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ORIGINAL ARTICLE/SHORT PAPER

An agar nutrient solution technique as a screening tool for tolerance to zinc deficiency and iron toxicity in rice

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Abstract

Studies examining iron (Fe) toxicity and zinc (Zn) deficiency in rice have shown that screening experiments in nutrient solutions are of limited use because the rankings of genotypes as tolerant or intolerant can be very different from the results obtained in field-screening experiments. A possible reason for such deviation is that crucial rhizosphere processes cannot be reproduced in nutrient solutions. The objective of the present study was to evaluate the suitability of low-concentration agar nutrient solutions (ANS) as an alternative screening tool. Agar was dissolved in boiling water and mixed with nutrient solution to achieve a final agar concentration of 0.1% (w/v). Zinc deficiency was induced by supplying Zn at a low concentration (0.1×10^{-3} μmol L^{-1}), while Fe toxicity was induced by supplying excess Fe^{2+} (200 mg L^{-1}). Three-week-old seedlings were transplanted into this medium. Symptoms of Zn deficiency and Fe toxicity developed more rapidly in ANS compared with conventional nutrient solutions (CNS). For Zn deficiency this was probably because of the development of Zn depletion zones as a result of the reduced convection in the viscous agar medium. In the case of Fe toxicity we observed far less Fe precipitation in ANS compared with CNS. Genotypic comparisons showed that the tolerance rankings obtained in ANS were very similar to the field tolerance rankings, whereas this was not the case in CNS. This was particularly evident with regard to the considerable root growth inhibition detected in intolerant genotypes when stress treatments were imposed in ANS.

Key words: genotypic screening, leaf bronzing, redox potential, rhizosphere, root growth.

INTRODUCTION

Zinc (Zn) deficiency and iron (Fe) toxicity are nutrient disorders that reduce rice (*Oryza sativa* L.) yields on millions of hectares worldwide (Dobermann and Fairhurst 2000; Ismail *et al*. 2007). In India alone approximately 10 Mha are considered Zn deficient (Singh *et al*. 2005), while Fe toxicity represents a serious constraint on 30–60% of the lowland rice area of West Africa (West African Rice Development Association 2001). Considerable effort has been spent on understanding plant adaptations to overcome such limitations, with the aim of breeding new varieties with improved tolerance to such nutrient disorders. One limiting factor in these efforts is a lack of rapid artificial screening methods that reliably reproduce the tolerance rankings observed in field trials (Neue *et al*. 1998; Shimizu *et al*. 2005).

A common problem in using nutrient solutions to study Fe^{2+} toxicity is the tendency of ferrous iron to be rapidly oxidized with subsequent precipitation. Shimizu *et al*. (2005) proposed lowering solution temperatures to 20°C to reduce Fe precipitation. However, even with this modification, the selection of tolerant genotypes in solution was not successful because the selected genotypes did not show increased tolerance to Fe toxicity under field conditions (Nozoe *et al*. 2008). Similarly unsatisfactory results were obtained with low-Zn nutrient solutions. A comparison of data from field experiments conducted on a Zn-deficient soil with screens in chelator-buffered solutions showed that tolerance rankings between the different genotypes differed considerably between the two screening methods (Wissuwa *et al*. 2006).

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The reasons for the failure of nutrient solutions as reliable screening tools will vary depending on the nutrient disorder, for example, Zn deficiency or Fe toxicity; however, a common feature of both of these stresses is that they result from complex rhizosphere processes in flooded soils (Kirk 2004; Neue *et al*. 1998). Rice and other plant species are known to actively change their rhizosphere through leakage or excretion of oxygen, protons and organic compounds, such as organic acids or mucilage. Genotypic differences in tolerance to soil-related stresses have typically been associated with differences in the excretion rates of these beneficial compounds (Hoffland *et al*. 2006; Kirk 2004). One of the inherent limitations of nutrient solutions as a screening tool is that they are unable to realistically reproduce complex rhizosphere processes or allow genotypes to lastingly alter rhizosphere conditions. Any beneficial root exudates will not remain close to the roots for an extended period of time because convection and unhindered diffusion prevents the establishment of a rhizosphere in solution (Wiengweera *et al*. 1997).

The ability to conduct genotypic comparisons in a less liquid medium would allow for the establishment of a quasi-rhizosphere. Agar medium has been used to study anatomical changes in rice roots exposed to excess Fe^{2+} (Green and Etherington 1977) and to visualize plant-induced rhizosphere changes (Marschner and Römheld 1983). However, these studies used a solidified agar medium (0.5–0.75% agar, w/v) that may not be an ideal screening tool. More recently, Wiengweera *et al*. (1997) suggested using low-concentration agar nutrient solutions (0.1% agar, w/v) as a way to limit convection within solutions. Subsequently this method has been used in studies of radial oxygen loss and root morphological changes in rice (Colmer 2003; Insalud *et al*. 2006), but to the best of our knowledge these low-concentration agar solutions have not been used in routine evaluations of genotypic responses to nutrient deficiencies or toxicities.

The objective of the present study was to assess the suitability of low-concentration agar nutrient solutions (ANS) in screening rice for tolerance to Zn deficiency and Fe toxicity. In particular we tested: (1) whether rice shows near normal growth in agar nutrient solution, even for screening periods of up to 2 weeks, (2) whether conditions around rice roots (pH, Eh) are measurably altered by the plant, indicating the development of a quasi-rhizosphere, (3) whether genotypic differences detected in ANS resemble field tolerance rankings more closely than conventional nutrient solutions (CNS). One important consideration in our study was to develop a simple screening method that avoids costly inputs and could, therefore, be used in less affluent rice growing regions of the world.

MATERIALS AND METHODS

Growth conditions

Seeds were surface sterilized with 5% $NaClO_3$ and 0.15 mol L^{-1} KPO_4, washed thoroughly with deionized water and soaked in water at 30°C in the dark for 3 days until germination. Germinated seeds were placed in mesh floating on 0.3 mmol L^{-1} $CaCl_2$ solution. After 1 week, uniform rice seedlings were transplanted to half-strength modified Yoshida solution (Yoshida *et al*. 1976) with the following composition at full strength: NH_4NO_3 1.42 mmol L^{-1}, $KH_2PO_4 \times 2H_2O$ 0.05 mmol L^{-1}, K_2SO_4 0.5 mmol L^{-1}, $CaCl_2 \times 2H_2O$ 1 mmol L^{-1}, $MgSO_4 \times 7H_2O$ 1 mmol L^{-1}, $MnCl_2 \times 4H_2O$ 9 μmol L^{-1}, $(NH_4)_6Mo_7O_{24} \times 4H_2O$ 0.07 μmol L^{-1}, H_3BO_3 18.5 μmol L^{-1}, $CuSO_4 \times 5H_2O$ 0.16 μmol L^{-1}, Fe-ethylenediaminetetraacetic acid (FeEDTA) 36 μmol L^{-1}, $ZnSO_4 \times 7H_2O$ 0.15 μmol L^{-1}; for plants in the Zn experiments this solution was prepared without Zn. This pre-treatment period lasted for 2 weeks. Three-week-old rice seedlings, with four leaves per plant, were transferred to treatment trays that had tightly fitted lids into which holes had been drilled to fit plants at the desired planting density (12 holes per 12-L tray for ANS; 20 holes per 45-L tray for CNS). Two seedlings per hole were fastened in place with soft sponges so that seedling roots were uniformly immersed for 2 cm. Plants were grown in a growth chamber with an 11 h dark period and a night temperature of 24°C. A 150-min transition period between darkness and daytime conditions was used (this period was also used for day–night transition): temperatures and light intensities gradually increased until a maximum light intensity of 300–350 μmol m^2 sec^{-1} at 30°C was reached and this was maintained for 8 h.

Nutrient solution preparation

For experiments with ANS, 12-L black plastic trays measuring 37 cm × 25 cm × 14 cm (length × width × depth) were used and filled with 6 L of deionized water followed by 2.4 L of 5× strength stock nutrient solution (modified Yoshida solution). Concentrations of the Zn and Fe treatment factors varied according to the treatment specifications (see below). Zinc and Fe were added separately (in 1 L of water). Subsequently, 12 g of agar was dissolved in 1 L of boiling deionized water, allowed to cool to approximately 60°C and then added to the trays (the final agar concentration was 0.1% [w/v]). The trays were filled to 12 L with deionized water and the pH of the medium was adjusted to pH 5.6–5.7. Forty-five liter trays measuring 46 cm × 33 cm × 30 cm (length × width × depth) were used for CNS treatments because previous experiments had shown that a larger volume offered some buffer against pH fluctuations.

The same 1× strength modified Yoshida solution (final concentration) that was used in ANS was also used in CNS. The pH of the nutrient solutions (CNS) was adjusted to 5.5 every other day.

Plant material and treatments

Genotypes IR70617, IR24637 (both tolerant), Milyang23 (intermediate), IR64 and SZH2 (both intolerant) were selected for experiments with excess Fe^{2+} because they had previously shown a broad range of responses to Fe toxicity in the field (M. Wissuwa, unpubl. data, 2006). In all control treatments, Fe was supplied as FeEDTA (50 μmol L^{-1}). FeEDTA was replaced by $FeSO_4$ in the excess Fe^{2+} treatments, either at concentrations of 300 mg Fe^{2+} L^{-1} (CNS) or 200 mg Fe^{2+} L^{-1} (ANS). Previous tests had shown that these two concentrations produced roughly equal stress levels. Two additional subtreatments were prepared within the excess Fe treatment. To buffer the anticipated drop in pH and to provide organic matter that would allow microbial degradation with ensuing consumption of O_2, either succinate (as 1 mmol L^{-1} sodium succinate) or starch (1 g L^{-1}) was added to the ANS.

Experiments on Zn deficiency were conducted with genotypes IR74 (intolerant) and RIL46 (tolerant); these genotypes were classified based on results from field experiments (Ismail *et al.* 2007). Plants were supplied with two Zn treatments; either 0.1×10^{-3} μmol L^{-1} $ZnSO_4$ (deficient) or 1.5 μmol L^{-1} $ZnSO_4$ (control).

Data collection and analysis

Changes in redox potential and pH were monitored over the 2-week growth period using a handheld pH meter (Model pH82; Yokogawa, Tokyo, Japan) and platinum glass electrodes. In the ANS treatments we differentiated between the "rhizosphere" and the bulk agar (at a distance of 8 cm from the nearest plant). Bulk agar measurements were taken repeatedly with stationary platinum glass electrodes (Eh) and by inserting a pH electrode through extra holes in the lid. For measurements of "rhizosphere" Eh, platinum glass electrodes were placed inside the root zone at depths of 2–7 cm. Rhizosphere pH was measured in a similar way using a needle-shaped micro pH electrode (Lazar Research Laboratories, Los Angeles, CA, USA). Rhizosphere measurements were repeated 8–10 times to obtain a representative value, while bulk values are the means of four measurements.

The extent of leaf bronzing was scored at harvest and was based on a scale of 0 to 9 according to Wissuwa *et al.* (2006). Shoots and roots were separated and oven-dried at 70°C for 3 days prior to weighing. For the analysis of mineral composition, the samples were burned in a muffle furnace for 6 h at 550°C, dissolved in 2 mol L^{-1} HCl, filtered and analyzed by inductively coupled plasma atomic emission spectroscopy. All experiments were conducted in a randomized complete block design with three or four replicates.

RESULTS AND DISCUSSION

Treatment effects on plant growth and related parameters

Rice seedlings showed normal growth without visual symptoms of nutrient deficiency or toxicity in full-strength (control) Yoshida nutrient solutions containing 0.1% agar (ANS). However, we observed a 20% reduction in plant dry weight in the control ANS relative to the full-strength conventional nutrient solution (CNS); an interaction between the growth medium and genotype was not detected, indicating that all genotypes showed a similar reduction in dry weights in ANS (data not shown). Treatment effects were highly significant in ANS and shoot dry weights were reduced by approximately 50% in the low-Zn treatment and by approximately 43% in the excess-Fe treatment relative to the respective controls (Table 1). Shoot Zn concentrations in the low-Zn treatment decreased to 9.0 mg kg^{-1} which is well below the threshold level of 15–20 mg kg^{-1} considered indicative of Zn deficiency (Dobermann and Fairhurst 2000; Sakal *et al.* 1982). A clear effect of the treatment was also seen in the excess Fe treatment where shoot Fe concentrations increased more than fourfold to 840 mg kg^{-1}, which is in the range typically associated with Fe toxicity (Becker and Asch 2005). These changes in Zn and Fe tissue concentrations confirmed that the reductions in dry weight were caused by the treatment factors. Furthermore, the leaves of plants in the low-Zn and excess-Fe treatments showed the typical symptoms (bronzing) associated with both stresses (Table 1).

Changes in pH and redox potential in the agar nutrient solution

We hypothesized that a screening system using viscous agar would be characterized by low oxygen diffusion, and that the absence of convective mixing would more closely resemble the conditions in paddy soils compared with conventional nutrient solutions. Monitoring pH and Eh showed that the erratic changes in pH typically encountered with the addition of excess Fe^{2+} were avoided in ANS (Fig. 1). Within 48 h the pH in CNS containing 300 mg L^{-1} Fe^{2+} decreased from an initial pH of 5.6 to below pH 4.0 and reached a level that can be toxic to rice roots. Therefore, it was necessary to adjust the pH every 48 h in CNS. In contrast, the pH in ANS decreased less rapidly and remained in the

Table 1 Effects of simulated Zn deficiency and Fe toxicity in agar nutrient solution on plant dry weight, leaf bronzing and tissue Zn and Fe concentrations

Trait	Low Zn (0.1 nmol L^{-1} $ZnSO_4$)	Control (1.5 μmol L^{-1} $ZnSO_4$)	HSD	Excess Fe^{2+} (200 mg L^{-1} $FeSO_4$)	Control (36 μmol L^{-1} FeEDTA)	HSD
Shoot dry weight (mg)	165.1	317.3	***	250.1	435.2	***
	(12.5)	(11.5)		(11.2)	(28.3)	
Leaf bronzing	2.9	0	***	4.6	0	***
	(0.8)			(0.4)		
Shoot Zn (mg kg^{-1})	9.0	59.4	***	35.3	31.3	ns
	(0.7)	(5.7)		(5.1)	(2.5)	
Shoot Fe (mg kg^{-1})	251.2	133.9	**	839.2	188.6	***
	(7.6)	(11.0)		(49.4)	(11.5)	

$^{**}P < 0.01$; $^{***}P < 0.001$. Low-Zn experiments were conducted with genotypes IR74 and RIL46, with four replicate plants each; excess Fe^{2+} experiments were conducted with genotypes IR70617, IR24637, Milyang23, IR64 and SZH2, with four replicate plants each. Values in parentheses are standard errors (n = 8 for Zn; n = 20 for Fe). FeEDTA, Fe-ethylenediaminetetraacetic acid; HSD, Tukey's honestly significant difference test.

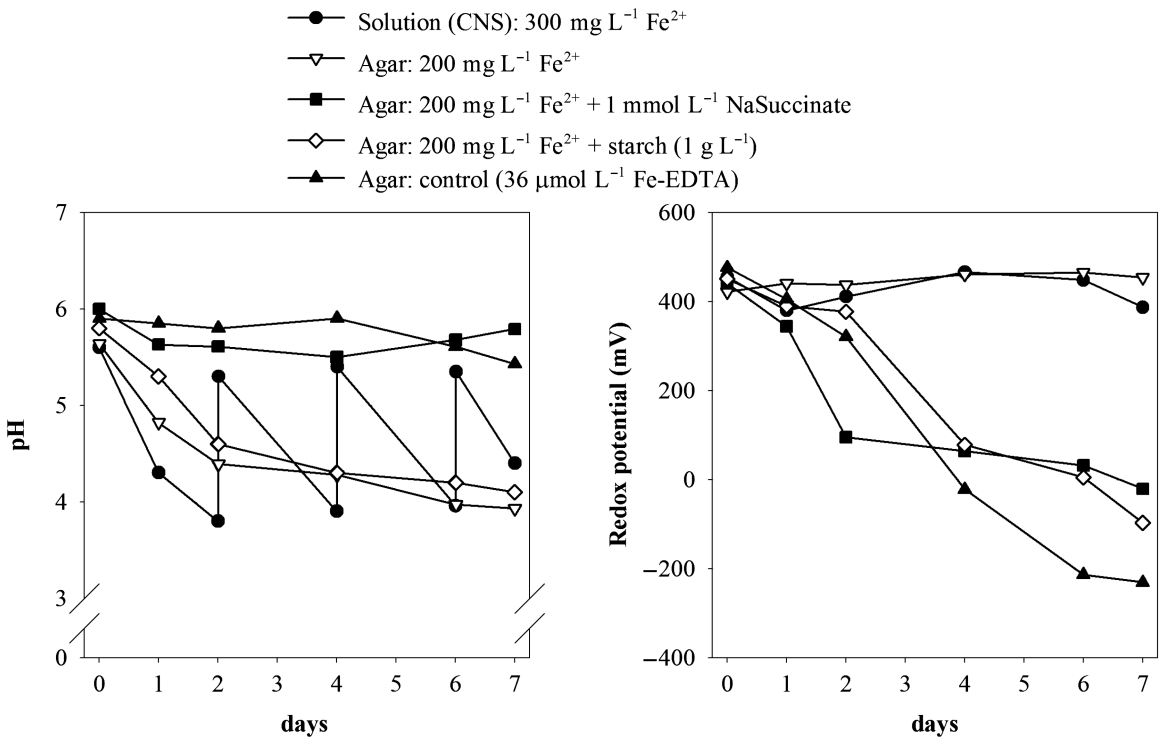

Figure 1 Changes in pH and Eh over time. The pH in the nutrient solution treatment (conventional nutrient solution [CNS] with 300 mg kg^{-1} Fe^{2+}) decreased rapidly and reached a level where the pH needed to be adjusted every 48 h (pH adjustment was conducted on days 2, 4 and 6).

physiologically relevant range of pH 4–5 for approximately 6 days. Adding 1 mmol L^{-1} sodium succinate to the ANS stabilized the pH at approximately pH 5.5–5.9.

No changes in the redox potential were seen in CNS over a 7-day period; values remained at approximately +400 mV for all CNS treatments (only the data for excess Fe are shown; Fig. 1). In contrast, the Eh in ANS typically dropped rapidly after 2–4 days and reached values below –200 mV after 7 days (Fig. 1; Table 2). The excess Fe treatment in ANS was an exception and did not follow this pattern; however, the addition of organic matter to ANS in the form of sodium succinate or starch overcame this limitation (Fig. 1). We hypothesize

Table 2 Changes in Eh between bulk agar and the "rhizosphere" in a Zn deficiency trial with a tolerant (RIL46) and intolerant (IR74) genotype after a 7-day treatment period

Variety	Zn level $ZnSO_4$	Eh "rhizosphere" mV	Eh bulk mV	Δ Eh mV
IR74	1.5 μmol L^{-1}	30.8 (53.2)	–204.8 (26.8)	235.6 (45.8)
IR74	0.1 nmol L^{-1}	63.4 (80.9)	–207.2 (33.6)	270.6 (52.3)
RIL46	1.5 μmol L^{-1}	–88.3 (43.2)	–204.8 (26.8)	116.5 (39.9)
RIL46	0.1 nmol L^{-1}	7.9 (67.4)	–207.2 (33.6)	215.1 (76.0)

Δ Eh denotes the difference between the bulk agar and rhizosphere Eh. Eh was measured by placing platinum electrodes inside the root zone at various depths; values for the rhizosphere represent the means of 8–10 measurements, while bulk values are the means of four measurements; values in parentheses are standard errors.

that the change in redox state was caused by oxygen depletion as a result of microbial degradation of organic material, including the agar itself, and that this process was partially inhibited by Fe toxicity.

To determine if a quasi-rhizosphere could be developed in ANS and to see whether genotypic differences in root oxidation power that could be associated with zinc efficiency existed, we measured Eh and pH within the root zone of two genotypes with contrasting tolerance to Zn deficiency in the field. The Eh in the root zone remained 116–270 mV higher compared with the bulk agar (Table 2) and the pH was approximately 0.5 units lower (data not shown), while genotypic differences were only significant in the high-Zn treatment where IR74 had larger roots. We conclude that Zn deficiency does not reduce root oxidation power and that genotypic differences in tolerance to Zn deficiency were probably not associated with higher rhizosphere oxidation. The processes responsible for changes in Eh and pH, that is, oxygen loss and proton release to balance excess cation uptake (Kirk 2004), are also expected to operate in CNS, but a quasi-rhizosphere could only be developed in ANS because of low convective mixing.

One limitation of using ANS in experiments exceeding 2 weeks is water loss from ANS as a result of transpiration. After 2 weeks of cultivation in ANS the solution level may decrease by 2–3 cm (depending on plant size), exposing the topmost parts of the roots to the air. We recommend renewing the solutions at 10–14-day intervals if the growth period needs to be extended, although this would interrupt continuous measurements of rhizosphere effects.

Genotypic differences in tolerance to Fe toxicity and Zn deficiency

Based on the results of screening experiments conducted at a field site known to be strongly affected by Fe toxicity (San Dionisio, Iloilo, Philippines), five contrasting genotypes were selected to evaluate the suitability of ANS as a screening tool for Fe toxicity. Genotypes IR70617 and IR24637 had been selected at the site and are considered to be highly tolerant (T) to Fe toxicity with significantly higher grain yield and lower leaf bronzing compared with the intolerant (S) genotypes IR64 and SZH (Table 3). Milyang23 is considered to be intermediate (M) in its tolerance.

Leaf bronzing developed after 3 days of exposure to 200 mg L^{-1} Fe^{2+} in ANS, whereas it took 5 days for symptoms to develop in CNS (with 300 mg L^{-1} Fe^{2+}). However, final leaf bronzing (after 14 days) was more severe in CNS, but genotypic differences were not as pronounced in CNS as in ANS (Table 3). The genotypic rankings based on dry matter accumulated after the exposure to excess Fe^{2+} also followed field-tolerance rankings more closely in ANS compared with CNS. In particular, genotype IR64 deviated from field tolerance rankings by showing tolerance to excess Fe^{2+} in CNS but not in ANS and this was particularly pronounced for root growth. Screening in ANS and CNS was done at different Fe^{2+} levels (200 vs 300 mg L^{-1} Fe^{2+}, respectively) because preliminary tests (data not shown) had revealed that these two concentrations produced roughly equal stress levels, which was confirmed here, and the average relative dry matter was 57% in ANS and 53% in CNS (Table 3). When the Fe^{2+} concentration was elevated to 300 mg L^{-1} in ANS in these preliminary tests, excessive stress led to plant mortality within a few days (data not shown). We observed Fe precipitates at the bottom of containers within 24 h in CNS and such precipitation made it difficult to maintain constant high iron concentrations in CNS (Shimizu *et al.* 2005). Iron precipitates were seen to a lesser extent in ANS and precipitates remained suspended in the vicinity of roots because of the viscous consistency of ANS. This difference may explain why lower Fe^{2+} concentrations were required in ANS to induce Fe toxicity.

In field studies on a highly Zn deficient soil genotypic differences in tolerance to Zn deficiency were detected and IR74 was intolerant (Wissuwa *et al.* 2006). Furthermore, Wissuwa *et al.*'s (2006) study showed that

Table 3 Phenotypic response of five rice genotypes to Fe toxicity in a field trial in comparison to their performance in experiments conducted with excess Fe^{2+} in agar nutrient solution or conventional nutrient solution

		Grain yield	Leaf-bronzing score†			ShDM added‡		RDM added‡		Relative TDM§	
		Field	Field	ANS	CNS	ANS	CNS	ANS	CNS	ANS	CNS
Genotype	Rating	(t ha^{-1})				(g $plant^{-1}$)		(g $plant^{-1}$)		(%)	
IR70617	T	3.24	2.5	3.6	4.5	0.195	0.212	0.060	0.092	54.2	48.4
IR24637	T	3.04	3.0	2.5	6.3	0.183	0.171	0.081	0.089	68.1	52.4
Milyang23	M	2.02	4.5	5.0	5.7	0.154	0.177	0.041	0.067	63.7	56.2
IR64	I	1.57	6.3	5.5	7.5	0.138	0.199	0.041	0.093	50.2	61.0
SZH2	I	1.65	7.9	8.0	8.5	0.094	0.100	0.027	0.039	49.1	48.1
HSD		0.73	1.9	1.2	1.7	0.053	0.067	0.016	0.030	16.3	18.8

†Visual score of the extent of leaf bronzing; where 0 = no bronzing and 9 = all but the youngest emerging leaf severely bronzed. ‡Increase in shoot dry matter (ShDM) or root dry matter (RDM) during the treatment period. Plants had been sampled and weighed just before adding excess Fe^{2+} and after the 2-week treatment period. §Total dry matter (TDM) in the excess Fe^{2+} treatments relative to the respective control. Based on field screening results genotypes were rated as tolerant (T), intermediate (M) or intolerant (I) to Fe toxicity. ANS, agar nutrient solution; CNS, conventional nutrient solution; HSD, Tukey's honestly significant difference test at $P < 0.05$.

Table 4 Effect of Zn deficiency on the relative dry weight of two rice genotypes with contrasting Zn-deficiency tolerance

Growth medium	Zn (μmol L^{-1})	Genotype	Relative shoot dry weight† (%)	Relative root dry weight‡ (%)	Leaf bronzing
ANS	0.1×10^{-3}	IR74	45.0	44.7	3.5
		RIL46	60.5	95.2	0.0
Field	Deficient†	IR74	31.5	35.7	4.7
		RIL46	38.8	52.4	0.8

†Diethylene triamine pentaacetic acid extractable Zn: 0.86 mg Zn kg^{-1}. ‡Dry matter relative to the respective control. For the agar nutrient solution (ANS) experiments the control contained 1.5 μmol L^{-1} Zn and 1 mmol L^{-1} HCO_3^-; in the field experiment the control plot was fertilized with 15 kg ha^{-1} of Zn (as $ZnSO_4$); genotype IR74 is intolerant and RIL46 is tolerant.

low-Zn nutrient solutions were not suitable to reproduce the tolerance rankings observed in the field: IR74 had a higher relative dry weight in low-Zn nutrient solution compared with several genotypes that were more tolerant than IR74 in the field. In contrast, the present experiments in ANS with IR74 and an IR74-derived recombinant inbred line (RIL46, which was highly tolerant in the field) were able to reproduce the much higher tolerance of RIL46. Leaf bronzing symptoms were absent in RIL46, but were very pronounced in IR74 (Table 4). Furthermore, the relative dry weight of RIL46 was higher in the low-Zn ANS treatment compared with the intolerant genotype IR74 and this difference was particularly obvious for relative root weight.

Conclusions

Based on our experience using ANS we conclude that ANS offers several advantages compared with CNS:

- Fe toxicity is induced more rapidly and at lower Fe^{2+} concentrations in ANS, possibly because low convection and O_2 diffusion reduce Fe precipitation and because the precipitates remain suspended in the vicinity of the roots because of the viscous state of ANS. Similarly, Zn deficiency developed more rapidly in ANS because reduced convection prevents rapid replacement of absorbed Zn, thereby creating Zn-depletion zones around roots
- Plant-induced changes (Eh, pH) around roots were maintained in ANS, creating a quasi-rhizosphere in which genotypes may have lastingly altered conditions to their advantage. It is interesting to note that the genotypic differences in ANS were most pronounced for relative root growth. To maintain root growth under stress is one key tolerance mechanism and in this regard ANS may offer an important advantage over CNS
- The method is simple and labor efficient, particularly if a large number of genotypes need to be screened. Furthermore, the method does not require inputs such as pH buffers, chelators or N_2 gas that can

represent a considerable cost factor in the developing world, assuming they are even available.

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