Effect of soil moisture stress at booting and flowering stages on pollen development, pollination and fertilization in upland NERICA cultivars

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Abstract

Spikelet sterility induced by soil moisture stress during reproductive development greatly limits grain yield in upland rice. This study aimed to elucidate differences in responses to soil moisture stress for pollen development, pollination and fertilization among upland rice cultivars. A greenhouse experiment with a split-plot design was performed for five different soil moisture treatments (T1 to T5) as the main plots and three cultivars (NERICA 1, NERICA 4 and Yumenohatamochi) as subplots, each with three replicates. Plants in pots were grown under well-watered condition (T1) and various moisture stress conditions: moderate at the booting stage (T2), severe at the booting stage (T3), moderate at the flowering stage (T4) or severe at the flowering stage (T5). During the 9-day stress period, soil moisture was maintained at -10 to -20 kPa for moderate moisture stress or -20 to -49 kPa for severe moisture stress under controlled irrigation. NERICA 1 had fewer differentiated microspores and developed pollen grains in T2 and T3 and showed poorer anther dehiscence and fewer pollen grains on the stigma than did NERICA 4 and Yumenohatamochi. NERICA 4 showed a lower percentage of basal dehiscence in T4 and T5, causing fewer pollen grains to be deposited on the stigma than for NERICA 1 and Yumenohatamochi. The results indicate that the highly sensitive process of fertilization are pollen development in NERICA 1 and pollination in NERICA 4 under soil moisture stress.

Keywords: Anther dehiscence; drought; pollen grain; rice; spikelet sterility.
Abbreviations: DW_dry weight; FW_fresh weight; IKI_iodine potassium iodide; NERICA_New Rice for Africa; RWC_relative water content; SSA_Sub-Saharan Africa; SWC_soil water content; T1_well-watered; T2_moderate moisture stress at the booting stage; T3_severe moisture stress at the booting stage; T4_moderate moisture stress at the flowering stage; T5_severe moisture stress at the flowering stage; TW_turgid weight.

Introduction

In Sub-Saharan Africa (SSA), rice has risen in importance as consumption has continuously increased (Gajigo and Denning, 2010) and, thus, it is necessary to expand rice production. Rice is mainly grown in rainfed uplands or wetlands in SSA where insufficient rainfall leads to soil moisture stress and limits rice productivity. To improve production under these conditions, new strains of rice have been developed. Since NERICAs, inter-specific progenies of *Oryza sativa* L. and *Oryza glaberrima* Steud., were developed in 1994 (Jones et al., 1997), cultivation reached about 700,000 ha in SSA by 2009 (Diagne et al., 2010). These strains are expected to be well adapted to drought-prone areas, showing relatively high yields even under soil moisture stress (Somado et al., 2008). Nevertheless, a multi-location trial with 14 NERICA cultivars in Tanzania (Sekiya et al., 2013) and a long-term experiment with cultivar NERICA 4 in Uganda (Tsuboi et al., 2018) revealed the close relationship between yield and precipitation, indicating that soil moisture stress reduces grain yield in NERICAs. Thus, recovery of yield reduction in NERICAs due to soil moisture stress is required to improve rice productivity in SSA. Sekiya et al. (2013) and Tsuboi et al. (2018) demonstrated that

compared to other growing periods, soil moisture stress during periods of active meiosis and anthesis cause the largest reduction in grain yield with low ripening ratio having the biggest contribution to yield in NERICAs. Moreover, spikelet sterility, which controls ripening ratio, was identified as the factor significantly affecting grain yield in NERICA 4 experiencing soil moisture stress from panicle initiation to anthesis (Alou et al., 2018). These observations suggest that, in NERICAs, achieving spikelet fertilization is essential for reaching higher yield under soil moisture stress.
In rice, soil moisture stress during reproductive development, particularly at meiosis and anthesis, causes severe spikelet sterility (Matsushima, 1962; Saini and Westgate, 1999). Generally, spikelet sterility induced by stress at meiosis mainly leads to insufficient development of pollen grains in anthers (Sheoran and Saini, 1996; Nguyen and Sutton, 2009; Saragih et al., 2013), while stress at anthesis induces failure of pollination (Ekanayake et al., 1990; Liu et al., 2006; Rang et al., 2011) and pollen germination (Ekanayake et al., 1990; Liu et al., 2006; Fu et al., 2011; Rang et al., 2011). Further, anther indehiscence due to soil moisture stress at anthesis is considered to cause

poor pollination and consequently result in spikelet sterility (Ekanayake et al., 1990; Liu et al., 2006; Rang et al., 2011; He and Serraj, 2012). Several reports have pointed out that genetic variation in pollen development (Fu et al., 2011) and pollination (Liu et al., 2006; Fu et al., 2011) are related to drought tolerance. However, the physiological and morphological characteristics of reproductive processes under soil moisture stress have not been extensively analyzed in NERICAs.

In our previous study, differences in ripening ratio were observed among NERICAs under soil moisture stress at the booting stage (Iwata-Higuchi et al., 2019), suggesting the possibility of cultivar differences in spikelet sterility. Thus, we here investigate the effect of soil moisture stress in NERICAs at the booting or flowering stage on fertilization processes. In the present investigation, pollen development and pollination in NERICA 1 and NERICA 4 were characterized and compared with Yumenohatamochi, a Japanese drought-resistant upland cultivar. Among NERICA cultivars, NERICA 1 (Atera et al., 2011; Sekiya et al., 2013) and NERICA 4 (Kinyumu, 2009; Nassir et al., 2017) have been reported to show higher yields under drought conditions in different countries and are widely grown as adopted or certified cultivars in SSA (Diagne et al., 2010). We discussed differences between fertilization responses in these two leading NERICAs due to soil moisture stress.

Results

SWC and leaf RWC

Mean soil water content (SWC) and leaf relative water content (RWC) decreased during stress periods in all soil moisture stress treatments (T2 to T5) but no significant differences were found among cultivars and in the interaction between treatment and cultivar (Table 1). Moisture stress at the flowering stage (T4, T5) tended to cause lower SWC and leaf RWC than at the booting stage (T2, T3).

Pollen development, pollination and spikelet fertility

The numbers of differentiated microspores and developed pollen grains tended to be reduced in T2 and T3, except for those of NERICA 4 which were hardly reduced (Fig. 1A). The largest and significant reductions in the number of differentiated microspores were observed in NERICA 1 under T3. In T3, NERICA 1 had significantly fewer differentiated microspores than NERICA 4 and Yumenohatamochi. In contrast, the number of differentiated microspores was less affected in T4 and T5, and there were no significant differences among cultivars.

With an increase in undeveloped pollen grains (Fig. 1A), moisture stress tended to reduce the percentage of developed pollen grains for all cultivars (Fig. 1B). Compared to T1, the percentage of developed pollen grains of NERICA 1 and NERICA 4 was significantly lower in T3 and T5, respectively. NERICA 1 had a significantly lower percentage of developed pollen grains than NERICA 4 and Yumenohatamochi in T2 and T3, whereas there were no clear differences among cultivars for T4 and T5.

The number of deposited pollen grains decreased greatly in T2 to T5 (Fig. 1C). In T1, Yumenohatamochi had significantly more pollen grains on the stigma than NERICA 1 and NERICA 4, and a similar tendency (differences not significant) was observed for other stress treatments, except for T5. NERICA 1 showed slightly fewer deposited pollen grains than NERICA 4 for moisture stress at the booting stage (T2, T3), while NERICA 4 had fewer deposited pollen grains than NERICA 1 under moisture stress at the flowering stage (T4, T5), although the differences were not significant.

All stress treatments considerably reduced spikelet fertility (Fig. 1D), and differences compared to T1 were significantly lower for all cultivars in T3, T4 and T5, as well as NERICA 4 in T2. Spikelet fertility tended to be lower in T4 and T5 than in T2 and T3. The largest reductions were observed for NERICA 4 under T5, and NERICA 4 tended to have lower spikelet fertility than NERICA 1 and Yumenohatamochi in T4 and T5. Spikelet fertility of NERICA 1 was slightly lower than that of NERICA 4 and Yumenohatamochi in T2 and T3. However, these cultivar differences in spikelet fertility were not statistically significant.

Anther dehiscence

Stress treatments tended to reduce the percentage of basal dehiscence for NERICA 1, and NERICA 4 showed a significantly lower percentage of basal dehiscence in T4 and T5 than in T1 (Fig. 2A). Although the differences were not significant, the percentage of basal dehiscence in NERICA 4 was lower than in the other cultivars in T4 and T5. NERICA 1 had a significantly lower percentage of basal dehiscence than NERICA 4 and Yumenohatamochi in T2. Compared to NERICA 1 and NERICA 4, Yumenohatamochi tended to show a higher percentage of basal dehiscence both under well-watered condition (T1) and all stress treatments (T2 to T5). The length of anther dehiscence differed among cultivars and there was no clear effect of treatment (Fig. 2B). NERICA 1 and NERICA 4 showed a significantly smaller basal dehiscence than Yumenohatamochi in T1, in which was also evident in T2 and to a lesser extent in T3 to T5. In addition, the length of basal dehiscence of NERICA 4 tended to be shorter in T4 and T5 than that of the other cultivars.

The number of deposited pollen grains was positively correlated with the percentage of basal dehiscence for all cultivars (r = 0.455, p < 0.01, Fig. 3A), but there was no significant relationship between the number of deposited pollen grains and the length of basal dehiscence (Fig. 3B).

Discussion

Meiosis and anthesis are regarded as two stages that are highly sensitive to soil moisture stress in rice (Saini and Westgate, 1999). In the present experiment, soil moisture stress during meiosis (booting stage, T2, T3) and anthesis (flowering stage, T4, T5) reduced spikelet fertility (Fig. 1D) as leaf RWC decreased (Table 1). The number of differentiated microspores, the percentage of developed pollen grains, and the number of deposited pollen grains were significantly reduced by moisture stress at the booting and/or flowering stages, and the effects of soil moisture stress on these parameters differed among NERICA 1, NERICA 4 and Yumenohatamochi (Fig. 1 A, B, C).

Moisture stress at the booting stage (T2, T3) reduced the number of differentiated microspores and lowered the percentage of developed pollen grains in NERICA 1 more than in NERICA 4 and Yumenohatamochi (Fig. 1A, B). These observations suggest that water deficit inhibits pollen differentiation and development in NERICA 1 more severely than in the other cultivars. In addition, NERICA 1 had poorer anther dehiscence (Fig. 2A) and tended to have relatively

Table 1. Mean volumetric soil water content (SWC) and leaf relative water content (RWC) during moisture stress periods.

| Treatment	Cultivar	SWC (%)		Leaf RWC (%)	
T1	YHM	64.6	a	87.0	a
	N1	61.6	a	89.7	a
	N4	60.3	a	90.3	a
T2	YHM	12.4	b	82.2	ab
	N1	12.8	b	82.3	ab
	N4	12.6	b	77.0	ab
T3	YHM	12.0	b	72.1	ab
	N1	12.4	b	75.6	ab
	N4	12.1	b	76.3	ab
T4	YHM	9.8	b	70.2	ab
	N1	11.1	b	76.3	ab
	N4	11.9	b	76.0	ab
T5	YHM	9.1	b	62.5	b
	N1	10.9	b	68.8	ab
	N4	9.6	b	63.9	b
T1		*62.2*	*A*	*89.0*	*A*
T2		*12.6*	*B*	*80.5*	*AB*
T3		*12.2*	*B*	*74.7*	*B*
T4		*10.9*	*B*	*74.2*	*BC*
T5		*9.9*	*B*	*65.1*	*C*
Treatment (T)		**		**	
Cultivar (C)		n.s.		n.s.	
TxC		n.s.		n.s.	

YHM: Yumenohatamochi, N1: NERICA 1 and N4: NERICA 4. SWC and leaf RWC of the control are means during the moisture stress periods of booting and flowering stages. ** and n.s. denote significant difference at $p < 0.01$ and not significant, respectively. Values followed by the same letter within each column do not differ at $p < 0.05$ by Tukey's test.
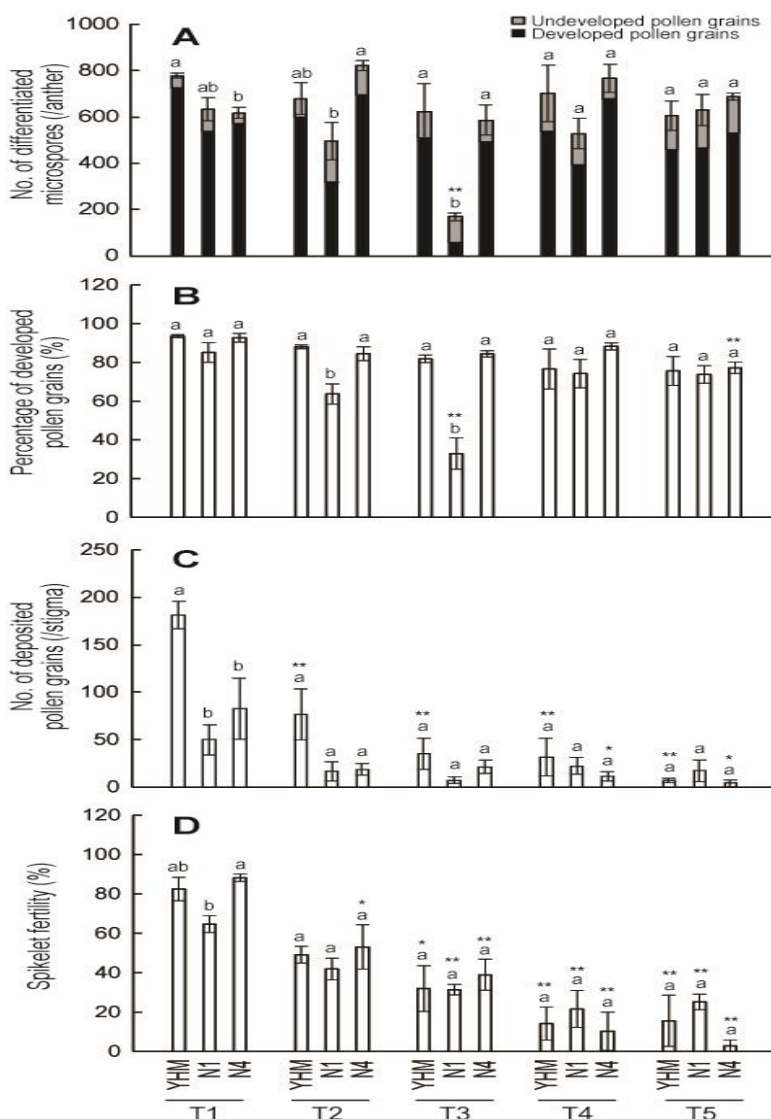

Fig 1. Effect of soil moisture stress on the number of differentiated microspores (A), percentage of developed pollen grains (B), number of deposited pollen grains (C) and spikelet fertility (D). Abbreviations are the same as for Table 1. * and ** denote significant differences from values in T1 of the same cultivar at $p < 0.05$ and $p < 0.01$, respectively, by Dunnett's test. Bars with the same letter within each treatment do not differ at $p < 0.05$ by LSD test.

1937
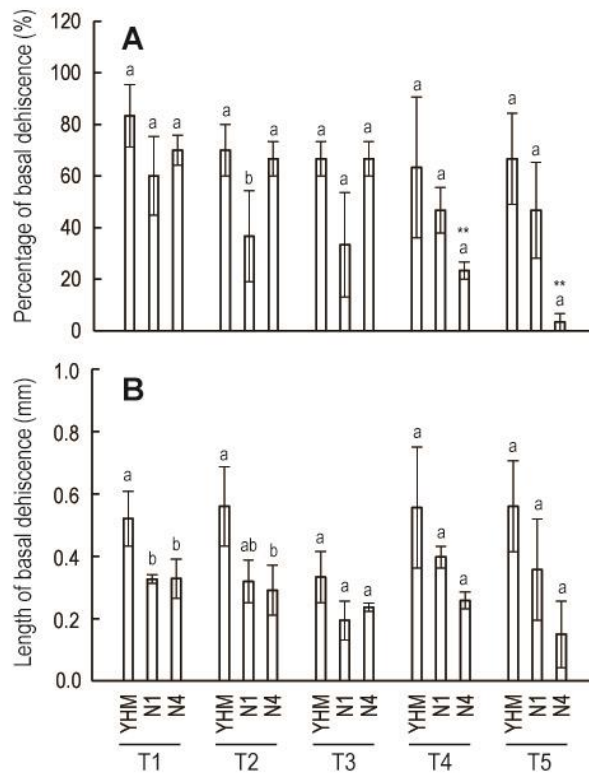
Fig 2. Effect of soil moisture stress on the percentage (A) and length (B) of basal dehiscence. Abbreviations are the same as for Table 1. ** denotes significant difference from values in T1 of the same cultivar at $p < 0.01$ by Dunnett's test. Bars with the same letter within each treatment do not differ at $p < 0.05$ by LSD test.
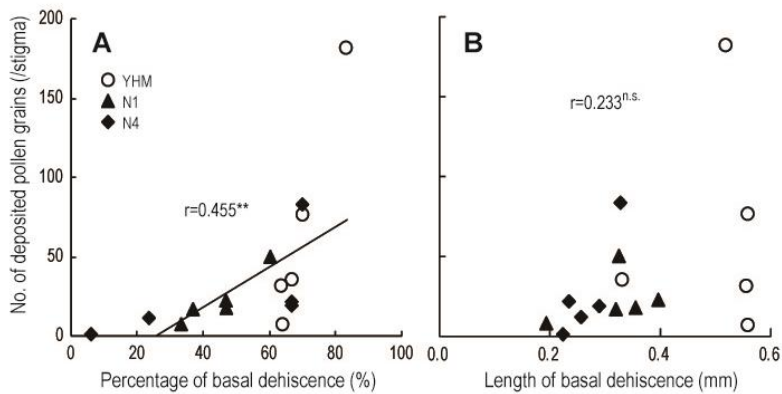
Fig 3. Relationships between the percentage (A) or length (B) of basal dehiscence and the number of deposited pollen grains on stigma. Abbreviations are the same as for Table 1. ** and n.s. denote significant difference at $p < 0.01$ and not significant, respectively.
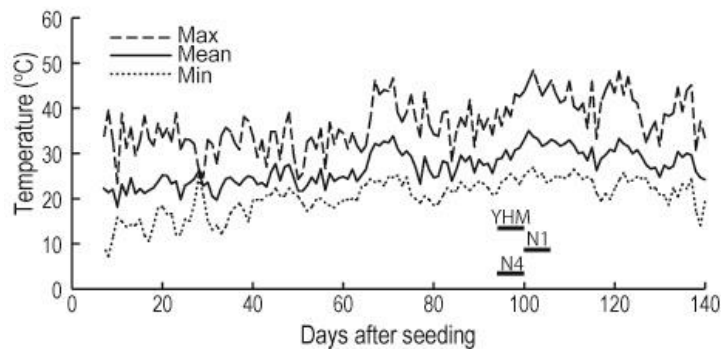
Fig 4. Daily mean, maximum and minimum temperature in the greenhouse during growing period. Bars with YHM, N1 and N4 indicate the range in heading date of Yumenohatamochi, NERICA 1 and NERICA 4, respectively.

fewer deposited pollen grains on the stigma at the booting stage (Fig. 1C) than NERICA 4 and Yumenohatamochi. Soil moisture stress inhibits pollen development, decreases the size of pollen grains (Liu et al., 2006) and reduces anther dehiscence (Matsui et al., 1999). The positive correlation between the percentage of basal dehiscence and the number of deposited pollen grains (Fig. 3A) suggests that insufficient pollen development causes poorer pollination in NERICA 1 than the other cultivars.

Starch accumulation in pollen grains plays an important role in pollen differentiation and development under soil moisture stress (Sheoran and Saini, 1996; Fu et al., 2011). A previous report (Sheoran and Saini, 1996) suggests that enzyme activities involved in sucrose cleavage and starch synthesis are depressed in pollen grains under soil moisture stress, while other investigations (Selote and Khanna-Chopra, 2004; Nguyen et al., 2010; Fu et al., 2011) indicate that insufficient defense against oxidative stress inhibits pollen development under soil moisture stress. Further investigation is therefore needed to analyze physiological mechanisms that cause differences in pollen differentiation and development between NERICA 1 and NERICA 4 under soil moisture stress.

Moisture stress at the flowering stage (T4, T5) hardly affected the number of differentiated microspores and the percentage of developed pollen grains (Fig. 1 A, B). The significant decline in the percentage of developed pollen grains for NERICA 4 in T5 is likely attributable to the stress treatment starting 2-6 days before heading, which partly suppresses pollen maturation. In contrast, moisture stress at the flowering stage markedly reduces the number of pollen grains deposited on the stigma (Fig. 1C), and the number of deposited pollen grains was smaller in NERICA 4 than in NERICA 1 and Yumenohatamochi. The percentage and the length of basal dehiscence of NERICA 4 tended to be inferior to those of NERICA 1 and Yumenohatamochi at the flowering stage (Fig. 2A, B), and the percentage of basal dehiscence was significantly related to the number of deposited pollen grains (Fig. 3A). These results suggest that the inferior anther dehiscence in NERICA 4 reduced the number of pollen grains deposited on the stigma as compared to the other cultivars. The present investigation failed to detect a significant correlation between the length of basal dehiscence and the number of deposited pollen grains on the stigma (Fig. 3B), which is inconsistent with the results of Matsui and Kagata (2003). This discrepancy is probably due to a portion of the pollen grains sticking to the inside the anthers despite the pores being sufficiently opened under conditions of drought (Liu et al., 2006).

NERICA 1 and NERICA 4 had lower percentages of basal dehiscence and smaller basal pores than Yumenohatamochi both under well-watered (T1) and stressed conditions (T2 to T5) (Fig. 2A, B). Similarly, NERICA 1 and NERICA 4 had fewer deposited pollen grains on the stigma than Yumenohatamochi under well-watered and stressed conditions (Fig. 1C). These results indicate that poorer basal dehiscence in NERICA 1 and NERICA 4 causes deposition of fewer pollen grains on the stigma irrespective of soil moisture stress. In the present experiment, plants may have been transiently exposed to high temperatures at anthesis (Fig. 4), and cultivar differences in anther dehiscence may have been affected by differences in tolerance to high temperature. However, as the length of basal dehiscence under high temperature strongly corresponds to that under normal conditions (Matsui et al., 2005), basal dehiscence ability in NERICAs might be genetically inferior to

that in a Japanese upland cultivar, irrespective of growing conditions.

In the present investigation, NERICA 1 and NERICA 4 tended to have lower spikelet fertility than the other cultivars under moisture stress at booting (T2, T3) and flowering (T4, T5) stages, respectively (Fig. 1D); however, these cultivar differences were not significantly demonstrated by this dataset. Although there were significant differences in differentiation and development of pollen grains and pollination among cultivars (Fig. 1A, B, C), spikelet fertility did not necessarily reflect these significant differences. Spikelet fertility is expressed as the product of fertilization components involved in the four main phases of fertilization (Satake and Shibata, 1992): microspore differentiation, microspore development, pollen shedding on the stigma, and pollen germination and fertilization. The last phase, which was not evaluated in the present experiment, may affect spikelet fertility, and investigations of pollen germination and fertilization are thus required to analyze differences in spikelet fertility under soil moisture stress.

Materials and Methods

Plants and growth conditions

Experiments were conducted in a greenhouse at the Agricultural and Forestry Research Center, University of Tsukuba, Tsukuba, Ibaraki, Japan in 2013. NERICA 1, NERICA 4 and Yumenohatamochi were used. Twenty pre-germinated seeds were sown in a circular pattern in each 1/5000 a Wagner pot filled with 3 kg of nursery soil containing 1.0 g N, 1.8 g P_2O_5 and 1.0 g K_2O on April 30. Each pot was top-dressed with 1.0 g N at 56 days after seeding. Tillers were removed as they emerged in order to obtain uniform main culms.

Air temperature in the greenhouse was measured and recorded every 20 min using a thermologger (TR-72U, T&D Corporation, Nagano, Japan). The daily mean, maximum and minimum temperatures throughout the growing period are shown in Fig. 4. During the heading period of the three cultivars, daily mean and maximum temperatures were 31.2 and 42.2°C, respectively.

Experimental design and treatment

In the experiment, five moisture treatments (T1-T5) were tested in three cultivars (NERICA 1, NERICA 4 and Yumenohatamochi) with three replicates each. A split-plot design was used with the treatments as the main plots and cultivars as subplots.

In T1, the soil was well-watered and maintained above field capacity at -3 kPa throughout the growing period. In the other four treatments, soil moisture stress was induced for 9 days at two different stages: booting stage stress started 9-12 days before heading (81-88 days after seeding) in T2 and T3 and flowering stage stress started 2-6 days before heading (94-100 days after seeding) in T4 and T5. The heading date for each cultivar was anticipated by observing the leaf number index and measuring the distance between auricles of the flag and penultimate leaves. Aside from periods of stress treatment, soil moisture in pots was maintained above field capacity.

Soil moisture stress management

Irrigation was performed three times daily (8:00, 11:00 and

14:00). To produce the stress periods for T2 to T5, water was withheld from the pots on the day before the start of the soil moisture stress treatment. To maintain the soil water potential from -10 to -20 kPa for moderate soil moisture stress (T2, T4) and from -20 to -49 kPa for severe soil moisture stress (T3, T5), the pots were watered until the water potential reached the upper bound of the specified range only when the water potential decreased below the lower bound.

Soil water potential was estimated by measuring volumetric SWC with a Hydrosense Soil Water Sensor (CD620, Campbell Scientific, UT, USA) at a depth of 0-10 cm based on a water retention curve. SWC was plotted against soil water potential measured with a soil water meter (PF-33, Fujiwara Scientific, Tokyo, Japan) on soil prior to seeding.

Measurements

To determine leaf RWC, the topmost fully expanded leaf blade was excised on 3, 6 and 9 days after starting the treatment. Fresh weight (FW) was quickly determined, and the leaf was soaked in distilled water for 12 h at 4°C and weighed to determine turgid weight (TW). Leaves were oven-dried for 48 h at 80°C before obtaining dry weight (DW). Leaf RWC was estimated according to Barrs and Weatherley (1962) as follows:

Leaf RWC (%) = [(FW - DW) / (TW - DW)] × 100

Spikelets from the uppermost four primary rachis branches on three panicles per pot were collected to investigate pollen grains just before anthesis. Five spikelets not including any that had already reached anthesis were sampled when the first spikelet with anthesis was observed. The spikelets were then immediately preserved with FAA fixative (50% ethanol, 5% acetic acid, 10% formaldehyde and 35% distilled water). The numbers of developed and undeveloped pollen grains per anther were determined with 30 anthers per pot by the method of Kariya et al. (1985). Anthers were removed from each fixed spikelet, crushed with a needle and then suspended in a 2 mL solution consisting of 50% ethanol, 2% glycerin, 40% iodine potassium iodide (IKI) and 8% distilled water. Drops of the pollen suspension were placed on a hemocytometer slide and IKI-stained and -unstained pollen grains in each 1 mm^2 cell were counted as developed and undeveloped grains, respectively, under an optical microscope (DM500, Leica Microsystems, Tokyo, Japan). Measurements were conducted four times per pot. The number of differentiated microspores per anther was determined as the sum of developed and undeveloped pollen grains. Additionally, the percentage of developed pollen grains was expressed as the percentage of the number of developed pollen grains to the number of differentiated microspores.

To examine pollination, just after anthesis (within spikelet opening), 15-20 spikelets from the uppermost four primary rachis branches per pot were sampled and fixed as described above. Three stigmas per pot were dissected from randomly selected spikelets and stained with cotton blue solution. The number of pollen grains deposited on each stigma was counted under an optical microscope. In addition, the percentage and length of anther dehiscence at the basal pore on the thecae, which contributes to reliable pollination (Matsui and Kagata, 2003), were estimated with ten anthers from different spikelets per pot under a stereo microscope (SZH, Olympus Corporation, Tokyo, Japan) using a micrometer.

At maturity, three panicles per pot were harvested and hand-threshed. Following the method by Kobata et al. (2010),

spikelets were separated in 80% ethanol solution (specific gravity, 0.86), sinking spikelets were counted as fertilized spikelets, and the spikelet fertility (%) was calculated.

Statistical analysis

Statistical analysis was performed using Statistix 9 software, version 9.0 (Analytical Software 2008, Tallahassee, FL, USA). Analysis of variance was conducted, and Tukey's test or the LSD test was carried out to check for significant differences at $p < 0.05$ in parameters among treatments and cultivars. To identify significant differences between the control and each treatment for the same cultivar, Dunnett's pairwise multiple comparison was used. Percentage data were analyzed after arcsine transformation.

Conclusion

The responses of pollen development and pollination to soil moisture stress at the booting and flowering stages differed among cultivars, and even within NERICAs. Soil moisture stress at the booting stage inhibited pollen differentiation and development more severely, causing poorer basal dehiscence and fewer pollen grains on the stigma in NERICA 1 than in NERICA 4 and Yumenohatamochi. In addition, a greater decline in basal dehiscence in NERICA 4 resulted in fewer deposited pollen grains on the stigma than in NERICA 1 and Yumenohatamochi under soil moisture stress at the flowering stage. These findings suggest that the sensitivity of the fertilization process to soil moisture stress differs among NERICAs. The underlying mechanisms of the different responses to soil moisture stress need to be elucidated in order to improve productivity of NERICAs under conditions of soil moisture stress.

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