.0 | 27.3 | 26.1 | | |
| | 5% LSD | *1.8* | | | | | |
| Panicle exsertion | N22-03911 | 96.9 | 87.5 | 40.5 | 29.4 | 63.6 | *10.9* |
| | N22-19379 | 91.4 | 91.2 | 28.7 | 24.7 | 59.0 | |
| | Apo | 96.4 | 88.6 | 34.5 | 30.4 | 62.5 | |
| | IR64 | 99.1 | 91.3 | 80.2 | 64.0 | 83.7 | |
| | Moroberekan | 100 | 94.1 | 67.1 | 55.9 | 79.3 | |
| | Mean | 96.8 | 90.5 | 50.2 | 40.9 | | |
| | 5% LSD | *9.7* | | | | | |

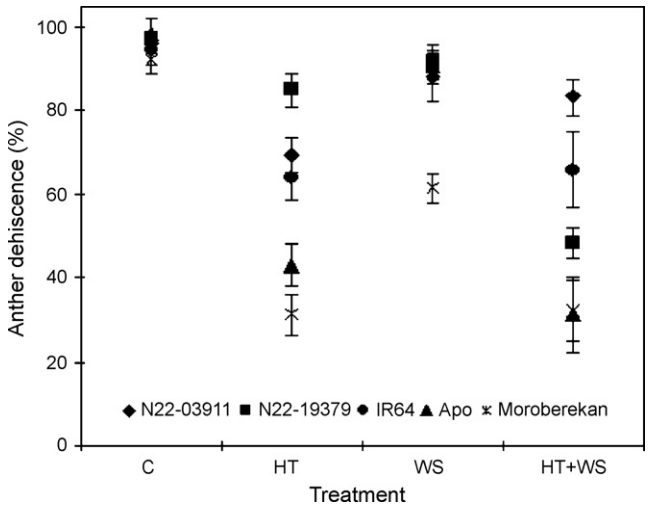


**Fig. 3.** Anther dehiscence affected by control (C), high temperature (HT), water stress (WS) and combined stress (HT + WS) in five rice genotypes ($n > 60$ anthers). Bars indicate ±SE.

superior panicle exsertion % ($P < 0.05$) compared to the other three genotypes.

### 3.2. Anther dehiscence

Anther dehiscence was highly affected by HT and clear genotypic differences were observed (Fig. 3). Both N22 accessions showed significantly higher anther dehiscence in all treatments ($P < 0.05$), whereas HT-sensitive genotype Moroberekan recorded the least anther dehiscence. Under WS, a significant difference was observed only with Moroberekan among the five genotypes. With HT + WS, anther dehiscence was affected similar to HT, indicating that the reduction in anther dehiscence was largely influenced by HT. Comparatively, one accession of N22 (19379) had lower anther dehiscence with HT + WS compared to HT, whereas Apo and Moroberekan were the most significantly affected genotypes ($P < 0.01$).

### 3.3. Germinated pollen on the stigma and spikelet fertility

The number of pollen on the stigma was significantly ($P < 0.01$) reduced by HT (72%), WS (31%), and HT + WS (71%), across both the experiments. HT ($P < 0.01$) and HT + WS ($P < 0.05$) significantly reduced the pollen count with N22 accessions having the highest pollen count (52 to 70) compared with the other three genotypes (<21%). The number of germinated pollen on stigma decreased significantly ($P < 0.01$) when plants were exposed to HT (81%), WS (59%), and HT + WS (84%) averaged over both experiments, compared with the control (Fig. 4). In both experiments, IR64 had the highest number of germinated pollen under control conditions. Among the stress treatments, WS had significantly higher ($P < 0.01$) pollen germination than HT and HT + WS, while no significant differences ($P > 0.05$) were noticed between HT and HT + WS. The HT + WS in Experiment 1 with 2 d of HT resulted in higher pollen germination% among N22 accessions while the difference disappeared when increasing duration to 4 d in Experiment 2 (Fig. 4). Comparatively, pollen germination was less sensitive to WS with 55% and 62% higher germination compared to HT and HT + WS, respectively. Under HT stress, the number of germinated pollen on the stigma was significantly higher in both N22 accessions ($P < 0.05$) compared to the other three genotypes.

Spikelet fertility was significantly ($P < 0.01$) reduced by HT, WS and HT + WS treatments ($P < 0.01$) (Fig. 4). Five days of HT reduced spikelet fertility by 81% in Experiment 1 and by 72% with four days of exposure in Experiment 2. On the other hand, a shorter (2 d) HT with WS had a decline of 63% and 80% with a longer (4 d) exposure of HT + WS. Spikelet fertility had the least decline (21%) under WS in both experiments. Two accessions of N22 showed significantly higher spikelet fertility ($P < 0.05$) under HT and HT + WS than the other three genotypes across both experiments.

Spikelet fertility on consecutive flowering days declined significantly with the duration of exposure to HT and HT + WS in both N22 accessions and Moroberekan. N22, however, had a significantly higher fertility on all four days in both HT and HT + WS (Fig. 5). Spikelet fertility decrease under HT was lower in N22-03911 (−0.047) and N22-19379 (−0.098) compared to Moroberekan (−0.189). On the other hand, spikelet fertility declined by 0.053 and 0.124 with both the accessions of N22 and Moroberekan, respectively, under HT + WS. Spikelet fertility in Moroberekan was reduced to 0% from the 2nd flowering day under both HT and HT + WS stress.

## 4. Discussion

In rainfed rice ecosystems, plants are often subjected to a combination of abiotic stresses, among which the simultaneous occurrence of HT and WS is more frequent (Mittler et al., 2001). Increasing the tolerance of rice during the most sensitive flowering stage to these stresses is an ideal adaptation strategy for highly variable future climates (Horie et al., 1996). Tolerance classically comprises elements of escape, that is rice genotypes can either escape or avoid HT during anthesis, by heading during the cooler periods of the season (macro-escape), by anthesing during cooler hours of early morning (micro-escape) (Jagadish et al., 2008), or by efficient transpiration cooling of canopy (Weerakoon et al., 2008). Absolute tolerance of stress is the ability of rice to carry out key physiological processes, such as anther dehiscence, pollination, pollen germination, and fertilization, under stress but still maintain high seed-set (Jagadish et al., 2008, 2010).

Phenological conditions at flowering under different abiotic stresses to a certain extent determine subsequent spikelet fertility. The plant water status under WS is commonly monitored using relative water content (Lafitte et al., 2006; Lafitte, 2002; Liu et al., 2006). The time taken to reach the target flag-leaf RWC of 50–60% was significantly different among genotypes (data not show), similar to Srinivasan et al., 2008. Imposing WS by reducing the RWC to 50–60% resulted in a significant decrease in spikelet fertility among the five genotypes compared with control conditions, but overall fertility was higher in response to WS. For example, IR64 recorded 66% spikelet fertility with 50–60% RWC in our studies while the same genotype had 33% fertility when exposed to 40–50% flag-leaf RWC (Liu et al., 2006). This indicates that the plants experienced a moderate terminal WS and the major factor reducing the fertility was HT.

Panicle exsertion has been suggested to be an important trait that ultimately reflects on the grain yield of rice plants when they encounter WS or combined HT + WS. PE is primarily influenced by peduncle elongation. The panicles partially exserted with both WS and HT + WS, had 29% and 37% of spikelets trapped in the leaf sheath, respectively, mainly due to shortened peduncle. There were strong positive relationship between panicle exsertion and peduncle length ($R^2 = 0.84$ and $R^2 = 0.74$ in Experiment 1 and 2, respectively). The negative effect of WS on panicle exsertion during anthesis and subsequently on yield was reported earlier (Jearakongman, 2005). Similarly, we found that spikelets inside the flag-leaf sheath showed complete sterility with both WS and HT + WS, which was partly responsible for the overall reduction in the spikelet fertility. Spikelets trapped within the leaf sheath result in an absence of anthesis and fertilization (Cruz and O'Toole, 1984;

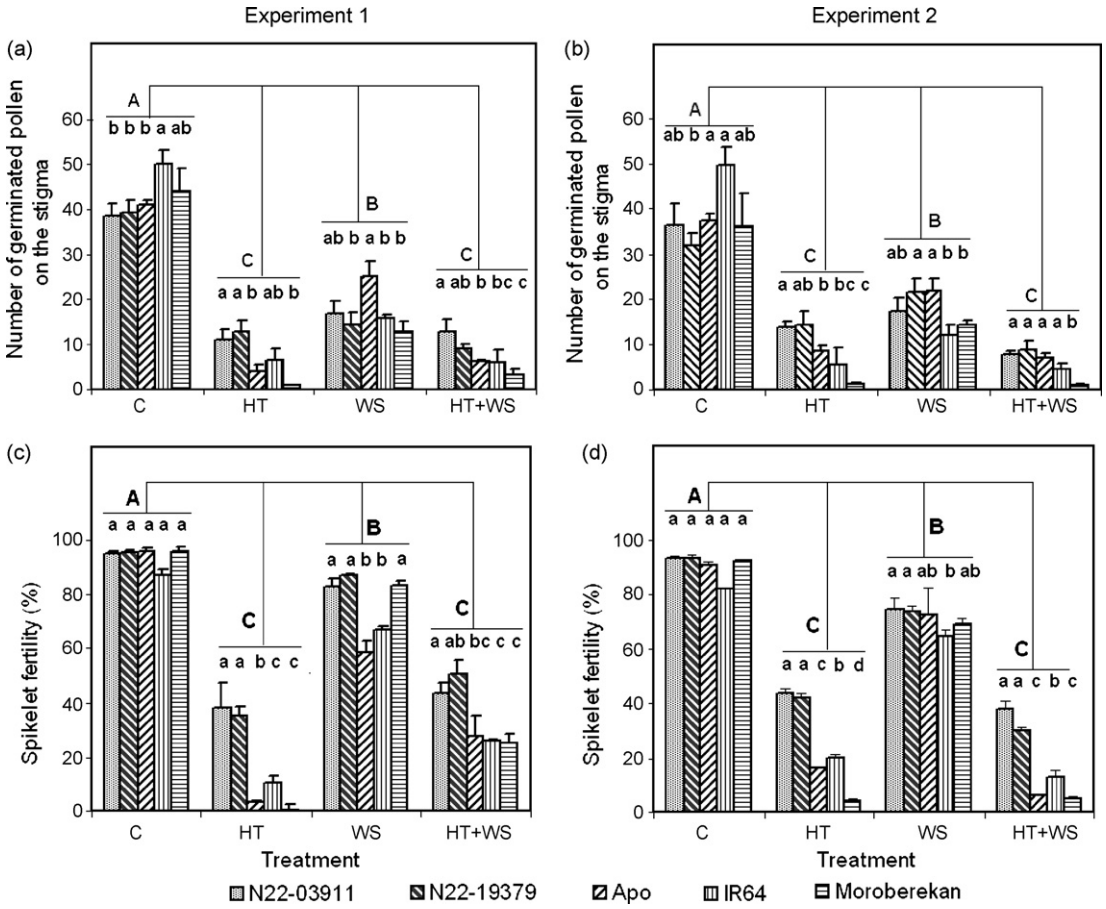


**Fig. 4.** Germinated pollen number on stigmas ($n > 15$ spikelets) and spikelet fertility ($n = 5, 7$) of five rice varieties under control (C), high temperature (HT), water stress (WS), and combined stress (HT + WS) in Experiment 1 (a and c) and Experiment 2 (b and d), respectively. The HT stress in Experiment 1 was for 5 d and 2 d for independent HT stress and HT + WS, respectively, while HT stress was held uniform for 4 d in Experiment 2. Capital letters indicate significance level at 1% and lowercase letters at 5%. Bars indicate ±SE.

O'Toole and Namuco, 1983) and, moreover, with HT + WS, the additional heat trapped within the leaf sheath, devoid of free air flow resulted in a much higher sterility.

The reproductive stage in rice is effected irreversibly by HT (Prasad et al., 2006) and WS (Liu et al., 2006) than the vegetative stage. Water deficit during reproductive stage significantly reduces pollen viability (Liu, 2003), spikelet fertility (Praba et al., 2009) and grain yield (Boonjung and Fukai, 1996). Similarly, we recorded a significant reduction in spikelet fertility under HT, WS and HT + WS. Independent HT and HT + WS induced abnormal anther dehiscence, resulting in a reduced number of germinated pollen on the stigma, consequently reducing fertilization, leading to spikelet sterility. Generally, higher anther dehiscence resulted in significantly more pollen on the stigma but, in highly sensitive genotypes like Moroberekan, even with higher anther dehiscence the pollen count (Fig. 6) decreased as noticed by Jagadish et al., 2010. This could be attributed to the asynchrony between the male (pollination) and female (stigma receptivity) reproductive organ mechanisms during anthesis, as seen in maize (Herrero and Johnson, 1981). Further, spikelets with at least 10 or >20 germinated pollen recorded high fertility, giving confidence to the previously identified critical number equated to seed-set (Jagadish

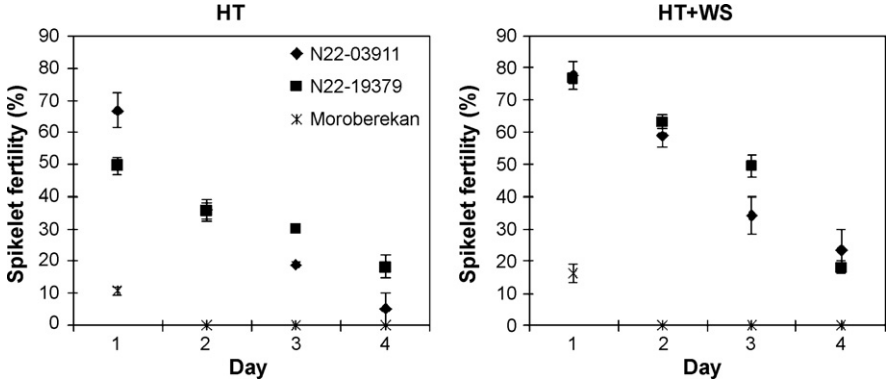


**Fig. 5.** Spikelet fertility on four consecutive flowering days in two N22 accessions and Moroberekan in Experiment 2. Individual spikelets flowering on different days were marked with 4 different-colored acrylic paints and fertility was recorded. Bars indicate ±SE.

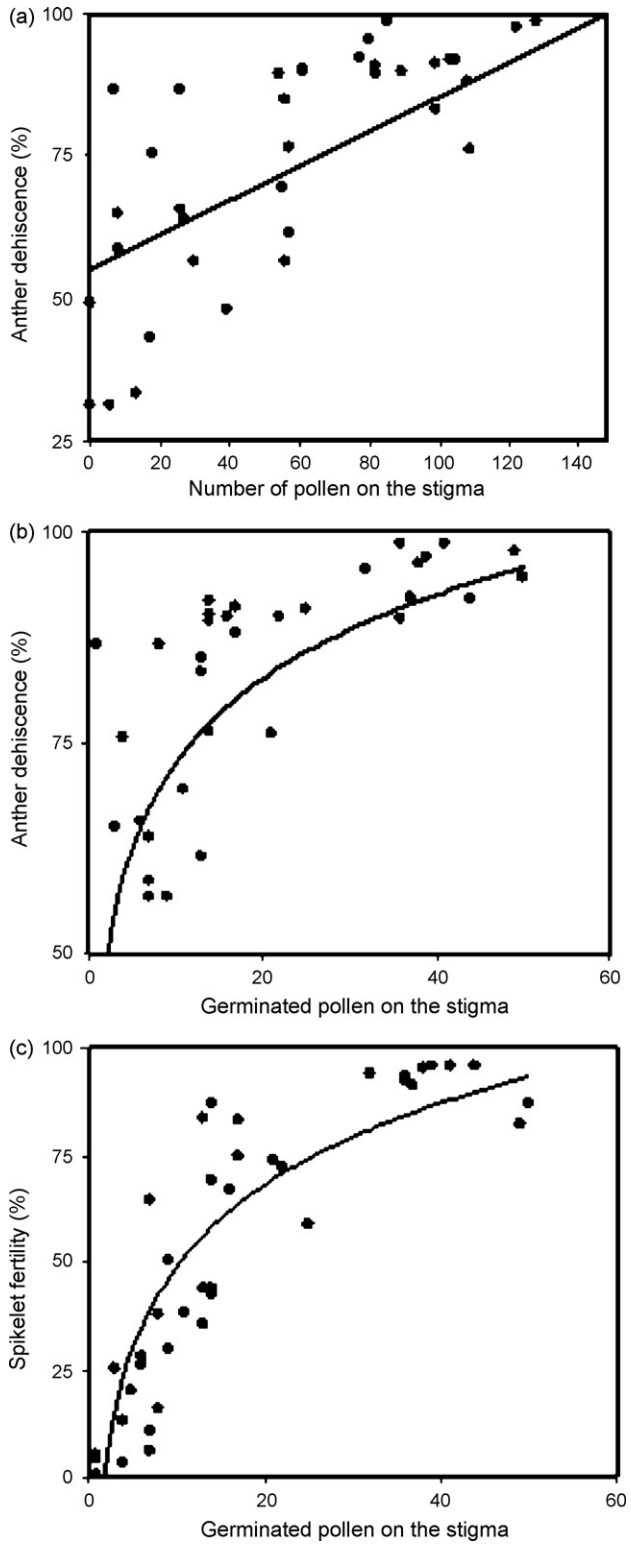


**Fig. 6.** Relationship between anther dehiscence (%) with pollen count on stigma (a: $R^2 = 0.43$, $y = 0.3025x + 55.039$, $n = 40$, $P < 0.001$) and number of germinated pollen on the stigma (b: $R^2 = 0.46$, $y = 14.40\,\mathrm{Ln}(x) + 39.37$, $n = 40$, $P < 0.001$); spikelet fertility and number of pollen germinated (c: $R^2 = 0.77$, $y = 27.65\,\mathrm{Ln}(x) - 14.77$, $n = 40$, $P < 0.001$).

et al., 2010). N22s in our present study showed higher anther dehiscence and thus more pollen and germinated pollen on the stigma compared with the other three genotypes, indicating true tolerance of HT and to HT + WS. Similar results were obtained from earlier reports of Mackill et al., 1982 and Satake and Yoshida, 1978 and more recently it was clearly shown that N22 had a significantly higher pollen count and pollen germination on the stigma compared to the most sensitive Moroberekan. Further in comparison, N22 had a normal rate of pollen tube growth compared to significantly slower pollen tube growth rate with the moderate tolerant IR64 (Jagadish et al., 2010) demonstrating the higher level of heat tolerance during anthesis.

Although an extended flowering period in response to the imposed stress treatments could be a potential alternative escape mechanism, plants encountering independent HT and in combination with WS did not have higher spikelet fertility even with the extended flowering period. Therefore, apart from the direct negative effect of HT coinciding with anthesis, resulting in spikelet sterility, the carry over effect on un-anthesised spikelets even after removal of stress also resulted in sterility. Following the spikelet marking approach the cumulative negative effect of prolonged HT stress on consecutive flowering days was tested. The linear decrease in spikelet fertility on consecutive flowering days either in HT- tolerant N22 or in sensitive genotype Moroberekan indicated a cumulative effect of HT stress. This further confirmed that HT stress given a day prior to anthesis affected the normal functioning of the pollen sac (anther) dehiscence and pollen viability (Matsui and Omasa, 2002). Therefore, the hypothesis that HT coinciding exactly at anthesis and not earlier would have an effect on fertility (Yoshida, 1981) is questionable. Moreover, Sato and Yokoya (2007) showed increased tolerance of rice seedlings of water deficit after prior exposure to HT at 42 °C for 24 h; over-expression of heat shock proteins increased tolerance to subsequent water deficit in *Nicotiana tabacum* (Cho and Hong, 2006) and water deficit and salt stress in *Arabidopsis thaliana* (Sun et al., 2001). Similarly, a higher spikelet fertility percentage in the HT + WS compared to independent HT was due to the acquired tolerance possibly due to increased heat shock proteins during the preceding WS resulting in higher thermo tolerance to subsequent HT stress. Physiological and molecular mechanisms resulting in this novel phenomenon in rice is presently being investigated.

In conclusion, the study has confirmed the true HT tolerance of N22 and its ability to withstand a combination of HT and WS. Hence, it is a potential candidate for breeding rice varieties capable of adapting to a range of abiotic stresses during flowering. We have demonstrated that the extended duration of HT or HT + WS had a negative cumulative effect on spikelet fertility and that this aspect has to be considered during germplasm screening for tolerance.

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