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Ashutosh Kumar Pandey

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Results from EDU treatments

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ABSTRACT

Ozone is considered to be a gaseous pollutant in the lower level of the atmosphere, the troposphere, causing a serious threat to global crop production in major agricultural regions of the world. Various modelling studies predict further increasing ozone levels, in South and East Asia in particular, thereby aggravating the damaging effects of ozone on agriculture. India's bread basket, the Indo-Gangetic Plains (IGP), have been classified as a 'hot spot' for air pollution, owing to the intense agriculture, land-use changes, industrialization, urbanization, population growth and favourable meteorological conditions, causing high emissions of precursors for ozone formation.

This thesis provides an insight into the current status of ozone studies and ozone risks in an agriculturally important region of India, i.e. IGP. Field experiments with local crop cultivars of mustard (*Brassica campestris* L.) and rice (*Oryza sativa* L.) were conducted in ambient ozone concentrations throughout the growing season. EDU (ethylenediurea) was used as a chemical protectant against the adverse effects of ozone. Both the mustard and the rice cultivars showed sensitivity to prevailing ozone concentrations suffering yield losses, thereby indicating the severity of the ozone-induced risk to agriculture in this region. Seven out of the 18 rice cultivars tested showed the best adaptability in high-ozone environments in terms of grain yield.

EDU-mediated protection against ozone stress was mainly due to the up-regulation of the antioxidative defence system, and its extent and timing varied with the developmental phase of the plant species and/or cultivars. The most responsive parameters in EDU treatments were lipid peroxidation, superoxide dismutase and catalase activities at the vegetative phase, and ascorbate and glutathione content at the flowering phase, under high ambient ozone conditions. These parameters can be used as the most useful indicator parameters, for practical ozone-tolerance screening in mustard and rice cultivars.

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CAB Thesaurus: Ozone, Air pollution, EDU (ethylenediurea), Mustard, Rice, Cultivars, Field experiments, Yield, Antioxidant defence system Indo-Gangetic Plains, India.

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LIST OF ABBREVIATIONS

A	Photosynthesis
AOT40	Accumulated O_3 exposure over the threshold of 40 ppb
APX	Ascorbate peroxidase
ASA	Ascorbate
b	Regression coefficient
CAT	Catalase
CH_4	Methane
CO	Oxides of carbon
DHA	Dehydroascorbate
EDU	Ethylenediurea
F_v/F_m	Ratio of variable to maximal chlorophyll fluorescence
GR	Glutathione reductase
g_s	Stomatal conductance
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HTAP	Hemispheric transport of air pollution
IGP	Indo-Gangetic Plains
MDA	Malondialdehyde
NBRI	National Botanical Research Institute
NO_x	Oxides of nitrogen
NPQ	Non-photochemical quenching
O_2	Oxygen
$^1O_2$	Singlet oxygen
O_3	Ozone
OH-	Hydroxide radical
OPLS-DA	Orthogonal projections to latent structure discriminant analysis
OTC	Open top chambers
PCA	Principal component analysis
ppb	Parts per billion

ppm	Parts per million
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TD-NMR	Time domain nuclear magnetic resonance
V	Volt
VOCs	Volatile organic compounds
WUE	Water use efficiency

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman numerals I–III.

I Oksanen E, Pandey V, Pandey AK, Keski-Saari S, Kontunen-Soppela S and Sharma C. 2013. Impacts of increasing ozone on Indian plants. *Environmental Pollution* 177: 189-200.

II Pandey AK, Majumder B, Keski-Saari S, Kontunen-Soppela S, Pandey V and Oksanen E. 2014. Differences in responses of two mustard cultivars to ethylenediurea (EDU) at high ambient ozone concentrations in India. *Agriculture, Ecosystems and Environment* 196: 158-166.

III Pandey A.K, Majumder B., Keski-Saari S, Kontunen-Soppela S, Mishra A, Sahu N, Pandey V and Oksanen E. 2015. Searching for common responsive parameters for ozone tolerance in 18 rice cultivars in India: results from ethylenediurea studies. *Science of the Total Environment* 532: 230–238.

The above publications have been included at the end of this thesis with their copyright holders’ permission.

AUTHOR’S CONTRIBUTION

Article I was a review paper written together with the supervisors. Ashutosh Kumar Pandey (AP) collected the present background information about the ozone studies in India, contributed to data presentation and participated in manuscript writing. For articles II, and III, AP participated in the planning and conducting of the experiments, performed the samplings, field measurements, laboratory analyses (antioxidant analyses) and data analyses, and wrote the manuscripts.

Contents

Original publications

1 Introduction

1.1 THE TWO OZONE LAYERS

Due to the sessile nature of plants, they are often subjected to various environmental challenges causing abiotic stresses. About 50% of agricultural losses worldwide are attributed to various abiotic stresses. Air pollution has become a serious environmental stress threatening crop production. Tropospheric ozone (O_3) alone or in combination with sulphur dioxide, and nitrogen oxides (NOx) may account for 90% of the crop losses that are due to air pollution (Heck et al., 1982; Felzer et al., 2007). O_3 is present in two different layers in the atmosphere: the upper stratospheric ozone layer (15–50 km above the surface) and the lower tropospheric ozone layer (0–15 km above the surface). In the stratospheric region, O_3 is present naturally, formed by the action of ultraviolet radiation from the sun on molecular oxygen (O_2), which in turn prevents the ultraviolet radiation from reaching the earth surface. While in the troposphere, O_3 is formed as a photochemical reaction of NOx and volatile organic compounds (VOCs), oxides of carbon (CO), and methane (CH_4), which are largely emitted by anthropogenic emissions. Only a minor fraction (approximately 10%) of tropospheric O_3 comes from stratospheric infusion (Avnery et al., 2011; Ainsworth et al., 2012). The two O_3-containing layers have different impacts on life on earth, especially on plants. High O_3 concentration can cause several growth, physiological and biochemical alterations, leading to reduced crop yield (Ashmore, 2005; Feng et al., 2007; Wilkinson et al., 2012)

1.2 PHYTOTOXIC EFFECTS OF OZONE ON PLANTS

O_3 enters the plant leaf through stomata, and due to its high oxidative capacity (redox potential +2.7 V), it rarely reaches the cell plasmalemma or cytoplasm itself. O_3 rapidly reacts with the water present in the intercellular spaces, generating reactive oxygen species (ROS), such as hydroxide (OH-) and superoxide (O_2-) radicals, and singlet oxygen ($^1O_2$) as well as hydrogen peroxide H_2O_2, thus resulting in oxidative stress (Heath 1987, Fiscus et al., 2005; Schraundner et al., 1998). These ROS are potentially damaging to proteins, lipids and nucleic acids (Ishida et al., 1999). O_3 causes visible injury to leaves, early senescence, increased leaf abscission, reduced stomatal conductance and net carbon dioxide (CO_2) assimilation, leading to reduced crop yield (Mudd 1996; Del Valle-Tascon and Carrasco-Rodriguez 2004). O_3 can even directly disrupt the normal metabolism as well as resource acquisition and allocation patterns, reducing growth and crop yield (Krupa and Manning 1988) (Fig. 1).

The balance between ROS production and detoxification is regulated primarily by (1) the antioxidative defence system, and (2) adjustments in the stomatal conductance, together with an array of other avoidance, defence, repair, and compensation processes that combat the negative effects of O_3-induced stress (Ashmore 2005; Fiscus et al., 2005; Overmyer et al., 2008). Several hypotheses have been proposed about the relationship between plant sensitivity and stomatal conductance rates. For example, Butler and Tibbitts, (1979) reported that in the O_3-tolerant cultivar of *Phaseolus vulgaris* stomata closed, while in the sensitive cultivar a small increase in stomatal conductance was observed in response to O_3 exposure. On the contrary, Crous et al. (2006) reported that the ozone-sensitive clone of white clover (*Trifolium repens* L.) showed a decline of 30% in stomatal conductance, while the ozone-tolerant clone was unaffected. Further, ozone-induced stomatal sluggishness, i.e. slower or less efficient stomatal response to normal environmental cues, have also been reported in different plant species (*Picea abies*, Keller and Hasler, 1987; *Helianthus annus*, Younglove et al., 1988; *Pinus ponderosa*, Grulke and Preisler, 2002; *Phaseolus vulgaris*, Paoletti and Grulke, 2010). However, the mechanism for sluggish response of stomata under high O_3 conditions is still not fully understood (Paoletti and Grulke, 2010; Hoshika et al., 2014).

1.3 THE BASIS FOR VARIABILITY IN OZONE TOLERANCE

There exists wide variability in ozone-tolerance in different plant species/cultivars (Mills et al., 2007; Biswas et al., 2008; Shi et al., 2009). Selection of ozone-resistant cultivars can prevent 64% of the global crop production losses otherwise expected to occur by 2030 (Avnery et al., 2013). Selection of ozone-tolerant cultivars can therefore be a significant step in improving agricultural production. However, our understanding of crop sensitivity to O_3 to date is imperfect (Wilkinson et al., 2012). For example, Picchi et al. (2010) reported that the wheat cultivars with the least ozone-induced injuries were the most adversely affected in yield, implying that a strong reduction in stomatal conductance both prevented the pollutant's entry and reduced the rate of carbon assimilation. Similarly, Sawada and Kohno (2009) demonstrated in 20 rice cultivars that visible injury symptoms in the foliage did not necessarily coincide with a negative impact on grain yield. The selection of indicator parameters for ozone-tolerance is therefore difficult, although attempts using different parameters, such as visible injury, chlorophyll b/a ratio and photosynthetic parameters have been used to rank cultivars for their tolerance (Betzelberger et al., 2010; Saitanis et al., 2014).

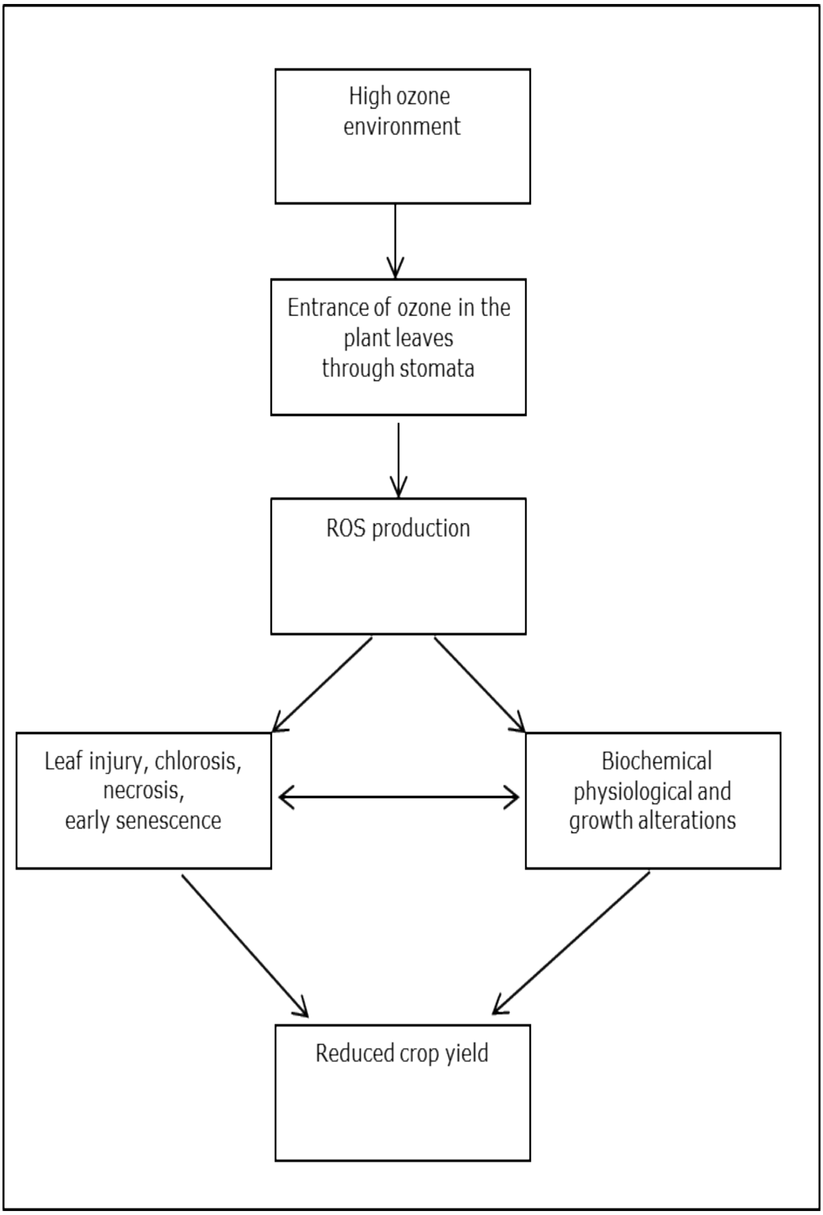

Figure 1 *Effect of ozone uptake on plants and its impact on crop yield*

1.4 IMPACT OF OZONE ON AGRICULTURE

O_3 is a serious threat to global agricultural production due to its high phytotoxicity and prevalence over agriculturally important regions (Emberson et al 2009; Van Dingenen et al., 2009). Further, not only atmospheric O_3 concentrations but also the transport of O_3 and its precursors (NOx and VOCs and CO) across the continents is of growing concern, especially in the northern hemisphere, as a threat factor to crop productivity (HTAP 2010). Globally, present O_3 concentrations ranging between 20–40 ppb, depending on the location, are expected to increase further at a rate of 0.5–2% year^{-1} (Vingarzan, 2004). Annual O_3-induced production losses for major agricultural commodities have been estimated at between $14 and $26 billion under present air quality legislation, and a further decline of 10% in the yields of ozone-sensitive crops is predicted by year 2030 (Van Dingenen et al., 2009; Avnery et al., 2011).

At regional levels, ambient O_3 concentrations seem to have levelled off or slightly decreased in North America and Europe (IPCC 2007). Klingberg et al. (2014) also reported that there is a substantial reduction in O_3 precursor emissions in Europe. On the contrary, O_3 levels are increasing and are predicted to increase further in South and East Asia due to rapid urbanisation and industrial development (Ohara et al., 2007; The Royal Society 2008). In Asia, modelling-based assessments of ozone-induced agricultural damage rely on European and North-American dose-response relationships. The estimation of agricultural losses may therefore not be accurate, and data on Asian plants and conditions are urgently needed. Emberson et al. (2009) reported that Asian cultivars of wheat and rice are more sensitive to O_3 as compared to North American cultivars. Overall, long term O_3 predictions and species-level information on O_3 sensitivity/tolerance are scarce (Wang and Mauzerall, 2004; Emberson et al., 2009). Recognising the importance of O_3 impacts on plants, North American and European countries have systematically assessed O_3 impacts on crop plants as well as the ways and means to ameliorate production and crop damage. However, in Asia and South East Asia, there is still a major problem dealing with the O_3 impacts, firstly because of the higher ozone-sensitivity of crop cultivars in this region (Emberson et al. 2009), and secondly, because the baseline data on O_3 impacts have not been systematically generated.

*1.4.1 **Agriculture and its importance in India***

India is ranked as second in farm output worldwide, supporting 18% of the world population on only 9% of the world's arable land and 2.3% of its geographical area. Agriculture in India employs 53% of the population and therefore plays an important role in the socio-economic development of the country (Indian Council of Agriculture Research, Vision 2030, 2011) (Fig. 2). The population of India, with ca. 1.2 billion people, is primarily dependent on food production within the country, and India also exports rice to other Asian and African countries. Thus agricultural losses in India may even affect food security globally (Burney and Ramanathan, 2014). In a recent report, Sachin et al. (2014) estimated that O_3-induced damage to wheat and rice crops, if prevented, would suffice to feed 94 million people.

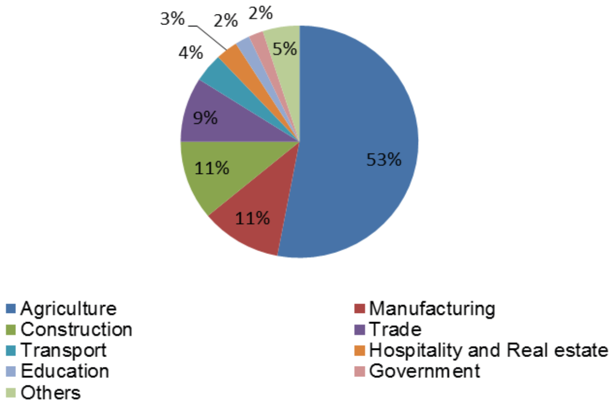

Figure 2. *Percentage employment across various sectors in India (Wikipedia)*

1.4.2 Importance of mustard and rice

Rapeseed-mustard (*Brassica spp.*) is grown as a major oilseed crop in 53 countries and across six continents, and is used mainly for cooking purposes. In India, mustard is mainly grown in the northern and eastern parts of the country (Fig. 3A). Despite being the second largest producer of rape-seed mustard (Hegde, 2005) after China, India still imports 40% of its annual edible oil needs (Boomiraj et al., 2010). Oilseed therefore plays an important role in the agricultural economy of India. Mustard is classified as moderately sensitive to O_3 (Mills et al., 2007).

Rice is the world's most important crop, feeding about 50% of the global population, and it is grown in 95 countries worldwide (IRRI; 2002, Maclean et al., 2002). India is the second largest producer of rice accounting for 21% of the world production after China (28%) (Fischer et al., 2014). Rice accounts for about 43% of total grain production in India and is grown almost throughout the country (Siddiq, 2000) (Fig. 3B). Mills et al. (2007) defined rice as moderately sensitive to O_3, although to date, as compared to wheat and soybean experiments, the number of field experiments is few, involving only a few cultivars. Feng and Kobayashi (2009) reported that rice can be equally sensitive to O_3 stress as other ozone-sensitive crops, such as wheat and soybean. Consequently, an accurate assessment of rice production losses in the areas suffering from high risk of ozone-induced damage is important for global food security. In India, IGP is one of most extensively cultivated areas, called a "bread-basket" of the country (Fig. 3A, highlighted region).

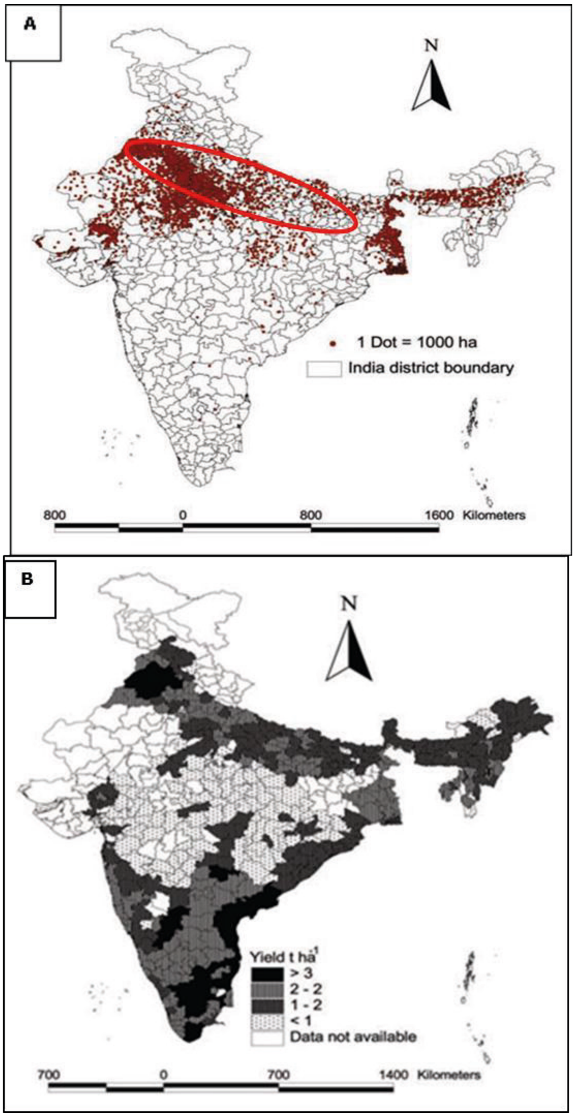

Figure 3. *Distribution of (A) mustard and (B) rice cultivation across different districts in India (Source: ICRAST report 2008), IGP area is highlighted in the map A.*

1.5 ASSESSMENT OF CROP PRODUCTION LOSSES

1.5.1 Methods for ozone experiments in field conditions

Several methods have been used to assess the phytotoxic effect of O_3 in field conditions; (1) Use of open-top chamber (OTC) or (2) open-air fumigation, and (3) indirect methods using chemical protectants, such as EDU (ethylenediurea; [N-(2-2-oxo-1-imidazolidinyl) ethyl]-N′-phenyl urea) (Fig. 4). The OTC method has some limitations, for example, constant wind speed and O_3 concentration, and elevated temperature, which due to the chamber effect may lead to the over- or under-estimation of the O_3 impact on plants (Manning and Krupa, 1992). Open-air fumigation systems are near to the true ambient outdoor conditions, (Karnosky et al., 2007; Watanabe et al., 2014), simulating the future effects of O_3 (ambient O_3 concentrations serve as control treatment, while elevated concentrations are the stress treatments) (Long et al., 2006; Betzelberger et al., 2010). However, under high prevailing O_3 concentrations it is a real challenge to provide ozone-free conditions for the field experiments. Moreover, this system is very expensive and requires high-level technology, and it is therefore very necessary to develop easy and economical methods for assessing the effect of O_3 in ambient field conditions, especially for developing countries where funding can also pose a major constraint for O_3 studies.

Among the indirect methods used, the most frequently studied chemical protectant against O_3 is EDU. Carnahan et al. (1978) first described EDU as a chemical protectant against O_3 stress. EDU is an alternative to open-top chambers and has been used as a tool for evaluating the impacts of O_3 on plants under ambient O_3 conditions (Paoletti et al., 2009; Manning et al., 2011). Although several other chemical protectants with antiozonant properties have been tested on plants over the years, for example phenylurea (Tomlinson and Rich, 1974), Santoflex (Gilbert et al., 1977) and Di-1-p-menthene (Vapor Gard) (Agathokleous et al., 2014), none of them were found to offer satisfactory protection against O_3 stress.

1.5.2 Ethylenediurea (EDU): a research tool for ozone stress

The structure of the EDU molecule (Fig. 4) contains four nitrogen atoms. However, EDU does not have any olefinic double bonds such as ascorbate and uric acids, which are considered to be potent O_3 scavengers and can directly trap O_3 as the primary ozonide (Enami et al., 2008 a, b). Although EDU contains nitrogen, the amount is not sufficient to provide any fertilising effects, as shown in hybrid *Populus* clones (Ainsworth et al., 1996) and *Pinus taeda* L. (Kuehler and Flagler, 1999). Neither do several other studies, for example, those by Paoletti et al. (2009), Feng et al. (2010) and Manning et al. (2011) support a role for nitrogen in EDU as providing protection against O_3 damage. EDU remains in the apoplast of the cells for more than eight days and does not enter the cell, implying that it does not directly affect the cell metabolism (Gatta et al., 1997). EDU is taken up by the roots and leaves but it is not transported to the newer or non-EDU treated leaves. EDU therefore needs to be applied on a regular schedule in order to protect plants against ozone-induced damage (Manning, 2000; Paoletti et al., 2014).

EDU has been used widely to evaluate ozone-induced losses in several crops, e.g., *Solanum tuberosum* L. (Eckardt and Pell, 1996), snap bean (Lee at al., 1997) bush beans (Tonneijck and Van Dijk, 1997) and *Triticum aestivum* L. (Tiwari et al., 2005; Singh et al., 2009). EDU can also reveal different responses to O_3 stress among the cultivars in ambient O_3 concentrations, as demonstrated, for example, in *Triticum aestivum* L. (Singh et al., 2009) *Trifolium repens* (Singh et al., 2010), *Vigna mung* (Singh et al., 2010) and *Raphanus sativus* (Pleijel et al., 1999). Feng et al., (2010) reported that application of EDU is easier in the case of crop species than in trees. Several experiments have proved that EDU may be used as a reasonable and inexpensive method for studying the impact of ambient O_3 on crops, especially in areas where electricity is a major constraint on continuous O_3 monitoring (Feng et al., 2010; Tiwari et al., 2005). EDU treatments can also be used to indicate the areas that are suffering from high O_3 pollution and in bio-monitoring programmes for mapping O_3 injuries to plants (Wang et al., 2007; Tiwari et al., 2005; Rai et al., 2015). Although an indirect method, the applicability of EDU is also supported by the observation that it does not have effects on plant growth in non-ozone conditions (Foster et al., 1983; Szantoi et al., 2007).

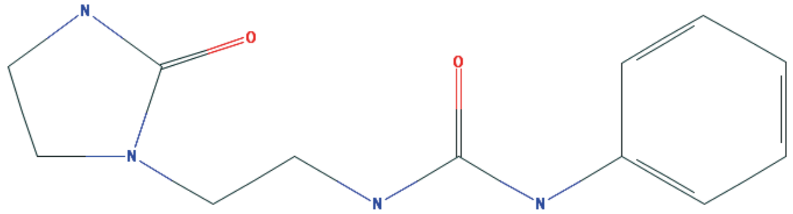

Figure 4. *Chemical structure of EDU (Source: Pubchem)*

1.6 AIMS OF THE THESIS

The aim of the study was to establish a reference point for O_3 research by identifying existing knowledge, knowledge gap areas, as well as to investigate O_3-induced risk in agriculture using EDU treatment and in two important crop plants, mustard (*Brassica campestris* L.) and rice (*Oryza sativa* L.), in field conditions throughout growing season in the agriculturally important region of IGP in India.

The specific aims of the study were:

1. To collect and collate the existing knowledge and data in order to understand the magnitude of potential O_3 problems in India, with respect to plant productivity and food production.

2. To screen local cultivars of mustard and rice for their O_3 sensitivity/tolerance.

3. To define the most responsive parameters for screening O_3-tolerant rice cultivars using EDU as a tool.

4. To elucidate the strategies adopted by different crops or cultivars under high ambient O_3 concentrations with EDU treatment.

2 Materials and Methods

Article I is a review paper summarising the present status of O_3 pollution and related research in India with regard to Indian crop plants. For articles II and III, a summary of the plant material, experimental sites and methodologies used in the field experiment are illustrated in Table 1.

2.1 REVIEW ARTICLE (I)

For the Review Article (I) all available data from modelled and measured O_3 and O_3 precursor concentrations over an Indian area were searched and collected (including satellite observations), and all published studies and data from experimental research with Indian plants were compiled. Besides the existing knowledge, gaps in knowledge concerning the O_3 risk in India were identified, forming a starting point for the experimental work of this thesis. The Review Article included altogether 98 references, including 14 experimental studies conducted in open-field conditions or using an open-top chamber facility as well as 10 studies where EDU was used.

Table 1. A summary of the plant material, experimental sites, and measurements in articles II and III.

Plant material	**Mustard**	**Rice**
Articles	II	III
Year of study	2012-2013	2011
Number of cultivars	Two	Eighteen
Time of measurement	Vegetative, flowering, harvest	Vegetative, flowering, harvest
EDU treatment levels	200 ppm, 400 ppm	300 ppm
EDU application method	Foliar spray	Foliar spray
NPK fertilisation dose (Kg ha^{-1})	80:40:40	120:60:60
Experimental condition	Ambient field conditions	Ambient field conditions
Geographical locations	NBRI (Lucknow) field site (26° 55′ N, 80° 59′ E)	Lucknow (26° 55′ N, 80° 59′ E) and Banthra (26°45' N, 80°53' E) field sites
Parameters measured	Growth, Photosynthetic pigments, Leaf gas exchange, Lipid peroxidation, Antioxidative enzymes, Antioxidants, Yield attributes	Soil physical properties, Growth, Photosynthetic pigments, Leaf gas exchange, Lipid peroxidation, Antioxidative enzymes, Antioxidants, Yield attributes

2.2 FIELD EXPERIMENTS (II, III)

In India the two major cropping seasons are based on the monsoon: (1) kharif, or the summer crop, grown during the months of July to October with the beginning of the monsoon rain, and (2) rabbi, or the winter crop, grown from October to April. The two important crop plants selected for the field experiments in this project were rice as a kharif crop and mustard as a rabbi crop. For both crop species the selected cultivars are widely grown by farmers in the IGP region in India. Both these crops also differ in their water requirements.

Field experiments were conducted at two different sites: the Botanical Garden of the National Botanical Research Institute (NBRI), located in the centre of Lucknow city, and the experimental site of NBRI in Banthra, located ca. 25 km from the main city of Lucknow (Fig. 5).

2.3 EDU APPLICATION

For the mustard experiment (II), two different concentrations of EDU (200 ppm and 400 ppm) were selected, while in the rice experiment (III), a single concentration of 300 ppm was used for the treatment. The entire foliage of each plant was sprayed until it was visibly saturated. The choice of these EDU concentrations and the method of application were based on the earlier literature suggesting that 200–400 ppm of EDU would be the most effective in protecting crop plants against O_3 stress (Feng et al., 2010).

2.4 OZONE MONITORING

Ambient O_3 concentration was monitored using the 2B Tech Ozone Monitor (106-L) on eight h d^{-1} (from 9.00 to 17.00) during both experiments (II, III).

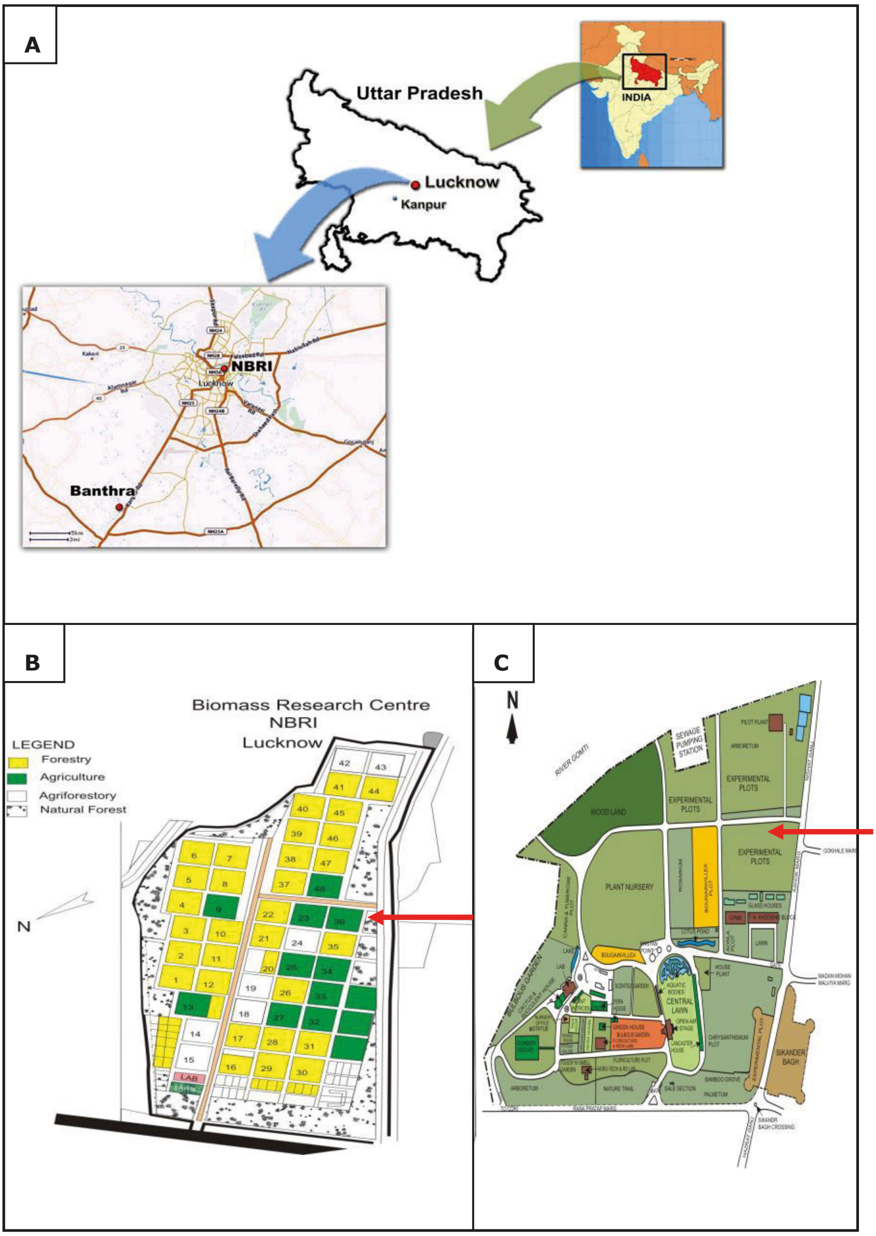

Figure 5. (*A) The location of experimental sites in India (B) Maps indicating the experimental plots at Banthra (C) and at the Lucknow site (Source: NBRI).*

2.5 ASSESSMENT OF VISIBLE OZONE INJURIES

The use of bio-indicator plants is an inexpensive and simple method for verifying the toxic levels of O_3 in the areas with high concentrations (Manning 1997). In our experiments, the tobacco cultivar Bel-W3 was used as a bio-indicator plant to indicate high prevailing O_3 concentrations at the field sites (Fig. 6A-C). However, the injury response cannot be used as qualitative measures to assess air quality for O_3 (Woodwell, 1989; Pandey et al., 2013). Foliar injury symptoms were recorded on mustard (Kranti) (Fig. 6D,E) and for all the 18 cultivars of rice (Fig. 7).

2.6 GROWTH, YIELD, PHYSIOLOGICAL AND BIOCHEMICAL MEASUREMENTS

Biomass was measured at three developmental phases (vegetative, flowering and harvest) (II, III), and chlorophyll content was measured using SPAD-502 (Konica-Minolta, Osaka, Japan) on five randomly selected plants at both the vegetative and the flowering phase (II). Gas exchange parameters, i.e. photosynthesis (A), stomatal conductance (g_s), ratio of variable to maximal chlorophyll fluorescence (F_v/F_m), water use efficiency (WUE) and non-photochemical quenching (NPQ) were measured using the Li-COR 6400 gas exchange portable photosynthesis system (Li-COR, Lincoln, Nebraska, USA) with a fluorescence chamber (LFC64000-40; 40 Li-COR) on three randomly selected plants per treatment and per cultivar. Biochemical parameters, such as lipid peroxidation (MDA content), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) activities, ascorbate (ASA), reduced dehydroascorbate (DHA), reduced glutathione (GSH), oxidised glutathione contents (GSSG) (II) and MDA content, SOD activity, CAT activity, and GSH and GSSG contents (III) were measured using the standard spectrophotometric techniques. Harvest parameters, for example, pods plant^{-1}, number of seeds five pods^{-1}, weight of 1000 seeds, seed weight plant^{-1} (II) and grain weight plant^{-1}, 1000 grains weight and inflorescence weight (III) were measured at the harvest phase (Table 1).

2.7 Seed oil content

The oil content of mustard seeds was measured using the minispec bench top Time Domain NMR (TD-NMR), which is a non-destructive method based on the detection of the hydrogen in the liquid phase of the sample (Jarret et al., 2011; Deblangey et al., 2013).

2.8 Statistical analysis

In the mustard experiment (II) the effect of the EDU treatment, the cultivar and their interactions were analysed using two-way ANOVA. In the rice experiment (III) the effect of EDU treatment, the cultivar and their interaction on all the measured parameters were analysed by non-parametric two-way ANOVA. All the three developmental phases (i.e. vegetative, flowering and harvest) were analysed separately. All the analyses were carried out with SPSS software (SPSS Inc., version 17.0). Principal component analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA), for the two sites separately, were conducted using Simca P+ software (12.0.1, Umetrics, Umeå, Sweden). Additionally, in the rice experiment (III), a linear regression technique was used to compare the performances of all the 18 rice cultivars, considering both experimental sites and in both treatments. This technique was originally developed by Finlay and Wilkinson (1963).

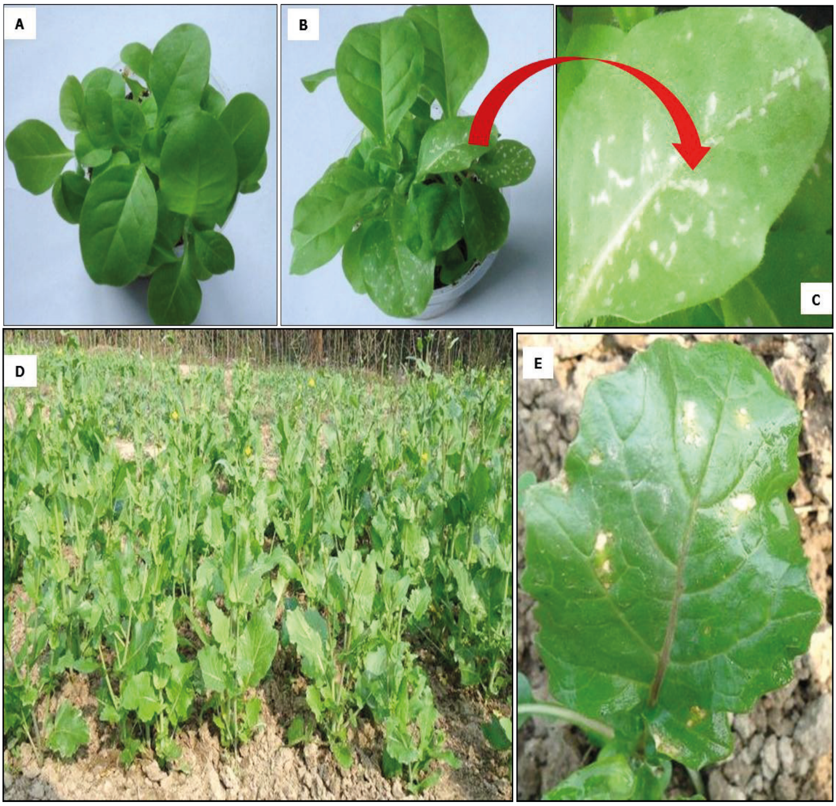

Figure 6. *Ozone injury symptoms on different plant species under ambient ozone concentration. (A) Bel B (ozone-tolerant); (B, C) Bel W3 (ozone-sensitive) varieties of tobacco plants* (Nicotiana tabacum L.) *(ozone concentration ranged between 26-78 ppb); (D) Mustard* (Brassica campestris L.) *grown under field conditions; (E) Kranti cultivar of mustard (ozone concentration ranged between 12–86 ppb).*

Figure 7. *Rice (Oryza sativa* L.*) seedling before the transplantation. (A). Rice seedling transplanted at the experimental site at Lucknow (B). Rice plants at the vegetative phase (C). Ozone injury symptoms on rice leaves on 18 different rice cultivars (D) (ozone concentration ranged between 7–83 ppb).*

3 Results and Discussion

The main results for each article are summarised below:

Article I

- There is great temporal and spatial variation in the reported O_3 concentrations over India. The highest concentrations prevail in the northern part of India, including the IGP area, particularly during the winter and spring months.
- The importance of carrying out experiments locally with reliable O_3 monitoring is highlighted since such data are limited.
- The need for cultivar screening is indicated, because so far species-level variability in O_3 sensitivity has been poorly addressed.

Article II

- Both tested mustard cultivars, Kranti and Peela sona, are sensitive to high ambient O_3 concentrations.
- The strategies adopted by the two cultivars against O_3 stress are different.
- EDU-mediated protection in mustard cultivars is mainly through the antioxidative defence system.
- EDU can serve as a useful tool for assessing crop production losses in the areas facing high ambient O_3 concentrations.

Article III

- Antioxidative defence plays a key role in EDU-mediated defence against high ambient O_3 concentrations in rice cultivars.
- EDU-induced positive effects are not strictly linked to crop yield.
- EDU treatment responses are cultivar, developmental phase, and site specific.
- Rice cultivars: NDR 359, HKR 47, Pusa Basmati 1, TCS-555, Narendra Usar 3, Pusa Sugandha 5, and Varadhan are the best yielding cultivars originating from the IGP area of India in the present study.

3.1 OZONE RESEARCH IN INDIA

The Review Article (I) included previous experimental work with the most important crop plants in India, i.e. wheat (*Triticum aestivum*), rice (*Oryza sativa*), mustard (*Brassica campestris*), palak (*Beta vulgaris*), linseed (*Linum usitatissimum*), mung bean (*Vigna radiata*), carrot (*Daucus carota*), white clover (*Trifolium repens*) and Black gram (*Vigna mungo*). Thereafter, several modelling studies (Tang et al., 2013; Elampari et al., 2013; Sinha et al., 2014; Burney and Ramamathan, 2014; Ghude et al., 2014; Debaje, 2014) and a few experimental studies with *Triticum aestivum* (Rai and Agrawal, 2014), *Zea mays* L. (Singh et al., 2014) and *Glycine max* (Rai et al., 2015) have added to the information about the ozone-induced agricultural risk of crop losses in India. Particularly in the IGP area, the vulnerability of plants to ozone-induced damage is well reported. However, the majority of the field experiments still involve only a few crop cultivars.

3.2 HIGH AMBIENT OZONE CONCENTRATIONS

In the mustard experiment, the plants were exposed to high ambient O_3 concentrations from the beginning of the experiment (II). Average 8-h ozone concentrations ranged between 12 and 86 ppb during the different developmental phases, and the accumulated O_3 exposure over the threshold of 40 ppb (AOT40) was 16 ppm h at the end of the experiment. In the rice experiment (III) the plants were exposed to high ambient O_3 concentrations particularly during the flowering and the harvest phase, with an average of 8-h O_3 concentration ranging between 7-83 ppb. The vegetative phase occurred with the onset of the monsoon period, which is characterised by heavy rain leading to the washout of the O_3 precursors (Fig. 8). Similar O_3 concentrations have been reported during the monsoon season (June to September) by Jain et al. (2005), Ghude et al. (2008), Ali et al. (2012). O_3 is a secondary air pollutant, and its formation depends on various meteorological conditions, being favoured by high radiation, high temperature, and low relative humidity (Singh et al., 2014). Earlier studies have indicated that crop plants are most vulnerable to ozone-induced damage especially during the flowering phase and seed maturity phase (Heagle, 1989; Mulholland et al., 1998; Lee et al., 1988; Pleijel et al., 1998). Both our field experiments showed high O_3 concentrations during this phase of crop growth and therefore a high impact of O_3 could be expected.

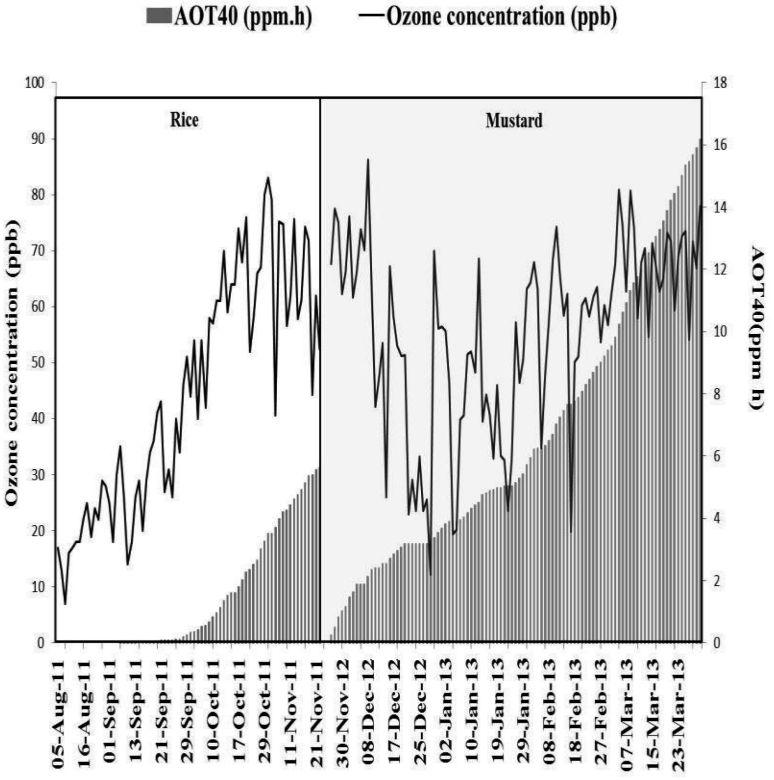

Figure 8. *Daily ozone concentration (ppb, 8-h average) and AOT40 (ppm h) indices during the growing season for rice* (Oryza sativa L.) *(27August 2011-22 November 2011 and mustard* (Brassica campestris L.) *(27 November 2012 - 1 April 2013).*

3.3 TOWARDS MORE ACCURATE OZONE RISK ASSESSMENT

India covers about 3 million km^2 of the world land area of which 53 % is arable land. Due to its vast land cover and varied agro-climatic conditions, (Table 2) (Indian Agriculture Statistics Research Institute, Agriculture Research Data Book, 2014), differences in O_3 tolerance among the cultivars is expected (I). It is important to establish dose-response curves under different natural field conditions using local crop cultivars. To assess agricultural losses, the data from local field experiments, particularly on staple crops, would improve the present modelling approaches used for O_3 risk assessment, which still rely on the European and North-American dose-response relationships (Emberson et al., 2009). Mills et al., (2007) set the critical level for rice (AOT 40) for 5% yield reduction at 12.82 ppm h. However, much higher yield losses for rice have been reported in later studies from the Indian and Pakistan areas. For example, Rai et al., (2010), showed a rice yield reduction of 10–15% at AOT40 values of 2.1 ppm h in the IGP region. Similarly, Wahid et al. (1995) reported 37–42% yield reductions in two rice cultivars growing under a mean O_3 concentration of 35 ppb throughout the one growing season in Pakistan. Our results also indicate that the critical O_3 exposure is exceeded in the IGP area of India for crops, and that tropical rice cultivars show a higher sensitivity to O_3-induced damage. AOT40 exposure at the end of the mustard experiment (II) was 16 ppm h, with both cultivars (Kranti and Peela sona) showing an increase of 4–5% in seed oil content when treated with 400 ppm EDU. In the rice experiment (III) an AOT40 exposure of 5.6 ppm h was recorded. At Lucknow site, the net yield increase was 25% (calculated for all 18 cultivars) in 300 ppm EDU treatment, but the complex soil-water status-EDU interactions caused a decrease in net yield at the Banthra site, hampering O_3 exposure response estimations from this experiment.

Besides the urgent need for nation-wide O_3 monitoring in order to form reliable dose-response curves, and the need for experiments involving local cultivars, the Review Article (I) indicates the importance of studies where edaphic factors, crop phenology, agricultural practices and other air pollutants have been incorporated and considered. These findings were used as background knowledge for our field experiments, where local mustard and rice cultivars were screened for ozone-sensitivity in a high O_3 environment.

Table 2. *List of agro-climatic regions and the different states presenting them in India (Source: IASRI, Agriculture data book, 2014).*

S.No	**Agro-climatic regions/zones**	**States represented**
1	Western Himalayan regions	Himachal Pradesh, Jammu and Kashmir, Uttarakhand
2	Eastern Himalayan region	Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, Tripura, West Bengal
3	Lower Gangetic plains region	West Bengal
4	Middle Gangetic plain region	Uttar Pradesh, Bihar
5	Upper Gangetic plain region	Uttar Pradesh
6	Trans Gangetic plain region	Chandigarh, Delhi, Haryana, Punjab, Rajasthan
7	Eastern plateau and hills region	Chhattisgarh, Jharkhand, Madhya Pradesh, Maharashtra, Orissa, West Bengal
8	Central plateau and hills region	Madhya Pradesh, Rajasthan, Uttar Pradesh
9	Western plateau and hills region	Madhya Pradesh, Maharashtra
10	Southern plateau and hill region	Andhra Pradesh, Maharashtra
11	East coast plains and hills region	Andhra Pradesh, Karnataka, Tamil Naidu
12	West coast plains and ghat region	Goa, Karnataka, Kerala, Maharashtra, Tamil Nadu
13	Gujarat plains and hills region	Gujarat Dadra, Nagar Haveli, Daman and Diu
14	Western dry region	Rajasthan
15	Island region	Andaman and Nicobar Islands, Lakshdweep

3.4 ROLE OF EDU IN COMBATING OZONE STRESS

3.4.1 Effect of EDU on Gas-exchange

The gas-exchange parameters, g_s, and A, did not show any significant effect with EDU treatment in either experiments (II, III). This is in accordance with most of the earlier studies, for example, in *Fagus sylvatica* (Ainsworth and Ashmore, 1992), in hybrid *Populus* clones (Ainsworth et al., 1996), *Pinus taeda* L. (Kuehler and Flagler, 1999), *Solanum tuberosum* L. (Hasan et al., 2006), and in the meta-analysis of 15 crop species by (Feng et al., 2010). These findings indicate that EDU does not operate by causing stomatal closure and thus restricting the O_3 uptake, but its effects are only seen after entry into the plants (Bennett et al., 1984). However, Singh et al. (2009) reported increased g_s in a sensitive cultivar of wheat with the EDU treatment.

In the mustard experiment (II), the only exception to the lacking effects of EDU treatment in gas-exchange parameters was decreased NPQ, indicating that EDU-mediated protection against O_3 stress occurs through improving photosynthetic capacity for electron transfer, less energy being dissipated as heat (Muller et al., 2001). A meta-analysis showed increased A with EDU treatment (Feng et al., 2010). This may partly be explained by the senescence-delaying capacity of EDU and also its ability to act like the plant hormone cytokinin, which can retard chlorophyll degradation in leaves (Lee and Chen, 1982; Tonneijck and Van Dijk, 1997). Similar results were observed in the mustard experiment (II), as chlorophyll content slightly increased in the Peela sona cultivar at the vegetative and the flowering phase with EDU treatment.

In the rice experiment (III) the plants retained higher g_s throughout the developmental phase at the Banthra site, which may be related to better water availability because of the higher water retention capacity of the soil (due to a higher percentage of silt and clay). Thus, plants at the Banthra site may have suffered higher O_3 exposure, even though the O_3 regimes at both the experimental sites (Lucknow and Banthra) were similar. The inherent rate of g_s has been associated with the variability of O_3

sensitivity in plants (Biswas et al., 2008; Brosche et al., 2010). Together with g_s, other environmental factors, such as temperature, vapour pressure deficit and soil moisture also influence the actual O_3 uptake by plants (Weber et al., 1993; Agrawal and Agrawal, 1999; Emberson et al., 2000; Karlsson et al., 2004), affecting their vulnerability to ozone-induced damage.

3.4.2 EDU mediated antioxidative defence against ozone stress

In both field experiments with mustard (II) and rice (III), it was evident that in plants treated with EDU, the antioxidative defence system played an important role in ameliorating the negative effects of O_3, as compared with ambient-O_3 plants. The extent and response time of antioxidative defence varied among the cultivars, developmental phases and experimental sites with EDU. Ranieri and Soldatini (1995) reported that in snapbean (*Phaseolus vulgaris*) EDU-induced protection by increased level of antioxidative enzyme activity and antioxidants varied with the developmental phase of the plants.

In the mustard experiment (II), MDA content (an indicator for membrane damage) was significantly reduced in both cultivars, particularly in Kranti at 400 ppm EDU concentration, at both the flowering and the vegetative phases. Increased MDA content has been shown to correlate with severity of O_3 exposure (Calatayud et al., 2004; Biswas et al., 2008). However, the Kranti cultivar showed more pronounced antioxidative defence in response to EDU treatment as compared to Peela sona, as evidenced by its significant up-regulation of antioxidative enzyme activities (SOD, APX, and GR) and antioxidant contents (ASA and GSH), particularly at the flowering phase with 400 ppm EDU concentration (Figure 9). Similarly, the rice experiment (III) indicated the key role of the antioxidative defence system (SOD, CAT, and GSH) in response to EDU treatment under high ambient O_3 concentrations for all 18 cultivars and both experimental sites. In accordance with this finding, Rai et al., (2015) also reported an 11–40% increase in the activities of antioxidative enzymes SOD, APX and GR in soybean cultivars at a corresponding 400 ppm EDU concentration. Studies on *Solanum tuberosum* L. (Hasan, 2006) and *Triticum aestivum* L. (Singh et al., 2009) also showed increased activity of SOD with EDU treatment. SOD is the primary defence enzyme for scavenging the superoxide radical produced during oxidative stress, while CAT and APX subsequently detoxify H_2O_2 to form a harmless end product. Besides these antioxidative enzymes (SOD, CAT, APX and GR), various non-enzymatic antioxidants (ASA, GSH) play an important role in the plant defence system against ROS and maintain a cellular redox balance, preventing higher accumulation of ROS, which may lead to irreversible metabolic dysfunctions and cell death (Noctor and Foyer, 1998; Halliwell and Gutteridge, 2007; Sharma et al., 2012; Ueda et al., 2013). Ascorbic acid, in particular, is a well know antioxidant and a cellular reductant which can directly scavenge O_3 in the apoplast or act as an electron donor to APX in the Halliwell-Asada pathway thus attenuating the generation of ROS (Burkey et al., 2006). Higher content of GSH with EDU treatment has been reported in *Phaseolus vulgaris* (Lee et al., 1997) and potato (*Solanum tuberosum* L.) (Hassan, 2006).

Paoletti et al., (2014) confirmed by means of the gene expression technique that 300 ppm EDU treatment may halt ozone-induced ROS generation within 24 h from the start of the exposure in snapbean (*Phaseolus vulgaris*), thus preventing the downstream cascade mechanism that leads to increased H_2O_2 production, impaired gas exchange and occurrence of leaf lesions. This result indicates the efficient role of EDU-mediated protection against O_3 stress, which was also found for mustard (II) and rice (III) in the present studies.

Concluding from the antioxidative studies in both mustard and rice, our results indicate that MDA content, SOD and CAT activities at the vegetative phase, as well as ASA and GSH content at the flowering phase are the most useful indicator parameters for practical O_3 tolerance screening.

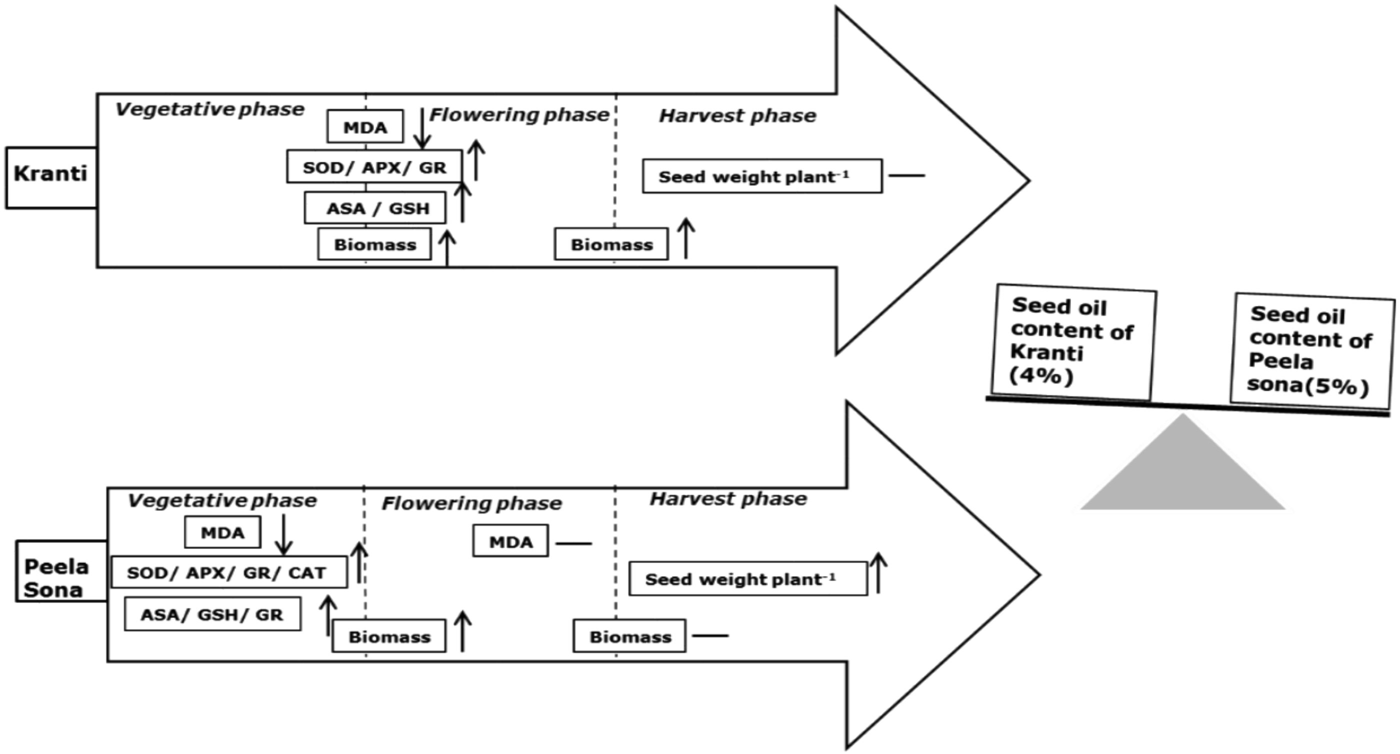

Figure. 9 *The effect of EDU-mediated differential strategy in mustard (*Brassica campestris L.*) cultivars Kranti and Peela sona on biomass and antioxidative defence and final yield output (seed oil content). An increase, decrease or no significant change in different parameters are marked with symbols ↑ , ↓ , –, respectively. MDA (malondialdehyde), SOD (superoxide dismutase), CAT (catalse), APX (ascorbate peroxidase), GR (glutathione reductase), ASA (ascorbate), GSH (glutathione).*

3.6 LARGE CULTIVAR DIFFERENCES

Although both mustard cultivars (Kranti and Peela sona) had a similar life span of 125 days, their biomass accumulation differed particularly after the flowering phase, indicating major developmental differences. Kranti continued to invest more of its resources to accumulation of biomass until the final harvest phase, while Peela sona did not show any significant changes in biomass between the flowering and the harvest phase, as it invested more in seed production. Further, the EDU treatment effect was significant only at the harvest phase, as it increased the biomass by 78% and 98% in Kranti and Peela sona, respectively, at 400 ppm EDU (Fig. 9). Earlier studies with wheat (*Triticum aestivum* L.) have demonstrated that ozone-induced adverse effects on biomass growth and yield attributes can only be measured after the final harvest phase, as the effects are often cumulative across the full growing season (Singh and Agrawal, 2010; Wilkinson et al., 2012). Despite the major differences between the mustard cultivars (II) as regards growth behaviour and allocation to seed production, the impact of EDU on seed oil content was almost similar. Seed oil content in Kranti and Peela Sona increased by 4% and 5% respectively with 400 ppm EDU concentration (Fig. 9).

The rice experiment (III) with 18 cultivars and 24 test parameters demonstrated the complexity of ozone-tolerance screening when large number of cultivars and parameters are included. Different cultivars showed varied responses, depending on the experimental site and the EDU treatment. For example, at the Lucknow site, most of the cultivars showed increased catalase activity, but at the Banthra site, decreased activity. Despite these facts, using the linear regression method we were able to classify all the 18 cultivars into 4 groups based on their yield performances: (1) generally well adapted cultivars (above-average yield) NDR 359, HKR 47, Pusa Basmati 1, TCS-555, Narendra Usar 3, Pusa Sugandha 5, and Varadhan; (2) poorly adapted (below-average yield) MTU 7029, Sarjoo 52, BPT 5204, PANT 12, PR 113, Narendra-97, Barani Deep and Narendra

Lalmati; (3) adapted to low-yield conditions NDR 8002 and Shusk Samrat; and (4) very sensitive cultivars (adapted to high-yield conditions) Swarana-Sub-1. Sawada and Kohno (2009) also demonstrated a wide variability in O_3 sensitivity in 20 rice cultivars using an OTC experiment. In their study, six out of 20 cultivars suffered significant yield reduction, while the remaining cultivars (except one) showed no significant changes in yield due to high O_3 exposure.

Overall, our results (II and III) demonstrate that long term experiments extending over full growing seasons with several cultivars are also needed in the case of for crop plants, to ensure realistic O_3 risk assessment.

3.7 IMPORTANCE OF TESTING IN DIFFERENT ENVIRONMENTS

Although there were no major differences in ambient O_3 concentrations between the two experimental sites, Lucknow and Banthra, the discriminant analysis (OPLS-DA) of the rice experiment with 18 cultivars revealed that the EDU treatment gave a site-specific response (III). At the Lucknow site, cultivars showed no significant change in biomass, while at Banthra site, the shoot weight plant^{-1} increased at the final harvest phase in response to EDU. An EDU-mediated increase in above-ground biomass was also reported in a meta-analysis including 15 crop species by Feng et al. (2010). However, all previous experiments have been conducted without the site replications. In our study the yield responses were more varied at the Banthra experimental site, 14 of 18 cultivars showing no change, while four cultivars showed a decreased yield with EDU treatment (III). In our experiment, the differences between the experimental sites as regards soil properties most likely led to the differential response to EDU: at the Banthra site a higher percentage of silt and clay was detected, resulting in higher water retention capacity and water availability, which in turn promotes O_3 uptake and the action of EDU. Therefore even in similar O_3 concentrations, during the experiment the response of cultivars varied between the experimental sites. Thereby, our results indicate that several sites or careful characterisation of soil properties are needed for field testing if EDU is to be used as a tool for O_3 sensitivity screening.

4 Conclusions and future prospects

The present thesis highlights the importance of O_3 research in the IGP region of India, the most important crop cultivation area of the country. In addition, it brings a better understanding of the present status, knowledge gaps and practical implications for farmers of reducing the adverse impact of O_3 on crop production, taking mustard (II) and rice (III) as the study cases. The results bring new knowledge about the responses of two agriculturally important crops (mustard and rice), to EDU treatment under high ambient O_3 conditions. The main conclusion and the future prospects can be summarised as follows:

- O_3 concentrations in the IGP area are so high that a negative impact on crop plants can be detected, as suggested by the previous information compiled in the Review Article (I) and by our experiments with mustard (II) and rice (III)
- The cultivars have different strategies for defending against O_3 stress. Our experiments with rice and mustard bring new knowledge about defence strategies, as compared to the previous results presented in the Review Article (I).
- EDU is a useful tool for O_3 risk determination, but the limitations of the EDU method, e.g. site-specific effects (rice experiment) and lack of correlation between the EDU response and the seed yield (II and III), must be known.
- To accurately evaluate the ozone-induced damage, a more comprehensive study involving several local

cultivars and regular O_3 monitoring at the experimental site is further warranted.

- Because EDU did not affect the gas-exchange parameters, such as *A* or *gs* directly (II, III), it becomes a useful tool for estimating the effect of O_3 inside the leaves, after entering the leaves; it does not capture the effect of O_3 on the stomatal aperture.
- EDU-mediated protection is mainly conferred by the up-regulation of the antioxidative defence system, which varies among the crops/cultivars during their development.
- The most responsive parameters for both mustard (II) and rice (III) with EDU treatment were MDA content, SOD, CAT activities at the vegetative phase, and ASA and GSH content at the flowering phase. These indictors can be used for the screening of O_3-tolerant cultivars and further utilised for breeding purposes.
- Both the cultivars of the mustard experiment (II) showed sensitivity to the ambient ozone, whereas in the rice experiment (III), 7 out of 18 cultivars can be recommended for cultivation in high O_3 conditions in the IGP regions.

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ASHUTOSH KUMAR PANDEY

Responses of mustard (Brassica campestris) and rice (Oryza sativa) cultivars to tropospheric ozone in India

Tropospheric ozone is causing a serious threat to crop production in major agricultural regions of the world. This thesis provides an insight into the current status of ozone research in an agriculturally important region i.e., Indo-Gangetic Plains in India, and studies the effects of high ambient ozone concentrations on cultivars of two important crop species, mustard and rice. This knowledge can be used for formulating strategies in providing food security in regions with high ambient ozone concentrations.

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