Exploring ethylenediurea (EDU) as an ozone biomonitoring and screening tool for rice (*Oryza sativa* L.)

Dissertation

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Contents

1 Summary

1.1 Summary

Tropospheric ozone concentrations are rapidly rising in many developing parts of the world, thereby threatening food security. Therefore, breeding for adapting rice varieties is essential in Asia, especially in the Indian subcontinent, to cope with ozone stress and to secure staple food supply. Genotypic variation can play a key role in successful ozone tolerance/resistance breeding. However, ozone impact evaluation on crops and field screening experiments in these highly ozone affected areas are scarce, as the establishment of field-based ozone fumigation facilities can be technically challenging or very expensive. Alternatively, ethylenediurea (EDU) has been proposed as a chemical applied as a foliar spray to assess the effects of ozone in large-scale field experiments. However, little has been known about its confounding effects on rice in the absence of ozone, and the protection mechanisms against ozone phytotoxic effects. Therefore, a series of agronomic and physiological experiments were performed including transcriptomics (RNA-Seq) and scanning electron microscopy (SEM), to test the suitability and feasibility of EDU as a future ozone biomonitoring tool for field crops. Four different treatments, (i) control (below the damage threshold level, 40 ppb), (ii) control+EDU, (iii) ozone (average 77 to 108 ppb for 7 h day^{-1}), and ozone+EDU were assigned to rice genotypes ranked *a priori* in terms of ozone tolerance. Application of EDU did not affect plants in the absence of ozone, but it alleviated negative effects of ozone on plant morphology, leaf symptom formation, spectral reflectance indices such as the normalized difference vegetation index (NDVI), SPAD value, lipid peroxidation, photosynthetic parameters, panicle number, spikelet sterility as well as biomass and grain yields in the sensitive genotypes. RNA-sequencing and SEM were conducted using the Bangladeshi high yielding rice variety BR28 which was highly affected by ozone (37 percent grain yield reductions) and showed consistent recovery by EDU applications. Transcriptome profiling revealed that several thousand genes responded to ozone treatment, but almost none responded to EDU application. The dominant trend of significant interactions between ozone treatment and EDU application for the ozone responsive genes was the ozone mediated up-regulation mitigated by EDU application. These transcriptional patterns suggested that EDU did not enhance stress defense pathways in plants, but rather acted as a surface protectant against upstream physiological stress reactions. Additional experiments indicated that EDU might have ozone degrading properties due to abiotic chemical interactions between ozone and EDU. Further, SEM image analyses displayed the presence of EDU deposits on treated leaf surfaces. We further demonstrated that EDU application did not alleviate the reaction of plants to a number of other abiotic stresses *i.e.* iron toxicity, zinc deficiency and salinity. In conclusion, EDU is a surface protectant that specifically mitigates ozone stress without interfering with the plants' stress response systems. These properties, together with its ease of application, make it very suitable for biomonitoring and screening studies of ozone damage to field crops in developing countries.

1.2   Zusammenfassung

Die troposphärischen Ozonkonzentrationen steigen in vielen Entwicklungsländern rapide an und bedrohen damit die Ernährungssicherheit. Deshalb ist die Züchtung zur Anpassung von Reissorten in Asien, insbesondere auf dem indischen Subkontinent, unerlässlich, um dem Ozonstress zu begegnen und die Versorgung mit Grundnahrungsmitteln zu sichern. Genotypische Variationen können eine Schlüsselrolle für eine erfolgreiche Ozonverträglichkeits-/Widerstandszüchtung spielen. Allerdings sind Bewertung der Ozonauswirkungen auf Pflanzen und Screening-Experimente in diesen stark ozonbelasteten Gebieten selten, da der Aufbau von feldbasierten Ozonbegasungsanlagen technisch anspruchsvoll oder sehr teuer sein kann. Alternativ wurde Ethylendiurea (EDU) als Chemikalie vorgeschlagen, die als Blattspray eingesetzt wird, um die Auswirkungen von Ozon in groß angelegten Feldversuchen zu beurteilen. Über seine Wirkung auf Reis in Abwesenheit von Ozon und die Schutzmechanismen gegen phytotoxische Wirkungen von Ozon ist jedoch wenig bekannt. Daher wurden eine Reihe von agronomischen und physiologischen Experimenten durchgeführt, darunter transkriptomische Analytik (RNA-Seq) und Rasterelektronenmikroskopie (REM), um die Eignung von EDU als zukünftiges Ozon-Biomonitoring-Tool für Feldkulturen zu testen. Vier verschiedene Behandlungen, (i) Kontrolle (unterhalb der Schadschwelle, 40 ppb), (ii) Kontrolle+EDU, (iii) Ozon (durchschnittlich 77 bis 108 ppb für 7 h pro Tag) und Ozon+EDU wurden bei Reisgenotypen getestet, die in Bezug auf die Ozonverträglichkeit *a priori* eingestuft wurden. Die Anwendung von EDU wirkte sich in Abwesenheit von Ozon nicht auf Pflanzen aus, aber sie milderte negative Auswirkungen von Ozon auf die Pflanzenmorphologie, die Bildung von Blattsymptomen, spektrale Reflexionsindizes wie den normierten differenzierten Vegetationsindex (NDVI), den SPAD-Wert, die Lipidperoxidation, photosynthetische Parameter, die Rispenzahl, die Sterilität der Ährchen, sowie die Biomasse- und Getreideerträge in den empfindlichen Genotypen. Die RNA-Sequenzierung und die REM wurden mit der bangladeschischen Hochertragsreissorte BR28 durchgeführt, die stark von Ozon betroffen war (37 Prozent Ertragsminderung) und eine fortlaufende Regenerieung durch die EDU-Anwendungen zeigte. Die Transkriptomprofilierung ergab, dass mehrere tausend Gene auf die Ozonbehandlung ansprachen, aber fast keines reagierte auf die EDU-Anwendung. Der Trend, welcher sich bei der signifikanten Wechselwirkung zwischen der Ozonbehandlung und der EDU-Anwendung abzeichnete, erfolgte auf Grund einer durch Ozon induzierten Hochregulierung der auf Ozon reagierenden Gene, welche durch die EDU-Anwendung abgeschwächt wurde. Diese Transkriptionsmuster deuteten darauf hin, dass EDU die Stoffwechselwege der Stressabwehr in den getesteten Pflanzen nicht direkt beeinflusst, sondern als Oberflächenschutz gegen physiologische Stressreaktionen wirkt. Zusätzliche Experimente zeigten, dass EDU aufgrund von abiotisch-chemischer Wechselwirkungen zwischen Ozon und EDU ozonabbauende Eigenschaften aufweisen könnte. Darüber hinaus zeigten REM-Bildanalysen das Vorhandensein von EDU-Ablagerungen auf behandelten Blattoberflächen. Wir konnten weiterhin zeigen, dass die EDU-Anwendung die Reaktion der Pflanzen auf eine Reihe anderer abiotischer Stresse wie Eisentoxizität, Zinkmangel und Salzstress nicht verringert. Zusammenfassend lässt sich sagen, dass EDU ein Oberflächenschutzmittel ist, das gezielt den Ozonstress mildert, ohne die Stressreaktionssysteme der Pflanzen direkt zu beeinflussen. Diese Eigenschaften und die einfache Anwendung, sorgen dafür, dass EDU für Biomonitoring und Screening-Studien von Ozonschäden an Feldkulturen in Entwicklungsländern sehr gut geeignet ist.

2 Introduction

2.1 Tropospheric ozone: a major threat to global crop production

Tropospheric ozone is one the most important environmental pollutants that is currently having an adverse effect on vegetation, human health, and agricultural crop production (Ainsworth et al., 2012; Li et al., 2017; Mills et al., 2018). This secondary air pollutant is formed through photochemical reactions between primary air pollutants such as nitrogen oxides (NOx), volatile organic compounds (VOCs), carbon monoxide (CO), and methane (CH_4), which are known as ozone precursor gases (Figure 1.1; The Royal Society, 2008). A wide range of ozone precursor gas sources are natural or the result of human activities like energy generation, transportation, fossil-fuel combustion, industrialization, urbanization, deforestation, rapid population, and economic growth (Brauer et al., 2016; Cho et al., 2011; IPCC, 2014). The phytotoxic effect of high tropospheric ozone on different crop species is well documented by several scientists, and clearly indicates the remarkable crop yield loss globally. For instance, annual yield reduction of ca. 13% in soybean, 7% in wheat, 5% in rice, and 6% in maize (Mills et al., 2018), resulting in an annual economic loss of US$ 14–26 billion (Van Dingenen et al., 2009) are estimated due to ozone pollution using global crop models. Ozone damage occurs in plants directly through oxidative stress and indirectly through its role as a major greenhouse gas (Ainsworth, 2017). It is a highly reactive molecule and degrades rapidly into various reactive oxygen species (ROS) after entering the leaf apoplast primarily through stomata (Krasensky et al., 2017). The ROS include singlet oxygen ($^1O_2$), hydrogen per oxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals ($^{\cdot}OH$); which promote oxidative burst and ultimately lead to cell death (Ainsworth et al., 2017). These ROS can also interfere with various enzymatic processes, which ultimately produce visible necrotic symptoms on leaves and causes damage to membrane lipids (Kangasjärvi et al., 2005). Therefore, elevated tropospheric ozone directly affects photosynthetic carbon assimilation, stomatal conductance, and reduces crop yields, and quality (Ainsworth et al., 2012; Emberson et al., 2018).
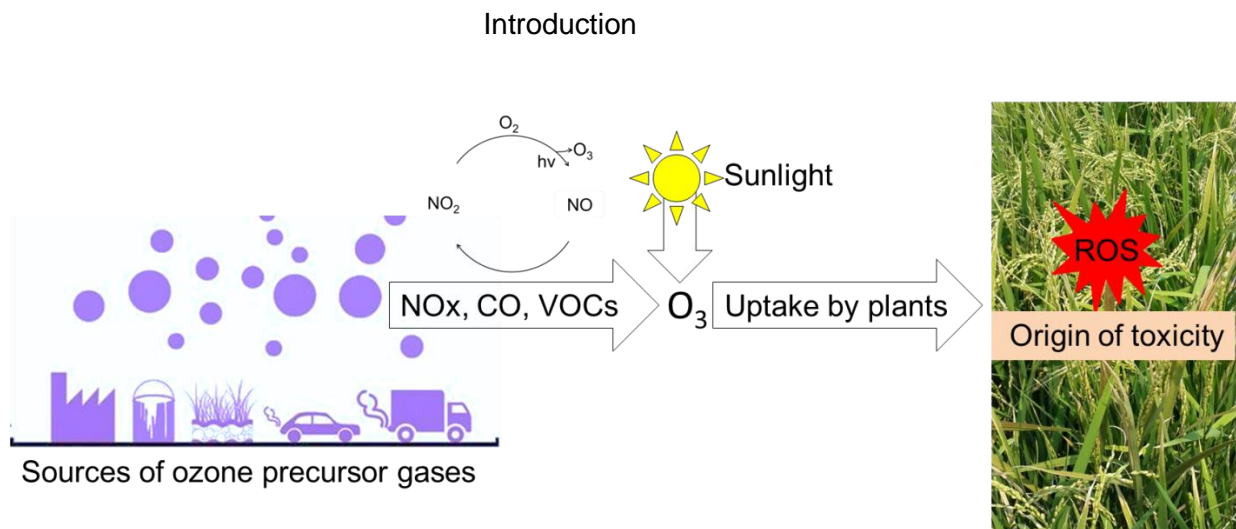
Figure 1.1 A simplified view of the major sources and formation of tropospheric ozone. NOx, nitrogen oxides; CO, carbon monoxide; VOCs, volatile organic compounds; O_3, ozone; ROS, reactive oxygen species (adapted from The Royal Society (2008) and http://www.ccacoalition.org/ru/slcps/tropospheric-ozone).

2.2   Emergence and distribution of tropospheric ozone

Ozone is mainly evolved in two phases of the Earth's atmosphere; the stratosphere and troposphere. Stratospheric ozone (which comprises of ca. 90% of total ozone) lies between 10 and 17 km above the Earth's surface and extends up to ca. 50 km, which is commonly known as the ozone layer (WMO, 2014). The remaining ozone in the lower atmospheric region is the tropospheric or ground-level ozone. The ozone layer in the stratosphere plays an important and beneficial role by absorbing most of the harmful ultraviolet rays (UV-B) coming from the solar radiation (Donahue, 2011). In contrast, elevated tropospheric ozone has been shown to be harmful to human health, vegetation and crop production (Ashmore et al., 2006; Dentener et al., 2006; Fuhrer et al., 2016). While ozone concentration in the stratosphere is depleting, tropospheric ozone is increasing rapidly due to higher emissions of its precursor gases as well as global warming (Ashmore, 2005; Tai and Val Martin, 2017). Hot sunny weather can accelerate the formation of ozone, and therefore, tropical regions are at high risk of pollution due to its favorable environmental conditions and high emissions of precursor gases (Jain et al., 2005; Tiwari et al., 2008). However, crop damage caused by air pollution was first reported in the USA in 1940s (Middleton et al., 1950), and the impacts of tropospheric ozone pollution was first recognized in California during the 1950s as the cause of 'weather fleck' in tobacco (Haagen-Smit, 1952). The damage symptom of the plant was further reproduced in the laboratory by

the reaction of organic trace gases or car exhaust with nitrogen oxides (NOx) in presence of sunlight (Haagen-Smit and Fox, 1954; Heggestad and Middleton, 1959). However, increasing tropospheric ozone concentrations and its damaging effects on plants and vegetation in other parts of North America, Europe and Japan were gradually reported by 1970s (The Royal Society, 2008).

Several projections indicated that in the past few decades, rising tropospheric ozone concentrations were controlled by adopting various air-quality regulations in the developed countries such as in Europe (Chang et al., 2017). In contrast, the ozone pollution scenarios are rather severe in developing countries, especially rice producing countries in Asia which are currently marked as ozone hotspot (Mills et al., 2018). The pollution scenario will be exacerbated in the 21st century because of rapid economic and population growth, higher emission of precursor gases and lack of air-quality regulations (Brauer et al., 2016; Maas and Grennfelt, 2016). During the summer season, high tropospheric ozone concentrations (ca. 100 ppb), far above the damage threshold level (40 ppb) have been reported in many parts of China, India, Pakistan, and Bangladesh (Brauer et al., 2016; Deb Roy et al., 2009; Ran et al., 2009). However, ozone concentrations less than 40 ppb can also have adverse effects on sensitive vegetation (Agathokleous et al., 2015; Sugai et al., 2018). Therefore, several ozone phytotoxicity metrics mainly based on ozone exposure or the accumulated stomatal ozone flux have been proposed and used so far globally (Musselman et al., 2006; Pleijel et al., 2004); AOT40, SUM06, and W126 are the ozone exposure based metrics, and the flux based available metrics are DO$_3$SE, AFstY, and POD$_Y$ (CLRTAP, 2017; Emberson et al., 2001; Karlsson et al., 2004; Wang and Mauzerall, 2004). Nevertheless, ozone risk assessment based on modelled stomatal uptake or flux provides a stronger indication of ozone effects in Europe compared to those based on concentrations (Mills et al., 2011; Pleijel et al., 2004; Simpson et al., 2007). However, AOT40 (accumulated ozone exposure over a threshold of 40 ppb) is the most widely reported critical index and is adopted by several regulatory agencies for its simple calculation method and suitability for highly polluted regions (Agathokleous et al., 2018a). Changes in daily maximum ozone concentration were estimated to be highest in Bangladesh, India, and Pakistan (ca. 20%) compared with global (ca. 9%) between 1990 and 2013 (Figure 1.2). On the other hand, China is the largest ozone precursor gases (NOx) emitter in Asia (Feng

et al, 2015). Therefore, it is obvious that crops and vegetation are endangered by current tropospheric ozone concentrations in many parts of the world especially in Asia and will be the major menace in the near future due to its rising trends.
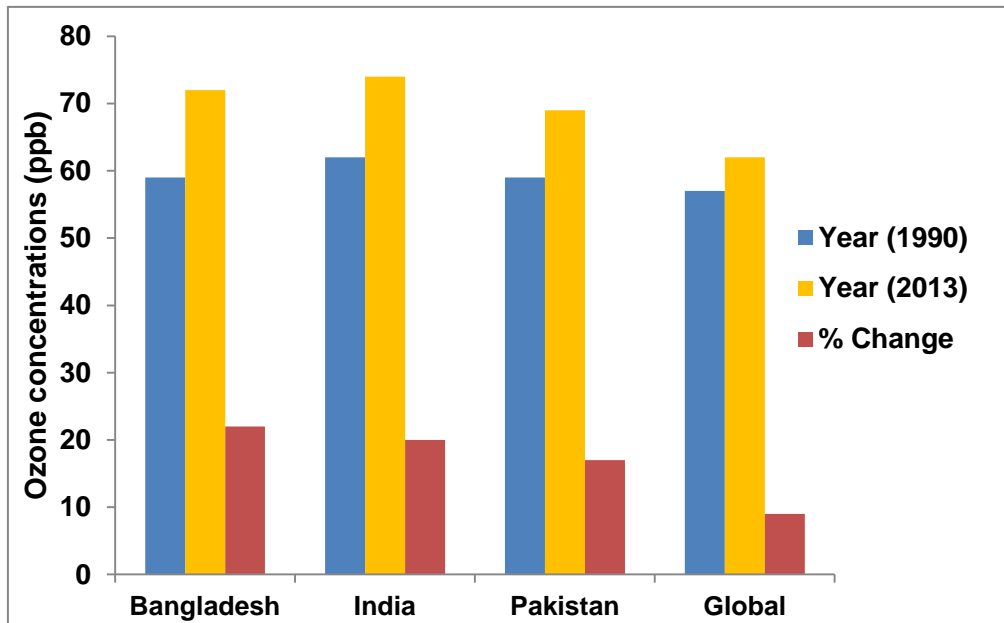
Figure 1.2 Changes of average 1 h daily maximum ozone (ppb) concentrations between the year of 1990 and 2013 (adapted from Brauer et al. (2016).

2.3 Rice: staple food crop of half of the global population

Rice (*Oryza sativa* L.) is the most widely grown and consumed food crop in Asia (McCouch et al., 2016). It is a diploid species (n=12) and a self-pollinated crop. Rice is classified in the genus *Oryza* and belongs to the grass (poaceae) family. Two different types of cultivated rice are available globally; *Oryza sativa* which is grown and popular in Asia, also known as Asian rice, and another *Oryza glaberrima*, grown in several areas in Africa, also known as African rice. The genus *Oryza* has several wild relatives, among them *O. rufipogon, O. nivara and O. barthii are* important (IRRI Ricepedia, 2018). Several studies revealed that Asian rice was domesticated ca. 10,000 years ago in the form of its ancestor wild rice *O. rufipogon* in China, whereas African rice ca. 3000 years ago from the ancestor *O. barthii* along the river Niger (Huang et al., 2012; Kovach et al., 2007; Wang et al., 2014). *O. sativa* has two popular cultivated sub species, *O. sativa spp. japonica* (sticky, short-grained) and *O. sativa spp. indica* (nonsticky, long-grained)*.* Ancient *japonica* sub species was first domesticated in South China near the Pearl River (Huang et al., 2012). On the other

hand, *indica* was developed and domesticated from the crosses between *japonica* and wild relatives in South and Southeast Asia as it is genetically intermediate between cultivated rice and wild rice from South China (Huang et al., 2012). The rice genome size (*O. sativa spp. japonica cv.* Nipponbare) is ca. 430 Mb and full sequences are publicly available through the rice annotation project database (RAP-DB, http://rapdb.dna.affrc.go.jp/) and rice genome annotation project (RGAP, http://rice.plantbiology.msu.edu/). In addition, the largest genome sequence databases for ca. 3000 rice accessions are publicly available in the International Rice Genebank Collection in IRRI (Li et al., 2014; Wang et al., 2018). Therefore, rice is suitable for any genetic modifications and used as a model plant for the study of cereal crops biology.

Rice is a carbohydrate-rich food (80% by weight) crop which also contains other nutrients, such as in 100 gm of rice contents 7.13 gm protein, 0.12 gm sugar, 2.88 mg vitamin B complex, 28 mg calcium and 115 mg of both phosphorus and potassium (IRRI Ricepedia, 2018; USDA, 2018). There are mainly three phases in the rice life cycle *i.e.* vegetative, reproductive and ripening. The duration from seed to maturity varies among the varieties, and generally lies between 80 and 180 days (IRRI Ricepedia, 2018). Rice is ranked the third highest (ca. 750 million tonnes) globally produced agricultural commodity after maize and sugarcane (FOASTAT, 2018). The production (ca. 90% of the global rice) and

Figure: Rice plant (derived from http://www.ricehub.org

consumption is dominated by Asian countries. Among them, China is the leading rice producing country followed by India (2nd) and Bangladesh (4th), and interestingly, these three countries along with Pakistan produced more than half (ca. 60%) of the global rice (Figure 1.3 (a) and (b), FAOSTAT, 2018).
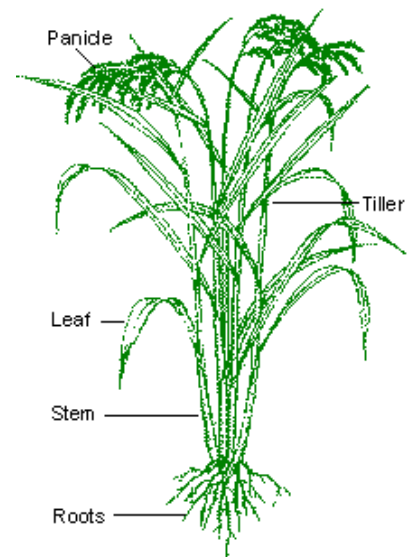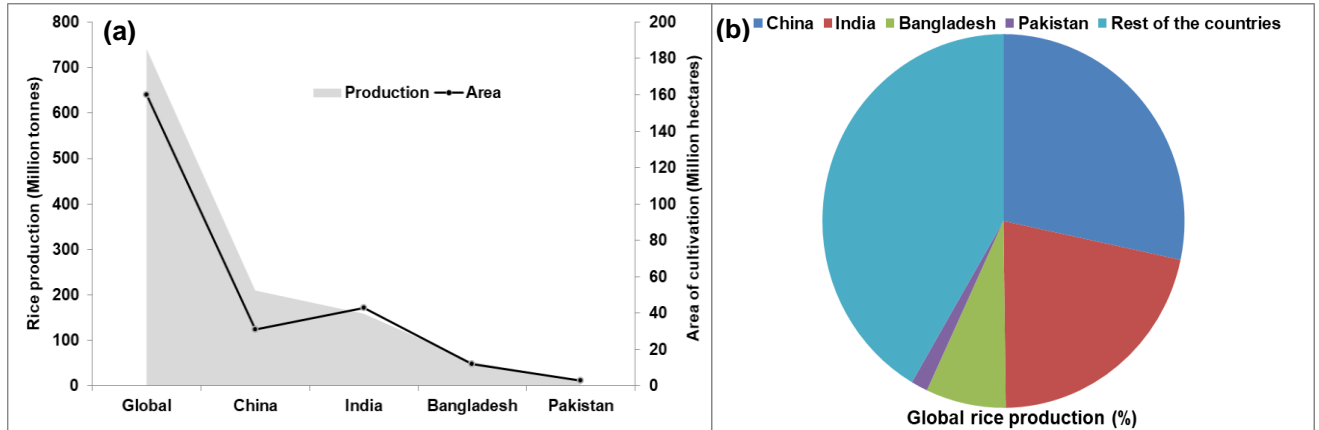
Figure 1.3 Rice production and cultivation scenario of leading Asian countries. (a) The plot area shows rice production in million tonnes and cultivated area in million hectares. (b) Chart showing % rice production by leading Asian countries compared to global. The chart is derived from global crop production databases of Food and Agricultural Organization (FAOSTAT (2018).

2.4 Tropospheric ozone: major constraint of Asian rice production

Elevated levels of ozone have detrimental effects on plant physiological, and genetic factors, affecting numerous metabolic pathways, which leads to adverse results such as reduced photosynthesis, cell death, inhibited plant growth and development, decreased stomatal conductance, accelerated senescence, and altered antioxidant system (Feng and Kobayashi, 2009; Frei et al., 2010; Ueda et al., 2015; Wilkinson et al., 2012). Moreover, numerous studies that are mostly conducted in a controlled environment, have extensively documented the phytotoxic effects of ozone on rice growth, development, yield, and quality (Ainsworth, 2008; Frei, 2015). It is projected that global ozone concentrations already reached ca. 50 ppb in 2000 (Fiscus et al., 2005). On the other hand, a meta-analysis estimated that ca. 18 percent rice yield reduction may occur with 31 to 50 ppb of ozone, which will further result in a 10% additional loss of 51 to 75 ppb (Feng and Kobayashi, 2009). It is also estimated that more than 10% of regional loss (Ainsworth, 2008; Van Dingenen et al., 2009), as well as ca. 20% of East Asian (Chen et al., 2011) and ca. 15% of Indian (Debaje, 2014; Mills et al., 2018), rice yield damage is caused by ozone pollution each year. On the other hand, rice yield reduction by ca. 50 percent was reported in several controlled experiments due to the adverse effects of ozone on yield-contributing components

compared to control (Akhtar et al., 2010; Rai et al., 2010; Sawada and Kohno, 2009). For instance, lower number of spikelets per panicle and individual grain mass (Frei et al., 2012; Wang et al., 2012), higher spikelet sterility (Yamaguchi et al., 2014) and lower number of tiller (Akhtar et al., 2010; Frei et al., 2008). In addition, a recent modeling study determined the total annual rice production loss in India to be 6.7 million tonnes (corresponding to 6.3 percent) due to ozone pollution, in which the highest reduction (2.6 million tonnes) was estimated in the eastern part, considering the average yield between the year 2011 and 2014 (Lal et al., 2017). Therefore, rising tropospheric ozone is one of the major threats for growing rice in Asia.
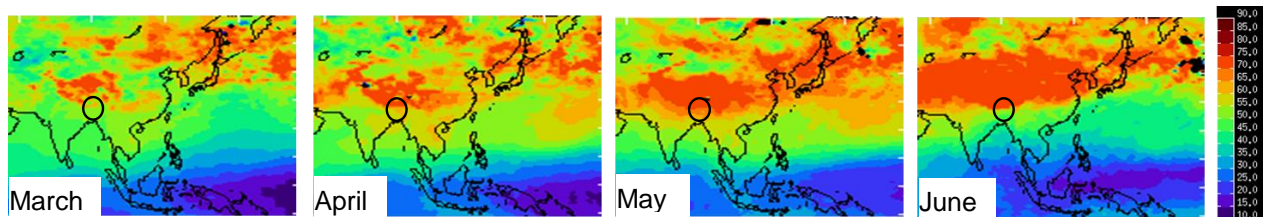
Figure 1.4 Monthly average tropospheric ozone volume mixing ratio in Asia in the year of 2016 (peak ozone periods (March to June) in Indian subcontinents). The color scale indicates the density of ozone divided by the density of all constituents in a unit volume of air (ppbv) in the total tropospheric column. Monthly mean maps were derived and adapted from Ziemke et al. (2006). Maps were then compiled and modified in August 2018 from http://acd-ext.gsfc.nasa.gov/Data_services/cloud_slice/ Black circle indicates Bangladesh as a representative of the Indian subcontinent developing country.

From the different projections, it is clear that current rice production in South Asia especially in the developing countries of Indian subcontinent such as India, Bangladesh, Pakistan, Myanmar, and Nepal, is already severely affected by elevated ozone stress (Frei, 2015; Lal et al., 2017). Ozone levels will rise further until the middle of the 21st century due to the higher emissions of precursor gases, favorable environmental conditions for ozone formation and lack of regulations (Lei et al., 2012; Williams et al., 2014). 'Business as usual' scenarios also suggest that tropospheric ozone will severely increase, particularly in the Indian subcontinent (Pozzer et al., 2012; Van Dingenen et al., 2009). The peak ozone episodes of the Indian subcontinent, especially in Bangladesh occurred during the hot and humid summer between the months of March and June (Figure 1.4; Ziemke et al., 2006). Besides

this, rice is produced almost all year round during two major seasons; the wet season (June to November) generally known as Aman rice, in which high ozone level can overlap with the crucial early vegetative stages including tillering. The other is dry season (December to May) called Boro rice, in which reproductive growth stages (flowering and grain filling) can encounter with high ozone episode (Frei, 2015). Therefore, both rice growing seasons in these regions are at high risk, as different growth phases of rice cultivation encounter and overlap with peak ozone episodes; this represents a critical threat for rice yield and production (Frei, 2015). However, field-based ozone damage evaluation is still lacking in these highly ozone prone areas. Therefore, it is of paramount importance to develop and/or validate research tools to monitor and evaluate ozone induced damage in crops and vegetation.

2.5   Ozone impacts evaluation systems

Adverse effects of ozone on plants and vegetation are mainly evaluated through sun-lit plant growth chamber, open top chambers (OTC), and free air concentration enrichment with ozone (FACE-O_3), experimental systems so far (Kobayashi, 2015). Sun-lit are environmentally controlled chambers in which ozone concentration can be maintained at desired levels for the purpose of better understanding of the mechanism of ozone damage at smaller scale (Kobayashi 2015). The OTC, first introduced in the early 1970s by Heagle et al. (1973), are the most widely used environmentally controlled system in which ozone concentration is maintained at a desired level, with artificially generated air blowing with ozone into the chamber compared to control (Figure 1.5 (a); Frei, 2015). FACE is another promising and more realistic; a chamber-less system for field conditions at which air is enriched with elevated ozone and is released into the wind through rings of pipes just above the canopy at a certain increased level compared with the ambient ozone concentration (Figure 1.5 (b); Morgan et al. 2004). Nevertheless, both OTC and FACE-ozone systems have a number of limitations. For example, OTC may interfere in the ozone impacts by modifying the microclimate (especially, temperature, humidity, CO_2 concentration) due to 'chamber effects' which can differ from the actual field conditions (Piikki et al., 2008; Kobayashi, 2015). Moreover, it is small in dimensions and usually not suitable for larger scale applications (Macháčová, 2010). On the other hand, FACE–ozone offers a unique opportunity to screen large numbers of genotype

in field conditions but it can only compare between current ambient and elevated ozone concentrations (Ainsworth et al., 2014; Pleijel, 2011). In addition, very few studies have been conducted so far using FACE as only three FACE-ozone system are available globally, mainly in the USA for soybean (Morgan et al., 2004), in China for rice and wheat (Shi et al., 2009; Tang et al., 2011), and in Italy for tree species (Paoletti and Carriero, 2016). Moreover, crop sensitivity towards ozone may differ between OTC and FACE systems (Feng et al., 2018). However, both systems require infrastructure, continuous electricity supply and are expensive, technically challenging and laborious to maintain. Therefore, they are not easy to use and establish in many areas of the highly ozone affected developing countries in the Indian subcontinent (Kobayashi, 2015; Oksanen et al., 2013).
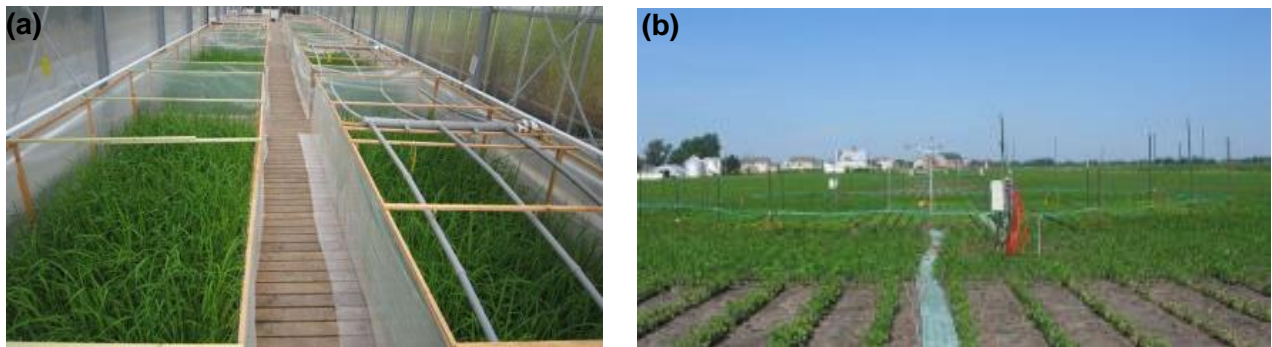
Figure 1.5 Photographs of open top chambers (OTC) and free air concentration enrichment (FACE) experimental system. (a) OTC (source: collected from Dr. Michael Frei), (b) FACE (source: Ainsworth (2017).

As an alternative of ozone biomonitoring tools, several chemicals have been tested so far (Saitanis et al., 2015). Among them, ethylenediurea (N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea), abbreviated as EDU), is the most widely studied and long established chemical compound to evaluate ozone impacts on different plants and crop species (Figure 1.6; Feng et al., 2010; Manning et al., 2011; Paoletti et al., 2009). EDU was first introduced by Carnahan et al. in 1978 and successfully used to protect bean plants against ozone induced visible leaf injury. After that, numerous studies reported and confirmed the EDU-mediated protection against ozone in a number of plant species, specifically in sensitive genotypes. The applications of EDU are mainly carried out through spraying and soil drenching, but stem injections are also reported in few cases (Manning et al., 2011; Paoletti et al.,

2007). However, the actual mode of action and prevention mechanisms against ozone induced phytotoxic effect is still unclear (Agathokleous, 2017; Tiwari, 2017). Two possible hypotheses are discussed so far regarding the EDU mediated protection; EDU may facilitate to scavenge ozone detrimental activity through a direct effect on physiological parameters, or by an antioxidant mediated defense system (Manning et al., 2011; Pandey et al., 2015).
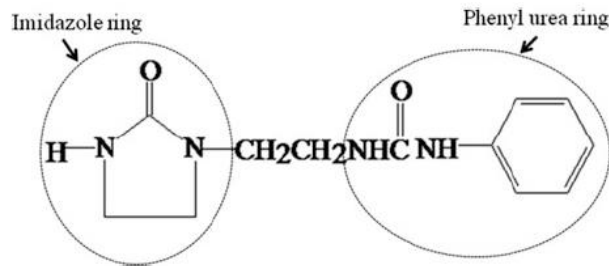
Figure 1.6 Structural formula of ethylenediurea (EDU), chemical formula $C_4H_{10}N_4O_2$ (derived from Singh et al. (2015).

To our knowledge, EDU studies in controlled conditions and in-depth physiological studies including transcriptomics approaches are lacking. Therefore, it is essential to investigate the constitutive effects of EDU *i.e.* whether EDU has any growth regulating or promoting activities in the absence of ozone as it contains ca. 22% nitrogen (Godzik and Manning, 1998). On the other hand, a number of studies have explored the transcriptomic responses of plants to ozone through microarrays and RNA-sequencing. In a comparative microarray study using two chromosome segment substitution rice lines, genes involved in programmed cell death (ethylene or jasmonic acid biosynthesis, mitogen-activated protein (MAP) kinase and disease resistance, and the detoxification of ROS (catalase and peroxidase) were investigated and identified as potential ozone responsive genes (Frei et al., 2010). Moreover, a number of genes are validated and declared as ozone responsive with the help of high-throughput sequencing technique (RNA-Seq). For instance, genes associated with photosynthesis and respiration, oxidative stress, defense response, protein ubiquitination and organic acid biosynthesis in soybean (Waldeck et al., 2017), cellular integrity and metabolism (ascorbate-glutathione metabolism, hormone, wax and cutin biosynthesis) in pak choi (Zhang et al., 2017) and antioxidant metabolism (ascorbate-glutathione cycle genes) in legume crops (Yendrek et al., 2015).

Therefore, it is obvious that large numbers of genes are ozone responsive in different plant species. In addition, next generation deep sequencing techniques (e.g., RNA-Seq) offer unique opportunities to reveal unbiased, highly reproducible and a far more precise measurement of whole genome transcripts and have a large dynamic range compared to the other closest sequencing platforms such as microarrays (Hurd and Nelson, 2009; Wang et al., 2009). Thus, RNA sequencing provides an excellent tool to explore global physiological responses to external stimuli such as ozone, EDU application, and their interaction. Taken together, we designed and performed a series of experiments including in-depth physiological, biochemical, transcriptome (RNA-sequencing), scanning electron microscopy (SEM) and other related approaches including four different treatments *i.e.* control, control+EDU, ozone and ozone+EDU. For these purposes, we used *a priori* ranked different contrasting ozone responsive rice genotypes. We also performed an additional experiment to investigate the specificity of EDU towards different stresses *i.e.* salinity, iron toxicity, and zinc deficiency. To this end, all conducted experiments were primarily aimed at investigating and validating the suitability of EDU as an ozone biomonitoring tool through its functional characterization.

2.6   Aims of this study

This study mainly aimed to test the suitability and feasibility of EDU as a future ozone biomonitoring tool for rice specifically for the highly ozone affected developing countries in Indian subcontinent, which has a lack of conventional ozone experimental facilities such as OTC and FACE-O_3.

In detail, the following objectives were investigated

1. Differential responses of rice towards ethylenediurea (EDU) under ozone stress
   1.1 Assessment of confounding effects of EDU in absence of ozone
   1.2 Evaluation of the suitability of EDU as an effective screening tool to differentiate ozone sensitivity

2. Protection mechanisms of EDU against ozone phytotoxicity (insights into the mode of action)
   2.1 Investigation of EDU fertilization or growth regulating activities
   2.2 Elucidation of direct/indirect effects of EDU on the activation of ozone stress defense pathways genes
   2.3 Alleviation of ozone damage effects with EDU application via indirect protection mechanisms
   2.4 Mitigation of the negative effects of the stresses other than ozone by EDU application

3   Results

3.1   Confounding effects and differential responses of EDU

A season-long OTC experiment was conducted in a climate controlled glasshouse near to Bonn (Campus Klein-Altendorf, University of Bonn). Three contrasting rice genotypes were used, Nipponbare (NB) and BRRI dhan28 (BR28) were ozone sensitive (Jing et al., 2016; Akhtar et al., 2010) and L81, an ozone tolerant introgression line which carries two ozone tolerant quantitative trait loci (QTL) from the *Aus* landrace Kasalath in NB background (Wang et al., 2014; Frei et al., 2008, 2010). Four different treatments were assigned, (i) control, (ii) control+EDU, (iii) elevated ozone, and (iv) elevated ozone+EDU, including at least three replicates in each treatment. The actually measured average elevated ozone concentration was 77 ppb (7 h day^{-1}) and in control conditions 16 ppb. EDU was sprayed at a concentration of 300 ppm once a week and control plants were treated with same amount of water instead of EDU. Different growth, physiological, biochemical and yield parameters were measured regularly at different growth phases of rice plants.

3.1.1   EDU effects on plants with and without ozone stress

We measured a number of different growth, physiological and yield components *i.e.* plant height, tiller number, leaf greenness (SPAD), stomatal conductance, lipid peroxidation (MDA content), spectral reflectance indices (NDVI, SR, PRI and ARI), leaf symptoms (LBS), grain yield, panicle number, spikelet sterility and biomass of a *priori* ranked ozone responsive genotypes to investigate the effects of EDU in presence and absence of ozone. In a total 26 measured variables (growth and yield components) were significantly responded towards treatment and/or genotype by treatment interactions. A drastic effect of elevated ozone was seen in most of the measured variables when averaged over the performance of all genotypes between control and ozone (without EDU treatment). But the primary aim of this study was not to evaluate the negative effects of ozone rather to investigate the contrasting effects of EDU on plants in control and ozone condition with and without EDU application. EDU application had almost no effects on control plants, but significantly mitigated ozone effects in 9 out of 26 growth, physiological, and yield parameters when averaged across all genotypes. Although none of the yield components completely

recovered by EDU application compared to control, a clear positive effect of EDU on ozone afflicted plants was observed in the investigated genotypes. For instance, in the most important trait grain yield, 26% yield reduction was estimated due to ozone compared with control in averaged over for all genotypes. In contrast, only 16% yield loss was determined in ozone+EDU treated plants which clearly indicated the alleviation of negative effects of ozone with a recovery of 10% of the original yield.

3.1.2 Differential responses towards EDU application

The investigated genotypes contrasting ozone response barely responded differentially to EDU application in control conditions, but differential responses were common in ozone with and without EDU treatment. We determined that ca. 50% of measured variables (13 out of 22 and 12 out of 26) were EDU responsive in the sensitive genotypes BR28 and NB, respectively in presence of ozone. On the other hand, only ca. 15% variables (4 out of 26) were EDU responsive for the tolerant genotype L81. Moreover, none of the yield components responded to EDU application for tolerant L81. In contrast, a significant effect and alleviation by EDU application for foliar injury, lipid peroxidation, leaf greenness (vegetation indices NDVI, SR), panicle number, and grain yield were seen only in the ozone sensitive genotypes BR28 and NB. However, the widely cultivated Bangladeshi modern-variety BR28 exhibited the most drastic response to ozone, which was most consistently recovered by the EDU application. For instance, we calculated the grain yield in control condition for BR28 with and without EDU application ca. 7 tons/ha, while 37% yield reduction was estimated due to ozone stress. On the other hand, 25% yield loss was displayed in ozone+EDU condition with a recovery of 12% of the original yield by EDU application.

These results and experimental details are described in the following publication

Ashrafuzzaman, M., Lubna, F. A., Holtkamp, F., Manning, W. J., Kraska, T., & Frei, M. (2017). Diagnosing ozone stress and differential tolerance in rice (*Oryza sativa* L.) with ethylenediurea (EDU). Environmental Pollution*, 230*, 339–350.

3.2 Protection mechanisms (mode of action) of EDU against ozone phytotoxicity

A series of experiments including growth, physiology, transcriptomics (RNA-Seq), scanning electron microscopy (SEM) and other related experiments were performed to explore the mode of action of EDU. Four different contrasting ozone responsive genotypes were used, Bangladeshi ozone sensitive mega varieties BR28 and BINA11, ozone sensitive Japanese NB genotype and ozone tolerant introgression line L81. Four different treatments were assigned, (i) control, (ii) control+EDU, (iii) elevated ozone, and (iv) elevated ozone+EDU, including at-least three replicates in each treatment. The reordered average ozone concentration in the two fumigation studies was ranged from 107 to 108 ppb (7 h day^{-1}) and in control conditions 17 to 37 ppb. EDU was sprayed at a concentration of 300 ppm once a week and control plants were treated with same amount of water instead of EDU. RNA-sequencing and SEM was conducted using the ozone sensitive and EDU responsive genotype BR28, including at least three replicates in each treatment. Finally, an additional experiment was performed to test the EDU specificity in a diverse set of stresses (iron toxicity, salinity and zinc deficiency) using the mega rice variety IR64 along with BR28 and NB.

3.2.1 Nitrogen fertilization and constitutive activity of EDU

Leaf nitrogen contents were measured to investigate whether EDU is a potential source of nitrogen and/or acting as a growth promoting agent. Leaf nitrogen content did not respond significantly to the treatment in any of the investigated genotypes (control vs control+EDU and ozone vs ozone+EDU). However, nitrogen content was significantly declined in ozone in the sensitive variety BR28. On the other hand, significant ozone stress effects and alleviation by EDU application were seen in different measured growth and physiological parameters (LBS, MDA, tiller number, shoot length, shoot dry weight), specifically in the sensitive genotypes BR28, BINA11 and NB. In contrast, we did not observe any significant differential response in control plants with and without EDU applications. In addition, in depth physiological measurements (photosynthesis, stomatal conductance, Vcmax and Jmax) of the ozone sensitive and EDU responsive BR28 variety also confirmed the amelioration of the deleterious effects of ozone except for stomatal conductance while constitutive effects were absent in the control plant with and without EDU application.

3.2.2 Unravelling EDU mediated transcriptomic responses

In this study, the transcriptomics of BR28 genotype were compared in three biological replicates with four different treatments *i.e.* control, control+EDU, ozone and ozone+EDU. From the isolated RNA, Illumina cDNA libraries were constructed and sequenced using Illumina HiSeq4000 sequencer. Finally, the reads were aligned and mapped uniquely to the rice reference genome of Nipponbare after trimming and removal of stacked reads. A total of 23,208 rice genes were nominated for the further analyses, based on the expressed genes in at least one of the four treatments. For the validation of gene expression data from the RNA-Seq, qRT-PCR in a subset of 18 different stress responsive genes and a multidimensional scaling (MDS) plot analyses were conducted. The qPCR and RNA-Seq data were in a good agreement (R^2=0.81) and the control and control+EDU samples were clearly separated from the ozone and ozone+EDU in the MDS plot. We conducted a two-way ANOVA to analyze gene expression patterns including the factors ozone treatment (with the levels control and ozone), and EDU application (with the levels with or without EDU) and also determined pair-wise contrasts in each possible pair of the experimental conditions. We calculated the differentially expressed genes (DEGs), including both of the cut off thresholds level 5% and 10% false discovery rate (FDR). 933 ozone responsive genes were identified at 5% FDR and it increased to 4676 at 10% FDR level. In contrast, only 10 and 7 DEGs responded to EDU treatment and ozone x EDU interaction, respectively at both 5% and 10% FDR level, which clearly indicated that EDU had almost no direct effects on the global gene expression pattern of the investigated rice genotype. In addition, around 74% identified ozone-responsive DEGs (FDR <0.05) in this study were identical to those reported in a previous microarray study by Frei et al. (2010). Interestingly, no DEGs were identified between the pairwise contrast control vs control+EDU treatment at both FDR, which further indicated the lack of constitutive effects of EDU on global gene expression profile. In addition, almost no DEGs were identified in the control vs ozone+EDU and control+EDU vs ozone+EDU conditions. In contrast, a large number of DEGs were identified between control vs ozone (3182) and control+EDU vs ozone (3367) at 10% FDR which reflected the drastic effect of ozone on global gene expression profile in rice.

Gene ontology (GO) enrichment analysis was conducted separately for the down-regulated and up-regulated ozone responsive 933 DEGs at 5% FDR. Catalytic activity, various types of binding, ligase, kinase and transferase activity were the dominant GO terms in the category 'molecular function'. Catalytic activity was the only significant GO term for the down-regulated genes. Heat maps were generated to summarize the expression profiles for EDU responsive, ozone x EDU interaction and the pairwise contrast analysis ozone vs ozone+EDU DEGs along with functional annotation. The predominant pattern in the expression of these genes was an induction in the ozone treatment, which was offset by the application of EDU. Among these, few typical stress-responsive genes were displayed such as glutathione-S-transferase (LOC_Os01g27480), drought-induced protein (LOC_Os01g48190) or immediate-early fungal elicitor protein CMPG1 (LOC_Os03g13740).

3.2.3 Elucidation of EDU mediated indirect protection

Due to the absence of any direct effect of EDU on rice global gene expression profile, an additional experiment was conducted to test the hypothesis whether EDU has any direct abiotic chemical interaction with ozone. Generated ozone (600 mg/h) was first percolated through water and water+EDU (300 ppm) and then blown with a fan into plastic pipes vertically distributed on an empty open-top chamber. The accumulated ozone concentrations were measured at 1 min interval with a handheld ozone sensor (series 500; Aeroqual Ltd. Auckland, New Zealand). Significant reductions (ca. 15 percent) of ozone concentrations were observed in water+EDU treatment compared with only water, which indicates the potential of EDU to limit the concentrations by abiotic chemical interactions.

3.2.4 EDU fate on leaf surfaces

EDU can only reduce ozone concentrations if enough remnants are present on the leaf surface. For this, we performed scanning electron microscopy (SEM) of leaves in control and ozone with and without EDU applications (1 d and 7 d after the treatment). Interestingly, visible deposits of amorphous crystal structures similar to EDU crystals on a glass plate were displayed on the ozone+EDU treated leaves 7 d after EDU application which was absent in control. Therefore, an additional experiment including leaf surfaces treated with 600 and 300 ppm of EDU was conducted to quantify and further analyses of the EDU deposits. A number of

different shape and sized EDU amorphous particles (ca. 400 deposits cm^{-2} leaf area) were seen on 1 d and 7 d after EDU treated leaf which was absent in control leaf treated with only water. We also observed the spreading of the EDU particles on leaf surface which might cover almost all the leaf area.

3.2.5   Investigation of EDU specificity

Finally, we designed and performed an experiment to test whether EDU can only mitigate ozone stress and/or other stresses as well. Therefore, three stress conditions were tested, *i.e.* Fe toxicity, Zn deficiency and salinity. Almost all measured growth and physiological parameters (visible leaf symptoms, shoot and root length, shoot and root dry weight and SPAD value) were significantly affected by stresses compared to control when averaged over all investigated genotypes. In contrast, no significant differences were observed between stress and stress+EDU conditions. However, individual rice genotypes responded differentially to the stresses, and BR28 exhibited more consistent damage against stresses compared to the other two genotypes.

These results and experimental details are described in the following publications

Ashrafuzzaman M., Haque Z., Ali B., Mathew B., Yu P., Hochholdinger F., … Frei M. (2018) Ethylenediurea (EDU) mitigates the negative effects of ozone in rice: insights into its mode of action. Plant, Cell & Environment. 1-17.

4   Discussion

We employed an average ozone concentration of 77 to 108 ppb during the treatment period (7 h day^{-1}) in a series of OTC experiments. Several rice producing Asian countries especially on the Indian subcontinents are already experiencing a high level of ozone exceeding this concentration in field during the cropping seasons, due to increased levels of ozone precursor pollutants (Brauer et al., 2016; Deb Roy et al., 2009). Several projections estimated that crop yields and productions especially rice and wheat are greatly hampered in these highly ozone affected areas (Frei, 2015; Lal et al, 2017; Mills et al., 2018). Nevertheless, lack of field-based evaluation due to insufficient experimental facilities is a limiting factor for the actual ozone impact assessment. Therefore, it is of paramount importance to test all possible alternative tools for ozone impacts evaluation in these highly ozone prone areas as well as to speed up the ozone tolerance or resistant breeding program. To elucidate the confounding effects and mode of action of EDU against ozone phytotoxicity, following three hypotheses were tested.

4.1   Hypothesis 1: EDU does not have any constitutive effects on crops while mitigating phytotoxic effects of ozone

Absence of differential responses in control plants with and without EDU applications were observed in almost all of the measured traits (a number of growth, physiological, biochemical and yield parameters). Besides this, none of the genes were differentially expressed between control and control+EDU conditions. In addition, EDU spraying did not affect leaf nitrogen content in rice genotypes which was also confirmed in willow plants (Agathokleous et al., 2018b). But, it has been mentioned earlier by several researchers that EDU might act as a foliar fertilizer due to high percentage (ca. 22%) of nitrogen contents (Godzik & Manning, 1998; Manning et al., 2011). However, EDU application dose and nutrients status of the plant growing medium can play a crucial role for the nitrogen contribution. For instance, high concentrations of EDU (above 800 ppm) contributed to increased leaf nitrogen content of willow plants grown in low nitrogen and organic matter free soil (Agathokleous et al., 2016a). Another important aspect of EDU application is the toxicology *i.e.* effects towards environment, flora and fauna. Acute toxicological tests were conducted with EDU in

rat at oral administration LD_{50} (14000 mg/kg) and no irritation and injury was reported in skin (Guinea pig) and eye (rabbit), respectively (Manning et al., 2011). However, high doses of EDU application (> 1000 ppm) might have adverse effects on plants (Agathokleous et al., 2016b; Elagöz and Manning, 2005). On the other hand, a drastic effect of ozone was observed in the morphology of the experimental rice genotype which was partly offset by EDU applications in the sensitive genotypes, specifically BR28 (Figure 4.1).

Control Control+EDU Ozone   Ozone+EDU

Figure 4.1 Morphology of the control and ozone rice plant with and without EDU application of the ozone sensitive BR28 genotype (photographs were taken after 14 d (7 h day^{-1}) of elevated ozone treatment. Average measured elevated ozone concentration was 107 ppb and in control condition 17 ppb.

4.2 Hypothesis 2: EDU mediated protection is regulated by surface protection rather than direct effects on plant physiology

Ozone primarily enters through leaf stomata and rapidly degrades into different ROS elements in the apoplast (Kangasjärvi et al., 2005). On the other hand, EDU applied by available methods (mainly through the spray and soil drench) is rapidly translocated into leaf apoplastic regions and persists for eight days or more (Gatta et al., 1997; Pasqualini et al., 2016). Therefore, EDU might have an important role in regulating ozone stress defense pathways, which could be responsible for the EDU mediated protection such as stimulation of antioxidants (Pandey et al., 2014, 2015). But our transcriptomic analyses showed the reverse phenomenon, and almost none of the genes responded significantly to EDU application (only 10 and 7 genes for EDU and ozone x EDU interaction, respectively at FDR 10%). In contrast, several thousand genes were identified as ozone responsive at 10% FDR. Around 74 percent of these differentially expressed genes (DEGs) were also identical in a previous microarray study in rice which was mainly involved in ethylene or jasmonic acid metabolism, general disease resistance and antioxidant pathways (Frei et al., 2010). It is thus obvious that rice global gene expression profile was barely affected by EDU application, which is clearly indicated the absence of any direct EDU mediated protection in downstream physiological stress reactions (Figure 4.2).
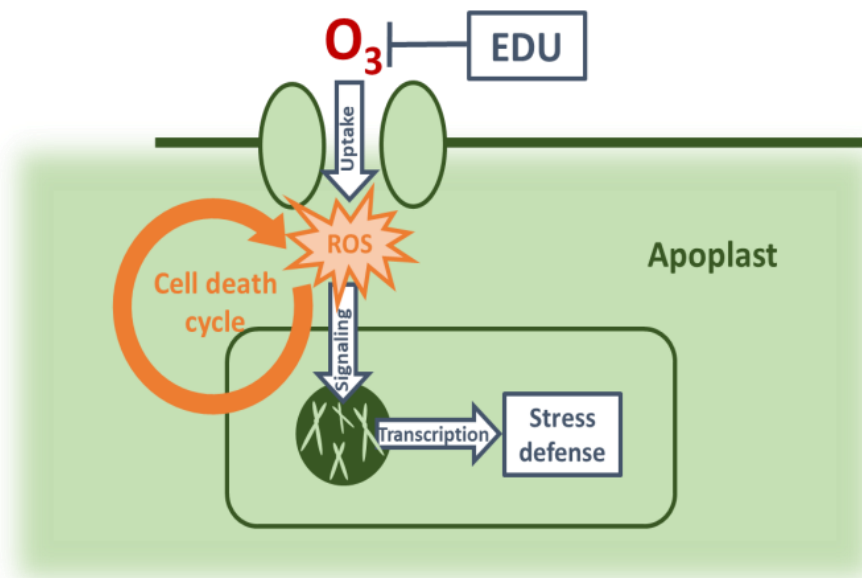
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Figure 4.2 Hypothetical mode of action of EDU in ozone afflicted plants

EDU did not affect stomatal conductance in the sensitive BR28 genotype. This indicates that stomatal closure as the first line defense against ozone stress did not contribute to mitigating ozone effects, which was also observed earlier in snap bean (Paoletti et al., 2014). It is therefore plausible that EDU alleviated harmful effects of ozone through a passive surface or apoplastic protection effect upstream of any defense reactions (Figure 4.2) *i.e.* by limiting the entry into the plant or by degrading the ozone itself. Similar gene expression pattern between pairwise contrast, control vs ozone+EDU and control+EDU vs ozone+EDU of the RNA-Seq analyses supported this hypothesis. Moreover, significantly lower accumulation of ozone, which was first percolated through EDU+water (300 ppm) compared with only water further consolidated this assumption.

It is therefore plausible that EDU might have ozone degrading properties which ultimately limit and inhibit the entry of ozone into plants by an abiotic chemical interaction between EDU and ozone. Tuazon et al. (1994), reported similar findings by determining the gas-phase rate of coefficients of several amines towards ozone. This might be true if enough EDU residues are present on leaves. A number of amorphous EDU deposits including a spreading phenomenon were observed 1 d and 7 d after EDU treated leaf surface, which indicated the persistence of EDU. On the other hand, non-stomatal ozone uptake and deposition was also recorded as a dominating pathways compared to stomatal uptake in many plant species (Horváth et al., 2017; Kanagendran et al., 2017). Consequently, leaf surface structures can play a critical role to uptake ozone and reduce the toxicity as a chemical barrier by decomposing it before entering into the leaf (Horváth et al., 2017; Li et al., 2018; Oksanen, 2018). For instance, glandular trichomes can directly limit the ozone concentration at the leaf surface by increasing the emissions of volatile organic compounds in a diverse species (Li et al., 2018), and in tobacco (Kanagendran et al., 2017). Therefore, it is possible that EDU interferes with these surface protection mechanisms by interacting with ozone directly or accelerates the ozone degrading processes. This phenomenon might be interfered in the stronger protection of EDU by foliar spray method compared to soil drench application (Agathokleous et al., 2016c).

4.3   Hypothesis 3: EDU does not mitigate the effect of stresses other than ozone

Finally, we tested the specificity of EDU whether it responded towards other commonly available stresses in the field to consolidate the use of EDU as an ozone biomonitoring tool. In Asian rice growing countries including Bangladesh and India, multiple stresses such as salinity or nutrient disorders can coincide with ozone during the crop growing phase (Frei, 2015; Gregorio et al., 2002). However, none of the tested stress conditions in our study (iron toxicity, salinity, and zinc deficiency) responded significantly in rice due to EDU application, which was also confirmed earlier for moderate drought stress in poplar plants (Xin et al., 2016). Therefore, our experimental result clearly demonstrated that the EDU effect was ozone specific and actively alleviated ozone mediated deleterious effects in rice plants.

4.4   Lesson for future EDU research

Lack of available germplasm from the highly ozone-prone areas and translation of controlled experiments into the field condition are the major limiting factors for ozone tolerance breeding (Ainsworth, 2017). On the other hand, ozone-caused yield reductions are under-estimated especially in Asian highly ozone affected areas due to lack of farmers' awareness and absence of simple diagnostic tools compared with other stresses such as salinity, heat, and drought (Frei, 2015). Nevertheless, a few studies have been conducted so far in India with EDU applications, mainly in rice (Pandey et al., 2015), and wheat (Gupta et al., 2018), but no studies have been reported from Bangladesh. So, there is an enormous scope to explore the ozone impacts evaluation in plants and vegetation using EDU as a diagnosing tool. Moreover, two Bangladeshi mega-varieties BR28 and BINA11 showed high sensitivity to ozone in our study, in accordance with earlier predictions by Emberson et al. (2009), that South Asian crop varieties are more sensitive to ozone compared to North American ones. It will presumably be very difficult to adopt the air quality regulations aimed to minimize the emission of ozone precursor gases in most of the Asian countries, specifically Indian subcontinent due to rapid population and economic growth. In contrast, it is projected that 12% ozone caused yield reduction can be recovered by cultivating more ozone tolerant or resistant crop varieties in 2030 relative to the damage that occurred in 2000 (Avnery et al., 2013). Therefore, EDU mediated ozone tolerance breeding in crops can be an effective strategy to enhance crop yield and production, which will contribute to the food security of many densely populated countries in Asia. In conclusion, our experimental result encourages the use of EDU as an ozone biomonitoring tool to screen ozone tolerance (less EDU-responsive) and sensitivity (more EDU-responsive), which can facilitate the breeding schemes by generating ample of contrasting breeding materials from the ambient field. However, further studies are warranted for the commercial and cheap production of EDU and to explore its toxicological aspects.

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6 Publications

6.1 <u>Ashrafuzzaman, M.,</u> Lubna, F. A., Holtkamp, F., Manning, W. J., Kraska, T., & Frei, M. (2017). Diagnosing ozone stress and differential tolerance in rice (*Oryza sativa* L.) with ethylenediurea (EDU). Environmental Pollution, 230, 339–350.

Own contribution: Leading the experiment and carried out whole work with the help of F.H. and F.A.L. and partly contributed in the conceptual design. I prepared all tables, figures and graphs. I interpreted the data and wrote the manuscript with the help of M.F. The manuscript was revised by M.F. and all other co-authors.

6.2 <u>Ashrafuzzaman M.,</u> Haque Z., Ali B., Mathew B., Yu P., Hochholdinger F., … Frei M. (2018) Ethylenediurea (EDU) mitigates the negative effects of ozone in rice: insights into its mode of action. Plant, Cell & Environment. 1-17.

Own contribution: I carried out all the experiments with the help of Z.H. and partly contributed in the conceptual design. I prepared all tables, figures and graphs. I interpreted the data and wrote the manuscript with the help of M.F. RNA-Seq data analysis contributed by P.Y. and H.J.E. contributed to scanning electron microscopy. The manuscript was revised by M.F. and all other co-authors.

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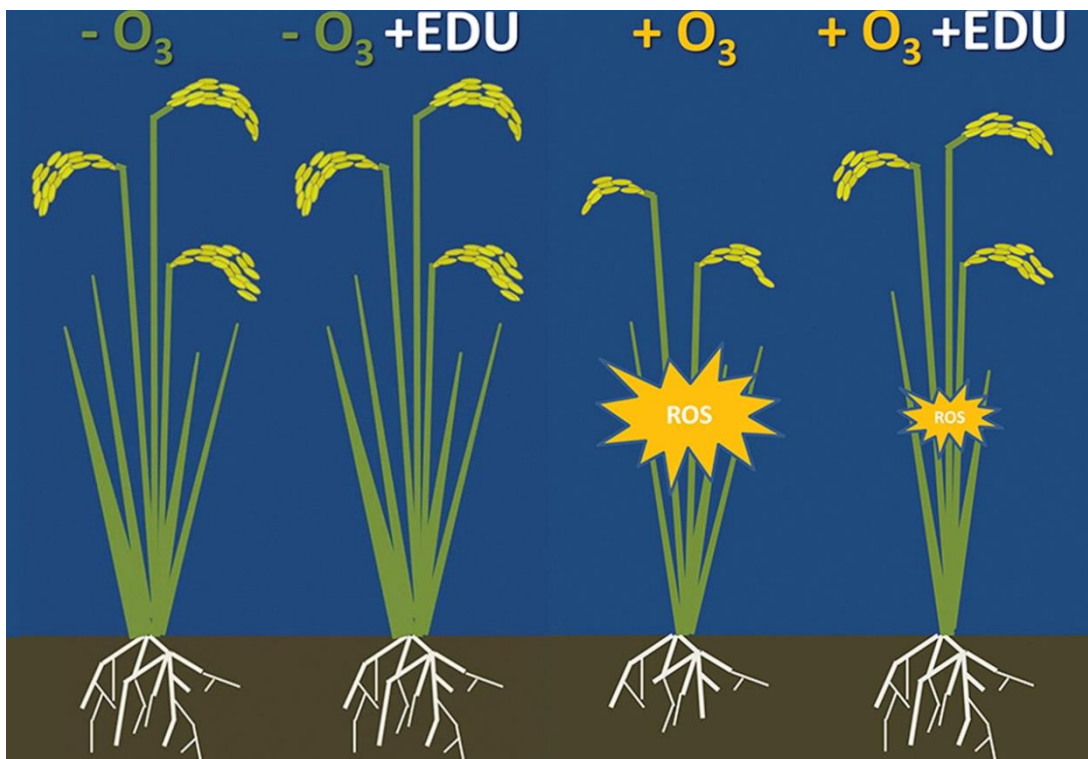**Diagnosing ozone stress and differential tolerance in rice (*Oryza sativa* L.) with ethylenediurea (EDU)**

Ashrafuzzaman, M., Lubna, F. A., Holtkamp, F., Manning, W. J., Kraska, T., & Frei, M.

Highlights

- Ethylenediurea (EDU) was tested as a screening tool for ozone response in rice.
- EDU does not have any constitutive effect on rice plants in ozone free environment.
- Tolerant and sensitive genotypes responded differentially towards EDU.
- EDU can be used as an effective screening and ozone biomonitoring research tool.

Graphical abstract

Diagnosing ozone stress and differential tolerance in rice (*Oryza sativa* L.) with ethylenediurea (EDU)[☆]

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ABSTRACT

Rising tropospheric ozone concentrations in Asia necessitate the breeding of adapted rice varieties to ensure food security. However, breeding requires field-based evaluation of ample plant material, which can be technically challenging or very costly when using ozone fumigation facilities. The chemical ethylenediurea (EDU) has been proposed for estimating the effects of ozone in large-scale field applications, but controlled experiments investigating constitutive effects on rice or its suitability to detect genotypic differences in ozone tolerance are missing. This study comprised a controlled open top chamber experiment with four treatments (i) control (average ozone concentration 16 ppb), (ii) control with EDU application, (iii) ozone stress (average 77 ppb for 7 h daily throughout the season), and (iv) ozone stress with EDU application. Three contrasting rice genotypes were tested, *i.e.* the tolerant line L81 and the sensitive Nipponbare and BR28. The ozone treatment had significant negative effects on plant growth (height and tillering), stomatal conductance, SPAD value, spectral reflectance indices such as the normalized difference vegetation index (NDVI), lipid peroxidation, as well as biomass and grain yields. These negative effects were more pronounced in the *a priori* sensitive varieties, especially the widely grown Bangladeshi variety BR28, which showed grain yield reductions by 37 percent. EDU application had almost no effects on plants in the absence of ozone, but partly mitigated ozone effects on foliar symptoms, lipid peroxidation, SPAD value, stomatal conductance, several spectral reflectance parameters, panicle number, grain yield, and spikelet sterility. EDU responses were more pronounced in sensitive genotypes than in the tolerant L81. In conclusion, EDU had no constitutive effects on rice and partly offset negative ozone effects, especially in sensitive varieties. It can thus be used to diagnose ozone damage in field grown rice and for distinguishing tolerant (less EDU-responsive) and sensitive (more EDU-responsive) genotypes.

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1. Introduction

Tropospheric ozone (O₃) poses a major emerging threat to global crop production due to its high phytotoxicity (Tai et al., 2014; Agathokleous et al., 2015a; Ainsworth, 2016). It is one of the most widespread secondary air pollutants, formed through photochemical reactions of precursor gases such as nitrous oxides, volatile organic compounds, carbon monoxide and methane (The Royal Society, 2008; Paoletti et al., 2014). Detrimental effects of current ambient ozone levels on vegetation including crop growth and yields have been estimated to cause global economic losses in the range of 14—26 billion US$ (Ashmore, 2005; Feng and Kobayashi, 2009; Van Dingenen et al., 2009; Mills and Harmens, 2011; Avnery et al., 2013). In addition, several model projections predicted that East and South Asian developing countries (including Bangladesh) will be most strongly affected by tropospheric ozone in the coming decades due to insufficient environmental legislation, rapid economic growth and industrial development (Emberson et al., 2009; Van Dingenen et al., 2009;

Avnery et al., 2011; Pozzer et al., 2012; IPCC, 2014). Among the ten most populated countries in the world, the change in daily maximum average ozone concentration was the highest (21.3%) in Bangladesh, between 1990 (59.4 ppb) and 2013 (72.0 ppb) (Brauer et al., 2016). This trend occurred due to higher emission of ozone precursor gases (Chakraborty et al., 2015; Brauer et al., 2016) arising from unprecedented increases of motor vehicles, conventional brick-making kilns, and fossil fuels combustion without any regulation, in combination with favorable climatic conditions for ozone formation (Akhtar et al., 2010).

Ozone adversely affects crop productivity directly through oxidative damage to cells and indirectly as a greenhouse gas accelerating global warming (Ainsworth, 2016). Rice is the most important staple food crop in Asia, including Bangladesh (Akhtar et al., 2010; Alexandratos and Bruinsma, 2012), the 4th largest rice growing country globally (FAOSTAT, 2013). An estimated 3.7% of global and more than 10% of regional rice yields are lost due to rising ozone, which will exacerbate in near future with further increases in ozone levels (Ainsworth, 2008; Van Dingenen et al., 2009; Frei, 2015). In South Asia rice is cultivated almost year round with two major seasons, and both growing seasons can overlap with the peak ambient ozone episodes (Frei, 2015). Although no field studies have been done so far to determine ozone impacts on rice production, significant yield reduction of Bangladeshi rice cultivars have been reported in controlled environment systems due to elevated ozone (chronic stress, ozone concentration was 60 and 100 ppb) (Akhtar et al., 2010). Therefore, it is essential to develop an effective and reliable research tool which can be used to quantify ozone impacts in the field and to screen a wide range of genotypes grown in ozone affected developing areas such as Bangladesh.

Three types of experimental systems have been used so far for the quantification of ozone impacts on plants: sun-lit plant growth chambers, open top chambers (OTC) and free air concentration enrichment (FACE) (Frei, 2015; Kobayashi, 2015). Sun-lit plant growth chambers are environmentally controlled chambers in which ozone concentrations can be maintained at desired levels for the purpose of better understanding mechanism of ozone damage (Kobayashi, 2015). OTC were first introduced in the early 1970s by Heagle et al. (1973), and are widely used controlled-environment systems, in which ozone concentrations are maintained at desired levels with artificially generated air blowing ozone into the chambers (Frei, 2015). They have been criticized for causing a 'chamber effect' thereby modifying the ozone impacts by changing the microclimate (especially, temperature, humidity, CO_2 concentration), which can differ from the field conditions (Morgan et al., 2006; Piikki et al., 2008; Kobayashi, 2015). Another promising and more realistic approach is free air concentration enrichment (FACE), a chamber-less system for evaluating plant performance in field conditions (Morgan et al., 2004). Despite that, only few studies have been conducted so far with FACE-ozone systems, for example in the USA for soybean (Morgan et al., 2004) and in China for rice and wheat (Shi et al., 2009; Feng et al., 2011, 2016; Tang et al., 2011). Maintaining FACE experiments is expensive, technically challenging and laborious (Frei, 2015). Therefore, this approach is less suitable for developing countries such as Bangladesh due to lack of reliable electricity and infrastructure facilities (Oksanen et al., 2013; Kobayashi, 2015). FACE also has the limitation that it only compares ambient to elevated ozone levels, but unlike chamber experiments offers no possibility of estimating effects of current ambient ozone levels (Pleijel, 2011).

As an alternative to FACE systems, a synthetic chemical, ethylenediurea (N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea, abbreviated as EDU) also termed as antiozonant, has been proposed to evaluate the differential responses of plants and crop species towards ozone damage (Paoletti et al., 2009; Feng et al., 2010; Manning et al., 2011; Agathokleous et al., 2015b). The EDU protection against ozone injury was first reported in bean plants (Carnahan et al., 1978). However, the actual mode of action and prevention mechanisms of EDU against phytotoxic effects of ozone still remain elusive (Paoletti et al., 2009; Manning et al., 2011). It is suggested that EDU may facilitate the scavenging of detrimental ozone activity through a direct effect on physiological parameters, or by antioxidant-mediated defense reactions (Manning et al., 2011; Pandey et al., 2015). As EDU can be applied flexibly by spraying on plants, it may constitute a convenient tool to screen a large number of rice genotypes in field experiments. However, this approach assumes that EDU has an ozone-specific, but no constitutive effect on plants. With few exceptions (such as a study on clover by Karlsson et al., 1995) most EDU studies have been conducted in ambient ozone conditions without any ozone-free control. Therefore, prior to the use in large scale field experiments, specific EDU doses applied to crops should be validated to mitigate ozone effects, without having any effects on plants in the absence of ozone.

To this end, the current study was designed to test contrasting rice genotypes (two *a priori* ozone sensitive and one ozone-tolerant), and to explore the constitutive effect of EDU in the absence or presence of ozone on physiological parameters, growth and rice yield components. Our specific hypotheses were that (i) EDU will play an important role in protecting ozone-stressed plants against phytotoxicity without any constitutive effect, *i.e.* control plants will not be responsive towards EDU; (ii) ozone sensitive genotypes will be more responsive towards EDU in presence of ozone than the tolerant genotype.

2. Materials and methods

2.1. Plant materials and growth condition

The experiment was carried out in a climate controlled glasshouse near Bonn (Campus Klein-Altendorf, University of Bonn), Germany, from April to October 2016. Three different rice genotypes were used for this study (i) Nipponbare (NB), an ozone sensitive Japanese modern rice variety (Jing et al., 2016) (ii) L81, an ozone tolerant introgression line (Wang et al., 2014), which carries two ozone tolerant quantitative trait loci (QTL) from the *Aus* landrace Kasalath (Frei et al., 2008, 2010) in the genetic background of NB (iii) BRRI dhan28 (BR28), an ozone sensitive and high yielding modern rice variety developed by the Bangladesh Rice Research Institute, which is widely cultivated by Bangladeshi farmers (Akhtar et al., 2010). Seeds were germinated in the dark for 3 d at 28 °C and then transferred to a glasshouse under natural light. Seedlings were placed in a mesh floating on solutions containing 0.5 mM $CaCl_2$ and 10 μM $FeCl_3$ until transplantation. Three-week-old seedlings were transplanted into three experimental polders measuring 6 m × 2 m filled with a local clay-silt luvisol soil with 16% clay, 77% silt, 7% sand, 1.2% organic carbon and pH 6.3 (Ueda et al., 2015a). Constant water level of at least 3 cm was maintained from 10 d after transplanting throughout the growth season. The polders were previously sealed with PVC sheets at 50 cm soil depth (Frei et al., 2016) and had been used for flooded rice cultivation in three consecutive years before this experiment. Temperature and relative humidity were measured continuously at 2-min intervals (sensor type 224.401, RAM GmbH Mess-und Regeltechnik, Herrsching, Germany), and CO_2 was measured at 10-min intervals (sensor type GMT 222EONOAON1AOB, Vaisala, Helsinki, Finland), respectively. The average daytime (7.00 h–19.00 h) and nighttime (19.00 h–7.00 h) temperatures were 27.8 °C and 20.6 °C, average relative humidity was 54.5% and 81.8%, respectively. The average

CO_2 concentration was 455/578 ppm (day/night) respectively. Artificial lighting was installed above the plots to ensure a minimum photosynthetic photon flux density of 400 μmol m^{-2} s^{-1} during the daytime.

Each polder (6 m × 2 m) was first subdivided into two 3 m × 2 m areas assigned to control and ozone treatment, respectively, and further subdivided into 1-m^2 subplots assigned to the individual genotypes (in a total 36 subplots in 3 polders). Within each control and ozone treatment area, three subplots (one for each genotype) were assigned to the EDU treatment and three to non-EDU treatment. Seedlings were planted following the straight row method at 20 cm × 20 cm spacing leading to 25 plants of each genotype in each subplot (IRRI, 2016). The middle 9 plants were used for measuring final yields, whereas the border plants were used for the destructive measurements (sample collection). To ensure sufficient nutrient supply, plants were provided with fertilizer at rates equivalent to 40 kg K ha^{-1} and 21 kg P ha^{-1} to each polder as basal fertilizer at the beginning of the season, and 60 kg N ha^{-1} (as urea, applied in three splits as for basal, 20 and 40 days after transplanting = DAT20 and DAT40). No chemical insecticides and pesticides were used during the whole growth period and weeds were uprooted manually.

The following treatments were implemented (i) Control, (ii) Control + ethylenediurea (EDU), (iii) Elevated ozone, and (iv) Elevated ozone + EDU. Each polder was surrounded by an open top chamber (1.30 m height) sealed with a transparent PVC sheet, with an additional sheet in the middle to separate control and ozone treatments. Starting from five weeks after transplanting, half of the control plants and half of the ozone treated plants were sprayed with ethylenediurea (EDU) at a concentration of 300 ppm once a week, as suggested in previous studies (Wang et al., 2007; Feng et al., 2010; Pandey et al., 2015). Weekly intervals were selected to ensure continuous effects, as EDU was reported to persist in the leaf apoplast for eight days or more (Paoletti et al., 2009). Approximately, 0.188, 0.300 and 0.375 g EDU were sprayed weekly per m^{-2} area (1 subplot) at the seedling, vegetative and reproductive growth stage of plants respectively, for complete saturation of canopies. Water was sprayed on the non-EDU treated plants instead of EDU.

2.2. Ozone treatment and monitoring

Five weeks after transplanting, ozone fumigation was started at a target level of 80 ppb for 7 h every day (9.00 h–16.00 h) to induce chronic stress. Comparable ambient levels were reported in many in Asian countries including Bangladesh, India and China (Yamaji et al., 2006; Feng et al., 2015; Frei, 2015). Ozone was generated by using custom-made ozone generators (UB 01; Gemke Technik GmbH, Ennepetal, Germany), in which dried air was passed through silica gels as an input. The ozone output was regulated by an ozone monitor (K100 W; Dr A. Kuntze GmbH, Meerbusch, Germany) and detected by an ozone sensor (GE 760 ozone; Dr A. Kuntze GmbH) placed inside the fumigation chambers. The generated ozone was first blown with a fan into a central plastic pipe, which was further connected with three parallel perforated pipes running above the plant canopy at a distance of 40 cm from each other. The ozone concentrations in different areas of the polders were monitored with an independent handheld ozone monitor (series 500; Aeroqual Ltd. Auckland, New Zealand) at 5-min intervals. The actually measured average daytime ozone concentration was 77 ppb ± 0.49 SE (standard error) in the ozone treatment whereas the average concentration in control conditions was 16 ppb ± 0.24 SE. The calculated AOT40 (accumulated exposure over a threshold of 40 ppb) values differed for the genotypes due to different harvest times and were 23.6, 30.6 and 33.7 ppm-h for BR28, L81 and NB, respectively.

Plant height (PH), tiller number (TN) and SPAD values were measured in two-week intervals. A SPAD 502 instrument (Konica Minolta, Osaka, Japan) was used for the SPAD value measurement. Three different points were measured at 20 cm distance from the tip of the last youngest fully expanded leaf of the three randomly selected plants from each subplot and the average of the three points was calculated. Stomatal conductance measurements were performed in several time points representing different growth phases. Measurements on the youngest fully expanded leaves of one selected plant in each sub-plot were performed on sunny days between 9:30 h to 12:00 h using a leaf porometer (Model SC1, Decagon Devices, Pullman, WA). Spectral reflectance of leaves was measured with a handheld spectro-radiometer PolyPen RP400 (Photon Systems Instruments, Drasov, Czech Republic). The measurements were performed on the youngest fully expanded leaves of three randomly selected plants in each sub-plot on sunny days. Four vegetation indices were determined, (i) Normalized difference vegetation index (NDVI) = $(R_{NIR}-R_{RED})/(R_{NIR} + R_{RED})$, (Rouse et al., 1974), (ii) Simple ratio index (SR) = R_{NIR}/R_{RED}, (Jordan, 1969), (iii) Photochemical reflectance index (PRI) = $(R_{531}- R_{570})/(R_{531}+R_{570})$ (Peñuelas et al., 1995), and (iv) Anthocyanin reflectance index (ARI) = $(R_{550})^{-1} - (R_{700})^{-1}$ (Gitelson et al., 2001). In the vegetation indices, R refers to reflectance and subscript indicates the wavebands in nanometers. For NIR and RED, we used a defined wave length of 780 nm and 630 nm respectively.

Visible leaf symptoms of ozone stress were quantified at final harvesting in the nine central plants of each subplot. The three rice genotypes differed in maturity period and were harvested separately. A leaf bronzing score (LBS) ranging from 0 to 10 was assigned to each plant to evaluate leaf symptoms (Ueda et al., 2015a), in which 0 indicated no ozone-induced symptoms in any of the leaves and the highest value 10 would indicate that the whole plant was severely damaged by ozone stress. For determination of biomass and yield components, the border plants were removed first and the panicles of the middle 9 plants were counted, separated from the shoots and dried at 60 °C for at least 72 h to complete dryness. The shoot samples (straw) were also dried at 60 °C and weighed. The total weight (grain with panicles) was measured and grains were separated from the panicles and weighed again for only grains. Thereafter, filled and unfilled spikelets were separated manually. Both filled and unfilled spikelets were counted using a seed counter (Chopin, Marcelin Berthelot, France). Grain sterility was calculated as the number of sterile spikelets relative to the total number of spikelets. Thousand kernel weight (TKW) was calculated from five subsamples of twenty randomly chosen kernels in each sample. Harvest index was expressed as the percentage of filled grains relative to total biomass. Finally, grain yield in kg per hectare was extrapolated based on the 0.36 m^2 area used for yield determination.

2.3. Biochemical assays

For biochemical measurements, the two youngest fully expanded leaves from randomly selected five border plants in each subplot were collected and pooled for one representative sample. Samples were taken twice in two different growth phases (vegetative and flowering). The collection of samples was performed in one day between 10:00 h and 12:00 h with liquid nitrogen and stored at −80 °C until further analysis.

2.3.1. Malondialdehyde (MDA) quantification

The amount of MDA was measured as described previously (Hodges et al., 1999; Höller et al., 2014) and used as an indicator of oxidative stress. Extraction was performed from approximately

100 mg of ground tissues with 1.5 mL of 0.1% (w/v) Trichloroacetic acid (TCA). Samples were then centrifuged at 4 °C and 20.000 g for 20 min and the supernatants were divided into two aliquots of 500 μL. These were mixed with same amount of 20% (w/v) TCA, 0.01% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 0.65% (w/v) thiobarbituric acid (TBA) was also added in one aliquot. The mixture was then heated to 95 °C for 30 min, and the absorbance measured at 440, 532, and 600 nm. Blank samples were also prepared with 0.1% (w/v) TCA solution instead of sample supernatant, and the absorbance was subtracted from each sample value.

2.3.2. Ascorbate (AsA) assay

Extraction and quantification of AsA were performed according to Ueda et al. (2013). The reduced AsA content was measured with the addition of 10 μL of 0.01 units μL^{-1} ascorbate oxidase to a mixture of 10 μL of extracted AsA and 80 μL of 0.1 M potassium phosphate buffer (pH 7.0) at 265 nm wave length. The oxidized AsA content was measured at 265 nm wave length after the addition of 10 μL of 4 mM dithiothreitol (DTT), a reducing agent to a mixture of 10 μL of extracted AsA and 80 μL of 0.1 M potassium phosphate buffer (pH 7.8). Based on the extinction coefficient of $\varepsilon = 14.3$ mM^{-1} cm^{-1} the AsA content was calculated.

2.4. Statistical analysis

All statistical analyses including analysis of variance (ANOVA) were performed using a mixed model analysis in PROC MIXED of SAS 9.4 (SAS Institute Inc. Cary, NC). A mixed model was designed (Piepho et al., 2003) with genotype, treatment and their interaction set as fixed effects, whereas polders and polder by genotype interactions were considered as random effects (Frei et al., 2011). Mean comparison was performed by Tukey's test for posthoc adjustment, and P values less than 0.05 were considered as significant.

3. Results

This section will focus on those traits which showed significant responses to ozone and EDU as indicated by significant treatment effects and/or genotype by treatment interactions. Significant effects were seen in 20 variables measured during the growth season (Table 1), and in six yield components (Table 2).

Due to differences in phenology, the three rice genotypes BR28, L81and NB were harvested at different time points at 127 DAT, 154 DAT and 166 DAT respectively. As a visible representation of oxidative stress, LBS showed highly significant effects on the treatment, genotype and interaction level (Table 1). Analysis of genotype specific treatment responses revealed significant EDU-response in symptom formation only for the sensitive genotypes BR28 and NB (Fig. 1). Significant increases in MDA due to ozone were seen in the sensitive genotypes BR28 and NB but not in the tolerant L81 (Fig. 2). Interestingly, EDU application mitigated ozone-induced lipid peroxidation as shown by significantly lower MDA concentration in the ozone + EDU treatment compared to the ozone treatment, while no effect of EDU on MDA was seen in the absence of ozone (Table 1, Fig. 2). Together these data demonstrated that ozone caused oxidative stress in the sensitive rice genotypes, which was mitigated by the application of EDU. We did not observe any significant treatment effect or treatment by genotype interaction in total ascorbate measurements (Supplementary Fig. S1).

Averaged over all three genotypes, tiller number did not significantly respond to the treatment (Table 1), although significant negative ozone-responses were seen in the sensitive BR28 and NB (Fig. 3). Only NB showed significantly enhanced tiller number due to EDU application compared to the ozone treatment (Fig. 3A).

Similarly, a significant treatment main effect on plant height was seen only on the last measuring day at DAT107, where ozone significantly reduced plant height (Table 1). The effects of ozone treatment on plant height increased progressively leading to significant height reductions towards the end of the growth in each genotype (Fig. 3). Only the tolerant L81 showed a significant response to EDU in terms of plant height (Fig. 3). In summary, plant growth traits were negatively affected only after prolonged ozone fumigation and were more responsive in the sensitive genotypes, while EDU mitigated negative ozone effects in some instances.

On most measuring days, negative effects of ozone on physiological traits were observed. SPAD value was significantly lower in the ozone treatment than in the control except for DAT95 (Table 1). On the last two measuring days, SPAD was significantly higher in EDU treated plants when compared to the ozone treatment (Table 1). Individual genotypes responded differently to the treatments. The sensitive BR28 exhibited an early negative response to the ozone treatment and significant alleviation due to EDU application even on DAT66. Similar responses were only seen at DAT115 and DAT133 in NB and L81 (Fig. 4A). Stomatal conductance was measured as a proxy for photosynthetic gas exchange. The treatment main effects were significant or close to significant (DAT93), while a significant ozone-mitigating effect of EDU was seen only on DAT106 when values were averaged over all three genotypes (Table 1). Analysis of treatment responses in individual genotypes revealed an early effect in BR28 at DAT93, followed by NB on DAT106, but only from DAT133 in L81. Positive effects of EDU application in the presence of ozone were seen in all three genotypes on different measuring days (Fig. 4B).

Vegetation indices NDVI and SR were measured as proxies for broadband greenness (chlorophyll contents, foliage greenness), PRI and ARI for the estimation of photosynthetic light use efficiency and stress related pigments (carotenoids and anthocyanins). Contrasting treatment effects for the selected indices were seen only after the plants were exposed to substantial amount of ozone at DAT109. Significant stress effects compared to control and (partial) recovery with the application of EDU were observed for all four selected reflectance indices based on the averaged performances of the three genotypes (Table 1). However, the sensitive genotypes BR28 and NB showed more pronounced responses towards EDU application compared to tolerant L81 (Fig. 5). Specifically, the application of EDU significantly mitigated ozone effect in both sensitive genotypes for the reflectance indices NDVI and SR (Fig. 5A and B). On the other hand, EDU significantly alleviated ozone effect for the indices PRI and ARI only for the sensitive genotype BR28 (Fig. 5C and D). No effect of EDU on spectral reflectance was detected in the absence of ozone for all genotypes. Moreover, EDU did not show any significant stress mitigation effect for the tolerant genotype L81 for all selected reflectance indices (Fig. 5). In general these observations of growth and physiological traits clearly suggested that EDU did not play any role as a growth promoting or regulating agent in control plants but specifically mitigated negative ozone effects on different measuring days in all genotypes, especially the sensitive ones.

After harvesting, several yield components were determined, six of which showed significant treatment effects due to declines in the ozone treatment compared to the control (Table 2). Similar to morphological and physiological traits, none of the yield parameters was affected by EDU application in the absence of ozone. Even within the ozone treatments, EDU did not significantly improve yield components when the averages of all three genotypes were compared (Table 2). However, this was different when the treatment responses of individual genotypes were observed (Fig. 6). The sensitive BR28 showed negative responses to ozone in all yield components except thousand kernel weight, but also a significant

Table 1
Statistical analysis (mixed model analysis using SAS 9.3) and treatment mean values (LS mean) of growth and physiological data collected from three different rice genotypes exposed to ozone and control conditions with or without the application of EDU.

| Traits	DAT	ANOVA results (Pr > F)			LS means (Treatment)			
		Treatment	Genotype	Interaction	Control	Control + EDU	Ozone	Ozone + EDU
Leaf Bronzing Score	Harvest time	<0.0001	0.0002	0.0005	n.d.	n.d.	4.3[a]	2.7[b]
MDA (nmol g^{-1}) FW	DAT76 and 106	<0.0001	0.2975	0.0137	13.7[b]	12.9[b]	19.8[a]	14.0[b]
Tiller number	DAT55	0.8434	0.0051	0.0002	9.9[a]	10.2[a]	9.4[a]	10.0[a]
	DAT93	0.6716	0.0450	0.0269	11.0[a]	10.9[a]	9.9[a]	10.3[a]
Plant height (cm)	DAT59	0.7318	0.0218	<0.0001	110.8[a]	109.4[a]	110.0[a]	110.7[a]
	DAT72	0.9492	0.0066	0.0321	123.2[a]	123.5[a]	124.1[a]	123.8[a]
	DAT85	0.1958	0.0033	<0.0001	129.0[a]	129.6[a]	126.9[a]	128.0[a]
	DAT107	<0.0001	0.0323	0.2359	148.9[a]	145.9[b]	142.0[c]	144.7[bc]
SPAD value	DAT66	0.0343	0.3559	0.0003	40.9[a]	40.8[ab]	38.9[b]	40.6[ab]
	DAT95	0.2894	0.1464	<0.0001	39.9[a]	39.2[a]	38.1[a]	39.1[a]
	DAT115	<0.0001	0.3160	<0.0001	37.3[a]	38.1[a]	31.2[c]	34.9[b]
	DAT133	<0.0001	0.2848	0.6594	36.3[a]	36.2[a]	33.6[c]	35.0[b]
Stomatal conductance (mmol m^{-2} s^{-1})	DAT93	0.0526	0.0007	<0.0001	316.5[a]	295.6[ab]	236.3[b]	278.0[ab]
	DAT106	<0.0001	0.0030	0.0531	418.4[a]	424.8[a]	276.4[b]	395.7[a]
	DAT133	0.0021	0.6883	0.1729	379.1[a]	351.6[a]	249.6[b]	313.5[ab]
	DAT148	0.0005	0.6312	0.2334	399.6[a]	387.6[a]	261.8[b]	305.9[b]
NDVI		<0.0001	0.3530	0.8331	0.7[a]	0.7[a]	0.6[b]	0.7[a]
SR	DAT109	<0.0001	0.0942	0.8174	7.4[a]	7.4[a]	5.3[b]	6.6[a]
PRI		<0.0001	0.0030	<0.0001	0.05[a]	0.05[a]	0.02[c]	0.04[b]
ARI		<0.0001	0.0003	<0.0001	0.1[c]	0.2[c]	0.6[a]	0.4[b]

LS means = least square means; DAT = days after transplanting. n.d. = not determined. LS mean values not sharing the same superscript letter are significantly different from each other at *P<0.05* Tukey-Kramer post-hoc comparison. NDVI=Normalized difference vegetation index, SR=Simple ratio index, PRI=Photochemical reflectance index, and ARI = Anthocyanin reflectance index. After DAT 127, all measurements were done only for NB and L81 genotypes because BR28 had already been harvested.

Table 2
Statistical analysis and treatment mean values (mixed model analysis using SAS 9.3) of yield components from three different rice genotypes exposed to ozone and control conditions with or without the application of EDU.

Traits	ANOVA Results (Pr > F)			LS means (Treatment)			
	Treatment	Genotype	Interaction	Control	Control + EDU	Ozone	Ozone + EDU
Panicle number (plant^{-1})	0.0003	0.0278	0.0480	8.8[a]	9.0[a]	7.9[b]	8.5[ab]
Grain yield (t ha^{-1})	<0.0001	0.0026	0.0020	4.9[a]	5.0[a]	3.6[b]	4.1[b]
Spikelet sterility (%)	0.0135	0.0081	0.5874	7.3[b]	9.0[ab]	13.1[a]	9.0[ab]
Straw yield (t ha^{-1})	0.0040	0.0006	0.4207	6.5[a]	6.8[a]	5.5[b]	5.9[ab]
Grains per panicle	0.0142	0.0009	0.3140	117.6[a]	112.1[ab]	101.4[b]	104.0[ab]
Thousand kernel weight (g)	0.0010	0.0069	0.5343	18.5[a]	19.2[a]	17.5[b]	18.4[ab]

LS means = least square means. LS mean values not sharing the same superscript letter are differ significantly from each other at *P<0.05* by Tukey-Kramer post-hoc comparison.
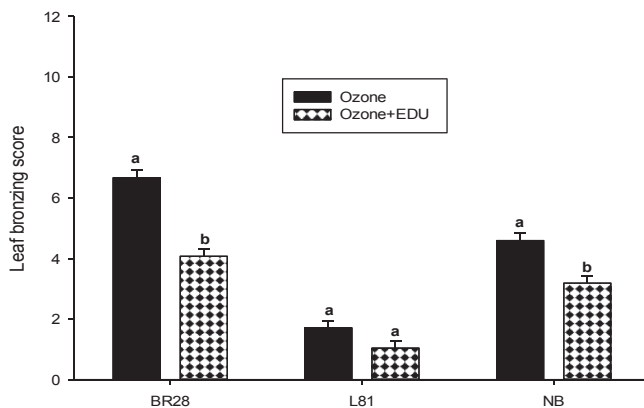

Fig. 1. Leaf bronzing scores of three rice genotypes in ozone stress with or without EDU application. Bars indicate mean value ± standard errors (n = 27). The letters above of the bars indicate pair-wise comparison (*P<0.05*) within the genotype (mean values not sharing the same letter are significantly different).
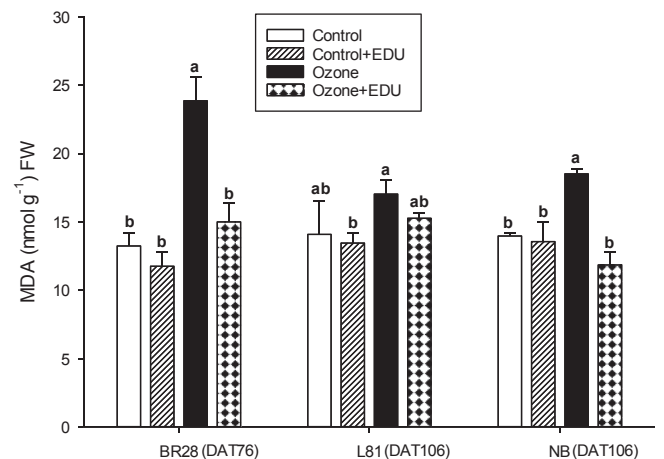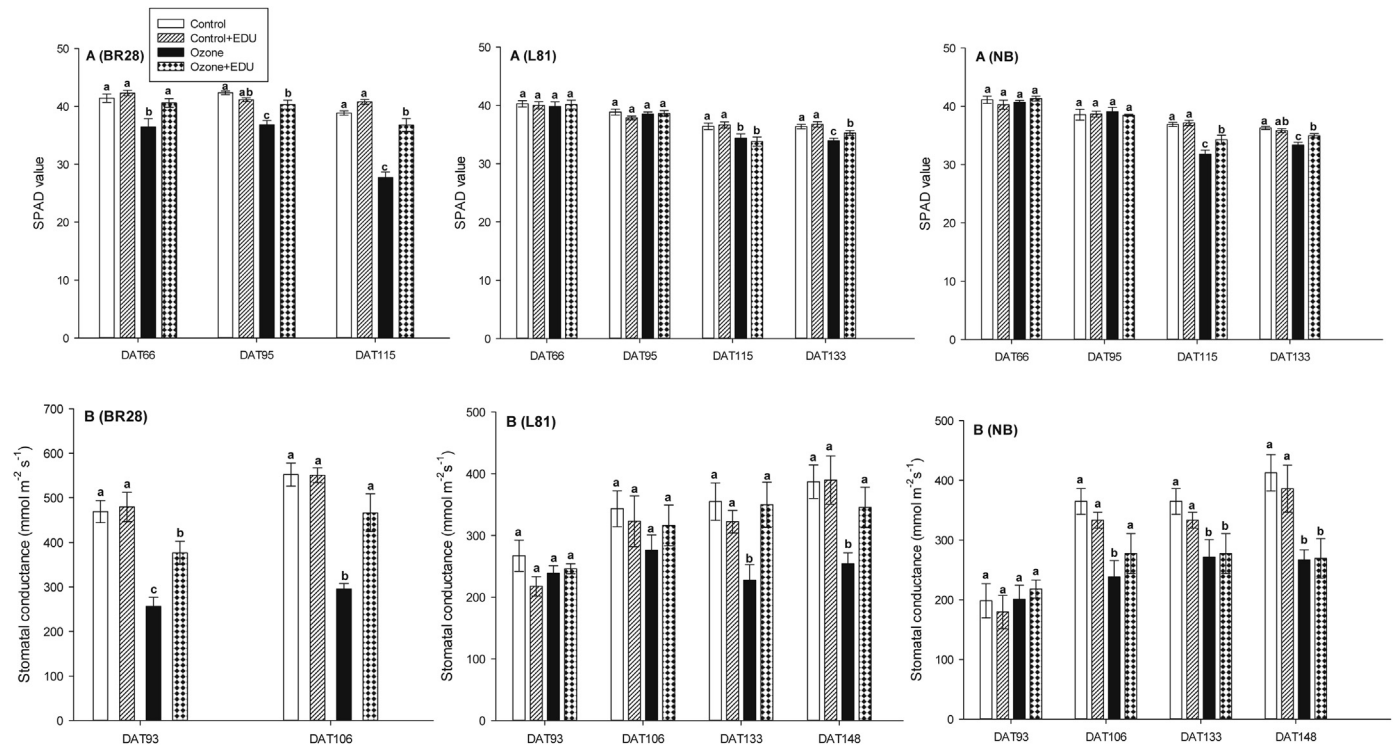

Fig. 2. Malondialdehyde (MDA) concentrations in leaves of three rice genotypes exposed to ozone or control conditions with or without the application of EDU. Bars indicate mean value ± standard errors (n = 3), fresh weight (FW). Y axis represent the genotype name along with sampling day (days after transplanting = DAT). Samples were taken when first symptoms appeared in each of the genotypes. The letters above of the bars indicate pair-wise comparison (*P<0.05*) within the genotype (mean values not sharing the same letter are significantly different).

mitigating effect of EDU in panicle number and grain yield. Similarly, the sensitive NB showed significant differences between control and ozone treatment (without EDU) in all yield components
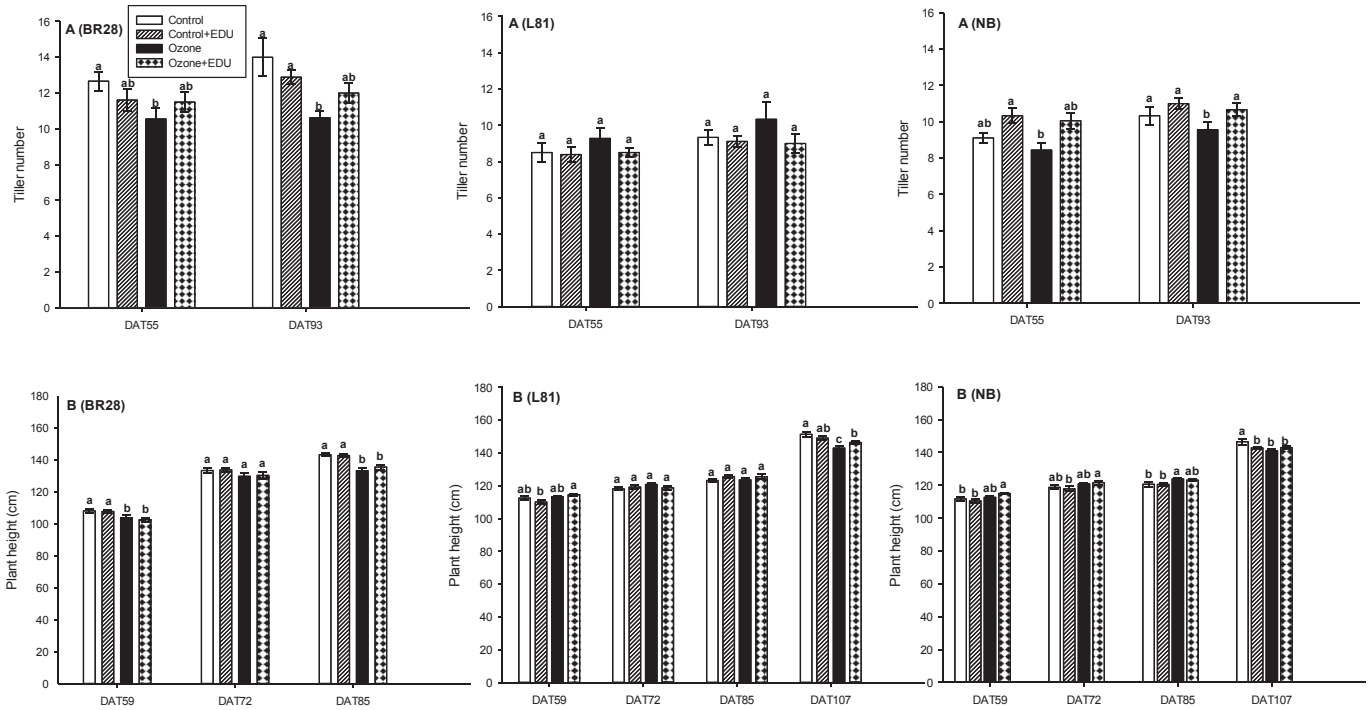
Fig. 3. Growth parameters of three rice genotypes exposed to ozone and control conditions with and without the application of EDU. Y axis represents different days after transplanting = DAT and bars indicate mean value ± standard errors (n = 18). Upper figure (A) and lower (B) represent tiller number and plant height respectively along with the respective genotype name. The letters above the bars indicate pair-wise comparison ($P<0.05$) within the genotype (mean values not sharing the same letter are significantly different). Plant height was not measured in BR28 at DAT107 because plants were maturing and tending to shed grains.

Fig. 4. Physiological parameters of three rice genotypes exposed to ozone and control conditions with and without the application of EDU. Y axis represents different days after transplanting = DAT and bars indicate mean value ± standard errors (SPAD and stomatal conductance, n = 9 and 3 respectively). Upper figure (A) and lower (B) indicate SPAD value and stomatal conductance along with the respective genotype name. The letters above the bars indicate pair-wise comparison ($P<0.05$) within the genotype (mean values not sharing the same letter are significantly different). Measurements were not taken from BR28 on DAT 133 and 148 because plants had already been harvested.
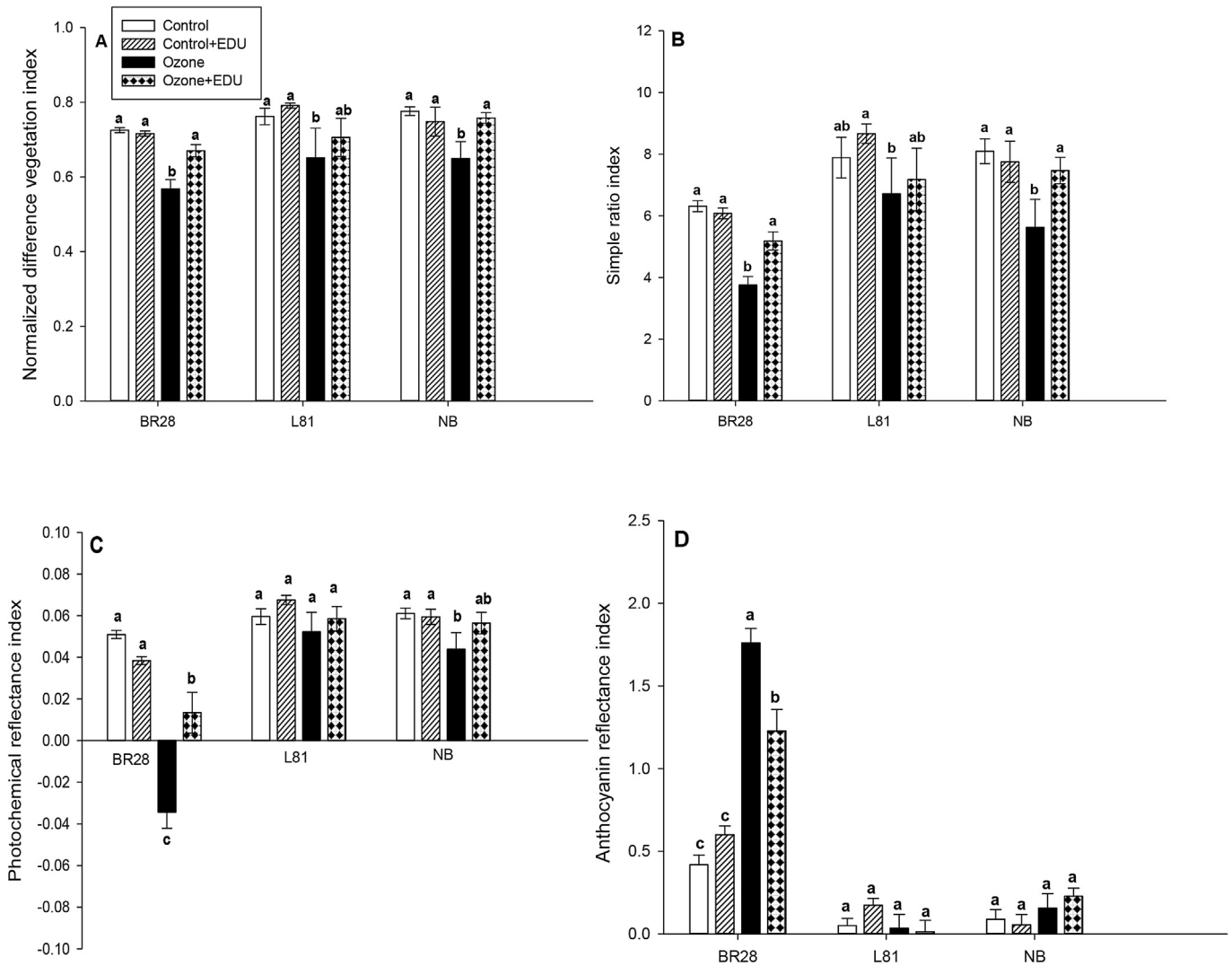
Fig. 5. Vegetation and photosynthetic light use efficiency indices (physiological parameters) based on the reflectance spectra of three rice genotypes exposed to ozone and control conditions with and without the application of EDU. Y axis represents different indices and bars indicate mean value ± standard errors (n = 9), X axis represents different rice genotypes. Letters above the bars indicate pair-wise comparison (*P<0.05*) within the genotype (mean values not sharing the same letter are significantly different).

except straw yield and thousand kernel weight, while EDU significantly mitigated ozone-induced effects on panicle number, grain yield and spikelet sterility (Fig. 6). In contrast, the tolerant L81 neither showed any significant ozone response in any of the measured yield component, nor any effect of EDU application (Fig. 6). Taken together, the analysis of yield components clearly confirmed the *a priori* tolerance ranking of the genotypes used in this study. It also showed that EDU might be suitable for differentiating between ozone sensitive (*i.e.* EDU responsive) and ozone tolerance (*i.e.* not EDU responsive) genotypes in field screening conditions, where ozone-free control treatments are lacking.

4. Discussion

4.1. Ozone levels and EDU concentration used in this study

In the present study, the average elevated ozone (O_3) concentration was 77 ppb during the 7 h treatment period (9.00 h–16.00 h). Many Asian rice growing countries including

China, India and Bangladesh are already experiencing ozone levels exceeding this concentration in ambient field conditions during cropping seasons due to increasing levels of ozone precursor pollutants (Ainsworth, 2016; Brauer et al., 2016). Currently, China is the largest emitter of the ozone precursor gas NOx in Asia and the daily 24 h average ozone concentration reaches more than 50 ppb in some regions during the crop growing season (Tang et al., 2013; Feng et al., 2015). In India, 8 h daily average concentrations of 100 ppb ozone have been reported earlier during the spring crop growing season (Roy et al., 2009). It was also estimated that in 2013, the daily maximum average ozone concentration was 72 ppb in Bangladesh (Brauer et al., 2016). A recent review of Frei (2015) clearly demonstrated that all rice growing seasons on the Indian subcontinent are at high risk of ozone damage. However, to our knowledge, no information is available about ozone impacts on crop production in Bangladesh and other rural areas due to the lack of infrastructure and electricity facilities (Oksanen et al., 2013; Kobayashi, 2015). Ethylenediurea (EDU) has been widely used for a long time in ambient fields to diagnose the effect of ozone
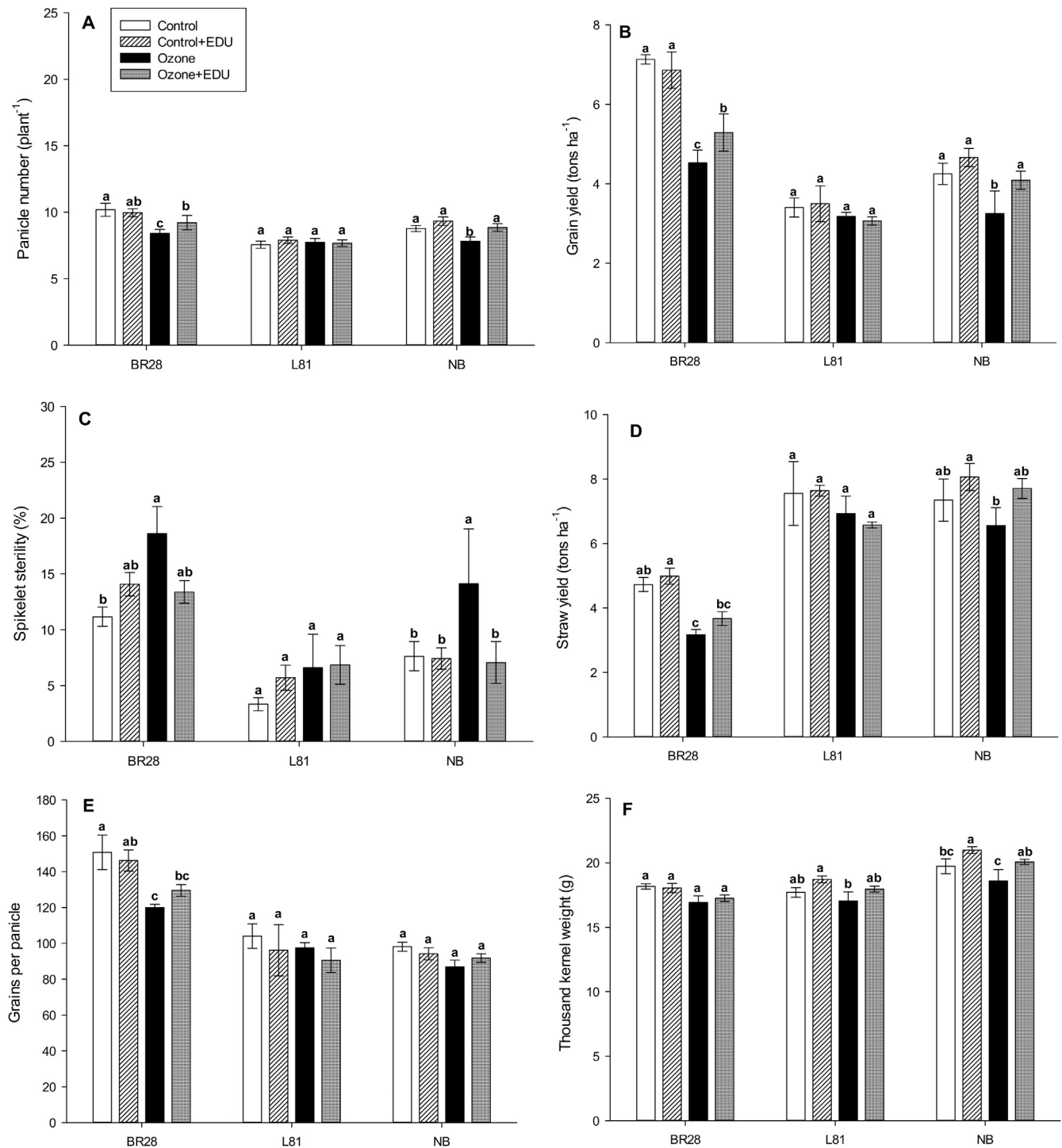
Fig. 6. Yields and yield components of three different rice genotypes exposed to four different treatments (control with and without ethylene diurea (EDU), ozone with and without EDU). Bars indicate mean value with ± standard errors (n = 3 for all traits except panicle number n = 27). The letters above of the bars represent pair-wise comparison at $P<0.05$ (mixed model analysis using SAS 9.3) within the genotype against treatments (mean values not sharing the same letter are significantly different).

(Manning et al., 2011; Agathokleous et al., 2015b). Foliar application is widely used for cereal crop species including rice because of the simplicity, reliability and effectiveness (Feng et al., 2010). A recent toxicological study of EDU revealed that 300 ppm concentration can be used effectively without any toxic effects (Agathokleous et al., 2016), whereas higher concentrations of EDU can have adverse effects on plants (Elagöz and Manning, 2005; Manning et al., 2011; Agathokleous et al., 2016). Taking into consideration

all previous findings, we sprayed EDU at 300 ppm concentration in rice plants at seven day intervals in presence and absence of ozone.

4.2. Effects of EDU on plants grown without and with ozone stress

The EDU approach assumes that the chemical alleviates ozone effects on crops, while having no constitutive effects on plants. However, most EDU experiments were conducted in the field with

ambient ozone conditions, lacking an ozone-free control (Manning et al., 2011). One major concern is whether EDU, a nitrogen containing compound, has any fertilization and/or growth regulating activity in plants irrespective of ozone. It contains around 22% of nitrogen (Godzik and Manning, 1998). Consequently, the amount of EDU applied in this study contained 6.9, 8.9 and 9.9 g nitrogen, equivalent to 1.9, 2.4 and 2.7 kg N ha^{-1} in the genotypes BR28, L81 and NB respectively. This amount of nitrogen via EDU is small compared to the nitrogen fertilizer (60 kg N ha^{-1}) applied as urea, suggesting that a foliar fertilizing effect can probably be neglected. This assumption is confirmed by the lack of response to EDU in the absence of ozone in most of the physiological or yield variables. Among 26 variables, significant differences between the control and control + EDU treatment were seen only in one variable (plant height, DAT107) (Table 1). None of the traits were significantly affected by EDU in BR28 and L81 in control conditions, while merely a slight plant height reduction (DAT107) and increase of thousand kernel weight was observed in NB in control plants with EDU treatment (Fig. 3B and Fig. 6F). Therefore, our experimental results indicated barely any constitutive effects of the applied EDU dose, and justify its use in studying specific ozone effects in rice as previously confirmed in potato (Foster et al., 1983) and tobacco plants (Godzik and Manning, 1998).

In contrast, EDU application had significant effects on a number of physiological, growth and yield traits in presence of ozone. For example, a 37 percent reduction of visible leaf injury and a 29 percent decrease in MDA concentration were observed (Supplementary Table S1). In snap bean around 20 percent of ozone caused foliar injury was offset by EDU application in ambient fields (Yuan et al., 2015). Similarly, EDU significantly mitigated ozone-induced lipid peroxidation in several crop species such as wheat (Singh et al., 2009), carrots (Tiwari and Agrawal, 2010), soybean (Rai et al., 2015) and the vegetative phase of rice (Pandey et al., 2015) in ambient fields. Although the actual function of EDU in protecting plants from ozone injury is still unclear, it was suggested that both direct effects on physiological parameters and indirect effects on the capacity to scavenge ROS may play a vital role (Tiwari and Agrawal, 2009; Pandey et al., 2014). In terms of growth parameters, EDU had positive effects on plant height in the ozone treatment, which confirms previous findings in mung bean (Agrawal et al., 2005) and tropical soybean (Rai et al., 2015) grown in an ambient rural site of India. In addition EDU stimulated tiller number in stress conditions similar to observations made in wheat (Tiwari et al., 2005). Also, stomatal conductance was enhanced by EDU application in the later growth phase (Fig. 4B). Stomatal responses to ozone have been interpreted as a response to inhibited carbon assimilation due to damage of photosynthetic enzymes (Paoletti and Grulke, 2005), or as direct involvement of ROS in stomatal aperture (Sierla et al., 2016). Because the positive effects of EDU occurred rather late in the development of the plants in our study, it seems more plausible that EDU mitigated chronic damage of the photosynthetic apparatus.

We also employed several non-invasive phenotyping techniques to estimate ozone and EDU effects on foliar pigments. Although vegetation indices are extensively used on the plant community or ecosystem level, they can also be employed at the individual plant level to detect early environmental stresses and leaf pigment status through non-destructive measurements (Sims and Gamon, 2002; Meroni et al., 2008, 2009; López-López et al., 2016). Leaf greenness is highly correlated with chlorophyll concentration and was represented in our measurements taken with a chlorophyll meter (SPAD), as well the spectral reflectance indices NDVI and SR (Sims and Gamon, 2002). Ozone-induced loss of chlorophyll in rice was consistent with previous measurements using destructive analytical techniques by Wang et al. (2014), while EDU application

mitigated ozone-induced chlorophyll degradation in our study (Fig. 4A and Fig. 5A, B). Another class of pigments affected by ozone is carotenoids. The PRI can be interpreted as an indirect estimation of chlorophyll/carotenoid concentration and photosynthetic radiation use efficiency (PRUE) (Garbulsky et al., 2011). Our results using rice thus confirm previous reports, where EDU had a positive effect on leaf chlorophyll and carotenoid concentrations in ozone-affected soybean (Rai et al., 2015) and wheat (Singh et al., 2009) in ambient fields. Anthocyanins are another class of photo-protective pigments that are often induced as a result of environmental stresses, including ozone (Foot et al., 1996; Gitelson et al., 2009). Their foliar concentration can be estimated non-destructively by employing the ARI (Gitelson et al., 2001). An increase in anthocyanins was thus particularly pronounced in the sensitive BR28, but significantly mitigated by the application of EDU (Fig. 5D). Together, these measurements suggested great potential of using remote sensing and non-destructive phenotyping techniques in detecting ozone effects on rice pigments, as well as detecting EDU responses.

As a consequence of enhanced growth and physiological traits, several yield components were positively affected by EDU application, e.g. higher panicle number (8%) and grain yield (14%). Various studies reported increased yield with the application of EDU in different crop species in ambient fields (Summarized by Singh et al., 2015), including rice (Pandey et al., 2015). Our controlled experiment including an ozone-free control allows for quantification of the EDU-induced yield loss recovery. When comparing yield losses in the ozone treatment (26% compared to control) to those in the ozone + EDU treatment (16% compared to control), a recovery of 10% of the original yield in control conditions was observed.

Taken together our data suggest a specific effect of EDU in protecting rice plants against ozone, although we cannot exclude the possibility that EDU might also protect plants from other stresses (*e.g.* salinity or drought), which were absent in our study but might occur in field conditions. A recent study suggested that EDU effectiveness against ozone was not altered by moderate drought in ambient field in sensitive poplar plants (Xin et al., 2016). Nevertheless, further research on non-specific stress-protective effects of EDU is required in the future.

4.3. Suitability of EDU to screen for ozone sensitivity/tolerance in rice

One of the objectives of this study was to test whether EDU would be a suitable tool for distinguishing ozone tolerant and sensitive rice genotypes, which could eventually be used for field-based screening and breeding. To this end, we used three rice genotypes *a priori* ranked in terms of ozone tolerance to investigate how they respond to EDU application. L81 is an ozone tolerant introgression line developed by Wang et al. (2014), containing two ozone tolerance QTLs, *OzT8* and *OzT9* (Frei et al., 2008, 2010) in the genetic background of Nipponbare. It was suggested that the pyramiding of these QTLs helps plants to maintain higher yield performance in ozone stress by enhancing the net photosynthetic rate, higher chlorophyll levels and biomass, and reductions in visible symptoms and lipid peroxidation (Wang et al., 2014). These advantages were confirmed in our present study. Furthermore, Ueda et al. (2015b), recently identified a novel gene, ozone-responsive apoplastic protein (*OsORAP1*), which was suggested to underlie the QTL, *OzT9*. Consistent with our hypothesis, contrasting responses of sensitive and tolerant genotypes to EDU application were observed with respect to growth, physiological and yield parameters. In fact, LBS, MDA, SPAD value, stomatal conductance, and spectral reflectance indices were significantly affected by EDU

application based on averaged performance of all genotypes (Table 1) in stress conditions. In addition, agronomically important traits such as panicle number, grain yield and spikelet sterility were EDU responsive in the sensitive genotypes BR28 and NB respectively, while only few traits, *i.e.* plant height, SPAD value and stomatal conductance were EDU responsive in the tolerant genotype L81 in the presence of ozone (Supplementary Table S2). Moreover, none of the yield components in the tolerant genotype L81 was affected in stress conditions by EDU application (Fig. 6). In addition, significant stress effects and mitigation by EDU application for foliar injury, lipid peroxidation, vegetation indices NDVI, SR, panicle number, and grain yield were seen only in the ozone-sensitive genotypes of BR28 and NB. According to our findings, the widely cultivated Bangladeshi mega-variety BR28 displayed the most drastic response to ozone, which was most consistently ameliorated by EDU application. This is consistent with previous studies suggesting that South Asian crop varieties are rather sensitive to ozone (Emberson et al., 2009; Feng et al., 2010). Taken together, our results thus suggest that ozone tolerance and sensitivity of different genotypes can be ranked based on EDU-responsiveness.

5. Conclusion

Ozone tolerance breeding approaches are greatly hampered by the lack of available germplasm from ozone affected areas and translation of laboratory experiments to the field (Ainsworth, 2016). The results of this study encourage the use of EDU as a tool to screen for ozone tolerance in rice, which can help to facilitate breeding schemes using large numbers of field grown plants. This can be an important step forward on the development of ozone tolerant rice varieties, which will contribute to the food security of many highly populated countries in Asia.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.06.055.

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Ethylenediurea (EDU) mitigates the negative effects of ozone in rice: insights into its mode of action

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Highlights

The application of ethylenediurea (EDU) as a foliar spray mitigated ozone stress in rice plants, without affecting their transcriptional profile directly. We concluded that EDU acts as a passive surface protectant that can be used to specifically monitor ozone damage in field experiments.

ORIGINAL ARTICLE

Ethylenediurea (EDU) mitigates the negative effects of ozone in rice: Insights into its mode of action

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Abstract

Monitoring of ozone damage to crops plays an increasingly important role for the food security of many developing countries. Ethylenediurea (EDU) could be a tool to assess ozone damage to vegetation on field scale, but its physiological mode of action remains unclear. This study investigated mechanisms underlying the ozone-protection effect of EDU in controlled chamber experiments. Ozone sensitive and tolerant rice genotypes were exposed to ozone (108 ppb, 7 hr day^{-1}) and control conditions. EDU alleviated ozone effects on plant morphology, foliar symptoms, lipid peroxidation, and photosynthetic parameters in sensitive genotypes. Transcriptome profiling by RNA sequencing revealed that thousands of genes responded to ozone in a sensitive variety, but almost none responded to EDU. Significant interactions between ozone and EDU application occurred mostly in ozone responsive genes, in which up-regulation was mitigated by EDU application. Further experiments documented ozone degrading properties of EDU, as well as EDU deposits on leaf surfaces possibly related to surface protection. EDU application did not mitigate the reaction of plants to other abiotic stresses, including iron toxicity, zinc deficiency, and salinity. This study provided evidence that EDU is a surface protectant that specifically mitigates ozone stress without interfering directly with the plants' stress response systems.

KEYWORDS

air pollution, food security, gene expression, global change, phenotyping, RNA-Seq, tolerance breeding

1 | INTRODUCTION

Increasing tropospheric ozone pollution poses a major threat to global crop production and food security (Tai, Martin, & Heald, 2014; Tai & Val Martin, 2017). Global yield losses due to ozone pollution have been estimated in a range of 4%–17% for wheat, 10%–14% for soybean, and 3%–6% for maize with a total estimated annual economic loss of US$ 12–21 billion based on global crop models (Avnery, Mauzerall, Liu, & Horowitz, 2011). Ozone was first recognized as a detrimental air pollutant in North America during the 1950s and gradually noticed in Europe and Japan in the 1970s (Haagen-Smit, 1952; The Royal Society, 2008). However, Asian countries, including Bangladesh, India, Myanmar, and Nepal, will be the most vulnerable in the upcoming decades due to rapidly growing populations and economies, and the lack of legislation to control the emission of ozone precursor gases such as nitrogen oxides (NOx), carbon monoxide, methane, and volatile organic compounds (Brauer et al., 2016; Emberson et al., 2009; Mills et al., 2018; Van Dingenen et al., 2009). Nevertheless, experimental evidence documenting crop responses and yield losses in these heavily ozone-affected areas is scarce

(Kobayashi, 2015; Oksanen et al., 2013). Rice (*Oryza sativa* L.) is the staple food crop for more than half of the world population and most widely grown and consumed in Asia (Alexandratos & Bruinsma, 2012; FAOSTAT, 2018). Around 4% of global and more than 10% of regional rice yield losses have been estimated due to ozone stress (Ainsworth, 2008; Van Dingenen et al., 2009). These yield losses will be exacerbated tremendously in the near future, as the rice cropping seasons overlap with the peak ambient ozone concentrations in South and East Asia, especially in India and Bangladesh, the second and fourth largest rice-producing countries globally (FAOSTAT, 2018; Frei, 2015). Therefore, it is of paramount importance to explore ozone tolerance and breeding options in rice to reduce yield losses as an effective strategy to secure food supply regionally, especially in Asia but also on the global scale.

Crop damage by ozone occurs directly through ozone-induced oxidative stress (Ainsworth, 2017). Plants take up ozone into the apoplast primarily through the stomata, where it rapidly degrades and generates reactive oxygen species (ROS) that interfere with various physiological and enzymatic processes (Kangasjärvi, Jaspers, & Kollist, 2005; Krasensky, Carmody, Sierla, & Kangasjärvi, 2017). Ozone impacts on crop plants are often investigated by using open-top chambers (OTC) or free air concentration enrichment with ozone (FACE-O_3; Feng, Uddling, Tang, Zhu, & Kobayashi, 2018; Frei, 2015). Both experimental systems require infrastructure and reliable electricity supply that is often not available in field locations of ozone-affected developing countries (Kobayashi, 2015; Oksanen et al., 2013). In addition, both experimental systems have their inherent weaknesses. OTC systems are usually small in dimension and therefore unsuitable for larger scale field experiments (Macháčová, 2010). In addition, they affect the microclimate plants are exposed to and may thus entail a "chamber effect" (Morgan, Mies, Bollero, Nelson, & Long, 2006; Piikki, De Temmerman, Högy, & Pleijel, 2008). FACE experiments can help to overcome these limitations, but they are more expensive, technically challenging to maintain, and can only compare between current ambient and elevated ozone concentrations (Frei, 2015; Pleijel, 2011). Moreover, a recent meta-analysis revealed that the assessment of crop sensitivity towards ozone can differ between OTC and FACE experiments (Feng et al., 2018). As an alternative research tool, the chemical compound ethylenediurea (EDU; N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N′-phenylurea), first introduced by Carnahan, Jenner, and Wat (1978), has been used for a long time to assess ozone impacts on plants (Feng, Wang, Szantoi, Chen, & Wang, 2010; Manning, Paoletti, Sandermann, & Ernst, 2011; Paoletti, Contran, Manning, & Ferrara, 2009). Its major advantage is the simplicity of application, as it is just repeatedly sprayed on leaves, which is assumed to protect plants from ozone damage, thereby providing a "control" without ozone damage. However, although EDU has long been established and numerous studies have provided evidence of its ozone-alleviating effect in diverse plant species, it has barely been critically tested in controlled conditions, and its mode of action is not well-understood (Agathokleous, 2017; Manning et al., 2011). To our knowledge, no study has analysed transcriptomic responses of any plant species to EDU in order to survey its physiological effects on plants.

A number of previous studies have reported transcriptomic responses of plants to ozone. A large number of genes involved in programmed cell death (ethylene or jasmonic acid biosynthesis, mitogen-activated protein kinase, and disease resistance) and detoxification of ROS (catalase and peroxidase) were identified as ozone responsive in a comparative microarray study using two chromosome segment substitution rice lines (Frei et al., 2010). More recent studies employed high-throughput sequencing techniques (RNA-Seq) to monitor transcriptomic responses to ozone. This led to the identification of ozone responsive genes and pathways associated with photosynthesis and respiration, oxidative stress, defence response, protein ubiquitination, and organic acid biosynthesis in soybean (Waldeck et al., 2017), cellular integrity, and metabolism (ascorbate (AsA)-glutathione metabolism, hormone, wax, and cutin biosynthesis) in pak choi (Zhang et al., 2017) and antioxidant metabolism (AsA-glutathione cycle genes) in legume crops (Yendrek, Koester, & Ainsworth, 2015). It is thus obvious that large numbers of genes are ozone responsive in different plant species. Fully quantitative transcriptome sequencing techniques such as RNA-Seq offer an excellent tool to explore global physiological responses to external stimuli such as ozone, EDU application, and their interaction.

In order to consolidate the use of EDU as a research tool in future studies, specifically in developing countries, this study aimed at unravelling the mode of action of EDU as an ozone-protectant based on transcriptome sequencing and a series of related experiments. To this end, a two-factorial experiment was conducted, in which an ozone-sensitive rice genotype was exposed to ozone with and without EDU application. Two alternative hypotheses were investigated: (a) If EDU had any direct effects on the activation of stress defence pathways, this would manifest in altered expression patterns of the associated genes. (b) Alternatively, if the protective effect of EDU was rather as a passive surface protectant, EDU would not directly affect gene expression patterns but rather mitigate the effects of ozone on the activation of stress response pathways.

2 | MATERIALS AND METHODS

2.1 | Experimental conditions

All experiments were carried out in greenhouses of the University of Bonn, Germany. Seeds were germinated at 30 °C in the dark. The seedlings were then transferred to a mesh floating on solutions containing 0.5 mM $CaCl_2$ and 10 μM $FeCl_3$ and placed under natural light in the greenhouse. After growing for 2 weeks, the seedlings were transplanted into 60-L plastic containers filled with half-strength modified Yoshida solution (Yoshida, Forno, Cock, & G. K., 1976). Transplanted seedlings were maintained in half-strength solution for 1 week, which was subsequently replaced weekly with full-strength solutions. The pH was adjusted twice a week to 5.5. Supplementary lighting was provided in the greenhouse from 7 a.m. to 8 p.m. every day to ensure a minimum photosynthetic photon flux density (PPFD) of 300 μmol m^{-2} s^{-1}. The minimum temperature of the greenhouse was set to 28/22 °C (day/night). Four different treatments were implemented: (a) control, (b) control+EDU, (c) stress, and (d) stress +EDU. In all experiments, at the time of stress treatments, half of the control plants and half of the stressed plants were sprayed with

300 ppm EDU once a week, as suggested in previous studies (Ashrafuzzaman et al., 2017; Pandey et al., 2015). Weekly intervals were selected to ensure a continuous effect of EDU, which can persist in the leaf apoplast for 8 days or more without entering the cell (Gatta, Mancino, & Federico, 1997; Paoletti et al., 2009). For this, approximately 75 mg EDU (250 ml of EDU solution) were sprayed weekly per 0.24 m^2 area (one container) for complete saturation of canopies. The same volume of water was sprayed on the non-EDU-treated plants instead of EDU.

2.2 | Plant materials

Five different rice genotypes were used in the three different experiments. Experiment 1 was carried out using two ozone sensitive rice genotypes, Nipponbare (NB) and Bangladesh Rice Research Institute dhan28 (BR28; Akhtar et al., 2010; Ashrafuzzaman et al., 2017; Jing et al., 2016), an ozone tolerant introgression line L81 (Wang et al., 2014), and a submergence tolerant genotype Bangladesh Institute of Nuclear Agriculture Dhan 11 (BINA11; IRRI, 2016). BR28 and BINA11 are Bangladeshi high-yielding modern rice varieties developed by the Bangladesh Rice Research Institute and Bangladesh Institute of Nuclear Agriculture, respectively, and NB is a Japanese japonica rice variety. The ozone tolerant L81 genotype carries introgressions of two ozone tolerant quantitative trait loci from the Aus landrace Kasalath (Frei et al., 2010; Frei, Tanaka, & Wissuwa, 2008) in the genetic background of NB. The in-depth physiological and transcriptome studies (RNA-Seq) in Experiment 1, and scanning electron microscopy (SEM), were conducted with the ozone sensitive and EDU-responsive genotype BR28 (Ashrafuzzaman et al., 2017). In Experiment 3, NB and BR28 genotypes were used along with world's most popular and widely grown rice variety IR64 (Ballini et al., 2007) in multiple stresses with and without the application of EDU.

The specific experiments were conducted as detailed below.

2.3 | Experiment 1

This experiment was carried out during the months of March and April 2017. The measured average daytime (7 a.m. to 8 p.m.) and night-time (8 p.m. to 7 a.m.) temperatures were 28 and 21 °C, average relative humidity was 41% and 54%, respectively. Four independent chambers were assigned to elevated ozone treatment, and control plants were placed in four identical chambers without ozone fumigation to ensure the same microclimate in both treatments. Plants were fumigated for 8 days at a target ozone concentration of 110 ppb. The recorded average daytime (9 a.m. to 4 p.m.) ozone concentration was 108 ± 0.46 ppb (average ± standard error) in the ozone treatment, whereas the average concentration in control (nonfiltered air) conditions was 37 ± 0.29 ppb. Control plants were exposed to ambient ozone concentrations, but in the control conditions, the ambient ozone concentrations were maintained below the damage threshold level (40 ppb). Two randomly selected plants of each genotype from each treatment chamber were collected, and shoots (whole plant without root) were pooled for one representative sample. Then, the samples were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction (transcriptome analysis) and biochemical analyses.

The collection of samples was performed in 1 day between 10 a.m. and 12 p.m. after the second round of EDU application (approximately 16 hr after EDU application). The samples were collected within 24 hr of EDU application because it is suggested that EDU can effectively inhibit ozone-induced ROS generation within 24 hr, which ultimately prevents leaf lesions formation (Paoletti et al., 2014).

Visible leaf symptoms of ozone stress were quantified as leaf bronzing score (LBS) ranging from 0 (no damage) to 10 (dead leaf), which was assigned to three fully expanded leaves (lower to higher) of each plant separately (Ueda et al., 2015). Tillers were counted before sample collection and harvesting, at which time shoot and root length was determined. Shoot samples were then placed in an oven at 60 °C for at least 72 hr for complete dryness, and dry weight was measured. Leaf nitrogen concentration was determined using an Elemental Analyser EuroEA 3000 Series (HEKAtech GmbH, Wegberg, Germany) after grinding dried samples to a fine powder.

2.4 | Experiment 2

This was carried out in the months of September and October 2017. The measured average daytime and night-time temperatures were 29 and 22 °C, and average relative humidity ranged from 42% to 55%, respectively. Two independent chambers were fumigated with elevated ozone, and control plants were placed in identical chambers without ozone fumigation. Plants were fumigated for 16 days at a target ozone concentration of 110 ppb. The measured average daytime (9 a.m. to 4 p.m.) ozone concentration was 107 ± 0.44 ppb in the ozone treatment whereas the average concentration in control conditions was 17 ± 0.33 ppb.

2.5 | Ozone treatment and monitoring

Ozone fumigation was conducted in OTC (1.5-m width × 1-m length × 1.3-m height) and (1-m width × 1-m length × 1.3-m height) covered by transparent plastic sheets (Ueda, Siddique, & Frei, 2015). Ozone was generated by using custom-made ozone generators (UB 01; Gemke Technik GmbH, Ennepetal, Germany) after drying air with silica gel. The generated ozone was first percolated through water to remove nitrogen oxides, and then ozone-enriched air was blown into the chambers and evenly distributed via perforated plastic tubes running above the plant canopy. The ozone output was regulated by an ozone monitor (K100 W; Dr. A. Kuntze GmbH, Meerbusch, Germany) and detected by an ozone sensor (GE 760 ozone; Dr. A. Kuntze GmbH, Meerbusch, Germany) placed inside the fumigation chambers. In addition, the ozone concentrations in different areas of the chamber were monitored with an independent handheld ozone monitor (series 500; Aeroqual Ltd. Auckland, New Zealand) at 2-min intervals.

2.6 | Experiment 3

This was carried out in the months of September and October 2017. The measured average daytime and night-time temperatures were 27 and 18 °C, respectively; average relative humidity was 40% (day) and 65% (night). Different stresses, that is, salt stress, iron toxicity, and zinc deficiency, were assigned to separate hydroponic tanks with and without the application of EDU including two replications per

treatment. EDU was applied in weekly intervals at 300 ppm concentrations as described in the experimental conditions section. During the first week, a half-strength nutrient solution was used, which was subsequently replaced with full-strength solutions. Seedlings were grown for 1 week on the full-strength solutions, and then the treatments were started. Plants were harvested after 14 days of salt and iron stress treatment, whereas the zinc deficiency treatment was continued for 21 days.

Leaf greenness was measured using a SPAD 502 instrument (Konica Minolta, Osaka, Japan) in three different points at 20-cm distance from the tip of the youngest fully expanded leaf of three randomly selected plants from each treatment, and the average of the three points was calculated. An LBS ranging from 0 to 10 was used to score the five fully expanded leaves (lower to higher) of each plant separately, and the average values were calculated to evaluate the leaf symptoms due to iron surplus and zinc deficiency stress (Höller, Meyer, & Frei, 2014; Wu et al., 2014). Visible leaf symptoms caused by salt stress was quantified using a modified salt scoring system (0 to 10) in an average of five fully expanded leaves (lower to higher) of each plant (Gregorio, Senadhira, & Mendoza, 1997). In both scales, the criteria were as follows: 0 indicated no stress symptoms in any part of the leaf, whereas 2, 4, 6, 8, and 10 define leaves with approximately 20%, 40%, 60%, 80%, and 100% damage due to the stress, respectively. At the time of harvesting, tiller numbers, shoot, and root length were measured. Shoot and root samples were oven-dried at 60 °C for at least 72 hr and weighed.

2.6.1 | Salt treatment

Salt (NaCl) treatment was applied in the salt setup after 7 days of growth of the plants with full strength nutrient solution in hydroponics. A stepwise 50-mM NaCl was added per day in the salt containers to reach the final salt concentration of 100 mM NaCl to avoid osmotic shock for the plants. The electrical conductivity of the salt-treated nutrient solution was maintained at 10 dSm^{-1}, and the treatment was continued for 14 days.

2.6.2 | Iron treatment

After 7 days of growth in full-strength nutrient solution in hydroponics, iron treatment was started with 300 ppm Fe^{2+} (as $FeSO_4.7H_2O$) over 14 days. In order to prevent Fe^{2+} from reoxidation and precipitation, solutions were percolated with N_2 gas for 15 min every 2 hr (Wu et al., 2014).

2.6.3 | Zinc (Zn) deficiency treatment

Zn deficiency treatment was started after the transfer of plants in hydroponics with half-strength nutrient solution containing no Zn and control with Zn. After 1 week, the nutrient solution was exchanged for full strength solution weekly without Zn (−Zn) for Zn deficiency treatment and control with Zn (+Zn). Double deionized purified water was used for Zn deficiency treatment to avoid any Zn contamination in the water. The treatment was continued for 21 days before harvesting the plants.

2.7 | Photosynthetic parameters

Midday ambient carbon assimilation rate/net photosynthesis (A), stomatal conductance (gs), and A/Ci curves were measured on the second youngest fully expanded leaf of each plant with at least three experimental replicates (Chen, Frei, & Wissuwa, 2011) for each treatment in Experiment 1, on days 6 and 7 after the start of ozone fumigation. All measurements were carried out on sunny days between 10 a.m. and 2 p.m. by using a portable photosynthetic gas exchange system (LI-6400XT, portable photosynthetic system, LI-COR, Inc., Lincoln, Nebraska, USA). Leaves were measured under a (PPFD) of 700 $\mu mol\ m^{-2}\ s^{-1}$, a CO_2 reference value of 400 ppm, a leaf temperature of 28 °C and relative humidity between 60% and 70%. For CO_2 response curves, net photosynthetic carbon assimilation rates (A) were measured under different intercellular CO_2 concentrations as follows: 400, 200, 100, 50, 400, 500, 600, 700, 800, and 1000 ppm (Chen et al., 2011). The equation of the A/Ci curve fitting model of Sharkey (2016) was used to calculate the maximum carboxylation rate of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; Vcmax) and the maximum electron transport rate (Jmax) by plotting A versus leaf intercellular CO_2 (Ci) concentrations.

2.8 | Biochemical analyses

The biochemical analyses were conducted in Experiment 1. The amount of malondialdehyde as an indicator of oxidative stress was determined as described previously (Hodges, DeLong, Forney, & Prange, 1999; Höller et al., 2014). Extraction was performed from approximately 100 mg of ground tissues with 1.5 ml of 0.1% (w/v) trichloroacetic acid (TCA). Samples were then centrifuged at 4 °C and 20.000 g for 20 min, and the supernatants were divided into two aliquots of 400 μl. These were mixed with the same amount of 20% (w/v) TCA and 0.01% (w/v) 2,6-di-tert-butyl-4-methylphenol, and 0.65% (w/v) thiobarbituric acid was added in one aliquot. The mixture was then heated to 95 °C for 30 min, and the absorbance was measured at 440, 532, and 600 nm. For the blank samples, 0.1% (w/v) TCA solution was added instead of sample supernatant, and the absorbance was subtracted from each sample value.

AsA analysis was performed according to Ueda, Wu, and Frei (2013). The reduced AsA content was determined with the addition of 10 μl of 0.01 units μl^{-1} AsA oxidase in a mixture of 10 μl of extracted sample and 80 μl of 0.1 M potassium phosphate buffer (pH 7.0) at 265-nm wavelength. The oxidized AsA content was measured at 265-nm wavelength with the addition of 10 μl of 4 mM dithiothreitol, a reducing agent in a mixture of 10 μl of extracted sample and 80 μl of 0.1-M potassium phosphate buffer (pH 7.8). Finally, AsA content was calculated on the basis of the extinction coefficient, ε, of 14.3 $mM^{-1}\ cm^{-1}$.

2.9 | Scanning electron microscopy

A first round of scanning electron microscopy (SEM) was conducted with samples collected 1 and 7 days after the EDU application (8 and 15 days after ozone fumigation) in Experiment 2. The measurements were performed on the upper leaf surface of the third youngest fully expanded leaf from the main tiller. Scanning electron imaging was

carried out on fresh, hydrated samples using a Cambridge Stereoscan S200 SEM (Cambridge Instruments, UK), equipped with secondary electron and backscattered electron detectors. Around 5 to 10 mm of the selected leaf pieces were cut out and mounted on SEM stubs with a conductive adhesive tab, then the pieces were sealed with conductive carbon glue. The SEM stubs with the samples were put in a Sputter Coater (SCD 040, Balzers Union, Liechtenstein) and coated with an approximately 30-nm thin layer of palladium. Then, the samples were inserted into the SEM for imaging. Crystalline EDU images were also collected by evaporating 1% EDU solution applied onto a glass slide.

Another round of SEM was performed using plants grown in hydroponic tanks for 2 weeks with three different treatments, that is, foliar spray of 600 ppm EDU, 300 ppm EDU, or water. SEM images were taken with four experimental and two analytical replicates 1 and 7 days after EDU applications on the upper leaf surface of second youngest fully expanded leaves of main tiller. Quantifications of amorphous EDU deposits were performed using images with 30× magnification and 0.06 cm^2 fixed leaf area.

2.10 | Transcriptome profiling

2.10.1 | RNA isolation and sample preparation

Two plants of BR28 genotype were selected randomly from each treatment chamber and shoots (whole plants without roots) were pooled together for one representative sample. RNA was extracted from three replicates per treatment, resulting in 12 samples in total. RNA extraction, purification, and quality determination from the pooled shoot samples were performed according to De Abreu Neto, Hurtado-Perez, Wimmer, and Frei (2016). Total RNA was extracted with a PeqGOLD Plant RNA extraction kit (Peqlab, Erlangen, Germany) including RQ1 DNase (Promega) treatment. For the determination of quantity, quality, and purity, the extracted samples were first analysed with Nanodrop 2000c (Thermo Fisher Scientific Inc., Wilmington, DE, USA), and integrity test was performed using 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA integrity number values >8.4 were subjected to transcriptome analyses (Figure S1).

2.10.2 | cDNA library construction and Illumina sequencing

cDNA libraries for Illumina sequencing were constructed according to the manufacturer (TruSeq DNA Sample Prep Kit V3; Illumina). For the quantification and qualification of the sample libraries, Agilent 2100 Bioanalyser and ABI StepOnePlus real-time PCR system were used. According to the manufacturer's guidelines (HiSeq 4000; Illumina), cluster preparation and paired-end read sequencing were performed. After the raw sequencing, adapter sequences were trimmed using the Trimmomatic program (Bolger, Lohse, & Usadel, 2014). Quality of the trimmed reads was checked by using the FastQC program (Andrews, 2010).

2.10.3 | Processing and mapping of Illumina sequencing reads

Raw sequencing reads were processed and subsequently mapped with CLC Genomics Workbench software (version 10.0.4). Reads with more than one mismatch in the adapter sequence were excluded, and low quality, ambiguous nucleotides of sequence ends, and adapter contaminations were removed by using quality trimming. Only ≥40-bp retained reads were further processed for analyses. The reads were initially mapped to the NB rice reference genome sequence (Os-Nipponbare-Reference-IRGSP-1.0, http://rapdb.dna.affrc.go. jp/download/irgsp1.html). To be mapped, at least 90% of each read had to fit with 90% similarity to the reference. Finally, IRGSP-1.0 gene model from the rice annotation project database (RAP-DB) were used for the gene annotation (https://plants.ensembl.org/Oryza_sativa/Info/Annotation). The expression level of each transcript was expressed as the fragments per kilobase of transcript per million mapped reads (FPKM) value, calculated on the basis of the number of mapped reads. The raw digital gene expression counts were normalized by using the following equation: FPKM = 10^9(C)/(N L), where FPKM (A) is the expression of gene A, C is the number of reads that uniquely aligned to gene A, N is the total number of reads that uniquely aligned to all genes, and L is the number of bases in gene A (Shen et al., 2014). A multidimensional scaling (MDS) analysis was conducted to display sample relationships based on the expressed genes using the plotMDS function of the Bioconductor package limma (Version 3.34.8) in R (Version 3.4.2 [2017-09-28]; Smyth, 2005). The distance between each pair of samples was calculated as the root mean square deviation for the top 500 genes with the largest standard deviations across all samples.

2.10.4 | Statistical analysis of differential gene expression

Genes were considered for the following analyses if they were represented by a minimum of five mapped reads in all three replicates of at least one sample and were declared as "expressed." Then, the total numbers of expressed genes were filtered to be expressed in at least one treatment. In a total, 23,208 rice genes were filtered with their respective expression value (FPKM) from the RNA-Seq analysis for the further statistical test. Expression data were then subjected to two alternative mixed model analyses using PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC, USA; Gibson & Wolfinger, 2004). In a one-way analysis of variance (ANOVA), treatment was set as fixed effect and chamber as a random effect. Linear contrasts between all four treatments (control, control+EDU, ozone, and ozone+EDU) were determined using the ESTIMATE statement. Second, a two-way ANOVA was conducted, in which ozone treatment, EDU treatment, and their interaction were set as fixed effects, and the chamber was set a random effect. False significances declared due to the multiple testing were corrected by determining false discovery rate (FDR) using SAS MULTTEST (Benjamini & Hochberg, 1995). Gene lists with differentially regulated genes were curated for FDR values <0.05 and <0.1. All the filtered genes with RAP-DB locus ID and annotations were further converted to the MSU rice genome annotation project database (MSU locus ID and annotations, version 7.0, http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/) by using R (Version 3.4.2) program scripts (R Core Team, 2016) and RAP-MSU_ID (http://rapdb.dna.affrc.go.jp/download/irgsp1.html) conversion file. The statistical analysis of the total filtered genes with their RAP-DB and MSU locus ID, annotations, respective expression values (FPKM), and fold changes were provided in Table S1.

All other experimental data with several genotypes were subjected to ANOVA by mixed model analysis in PROC MIXED of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The model included genotype, treatment, and their interaction as fixed effects, whereas chamber and chamber by genotype interactions as random effects (Frei, Kohno, Wissuwa, Makkar, & Becker, 2011). Mean comparisons were performed by Tukey's test for post hoc adjustment, and P values less than 0.05 were considered as significant.

2.10.5 | Data processing and gene ontology analysis

Gene ontology (GO) and pathway enrichment analysis of differentially expressed genes (DEGs) were conducted using the agriGO analysis toolkit, with the MSU locus ID and National Center for Biotechnology Information (NCBI) ID of rice as references (Tian et al., 2017). The heat map of DEGs with the gene expression value (FPKM) was performed by using ClustVis program (Metsalu & Vilo, 2015).

2.10.6 | Validation of RNA-Seq by quantitative RT-PCR

Reverse transcription and real-time PCR (300 ng of RNA) were conducted using GoScript Transcription System (Promega, Mannheim, Germany) and StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression of each sample was quantified employing the delta–delta CT quantification method (Ueda, Frindte, Knief, Ashrafuzzaman, & Frei, 2016) with analytical duplicates using at least three replicates per treatment and 18S rRNA (AK059783) as endogenous reference (Jain, Nijhawan, Tyagi, & Khurana, 2006). The efficiency of amplification of primer pairs was more than 80% (Table S2). Relative expression values from quantitative reverse transcription polymerase chain reaction (qRT-PCR) were then plotted against the FPKM values obtained from RNA-Seq analysis, and the correlation coefficient (R^2) was determined.

3 | RESULTS

3.1 | Phenotypic evaluation

After 8 days of ozone fumigation at 108 ppb in Experiment 1, sensitive rice genotypes exhibited leaf bronzing symptoms, which were significantly mitigated in three sensitive genotypes (BINA11, BR28, and NB) with the application of EDU (Figure 1a). A similar pattern was seen in lipid peroxidation, an indicator of oxidative stress (Figure 1b). We also determined the total AsA content but did not observe any significant treatment effect or treatment by genotype interaction (Figure S2). Averaged over all four rice genotypes, leaf nitrogen content did not respond significantly to the treatment, but nitrogen content was significantly reduced in ozone conditions in BR28. However, EDU application did not increase leaf nitrogen concentration in any of the investigated genotypes (Figure 1c). Tiller numbers were significantly reduced in ozone stress compared with control in all genotypes except for the tolerant L81, whereas significant mitigation of the effect on tiller number due to EDU application was seen only for the sensitive BR28 (Figure 1d). As a treatment main effect, negative ozone responses were observed for shoot length and shoot dry weight

for all rice genotypes, while EDU significantly mitigated the stress effect in shoot length for sensitive NB and BR28 genotypes (Figure 1e) and in shoot dry weight only for BR28 (Figure 1f).

Photosynthetic parameters were determined in the ozone-sensitive genotype BR28. All measured traits (midday carbon assimilation rate, stomatal conductance, Vcmax, and Jmax) were significantly affected in ozone condition compared with control (Figure 2a–d). EDU significantly alleviated negative stress effects for all measured photosynthetic parameters except for stomatal conductance compared with the ozone treatment, whereas no significant differential effects were seen between control and control+EDU treatments (Figure 2a–d). In summary, all measured biochemical, physiological, and growth parameters clearly demonstrated the effectiveness of EDU to mitigate the ozone-induced negative effects in sensitive rice genotypes. In addition, lack of significant differential response between control and control+EDU treatment in all measured traits including leaf nitrogen content suggested that EDU did not play any role as a constitutive growth promoting agent or as a nitrogen source.

3.2 | Global gene expression analysis (RNA-Seq)

BR28 was selected for transcriptome analysis, because it was highly sensitive to ozone and it responded positively to EDU application. A total of 23,208 rice genes were expressed in at least one of the four treatments and used for further analyses. To validate gene expression data from the RNA-Seq experiment, qRT-PCR analyses were conducted in a subset of 18 different stress responsive genes (Figure S3). The qPCR and RNA-Seq data were in a good agreement ($R^2 = 0.81$; Figure S4). Moreover, the control and control+EDU samples were clearly separated from the ozone and ozone+EDU in the MDS plot (Figure S5). To analyse gene expression patterns, we then conducted two-way ANOVA including the factors ozone treatment (with the levels control and ozone) and EDU application (with the levels with or without EDU) and then determined contrasts by comparing each possible pair of experimental conditions. For the determination of DEGs, we analysed both 5% and 10% FDR thresholds. This approach was taken because the number of DEG was substantially higher with a 10% FDR threshold for certain contrasts (e.g., control versus ozone). Even though the 10% FDR cut-off tolerates 10% false positives, the number of false negatives when looking only at the 5% FDR cutoff would have been much larger. Therefore, comparing both scenarios provided a more representative insight into global transcriptome responses. When controlling FDR at 5%, 933 DEGs were identified for ozone treatment in which 832 and 101 genes were up-regulated and down-regulated, respectively (Table 1). At 10% FDR, the number of ozone responsive DEGs increased to 4,676 (Table 1). In contrast, only ten and seven DEGs responded to EDU treatment or the ozone x EDU interaction at both 5% and 10% FDR level (Table 1), which clearly indicated that EDU barely affected the gene expression pattern of rice directly. Around 74% of the ozone-responsive DEGs (FDR < 0.05) identified in this study were identical (see Table S3 for the matched genes list) to those reported in a previous microarray experiment by Frei et al. (2010).

In the pairwise contrast analysis, no DEGs were identified between the control and control+EDU treatment at both FDR levels,
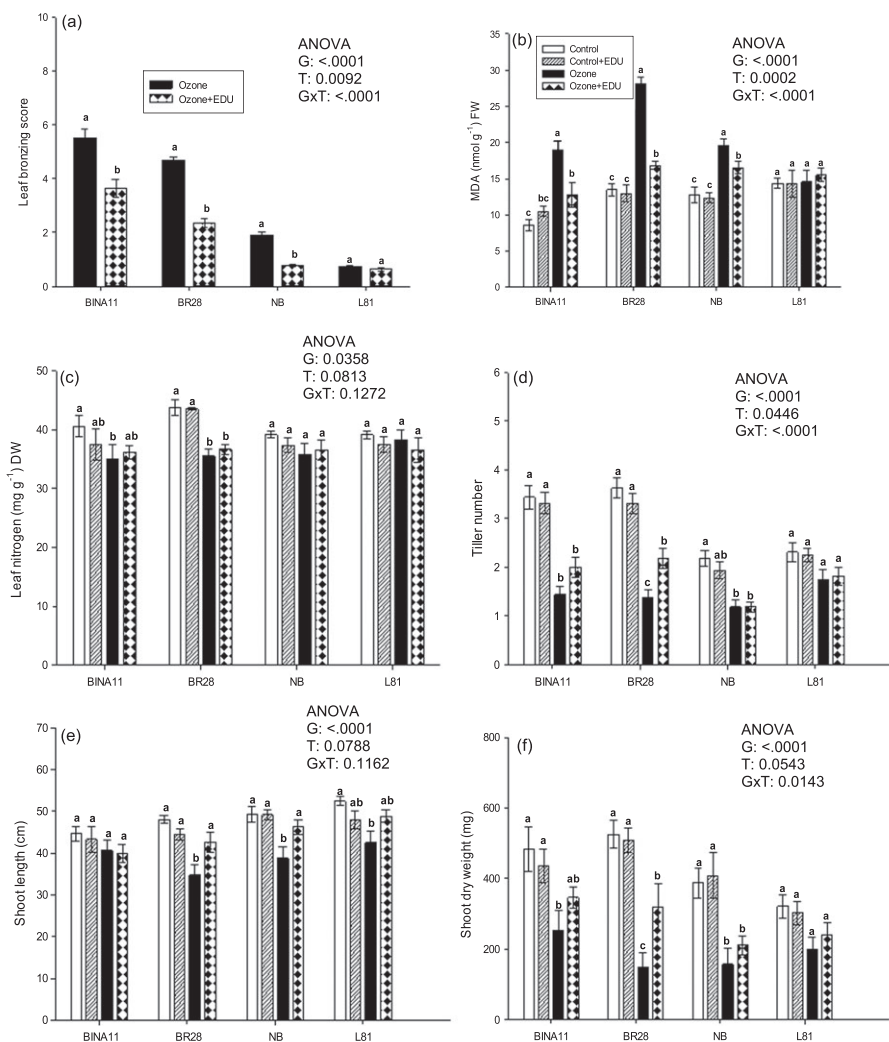
FIGURE 1 Biochemical, physiological, and growth parameters of four rice genotypes exposed to ozone and control conditions with and without the application of ethylenediurea. Bars indicate mean values with ±standard errors ($n = 16$ for leaf bronzing score, $n = 4$ for malondialdehyde and leaf nitrogen, and $n = 8$ for tiller number, shoot length, and shoot dry weight). The letters above of the bars represent pairwise comparison at $p < 0.05$ (mixed model analysis using SAS 9.4) within the genotype against treatments (mean values not sharing the same letter are significantly different). ANOVA: analysis of variance; FW: fresh weight; DW: dry weight; G: genotype; T: treatment; GxT: genotype and treatment interaction

which further confirms the lack of constitutive effects of EDU on global gene expression (Table 1). In addition, almost no DEGs occurred in the control versus ozone+EDU and control+EDU versus ozone +EDU comparisons at both 5% and 10% FDR (except for three DEGs between control+EDU vs. ozone+EDU at 10% FDR; Table 1). In contrast, a large number of DEGs occurred between control versus ozone (3,182) and control+EDU versus ozone (3,367) at 10% FDR (Table 1), further confirming the drastic effect of ozone on gene expression in rice. The number of DEG between the control+EDU versus ozone was even larger than that between control and ozone, presumably because residual amounts of ozone in the control treatment had an effect on gene expression that was offset by EDU application. When comparing the ozone and the ozone+EDU treatments, 23 DEGs were identified at 5% FDR and 59 at 10% FDR (Table 1).

A GO enrichment analysis was conducted for the 933 DEGs, which were responsive to ozone at 5% FDR. Catalytic activity, various types of binding, ligase, kinase, and transferase activity were the dominant GO terms in the category "molecular function" (Figure 3 and Table S4). Catalytic activity was the only significant GO term for the

down-regulated genes (Figure 3). Next, we investigated expression patterns and gene functions of gene sets that may help to explain physiological processes underlying the stress-mitigating effect of EDU. These include the EDU responsive DEGs (10 genes at 5% FDR), DEGs showing significant ozone x EDU interaction (seven genes at 5% FDR), and DEGs occurring in the contrast analysis between ozone and ozone+EDU (23 genes at 5% FDR). Heat maps were generated to summarize the expression profiles for these gene lists along with functional annotation (Figures 4–6). The predominant pattern in the expression of these genes was an induction in the ozone treatment, which was mitigated by the application of EDU (Figures 4 and 6). Among these genes were typical stress-response genes such as glutathione-S-transferase (LOC_Os01g27480, Figure 4) or a drought-induced protein (LOC_Os01g48190, Figure 6). A smaller number of genes with less informative annotation showed the opposite pattern, that is, down-regulation in the ozone stress treatment, which was offset by the application of EDU. Taken together, transcriptomic analyses clearly delineated that EDU had almost no direct effect on global gene expression profiles in rice but instead mitigated stress-responsive
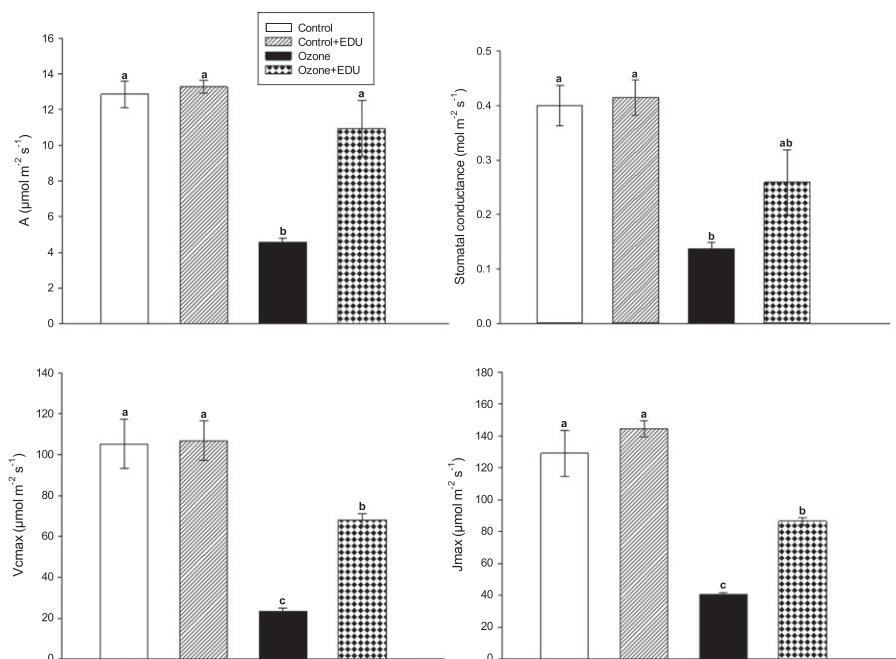
FIGURE 2 Measurement of photosynthetic parameters of ozone sensitive Bangladesh Rice Research Institute dhan28 genotype with and without the application of ethylenediurea (EDU) in control and ozone conditions. Bars indicate mean value with ±standard errors (n = 3). The letters above of the bars represent pairwise comparison at $p < 0.05$ (mean values not sharing the same letter are significantly different). (a) Midday ambient carbon assimilation rate/net photosynthesis rate (CO_2 concentration 400 ppm), (b) stomatal conductance (gs), and (c,d) photosynthetic biochemical capability as represented by the maximum carboxylation rate of Rubisco (Vcmax) and the maximum electron transport rate (Jmax). Vcmax and Jmax were calculated from the CO_2 response (A/Ci) curves. The measurement was taken on the second youngest fully expanded leaf of each plant between 10 a.m. and 2 p.m. using LI-6400XT

TABLE 1 Number of DEGs based on the treatment mean values (FPKM) of filtered (23,208) rice genes from the transcriptome (RNA-Seq) analysis of BR28 rice genotype exposed to ozone and control conditions with and without the application of EDU (statistical analysis were performed with mixed model analysis using SAS 9.4)

| | | Number of genes | | | | | |
| | | 5% FDR level (<0.05) | | | 10% FDR level (<0.10) | | |
Statistical test	Treatments	Up-regulated	Down-regulated	Total	Up-regulated	Down-regulated	Total
Two way ANOVA (n = 6)	Ozone	832	101	933	3,480	1,195	4,675
	EDU	2	8	10	2	8	10
	Interaction	n.d.	n.d.	7	n.d.	n.d.	7
Pairwise contrast (n = 3)	Control versus control+EDU	0	0	0	0	0	0
	Control versus ozone	15	1	16	2,490	692	3,182
	Control versus ozone+EDU	0	0	0	0	0	0
	Control+EDU versus ozone	497	21	518	2,700	667	3,367
	Control+EDU versus ozone+EDU	0	0	0	3	0	3
	Ozone versus ozone+EDU	3	20	23	5	54	59

Note. In two-way ANOVA, up-regulated and down-regulated genes were calculated on the basis of the response towards ozone and EDU application and their interaction. Pairwise contrast was determined by comparing each of the ozone/EDU treatment combinations. ANOVA: analysis of variance; BR28: Bangladesh Rice Research Institute dhan28; DEGS: differentially expressed genes; EDU: ethylenediurea; FPKM: fragments per kilobase of transcript per million mapped reads; FDR: false discovery rate; n.d., not determined.

gene regulation. Therefore, the protective effect of EDU is more likely to occur upstream of physiological stress responses, for example, by limiting ozone uptake at the leaf surface.

3.3 | SEM of rice leaves

In order to investigate the fate of EDU on leaf surfaces, we performed SEM of rice leaves exposed to different treatments in Experiment 2. First images were taken 1 day after the EDU application and after 8 days of ozone treatment, in which we did not observe any structural differences of leaf surfaces exposed to different treatments, that is, trichomes, cuticle, papillae (epidermis), silica cells, epicuticular wax layer, hairs, and stomata. Visible deposits of amorphous crystal structures similar to EDU crystals on a glass plate were seen on the epidermis of ozone+EDU-treated rice leaves (Figure S6a,b) 7 days after EDU application and 15 days after the start of the ozone treatment. In addition, glue-like amorphous sticky structures (Figure S6c) were observed on ozone+EDU-treated rice leaf surfaces but not in the control. We further quantified EDU deposits on leaf surfaces treated with 600 and 300 ppm of EDU (Figure 7a–d). Spreading of EDU particles was

FIGURE 3 Gene ontology enrichment analysis (molecular function) of differentially expressed ozone responsive genes (two-way analysis of variance and false discovery rate (FDR) < 0.05). Analysis was computed with 832 up-regulated and 101 down-regulated genes from AgriGO using rice NCBI ID as reference. Only significant enrichment terms (FDR < 0.05) with FDR under dependency multitest adjustment (Yekutieli) are shown in the figure [Colour figure can be viewed at wileyonlinelibrary.com]
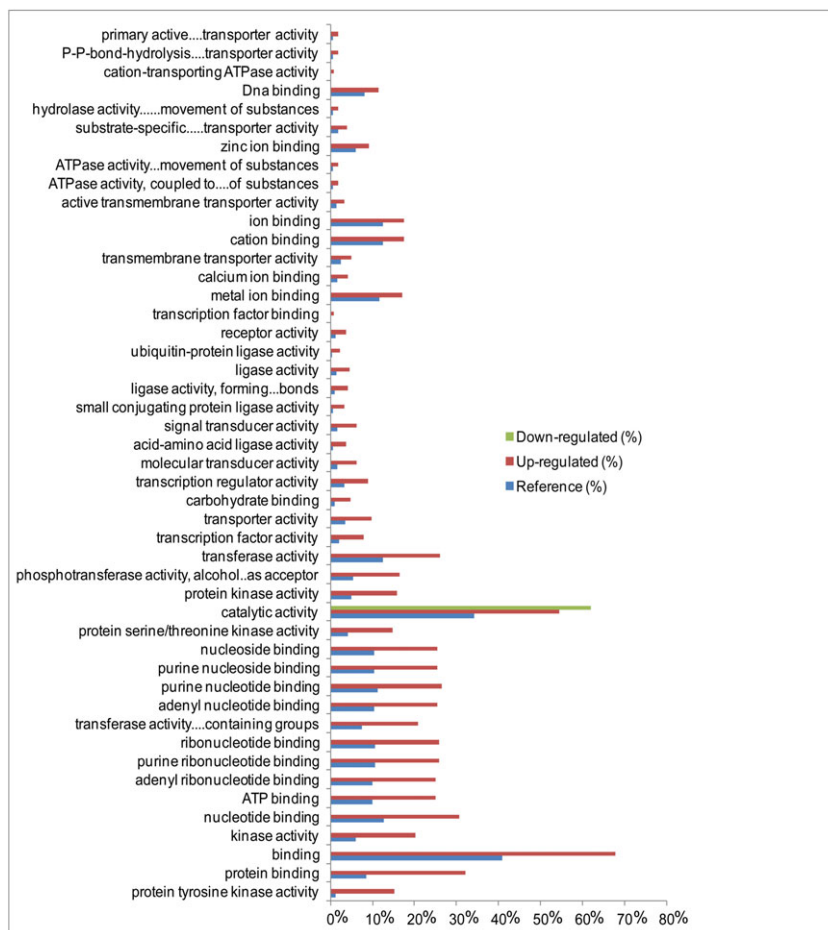
FIGURE 4 Relative expression of 10 differentially expressed ethylenediurea (EDU) responsive genes (false discovery rate < 0.05) based on with and without EDU application (eight down-regulated and two up-regulated genes in response to EDU). Statistical analysis (two-way analysis of variance) was performed with mixed model analysis using SAS 9.4 with mean expression value (FPKM) from RNA-Seq experiment. Locus name and annotations were used from MSU rice genome annotation project. FPKM: fragments per kilobase of transcript per million mapped reads [Colour figure can be viewed at wileyonlinelibrary.com]
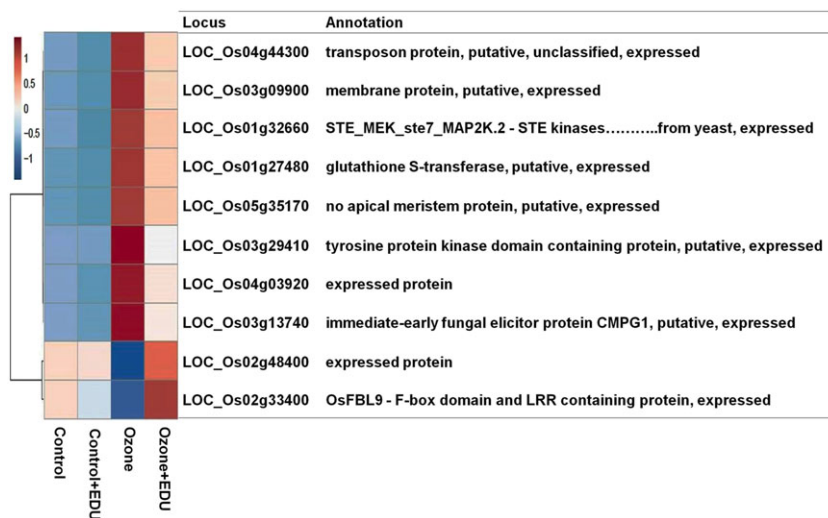
also seen on leaf surfaces 7 days after EDU applications (Figure 7e). On the other hand, we did not observe any deposits in control leaves treated with water instead of EDU (Figure 7f). For quantification, we categorized the deposits into large amorphous particles (LP; size approximately >300 μm) and small dot-like particles without any distinct shape (approximately <300 μm; Figure 7g). A significant reduction in the number of small particles was seen 7 days after EDU application in both treatments (Figure 7g). The spreading of EDU deposits might play an important role in decreasing the number of small particles.

3.4 | EDU and ozone interaction

Further, we tested the hypothesis that EDU directly interacts with ozone in an abiotic chemical reaction. Ozone produced by an ozone generator (600 mg/hr) was first percolated through water or water +EDU (300 ppm) solution and then blown with a fan into plastic pipes vertically distributed over an empty OTC (similar to the main experiment). The ozone concentrations were measured at 1-min interval with a handheld ozone sensor (series 500; Aeroqual Ltd. Auckland, New Zealand). We performed these measurements in two different
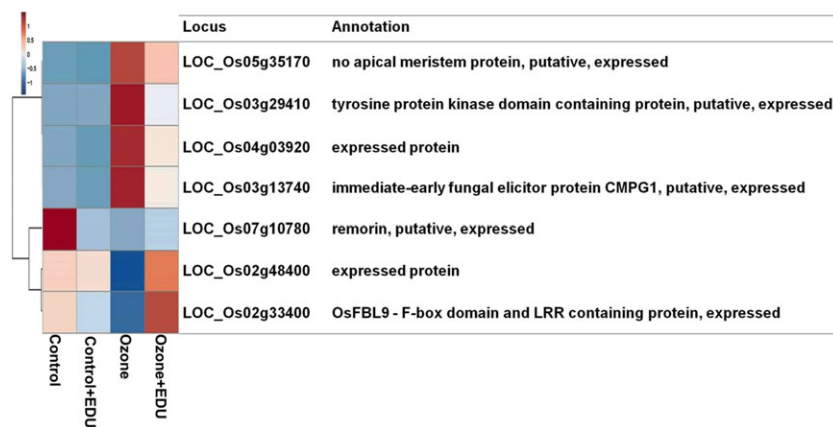
FIGURE 5 Relative expression of seven differentially expressed genes (false discovery rate < 0.05) based on interaction between ozone and ethylenediurea (EDU) application. Statistical analysis (two-way analysis of variance) was performed with mixed model analysis using SAS 9.4 with mean expression value (FPKM) from RNA-Seq experiment. Locus name and annotations were used from MSU rice genome annotation project. FPKM: fragments per kilobase of transcript per million mapped reads [Colour figure can be viewed at wileyonlinelibrary.com]
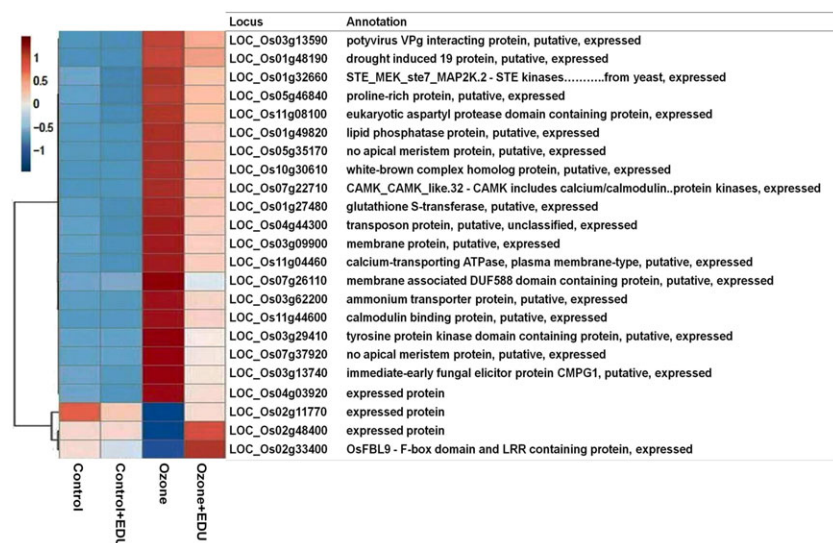
FIGURE 6 Relative expression of 23 differentially expressed genes (false discovery rate < 0.05) based on pairwise contrast between ozone and ozone+EDU treatment (20 down-regulated and three up-regulated genes in response to ethylenediurea [EDU]). Statistical analysis was performed with mixed model analysis using SAS 9.4 with mean expression value (FPKM) from RNA-Seq experiment. Locus name and annotations were used from MSU rice genome annotation project. FPKM: fragments per kilobase of transcript per million mapped reads [Colour figure can be viewed at wileyonlinelibrary. com]

days with six replications of each treatment. Significantly lower ozone concentration (approximately 15% decreased) was seen in the water +EDU (300 ppm) treatment compared with only water by student's *t* test (Figure S7). These data indicate that EDU potentially adds to the decomposition of ozone via chemical interactions.

3.5 | EDU and diverse stresses

Lastly, we conducted an experiment to exclude the possibility that EDU may act as an unspecific stress mediator instead of specifically mitigating ozone stress. Three stress conditions were tested, that is, Fe toxicity, Zn deficiency, and salinity. Excessive iron and salt significantly affected all measured growth and physiological parameters (except root length in salt) of rice in stress conditions compared with control when averaged over all three rice genotypes (Table 2). Moreover, visible leaf damage, significantly declined shoot and root dry weight, and SPAD value were observed due to the zinc deficiency treatment (Table 2). The individual rice genotypes responded differentially to the stresses, as BR28 exhibited more consistent damage against stresses compared with the other two genotypes (Table 2). Averaged over three genotypes, no significant differences were observed between stress and stress+EDU in visible leaf symptoms, shoot and root length, shoot and root dry weight, and SPAD value (Table 2). In conclusion, no genotype performed better in the stress

+EDU treatments compared with the stress treatments without EDU. Thus, the possibility that EDU mitigates any of the stresses investigated in this experiment can be excluded.

4 | DISCUSSION

In the present study, we employed an average ozone concentration of 110 ppb during the treatment period (9 a.m. to 4 p.m.). These concentrations are analogous to pollution scenarios currently experienced in several rice-producing Asian countries, where, for example, average daytime (8 hr) concentrations can exceed 100 ppb in the city of Pune, India (Roy, Beig, & Ghude, 2009), and where 1-hr maxima approach 130 ppb in Shanghai, China (Ran et al., 2009). Acknowledging the increasing trends of ground-level ozone observed in several Asian countries (Brauer et al., 2016), it is expected that the concentrations employed in this study will become increasingly representative of ambient ozone concentrations in future years, which will therefore exceed the damage threshold for rice (40 ppb) on a routine basis. Our data demonstrated that rice is quite sensitive to such ozone levels and reacts with a loss in biomass (Figure 1f), loss in photosynthetic capacity (Figure 2), visual damage (Figure 1a), and oxidative stress reflected in lipid peroxidation (Figure 1b). Likewise, this study illustrates substantial genotypic differences in the response to ozone.
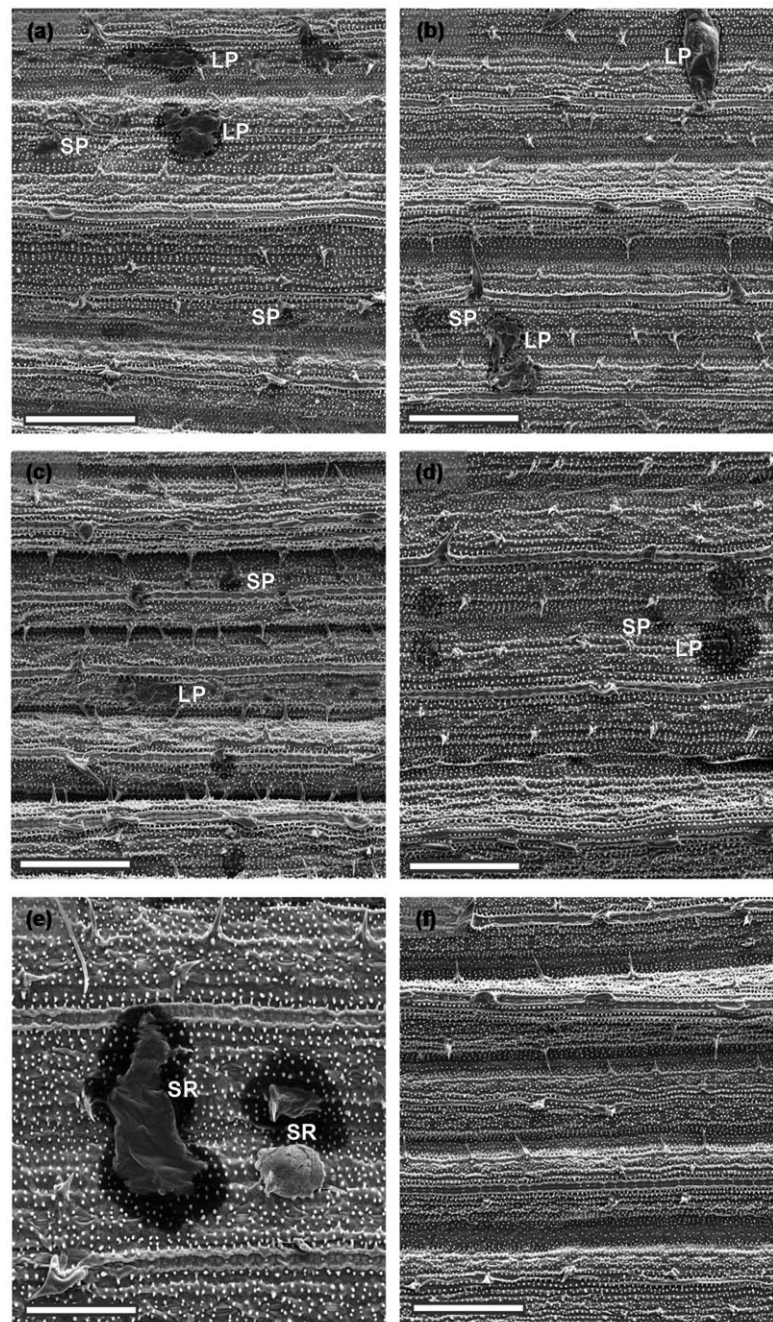
FIGURE 7 Scanning electron microscopy images and quantification of ethylenediurea (EDU) deposits on rice upper leaf surface (BR28 genotype). (a,b) Amorphous EDU particles on leaves treated with 600 and 300-ppm EDU 1 day after application; (c,d) amorphous EDU particles on leaves treated with 600 and 300-ppm EDU 7 days after application; (e) spreading of EDU deposits on leaf surface 7 days after application; (f) control leaf without EDU treatment, and (g) quantification of the number of deposited EDU particles per square centimetre leaf surface area, 1 and 7 days after applications of 600 and 300-ppm EDU. Scale bar a–d and f = 200 μm, and e = 100 μm. Bars indicate mean value with standard errors (n = 8). Bars not sharing the same letter within one treatment are significantly different at $p < 0.05$. LP: large particle (approximately >300 μm in size); SP: small particle (approximately <300 μm in size); SR: spreading of EDU deposits. BR28: Bangladesh Rice Research Institute dhan28
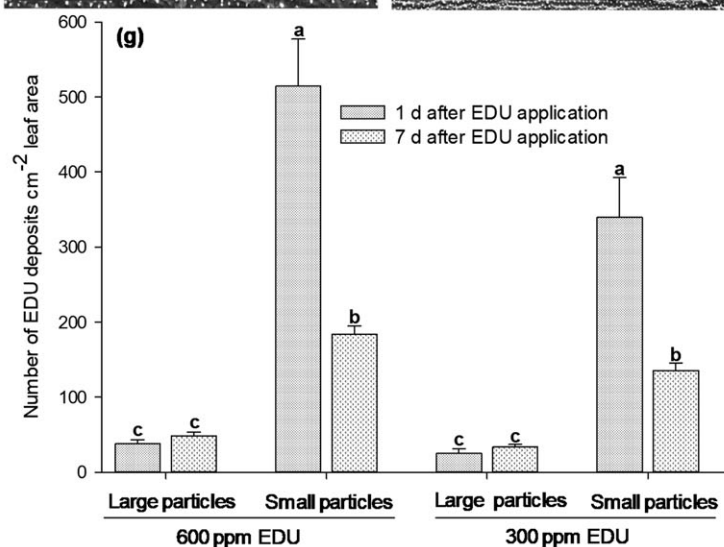
TABLE 2 Phenotypic effects of EDU application in diverse stress conditions

Trait	Genotype	LS means			
		Control	Control+EDU	Stress	Stress+EDU
Fe^{2+} toxicity					
LBS	BR28	n.d.	n.d.	5.5^a	5.9^a
	IR64	n.d.	n.d.	6.2^a	6.8^a
	Nipponbare	n.d.	n.d.	2.5^a	2.7^a
	Average	n.d.	n.d.	4.7^a	5.1^a
Shoot length (cm)	BR28	40.5^a	39.5^a	29.9^b	29.4^b
	IR64	26.8^a	28.1^a	24.9^a	24.1^a
	Nipponbare	46.4^a	45.3^a	35.1^b	32.6^b
	Average	37.9^a	37.6^a	30.0^b	28.7^b
Root length (cm)	BR28	22.3^a	19.1^a	12.0^b	13.0^b
	IR64	17.4^a	16.9^a	14.3^{ab}	11.5^b
	Nipponbare	15.4^{ab}	17.3^a	10.9^{bc}	10.3^c
	Average	18.3^a	17.8^a	12.4^b	11.6^b
Shoot dry weight (mg)	BR28	284.0^a	272.3^a	116.3^b	117.5^b
	IR64	100.3^a	107.0^a	89.3^a	80.0^a
	Nipponbare	168.5^a	157.3^a	102.0^b	94.8^b
	Average	184.3^a	178.8^a	102.5^b	97.4^b
Root dry weight (mg)	BR28	60.8^a	56.3^a	35.3^b	36.5^b
	IR64	21.8^a	22.3^a	23.5^a	21.8^a
	Nipponbare	24.3^a	24.8^a	23.5^a	24.8^a
	Average	35.6^a	34.4^a	27.4^b	27.7^b
SPAD value	BR28	29.4^a	29.1^a	23.2^b	20.6^b
	IR64	23.7^a	24.3^a	18.2^b	17.0^b
	Nipponbare	33.4^{ab}	34.0^a	29.5^b	29.7^b
	Average	28.8^a	29.1^a	23.6^b	22.5^b
Zn deficiency					
LBS	BR28	n.d.	n.d.	2.3^a	2.7^a
	IR64	n.d.	n.d.	7.1^a	7.5^a
	Nipponbare	n.d.	n.d.	2.6^a	3.0^a
	Average	n.d.	n.d.	4.0^a	4.4^a
Shoot length (cm)	BR28	48.5^a	47.1^a	40.5^{ab}	38.9^b
	IR64	27.9^a	26.5^a	26.4^a	25.5^a
	Nipponbare	48.3^a	48.0^a	46.9^a	45.6^a
	Average	41.5^a	40.5^a	37.9^{ab}	36.7^b
Root length (cm)	BR28	23.3^a	17.9^a	17.5^a	18.1^a
	IR64	18.8^a	16.1^a	18.4^a	16.8^a
	Nipponbare	16.3^a	16.9^a	16.9^a	15.1^a
	Average	19.4^a	17.0^a	17.6^a	16.7^a
Shoot dry weight (mg)	BR28	428.8^a	409.8^a	245.3^b	231.5^b
	IR64	136.0^a	107.0^a	112.0^a	92.8^a
	Nipponbare	272.0^a	279.0^a	211.3^{ab}	196.3^b
	Average	278.9^a	265.3^a	189.5^b	173.5^b
Root dry weight (mg)	BR28	103.8^a	90.5^a	45.8^b	49.3^b
	IR64	36.8^a	21.5^a	22.0^a	17.0^a
	Nipponbare	39.8^a	40.5^a	28.3^a	23.8^a
	Average	60.1^a	50.8^a	32.0^b	30.0^b
SPAD value	BR28	36.8^a	35.7^{ab}	31.6^{bc}	28.9^c
	IR64	24.9^a	24.3^a	24.1^a	21.6^a
	Nipponbare	35.6^a	34.9^{ab}	31.0^{ab}	30.3^b
	Average	32.4^a	31.6^a	28.9^b	27.0^b
Salinity					
Salt score	BR28	n.d.	n.d.	7.7^a	7.9^a
	IR64	n.d.	n.d.	8.4^a	8.4^a
	Nipponbare	n.d.	n.d.	7.6^a	8.0^a
	Average	n.d.	n.d.	7.9^a	8.1^a
Shoot length (cm)	BR28	40.5^a	39.5^a	32.5^b	32.0^b
	IR64	26.8^a	28.1^a	25.6^a	22.5^a
	Nipponbare	46.4^a	45.3^a	35.6^b	35.0^b
	Average	37.9^a	37.6^a	31.3^b	29.8^b
Root length (cm)	BR28	22.3^a	19.1^{ab}	18.0^{ab}	16.8^b
	IR64	17.4^a	16.9^a	17.0^a	15.5^a
	Nipponbare	15.4^a	17.3^a	15.4^a	16.1^a
	Average	18.3^a	17.8^a	16.8^a	16.1^a

TABLE 2 (Continued)

Trait	Genotype	LS means			
		Control	Control+EDU	Stress	Stress+EDU
Shoot dry weight (mg)	BR28	284.0[a]	272.3[a]	151.5[b]	142.3[b]
	IR64	100.3[a]	107.0[a]	85.5[a]	61.8[a]
	Nipponbare	168.5[a]	157.3[a]	104.8[b]	105.5[b]
	Average	184.3[a]	178.8[a]	113.9[b]	103.2[b]
Root dry weight (mg)	BR28	60.8[a]	56.3[a]	29.0[b]	28.8[b]
	IR64	21.8[a]	22.3[a]	14.5[a]	11.5[a]
	Nipponbare	24.3[a]	24.8[a]	16.3[a]	17.0[a]
	Average	35.6[a]	34.4[a]	19.9[b]	19.1[b]

Note. EDU: ethylenediurea; BR28: Bangladesh Rice Research Institute dhan28; LBS: leaf bronzing score; n.d.: not determined; LS: least square means of two replicates ($n = 4$) are shown; values not sharing the same superscript letter within one row differ significantly from each other by Tukey–Kramer post hoc comparison ($p < 0.05$).

Such genotypic differences provide the basis for adaptive breeding (Frei, 2015), which can only be exploited effectively if field screening tools such as EDU are applicable. This approach was previously shown to be agronomically sound in rice fumigation experiments, as it did not affect rice plants constitutively, but it mitigated rice yield losses in ozone stress (Ashrafuzzaman et al., 2017).

However, its physiological mode of action remained unclear. In our study, EDU did not affect stomatal conductance in the sensitive BR28 rice genotype (Figure 2b), indicating that stomatal closure as the first line of defence against ozone stress did not contribute to mitigating ozone effects. This phenomenon was also reported earlier in snap bean (Paoletti et al., 2014). EDU has also been criticized earlier because it might act as a foliar fertilizer, as it contains 22% nitrogen (Godzik & Manning, 1998; Manning et al., 2011). However, EDU spraying did not affect leaf nitrogen content in our study (Figure 1c), indicating that EDU was not a significant source of nitrogen, which was also confirmed in willow plants (Agathokleous et al., 2018). In contrast, a previous study suggested that high concentrations of EDU (above 800 ppm) can increase leaf nitrogen content in willow plants grown in low nitrogen and organic matter free soil but did not show any toxicity effects (Agathokleous et al., 2016a). In order to get deeper insights into its physiological mode of action, we explored EDU responses in rice using a transcriptomic approach to test whether EDU induced active defence reactions. This would translate into the activation of defence pathways. Alternatively, if EDU acts as a passive surface protectant, the activation of ozone induced pathways would be offset.

A large number of ozone responsive rice genes were identified in the present RNA-Seq study (Table 1 and Figure 3). Around 74% of these DEGs were also identified in a previous microarray study. DEGs were involved in ethylene or jasmonic acid metabolism, general disease resistance, and antioxidant pathways (Frei et al., 2010). In contrast to those previous experiments, the aim of this study was not primarily to investigate ozone responsive genes but rather to elucidate genes and pathways responsible for ozone mitigating effects of EDU. Only very few genes responded significantly to EDU application in our RNA-Seq analysis (Table 1). The number of DEGs for EDU and EDU x ozone interaction was negligible compared with ozone responsive genes (4675) at 10% FDR. Therefore, the rice global gene expression patterns were barely affected by the EDU application and provide evidence that it does not have any direct physiological effects on plants. This is in contrast to previous assumptions that EDU might elicit

defence reactions in plants such as stimulating antioxidants (Pandey et al., 2015, 2014). It is therefore plausible that EDU mitigated deleterious ozone effects through a passive surface or apoplastic protection effect upstream of any defence reaction, for example, by hindering it from entering into the plant or by decomposing ozone or ROS derived from it. This idea is supported by the fact that ozone-treated plants sprayed with EDU had very similar gene expression pattern as plants in the control (Table 1). However, EDU applications have also been effective when applied as soil drench (Feng et al., 2010; Manning et al., 2011), which may be due to rapid translocation through the xylem vessels into the leaf aploplast where it remains for 8 days or more (Gatta et al., 1997; Paoletti et al., 2009) and could provide surface protection. Nevertheless, a recent comparative study demonstrated that foliar spray was more effective in willow plants than soil drench (Agathokleous et al., 2016b).

In order to monitor the fate of EDU on leaf surfaces, we conducted SEM of rice leaves to explore the possibilities of integration or deposition of EDU crystals on leaf surfaces (trichomes, cuticle, and epidermis), which can modify or inhibit the entry of ozone through leaves and ultimately protect the plants from ozone stress. EDU application did not visibly affect the leaf structure, but interestingly, amorphous solid EDU structures were observed on leaf surfaces (Figures 7 and S6). This phenomenon also confirmed the persistence of EDU in leaves for several days (Gatta et al., 1997). Amorphous sticky structures and spreading of EDU deposits were observed on EDU-treated leaf surfaces, although their function is not well-understood. Leaf surface structures such as trichomes and cuticles can play a critical role in ozone uptake (nonstomatal uptake) and reduce ozone toxicity as a chemical barrier decomposing ozone before entering into the leaf (Horváth et al., 2017; Jud et al., 2016; Oksanen, 2018). Recent studies also confirmed that glandular trichomes can directly limit the ozone concentration at the leaf surface by increasing the emissions of volatile compounds in diverse species (Li et al., 2018) and in tobacco (Kanagendran et al., 2017). Therefore, it is possible that EDU interferes with these surface protection mechanisms.

Alternatively, surface protection could also be explained by abiotic chemical reactions of EDU with ozone. This idea is supported by our experiment, in which ozone was percolated through EDU solution, leading to significantly reduced rates of ozone accumulation in OTC (Figure S7). The class of chemical reactions that are likely to responsible for this effect have been studied by Tuazon, Atkinson, Aschmann, and Arey (1994), who determined the gas-phase rate of coefficients of

several amines towards ozone and found that increasing the number of organic substitutions attached to an amine group strongly increased its reactivity towards ozone. On the basis of their analysis of reaction products, they concluded that the mechanism of reaction proceeds through an excited amine oxide intermediate, which in the case of a secondary amine (dimethylamine) leads to n-methyl methanimine formation. By analogy to the systems studied by Tuazon et al. (1994), it is expected that reactions occur at the two identical secondary amine sites, and by applying the same mechanism, leads to the reaction scheme presented in Figure S8. Presumably such reactions proceed on aqueous films on plants surfaces, given that under ambient conditions, water forms a film on all surfaces (Verdaguer, Sacha, Bluhm, & Salmeron, 2006) and that EDU is moderately soluble in water (Carnahan et al., 1978). This may also accelerate the rate of reaction by mobilizing reactants and reactive sites, which would otherwise be confined by a solid crystalline lattice. Furthermore, the reaction rate with EDU may be more rapid than with dimethylamine, and any reaction occurring on the surface (as in the fumigation experiments) is likely to be faster still, primarily as a consequence of the large surface concentrations of EDU encountered by gas-phase ozone molecules. If the reaction produces a water molecule rather than sequential production of OH and HO_2, then this reaction would be very effective at reducing the concentration of oxidants in its local environment.

Apart from limited knowledge of its mode of action, the unknown effects of EDU in environmental stress conditions other than ozone represent another important constraint to its wider application. In other words, if EDU was an unspecific stress remedy, its positive effects on plants could not be associated with ozone damage alone. This question is of high relevance in biomonitoring field studies, where multiple stresses such as salinity or nutrient disorders can coincide with ozone in rice growing Asian countries such as Bangladesh and India (Frei, 2015; Gregorio et al., 2002; Lafitte, Ismail, & Bennett, 2004). However, we did not observe stress mitigating effect of EDU application in any of the stress conditions tested in this study (Table 2). These results are in line with a previous study, in which the effectiveness of EDU was not altered by moderate drought in the ambient field that was confirmed in poplar plants (Xin et al., 2016).

Taken together, our results encourage the use of EDU as a tool for diagnosing ozone tolerance and response in field grown plants. It is estimated that ozone-induced global crop production can be recovered by 12% in 2030 relative to damage occurred in 2000 by cultivating more ozone tolerant or resistant crop varieties (Avnery, Mauzerall, & Fiore, 2013). Therefore, EDU can be used as an effective strategy to facilitate ozone tolerance breeding that may ultimately reduce the damage caused by ozone and help to ensure global food security.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.
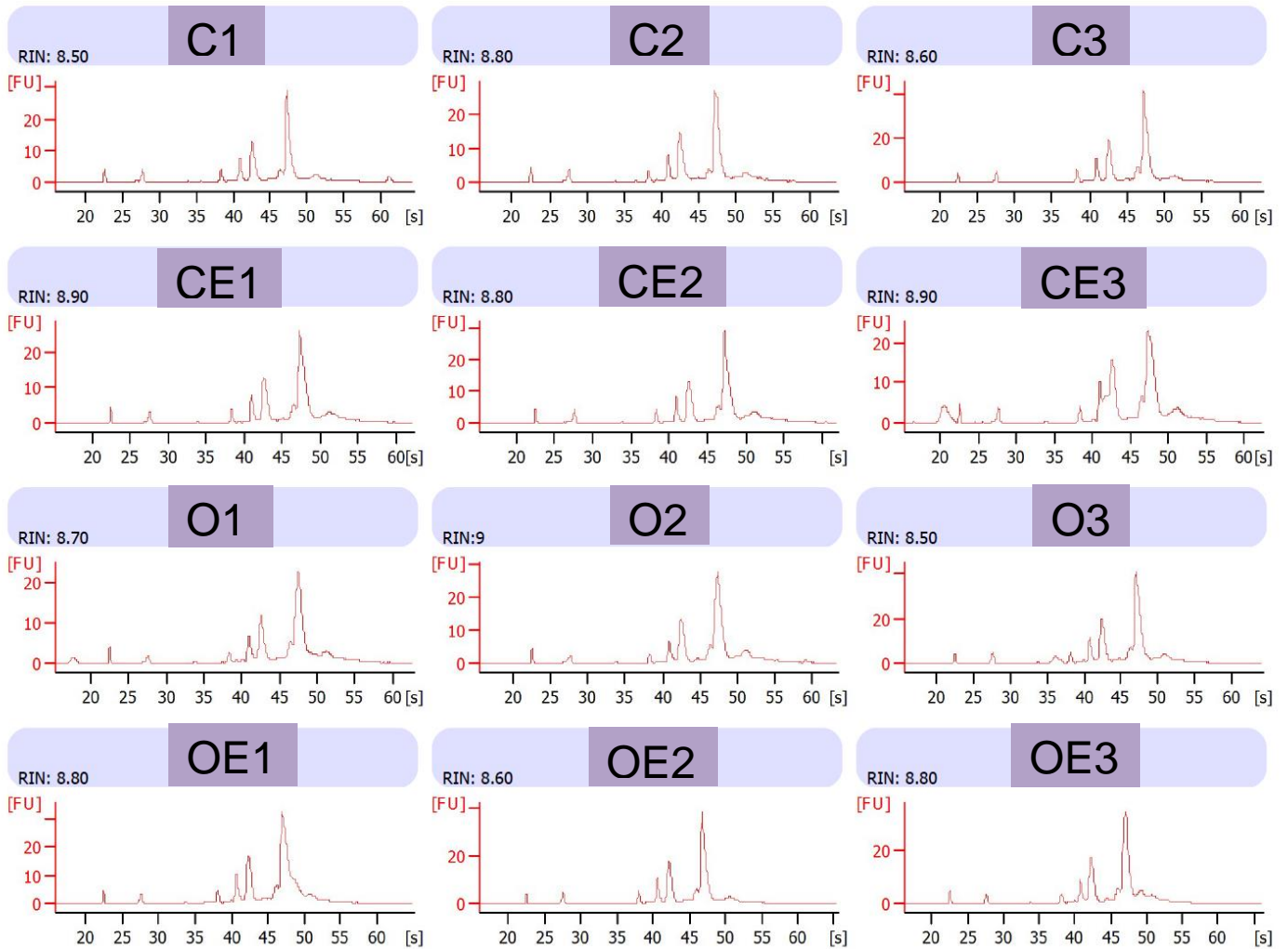
Figure S1 RNA quality assessment of 12 rice samples exposed to ozone and control conditions with and without the application of EDU. The RNA was extracted from frozen pooled shoot samples (whole plant without root) and the quality analysis was conducted by using 2100 Bioanalyzer (Agilent Technologies). C, control; CE, control+EDU; O, ozone; OE, ozone+EDU and numerical values indicate the number of replications.
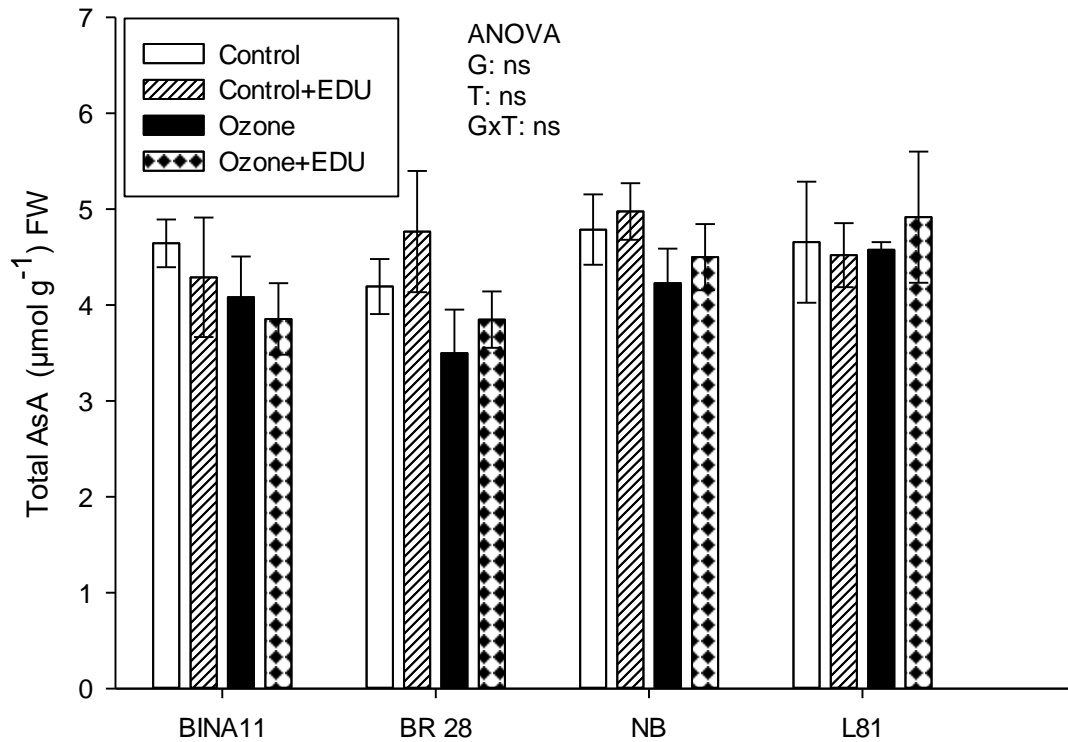
Figure S2 Total ascorbate (AsA) concentrations of four rice genotypes exposed to ozone and control conditions with or without EDU application. Bars indicate mean value ± standard errors ($n = 3$). Y axis represents the genotype name. FW, fresh weight; G, genotype; T, treatment; GxT, genotype and treatment interaction; ns, not significant.
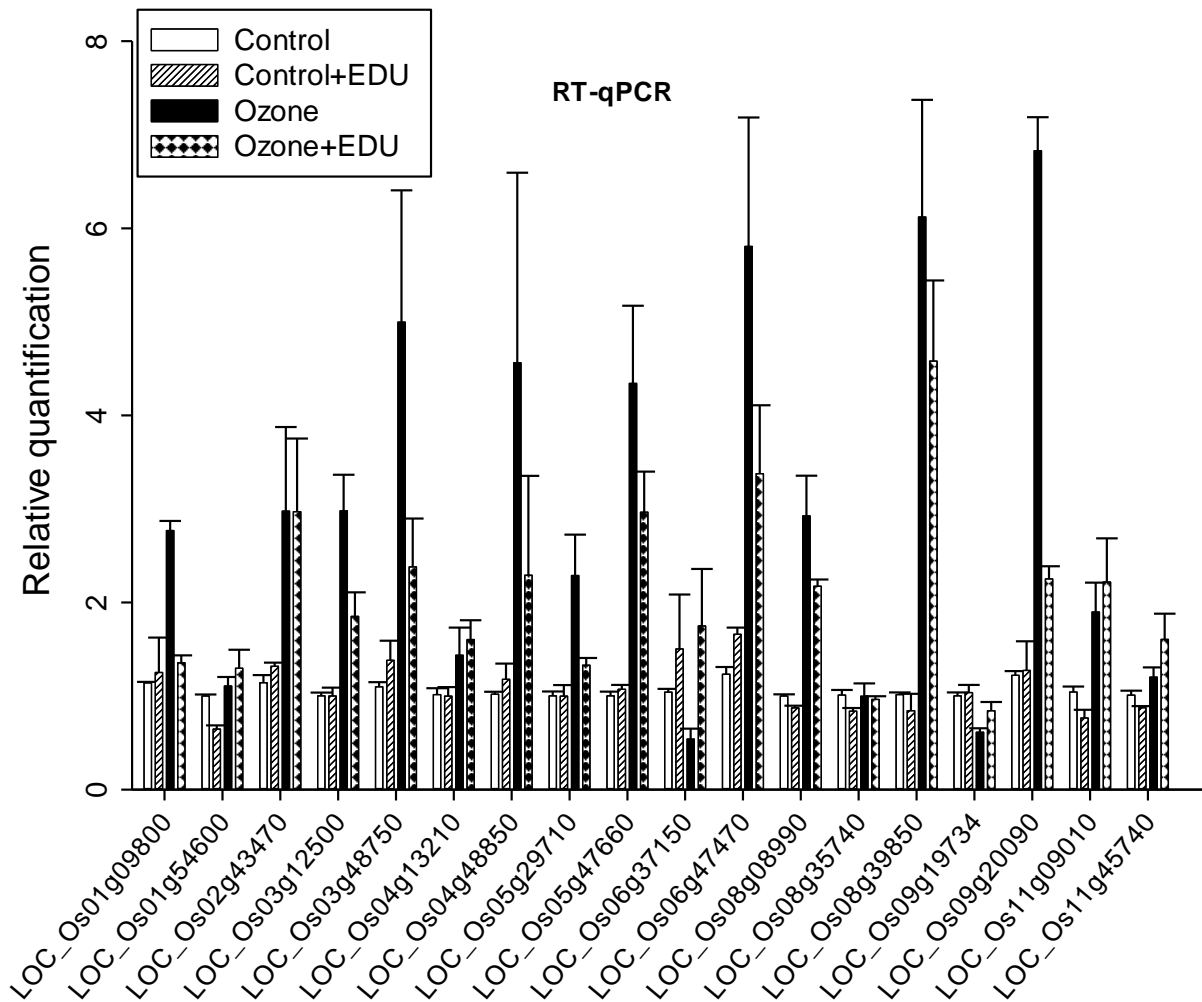
Figure S3 Relative expression of selected 18 rice genes in control and ozone treatment with and without the application of EDU along with their MSU locus ID used for validation of RNA-Seq data by RT-qPCR. Bars indicate mean value with standard errors ($n = 3$). The 18S rRNA was used as endogenous reference and the control treatment was used as the calibrator to express relative expression using the ΔΔCT method.

$y = 0.6717x + 0.2628$
$R^2 = 0.8117$
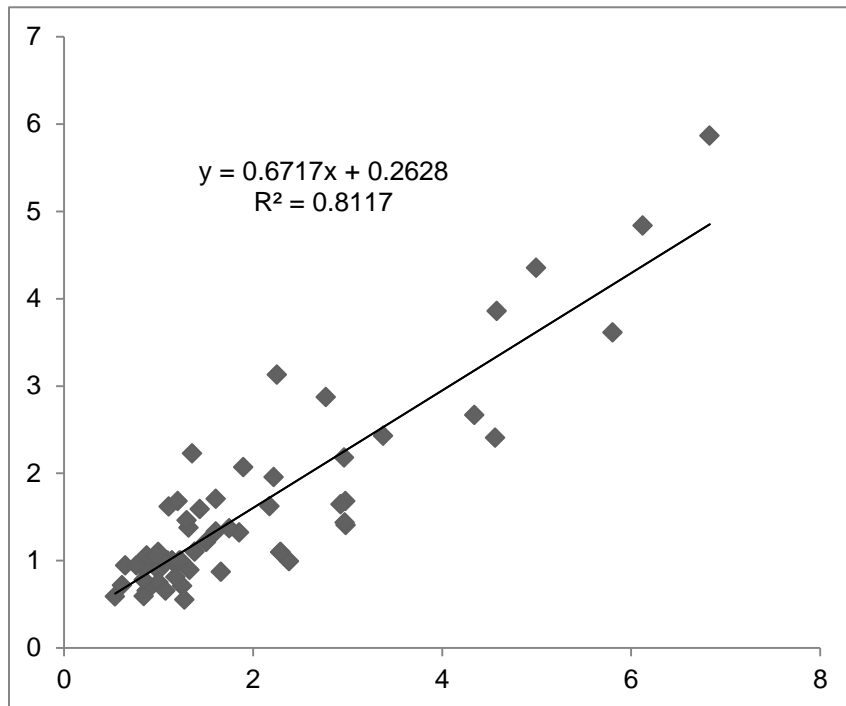
Figure S4 Validation of RNA-Seq data by RT-qPCR with 18 selected genes. Data from both RNA-Seq and qRT-PCR were normalized by setting the expression level in the control as 1, and mean values of relative expression of each gene in four different treatments (control, control+EDU, ozone and ozone+EDU) of RNA-Seq and qPCR data were plotted.
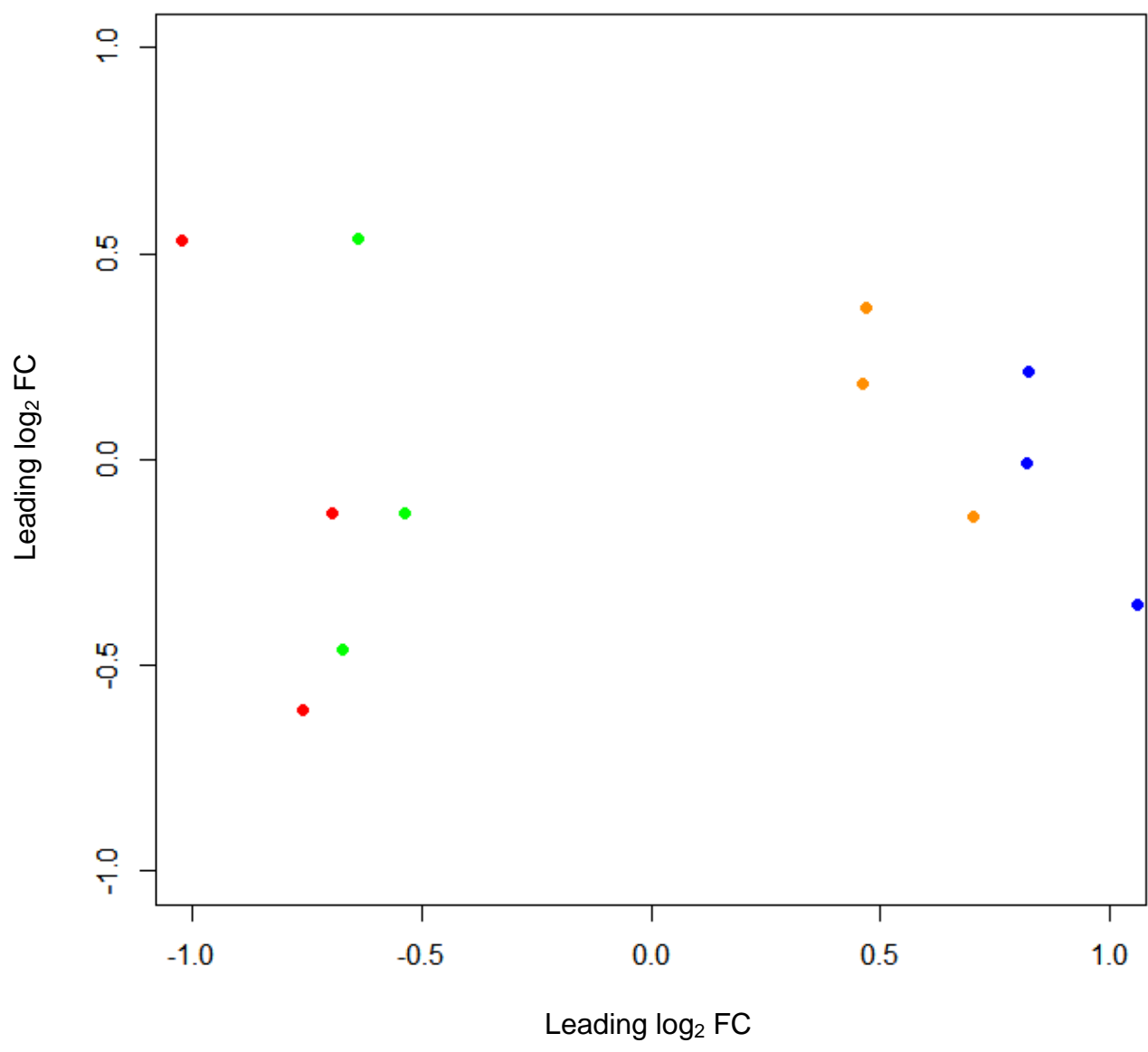
Figure S5 Multidimensional scaling plot of 12 RNA-Seq samples of BR28 genotype. Each treatment and replicates are highlighted by a different color (green = control, red = control+EDU, blue = ozone, orange = ozone+EDU).
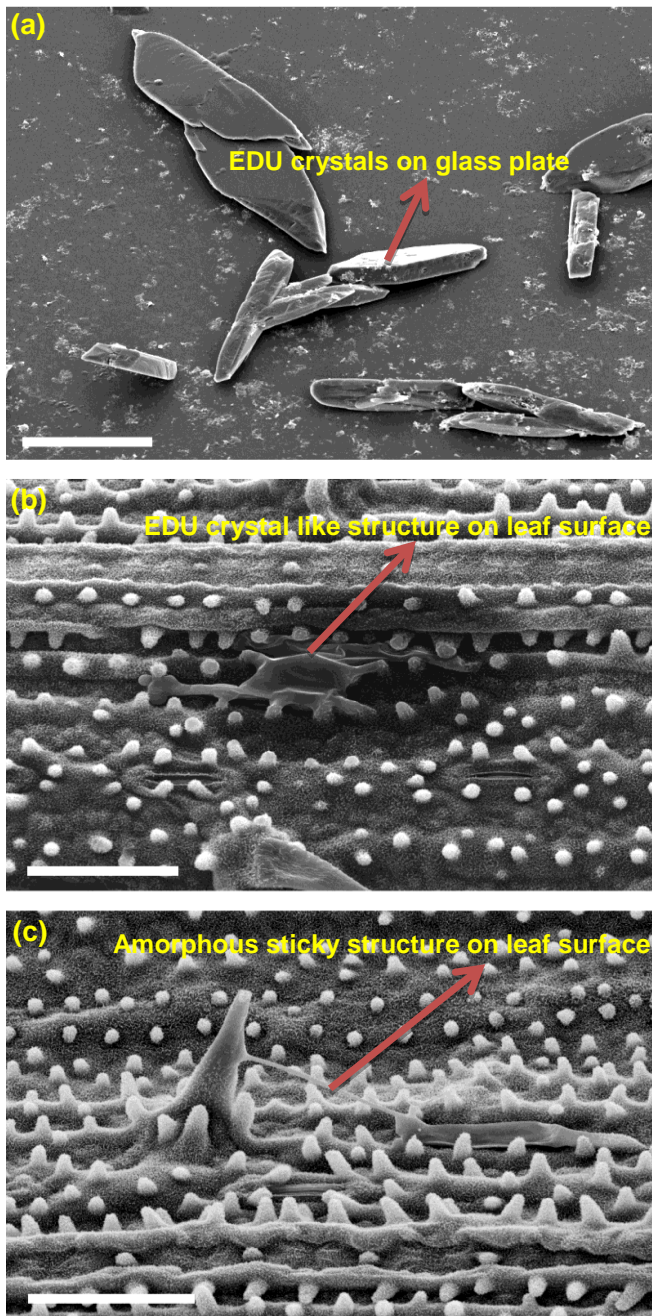
Figure S6 Scanning electron microscopy images (close view) of EDU and ozone+EDU treated rice upper leaf surface (BR28 genotype). (a) EDU crystals on a glass slide (1% EDU solution); (b) and (c) crystal like structure (similar to EDU crystal) and amorphous sticky structure on ozone+EDU treated leaf surface 7 d after EDU application (15 d ozone treatment). Scale bar (a) = 100 μm, (b) and (c) = 20 μm.
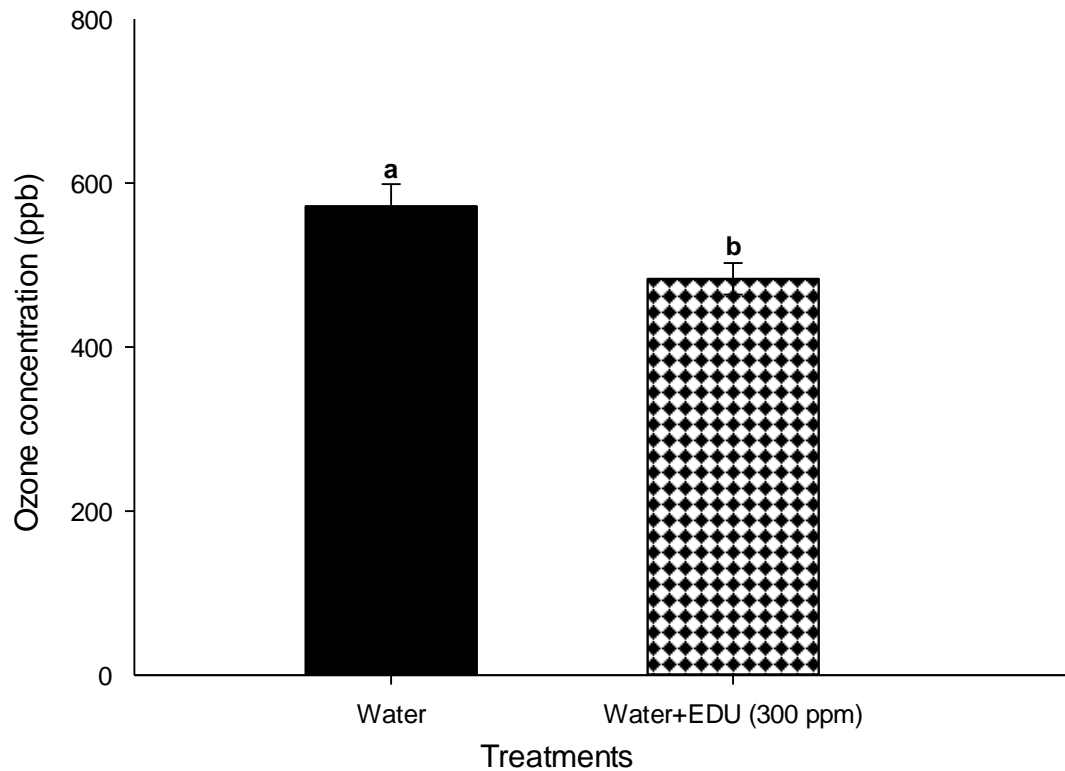
Figure S7 The measured ozone concentrations in the open top chamber (empty) with an independent handheld ozone monitor (series 500; Aeroqual Ltd. Auckland, New Zealand) at 1-min intervals. Bars indicate mean value ± standard errors ($n = 36$ with 6 replicates in each treatment). The generated ozone was first passed through water or 300 ppm EDU mixed with water. Different letters above the bars represent significant differences between the treatments (statistical comparison t-test) at $P < 0.05$ level of significance.
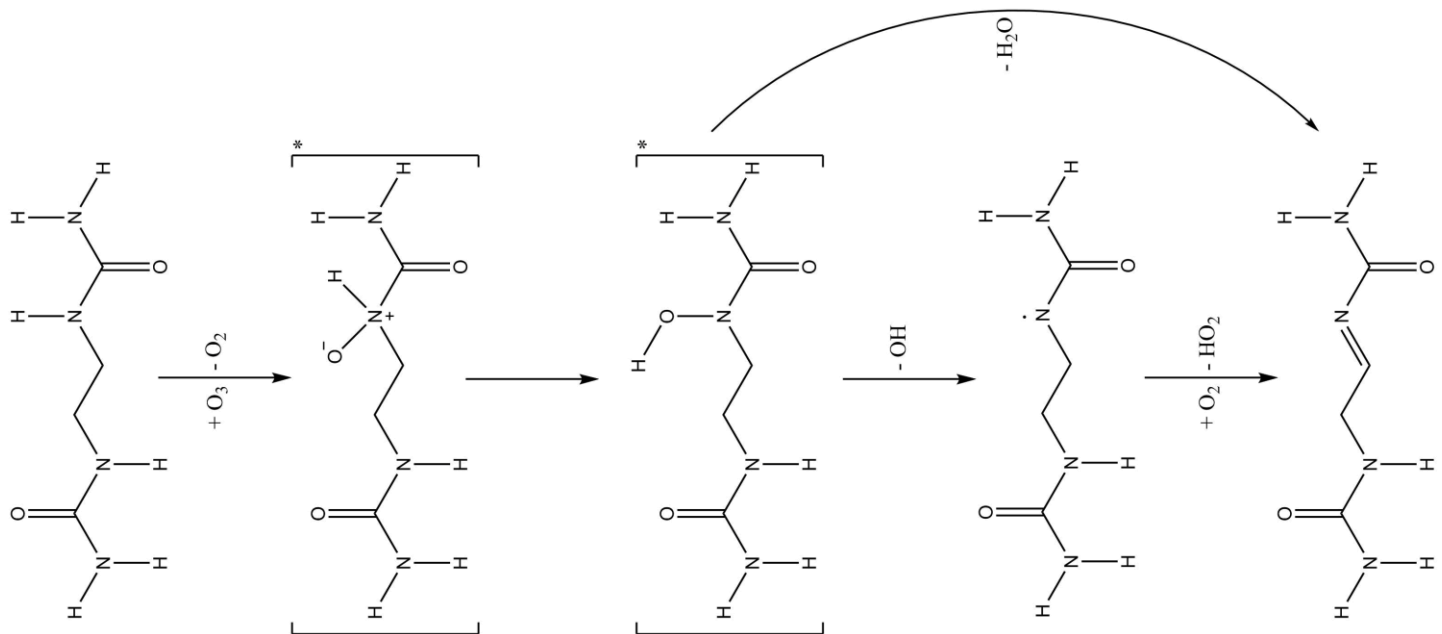
Figure S8 Hypothetical chemical reaction scheme of EDU and ozone interaction.

6.3   Publications unrelated to this thesis

Ueda, Y., Frindte, K., Knief, C., Ashrafuzzaman, M., & Frei, M. (2016). Effects of elevated tropospheric ozone concentration on the bacterial community in the phyllosphere and rhizoplane of rice. PLOS ONE, 11(9), e0163178.

Ali, B., Pantha, S., Acharya, R., Rashid, B., Ueda, Y., Wu, L.B., Ashrafuzzaman, M., Ishizaki, T., Wissuwa, M., Freitag, L., Peiter, E., Bulley, S., and Frei, M., (2018). Abiotic stress tolerance of transgenic cereal crops with enhanced ascorbate biosynthesis (Submitted)

6.4   Conference participation

Poster presentation

Test of ethylenediurea (EDU) as a biomonitoring and screening tool to assess ozone damage in rice (*Oryza sativa* L.)
3rd Asian Air Pollution Workshop (AAPW-3)
20-22 October, 2017. Tokyo, Japan

Oral presentation

Insights into the mode of action of ethylenediurea (EDU) as an antiozonant in rice (*Oryza sativa* L.)
International Conference on Ozone and Plant Ecosystems
21-25 May, 2018. Florence, Italy

Participated actively and contributed as volunteer in the organizing committee

Tropentag international conference
 20-22 September 2017, Bonn, Germany

6.5   Awards

IPID4all (International promovieren in Deutschland–für alle), International conference participation grant, Bonn Graduate Center, Bonn, Germany (2017)

International conference participation grant**,** Theodor-Brinkmann-Graduate School. Bonn, Germany (2018)

7   Curriculum vitae

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