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Research Report

The effects of multifactorial stress combination on rice and maize

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Abstract

The complexity of environmental factors affecting crops in the field is gradually increasing due to climate change-associated weather events, such as droughts or floods combined with heat waves, coupled with the accumulation of different environmental and agricultural pollutants. The impact of multiple stress conditions on plants was recently termed "multifactorial stress combination" (MFSC) and defined as the occurrence of 3 or more stressors that impact plants simultaneously or sequentially. We recently reported that with the increased number and complexity of different MFSC stressors, the growth and survival of Arabidopsis (*Arabidopsis thaliana*) seedlings declines, even if the level of each individual stress is low enough to have no significant effect on plants. However, whether MFSC would impact commercial crop cultivars is largely unknown. Here, we reveal that a MFSC of 5 different low-level abiotic stresses (salinity, heat, the herbicide paraquat, phosphorus deficiency, and the heavy metal cadmium), applied in an increasing level of complexity, has a significant negative impact on the growth and biomass of a commercial rice (*Oryza sativa*) cultivar and a maize (*Zea mays*) hybrid. Proteomics, element content, and mixOmics analyses of MFSC in rice identified proteins that correlate with the impact of MFSC on rice seedlings, and analysis of 42 different rice genotypes subjected to MFSC revealed substantial genetic variability in responses to this unique state of stress combination. Taken together, our findings reveal that the impacts of MFSC on 2 different crop species are severe and that MFSC may substantially affect agricultural productivity.

Introduction

Global warming and climate change are subjecting plants to an increased frequency and intensity of different abiotic stressors that include droughts, heat waves, floods, and cold snaps (Bailey-Serres et al. 2019; IPCC 2021; Zandalinas et al. 2021a). In many instances these stressors occur together, for example during episodes of drought and heat waves (e.g. Mittler 2006; Mittler and Blumwald 2010; Zhang and Sonnewald 2017; Alizadeh et al. 2020; Cohen et al. 2021). On top of these abiotic stresses and their combinations, are different man-made pollutants, such as heavy metals, microplastics, and pesticides, that affect plant growth and reproduction (Rillig et al. 2019, 2021; Zandalinas et al. 2021a). These could occur together with some of the different abiotic stresses and their combinations, highlighted above (Rillig et al. 2019, 2021; Zandalinas et al. 2021a;

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Zandalinas and Mittler 2022). In addition to these stressors are also climate-driven changes in the dynamics and distribution of different pathogen and insect populations that impact plants (Hamann et al. 2021; Kim et al. 2021). These conditions could be further augmented by poor nutrient content of different soils, as well as by a decrease in the complexity of soil microbiota. The microbiome diversity of soils was, for example, shown to decline with an increase in the number of different climate change-driven stressors present in our environment (Rillig et al. 2019, 2021). The potential impact of the different complex abiotic and biotic stress conditions, described above, on plants was recently termed "multifactorial stress combination" (MFSC) and defined as the occurrence of 3 or more different stressors that impact a plant simultaneously or sequentially (Rillig et al. 2019, 2021; Zandalinas et al. 2021a, 2021b).

We recently reported that with the increased number and complexity of different stressors, occurring together during a MFSC, the growth and survival of Arabidopsis (*Arabidopsis thaliana*) seedlings declines, even if the level of each individual stress is low enough to have no significant effect on plants (Zandalinas et al. 2021a, 2021b). This finding is extremely alarming since it reveals that a MFSC of different low-level stressors (some already existing at different regions around the globe) could impact crops and cause a dramatic reduction in overall growth (Zandalinas and Mittler 2022). However, whether MFSC would similarly impact commercial crop cultivars is largely unknown. Here, we reveal that a MFSC of 5 different low level abiotic stresses (salinity, heat, paraquat, phosphorus deficiency, and cadmium), applied in an increasing level of complexity, has a significant negative impact on the growth and biomass of a commercial rice (*Oryza sativa*) cultivar and a maize (*Zea mays*) hybrid. To increase our understanding of the molecular mechanisms associated with the response of commercial cultivars to MFSC, we conducted proteomics, element content, and mixOmics analyses of MFSC responses in rice, as well as studied the physiological response of 42 different rice genotypes subjected to MFSC. Our study identified a set of proteins that negatively or positively correlate with the impacts of MFSC on rice seedlings, as well as revealed a large diversity in responses of different rice genotypes to MFSC. We further report that different pathways involved in maintaining reactive oxygen species (ROS), secondary metabolism, and iron homoeostasis are upregulated in rice seedlings subjected to MFSC. Taken together, our findings reveal that the impacts of MFSC on 2 different crop species are severe, and that MFSC may have a substantial impact on agricultural productivity.

Results and discussion

Impact of MFSC on growth and biomass of rice and maize seedlings

To determine whether MFSC would affect the growth and biomass of agricultural crops, we obtained seeds of a commercial rice cultivar (*O. sativa* var. Diamond) and a maize (*Z. mays* var. P1151AM) hybrid and studied their growth and biomass in response to MFSC. Seedlings were grown for 21 d under a combination of 5 different growth conditions (low-level stressors) that include: salinity (50 mM NaCl), cadmium (400 μM $CdCl_2$), paraquat (50 μM), heat stress (HS) (42 °C/36 °C or 40 °C/32 °C, day/night temperature, for rice or maize, respectively), and phosphorus deficiency. Control rice and maize plants were grown at 30 °C/26 °C, day/night temperature. Each condition was applied individually and in all possible combinations, as previously reported for Arabidopsis (Zandalinas et al. 2021b). As shown in Fig. 1, with the increase in the number and complexity of MFSCs, plant height, growth rate, and biomass of both rice and maize seedlings significantly declined. These findings suggest that while each of the different stresses had a minimal effect on rice and maize seedlings when applied individually, the cumulative effect of all 4 or 5 low-level stressors drastically reduced rice and maize seedling's growth and biomass (Fig. 1; Supplemental Fig. S1). Taken together, our findings reveal that, like Arabidopsis, commercial crop cultivars, in this case rice and maize, are negatively impacted by MFSC. This finding is important since Arabidopsis, which is extensively used in laboratory studies, is very different from commercial cultivars of crops used for agriculture (Mittler and Blumwald 2010). In addition, while Arabidopsis is a dicot, rice and maize are monocots, showing that the negative effects of MFSC on plants are broad and can also apply to monocots. Although the different growth conditions used in this study may or may not occur in the natural or agricultural environment of different rice and maize genotypes, they nevertheless highlight the key principle of MFSC: With the increased number and complexity of different low-level stressors, occurring together during a MFSC, the growth of seedlings declines (Fig. 1; Zandalinas et al. 2021a, 2021b; Zandalinas and Mittler 2022).

Element content and survival of rice seedlings subjected to MFSC

To further study the effects of MFSC on a commercial crop cultivar, we focused on rice and measured the total content of nitrogen, phosphorus, potassium, calcium, magnesium, and chlorophyll in rice seedlings subjected to the same MFSC conditions described in Fig. 1 and Supplemental Fig. S1 (Fig. 2, A to G; Supplemental Figs. S2 and S3). While the levels of nitrogen, phosphate, potassium, and calcium decreased in plants subjected to MFSC, the levels of magnesium and total chlorophyll did not (Fig. 2, A to G). Taken together, the results shown in Fig. 2, A to G reveal that plants subjected to MFSC could be impaired in the uptake and/or retention of essential elements such as nitrogen, potassium, phosphate, and calcium, potentially impairing their growth and biomass accumulation. In addition, they reveal that unlike the dicot model plant Arabidopsis (Zandalinas et al. 2021b), the levels of chlorophyll did not decline in rice seedlings in response to MFSC (Fig. 2G).

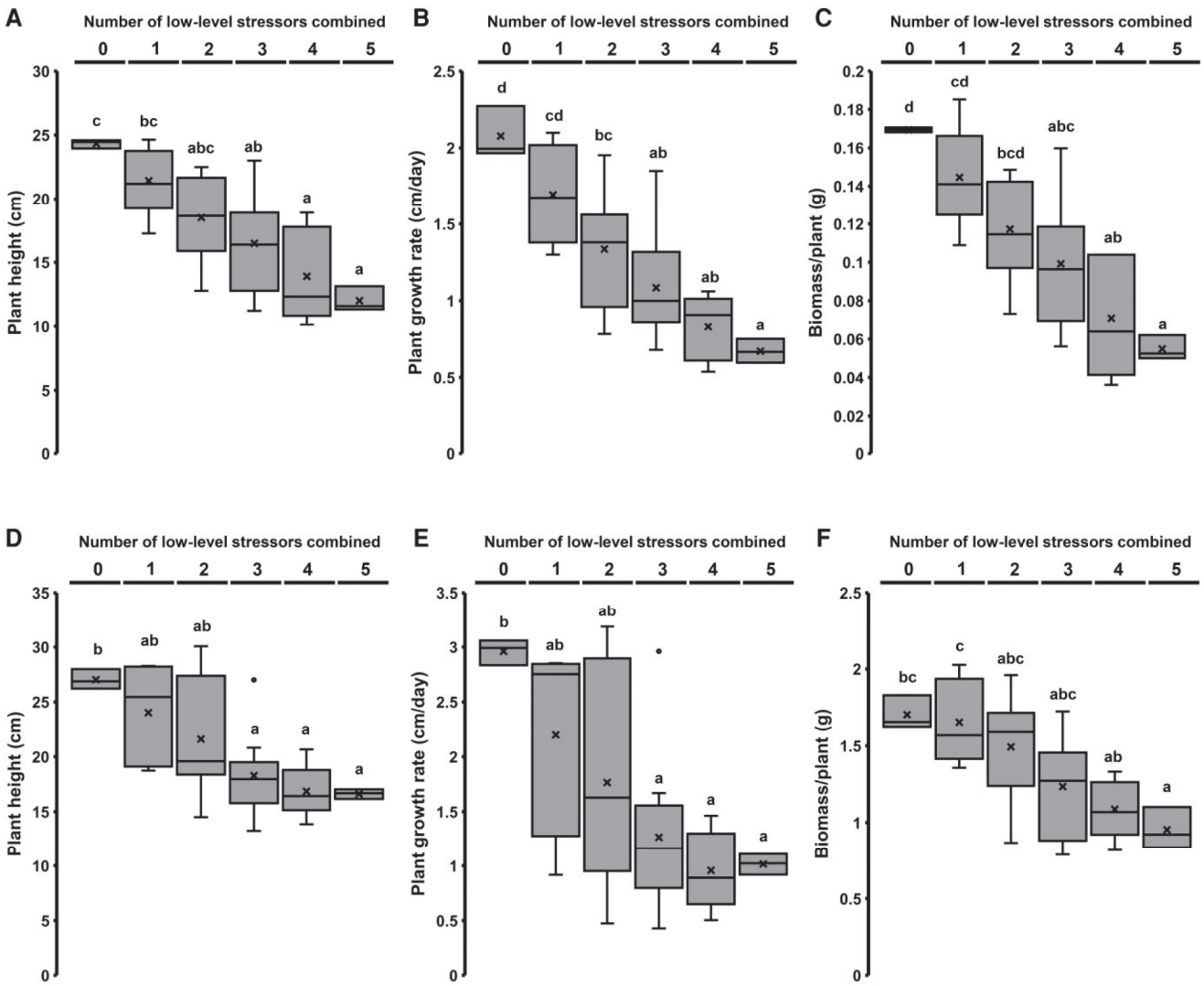

Figure 1. The impact of multifactorial stress combinations on the height, growth rate, and biomass of commercial rice (*Oryza sativa*) and maize (*Zea mays*) seedlings. The effects of multifactorial stress conditions (heat, salt, phosphorus deficiency, cadmium, and paraquat) applied in all different combinations (up to a combination of all 5 factors) were determined on the height, growth rate, and biomass of rice (**A** to **C**) and maize (**D** to **F**) seedlings. Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles (the 25th and 75th percentiles, respectively), and whiskers above and below the box plot indicate 1.5 times the interquartile range. Results presented for each stress/stress combination are shown in Supplemental Fig. S1. Statistical analysis was performed by 1-way ANOVA followed by a Tukey post hoc test (different letters denote statistical significance at $P < 0.05$).

To study the effects of MFSC on the survival of rice seedlings we used 2 different sets of growth conditions: (i) the MFSC conditions used above in Fig. 1A and Supplemental Fig. S1 (Fig. 2H; Supplemental Fig. S4A) and (ii) a different set of MFSC conditions that controlled for the possible interactions between paraquat and light [salinity (50 mM NaCl), admium (400 μM $CdCl_2$), paraquat (50 μM), HS (40 °C/34 °C day/night temperature), and low light (LL) (150 μmol photons m^{-2} s^{-1}); Control plants were grown at 30 °C/26 °C day/night temperature; 700 μmol photons m^{-2} s^{-1}; Fig. 2I; Supplemental Fig. S4B]. The reason for using LL as a growth condition was to control for the potential interactions between paraquat and high light intensities that can cause plant death (Zandalinas et al. 2021b). LL intensity of 150 μmol photons m^{-2} s^{-1} is also considered a low-level stressor, as it provides rice plants with limited light energy for photosynthesis and growth (Yamori et al. 2016). As shown in Fig. 2, H and I and Supplemental Fig. S4, each of the different individual growth conditions, as well as all the different 2- and 3-factor combinations, had no significant effect on the survival of rice. In contrast, the 2 different combinations of 5 low-level MFSCs had a significant effect on seedling survival, reducing it by about 40% (Fig. 2, H and I), and the different 4 low-level stress combinations shown in Fig. 2I had a significant effect of seedling survival reducing it by about 25%. These findings suggest that while each of

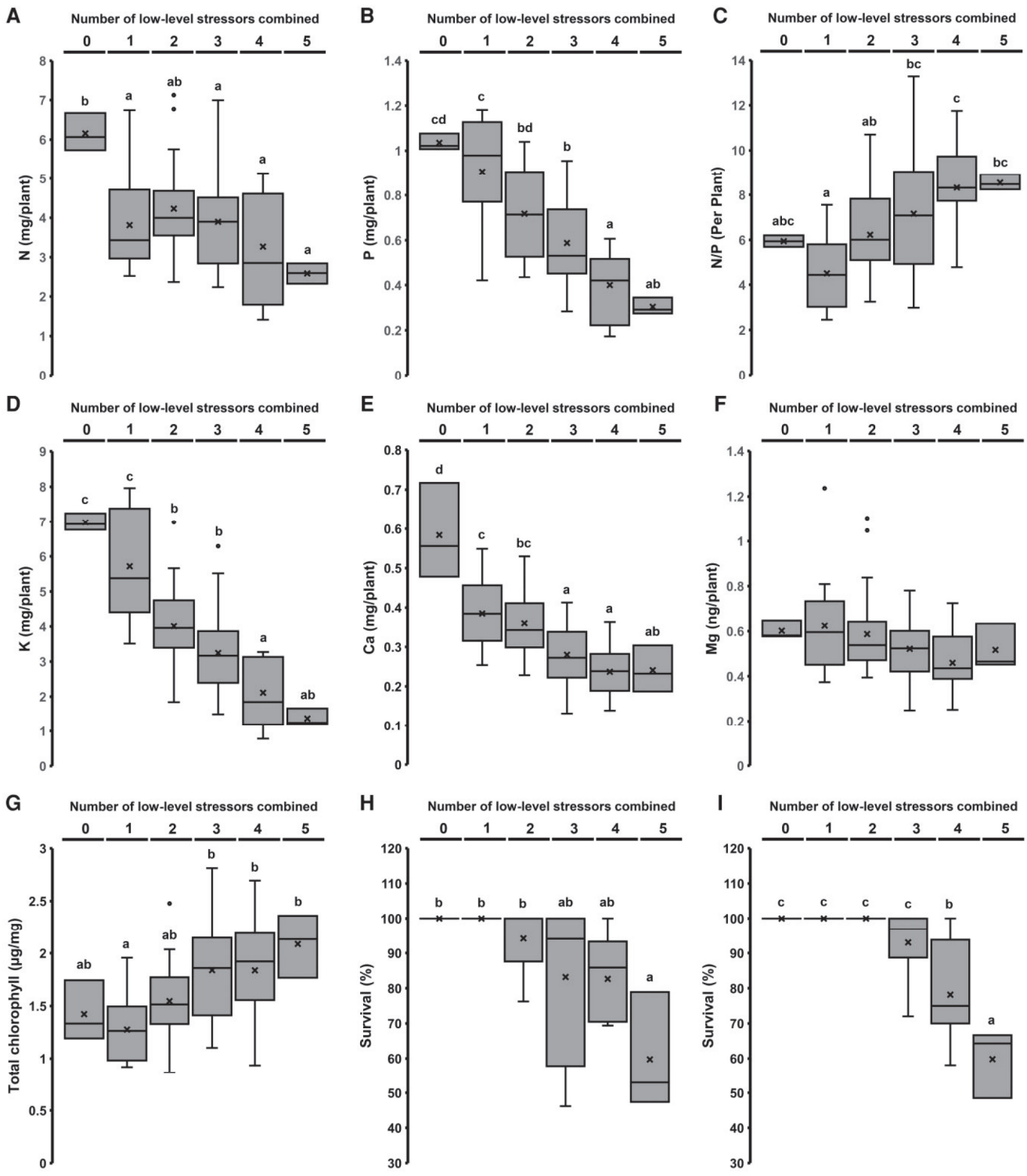

Figure 2. The impact of multifactorial stress combinations on survival, and element and chlorophyll content of rice seedlings. The content of nitrogen, phosphorus, potassium, calcium, and magnesium per plant, total chlorophyll, and precent survival, under multifactorial stress conditions (heat, salt, phosphorus deficiency, cadmium, and paraquat) applied in all different combinations (up to a combination of all 5 factors) was determined in rice seedlings (**A** to **H**). As described in the text, seedling survival was further tested in response to a different set of multifactorial stress combinations that included a lower light intensity (**I**). Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles (the 25th and 75th percentiles, respectively), and whiskers above and below the box plot indicate 1.5 times the interquartile range. Results presented for each stress/stress combination are shown in Supplemental Figs. S2 to S4. Statistical analysis was performed by 1-way ANOVA followed by a Tukey post hoc test (different letters denote statistical significance at $P < 0.05$). Ca; calcium; Cd, cadmium; K, potassium; Mg, magnesium; N, nitrogen; P, phosphorus.

the different stress conditions used had a negligible effect on seedling survival when applied individually, the cumulative effect of all 4 or 5 low-level stressors significantly reduced seedling survival. Although our study was conducted with seedlings and did not evaluate grain yield per plant, a 25% to 50% decrease in biomass accumulation (Fig. 1C) and a 25% to 40% decrease in seedling survival (Fig. 2, H and I) are likely to substantially decrease overall production of grain per unit area/field in rice.

Proteomics analysis of MFSC in rice seedlings

To gain better understanding of the molecular responses of a commercial cultivar to MFSC, and identify different proteins associated with MFSC in a crop plant (that could be used as an initial reference in breeding efforts), we conducted proteomics analysis of rice seedlings subjected to the MFSC shown in Fig. 1, A to C and Supplemental Fig. S1. As shown in Supplemental Table S1, proteomics coverage was in the range of 4,100 to 4,600 identified proteins per treatment. To determine the effects of each stress, the abundance of the proteins identified in each treatment were compared to that of the control, and only proteins that had a significant change in their abundance (up- or downregulated), compared to control in each treatment were considered for further analysis (Supplemental Tables S1 to S32). Interestingly, under the conditions we used, paraquat and phosphorus deficiency resulted in a low number of proteins altered compared to control (2 for paraquat and 8 for phosphorus deficiency). However, when these 2 stresses were combined (paraquat + phosphorus deficiency), 157 proteins were altered in their abundance. Similar findings were obtained with other individual low-level stresses and their combination [e.g. cadmium (78) and paraquat (2), and their combination (145), and cadmium (78) and phosphorus deficiency (8) and their combination (253); Supplemental Table S1]. These findings suggest that with the increased complexity of some stresses, the response of plants increases, potentially indicating that some stresses may have a synergistic effect on each other (Zandalinas and Mittler 2022). Of the different individual stresses used, HS had the highest impact on protein expression with over 1,500 proteins altered in their abundance (Supplemental Table S1). This finding is consistent with the extensive impact of HS conditions on protein abundance in crops, including rice (e.g. Zou et al. 2011), and suggests that global warming is likely to play a key role in future responses of crops to MFSCs.

To compare the overlap between the different stress treatments, we generated UpSet plots for all 2-, 3- (Supplemental Fig. S5), 4-, and 5-stress combinations (Fig. 3A). This analysis revealed that the expression of 332 proteins was common to all 4- and 5-stress combinations, that resulted in the most severe impact on plant height, growth rate, biomass, and survival (Figs. 1, A to C and 2, H and I). Gene ontology (GO) annotation term analysis of this group of proteins revealed that they were enriched in redox, catabolic metabolism, ROS scavenging, chaperone activity, iron-sulfur metabolism, and other functions (Fig. 3B; see full list of GO terms in Supplemental Table S33). To further study, the abundance of ROS scavenging enzymes in our dataset, we generated a heatmap for the abundance of the key ROS scavenging enzymes ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (Mittler et al. 2022), found in all samples by our proteomics analysis (Fig. 3C; Supplemental Table S34). This analysis revealed that the abundance of many ROS scavenging enzymes (e.g. APX1, APX4, GR, and CAT-B) was elevated in samples from plants subjected to 4- or 5-stress combinations, compared to plants subjected to single stress conditions, or simple combinations of 2 and 3 stresses (Fig. 3C). The identification of ROS and iron-sulfur metabolism categories in rice plants subjected to 4 and 5 stressors combined (Fig. 3, B and C) is in agreement with our previous transcriptomic study in Arabidopsis plants that identified these 2 categories as enriched in plants subjected to MFSC, as well as revealed that mutants deficient in ROS scavenging or signaling (*apx1* or respiratory burst oxidase homolog D; *rbohD*), or in balancing iron and ROS levels (plants with suppressed expression of AtNEET; AT5G51720) were less tolerant to MFSC (Zandalinas et al. 2021b). As ROS metabolism and NEET proteins, which regulate iron and ROS metabolism, are conserved among eukaryotic organisms (Nechushtai et al. 2012; Mittler et al. 2019, 2022), augmenting the ability of different crops to scavenge ROS or balance ROS and iron levels could be a viable strategy to increase their resistance to MFSC. Further studies are needed to determine the role of these pathways, as well as other pathways identified by our analysis (Fig. 3; Supplemental Tables S1 to S34) in augmenting the tolerance of plants to MFSC.

To further dissect the molecular response of rice seedlings to MFSC, we utilized the multivariate tool mixOmics (Rohart et al. 2017). As inputs, we used all proteins altered in response to all 1-, 2-, and all MFSCs (Fig. 3), the growth phenotypes of rice seedlings (Fig. 1, A to C; Supplemental Fig. S1, A to C), and the element content of seedlings subjected to MFSC (Fig. 2A to F, Supplemental Fig. S2). As shown in Fig. 3D and Supplemental Table S35, this analysis yielded putative interactions between specific proteins expressed during MFSC, and specific phenotypes. Among the proteins that positively correlated with the response of rice seedlings to MFSC were phenylalanine ammonia-lyase (PAL), peroxidase, phospholipase A1, and a gibberellin-response protein (Supplemental Table S35).

Taken together, our omics studies of MFSC in Arabidopsis (Zandalinas et al. 2021b) and rice (Fig. 3; Supplemental Fig. S5) demonstrate that the response of plants to each different stress combination contains unique transcripts and proteins, and that only a few pathways are common to all different 4- and 5-stress combinations (e.g. ROS, iron metabolism, PAL-associated secondary metabolism, and chaperones). These could serve as a starting point in the search for genes that could augment the tolerance of different plants to different types of MFSCs (Rivero et al. 2022; Zandalinas and Mittler 2022).

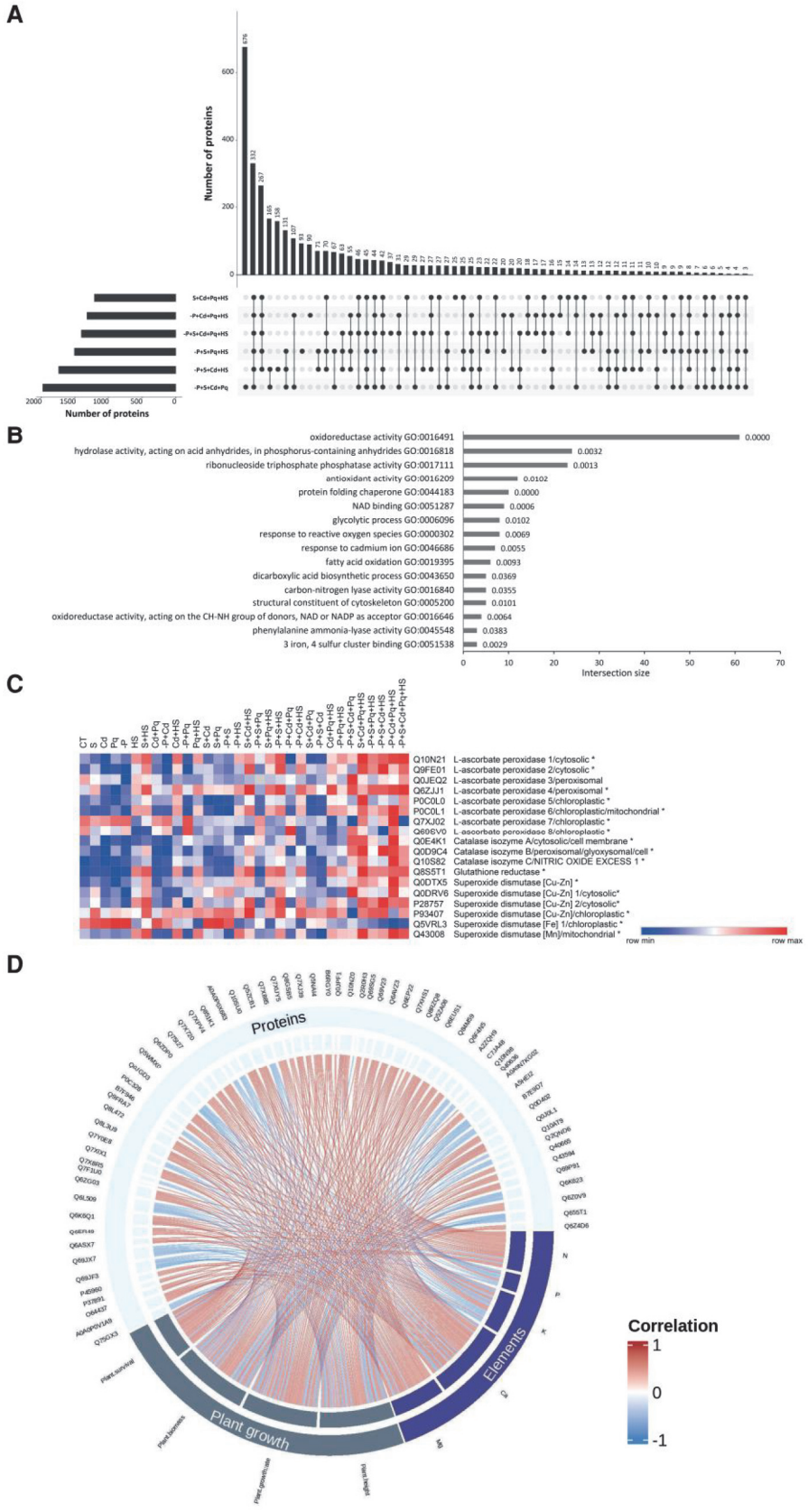

Figure 3. Proteomics and mixOmics analyses of multifactorial stress combination in rice seedlings. **A)** An UpSet plot showing the overlap between proteins significantly altered in their expression in all the different 4- and 5-stress combinations (UpSet plots for all 2- and 3-combinations are shown in Supplemental Fig. S5). **B)** Selected GO enrichment analysis terms for the 332 proteins common to all 4- and 5-combinations shown in

(continued)

Variability in the response of rice genotypes to MFSC

To begin addressing the genetic diversity associated with rice responses to MFSC, we subjected a panel of 42 different rice genotypes (Supplemental Table S36; obtained from different regions around the globe; https://npgsweb.ars-grin.gov/gringlobal/) to a MFSC of 5 different stresses (Fig. 1, A to C), and studied their growth, biomass accumulation, and survival. The response of each genotype was compared to unstressed conditions and expressed in % of control, and the relative response of each genotype was compared to that of the *O. sativa* var. Diamond cultivar (Figs. 1 to 3). As shown in Fig. 4, different rice genotypes demonstrated a high variability in their response to MFSC (that did not seem to be related to their country of origin). As evident from the effects of MFSC on the growth rate of the different genotypes, 3 of the 4 rice accessions with *Oryza glaberrima* background obtained from West Africa (*O. glaberrima Steud. CG 14*; *O. glaberrima Steud. TOg 5603*; and a hybrid of *O. sativa* and *O. glaberrima*, *O. hybr.* WAB450-I-B-P-23-HB; Fig. 4B), were found to have higher tolerance to MFSC. This finding identified *O. glaberrima*, that is thought to have been domesticated from the African wild rice species *Oryza barthii* (Wang et al. 2014), and serves as a rich reservoir of genes for tolerance to various biotic and abiotic stresses (Futakuchi et al. 2012; Sikirou et al. 2018), as a potential source for rice genes associated with tolerance to MFSC.

Conclusions

Our findings highlight the growing risk global warming, climate change, and industrial pollution pose to agriculture. The impacts of MFSC on plants, documented under controlled environmental conditions (Figs. 1 and 2; Zandalinas et al. 2021b; Pascual et al. 2023), highlight the urgent need for studies that quantify the impact of complex multifactorial stress combinations under real-world conditions. Additional studies are also needed to determine the role of the different pathways identified by our proteomics (Supplemental Tables S1 to S32 and Fig. S5; Fig. 3) and transcriptomics (Zandalinas et al. 2021b) analyses in augmenting the tolerance of different crops to MFSC. If we will not act to enhance the tolerance of our crops, and/or reduce the number, complexity, and intensity of different stressors affecting them, future episodes of MFSC could have a devastating impact on agriculture, potentially even leading to the destabilizing of multiple societies (Lobell et al. 2011; Challinor et al. 2014; IPCC 2021; Zsögön et al. 2021; Zandalinas et al. 2021a).

Materials and methods

Plant growth and stress treatments

All experiments were conducted in 4 identical growth chambers (BDR16, Conviron, Canada) under controlled growth conditions, and seedlings were randomized into the different growth conditions as described in Sinha et al. (2022). Rice (*O. sativa* var. Diamond) and maize (*Z. mays* hybrid P1151AM) seeds, obtained from Tanner Seed Co. (MO, USA) and Pioneer (Johnston, IA, USA), respectively, were germinated in peat and vermiculite growth media (1:1 mix) soaked in ¼ strength Hoagland solution with or without ammonium phosphate (Caisson Labs, Cat no. HOP02, Smithfield, UT, USA), for control (CT) and phosphorus deficiency (-P), respectively. For CT conditions, rice and maize seedlings were grown at 30 °C/26 °C day/night temperature, 700 μmol photons m^{-2} s^{-1} light intensity, and 14/10 h day/night photoperiod. For salinity (S), cadmium (Cd), and/or paraquat (Pq) stresses, seeds were germinated in growth media mix soaked in ¼ Hoagland with 50 mM NaCl (Fisher Scientific, Hampton, NH, USA), 400 μM $CdCl_2$ (Sigma-Aldrich, St. Louis, MO, USA), and/or 50 μM paraquat (Sigma-Aldrich). For salinity, cadmium, and/or paraquat, without phosphorus stresses, seeds were germinated in growth media mix soaked in ¼ Hoagland without phosphate with 50 mM NaCl, 400 μM $CdCl_2$, and/or 50 μM paraquat. Briefly, 40 g of vermiculite and peat mix (1:1) was soaked with 160 mL of ¼ Hoagland solution (with/without stressors) and filled into free-draining pots of 12 × 8 × 6 cm dimension (length × width × height). Twenty or 12 rice or maize seeds were planted in each pot, respectively (1 cm below soil surface). Pots were watered one more time with the above-mentioned stressors and their combinations. Afterwards, seedlings were periodically watered with deionized water and once a week with ¼ Hoagland with or without phosphate for the respective treatments, avoiding excessive watering. For CT, or stress treatments without HS, seedlings were grown at 30 °C/26 °C day/night temperature, 700 μmol photons m^{-2} s^{-1}, 14/10 h day/night photoperiod. For HS and the different combinations that included HS, rice plants were germinated and grown under 42 °C/36 °C day/night

Figure 3. (Continued)
(A) (a complete list of GO terms for the 332 proteins is shown in Supplemental Table S33). **C)** Heatmap for the abundance of different ROS-scavenging enzymes in all stress treatments used for the study (Supplemental Table S34). Asterisk denotes significant difference in abundance of the proteins compared to control ($P < 0.05$) in response to 3 stresses or more. **D)** MixOmics analysis linking specific proteins expressed in rice seedlings during multifactorial stress combination with plant growth parameters and element content (a list of all proteins positively or negatively correlated with growth and element content during multifactorial stress combination is shown in Supplemental Table S35). Benjamini–Hochberg with an FDR ≤0.05 was applied for proteomics analysis to determine significance. Summary of all proteomics results is shown in Supplemental Table S1, and all proteins significantly altered in their abundance in each treatment are shown in Supplemental Tables S2 to S32. Ca; calcium; Cd, cadmium; CT, control; HS, heat stress; K, potassium; Mg, magnesium; N, nitrogen; P, phosphorus; Pq, paraquat; -P, phosphorus deficiency; ROS, reactive oxygen species; S, salt.

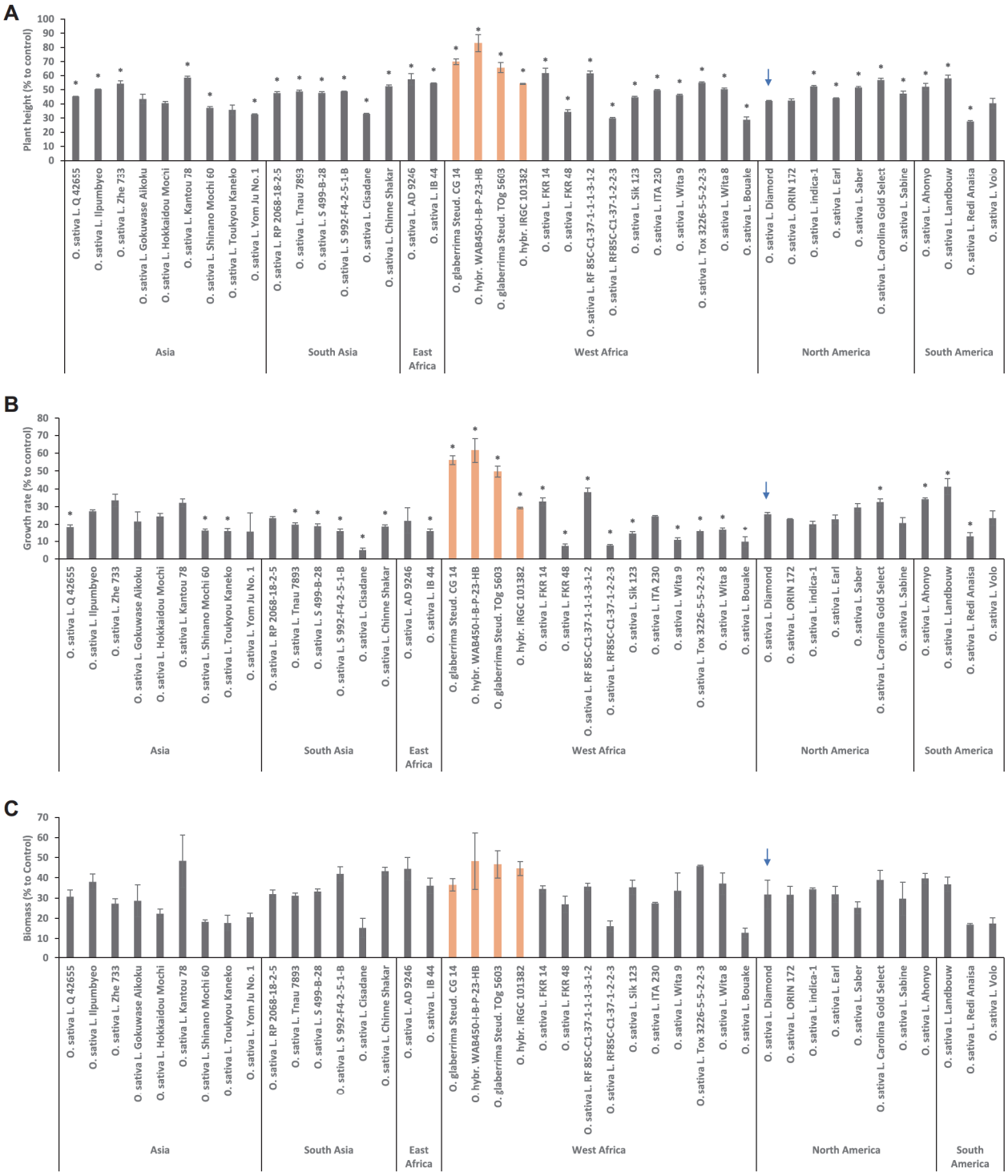

Figure 4. The impact of multifactorial stress combinations on the biomass, growth rate, and height of seedlings belonging to selected rice genotypes. The effects of multifactorial stress conditions (heat, salt, phosphorus deficiency, cadmium, and paraquat applied simultaneously) compared to control (no-stress) were determined on the height, growth rate, and biomass of rice seedlings (**A** to **C**; presented as precent of control). Results are shown as average and SE for each treatment separately. Statistical differences between each cultivar or species and the *O. sativa* var. *Diamond* cultivar (used for the analyses shown in Figs. 1 to 3; arrow), $*P < 0.05$, was determined with Student's *t*-test). A list of the *sativa*, *glaberrima*, and hybrid *Oryza* genotypes is shown in Supplemental Table S36. O., *Oryza*.

temperature, 700 μmol photons m^{-2} s^{-1}, 14/10 h photoperiod, while maize plants were germinated and grown at 40 °C/32 °C day/night temperature under the same light conditions. For LL stress conditions, rice seedlings were subjected to LL (150 μmol photons m^{-2} s^{-1}, 30 °C/26 °C day/night temperature, 14/10 h photoperiod). While the use of Hoagland solution without ammonium phosphate to induce -P conditions caused some decrease in N availability (in the form of ammonium) to plants, these conditions did not cause a strong decline in rice or maize seedling growth/growth rate (Supplemental Fig. S1), suggesting that the decreased N availability did not result in a substantial stress to plants. Rice and maize MFSC experiments were carried out separately using the same growth chambers.

Physiological measurements

Seedlings were grown for 21 d and scored for plant height at 10 and 18 d as described in Zandalinas et al. (2021b). Plant growth rate was calculated from the 2 height measurements of Day 10 and 18. Survival and shoot biomass were scored at Day 21 as described in Zandalinas et al. (2021b) and Sinha et al. (2022) by weighing individual plants (shoot biomass) and scoring for survival.

Measuring the impact of MFSC on different rice species and cultivars

Seeds of 42 different genotypes from *sativa*, *glaberrima*, and hybrid *Oryza* (Supplemental Table S36) were obtained from U.S. National Plant Germplasm System collection (GRIN-Global; https://npgsweb.ars-grin.gov/gringlobal/). Seeds (15 seeds per pot) were germinated in peat and vermiculite growth media under controlled or 5-stress MFSC as described above (Fig. 1, A to C). Plant height was measured at 15 and 20 d post seed germination to calculate plant growth rate. Total biomass and survival were measured at 25 d postgermination as explained above. Experiments were conducted in 3 biological repeats.

Element analysis of rice shoots

Rice shoots were collected at 15 d postgermination from control and all stress treatments (Fig. 1, A to C). Twenty rice shoots from each replicate of each treatment were pooled and flash frozen in liquid nitrogen. Samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Nitrogen, phosphorus, calcium, potassium, and magnesium element concentration analyses were performed in the Soil and Plant Testing Laboratory, University of Missouri, Columbia, USA (https://extension.missouri.edu/programs/soil-and-plant-testing-laboratory). Element concentrations were determined using 0.5 g (fresh weight; FW) of ground shoot tissue according to Jones (2001) and content per plant was calculated by multiplying concentration by shoot FW.

Chlorophyll

Chlorophyll concentration was measured in rice shoots according to Moran (1982), with some modifications. Fifty milligrams of rice shoot powder obtained as described above were incubated in 5 mL of *N*,*N*-dimethylformamide (DMF) at 4 °C in the dark for 5 d. The absorbance of the DMF extract was read at 603, 647, and 664 nm in a spectrophotometer. Clean DMF (1 mL) was used as blank.

Proteomics analysis

Rice shoots (cv. Diamond) were collected at Day 15 postgermination from CT and all stress treatments (Supplemental Table S1). About 20 rice shoots from each treatment were pooled and flash frozen in liquid nitrogen as an individual biological replicate, and the entire experiment contained 3 biological replicates per treatment. Total protein was isolated using the phenol extraction protocol described in Mooney and Thelen (2004), and protein pellets were resuspended in 6 M urea, 2 M thiourea, and 100 mM ammonium bicarbonate. Protein concentration was determined using Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). Thirty micrograms of proteins from each sample were reduced, alkylated, and digested as described in Zandalinas et al. (2020). An EvoSep One liquid chromatography system coupled to a modified trapped ion mobility spectrometry quadrupole time-of-flight mass spectrometer (timsTOF Pro 2, Bruker Daltonik, GmbH, Germany) was used for all proteomics analyses as described by Zandalinas et al. (2020).

Proteomics data analysis

The FragPipe computational platform (version 18.0) with MSFragger (version 3.5), Philosopher (version 4.4.0), and EasyPQP (version 0.1.33) components were used to build the spectral library, and the protein sequence database *O. sativa* subsp. *japonica*, UniProt-UP000059680-48,899, was used for protein identification (Tyanova et al. 2016; Kong et al. 2017; da Veiga et al. 2020). dia-PASEF raw data were analyzed with DIA-NN version 1.8 (Demichev et al 2020). Data were exported from DIA-NN for further analysis using Perseus version 1.6.15.0. Differential expression analysis by 2-sided unpaired *t*-test was performed between each treatment and the CT (Zandalinas et al. 2020). Benjamini–Hochberg correction for multiple hypothesis testing was applied, with FDR ≤0.05 reported as significant. KEGG and quantification of significantly represented GO terms (*q*-value 0.05) were conducted using g:profiler (https://biit.cs.ut.ee/gprofiler/gost). Upset plots were created in upsetr (https://gehlenborglab.shinyapps.io/upsetr). Heatmaps were generated according to Sinha et al. (2022).

MixOmics analysis

The mixOmics (Rohart et al. 2017) R package was used to correlate the proteins with plant growth data (plant height, plant growth rate, plant biomass, plant survival) and elements data (nitrogen, phosphorus, potassium, calcium, magnesium). The proteins significantly differentially expressed compared to control with *q*-value <0.05 and log2-fold change (log2FC) of 2 or more for all comparisons were

selected for the analysis. Data integration and classification were carried out by Data Integration Analysis and Biomarker discovery using Latent variable approaches for Omics studies (DIABLO). The N-integration Sparse Partial Least Square Discriminant Analysis (SPLS-DA) approach was used with function block.splsda to identify signatures composed of highly correlated variables across the multiple matrix sets, which enable us to detect a confident relationship among the datasets. The circos correlation matrix generated by the function circosplot was imported to the circlize (Gu et al. 2014) R package to create the circos plot and 0.7 correlation was used as the cutoff.

Statistical analysis

All experiments were conducted with 3 biological repeats. Each biological repeat was conducted with 3 technical repeats. Each technical repeat included 20 or 12 seedlings of rice or maize, respectively, per treatment (20 rice seedlings per repeat for proteomics). Treatments and growth chambers were randomized with each biological repeat. Statistical analysis for box plots in Figs. 1 and 2 was performed using 1-way ANOVA followed by Tukey post hoc test (different letters denote statistical significance at $P < 0.05$; Zandalinas et al. 2021b). Statistical analysis for Figs. 3C and 4, and Supplemental Figs. S1 to S4 was performed using Student's *t*-test (asterisks denote statistical significance at $P < 0.05$ compared to control).

Accession numbers

Proteomics data from this article can be found in the Pride (https://www.ebi.ac.uk/pride/) database under accession number PXD039065.

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Author contributions

R.S., M.A.P.V., T.T.N., L.S.P., A.M.O., Z.L., and B.S. performed experiments and analyzed the data. R.M., F.B.F., R.S., T.J., M.A.P.V., and S.I.Z. designed experiments, analyzed the data, and/or wrote the manuscript.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. The impact of multifactorial stress combinations on the height, growth rate, and biomass of commercial rice (*Oryza sativa*) and maize (*Zea mays*) seedlings (in support of Fig. 1).

Supplemental Figure S2. Nitrogen, phosphorus, potassium, calcium, and magnesium content per plant under control and MFSC conditions.

Supplemental Figure S3. The impact of multifactorial stress combinations on chlorophyll content of rice (*Oryza sativa*) seedlings.

Supplemental Figure S4. The impact of multifactorial stress combinations on the survival of rice (*Oryza sativa*) seedlings.

Supplemental Figure S5. Proteomics analysis of multifactorial stress combination in rice seedlings.

Supplemental Table S1. Summary of proteomics results for the multifactorial stress combination analysis in rice

Supplemental Table S2. List of proteins differentially expressed in rice shoot grown under salt stress (S) compared to control (CT)

Supplemental Table S3. List of proteins differentially expressed in rice shoot grown under cadmium stress (Cd) compared to control (CT)

Supplemental Table S4. List of proteins differentially expressed in rice shoot grown under paraquat stress (Pq) compared to control (CT)

Supplemental Table S5. List of proteins differentially expressed in rice shoot grown under phosphorus deficiency (-P) compared to control (CT)

Supplemental Table S6. List of proteins differentially expressed in rice shoot grown under heat stress (HS) compared to control (CT)

Supplemental Table S7. List of proteins differentially expressed in rice shoot grown under combination of salt and heat stress (S + HS) compared to control (CT)

Supplemental Table S8. List of proteins differentially expressed in rice shoot grown under combination of cadmium and paraquat stress (Cd + Pq) compared to control (CT)

Supplemental Table S9. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency and cadmium stress (-P + Cd) compared to control (CT)

Supplemental Table S10. List of proteins differentially expressed in rice shoot grown under combination of cadmium and heat stress (Cd + HS) compared to control (CT)

Supplemental Table S11. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency and paraquat stress (-P + Pq) compared to control (CT)

Supplemental Table S12. List of proteins differentially expressed in rice shoot grown under combination of paraquat and heat stress (Pq + HS) compared to control (CT)

Supplemental Table S13. List of proteins differentially expressed in rice shoot grown under combination of salt and cadmium stress (S + Cd) compared to control (CT)

Supplemental Table S14. List of proteins differentially expressed in rice shoot grown under combination of salt and paraquat stress (S + Pq) compared to control (CT)

Supplemental Table S15. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency and salt stress (-P + S) compared to control (CT)

Supplemental Table S16. List of proteins differentially expressed in rice shoot grown under combination of

phosphorus deficiency and heat stress (-P + HS) compared to control (CT)

Supplemental Table S17. List of proteins differentially expressed in rice shoot grown under combination of salt, cadmium, and heat stress (S + Cd + HS) compared to control (CT)

Supplemental Table S18. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, salt, and paraquat stress (-P + S + Pq) compared to control (CT)

Supplemental Table S19. List of proteins differentially expressed in rice shoot grown under combination of salt, paraquat, and heat stress (S + Pq + HS) compared to control (CT)

Supplemental Table S20. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, salt, and heat stress (-P + S + HS) compared to control (CT)

Supplemental Table S21. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, cadmium, and paraquat stress (-P + Cd + Pq) compared to control (CT)

Supplemental Table S22. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, cadmium, and heat stress (-P + Cd + HS) compared to control (CT)

Supplemental Table S23. List of proteins differentially expressed in rice shoot grown under combination of salt, cadmium, and paraquat tress (S + Cd + Pq) compared to control (CT)

Supplemental Table S24. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, salt, and cadmium stress (-P + S + Cd) compared to control (CT)

Supplemental Table S25. List of proteins differentially expressed in rice shoot grown under combination of cadmium, paraquat, and heat stress (Cd + Pq + HS) compared to control (CT)

Supplemental Table S26. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, paraquat, and heat stress (-P + Pq + HS) compared to control (CT)

Supplemental Table S27. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, salt, cadmium, and paraquat stress (-P + S + Cd + Pq) compared to control (CT)

Supplemental Table S28. List of proteins differentially expressed in rice shoot grown under combination of salt, cadmium, paraquat, and heat stress (S + Cd + Pq + HS) compared to control (CT)

Supplemental Table S29. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, salt, paraquat, and heat stress (-P + S + Pq + HS) compared to control (CT)

Supplemental Table S30. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, salt, cadmium, and heat stress (-P + S + Cd + HS) compared to control (CT)

Supplemental Table S31. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, cadmium, paraquat, and heat stress (-P + Cd + Pq + HS) compared to control (CT)

Supplemental Table S32. List of proteins differentially expressed in rice shoot grown under combination of phosphorus deficiency, salt, cadmium, paraquat, and heat stress (-P + S + Cd + Pq + HS) compared to control (CT)

Supplemental Table S33. GO and KEGG enrichment (Fig. 3B) of differentially expressed proteins common in 4 and 5 stress combinations (Fig. 3A)

Supplemental Table S34. The protein abundance values used in the heatmap (Fig. 3C)

Supplemental Table S35. MixOmics analysis linking specific proteins expressed in rice seedlings during multifactorial stress combinations with plant growth parameters and element content (Fig. 3D)

Supplemental Table S36. List of rice cultivars used to study the MFSC impact (Fig. 4)

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Conflict of interest statement. None declared.

Data availability

The data that support the findings of this study are available in the text, figure, and Supplemental material of this article. Proteomics data were deposited in Pride (https://www.ebi.ac.uk/pride/), under the following accession number: PXD039065.

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