Ultrasonic seed treatment and Cu application modulate photosynthesis, grain quality, and Cu concentrations in aromatic rice

Z. MO[*,**], Q. LIU[*], W. XIE[*], U. ASHRAF[***], M. ABRAR[#], S. PAN[*,**], M. DUAN[*,**], H. TIAN[*,**], S. WANG[*,**], and X. TANG[*,**,+]

College of Agriculture, South China Agricultural University, Guangzhou, 510642, Guangdong, China[*]
Scientific Observing and Experimental Station of Crop Cultivation in South China, Ministry of Agriculture, Guangzhou, 510642, Guangdong, China[**]
Department of Botany, Division of Science and Technology, University of Education, Lahore, 54770, Punjab, Pakistan[***]
State Key Laboratory of Grassland Agroecosystem, School of Life Sciences, Lanzhou University, Lanzhou, China[#]

Abstract

The present study investigated the effects of ultrasonic seed treatment and Cu application on grain yield, photosynthesis, grain quality, and Cu contents of aromatic rice. The ultrasonic treated and nontreated seeds of two aromatic rice cultivars, *i.e.*, Nongxiang 18 and Meixiangzhan 2, were sown in Cu-contaminated soil with 1.5 and 3.0 g(Cu) m^{-2} whereas the soil without Cu application was taken as control (CK). Ultrasonic seed treatment and Cu application increased grain yield by 2.7–33.5% in Nongxiang 18 and by 12.9–20.2% in Meixiangzhan 2 owing to substantial improvements in morphophysiological and yield-related traits. Both ultrasonic seed treatment and Cu application also improved the 2-acetyl-1-pyrroline contents as a result of increased proline contents and proline dehydrogenase activity. The Cu contents in plant parts were higher in Cu-containing soil than that of CK. Overall, ultrasonic seed treatment and optimum Cu application improved aromatic rice grain yield, dry biomass, aroma, and grain quality of aromatic rice.

Additional key words: 2-acetyl-1-pyrroline; antioxidant; copper; grain yield; growth; plant biomass.

Introduction

Rice is an important cereal crop that is largely consumed globally (Ashraf *et al.* 2017, 2018a). Rice production has been significantly affected by various external factors including soil contamination with heavy metals (De Britto *et al.* 2011, Ashraf *et al.* 2015, Ashraf and Tang 2017, Liu *et al.* 2019a,b). Some metals, *e.g.*, Mn, Cu, Zn, Mo, and Ni are essential and/or beneficial micronutrients for microorganisms, plants, and animals (Nedelkoska and Doran 2000, Li *et al.* 2016) and play essential roles in many physiological processes and plant metabolism. Plants respond differently to different concentrations of metallic ions in soil (Singh *et al.* 2007, Ashraf *et al.* 2018b), however, several of these ions in trace amounts are required for normal growth and development of plants (De Britto *et al.* 2011). At high concentrations, almost all heavy metals have significant phytotoxic effects on plants (Nedelkoska and Doran 2000) as well as inhibited activities of enzymes involved in photosynthetic reaction in plants (Singh *et al.* 1997, Wang and Zhou 2005, Smirnova *et al.* 2006).

Previous studies demonstrated that Cu and other micronutrients are essential for normal growth of plants (Cook *et al.* 1998). Among the micronutrients, Cu plays substantial role in crop growth by increased tillering ability and pollen viability of the crop (Das 2014). Cu is also considered as an essential element for all living organisms including plants and plays an important role in the photosynthetic reactions (Singh *et al.* 2007, Adhikari *et al.* 2012) and is also associated with proteins and enzymes involved in electron transfer and redox reactions (Burkhead *et al.* 2009). However, excess Cu is toxic to plants and results in a wide range of biochemical and physiological disturbances in photosynthesis, pigment synthesis, nitrogen and protein metabolism, membrane integrity, and mineral uptake (Panda 2008). Moreover, Cu activates several enzymes and contributes to RNA synthesis and improves the efficiency of photosystems (Adhikari *et al.* 2012). It is required as a structural and catalytic component of several proteins and enzymes involved in electron transfer, oxygenation reactions, and charge accumulation (Cook *et al.* 1998). Cu deficiency reduces the synthesis of plastocyanin and cytochrome oxidase, which results in growth inhibition and decreased photosynthesis and respiration (Ayala and

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[+]Corresponding author; e-mail: tangxr@scau.edu.cn
Abbreviations: 2AP – 2-acetyl-1-pyrroline; PDH – proline dehydrogenase; P_N – photosynthetic rate; POD – peroxidase; SOD – superoxide dismutase.
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Sandmann 1988, Ayala *et al.* 1992). Cu is also essential for PSII activity (Ayala *et al.* 1992) to ensure the correct contents and composition of pigments and polypeptides in PSII (Ayala *et al.* 1992). It also serves as a structural component of an active PSII complex (Arvidsson *et al.* 1993), however, at higher Cu concentration, the seed germination and plant growth were severely inhited (Adhikari *et al.* 2012). Excess Cu is phytotoxic and causing stunted growth, chlorosis, and malformation of the roots in plants (Punz and Sieghardt 1993). The use of physical methods for plant growth stimulation attracts renowned scientists as an alternative of chemical amelioration, which offer ways to improve food quality without impairing its safety (Aladjadjiyan 2011).

Several studies have revealed that some techniques are being used to enhance the seed germination and seedling growth of crop plants. Those techniques usually include priming, hardening, humidification, growth regulators, and dry heat treatments (Akinola *et al.* 2000, Ashraf and Foolad 2005, Farooq *et al.* 2006), however, such techniques are time-consuming and laborious. In contrast, ultrasound seed treatment is a swift technique and requires only an ultrasound generator which can cause heating effect, mechanical effect or even chemical effects for short period of time. Ultrasound is a mechanical wave having frequency higher than 20 kHz (Aladjadjiyan 2011). Besides industrial use of ultrasonic waves, this technique becomes famous in the field of agriculture and food industry due to its wide range application (Benedito *et al.* 2002). For example, ultrasonic treatment of seeds was used for industrial purposes such as oil extraction and malts preparation (Kobus 2008, Yaldagard *et al.* 2008a,b). In biotechnology processes and the food industry, ultrasonically stimulated seed germination and better stand establishment offers the possibility to increase productivity for large-scale farm crops (Liu *et al.* 2003, Yaldagard *et al.* 2008a). Ultrasound treatments have been reported to stimulate germination in many crops, such as bean (Wang *et al.* 2012), corn (Flórez *et al.* 2007), barley (Yaldagard *et al.* 2008a), fern (Wang *et al.* 2012), chickpea and pepper (Goussous *et al.* 2010, Shin *et al.* 2011), fodder beans (Aladjadjiyan 2011), radish (Aladjadjiyan 2011), rice cells (Liu *et al.* 2003), carrot (Aladjadjiyan 2002, 2011), and temperate *Cymbidium* species (Chio and Chung 1991, Yaldagard *et al.* 2008a). It has been demonstrated that ultrasonic treatment for 2 s could promote the growth of carrot cells whereas cells were destroyed/damaged when exposed for more than 2 s (Bochu *et al.* 1998). Previous results indicated the effects of the ultrasonic seed treatment on seed germination depends on frequency of ultrasonic waves and exposure time as well as on the species and cultivars. For instance, ultrasound frequencies ranging 15–100 kHz with an exposure time of 1 to 60 min were found to be quite suitable for seed treatment of various crops (Goussous *et al.* 2010, Aladjadjiyan 2011). Hence, ultrasounic seed treatment induces growth variations in plants at appropriate intensity and time period, however, effects of ultrasonic seed treatment and Cu application on the performance of rice have been rarely investigated. The present study was therefore conducted to assess the effects of ultrasound seed treatment along with Cu application on the photosynthesis, growth, yield, and grain quality of aromatic rice.

Materials and methods

Field and treatment conditions: A field experiment was conducted from March to July 2014 at the Experimental Research Farm, College of Agriculture, South China Agricultural University, Guangzhou, China. This region has subtropical type of climate with yearly average temperature of 21–29°C (Ashraf and Tang 2017). Two widely cultivated aromatic rice cultivars, *i.e.*, Nongxiang 18 and Meixiangzhan 2, were sown in small plots and covered with plastic nets. The soil properties in the plots were as follows: 23.34 mg(organic matter) kg^{-1}, 1.139 mg(total nitrogen) kg^{-1}, 1.136 mg(total phosphate) kg^{-1}, 24.41 mg(total potassium) kg^{-1}, 114.27 mg(available nitrogen) kg^{-1}, 61.34 mg(available phosphate) kg^{-1}, 127.04 mg(available potassium) kg^{-1}, and 0.34 mg(available Cu) kg^{-1}.

The ultrasonic treated and nontreated seeds of two aromatic rice cultivars, *i.e.*, Nongxiang 18 and Meixiang-zhan 2, were sown in soil contaminated with 1.5 and 3.0 g(Cu) m^{-2}, whereas the soil without Cu application was taken as control (CK). Cu in the form of $CuSO_4 \cdot 5H_2O$ was applied for ultrasonic treatment, the seeds were placed in the equipment at 20–40 KHz for 30 min, the ultrasonic seed processor (*JD-1L*) was supported by the *New Power Ultrasonic Electronic Equipment Co.*, *Ltd.*, Guangzhou, China. The experimental treatments are described in the following table.

Treatment	Description
CK	0 g(Cu) m^{-2} + untreated seeds
T1	0 g(Cu) m^{-2} + ultrasonic-treated seeds
T2	1.5 g(Cu) m^{-2} + untreated seeds
T3	1.5 g(Cu) m^{-2} + ultrasonic-treated seeds
T4	3.0 g(Cu) m^{-2} + untreated seeds
T5	3.0 g(Cu) m^{-2} + ultrasonic-treated seeds

The experiment was arranged at randomized complete block design (RCBD) with 24 m^2 net plot size in triplicate. The seeds were sown on 11 March, 2014 in a special type of PVC tray container with 2–3-cm soil layer for nursery growth. Twenty-seven-d-old seedlings were transplanted into each plot with one seedling per hill. Urea (39 g m^{-2}), calcium superphosphate (75 g m^{-2}), and KCl (20 g m^{-2}) were applied to each experimental unit. During the rice growth period, the pest, disease, and weeds were controlled to avoid yield loss whereas the plots were flooded with 2–4-cm water layer throughout the growth period.

Plant sampling: At tillering, flowering, and maturity, three plants were harvested from each plot, rinsed with tap water, then with distilled water to remove deposits, and then separated into stems, leaves, and panicles and oven-dried at 70°C to constant mass for measurement of dry biomass and Cu content. The top fully expanded leaves at tillering stage and the flag leaves at flowering stage and maturity were collected and immediately put into the liquid N_2 for 2 min, then stored at –80°C for determination of proline dehydrogenase (PDH) activity and proline contents. The mature grains were separated into two sets, one set of fresh grains was stored at –80°C for measurement of 2-acetyl-1-pyrroline (2AP) content, the rest of the grains was sun-dried for grain quality measurements.

Cu content: The Cu contents in leaves, stem sheath, and grains were measured by using the (HCl+Vc) soaking extracting method (Zhou *et al*. 1997) and measured by using atomic absorption spectrophotometer (*AA-6300C*, *Shimadzu*, Japan).

Net photosynthetic rate (P_N) and SPAD values: The top fully expanded leaves at tillering stage and the flag leaves at flowering stage and maturity were selected to measure the net photosynthetic rate (P_N) by using the *LI-6400* photosynthetic determination system (*LI-COR Inc*., Lincoln, NE, USA). Three leaves were measured for each treatment and three replicates were measured for each plot, the measurements were conducted between 9:30–11:00 h on a sunny day. The light intensity, leaf temperature, CO_2 concentration, and relative humidity during the measurement were 1,000 μmol(photon) m^{-2} s^{-1}, 400 μmol(CO_2) mol^{-1}, 25°C, and about 25%, respectively. The top fully expanded leaves at tillering stage and the flag leaves at flowering stage and maturity were selected to measure SPAD values by using *SPAD-502* chlorophyll meter (*Konica Minolta*, Japan). The measurements were conducted at the top 1/3, middle, and lower 1/3 of each leaf and the mean was calculated as one SPAD reading. Ten leaves for each replication in each plot were measured.

Proline dehydrogenase (PDH) activity and proline content: The PDH (EC 1.5.5.2) activity in leaves was measured according to the methods of Tateishi *et al*. (2005) and Ncube *et al*. (2013). The absorbance of the colored supernatant complex was read at 440 nm by using UV-VIS spectrophotometer *UV-2550* (*Shimadzu*, Japan). The absorbance change of 0.1 in one min was defined as one unit of PDH activity, and expressed as U g^{-1}(FM) (similarly hereinafter). The proline content was measured by method of Bates *et al*. (1973) by using ninhydrin method, the absorbance was read at 520 nm and the proline content was expressed as μg g^{-1}.

Superoxide dismutase (SOD) and peroxidase (POD) activity: The SOD (EC 1.15.1.1) activity was measured by using the nitroblue tetrazolium (NBT) photochemical reduction method according to Beyer and Fridovich (1987). One unit (U) of SOD activity was defined as the amount of enzyme that induced 50% inhibition of the initial rate of reduction of NBT. The absorbance was read at 560 nm by using UV-VIS spectrophotometer *UV-2550* (*Shimadzu*, Japan). The SOD activity was expressed as U g^{-1}(FM). The POD (EC 1.11.1.7) activity was determined using the method described by Fang and Kao (2000) and Li *et al.* (2019). The absorbance was determined at 470 nm with four intervals and 30 s for each interval. The change in absorbance every minute by 0.01 was defined as one unit (U) of activity. The POD activity was expressed as U g^{-1}(FM).

The 2AP content and grain quality: The 2AP content was measured as described by Mo *et al.* (2015, 2019) by using synchronization of distillation and extraction method (SDE) combined with *GCMS-QP2010 Plus* (*Shimadzu*, Japan). About 1 kg of grains for quality measurement was obtained from each sample after they were stored at room temperature for three months to make sure the grain quality is stable. Brown rice rate was estimated using a rice huller (*THU-35B*, *Sato Machinery Co*., *Ltd*., Jiangsu, China). Milled rice and head milled rice rate was estimated using a Jingmi testing rice grader (*JNMJ3*, *Taizhou Liang Yi*, Zhejiang, China). Percentage of grains with chalkiness and chalkiness degree was estimated using a *SDE-A* light box (Guangzhou, China). Amylose and protein contents of grains were determined using *Infratec1241* grain analyzer (*Foss Tecator Co.*, *Ltd*., Denmark).

Grain yield and yield-related traits: At maturity, the mature grains were harvested in 1 m^2 in each plot, sun-dried, then weighed and adjusted to ~ 14% moisture content recorded as grain yield. Panicle numbers in each plot were determined by counting the panicle of 50 hills. The grains of representing plant were harvested and manually separated from the leaves and stem sheath, then the filled grain numbers and total grain numbers were recorded to evaluate filled grain percentage.

Statistics analysis: Data were processed by using *Excel 2010* and the analysis of variance (*ANOVA*) was performed by using *Statistics version 8.0*. The comparisons of means between the treatments were conducted by least significant difference (LSD) test at P=0.05 significance level.

Results

Grain yield and yield-related traits: Ultrasonic seed treatment and Cu application increased grain yield, panicle number per m^2, grain number per panicle, and filled grain percentage. For Nongxiang 18, the grain yield, panicle number per m^2, grain number per panicle, and filled grain percentage increased by 2.7–33.5%, 0.8–12.8%, 4.6–19.3%, and 0.5–20.0%, respectively. For Meixiang-zhan 2, the grain yield, panicle number per m^2, grain number per panicle, and filled grain percentage increased by 12.9–20.2%, 1.1–28.0%, 2. 9–16.3%, and 1.9–11.0%, respectively. Ultrasonic seed treatment resulted in higher panicle number per m^2, grain number per panicle; filled grain percentage, and grain yield (Fig. 1).

Leaves and stem sheath dry biomass: Ultrasonic seed treatment and Cu application increased leaves and stem sheath dry biomass of aromatic rice. For Nongxiang 18, all the treatments increased leaf dry biomass at tillering, flowering, and maturity in a range of 26.3–61.8%, 5.1–30.7%, and 1.1–27.3%, respectively. The stem sheath dry biomass at tillering stage, flowering stage, and maturity of Nongxiang 18 increased in a range of 4.1–50.0%, 12.3–35.7%, and 12.0–25.9%, respectively. For Meixiangzhan 2, all the treatments increased leaf dry biomass at tillering stage, flowering stage, and maturity in a range of 2.2–15.1%, 4.7–62.1%, and 10.3–47.4%, respectively. The stem sheath dry biomass at tillering, flowering, and maturity of Meixiangzhan 2 increased in a range of 7.5–33.8%, 8.5–57.4%, and 5.1–26.5%, respectively. Moreover, the ultrasonic seed treatment resulted in higher leaves and stem sheath dry biomass than that of untreated seeds (Table 1S, *supplement*).

Net photosynthetic rate and SPAD value: Compared with CK, all treatments improved the net photosynthetic rate (P_N) and SPAD value in leaves of aromatic rice. Ultrasonic seed treatment increased P_N at tillering and maturity stage and enhanced SPAD value in the leaves of aromatic rice. At tillering stage, the P_N significantly increased in Nongxiang 18 (4.1–25.0%) and Meixiangzhan 2 (6.8–32.8%). At maturity, the P_N was improved in Nongxiang 18 (3.3–72.5%) and Meixiangzhan 2 (0.2–33.3%). Under similar Cu application treatment, ultrasonic seed treatment showed lower P_N at flowering stage, *i.e.*, T3 < T2 and T5 < T4. Furthermore, ultrasonic seed treatment and Cu application significantly increased SPAD value at flowering stage and maturity (Table 1).

Antioxidant enzyme activities: Ultrasonic seed treatment increased the SOD and POD activity in leaves of aromatic rice. For instance, compared with CK, all treatments improved the SOD and POD activity in the leaves of aromatic rice. The SOD activity was increased at tillering stage (12.5–87.9% for Nongxiang 18 and 6.8–50.7% for Meixiangzhan 2), flowering stage (26.0–84.9% for Nongxiang 18 and 9.8–23.9% for Meixiangzhan 2), and maturity (38.6–82.1% for Nongxiang 18 and 2.2–61.0% for Meixiangzhan 2). The increment in POD activity at tillering, flowering, and maturity stage was 4.4–33.2%, 21.0–70.8%, and 3.4–28.4% for Nongxiang18, respectively; and 11.0–42.0%, 3.2–121.5%, and 7.8–67.3% for Meixiangzhan 2, respectively. Overall, ultrasonic seed treatment caused higher SOD activity and POD activity in leaves than that of untreated seeds (Table 2).

2-acetyl-1-pyrroline content: Compared with CK, the T4 and T5 treatment significantly increased the 2AP contents in grains by 13.1 and 88.1% for Nongxiang 18, respectively, and by 20.5 and 50.2% for Meixiangzhan 2, respectively. For Nongxiang 18, ultrasonic seed treatment and Cu application improved 2AP content in grains while for Meixiangzhan 2, the ultrasonic seed treatment decreased 2AP content in grains after Cu application. Ultrasonic seed treatment had higher 2AP contents in grains for Nongxiang 18 than that of untreated seeds (Fig. 2).

Proline content and PDH activity: Ultrasonic seed treatment and Cu application enhanced the proline content in grains. For Nongxiang 18, the proline content increased from 2.7 to 122.0%. For Meixiangzhan 2, an increment

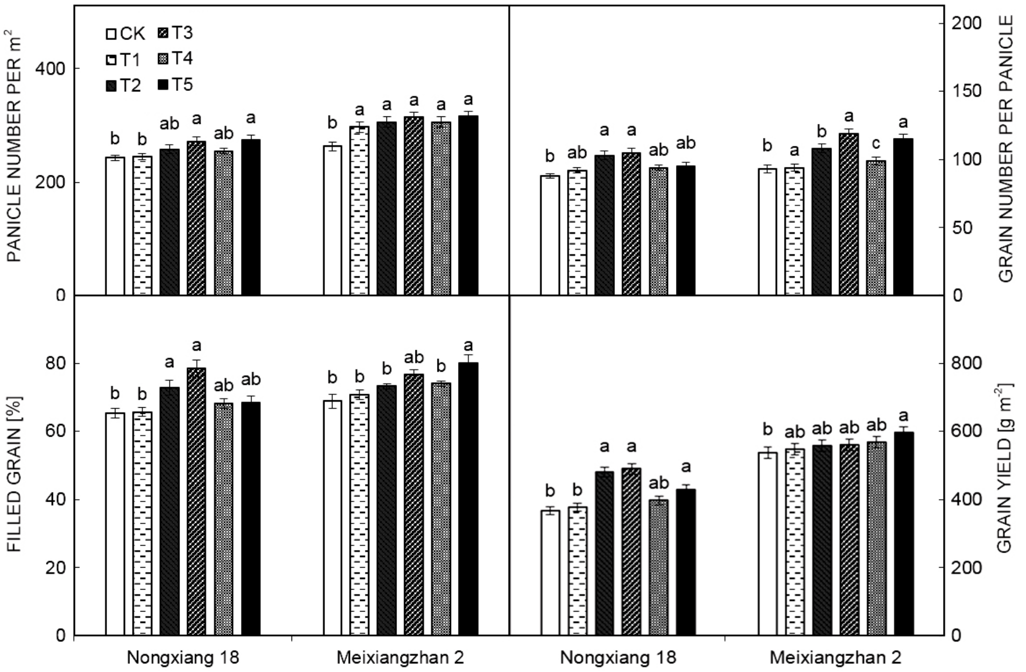

Fig. 1. Effects of ultrasonic seed treatment and Cu application on (*A*) panicle number m^{-2}, (*B*) grain numer per panicle, (*C*) filled grain percentage, and (*D*) grain yield of aromatic rice. Vertical bars with *different lowercase letters* above are significantly different at *P*=0.05 by LSD test. Capped bars represent SD. CK: 0 g(Cu) m^{-2} + untreated seeds; T1: 0 g(Cu) m^{-2} + ultrasonic-treated seeds; T2: 1.5g(Cu) m^{-2} + untreated seeds; T3: 1.5g(Cu) m^{-2} + ultrasonic-treated seeds; T4: 3.0 g(Cu) m^{-2} + untreated seeds; T5: 3.0 g(Cu) m^{-2} + ultrasonic-treated seeds.

Table 1. Effects of ultrasonic seed treatment and Cu application on net photosynthetic rate (P_N) and SPAD value in leaves of aromatic rice. Mean ± SD in the same column followed by *different lowercase letters* for the same variety differ significantly at P=0.05 by LSD test. CK: 0 g(Cu) m^{-2} + untreated seeds; T1: 0 g(Cu) m^{-2} + ultrasonic-treated seeds; T2: 1.5g(Cu) m^{-2} + untreated seeds; T3: 1.5g(Cu) m^{-2} + ultrasonic-treated seeds; T4: 3.0 g(Cu) m^{-2} + untreated seeds; T5: 3.0 g(Cu) m^{-2} + ultrasonic-treated seeds.

Cultivar	Treatment	P_N [µmol(CO_2) m^{-2} s^{-1}]			SPAD value		
		Tillering	Flowering	Maturity	Tillering	Flowering	Maturity
Nongxiang 18	CK	25.39 ± 0.06^d	16.69 ± 0.21^d	22.54 ± 0.17^e	41.51 ± 0.17^c	37.52 ± 0.11^d	32.45 ± 0.16^e
	T1	26.43 ± 0.21^c	19.73 ± 0.09^c	23.29 ± 0.18^{de}	41.60 ± 0.13^c	38.32 ± 0.17^c	33.71 ± 0.11^d
	T2	27.42 ± 0.12^b	25.55 ± 0.20^a	27.66 ± 0.17^{cd}	42.63 ± 0.06^b	40.45 ± 0.21^b	35.65 ± 0.33^c
	T3	27.62 ± 0.18^b	21.59 ± 0.17^b	32.35 ± 0.45^b	42.66 ± 0.26^b	42.83 ± 0.15^a	36.70 ± 0.17^b
	T4	31.40 ± 0.36^a	26.03 ± 0.34^a	31.12 ± 1.87^{bc}	43.47 ± 0.46^a	43.02 ± 0.51^a	36.87 ± 0.47^b
	T5	31.73 ± 0.21^a	20.41 ± 0.35^c	38.88 ± 2.96^a	43.62 ± 0.13^a	43.28 ± 0.19^a	40.23 ± 0.13^a
Meixiangzhan 2	CK	22.82 ± 0.02^d	16.45 ± 0.19^e	20.58 ± 0.03^e	40.38 ± 0.13^c	38.16 ± 0.19^d	26.47 ± 0.14^e
	T1	24.36 ± 0.08^c	18.69 ± 0.19^{cd}	20.61 ± 0.01^e	41.02 ± 0.05^{bc}	39.39 ± 0.07^c	27.78 ± 0.04^d
	T2	24.47 ± 0.23^c	22.14 ± 0.70^a	21.14 ± 0.14^d	41.49 ± 0.21^{bc}	40.79 ± 0.44^b	29.99 ± 0.31^c
	T3	28.29 ± 0.18^b	18.35 ± 0.12^d	23.39 ± 0.13^c	41.94 ± 0.91^b	41.53 ± 0.20^b	31.47 ± 0.05^b
	T4	28.80 ± 0.24^b	20.63 ± 0.07^b	25.43 ± 0.15^b	41.33 ± 0.25^{bc}	42.49 ± 0.31^a	31.51 ± 0.20^b
	T5	30.31 ± 0.14^a	19.40 ± 0.19^c	27.44 ± 0.28^a	43.60 ± 0.09^a	42.59 ± 0.06^a	32.70 ± 0.13^a

Table 2. Effects of ultrasonic seed treatment and Cu application on superoxide dismutase (SOD) and peroxidase (POD) activity in the leaves of aromatic rice. Mean ± SD in the same column followed by *different lowercase letters* for the same variety differ significantly at P=0.05 by LSD test. CK: 0 g(Cu) m^{-2} + untreated seeds; T1: 0 g(Cu) m^{-2} + ultrasonic-treated seeds; T2: 1.5g(Cu) m^{-2} + untreated seeds; T3: 1.5g(Cu) m^{-2} + ultrasonic-treated seeds; T4: 3.0 g(Cu) m^{-2} + untreated seeds; T5: 3.0 g(Cu) m^{-2} + ultrasonic-treated seeds.

Cultivar	Treatment	SOD activity [U g^{-1}(FM)]			POD activity [U g^{-1}(FM)]		
		Tillering	Flowering	Maturity	Tillering	Flowering	Maturity
Nongxiang 18	CK	66.79 ± 0.99^e	98.12 ± 2.19^e	98.98 ± 2.45^d	23.12 ± 0.70^c	13.94 ± 0.14^e	16.42 ± 0.37^d
	T1	75.16 ± 1.99^d	123.58 ± 2.47^d	137.19 ± 1.01^c	24.13 ± 0.39^c	16.87 ± 0.39^d	16.97 ± 0.10^{cd}
	T2	106.09 ± 2.03^c	124.95 ± 1.44^d	139.10 ± 9.64^{bc}	27.82 ± 2.90^{abc}	18.82 ± 0.48^c	17.41 ± 0.23^{bcd}
	T3	123.63 ±1.18^a	134.76 ± 2.62^c	180.23 ± 2.28^a	29.39 ± 0.67^{ab}	19.64 ± 0.18^c	21.08 ± 0.58^a
	T4	114.54 ±1.56^b	173.44 ± 0.93^b	145.41 ± 1.93^{bc}	24.60 ± 0.67^{bc}	21.29 ± 0.48^b	17.72 ± 0.60^{bc}
	T5	125.48 ±1.61^a	181.45 ± 1.74^a	151.75 ± 0.16^b	30.80 ± 2.55^a	23.81 ± 0.32^a	18.47 ± 0.26^b
Meixiangzhan 2	CK	115.46 ± 0.22^f	98.11 ± 0.72^e	102.72 ± 1.15^d	15.56 ± 0.22^e	8.82 ± 0.66^d	10.39 ± 0.07^e
	T1	123.36 ± 1.61^e	107.77 ± 1.15^d	104.99 ± 0.47^d	17.31 ± 0.16^d	9.10 ± 0.29^d	11.20 ± 0.27^d
	T2	145.41 ± 1.23^d	114.14 ± 2.14^c	135.00 ± 1.13^b	18.54 ± 0.16^c	12.55 ± 0.18^c	12.57 ± 0.23^b
	T3	157.60 ± 1.11^c	144.72 ± 1.08^a	165.37 ± 4.27^a	20.39 ± 0.11^b	15.51 ± 0.18^b	17.38 ± 0.12^a
	T4	169.81 ± 0.91^b	111.12 ± 2.88^{cd}	106.14 ± 1.37^{cd}	19.84 ± 0.21^b	16.32 ± 0.33^b	11.83 ± 0.06^c
	T5	173.95 ± 1.87^a	121.59 ± 0.81^b	114.76 ± 5.75^c	22.10 ± 0.79^a	19.54 ± 0.23^a	12.53 ± 0.18^b

in proline content was observed from 10.1 to 59.9%. Ultrasonic seed treatment had the higher proline content in grains than that of untreated seeds (Fig. 1S, *supplement*).

Ultrasonic seed treatment and Cu application enhanced proline contents in leaves of aromatic rice. For Nongxiang 18, compared to CK, all treatments significantly increased the proline contents in leaves at tillering stage with a range from 29.3 to 110.4%. The T3, T4, and T5 treatments significantly increased proline content in leaves at flowering stage by 56.2, 101.7, and 126.5%, respectively; T2, T3, T4, and T5 treatments significantly increased proline content in leaves at maturity by 22.1, 68.4, and 75.4%, respectively. For Meixiangzhan 2, compared with CK, all treatments significantly increased the proline contents in leaves at tillering and flowering stage, whereas the T3, T4, and T5 treatments significantly increased the proline content in leaves at maturity by 37.4, 27.9, and 52.4%, respectively (Table 2S, *supplement*). Furthermore, ultrasonic seed treatment and Cu application improved PDH activity in leaves of aromatic rice. For Nongxiang 18, compared with CK, the T2, T3, T4, and T5 treatments significantly increased PDH activity in leaves at tillering and flowering stage whilst the T3 treatment significantly increased the PDH activity in leaves at maturity by 14.1%. For Nongxiang 18, compared with CK, all treatments significantly increased the PDH activity in leaves at tillering stage and maturity. The T3, T4, and T5 treatments significantly increased PDH activity in leaves at flowering stage by 6.4, 7.4, and 8.3%, respectively. Ultrasonic seed treatment resulted in higher proline content and PDH activity in leaves than that of untreated seeds (Table 2S).

Grain quality: Ultrasonic seed treatment and Cu application improved brown rice rate, milled rice rate, head milled rice rate, protein content, and amylose content, decreased chalky rice percentage, and chalkiness degree. For Nongxiang 18, compared with CK, all treatments significantly improved the brown rice rate, head milled rice rate, but significantly decreased chalky rice percentage and chalkiness degree. The T2, T3, T4, and T5 treatments enhanced milled rice rate and protein content, whereas the T3, T4, and T5 treatment significantly enhanced the amylose content. For Meixiangzhan 2, compared to CK, all treatments substantially improved the brown rice rate, milled rice rate, head milled rice rate, but decreased chalky rice percentage and chalkiness degree. Moreover, the T2, T3, T4, and T5 treatment significantly increased protein content whilst T4 and T5 treatment significantly increased amylose content (Table 3).

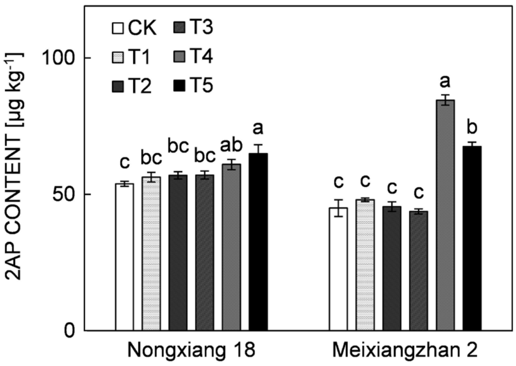

Fig. 2. Effects of ultrasonic seed treatment and Cu application on 2-acetyl-1-pyrroline (2AP) content in grains of aromatic rice. Vertical bars with *different lowercase letters* above are significantly different at *P*=0.05 by LSD test. Capped bars represent SD. CK: 0 g(Cu) m^{-2} + untreated seeds; T1: 0 g(Cu) m^{-2} + ultrasonic-treated seeds; T2: 1.5g(Cu) m^{-2} + untreated seeds; T3: 1.5g(Cu) m^{-2} + ultrasonic-treated seeds; T4: 3.0 g(Cu) m^{-2} + untreated seeds; T5: 3.0 g(Cu) m^{-2} + ultrasonic-treated seeds.

Cu content: Ultrasonic seed treatment and Cu application significantly increased the Cu content in grains. For Nongxiang 18, compared with CK, T1, T2, T3, T4, and T5 treatment significantly increased Cu content by 11.2, 19.6, 20.7, 26.9, and 29.7%, respectively. For Meixiangzhan 2, significant increase in Cu content for T1, T2, T3, T4, and T5 was observed by 31.1, 39.6, 43.5, 91.9, and 100.4%, respectively. Ultrasonic seed treatment had the higher Cu content than that of untreated seeds (Fig. 3).

Ultrasonic seed treatment and Cu application increased the Cu content in leaves and stem sheath of aromatic rice. For Nongxiang 18, compared with CK, the T2, T3, T4, and T5 treatment significantly increased the Cu content in leaves at tillering and flowering stage and in stem sheath at tillering. The T2, T3, and T4 treatment significantly increased the Cu contents in leaves and in stem sheath at maturity, while T3, T4, and T5 treatment substantially increased the Cu contents in stem sheath at flowering stage. Under 30 g(Cu) m^{-2}, the ultrasonic seed treatment decreased Cu contents in leaves and stem sheath when compared to that of untreated seed. For Meixiangzhan 2, compared with CK, all treatments significantly increased Cu contents in leaves at flowering and maturity stage and in stem sheath at tillering and maturity stage. Moreover, the T4 and T5 treatment significantly increased the Cu contents in leaves, whereas T2, T3, T4, and T5 treatment significantly increased the Cu contents in stem sheath at flowering stage. Under 30 g(Cu) m^{-2}, ultrasonic seed treatment decreased the Cu content in leaves at flowering and maturity stage and decreased the Cu content in stem sheath at tillering and flowering stage when compared to that of untreated seeds (Table 4).

Table 3. Effects of ultrasonic seed treatment and Cu application on grain quality of aromatic rice. Mean ± SD in the same column followed by *different lowercase letters* for the same variety differ significantly at *P*=0.05 by LSD test. CK: 0 g(Cu) m^{-2} + untreated seeds; T1: 0 g(Cu) m^{-2} + ultrasonic-treated seeds; T2: 1.5g(Cu) m^{-2} + untreated seeds; T3: 1.5g(Cu) m^{-2} + ultrasonic-treated seeds; T4: 3.0 g(Cu) m^{-2} + untreated seeds; T5: 3.0 g(Cu) m^{-2} + ultrasonic-treated seeds.

Cultivar	Treatment	Brown rice rate [%]	Milled rice rate [%]	Head milled rice rate [%]	Chalky rice percentage [%]	Chalkiness degree [%]	Protein content [%]	Amylose content [%]
Nongxiang 18	CK	75.38 ± 0.13c	61.09 ± 0.17^{c}	48.54 ± 0.23^{d}	9.67 ± 0.33^{a}	1.51 ± 0.05^{a}	8.50 ± 0.03^{e}	17.50 ± 0.06^{c}
	T1	76.61 ± 0.25^{b}	61.47 ± 0.06^{c}	51.66 ± 0.27^{c}	6.00 ± 0.00^{b}	0.90 ± 0.03^{b}	9.03 ± 0.06^{de}	17.57 ± 0.03^{bc}
	T2	76.53 ± 0.11^{b}	62.50 ± 0.18^{b}	53.12 ± 0.05^{b}	4.33 ± 0.33^{c}	0.43 ± 0.02^{c}	9.30 ± 0.15^{cd}	17.97 ± 0.00^{bc}
	T3	76.16 ± 0.02^{b}	62.62 ± 0.23^{b}	53.69 ± 0.34^{ab}	3.00 ± 0.58^{d}	0.29 ± 0.06^{d}	9.87 ± 0.15^{c}	18.33 ± 0.33^{b}
	T4	77.58 ± 0.22^{a}	63.56 ± 0.15^{a}	53.94 ± 0.27^{a}	2.33 ± 0.33de	0.17 ± 0.02^{e}	10.70 ± 0.32^{b}	19.77 ± 0.06^{a}
	T5	76.34 ± 0.04^{b}	63.65 ± 0.22^{a}	53.31 ± 0.19^{ab}	1.33 ± 0.33e	0.08 ± 0.02^{e}	11.63 ± 0.13^{a}	19.97 ± 0.34^{a}
Meixiangzhan 2	CK	73.47 ± 0.24^{d}	61.24 ± 0.07^{d}	48.51 ± 0.13^{c}	8.67 ± 0.33a	1.04 ± 0.09^{a}	8.60 ± 0.00^{c}	17.53 ± 0.00^{c}
	T1	74.59 ± 0.23^{c}	62.11 ± 0.02^{c}	52.36 ± 0.18^{b}	7.00 ± 0.58^{b}	0.81 ± 0.04^{b}	8.87 ± 0.09^{bc}	17.63 ± 0.03^{c}
	T2	75.67 ± 0.19^{b}	62.53 ± 0.04^{b}	52.30 ± 0.15^{b}	5.67 ± 0.33^{bc}	0.49 ± 0.01^{c}	9.33 ± 0.06^{b}	18.03 ± 0.32^{c}
	T3	74.35 ± 0.11^{c}	62.49 ± 0.09^{b}	53.16 ± 0.06^{a}	5.33 ± 0.67^{c}	0.41 ± 0.07^{c}	9.37 ± 0.09^{b}	18.23 ± 0.32^{bc}
	T4	76.59 ± 0.18^{a}	63.65 ± 0.17^{a}	52.35 ± 0.24^{b}	3.00 ± 0.58^{d}	0.20 ± 0.04^{d}	10.63 ± 0.06^{a}	19.13 ± 0.03^{ab}
	T5	75.77 ± 0.14^{b}	62.48 ± 0.19^{b}	52.70 ± 0.09^{ab}	2.67 ± 0.33^{d}	0.14 ± 0.01^{d}	11.13 ± 0.10^{a}	19.33 ± 0.32^{a}

Discussion

Ultrasonic waves may stimulate various plant processes and cause structural and functional modifications in plants; however, its stimulatory and/or inhibitory effects depend on intensity and duration of the treatment (Rao *et al.* 2018). Cu is considered as an essential element for plants and plays an important role in the photosynthetic reactions being associated with proteins and enzymes involved in electron transfer and redox reactions (Burkhead *et al.* 2009, Adhikari *et al.* 2012). Trace amounts of Cu stimulate plant growth and improve photosynthetic efficiency of plants and improve rice tillering (Adhikari *et al.* 2012, Das 2014). Ultrasonic seed treatment and Cu application increased yield and related attributes of rice, *e.g.*, panicle number per m^2, grain number per panicle, and filled grain percentage in both rice cultivars (Fig. 1). Ultrasonic seed treatment and Cu application also increased leaves and stem sheath dry biomass of aromatic rice (Table 1S). These results corroborate with the previous findings of Das (2014),Nie *et al.* (2013), and Rao *et al.* (2018). Moreover, ultrasonic seed treatment and Cu application increased P_N and enhanced SPAD value of aromatic rice. However, under similar Cu application treatment, ultrasonic seed treatment showed lower P_N at flowering stage (Table 1). Ultrasonic seed treatment may cause physiobiochemical changes in seeds, *e.g.*, elongation in coleoptiles and mesocotyl, thus accelerated seminal roots growth which may resulted in increased absorption of water and other mineral nutrients that ultimately led to enhanced growth and development of crop plants (Kratovalieva *et al.* 2012). In general, ultrasonic seed treatment enhanced crop stand establishment which resulted in superior growth and yield production. On the other hand, Cu plays an active role in nitrogen, hormone, and protein metabolism, as well as enhances photosynthesis and auxin production, and ultimately rice growth. Thus, the positive effects of Cu on rice grain yield observed in the current experiment might be attributed to development of the pollen availability, grain formation, and grain filling by improving the enzyme activities. Moreover, the uptake of Cu increased the photosynthetic pigments in the crop resulting in the enhanced yield and yield-related attributes. Recently, Rao *et al.* (2019) reported that ultrasonic seed treatment improved the root and shoot biomass of rapeseed under Cd stress conditions.

Plants have established a complex enzymatic and nonenzymatic antioxidative defense mechanism to negate the oxidative stress caused by external environmental factors (Ashraf *et al.* 2015). Generally, SOD performs as the first line of defense system altering O^{2-} into H_2O_2, and then CAT and APX detoxify H_2O_2, thereby preventing the formation of hydroxyl radicals (Chen *et al.* 2013).

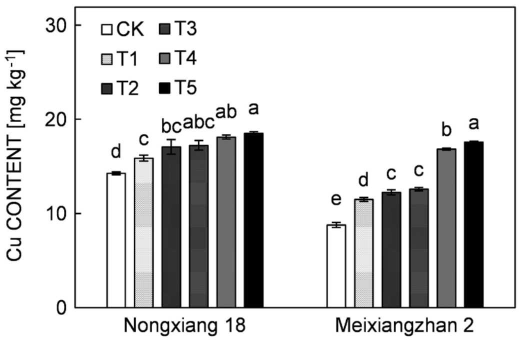

Fig. 3. Effects of ultrasonic seed treatment and Cu application on Cu content in grains of aromatic rice. Vertical bars with *different lowercase letters* above are significantly different at P=0.05 by LSD test. CK: 0 g(Cu) m^{-2} + untreated seeds; T1: 0 g(Cu) m^{-2} + ultrasonic-treated seeds; T2: 1.5g(Cu) m^{-2} + untreated seeds; T3: 1.5g(Cu) m^{-2} + ultrasonic-treated seeds; T4: 3.0 g(Cu) m^{-2} + untreated seeds; T5: 3.0 g(Cu) m^{-2} + ultrasonic-treated seeds.

Table 4. Effects of ultrasonic seed treatment and Cu application on Cu content [mg kg^{-1}] in leaves and stem sheath of aromatic rice. Mean ± SD in the same column followed by *different lowercase letters* for the same variety differ significantly at P=0.05 by LSD test. CK: 0 g(Cu) m^{-2} + untreated seeds; T1: 0 g(Cu) m^{-2} + ultrasonic-treated seeds; T2: 1.5g(Cu) m^{-2} + untreated seeds; T3: 1.5g(Cu) m^{-2} + ultrasonic-treated seeds; T4: 3.0 g(Cu) m^{-2} + untreated seeds; T5: 3.0 g(Cu) m^{-2} + ultrasonic-treated seeds.

Cultivar	Treatment	Leaves			Stem sheath		
		Tillering	Flowering	Maturity	Tillering	Flowering	Maturity
Nongxiang 18	CK	28.39 ± 0.27^d	12.38 ± 0.08^e	7.59 ± 0.24^d	14.55 ± 0.10^c	10.58 ± 0.07^d	8.72 ± 0.02^c
	T1	28.52 ± 0.39^d	12.79 ± 0.14^e	7.84 ± 0.33^d	15.31 ± 0.22^{bc}	10.73 ± 0.19^{cd}	8.77 ± 0.44^c
	T2	28.74 ± 0.16^{cd}	15.84 ± 0.45^d	12.28 ± 0.17^c	15.60 ± 0.43^b	11.24 ± 0.13^{cd}	10.56 ± 0.20^b
	T3	30.17 ± 0.52^b	17.48 ± 0.18^c	20.99 ± 0.88^a	16.18 ± 0.03^b	12.30 ± 0.27^b	11.79 ± 0.12^a
	T4	31.71 ± 0.52^a	19.58 ± 0.27^a	13.95 ± 0.49^b	18.18 ± 0.41^a	14.55 ± 0.50^a	11.32 ± 0.26^a
	T5	29.71 ± 0.15^{bc}	18.42 ± 0.25^b	8.39 ± 0.05^d	17.67 ± 0.32^a	11.44 ± 0.03^c	8.54 ± 0.22^c
Meixiangzhan 2	CK	26.43 ± 0.23^b	16.41 ± 0.20^d	11.45 ± 0.14^e	16.28 ± 0.22^c	13.32 ± 0.32^d	10.16 ± 0.24^e
	T1	26.55 ± 0.25^b	18.49 ± 0.17^c	12.70 ± 0.34^d	17.11 ± 0.02^b	13.59 ± 0.29^d	11.71 ± 0.78^d
	T2	26.57 ± 0.03^b	19.05 ± 0.31^{bc}	13.35 ± 0.17^d	17.54 ± 0.05^b	14.44 ± 0.19^c	14.59 ± 0.27^c
	T3	26.73 ± 0.22^b	19.23 ± 0.17^b	15.55 ± 0.04^c	19.18 ± 0.51^a	15.89 ± 0.07^b	15.83 ± 0.35^b
	T4	28.39 ± 0.27^d	21.45 ± 0.22^a	20.35 ± 0.46^a	19.68 ± 0.13^a	17.62 ± 0.24^a	16.32 ± 0.01^b
	T5	28.52 ± 0.39^d	19.59 ± 0.17^b	18.65 ± 0.15^b	19.03 ± 0.19^a	15.74 ± 0.17^b	18.39 ± 0.18^a

Ultrasonic seed treatment and Cu application increased SOD and POD activity in leaves of aromatic rice (Table 2). Similar results were reported in other studies, which stated that ultrasound seed treatments can assist plants to reduce ROS by increasing the activities of antioxidant enzymes (Chen *et al.* 2013, Rao *et al.* 2018). Proline, being involved in osmoregulation mechanism, protects and stabilizes essential cellular structures and photosynthetic apparatus. Biosynthesized and accumulated proline induces stress tolerance and prevents electrolyte leakage by scavenging ROS and lipid peroxidation inhibition (Kaur and Asthir 2015). Ultrasonic seed treatment had higher proline and PDH content in leaves than that of untreated seeds (Table 2S). Similar results were reported in some other studies (Rao *et al.* 2018). It is also reported that foliar applied proline activates protective mechanisms in plants (Emamverdian *et al.* 2015).

Ultrasonic seed treatment resulted in the higher 2AP contents in grain of Nongxiang 18 than that of untreated seeds (Fig. 2). The 2AP is considered critical for the aroma and flavor of aromatic rice (Yang *et al.* 2007). Proline is a key compound that is involved in the 2AP biosynthetic pathway (Mo *et al.* 2016). Former studies suggest that the content of 2AP is correlated to the content of proline (Sansenya *et al*. 2018). Thus, the possible mechanism lying behind enhanced aromatic compounds may be that ultrasound seed treatments induce physiological adjustments in rice crop causing alterations in microstructures, activities of cellular enzymes, and plant metabolism (Yusaf 2015, Chemat *et al*. 2017, Rao *et al.* 2018).

Furthermore, ultrasonic seed treatment and Cu application improved grain quality, *i.e*., brown rice rate, milled rice rate, head milled rice rate, protein content and amylose content, decreased chalky rice percentage and chalkiness degree (Table 3). The positive effect of Cu on grain quality of rice observed in the present study might be attributed to enhanced enzymatic activities involved in physiobiochemical activities as well as those involved in rice aroma formation. Ultrasonic seed treatment and Cu application significantly increased Cu content in grains (Fig. 3), leaves, and stem sheath of aromatic rice (Table 4). Contrary to our fingdings, Rao *et al.* (2019) reported that ultrasonic seed treatment of *Brassica napus* reduced Cd accumulation in different plant parts. The higher Cu accumulation in different plant parts under ultrasonic treatment might be the dependent on plant types, nature, concentration, and availability of metals in soil solution. However, further studies are needed to reveal the dilemma regarding the roles of ultrasonic seed treatment in plants grown in metal-contaminated soil.

In conclusion, ultrasonic seed treatment and Cu application increased grain yield in Nongxiang 18 (2.7–33.5%) and Meixiangzhan 2 (12.9–20.2%) as a result yield-related components and modifications in physiobiochemical attributes. Ultrasonic seed treatment and Cu application also modulated 2AP accumulation with increased proline contents in grains and leaves, and PDH activity in leaves. Additionally, ultrasonic seed treatment and Cu application improved grain quality attributes whereas the Cu contents were found higher in grains, leaves, and stem sheath under Cu applied plots. Overall, ultrasonic seed treatment is benefitial for modulating the growth, aroma, grain quality, and Cu concentration in aromatic rice.

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