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# IRRIGATION MANAGEMENT RISKS AND ZN FERTILIZATION NEEDS IN ZN BIOFORTIFICATION BREEDING IN LOWLAND RICE

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SUMMARY

As zinc (Zn) fertilizer and water management affect the expression of Zn-enriched grain traits in rice, we studied the effect of Zn fertilizer and water management on Zn uptake and grain yield of different biofortification breeding lines and the possible biases in selection for high grain Zn content. The first field experiment showed that longer duration genotypes had higher grain Zn uptake rate than shorter duration genotypes during grain filling. In the first greenhouse experiment, neither application of Zn fertilizer at mid-tillering nor application at flowering significantly increased the grain Zn concentration. In the second greenhouse experiment, application of alternate wetting and drying (AWD) significantly increased the available soil Zn and plant Zn uptake but not grain Zn concentration. Terminal drying (TD) did not increase the available soil Zn or grain Zn contents. The second field experiment confirmed that differences in TD were not important in understanding differences between genotypes. Zn application is not always necessary to breeding trials unless there is a severe Zn deficiency and there is no need to carefully regulate TD prior to harvest.

## INTRODUCTION

Biofortification is the process of breeding food crops that are rich in bioavailable micronutrients like zinc (Zn). It uses traditional breeding practices and modern biotechnology to develop micronutrient-dense staple crops like rice (Nestel *et al.*, 2006). Rice is 'biofortified' by loading higher levels of Zn into the edible parts of the seeds during growth (http://www.harvestplus.org). Stein *et al.* (2006) showed that Zn biofortification of rice and wheat through plant breeding has a potential to reduce by 20–51% the annual loss of healthy human life years in India caused by Zn deficiency. The effectiveness of biofortification can be augmented with Zn fertilizer application which is sometimes called agronomic biofortification (Cakmak, 2008). Germplasm screening at the International Rice Research Institute (IRRI) found that the Zn concentration in brown rice (n=1,138) ranges from 13.5 to 58.4 mg $kg^{-1}$, suggesting

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**Abbreviations:** AWD – Alternate wetting and drying; CF – Continuous flooding; TD – Terminal Drying.

the genetic potential of increasing the concentration of Zn in rice (Gregorio, 2002). High soil Zn alone will not ensure high grain Zn content; it is the genetic potentiality of the breeding lines that will lead to better uptake, translocation and loading of Zn into the grains.

There is disagreement on whether or not Zn fertilizer is needed during biofortification breeding trials. Some believe that it is important to apply the recommended fertilizer rates in breeding trials, including Zn in regions where it is recommended, to avoid nutrient deficiencies that might prevent the genotypes from reaching their potential for grain yield and grain Zn concentration. Others believe that it is important to know how the biofortified genotypes perform without Zn fertilizer because there is evidence that farmers do not always apply Zn even when it is recommended. However, it may be a moot point because the evidence for the effectiveness of soil-applied Zn fertilizers is mixed at best, with many studies showing that they are ineffective at increasing grain Zn content (Phattarakul *et al.*, 2012) and only a few showing limited effectiveness with increases of 2 to 3 mg $kg^{-1}$ in grain Zn concentration (Rehman *et al.*, 2012; Shivay *et al.*, 2008; Wang *et al.*, 2014). There is some evidence that the timing of Zn fertilizer application to soil might affect plant Zn uptake, with a higher total Zn uptake when Zn is topdressed rather than applied basally. It has also been shown that there is genotypic variation in rice plant responsiveness to Zn fertilization (Jiang *et al.*, 2008). Then, it would be helpful to have a recommendation for Zn fertilization during breeding trials that would minimize potential biases from Zn management and would reveal genetic variation.

Farmers typically allow rice fields to dry for the last two weeks prior to harvest to make movements in the field easier during harvest operations. This terminal drying (TD) is commonly practiced in rice breeding trials but with the added complication of screening genotypes with varying growth durations. In order to keep the later-maturing genotypes flooded during the sensitive time around flowering, TD is timed with the end of the long-duration genotypes, meaning that the earlier-maturing genotypes are harvested in standing water. Because Zn is more available in aerobic (high redox potential) than anaerobic (low redox potential) soil (Johnson-Beebout *et al.*, 2009a), this experimental design may be biased against the short-maturing genotypes since they are kept in anaerobic conditions during the time when the grains are filling (and accumulating Zn) rather than being allowed the normal TD that they would get in a farmer's field. Alternative experimental designs (e.g., staggered sowing dates or grouping genotypes by growth duration) may also be problematic as these require phenotypic data about growth duration, which is not always available. Therefore, it is important to understand the potential magnitude of this 'growth duration by TD'" bias before recommending a major change in experimental design.

In addition to the TD factor, water management during the main part of the season has been shown to influence rice Zn uptake, with aerobic soil (e.g., alternate wetting and drying [AWD] or mid-season drainage) resulting in better soil Zn availability and increased plant uptake than continuously flooded (CF) soil (Tuyogon *et al.*, 2016). It may be interesting to include more aerobic water management in Zn biofortification breeding trials as this may help genotypes to reach their genetic potential for grain

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Zn enrichment. However, as with TD, the growth duration differences of genotypes make it difficult to time the alternating wet and dry periods during a breeding trial. The 'safe' (i.e., without risk of yield loss) AWD protocol requires flooding the soil during the first 10 days after transplanting and for 10 days during flowering, while during the rest of the season, irrigation is only applied when water is 15 cm below the soil surface (http://www.knowledgebank.irri.org/ewatermgt) (Lampayan *et al.*, 2015). Therefore, it would not be practical to attempt a perfect implementation of AWD in breeding trials with wide maturity differences among genotypes, but it is important to understand how variations in vegetative-stage water management affect the expression of grain Zn concentration.

Our study aims to provide a recommendation for optimal water and Zn fertilizer management in breeding trials when selecting for high grain Zn and to investigate potential biases in breeding trials due to differing water statuses during the grain-filling stage of genotypes with different growth durations. The specific objectives of this study are to (1) determine the effect of Zn fertilization during the vegetative or reproductive stage on the grain Zn concentration of a genetically diverse set of Zn biofortification rice breeding lines, and (2) determine the effect of water management during vegetative and reproductive stages on the grain Zn concentration of the same genotypes. We hypothesized that (i) plants with Zn fertilizer applied will have higher plant Zn uptake and, therefore, grain Zn concentration than those with no fertilizer applied, and (ii) plants grown in moderately aerobic soil during the vegetative stage (e.g., AWD) or during the grain-filling stage (e.g., TD) will have higher plant Zn uptake and, therefore, grain Zn concentration than those grown in continuously anaerobic soils.

## MATERIALS AND METHODS

### *Field experiment 1*

The first field trial was designed as a replicated yield trial of advanced biofortification breeding lines, parents and check varieties. We observed the differences in water depth during the grain-filling stage and collected additional data to determine if these differences caused sufficient problems with data interpretation to merit further investigation. This breeding trial was conducted in the dry season of 2009 of the lowland section of the IRRI experiment farm in Los Baños, Laguna, Philippines (14.16°N, 121.26°E). The soil is an Aquandic Epiaquoll (Dobermann *et al.*, 2000). Twenty-one genotypes (Table 1) were grown in a randomized complete block design (RCBD), with three replicates. Irrigation was started 4 days after transplanting and the field was maintained CF (with water depth above the soil varying from 3 to 5 cm) until the TD period 3 weeks prior to the latest harvest, which did not start until after the harvest of the earliest-maturing genotypes. Fertilizer management involved applying the following rates of nitrogen (N), phosphorus (P), potassium (K) and Zn: 140 kg $ha^{-1}$ of N, 30 kg $ha^{-1}$ of P, 30 kg $ha^{-1}$ of K and 5 kg $ha^{-1}$ of Zn to the soil basally. Also, 40 kg $ha^{-1}$ of N was applied at 40 days after seeding and another 60 kg $ha^{-1}$ of N was applied at 60 days after seeding.

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Table 1. List of genotypes used for each experiment.

| First field experiment | Two greenhouse experiments [*abbreviations*] | Second field experiment |
|---|---|---|
| *Parents of biofortification breeding programme* | | |
| IR69428-6-1-1-3-3 | IR 69428-6-1-1-3-3 [*IR 69428*] | IR 69428-6-1-1-3-3 |
| SWHOO (25-1) | SWHOO (25-1) [*SWHOO*] | |
| Areumbyeo (IR) | Areumbyeo (IR) [*Are IR*] | |
| Areumbyeo (KR) | Areumbyeo (KR) [*Are KR*] | |
| Ilpumbyeo | | |
| Daesanbyeo | | |
| Joryeongbyeo | | |
| Hwaseongbyeo | | |
| IR 68144-2B-2-2-3-1-120 | IR 68144-2B-2-2-3-1-120 [*IR 68144*] | |
| IR 68144-2B-2-2-3-1-166 | | IR 68144-2B-2-2-3-1-166 |
| IR 68144-2B-2-2-3-1-127 | | |
| | | A69-1* |
| | | IR55179-3B-11-3* |
| *Elite biofortification lines* | | |
| IR75862-206-2-8-3-B-B-B | IR 75862-206-2-8-3-B-B-B [*IR 75862*] | IR 84848-84-1-3-2 |
| IR75862-221-2-1-2-B-B-B | | IR 83286-22-1-2-1-1 |
| IR 75920-3-3-4 | | IR 84020-84-2-3-2 |
| IR 78809-2B-31-2-2 | | IR 85849-33-1-2-1-2 |
| IR 78809-2B-38-1-2 | | IR 84750-12-1-2-3-1 |
| IR 79805-2-2-2-2 | | IR 83663-20-3-2-2 |
| IR 79821-5-3-1-3 | | IR 85850-75-2-2-3-2 |
| IR 82247-5-3-3-2 | | IR 85850-47-1-1-3-1 |
| | | IR 91143 AC-239 |
| | | IR 91152 AC-317 |
| | | IR 91152 AC-443 |
| | | IR 91152 AC-819 |
| *Check varieties* | | |
| IR64 | | IR64 |
| PSBRc 28 | | PSBRc82 |
| | | NSICRc222 |
| | | NSIC158 |
| | | NSIC214 |

*These two lines are parents from the Zn-efficiency breeding programme rather than the biofortification programme.

Soil and plant samples were collected during the 50% flowering stage and 21 days thereafter. The soil samples were collected from the upper layer (0–2 cm) and lower layer (2–15 cm) of the soil beside the plant, which was also sampled at the same date, for a total of 126 soil samples taken within the breeding trial (21 genotype sub-plots × 3 replicates × 2 sampling times). Available soil Zn was analysed immediately using the modified DTPA extraction procedure (Johnson-Beebout *et al.*, 2009a). The moisture content of each soil sample was obtained to compute the dried soil Zn concentration. Plants were separated into the following parts: stem, leaves (which includes flagleaves and leaf blades), rachis (flowering and maturity only), unfilled grains (flowering and maturity only) and filled grains (maturity only). The plant samples were washed with tap water followed by reverse osmosis water and distilled water before separating them. The separated plant parts were dried in an oven at 80°C for 3 to 5 days or until constant weight was obtained. After taking the biomass, the dried samples

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were ground using a Wiley Mill except for the filled grain samples, which were only dehulled (JLG45, China). The processed plant samples were submitted to the Analytical Services Laboratory (ASL) at IRRI for analysis of plant Zn concentration. Plant samples were acid digested with 1:10 of $HClO_4$:$HNO_3$, and analysed for Zn by inductively coupled plasma – optical emission spectrometry (Perkin Elmer Inc. – Optima 5300DV, USA).

*Greenhouse experiments*

Two greenhouse experiments were conducted at IRRI during the dry season of 2010 to determine the effect of Zn fertilizer application at the mid-tillering or flowering stage on the grain Zn concentration (GH1) and to determine the effect of water management during the vegetative and reproductive stages on the grain Zn concentration (GH2) of genetically diverse genotypes. The Zn fertilizer management experiment (GH1) and the water management experiment (GH2) were designed as two separate experiments to enable the use of more genotypes than would have been manageable with a full factorial design. The experimental design of each experiment was a RCBD, with four replicates. The two factors for GH1 were genotypes (6 levels) and Zn fertilizer treatments (3 levels). The genotypes used were as follows: SWHOO, Are IR, Are KR, IR 68144, IR 69428 and IR 75862 (Table 1). The Zn treatments were as follows: Z0 (no Zn added), Z1 (20 kg $ha^{-1}$ of Zn at mid-tillering) and Z2 (20 kg $ha^{-1}$ of Zn at flowering). The two factors for GH2 were genotypes (same six as for GH1) and water management treatments (four levels). The water management treatments were as follows: continuously flooded through harvest (CF-nTD), continuously flooded with 2–3 weeks terminal drainage (CF-TD), alternate wetting and drying with CF from flowering to harvest (AWD-nTD) and alternate wetting and drying with flooding at flowering followed by 2–3 weeks terminal drainage (AWD-TD). CF treatments were irrigated daily to maintain a water depth of 3–5 cm above soil surface. AWD treatments were irrigated up to a 5-cm water depth and then allowed to dry until light cracking (with a water depth of 10–15 cm below soil surface) before re-irrigation of up to 5-cm water depth.

The soil used for both experiments was collected from a lowland rice field at IRRI, which is less than 1 km away from both field experiments. The soil was flooded when collected from the field and maintained under standing water during the process of homogenization and filling of pots. Sub-samples of the soil were air-dried for background characterization analysis by the Analytical Services Laboratory of IRRI (Table 2). Seeds were obtained from the Zn biofortification breeding programme of the Plant Breeding, Genetics and Biotechnology Division of IRRI. The dimensions of the pots used were 29.5 (top diameter) by 23.6 (bottom diameter) by 27 cm (height), while the weight of soil for each pot was 10.5 kg (field moist). Seeds were sown in seeding trays for 14 days. Three hills with one plant each for the two early-maturing genotypes, whereas four hills with two plants for each hill for the remaining genotypes were transplanted. Differences in the number of hills and plants were because of seedling availability.

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Table 2. Background characterization of soil for greenhouse experiments 1 and 2 and field experiment 2. Values represent mean ± standard deviation of three replicates.

| Parameter | Greenhouse experiments 1 and 2 | Field experiment 2 | Method description and references |
|---|---|---|---|
| pH ($H_2O$) | 6.4 ± 0 | | 1:2 soil:extractant ratio (Thomas, 1996) |
| pH (KCl) | | 4.6 ± 0.24 | |
| Cation exchange capacity, CEC ($cmol_c\ kg^{-1}$) | 28.90 ± 0.03 | 30.65 ± 0.74 | Ammonium acetate pH 7 (Sumner and Miller, 1996) |
| Available K ($cmol_c\ kg^{-1}$) | 0.63 ± 0.15 | 1.27 ± 0.05 | Ammonium acetate pH 7 (Helmke and Sparks, 1996) |
| Organic C ($g\ kg^{-1}$) | 13.5 ± 0.7 | 16.5 ± 1.4 | Potassium dichromate (Walkley and Black, 1934) |
| Available P ($mg\ kg^{-1}$) | 9.68 ± 3.20 | 10.35 ± 1.71 | Ammonium fluoride pH 2.6 (Bray and Kurtz, 1945) |
| Particle size: | | | |
| Sand (%) | 22 ± 2 | 23.67 ± 4.08 | Hydrometer (Gee and Bauder, 1979) |
| Silt (%) | 34.4 ± 1.3 | 39.5 ± 1.22 | |
| Clay (%) | 43.6 ± 1.3 | 36.83 ± 3.06 | |

Fertilization rates for NPK were the same for both experiments at 1.08, 0.51, 0.69 g per pot, which corresponded to 180, 40, 40 kg $ha^{-1}$, respectively. The total N amount of 1.08 g per pot was split in three applications, one basally followed by topdressing at mid-tillering and then at panicle initiation. Rice straw was added at a rate of 30 g per pot, corresponding to 6 t $ha^{-1}$ which provides a carbon source to promote the normal microbiologically-mediated decrease in redox potential (Johnson-Beebout *et al.*, 2009b). Eight pots were randomly selected within each experiment for monitoring the redox potential to verify if the water treatments caused the same chemical changes in the pots as was observed in the field (Tuyogon *et al.*, 2016), and three replicate platinum electrodes were installed in each of these pots, with the tips buried at a 2-cm depth below the soil surface. Redox potential readings were taken every Monday, Wednesday and Friday for the duration of the experiment with a pH/mV meter (Oakton Eutech Instruments, pH 110 Series, USA).

Soil and plant sampling of each pot was conducted three times for the two early-maturing genotypes during mid-tillering (combined with panicle initiation), flowering and maturity stage while four times for the four remaining genotypes, during mid-tillering, panicle initiation, flowering and maturity stages. The dates on which each growth stage was reached differed between genotypes. To analyse the available soil Zn, soil samples were collected from the upper layer (3 cm) of the soil beside the plant that was also sampled on the same date. The soil collected was extracted immediately (without drying) using the modified DTPA extraction procedure (Johnson-Beebout *et al.*, 2009a). Destructive plant sampling was conducted on the same dates when the soil samples were collected. One hill/plant per pot was sampled, leaving one hill for final harvest. Biomass dry weight and Zn concentration in each part were measured and analysed as described above.

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*Field experiment 2*

The Field Experiment 2 was done to determine the effect of Zn fertilizer application at panicle initiation on the grain Zn concentration of 21 genotypes, which includes advanced breeding lines, parents and check varieties. We also took additional measurements to confirm our observations about the complication of maturity differences with terminal water management. This field experiment was conducted in the dry season of 2013 in Block 506 at the IRRI experiment station. The experiment design was a split-plot with three replicates with Zn fertilizer treatments as the main plot and genotypes as the sub-plots. The two Zn fertilizer treatments were as follows: Z0 (no Zn added) and Z1 (10 kg $ha^{-1}$ added to soil at panicle initiation). The field was CF through the flowering stage and TD was implemented approximately 2 weeks prior to the harvest of the longest-duration genotypes. The range in genotype duration was 118 to 145 days from seeding. The fertilization rate used was 150-30-20 of NPK, with four splits of N as follows: 30 kg N $ha^{-1}$ basal, 40 kg N $ha^{-1}$ broadcast at mid-tillering, 40 kg N $ha^{-1}$ broadcast at panicle initiation and 40 kg N $ha^{-1}$ broadcast before heading. Soil was sampled from each sub-plot (126 total soil samples: 21 genotypes × 2 fertilizer treatments × 3 replicates) at the time of 50% flowering to assess the available Zn in the soil samples (as above). Biomass and Zn concentration were analysed for each plant part at mid-tillering, panicle initiation, flowering and harvest stage. Extractions, analysis and computations used were as above.

*Calculations and statistical analysis*

The Statistical Analysis System (SAS) version 9.1.2 (SAS Institute, 2003) software was used for analysis of variance (ANOVA) of the two field experiments and the two greenhouse experiments. All experiments were analysed separately, and data was transformed if necessary to meet normality criteria. When sampling date was a factor, a repeated-measures ANOVA was used with first-order autoregressive (ar(1)) covariance structure. Tukey–Kramer method was used to compare means ($p < 0.05$). Correlation analyses were conducted using the Statistical Tool for Agricultural Research (STAR) version 2.0.1, January 2014, IRRI.

## RESULTS

*Field experiment 1*

The available soil Zn of both the upper and lower layers of the soil of Field Experiment 1 was below 1 mg $kg^{-1}$ at the beginning of the monitoring period, but increased gradually up to 5 and 2.5 mg $kg^{-1}$ in the upper and lower layers, respectively, as TD progressed and the soil became more aerobic (Figure 1a). TD was not started until after the short-duration genotypes were harvested, so the available soil Zn during grain filling was lower for the early-maturing genotypes. The brown rice Zn uptake rate from flowering to 21 days after flowering (grain-filling stage) showed that genotypes with longer days to maturity tended to increase their brown rice Zn uptake rate compared to shorter duration genotypes (Figure 1b), and that the

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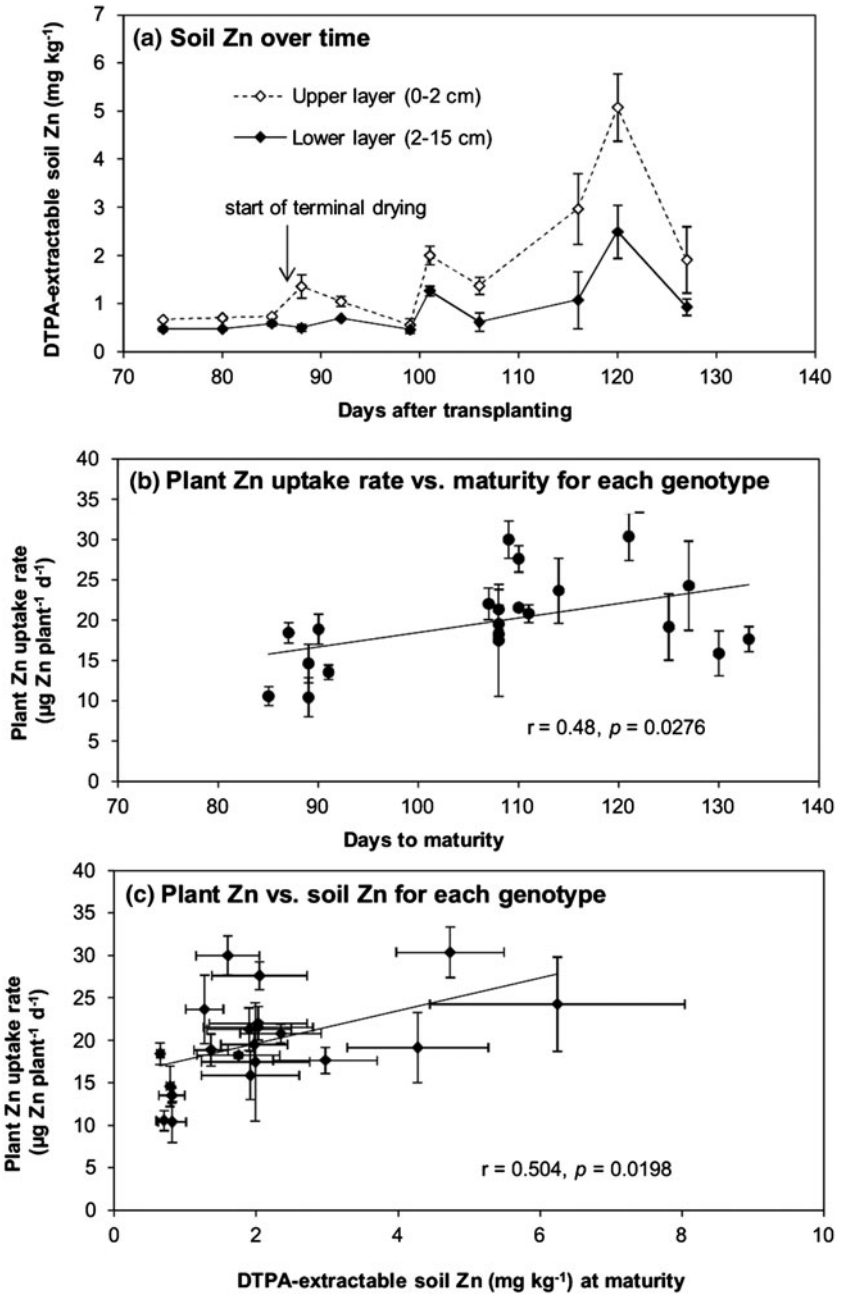


Figure 1. Possible systematic bias favouring late-maturing genotypes in field experiment 1, shown by (a) DTPA-extractable soil Zn at each date throughout the grain-filling stages of all genotypes; the arrow marks the beginning of the terminal drying period, indicating that the early-maturing genotypes were flooded all the way through harvest at maturity. (b) Pearson's correlation between plant Zn uptake rate (grain) during the 21 days after flowering and genotype maturity. (c) Pearson's correlation between plant Zn uptake rate (grain) in the 21 days after flowering and DTPA-extractable soil Zn measured at maturity for each genotype. Error bars indicate + standard error. In (a), data points represent the mean of all plots sampled on a date, while in (b) and (c), data points represent the mean of three replicates per genotype.

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Table 3. Grain yield and grain Zn concentration (brown rice) of four genotypes in greenhouse experiment 1.

| Genotype | Grain yield[†] | | | Grain Zn concentration[‡] |
|---|---|---|---|---|
| | Z0 | Z1 | Z3 | |
| | | g plant$^{-1}$ | | mg kg$^{-1}$ |
| SWHOO | 3.1 ef | 3.9 ef | 3.7 ef | 53.7 a |
| AreIR | 5.5 ef | 7.6 cde | 14.4 ab | 40.0 b |
| AreKR | 4.3 ef | 7.1 cdef | 6.4 def | 35.9 c |
| IR68144 | 16.2 a | 10.0 bcd | 11.3 bc | 38.0 bc |
| ANOVA *p*-values | | | | |
| Zn treatment | | 0.3066 | | 0.9794 |
| Genotype | | < 0.0001 | | < 0.0001 |
| Zn*genotype | | 0.0034 | | 0.2486 |

Z0 = no Zn added; Z1 = 20 kg Zn ha$^{-}$1 added at mid-tillering; Z2 = 20 kg Zn ha$^{-1}$ added at flowering.
[†]Different letters indicate significant differences between the 12 grain yield means (Tukey–Kramer, $p < 0.05$).
[‡]Different letters indicate significant differences between the four genotype means (Tukey–Kramer, $p < 0.05$).

Zn uptake rate was also correlated with plot-level available soil Zn as measured in the soil beside the sampled plant (Figure 1c).

*Experiment GH1: Zn fertilizer treatment*

Grain yield and brown rice Zn concentration are shown in Table 3 for four of the six genotypes. Data for the late-maturing genotypes at harvest is missing because a pest problem after flowering resulted in very low grain yield, making it inappropriate to compare their brown rice Zn concentration with the other genotypes. Significant differences were observed among the genotypes for all the Zn fertilizer treatments. Of the early-maturing genotypes, Are IR had a higher grain yield than SHWOO, while for the mid-maturing genotypes, IR 68144 had a higher grain yield than Are KR. The brown rice Zn concentration ranged from 35 to 57 mg kg$^{-1}$ among genotypes. Significant variation was found among genotypes but not among Zn treatments. The application of Zn fertilizer on either early or late season did not increase the brown rice Zn concentration for any of the genotypes.

Available soil Zn showed that the unfertilized pots had low but probably sufficient available soil Zn (Table 4). The addition of Zn fertilizer resulted in an increase in available soil Zn in the subsequent sampling for all genotypes and Zn treatments. The addition of Zn fertilizer at mid-tillering caused an increase in soil Zn availability that lasted throughout the experiment. ANOVA of the available soil Zn showed significant differences among Zn fertilizer treatments, sampling stages and their interaction. Only Are KR and IR 68144 had significantly increased their total Zn uptake at maturity upon application of Zn fertilizer (Figure 2). Zn fertilizer applied to the mid-maturing genotypes was mostly stored in the stems.

*Experiment GH2: water management treatments*

The grain yield and brown rice Zn concentration for Experiment 2 are shown in Table 5. Data for the late-maturing genotypes at harvest was not included because

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Table 4. DTPA-extractable soil Zn throughout the season for greenhouse experiment 1. Within each genotype maturity group, means with the same letter are not significantly different (Tukey–Kramer, $p < 0.05$). Note that all soil samples were taken prior to Zn fertilization at each sampling time.

| | Early-maturing genotypes | | | Mid-maturing genotypes | | | Late-maturing genotypes | | |
|---|---|---|---|---|---|---|---|---|---|
| Sampling time | Z0 | Z1 | Z2 | Z0 | Z1 | Z2 | Z0 | Z1 | Z2 |
| | mg Zn $kg^{-1}$ soil | | | | | | | | |
| Initial | 0.97 ± 0.43 | | | | | | | | |
| Mid-tillering | 1.39 cd | 1.20 d | 1.55 cd | 3.23 de | 2.30 e | 4.00 d | 2.40 de | 2.15 e | 2.66 de |
| Panicle initiation | –§ | – | – | 2.07 e | 11.8 b | 2.12 e | 2.86 cde | 9.40 a | 3.32 cd |
| Flowering | 2.00 cd | 6.54 b | 2.24 c | 2.93 de | 12.5 b | 2.13 e | 2.94 cde | 6.91 b | 3.14 cde |
| Harvest | 2.01 cd | 10.6 a | 11.9 a | 2.51 e | 8.05 c | 18.5 a | 3.98 c | 9.63 a | 11.1 a |
| ANOVA *p*-values | | | | | | | | | |
| Zn treatment | | <0.001 | | | <0.001 | | | < 0.001 | |
| Date | | <0.001 | | | <0.001 | | | < 0.001 | |
| Zn × date | | <0.001 | | | <0.001 | | | < 0.001 | |

Z0 = no Zn added; Z1 = 20 kg Zn $ha^{-1}$ added at mid-tillering; Z2 = 20 kg Zn $ha^{-1}$ added at flowering.
§For the early-maturing genotypes, the mid-tillering and panicle initiation sampling dates coincided, so there was only one sampling.

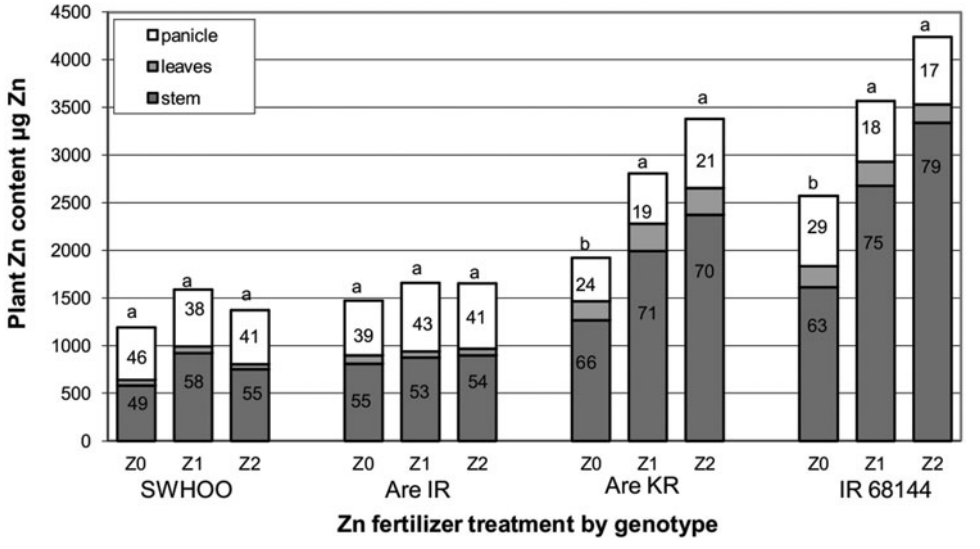


Figure 2. Plant Zn content at maturity (above-ground) in greenhouse experiment 1, showing each genotype and fertilizer-Zn treatment. The ANOVA of the sum of all above-ground plant parts showed significant ($p < 0.01$) effects of genotype, Zn treatment and their interaction. Columns with the same letter had no significant differences in above-ground plant Zn content (LSD, $p < 0.05$). The numbers inside the columns indicate the percent of total above-ground plant Zn in a plant part. Panicles included rachis, filled grains and unfilled grains. Z0 = no added Zn; Z1= 20 kg Zn $ha^{-1}$ applied at mid-tillering stage; Z2 = 20 kg Zn $ha^{-1}$ applied at flowering stage.

a pest infestation that occurred after flowering resulted in very low grain yield, so this was considered as missing data. Significant differences in grain yield among genotypes were observed ($p < 0.001$). The application of AWD water management treatment to Are KR significantly decreased the grain yield compared to the CF water

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Table 5. Grain yield and grain Zn concentration (brown rice) in greenhouse experiment 2. Within a column, different letters represent significant differences (Tukey–Kramer, $p < 0.05$). The non-significant water treatment effect is not shown.

| Genotypes | Grain yield | Grain Zn concentration |
|---|---|---|
| | g plant$^{-1}$ | mg kg$^{-1}$ |
| SWHOO | 6.5 c | 44.8 a |
| Are IR | 13.0 b | 35.1 b |
| Are KR | 20.8 a | 28.6 c |
| IR 68144 | 19.2 a | 32.6 b |
| ANOVA *p*-values | | |
| Water treatment | 0.2024 | 0.0923 |
| Genotype | <0.0001 | <0.0001 |
| Water × genotype | 0.1432 | 0.9972 |

Table 6. DTPA-extractable soil Zn throughout the season for greenhouse experiment 2. Within a maturity group, main effect means followed by the same letter are not significantly different (Tukey–Kramer, $p < 0.05$).

| | Early-maturing | Mid-maturing | Late-maturing genotypes | | | |
|---|---|---|---|---|---|---|
| Main effect | genotypes | genotypes | CF-nTD | CF-TD | AWD-nTD | AWD-TD |
| | mg Zn kg$^{-1}$ soil | | | | | |
| *Sampling time* | | | | | | |
| Initial | 1.15 ± 0.50 | | | | | |
| Mid-tillering | 1.10 b | 2.33 a | 1.85 ef | 1.94 ef | 3.00 ab | 2.86 abc |
| Panicle initiation | –¶ | 2.21 a | 1.74 ef | 2.37 bcde | 2.78 abc | 4.17 a |
| Flowering | 1.17 b | 2.05 a | 2.83 bcde | 2.89 abcd | 2.88 ab | 2.63 bcde |
| Harvest | 1.53 a | 1.19 b | 2.01 cdef | 1.90 def | 1.13 f | 2.31 bcde |
| *Water treatment* | | | | | | |
| CF-nTD | 0.86 c | 1.30 c | *(interaction shown above)* | | | |
| CF-TD | 1.05 bc | 1.73 bc | | | | |
| AWD-nTD | 1.30 b | 2.07 b | | | | |
| AWD-TD | 1.84 a | 2.68 a | | | | |
| ANOVA *p*-values | | | | | | |
| Water treatment | <0.0001 | <0.0001 | 0.0043 | | | |
| Sampling time | 0.0111 | <0.0001 | 0.0006 | | | |
| Water × time | 0.0698 | 0.4587 | 0.0245 | | | |

CF = continuous flooding; AWD = alternate wetting and drying; nTD = no terminal drying; TD = terminal drying.
¶For the early-maturing genotypes, the mid-tillering and panicle initiation sampling dates coincided, so there was only one sampling.

management treatment ($p < 0.01$). The brown rice Zn concentration for the ranged from 28 to 47 mg kg$^{-1}$ among genotypes. Significant differences in brown rice Zn concentration were found between genotypes but not between water management treatments.

The soil-available Zn data for Experiment 2 is shown in Table 6. The application of AWD increased the available soil Zn during flowering ($p \leq 0.001$) and harvest ($p \leq 0.001$) for the early-maturing genotypes while application of TD increased the available soil Zn for AWD treatment only. In the mid-maturing genotypes, AWD significantly increased the available soil Zn during mid-tillering ($p \leq 0.001$), flowering

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($p \leq 0.01$) and harvest ($p \leq 0.01$), but TD *vs.* nTD was significant only in the AWD treatment. For the late-maturing genotypes, AWD significantly increased the available soil Zn during mid-tillering ($p \leq 0.05$) and panicle initiation ($p \leq 0.001$) and TD *vs.* nTD was again significant only in the AWD treatment. The application of TD increased the available soil Zn for all genotypes with AWD applied at early season (Table 6) in spite of the fact that there was not a significant difference in redox potential (Supplementary Material Figure S1, available online at https://doi.org/10.1017/S0014479717000084), but did not make a difference in the CF soil for the main season. At mid-tillering, the application of AWD water treatment significantly increased the total Zn plant uptake of the two late-maturing genotypes, IR 69428 and IR 75862, but not of the early-maturing genotypes presumably because of the shorter duration of the treatment at this growth stage for the early-maturing genotypes (Figure 3a). At panicle initiation, AWD had increased the total Zn plant uptake of the genotypes Are KR, IR 68144, and IR 69428 but not IR 75862 (Figure 3b). At the flowering stage, most genotypes showed no significant difference between water management, but IR 75862 unexpectedly had a lower total Zn plant uptake when AWD water management was used (Figure 3c).

*Field experiment 2*

This study found no correlation between the number of days to maturity and the brown rice Zn uptake rate computed from flowering to harvest stage of 21 elite biofortification breeding lines (Figure 4).

## DISCUSSION

*Effect of terminal drying*

In the Field Experiment 1, in which we collected soil Zn data, the application of TD at the grain-filling stage was correlated with an increase in available soil Zn and in plant Zn uptake rate, confounding observations about genotype differences (Figure 1). Inherent genotypic differences between the early- and late-maturing genotypes could explain the same observation; hence, with this experimental design, it is not possible to know which the most important factor is. Due to differences in maturity between genotypes relative to the start of the TD period, there was a possible bias against the early-maturing genotypes because of the lower available soil Zn conditions during grain filling. A greenhouse experiment designed to compare TD with CF showed that TD had no effect on grain Zn at least for early- and mid-maturing genotypes (Figure 3). Therefore, in spite of the possible confounding influence observed in the Field Experiment 1, our greenhouse data indicated no compelling reason to try to maintain uniform water conditions during the grain-filling stage across genotypes with different maturity periods. We used a new experimental design in the Field Experiment 2 with an attempt to understand whether intentional manipulation of soil Zn supply would affect the genotype performance, and this time, we observed no correlation between the number of days to maturity and the brown rice Zn uptake rate or between soil Zn and brown rice Zn uptake (Figure 4). In this experiment, the

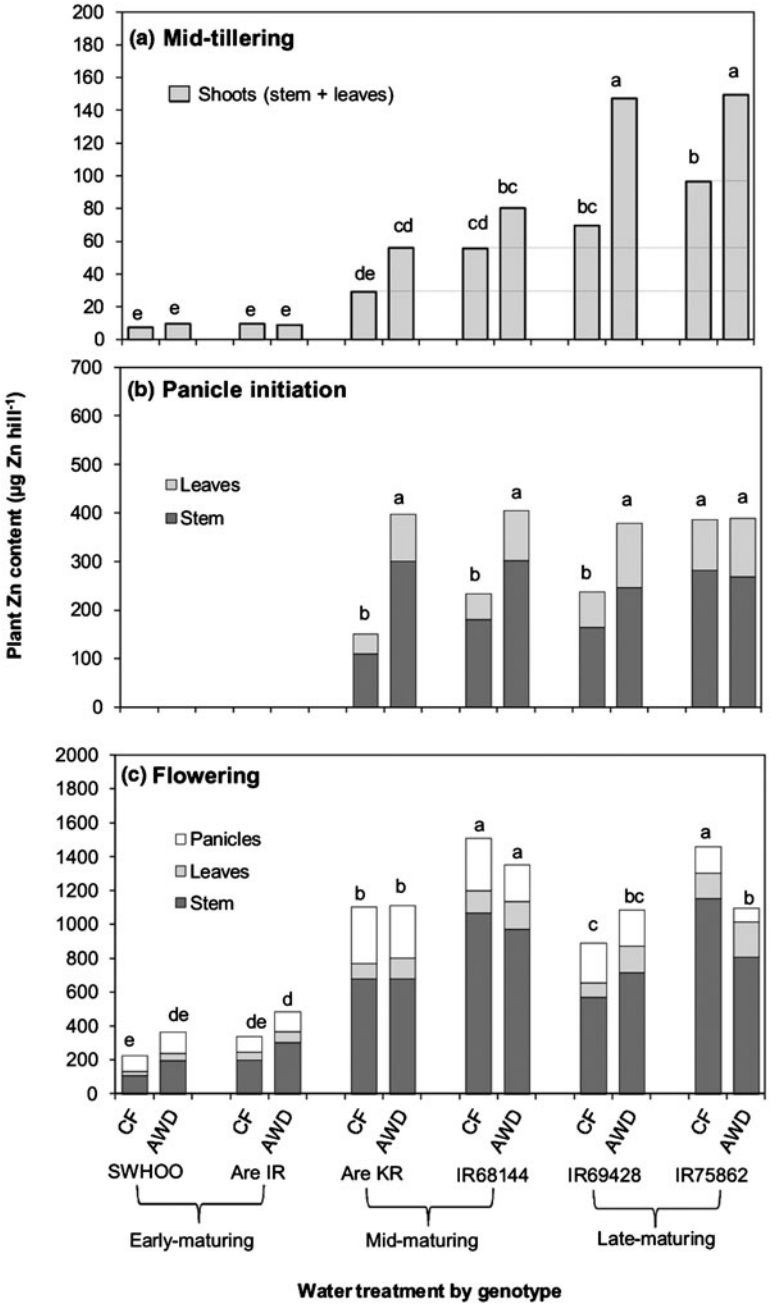


Figure 3. Plant Zn content (above-ground) of each genotype and water treatment in greenhouse experiment 2, showing various plant tissues at different growth stages: (a) mid-tillering; (b) panicle initiation; (c) flowering. The ANOVA of the sum of plant parts within each growth stage showed significant ($p < 0.001$) effects of genotype, water treatment and their interaction. Within a growth stage, letters above columns indicate significant differences in total above-ground plant Zn content (LSD, $p < 0.05$). Panicles included rachis and unfilled grains. CF = continuous flooding; AWD = alternate wetting and drying.

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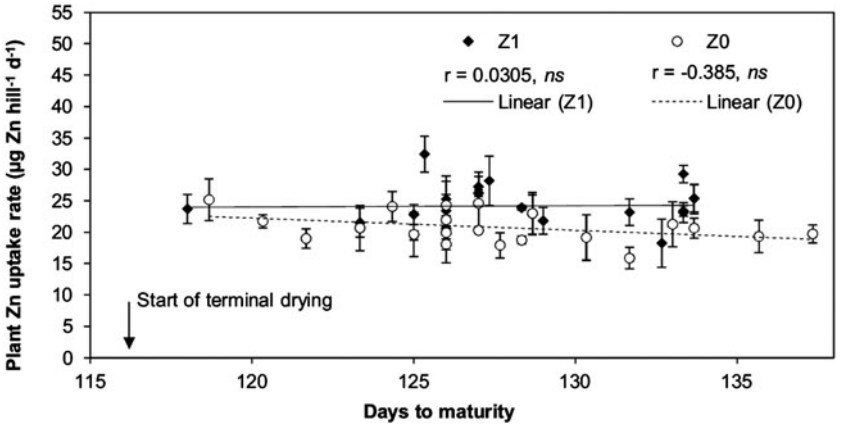


Figure 4. Lack of correlation between plant Zn uptake rate (grain) and genotype maturity in field experiment 2. Data points represent the mean of three replicate plots per genotype. Z0 = no added Zn; Z1 = 10 kg Zn $ha^{-1}$ applied at panicle initiation.

differences in days to maturity among the genotypes had a smaller range than in the Field Experiment 1.

*Effect of Zn fertilizer application*

The application of Zn fertilizer increased the available soil Zn in GH1 (Table 4). Upon the addition of Zn fertilizer in either early or late season, the soil was able to sustain high soil Zn throughout the experiment, in contrast to some studies in which a rapid decline in extractable soil Zn was found after fertilization (Bunquin *et al.*, 2016). Based on hypothesis (i), we expected that after the application of Zn fertilizer at the late season, plants would increase their Zn uptake and, therefore, increase their brown rice Zn concentration compared to the plants with no Zn fertilizer applied and the plants that received Zn fertilizer at the early season. Part of the hypothesis was confirmed in that some genotypes increased their total plant Zn uptake after Zn fertilizer was added both for early and late seasons. The mid-maturing genotypes significantly increased their plant Zn uptake after Zn fertilization at early and late seasons, while the early-maturing genotypes did not increase their Zn uptake for either Zn fertilizer applications at early or late season (Figure 2). However, the increase in plant Zn uptake did not result in a significant increase in brown rice Zn concentration at maturity, contrary to the second part of the hypothesis. The early-maturing genotypes had a lesser time of accumulating Zn from the soil even though the available Zn was increased. In genotypes that increased their plant Zn uptake but not brown rice Zn concentration upon Zn fertilization, the plants moved the extra Zn from the soil to the stem rather than to the grains. Treatments and genotypes possibly vary in the amount of Zn sequestered in their vegetative parts. In the Field Experiment 1, a uniform rate of 5 kg Zn $ha^{-1}$ was applied to the whole field, making it impossible to test the effect of Zn fertilization. In the Field Experiment 2, the genotypes did not respond to a mid-season (panicle initiation) Zn fertilization

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by increasing brown rice Zn concentration, presumably because any extra Zn was stored in other plant parts rather than in grains. The most common agronomic recommendation for Zn fertilization of rice is basal application at 5 to 10 kg Zn $ha^{-1}$, but this is usually ineffective at increasing brown rice Zn because the availability of the added Zn in the soil decreases rapidly (Tuyogon *et al.*, 2016). Alternative methods of Zn fertilization include topdressing by broadcasting $ZnSO_4$ later in the season (at panicle initiation or flowering) and foliar application in the early milk stage (Mabesa *et al.*, 2013). In summary, there is little data to support the practice of soil-applied Zn fertilization for the Zn biofortification of flooded rice.

*Water management*

In the greenhouse experiment (GH2), we demonstrated that Zn in the soil is more available in moderately aerobic conditions (Table 6) and that this difference in soil Zn leads to a difference in total plant Zn uptake (Figure 3) when the more aerobic water treatment (AWD) is applied throughout the main part of the cropping season. The increase in plant Zn uptake for the mid- and late-maturing genotypes at mid-tillering and panicle initiation (Figure 3) can be attributed to the increase in available soil Zn because of the AWD water management. No increase in plant Zn uptake was observed for the early-maturing genotypes in any sampling stage, which can be caused by the shorter time that the plants were exposed to more aerobic soil, hence, giving no time for the differences in plant uptake to become apparent. Another possible explanation is that these two particular short-duration genotypes were less susceptible to changes in soil Zn availability.

In the late part of the season, it was expected that the soil Zn would increase after the application of TD near the end of the season, but there were not always significant differences between TD and nTD (Table 6). With the late-maturing genotypes, the greenhouse air temperature was higher leading to faster evaporation of both TD and nTD treatments and resulting in an increase in redox potential for both treatments. Even in treatments which showed a difference between TD and nTD in available soil Zn, there were no differences in brown rice Zn concentration (Table 5), implying that the short duration of the TD treatments was not sufficient for the plants to be affected by soil Zn availability differences.

In summary, hypothesis (ii) was only partly confirmed. AWD did cause an increase in plant Zn uptake in four of the genotypes, but that increase in total plant Zn did not result in elevated brown rice Zn concentration. In contrast to our hypothesis, TD had no effect on brown rice Zn concentration, perhaps because the short time did not result in sufficient drying for changes in soil redox state at lower depths (Johnson-Beebout *et al.*, 2009b).

## CONCLUSIONS

When managing Zn biofortification breeding experiments for rice, it is important to understand the possible effects of Zn fertilizer and irrigation management on the goal of selecting Zn-enriched lines. Our recommendation for Zn fertilizer management

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is that it is usually unnecessary to apply any Zn, except in cases of very severe Zn deficiency. Foliar application has been shown to increase brown rice Zn concentration in diverse genotypes (Mabesa *et al.*, 2013), but it can mask the genotypic variation and it would be logistically impractical to manage early-milk-stage spraying in plots with genotypes of mixed maturity. Our recommendation for irrigation management is to avoid continuous submergence of the soil throughout the season (i.e., implement a mid-season drainage for one week during vegetative growth or allow the water depth to drop to <1 cm above the soil surface for part of the season), as this will enable the germplasm to reach their genetic potential for brown rice Zn, and then to keep the soil flooded before and after flowering to improve flowering and seed set. Although the practice of terminal drainage has the potential to confound genotypic differences in brown rice Zn when growth duration differences are large, our data showed that it is not necessary to stagger sowing times or otherwise manipulate the experimental design, because the available soil Zn in the last two weeks prior to harvest does not have a significant effect on grain Zn concentration. Another possible strategy to eliminate the terminal drainage complication is to maintain CF up until all genotypes are harvested. It is better to minimize the range in maturity within one trial, in case of any intentional or unintentional disturbance of available soil Zn that may result in some genotypes growing in a more favourable environment for a greater proportion of the growing season than others.

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## SUPPLEMENTARY MATERIAL

For supplementary material for this article, please visit http://doi.org/10.1017/S0014479717000084

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