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Full Length Research Paper

Development of a fast and reliable ozone screening method in rice (*Oryza sativa* L.)

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A rapid and reliable screening method for rice ozone tolerance was developed to enhance evaluation technique in determining the reaction of tolerant and susceptible genotypes at the early stage of growth. Plant growth parameters were measured to evaluate ozone effects on the growth of seedlings which can be later used as criteria for ozone tolerance. Plants were subjected to 0.3 and 1 ppm ozone using 15 and 30 day-old seedlings with 7 h exposure per day for 10 days. Our results show that the tolerant, moderately tolerant and susceptible cultivars can be classified at higher ozone concentration with younger seedlings by the leaf bronzing score after 10 days. Thus, screening for ozone tolerance could be done using 1 ppm concentration. For rapid screening, direct seeding and younger seedlings at 15 days are most desired to reduce time of growing in the pots or trays. Shoot dry weight was significantly reduced in the ozone-treated seedlings compared with the control plants. Higher reduction of shoot biomass was observed in 15 day-old seedlings at 34 - 66% and 38 - 54% in 0.3 and 1 ppm, respectively compared with 12 - 24% and 28 - 48% in the 30 day-old seedlings.

Key words: Fumigated chamber, leaf bronzing, ozone screening, rice.

INTRODUCTION

It has been reported that ground level ozone concentrations have increased during the last decades and affected rice growth and production. Yield reduction in crops due to ambient ozone can be between 5 - 35% at agriculturally important locations across South Asia which is about US$4 billion per annum for staple crops (Emberson and Buker, 2008). Krupa et al. (1998) reported that at least 12 major crop species are considered ozone-sensitive, many of which are classified as specialty crops (Kats et al., 1985; Kobayashi et al., 1995; Benton et al., 2000; Synder et al., 1991; Krupa et al., 1998; Burkey et al., 2005; Heagle, 1989). Ozone-sensitive plants exhibit foliar injury, decreased photosynthetic activity, reduction in shoot and root growth and long exposures can reduce yields, biomass and premature senescence (Heagle, 1989; Ariyaphanphitak, 2004; Burkey et al., 2007; Shi et al., 2009).

Efficient, reproducible and simple mass screening technique for selection of ozone tolerant genotypes are the most important considerations in breeding for ozone tolerance. Till date, there are few open-field studies conducted in evaluating genotypic differences among rice cultivars in response to ozone (Chen et al., 2008; Shi et al., 2009) perhaps due to resource limitations and uncontrolled environmental factors. Few studies have been conducted to specifically develop a protocol in screening genotypes for ozone tolerance. Hence, we combine different age of seedlings and ozone concentrations and improved evaluation criteria to come up with a more reliable method of screening. Considering the huge number of breeding materials for screening, a fast and reliable method for ozone screening is necessary to keep pace with the large breeding materials generated every year. Field screening for ozone effects or the free-air concentration enrichment (FACE) experiments can be done later in the yield evaluation of few selected genotypes similar to the study by Shi et al. (2009).

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Table 1. List of varieties used in the experiment and their known tolerance to ozone.

Variety	Type	Reaction (score)	Source
Cheongcheong	Tongil	Resistant (1)	Sohn et al. (2002)
Nongan	Japonica	Resistant (1)	Sohn et al. (2002)
Borami	Japonica	-	-
Chilbo	Japonica	-	-
Hanareum	Japonica	-	-
Dongjin	Japonica	Moderately susceptible (5)	Sohn et al. (2002)
		Susceptible	Hur et al. (2000)
Hwayeong	Japonica	Moderately susceptible (5)	Sohn et al. (2002)
		Moderately tolerant	Hur et al. (2000)
Ilmee	Japonica	Moderately susceptible (5)	Sohn et al. (2002)
		Tolerant	Hur et al. (2000)
Chucheong	Japonica	Susceptible (7-9)	Sohn et al. (2002)
Milyang 175	Japonica	-	-

Ozone is formed by photochemical oxidation when sunlight catalyses reactions between volatile organic compounds and carbon monoxides in the presence of nitrogen compounds. Ozone formation is highly dependent on temperature, relative air humidity and sun radiation time (Omidvari et al., 2008). APCEN (Air Pollution Crop Effect Network) estimated that ground level O_3 concentration in Asia in 2007 is 0.09 - 0.2 ppm making crops more vulnerable. In South Asia, particularly India where large areas of rice are grown, an hourly maximum O_3 concentration of 0.01 - 0.273 ppm have been recorded. Potentially damaging high concentration of O_3 has been found in other rice growing regions of Southeast Asia particularly Thailand, Indonesia, Philippines and Vietnam (Emberson, 2007). Ozone can reduce rice yields between 3 - 47% (Emberson and Buker, 2008) and therefore developing ozone tolerant rice is essential to address this problem.

This study was conducted to develop a fast, reliable and reproducible screening method for ozone tolerance and determine the appropriate age of seedlings for screening.

MATERIALS AND METHODS

Test materials and growth conditions

Experiments were conducted between January - May 2009 at the Functional Crop Resource Development Division, Department of Functional Crop, National Institute of Crop Sciences, Rural Development Administration, Korea. Ten varieties with various level of tolerance to ozone were used (Table 1) arranged in RCBD with 4 replications. Seeds were soaked in water for 3 days and sown in a tray (60 × 30 × 3.5 cm) and twenty seedlings were maintained with soilless medium of steam sterilized sand with peat compost and vermiculite. Seedlings were grown in a naturally lit glasshouse. Temperature in the glasshouse was maintained at 30 - 32°C/ 22 - 24°C day/night through heating and venting at daytime and heating at night.

Ozone fumigation

Ozone was generated by a high voltage discharge generator and monitored with a high concentration ozone analyzer (IN, USA, Inc.). 15 and 30 day-old seedlings were exposed separately to 0.3 and 1 ppm ozone concentrations for 10 consecutive days. The temperature inside the ozone chamber was maintained at 25°C at 40 - 60% RH. The time of ozone fumigation was performed from 900 – 1600 h with a photoperiod of 700 – 1800 h every day until the end of treatment. Photosynthetic Photon Flux Density (PPFD) inside the chamber was 400 μmol $m^{-2}sec^{-1}$. The control plants were maintained in a free-ozone environment in the glasshouse. Cultural management of the seedlings was similar in all treatments.

Evaluation of ozone symptoms

A standard visual scoring system described by Sohn et al. (2002) with modification was used in evaluating ozone symptoms (Table 2). Scoring was done 10 days after ozone treatment with the tolerant and intolerant check varieties planted alongside with the test entries. By this time, most of the leaves of the susceptible checks showed discoloration to reddish brown and the tolerant checks with slight yellowing of narrow and small reddish spots on lower leaves (Figure 1). The control plants are kept in the ozone-free conditions in the glasshouse and compared with the treated plants (Figure 2). Five plants were sampled from each treatment in each replication and oven dried at 80°C to constant weight. Leaf bronzing score and shoot dry weight were taken for data analysis.

RESULTS

Leaf bronzing

The typical ozone damage can be observed in the rice leaves with yellowing and narrow reddish spots in the older leaves. As the ozone effect progresses, reddish browning of the older leaves becomes more severe and the younger leaves are affected until all leaves turn into brown and later death of the plant. In developing a fast ozone screening protocol, we subjected 15 and 30 day-

Table 2. Standard scoring system described by Sohn et al., 2002 with modification.

Score	Description	Resistance (Sohn et al., 2002)	Tolerance modified
1	No injury	Resistant	Highly tolerant
3	Slight yellowing of narrow and small reddish spots on lower leaves	Moderately resistant	Tolerant
5	Older and lower leaves discolored to reddish brown	Moderately susceptible	Moderately tolerant
7	Most leaves discolored to reddish brown	Susceptible	Susceptible
9	Dead	Susceptible	Highly susceptible

Figure 1. Typical visual reaction of plants to 1 ppm ozone concentration 10 days after treatment. Red-brown color of older leaves is the most common symptom and death on highly susceptible cultivars.

old seedlings for 10 days using 0.3 and 1.0 ppm ozone concentrations in a controlled chamber. Our results show that the tolerant, moderately tolerant and susceptible cultivars can be classified at 1 ppm using 15 day-old seedlings by the leaf bronzing score (LBS) (Table 3). In the 0.3 and 1 ppm ozone concentration with 30 day-old seedlings in a controlled chamber, it appeared that there was inconsistency in the performance of the standard

Figure 2. Control plants were grown in the ozone-free glasshouse and compared with the test plants 10 days after ozone treatment.

Table 3. Leaf bronzing score (LBS) of different varieties at various ozone concentrations and seedling ages after 10 days of treatment.

Variety	0.3 ppm 15days	0.3 ppm 30 days	1 ppm 15 days	1 ppm 30 days
Cheongcheong	3 (T)	5 (MT)	4 (T)	7 (S)
Nongan	5 (MT)	6 (S)	4 (T)	6 (S)
Borami	3 (T)	4 (T)	4 (T)	3 (T)
Chilbo	4 (T)	6 (S)	4 (T)	5 (MT)
Dongjin	3 (T)	3 (T)	4 (T)	3 (T)
Hanareum	5 (MT)	7 (S)	5 (MT)	6 (S)
Hwayeong	5 (MT)	5 (MT)	7 (S)	5 (MT)
Ilmee	4 (T)	5 (MT)	6 (S)	4 (T)
Chucheong	6 (S)	7 (S)	6 (S)	6 (S)
Milyang 175	4 (T)	6 (S)	7 (S)	7 (S)

Legend: LBS = 1, Highly tolerant (HT); 3, Tolerant (T); 5, Moderately tolerant (MT); 7, Susceptible (S); 9, Highly susceptible (HS).

resistant check varieties Cheongcheong (Tongil type) and Nongan (Japonica type) compared with the treatment at 1 ppm ozone using 15 day-old seedlings which gave consistent LBS. In the open-top chamber field experiments, Sohn and Lee (1997) described that varietal resistance to ozone was more easily classified in 21 - 35 day-old seedlings than in 14 day-old rice plants when subjected to 0.3 ppm ozone for 2 - 4h. Sohn et al. (2002) reported varietal reactions to ozone in an open chamber field experiments using 21 day-old seedling at 0.3 ppm ozone concentration. Hur et al. (2000) had similar ozone test using 30 day-old seedlings using a lower ozone concentration range (0.1 - 0.2 ppm) but with conflicting results (Table 1). The differences of these results (Table

Table 4. Reaction to ozone injury of the varieties tested at 1 ppm concentration on 15 day-old seedlings in comparison with the previous results.

Variety	Type	Reaction 0.3 ppm	Source	Reaction 1.0 ppm
Cheongcheong	Tongil	R (1)	Sohn et al., 2002	T (4)
Nongan	Japonica	R (1)	Sohn et al., 2002	T (4)
Borami	Japonica	-	-	T (4)
Chilbo	Japonica	-	-	T (4)
Dongjin	Japonica	MS (5)	Sohn et al., 2002	T (4)
		S	Hur et al., 2000	
Hanareum	Japonica	-	-	MT (5)
Hwayeong	Japonica	MS(5)	Sohn et al., 2002	S (7)
		MT	Hur et al., 2000	
Ilmee	Japonica	MS (5)	Sohn et al., 2002	S (6)
		T	Hur et al., 2000	
Chucheong	Japonica	S(7-9)	Sohn et al., 2002	S (6)
Milyang 175	Japonica	-	-	S (7)

Legend: R, resistant; MS, moderately susceptible; S, susceptible; T, tolerant; MT, moderately tolerant.

4) could be attributed to the ozone concentration, age of seedlings, duration of the treatment and growth conditions of the plants in the field and glasshouse.

Shoot biomass

Shoot dry weight of each genotype was measured as the weight of ozone-treated plants and compared with the control plants. Shoot dry weight was reduced in the ozone-treated 15 and 30 day-old seedlings compared with the untreated seedlings (Figures 3 and 4). Generally, higher shoot biomass reduction of the varieties was observed in younger seedlings at 0.3 and 1 ppm ozone concentration with 33 - 66% and 38 - 54%, respectively compared with older seedlings with 12 - 24% and 28 - 48% and both treatments significantly reduced the seedling dry weight after ozone treatment. Ozone treatment significantly reduces the total biomass per plant in rice (Kobayashi et al., 1995; Jin et al., 2001; Frei et al., 2008). However, correlation coefficient analysis indicated in that there was a low negative correlation between leaf bronzing score and shoot biomass (r = -0.01 to -.048). This observation was similar to results presented by Frei et al. (2008) that relative dry weight had no association with LBS.

The genotypes in this study differed in their response to ozone with some genotypes such as Hwayeong, Milyang 175 and Chucheong showing very strong leaf symptoms while others showing reduction in shoot biomass (Figure 5). The tolerant genotypes like Borami, Cheongcheong, Dongjin, Chilbo and Nongan also had significant shoot biomass reduction.

DISCUSSION AND CONCLUSION

Leaf browning is the most common visual symptom of ozone injury and it is a commonly used parameter in evaluating genotypes for ozone tolerance. There are however, conflicting results on the evaluation of tolerance levels of the varieties used in ozone experiments. This is because of the different conditions that are applied when conducting the experiment. In our study in the controlled test chamber, we used extremely high (that is, 1ppm) and moderately low (that is, 0.3 ppm) concentration of ozone with different age of seedlings (that is, 15 and 30 day-old). Our results indicated that various responses to ozone of the varieties tested were observed at different treatment conditions (Table 3) . Higher concentration at 1 ppm increases the sensitivity of cultivars to ozone. This could be used as reliable indications of relative ozone damages (Hur et al., 2000) as manifested by the magnitude of ozone damage to photosynthetic activity in the plant. In analyzing the consistency of the performance of the resistant and susceptible check varieties by the leaf bronzing score, genotype performance was more discriminating at 1 ppm ozone concentration in the younger seedlings than in the 0.3 ppm ozone concentration of both young and older seedlings. Thus, tolerant and susceptible varieties can be easily classified and evaluated using 1 ppm concentration. Direct seeding method and use of 15 day- old seedlings are considered most appropriate in order to reduce time of growing.

A high level of ozone concentration affects dry matter production and yield of rice (Kobayashi et al., 1995; Ishioh et al., 2005; Chen et al., 2008) . Shoot biomass was greatly reduced in all varieties after ozone treatment (Figures 3 and 4). Frei et al. (2009) reported that rice biomass was reduced by 24% when subjected to 0.120 ppm ozone for 18 days. Our results show that at much higher ozone concentration of 1 ppm on 15 day-old seedlings, higher reduction of biomass was observed from 38 - 54% during 10 days of ozone treatment (Figure

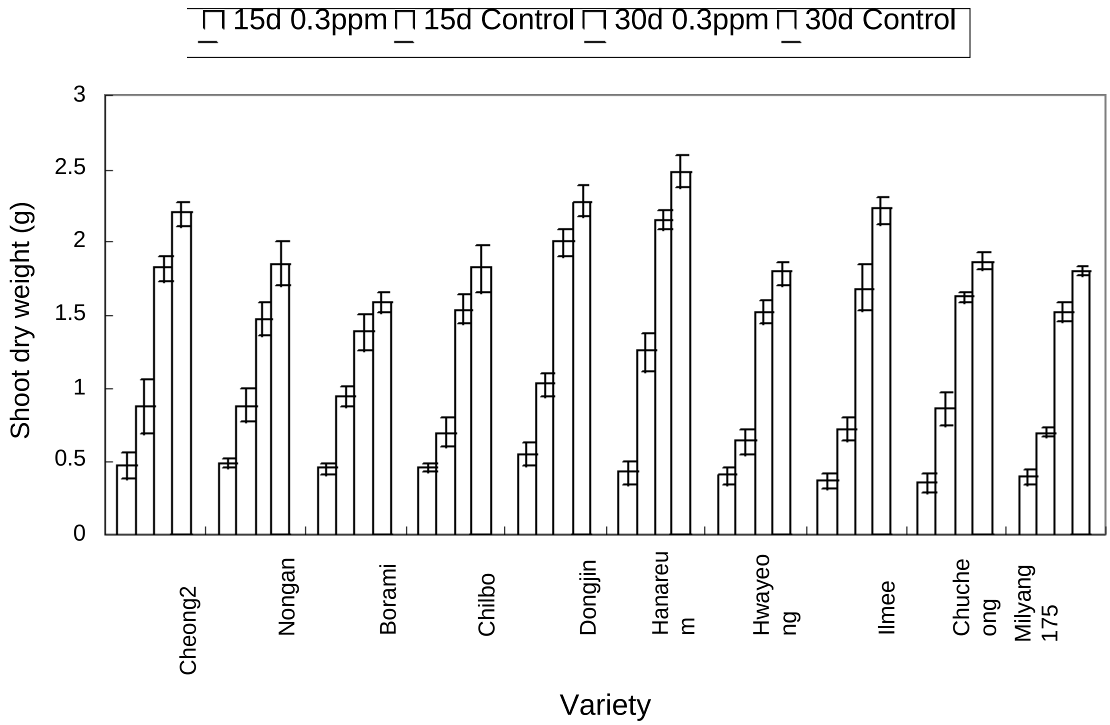

Figure 3. Shoot biomass of the 15 and 30 day-old seedlings (15 and 30 d) of different cultivars treated with 0.3 ppm ozone concentration for 10 days. The control plants were kept in an ozone-free environment. Vertical bars represent standard error of mean (n = 5).

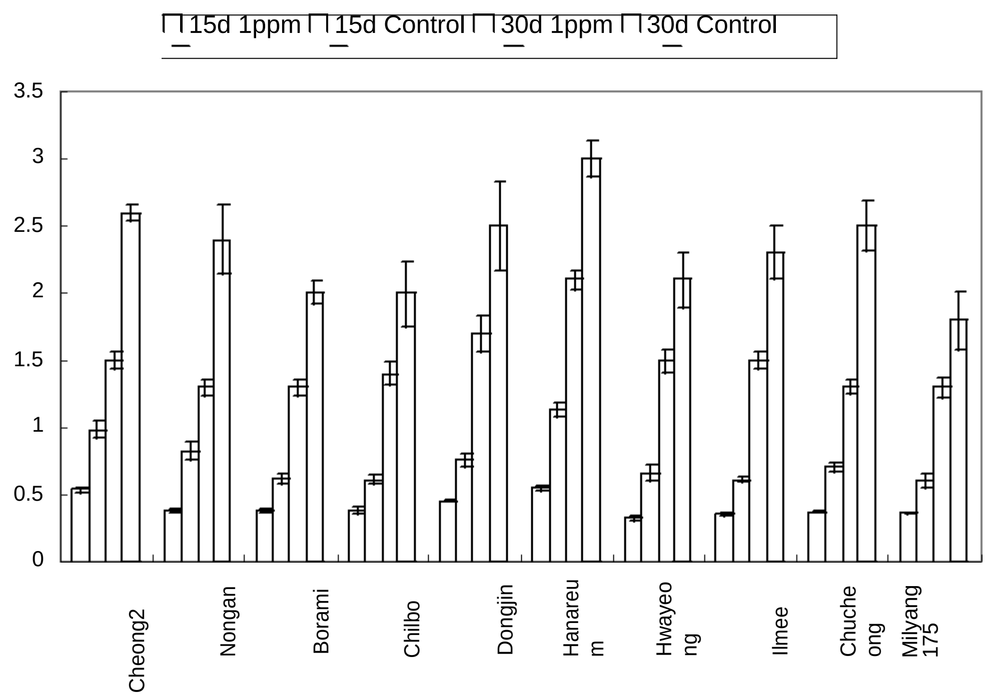

Figure 4. Shoot biomass of the 15 and 30 day-old seedlings (15 and 30 d) of different cultivars treated with 1 ppm ozone concentration for 10 days. The control plants were kept in an ozone-free environment. Vertical bars represent standard error of mean (n = 5).

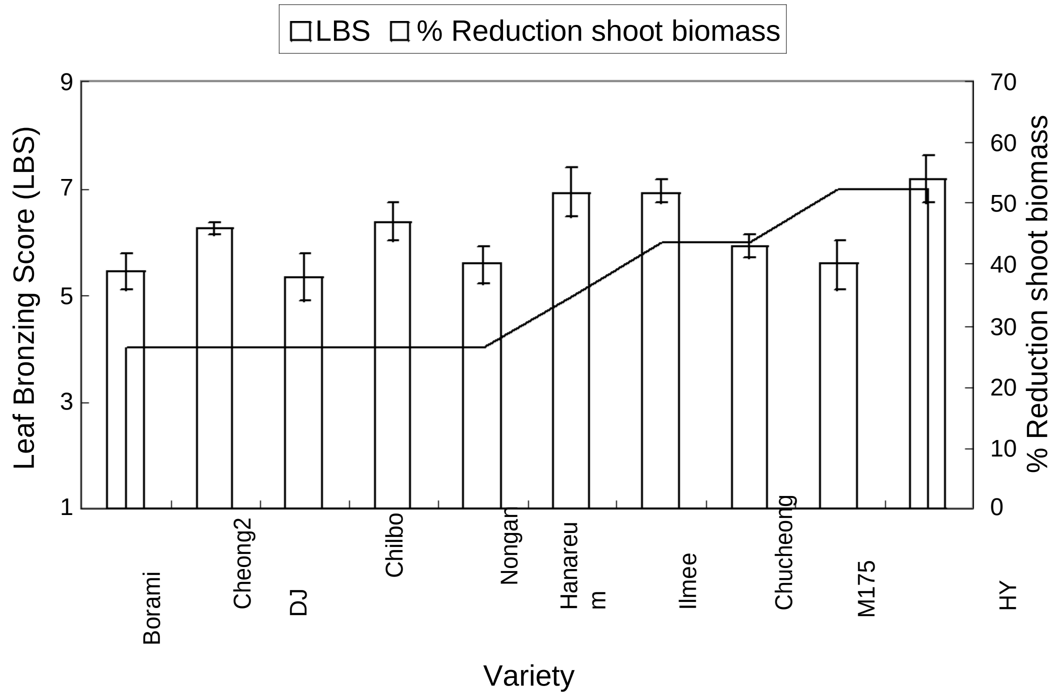

Figure 5. Effect of 10 day-exposure to 1 ppm ozone of 15 day-old seedlings of different genotypes, evaluated in terms of leaf bronzing and percentage shoot biomass reduction (shoot dry weight of the control relative to the treated plants).

5). This was attributed to the drying of older leaves as a result of ozone injury. Visible injury on the leaves can be easily detected after ozone treatment and thus photosynthetic activities in the plant are affected. Hur et al. (2000) described that photosynthetic activity is one of the sensitive responses, for which the chlorophyll pigment and leaf greenness can together provide reliable index of ozone injury. However, shoot biomass was not associated with the leaf bronzing score as shown by their negative correlation. This similar finding has been reported in ozone (Frei et al., 2008) and zinc deficiency (Wissuwa et al., 2006) experiments but leaf bronzing is still the generally accepted criterion in assessing tolerance to several abiotic stresses in crops (Frei et al., 2008). Other parameters like biochemical, enzymological and physiological aspects in assessing ozone tolerance will also be very useful to explain the mechanisms and biochemical pathways that are involved when plants are subjected to ozone stress. This however, was the limitation of the study. Here, we developed a faster way by which we can easily evaluate the symptoms of ozone stress specifically in rice seedlings and use the method to select breeding materials in a short period of time. Removal of susceptible genotypes in the early or late segregating population facilitates the selection process for a complex trait like ozone tolerance.

With the current concerns on stress- related ozone injury to rice production and increasing ground level ozone, there is a need to screen genotypic variation on O_3 sensitivity under controlled chamber condition or in open-field environments. Developing ozone tolerant rice is therefore essential to prevent yield losses in the future (Frei et al., 2008). However, there are many limitations in the conduct of experiments under open- field in terms of resources, manpower, space and time. A more advantageous screening technique was performed in this study that is fast and reliable especially when dealing with large population of genotypes and requires less amount of time in getting favorable results. Previous result also indicated that multiple loci were involved in the resistance of rice plants to O_3 stress and narrow heritability is low, hence it is more difficult to improve the trait by selection (Sohn et al., 2002). In this case, fast and reliable screening method becomes important and it can be done at early and late generation of breeding materials.

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