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Effects of salt and low light intensity during the vegetative stage on susceptibility of rice to male sterility induced by chilling stress during the reproductive stage

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ABSTRACT

We tested whether exposing rice plants to abiotic stress (salt or shade) during vegetative growth affects the chilling tolerance of reproductive organs, which is one of the most important traits for rice growing in a cool climate; we used two rice cultivars with different tolerance in two growing seasons. We divided the vegetative growth into three phases to clarify the most sensitive period: 7–22 days after transplanting (DAT), 23–38 DAT and 39–54 DAT. Chilling tolerance of the pre-stressed plants was based on the male sterility induced by low temperatures. Shade and salt stress during all three vegetative growth phases significantly reduced stomatal conductance. Shade decreased the specific leaf weight and the leaf sugar and starch contents, but salt had no significant effect, despite causing leaf damage. Low temperatures during the reproductive stage induced spikelet sterility in all plants, but the magnitude was greater in the salt- and shade-stressed plants of both cultivars, especially those stressed late during vegetative growth. The increased spikelet sterility caused by chilling was closely related to the reduction of the total spikelet number per panicle. This is the first study to show that salt and low light stress during vegetative growth increased the susceptibility of rice plants to chilling damage during panicle development.

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KEYWORDS

Chilling tolerance; panicle development; spikelet sterility; rice; vegetative stage

CLASSIFICATION

Crop Physiology

1. Introduction

Rice (*Oryza sativa* L.), which originated in a tropical environment, is sensitive to suboptimal temperatures, especially during the reproductive stage, when temperatures lower than 20 °C can induce male sterility, leading to severe yield losses (Matsushima et al., 1964; Shimono et al., 2002). The ability of chilling to induce male sterility is determined by inherited characteristics, and quantitative trait loci (QTLs) have been detected on most chromosomes: chromosome 1 (Andaya & Mackill, 2003; Kuroki et al., 2011; Takeuchi et al., 2001; Xu et al., 2008), 2 (Andaya & Mackill, 2003), 3 (Andaya & Mackill, 2003; Dai et al., 2004; Mori et al., 2011; Shirasawa et al., 2012; Suh et al., 2010), 4 (Saito et al., 2001; Xu et al., 2008), 5 (Andaya & Mackill, 2003; Shimono et al., 2016; Xu et al., 2008), 7 (Suh et al., 2010; Takeuchi et al., 2001; Xu et al., 2008; Zhou et al., 2010), 8 (Kuroki et al., 2011), 9 (Suh et al., 2010), 10 (Dai et al., 2004; Ye et al., 2010), 11 (Oh et al., 2004; Takeuchi et al., 2001) and 12 (Andaya & Mackill, 2003; Li et al., 1997; Shimono et al., 2016). However, most QTLs have not been annotated in

terms of their physiological or molecular roles in chilling tolerance and the complex nature of the regulatory mechanisms. The underlying mechanisms that control chilling tolerance are therefore not fully understood.

Recently, the inherited chilling tolerance of certain cultivars was reported to vary in response to the environmental conditions during vegetative growth. We found that the episode by low temperature at vegetative stage decreased the chilling tolerance (male sterility) (Shimono et al., 2007; Suzuki et al., 2015). This phenomena was commonly observed over nine rice cultivars at different conditions in a greenhouse (Shimono et al., 2007), in the field (Abe et al., 2013; Kanda et al., 2012; Shimono & Kanda, 2008; Shimono et al., 2011; Shimono et al., 2012), and under fully controlled-environment conditions (Matsumura et al., 2012; Suzuki et al., 2015). However, we found no studies that investigated whether the phenomena could be triggered by environmental cues other than temperature during vegetative growth.

In the field, additional environmental factors, such as solar radiation, which provides the energy used for

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photosynthesis, growth and stress responses (Yoshida, 1981), can fluctuate both seasonally and yearly. In the Tohoku region of northern Japan, one of the world's northernmost rice-growing areas, the *Yamase* wind frequently carries fog and chilling air into rice-producing areas during the growing season (Kanno, 1997), leading to low radiation intensity and chilling. The combined effects of low light intensity and chilling during the reproductive stage can increase spikelet sterility, although this is not inevitable (reviewed by Wada, 1992). However, there has been no study that determined whether low light intensity during vegetative growth affects the chilling tolerance of rice during the reproductive stage.

In the field, salt creates another important abiotic stress that can strongly affect plant growth by increasing water stress and by the direct effects of salt ions (Munns & Tester, 2008). This is likely to become an increasingly serious problem in paddy areas close to the sea, as sea levels rise under global warming (IPCC, 2007), although periodic disasters such as tsunamis can expose even inland fields to salt (Shimono et al., 2012). It is well known that salt stress during vegetative growth can damage the photosynthetic apparatus, cause stomatal closure and reduce growth (Munns & Tester, 2008). During the reproductive stage, salt can induce spikelet sterility (Tsuda, 2007). However, we found no studies of whether salt stress during the vegetative stage can affect chilling tolerance during the reproductive stage.

In the present study, our goal was to provide some of the missing data on impact of low light intensity and salt at vegetative stage on male sterility at the reproductive stage.

2. Materials and methods

2.1. Plant materials

We conducted pot experiments in 2012 and 2013 at Iwate University, Japan (39°42'N, 141°8'E). Seedlings of two rice cultivars with different chilling tolerances ('Hitomebore', with strong tolerance, and 'Sasanishiki', with weak tolerance) were transplanted on 16 May in both years. Plants were grown in 1/5,000-a Wagner pots (4 L size) at a density of 17 plants per pot, in open air in both years. The pots contained 3 L of commercial soil (Agro-Baido, Kanuma Co., Kanuma, Japan) with $N = 1.4$ g, $P_2O_5 = 3.6$ g and $K = 1.5$ g per pot. The plants were pruned to remove all tillers, leaving only the main stem, so as to provide a more uniform physiological status following the standard method of Satake (1976). The plants were grown submerged to a water depth of ca. 5 cm in six tubs (1.00 m × .67 m × .27 m in depth), with the water temperature controlled at 21.5 °C (2012) or

25.0 °C (2013). Temperatures were controlled with a 40-W heater and cooling coils connected to a CA-1115A recirculating pump (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) controlled by a CR-10 datalogger (Campbell Scientific, Inc., Logan, UT, USA), as shown in Supplemental Figure S1a. A Pt100 thermometer was used to measure water temperature (T_w). Water was circulated by a pump to minimize the spatial variation in T_w . Shimono et al. (2007, 2011) provide a detailed description of the set-up.

In 2012, we used 42 pots (= 714 plants) for the experiments: 21 pots for each cultivar, with 9 pots treated at low light intensity and 9 pots for the salt treatments (in each case, 3 T_w conditions during reproductive growth × 3 treatment phases during vegetative growth × 1 pot per treatment combination) and 3 pots for the control (3 T_w conditions during reproductive growth only × 1 pot per treatment). Figure 1 shows the timing and durations of the treatments during vegetative and reproductive growth; section 2.2 provides details of each treatment. In 2013, we used 36 pots (= 612 plants) for the experiments: 18 pots for each cultivar, with 6 pots at low light intensity and 6 pots for the salt treatment (in each case, 3 T_w conditions during reproductive growth × 1 treatment phase during vegetative growth × 2 pots per treatment combination) and 6 pots for the control (3 T_w conditions during reproductive growth only × 2 pots per treatment combination).

2.2. Pre-treatments during vegetative growth and treatments during the reproductive stage

Figure 1 illustrates the study design. During vegetative growth in 2012, the pre-treatments with low light intensity and salt were conducted during three 'phases', each of ca. 2 weeks; starting dates were 7–22 days after transplanting (DAT), 23–38 DAT and 39–54 DAT. The date of end of the treatments corresponded to periods between 40 and 8 days before panicle initiation (DBPI). In 2013, there was only one pre-treatment phase (from 29–42 DAT, ending at 11 DBPI) because of the most sensitive phase judging from the trial in 2012. Since we defined panicle initiation as the date when the young panicle reached a length of 1 mm (measured for 2–3 stems (=plants) at 2-d intervals), and it is known that panicle differentiation begins 7 days before panicle initiation (Yoshida, 1981), we assumed that our pre-treatments occurred before the panicle differentiation stage, even for the phase III treatments, which ended at 8 DBPI in 2012 and 11 DBPI in 2013.

In the shade treatment, black mesh sheets attached to frames (.7 m × 1.7 m × .9 m in height) reduced light intensity to 25% of the ambient photosynthetically active radiation, measured using a linear quantum

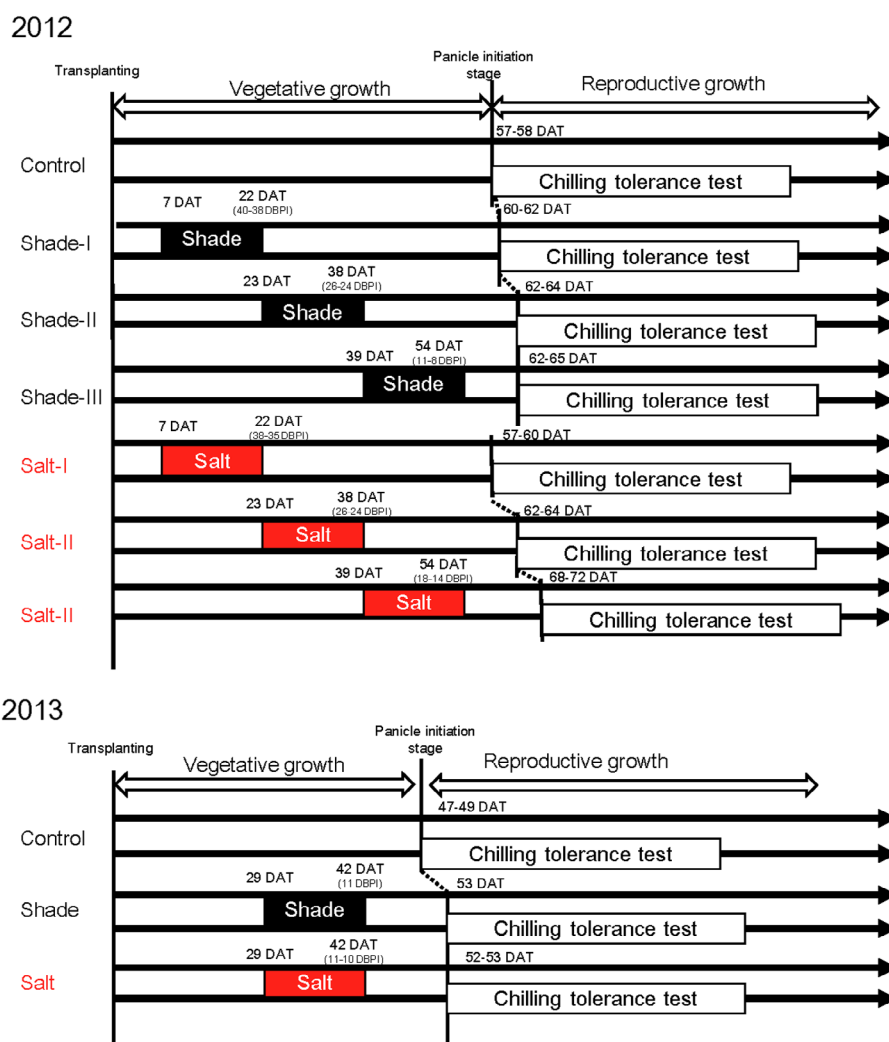


Figure 1. Timing and durations of the pre-treatments (shade or salt) during vegetative growth and of the treatments used in the chilling tolerance test during the reproductive stage. Timings represent the number of days after transplanting (DAT) and the number of days before panicle initiation (DBPI). Controls with no salt treatment; none, no chilling treatment.

sensor (Li-191R, Li-COR, Lincoln, NE, USA), as shown in Supplemental Figure S1b. In the salt treatment, NaCl was added to the water to achieve an electrical conductivity (EC) of 9.0 mS cm^{-1} , adjusted daily. EC was measured with an HI9813-6 EC meter (Hanna Instruments, Woonsocket, RI, USA). After the treatments, the pots were soaked in several changes of fresh water until the EC did not change.

During the reproductive growth (i.e. after panicle initiation), chilling treatments were applied to induce sterility (Supplemental Figure S1c). After the young panicle had reached a length of 1 mm, rice plants were transferred into deep water baths ($1 \text{ m} \times 1 \text{ m} \times 60 \text{ cm}$ deep) that were controlled at a temperature of $18.5 \text{ }^\circ\text{C}$ ($18.5 \pm .8 \text{ }^\circ\text{C}$, average \pm standard deviation of observed data) or $19.0 \text{ }^\circ\text{C}$ ($19.0 \pm .2 \text{ }^\circ\text{C}$) for both cultivars (in 2012) and of $18.5 \text{ }^\circ\text{C}$ ($18.5 \pm .3 \text{ }^\circ\text{C}$) or $19.0 \text{ }^\circ\text{C}$ ($18.7 \pm .2 \text{ }^\circ\text{C}$) for ‘Sasanishiki’ and

$18.0 \text{ }^\circ\text{C}$ ($17.8 \pm .3 \text{ }^\circ\text{C}$) or $18.5 \text{ }^\circ\text{C}$ ($18.5 \pm .3 \text{ }^\circ\text{C}$) for ‘Hitomebore’ (in 2013) by supplying cool water at ca. $15 \text{ }^\circ\text{C}$ through an electromagnetically controlled valve to uniformly expose all panicles to low temperature. Water depth was kept at 30 cm from the shoot base for exposing the developing panicles which initiated at shoot base and then lifted upward. Water was circulated by a high blow air pump (C-5BN, Techno Takatsuki, Osaka, Japan) to minimize spatial variation in T_w . The treatments were maintained until the mid-ripening stage. This methodology is widely used in breeding of new cultivars (Matsunaga, 2005) and in physiology experiments (e.g. Sakata et al., 2014).

2.3. Measurements

During the pre-treatments that were applied during vegetative growth, we measured stomatal conductance

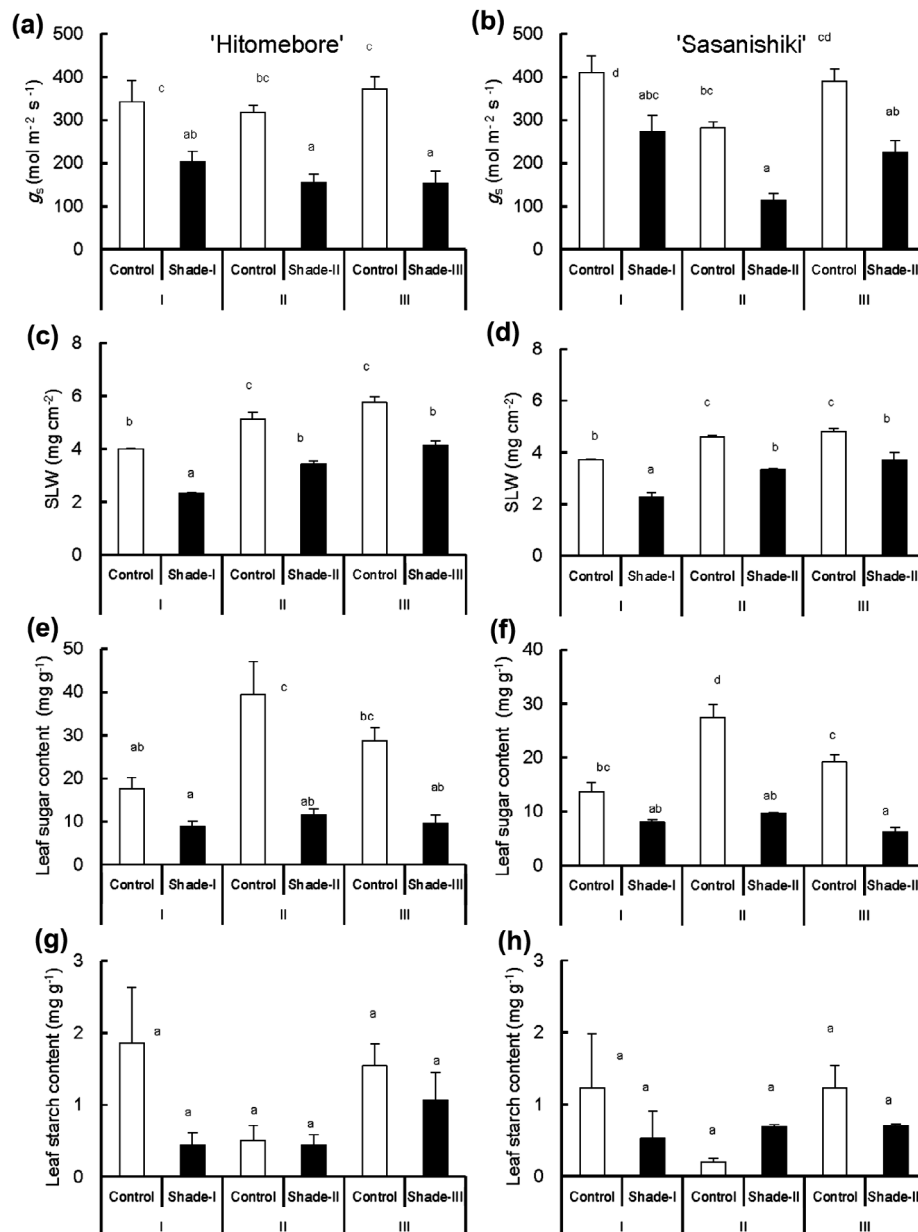


Figure 2. Leaf stomatal conductance (g_s), specific leaf weight (SLW), leaf sugar content and leaf starch content of two rice cultivars exposed to shade treatments during different phases of vegetative growth in 2012. Values are means \pm standard errors ($n = 3$). Controls with no shading; none, no chilling treatment. For a given cultivar and year, bars labelled with different letters differ significantly ($p < .05$).

(g_s) at the uppermost leaf at the lower surface in the morning between 09:00 h and 11:00 h with an SC-1 leaf porometer (Decagon Devices Co., Pullman, WA, USA) at 5~7 days before the end of the pre-treatment in fine weather. To measure the specific leaf weight (SLW; mg dry weight cm⁻²), we sampled the top three leaves at two days before the end of the pre-treatment; leaf area was measured with an AAM-9 leaf area meter (Hayashi-Denko, Tokyo, Japan); then, the leaves were dried at 80 °C for more than 72 h and weighed. The sugar and starch contents of the leaves were also measured

following the method of Suzuki et al. (2015). At harvest, we counted the total numbers of spikelets, fertile spikelet and sterile spikelets on each panicle, and used this data to calculate the percentage of spikelet fertility. Sterile spikelets were carefully identified by backlighting the heads with fluorescent lightbulbs; spikelets that showed no shadowy area (i.e. no developing embryo or grain) were considered to be sterile, following the method of Shimono et al. (2007).

We tested for statistically significant differences using multiple comparisons with Bonferroni's correction. We

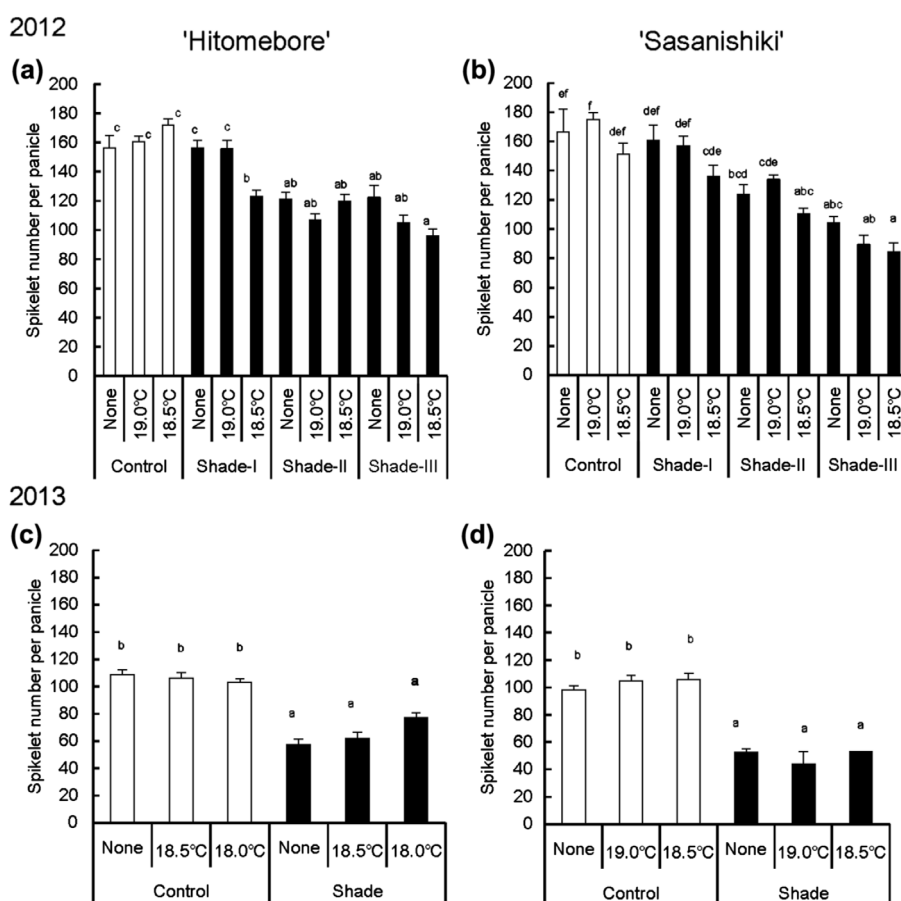


Figure 3. Spikelet number per panicle of rice cultivars exposed to shade treatments at different phases during vegetative growth. Values are means \pm standard errors ($n = 6-9$). Controls with no shading; none, no chilling treatment. For a given cultivar and year, bars labelled with different letters differ significantly ($p < .05$).

also performed linear regression analysis to describe the relationship between the change in spikelet fertility and the changes in spikelet number per panicle. All analyses were performed in Excel Statistics 2008 for Windows (SSRI Co., Tokyo, Japan). We used individual plants as replicates ($n = 6-9$ for spikelet fertility and spikelet number; $n = 3$ for SLW and sugar and starch).

3. Results

3.1. Shade treatment

In the shaded plants in 2012, g_s decreased significantly compared with the unshaded plants during each phase, by 41–59% in 'Hitomebore' and by 33–60% in 'Sasanishiki' (Figure 2(a) and (b)). SLW also decreased significantly compared with the unshaded plants during each phase, by 28–42% in 'Hitomebore' and by 23–38% in 'Sasanishiki' (Figure 2(c) and (d)). In fact, the shaded plants seemed to have flimsy leaves (Supplemental Figure S2). The leaf sugar content decreased compared with the unshaded plants in both cultivars, but the difference was significant only in the

Shade-II treatment in 'Hitomebore' and in the Shade-II and Shade-III treatments in 'Sasanishiki' (Figure 2(e) and (f)). The leaf starch content was not significantly affected in either cultivar (Figure 2(g) and (h)). Panicle initiation stage was delayed by 4–7 days for 'Hitomebore' and for 3–5 days for 'Sasanishiki' (Figure 1). It is visually difficult to identify the differences in plant morphology between shaded and unshaded plants at this stage when is the timing of start of chilling tolerance test during the reproductive stage (Supplemental Figure S3).

In 2012, the spikelet number per panicle at harvest decreased in many of the chilled plants that had been exposed to shade during vegetative growth, but the difference compared with the unchilled plants ('none') in any given phase was significant only in 'Hitomebore' plants chilled to 18.5 °C in the Shade-I treatment; none of the differences were significant in the 'Sasanishiki' plants (Figure 3(a) and (b)). The later the shading was applied in 2012, the greater was the reduction in spikelet number, but the difference was not always significant compared with the unshaded control. In 2013, there was no significant difference in the unchilled plants in either

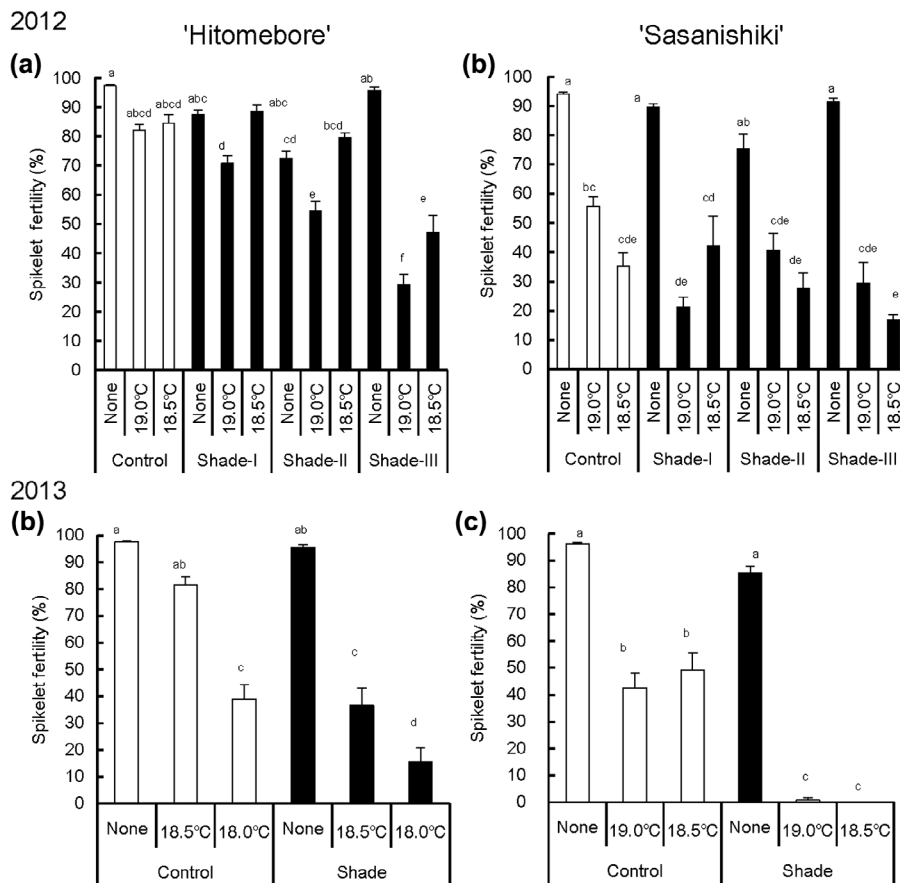


Figure 4. Spikelet fertility of rice cultivars exposed to the shade treatments at different phases during vegetative growth. Values are means \pm standard errors ($n = 6-9$). Controls with no salt treatment; none, no chilling treatment. For a given cultivar and year, bars labelled with different letters differ significantly ($p < .05$).

cultivar (Figure 3(c) and (d)). However, all shaded plants had significantly fewer spikelets than the corresponding unshaded control plants.

Spikelet fertility in the plants that were not chilled during the reproductive stage ('none') was not significantly affected by the shade treatments in both cultivars in both years, except for a significant decrease in the Shade-II treatment in 'Hitomebore' in 2012 (Figure 4). Chilling treatments during the reproductive stage significantly decreased spikelet fertility with greater magnitude for plants exposed to shade at the vegetative growth than control of not pre-treated plants. Thus, the susceptibility to chilling at the reproductive stage for inducing sterility was greater for the shaded plants than for unshaded plants.

3.2. Salt treatment

In 2012, the salt treatment decreased g_s compared with the unsalted control in all vegetative growth phases, by up to 53%, but the difference was significant only in 'Hitomebore' in Salt-II (Figure 5(a) and (b)). Leaves became bleached or brown in the salt treatments, indicating tissue damage (Supplemental Figure S2), but there was

generally no significant effect on SLW, sugar content, or starch content (Figure 5(c)–(h)). However, the following differences were significant in 'Sasanishiki': compared with the unsalted plants, SLW was greater in Salt-III, sugar content was greater in Salt-I and starch content was greater in Salt II. Panicle initiation stage was delayed by salt treatments by 2~10 days for 'Hitomebore' and by 0~15 days for 'Sasanishiki', and the magnitude was greater for later treatments (Figure 1). We can identify that plants treated with salt at phase III only showed leaves that were still brown, and not plants that were treated with salt at early stages of this stage (Supplemental Figure S3).

The spikelet number per panicle generally decreased due the salt treatment in both years, but not all of the differences were significant (Figure 6). In 'Hitomebore', Salt-III significantly decreased spikelet number compared with the unsalted plants, especially for chilled plants in 2012; in 'Sasanishiki', Salt-III significantly decreased spikelet number compared with the unsalted plants in both years.

Spikelet fertility in plants that were not chilled during the reproductive stage ('none') was not significantly affected by the salt treatments, except for a significant

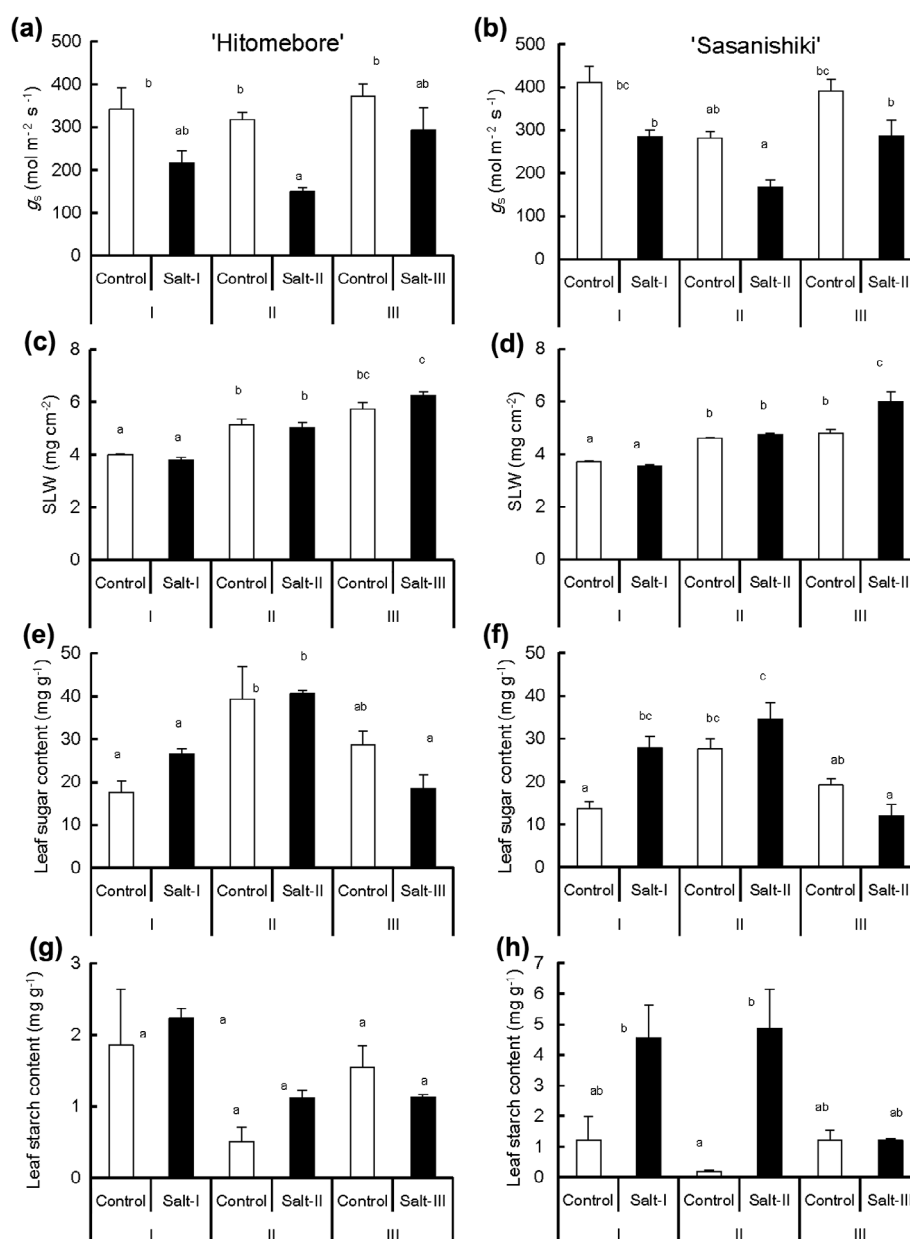


Figure 5. Leaf stomatal conductance (g_s), specific leaf weight (SLW), leaf sugar content and leaf starch content of rice cultivars exposed to salt treatments at different phases during vegetative growth in 2012. Values are means \pm standard errors ($n = 3$). Controls with no salt treatment; none, no chilling treatment. For a given cultivar and year, bars labelled with different letters differ significantly ($p < .05$).

decrease in 'Sasanishiki' in 2012 (Figure 7). Chilling treatments during the reproductive stage significantly decreased spikelet fertility with greater magnitude for plants exposed to salt at the vegetative growth period than plants of not pre-exposed, control. Even for 'Sasanishiki' in 2012, the reduction magnitude of spikelet fertility by chilling during the reproductive stage was greater for salt pre-treated plants during vegetative stage than control of non-pre-treated plants. Thus, the susceptibility to chilling at the reproductive stage for inducing

sterility was greater for the salted plants than for unsalted plants.

4. Discussion

We found salt or shade treatment during vegetative growth decreased the chilling tolerance during the reproductive growth, thereby decreasing spikelet fertility in both years in both cultivars, even their cold tolerance at the reproductive stage is largely different (Figures 4 and 7). This

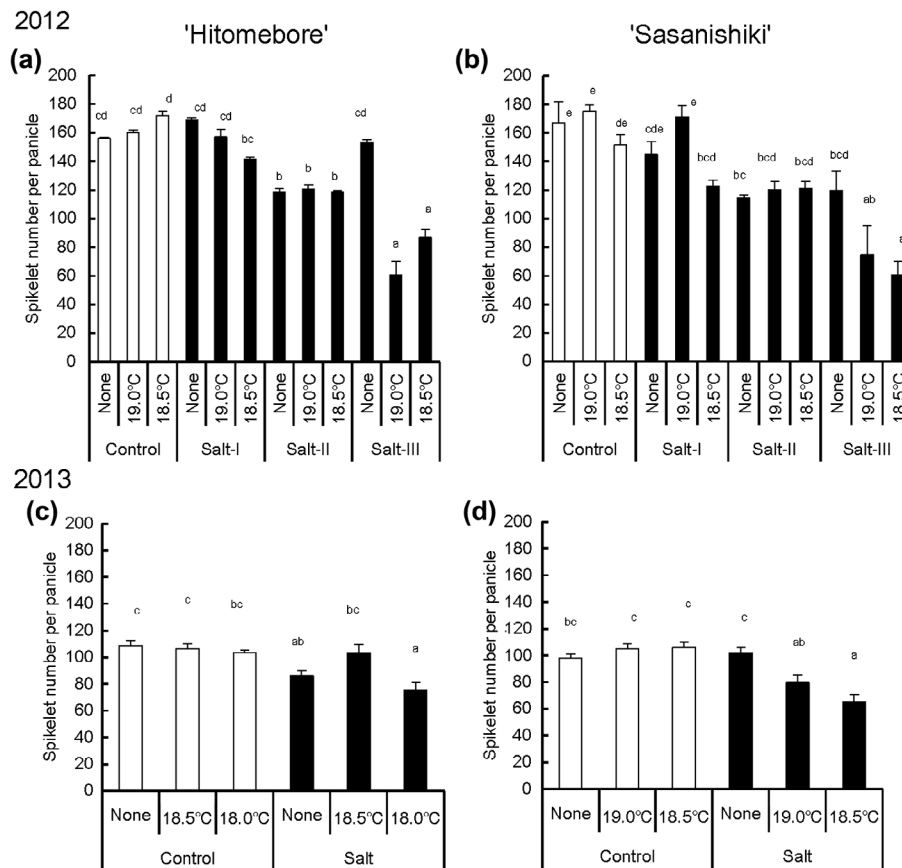


Figure 6. Spikelet number per panicle of rice cultivars exposed to salt treatments at different phases during vegetative growth. Values are means \pm standard errors ($n = 6-9$). Controls with no salt treatment; none, no chilling treatment. For a given cultivar and year, bars labelled with different letters differ significantly ($p < .05$).

phenomena was a similar response of our previous study, which examined the effects of low temperature stress during vegetative growth (Shimono et al., 2007). This is the first demonstration of negative impact of salt or shade stress at vegetative stage on the chilling tolerance at the reproductive stage. It is noteworthy that the responses of leaf morphology and sugar content to salt and shade differed depending on the phase of vegetative growth in which the plants experienced the stress (Figures 2 and 5; Supplemental Figures S2 and S3). Despite the different physiological mechanisms that underlie these responses, the phenotype (tolerance of chilling, expressed in terms of male sterility) changed similarly (Figures 4 and 7).

Figure 8 shows the relationship between the reduction of spikelet fertility and the relative change in spikelet number. We found a significant positive relationship between the two parameters; that is, as the spikelet number per panicle decreases, the spikelet fertility decreases. In our study, shade and salt stress during vegetative growth did not appear to improve chilling tolerance during the reproductive growth at either temperature.

Our results suggest that the reproductive growth of the plants was most sensitive to abiotic stress during phase III, which occurred between 39 and 54 DAT in 2012 (Figures 4 and 7). This phase was a period from 26–11 DBPI in 'Sasanishiki' and 23–8 DBPI in 'Hitomebore' for shade treatment, and from 29–14 DBPI in 'Sasanishiki' and 33–18 DBPI in 'Hitomebore' for salt treatment. Kanda and Shimono (2009) investigated the stage of vegetative growth when the pre-conditioning stress (low water temperature) produced the greatest effect on chilling tolerance of 'Sasanishiki' and 'Hitomebore' during the reproductive stage by dividing vegetative growth into five two-week intervals, and found that the plants were most sensitive between 16 and 11 DBPI, which overlapped with the present results, despite the use of different abiotic stresses. There are two possible explanations for this sensitivity: first is the magnitude of the damage since later growth stages generally experience more severe damage because of the higher transpirational demand by larger plants, combined with increased resistance to water uptake that is caused by

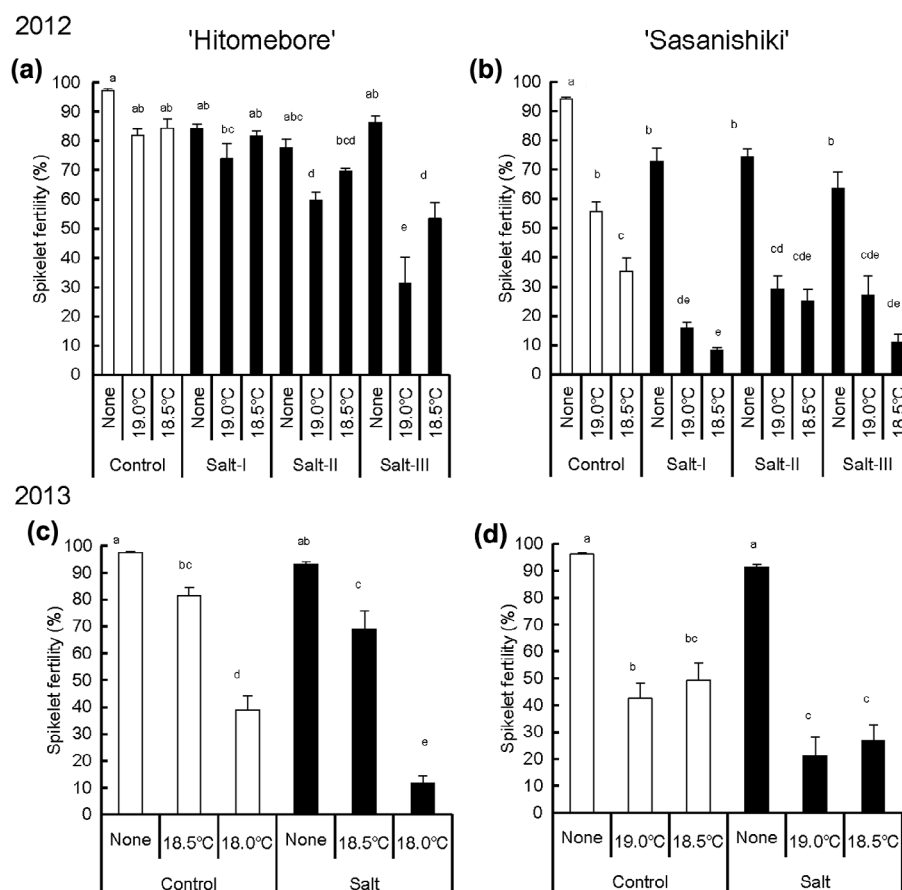


Figure 7. Spikelet fertility of rice cultivars exposed to salt treatments at different phases during vegetative growth. Values are means \pm standard errors ($n = 6-9$). Controls with no salt treatment; none, no chilling treatment. For a given cultivar and year, bars labelled with different letters differ significantly ($p < 0.05$).

the salty water. In terms of measured g_s in our study (Figure 3(a) and (b)) was less affected by salt treatment on later growth stage, but we observed severe damage of lower leaves at later growth stages (Figure S2). Second, the length of the recovery time after exposure of salt or shade might have different responsibilities. As shown in Figure S3, leaves of salt-treated plants at phase III were still bleached or brown at the start of chilling test. Also, different physiological stages may have different degrees of sensitivity, although underlying physiological and genetic mechanisms are not known.

In the present study, we did not measure physiological and molecular responses in developing pollen and anther. Abiotic stresses regulate plant responses through similar gene networks. Low temperature is commonly reported to induce responses via a network similar to that involved in drought and salt responses (Nakashima et al., 2009). In the case of low temperature at vegetative stage, Suzuki et al. (2015) described the molecular and physiological mechanisms responsible for the negative impacts of

pre-conditioning stress (low temperatures during vegetative growth) on chilling tolerance. A low temperature during vegetative growth can downregulate the expression of the stress response genes for ascorbate peroxidase (Os07g0694700), heat shock protein (OsHSP90.1, Os04g0107900) and FK506-binding protein (OsFKBP65, Os04g0352400), which would generally be upregulated to provide protection against chilling stress during the reproductive stage. Our shade or salt stress at vegetative stage might share a similar mechanism for affecting chilling tolerance at the reproductive stage. Further study will be required to confirm whether these changes occurred in our study system.

The present results have strong implications for rice production since they can be used to establish guidelines for growing rice during periods with low light intensity or in areas at risk of high salinity (Shimono et al., 2012). In these areas, farmers must take measures to improve the tolerance of chilling stress, such as using more tolerant cultivars and management practices that will mitigate the

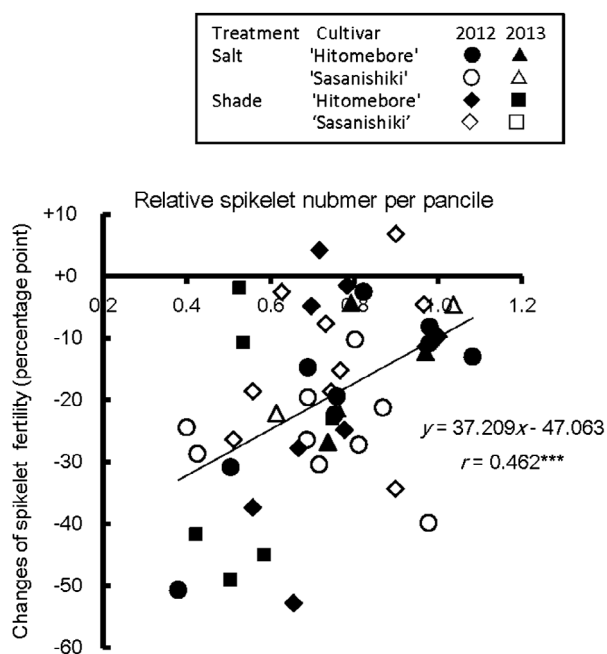


Figure 8. Relationship between the reduction of spikelet fertility (percentage points, value in the shade or salt treatment minus the control value at the corresponding chilling temperature during the reproductive stage) and the relative spikelet number (value in the shade or salt treatment divided by the control value at the corresponding chilling temperature during the reproductive stage) for rice cultivars exposed to shade or salt treatments at different phases during vegetative growth. *** $p < 0.001$.

effects of these stresses. Our results will also be useful in analyses that identify the cause of yield losses by identifying the effects of pre-conditioning by exposure to salt or low solar radiation on the response to chilling during the reproductive stage.

5. Conclusions

We found evidence that exposure of low light intensity and salt during vegetative growth can increase the susceptibility to low temperatures during the reproductive stage, leading to increased male sterility.

Disclosure statement

No potential conflict of interest was reported by the authors.

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