Assessment of Ethylene Diurea-Induced Protection in Plants Against Ozone Phytotoxicity

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Contents

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D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology Volume 233*, Reviews of Environmental Contamination and Toxicology 233,
DOI 10.1007/978-3-319-10479-9_4

1 Introduction

Rapid economic growth, industrialization, urbanization, and improper implementation of environmental regulations have contributed to increased tropospheric O_3 levels since preindustrial times, and this increase has produced a serious air pollution problem. Apart from being a hazardous air pollutant, O_3 has also been recognized as the third major (carbon dioxide and methane) green house gas in terms of additional radiative forcing and climate change (Forster et al. 2007). Because of its oxidative capacity, high O_3 levels in the atmosphere are detrimental to living organisms, including plants. Ozone is among the most damaging air pollutants to which plants are exposed, and produces substantive plant biomass and yield (seed weight) reductions (Thompson 1992; Agrawal et al. 2005; Manning 2005; Hassan 2006; Hassan and Tewfik 2006; Singh et al. 2009a, 2014; Wahid 2006 a, b; Sarkar and Agrawal 2010a, b; Tripathi and Agrawal 2013). The economic loss for 23 horticultural and agricultural crops from O_3 exposure was estimated to be approximately $6.7 billion for the year 2000 in Europe (Holland et al. 2006). Wang and Mauzerall (2004) anticipated economic losses of upto 9 % for four important cereal crops (viz., wheat, rice, maize and soybean) grown in China, South Korea and Japan. To minimize such crop losses many potential antioxidants (e.g., fungicides, insecticides, growth regulators and plant extracts) have been evaluated. Among these, the systemic antioxidant, ethylene diurea, –N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N′ phenylurea (popularly known as EDU) was found to be the most effective.

In this review, we address how O_3 is formed, and the phytotoxic losses it has produced over the past three decades. Moreover, we address and summarize the literature relating to efforts designed to identify antioxidants that can potentially protect vegetation from O_3 damage. We give special emphasis to the most competent and most studied of these synthetic antioxidants viz., ethylene diurea (EDU). We review EDU's effectiveness at the morphological, physiological and biochemical levels and its role in preventing yield losses in plants.

2 The Chemistry of O_3 Formation, and Its Uptake and Fate in Plants

Ozone is a secondary air pollutant that is not directly emitted to the atmosphere. It is formed by the reaction between primary air pollutants (viz., nitrogen oxides, hydrocarbons such as volatile organic carbons, carbon oxides, methane or non-methane volatile organic compounds, etc.) and solar radiation (Finlayson-Pitts and Pitts 1997). Nitrogen oxides (NOx–NO + NO_2) and volatile organic carbon

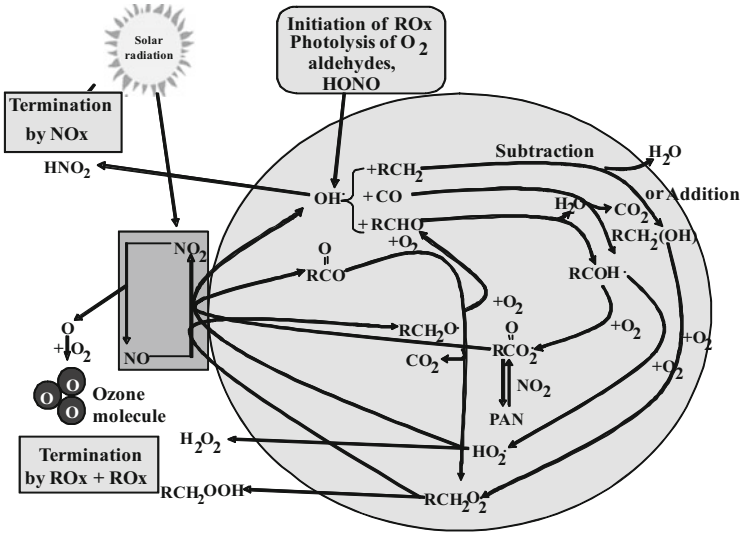

Fig. 1 The steps by which ozone is formed via photochemical processes in the troposphere (modified after Staehelin and Poberaj 2008)

(VOCs) emissions result from both natural and anthropogenic sources. Natural sources of NOx are from lightening discharges and from direct emissions from soil, whereas VOCs are released primarily from vegetation. Anthropogenic-sourced emissions of NOx result from combustion processes in motor vehicles, thermal power generation and various industrial activities. Volatile organic compounds are emitted when combustion processes are incomplete, e.g., from motor vehicle engines and from manufacturing and processing in the petrochemical industry, motor fuel production, and distribution and solvent use. In Fig. 1, we depict the steps by which O_3 is formed.

The emission of nitric oxide (NO_2) results from the reaction between nitrogen and oxygen present in atmosphere under high temperatures generally attained in the combustion chambers of engines. NO_2 thus produced is easily dissociated by the ultraviolet element of solar radiation into nitrogen monoxide and singlet oxygen ($^1O_2$). $^1O_2$ spontaneously reacts with molecular oxygen to give rise to O_3. Under these conditions, the life time of O_3 is very short, because the O_3 produced reacts with NO to produce NO_2 and O_2 again. Therefore, an equilibrium is established between O_3 formation and degradation (Lorenzini and Saitanis 2003). Alternatively, when non-methanic hydrocarbons react with NO, toxic PAN (peroxyacetyl nitrate—CH_3C (O) $OONO_2$) and other organic substances are formed. Thus, the ozone level is controlled by a complex set of photochemical reactions. There are also other chemical reactions involved in tropospheric O_3 formation that comprise a series of complex cycles, in which carbon-monoxide and VOCs are oxidized to form water vapor and carbon dioxide. The carbon monoxide oxidation results from the

presence of the hydroxyl radical ($OH^{\cdot}$). The resultant hydrogen atom rapidly reacts with oxygen to give a per-oxy radical ($HO_2^{\cdot}$). Peroxy radicals then react with NO to give NO_2, which is photolyzed to give the atomic oxygen. The atomic oxygen reacts with a molecule of oxygen to form O_3. The entire process represents a chain reaction, in which O_3 becomes photo dissociated by near ultraviolet radiation to form an excited oxygen atom. Excited oxygen again reacts with water vapor to regenerate $^{\cdot}OH$ radicals, which drives the chain process. O_3 destruction also results from photochemical reactions involving NO, HO_2 or $^{\cdot}OH$. Staehelin and Poberaj (2008) reported that the NOx species restrict ozone formation by having their concentration gradually decreased from the formation of HNO_3. Ozone concentrations are influenced by precursor availability, meteorological conditions and chemical reactions over local, regional and hemispherical distances (Lenka and Lenka 2012). In low latitude regions, O_3 generally exhibits a diurnal bell-shaped pattern, reaching peak concentration during mid day and early afternoon hours and gradually decreasing during late afternoon and evening hours (Lorenzini and Saitanis 2003). Although O_3 frequently originates in urban areas, it can be transported long distances to agricultural areas via prevailing winds.

It is known that atmospheric wind turbulence facilitates O_3 transport to the plant surface (Bennett and Hill 1973). The cuticle of plant leaves act as an impermeable barrier (Kerstein and Lendzian 1989), which restricts O_3 uptake. When O_3 enters plants it does so largely via stomata. When plants are exposed to ozone, stomatal conductance is reduced (Mansfield and Pearson 1996; Castagna et al. 2001; Guidi et al. 2001; Pasqualini et al. 2002; Degl'Innocenti et al. 2003; Singh et al. 2009a). How O_3 influences stomatal conductance (Paoletti and Grulke 2005) is uncertain, although it has been postulated that O_3 may act by altering abscisic acid signaling, or by generating an active oxygen species (AOS) burst directly in guard cells (Kangasjärvi et al. 2005). After entering through stomata, O_3 reaches the sub-stomatal cavity and air spaces in the leaf. O_3 does not persist in the apoplast for long and rapidly degrades to form various reactive oxygen species (ROS) and/or reacts with biomolecules present in the cell wall, apoplastic fluid or plasma membrane (Laisk et al. 1989; Mishra et al. 2013b). Diara et al. (2005) reported that extracellular H_2O_2 accumulation is one of the earliest detectable responses to O_3 exposure. The appearance of leaf lesions has also been correlated with H_2O_2 accumulation (Pellinen et al. 1999; Wohlgemuth et al. 2002). H_2O_2 disrupts photosynthesis and activates NAD(P)H-dependent oxidase (Park et al. 1998; Rao and Davis 1999), which leads to ROS accumulation. Alscher and Hess (1993) suggested that the superoxide radical is formed and produces leaf injury under O_3 exposure. Among other ROS, the hydroxyl radical is the most reactive of oxygen species. It reacts rapidly with proteins, lipids and DNA and causes cell damage (Iqbal et al. 1996). ROS leads to a chain of reactions, which cause significant effects on the cellular metabolism of the plants. In Fig. 2, we illustrate the entry of O_3 into plant leaves, its mechanism of toxicity and consequent defense responses.

ROS cause membrane damage and deleterious effects on the normal functioning of cells. To protect against the damaging effects of ROS, plants have developed and utilize several non-enzymatic (ascorbic acid, carotenoids, glutathione, α-tocopherol) and enzymatic antioxidants (superoxide dismutase, various peroxidases, catalases,

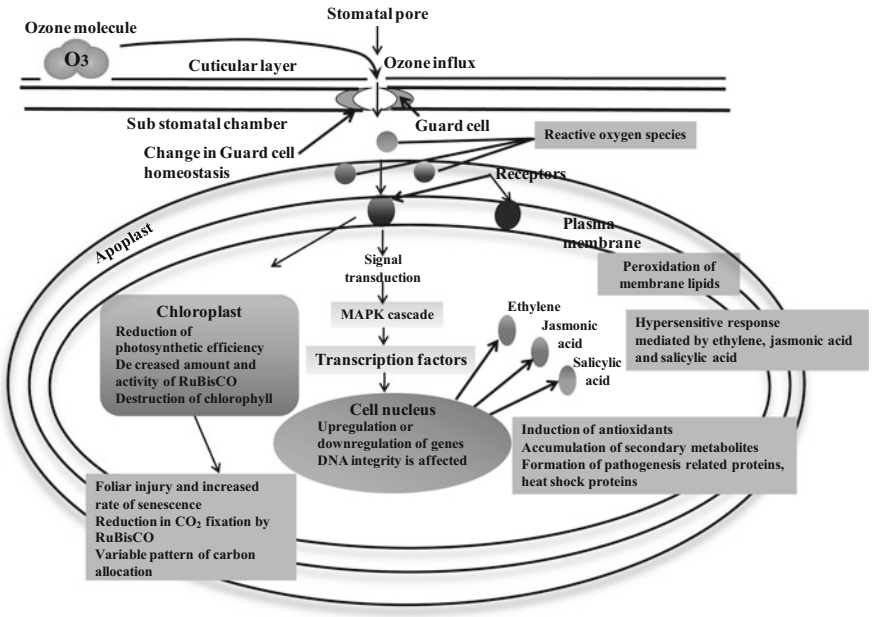

Fig. 2 Ozone uptake and its effect on cellular metabolism, signaling and cellular molecules

glutathione reductase, etc.) that exist in different cell compartments (Fig. 3). Carotenoids are non-photosynthetic pigments in leaves that help maintain the chlorophyll pool that is used against photooxidative damage by ROS. Glutathione (GSH), is the most abundant thiol in plants, and functions as an antioxidant that scavenges cytotoxic H_2O_2 and other ROS such as $OH^{\cdot}$, $O_2^{\cdot -}$ (Larson 1988). Ascorbic Acid (AA) is the most abundant low molecular weight antioxidant, is synthesized in leaf cells (Castillo and Greppin 1988; Smirnoff 2000) and forms the first line of defense against O_3 exposure (Polle et al. 1995). The ability of AA to donate electrons in a wide range of enzymatic and non-enzymatic reactions signifies it as being the main ROS detoxifying compound. In addition alpha-tocopherol is an important antioxidant for its ability to directly scavenge oxidizing radicals and to prevent chain propagation during lipid autoxidation, when plants are exposed to O_3 (Serbinova and Packer 1994).

Among enzymatic antioxidants, the primary one is superoxide dismutase (SOD), which represents a family of metalloenzymes that catalyze the dismutation of $O_2^{\cdot -}$ to H_2O_2 (Bowler et al. 1992, 1994). Another important enzyme is catalase (CAT), which exists primarily in peroxisomes and catalyzes the degradation of H_2O_2 (Willekens et al. 1995; Scandalios et al. 1997). Ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate (MDHAR) and dehydroascorbate reductase (DHAR) are enzymes of the ascorbate-glutathione cycle, and help to regulate ROS formed in the presence of O_3 (Biemelt et al. 1998; Jimenez et al. 1998; Noctor and Foyer 1998).

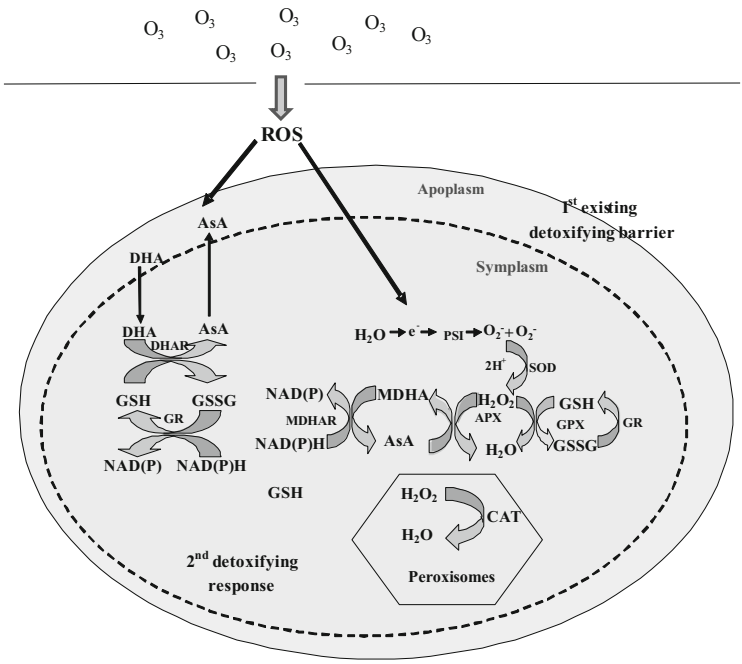

Fig. 3 The processes by which ROS are scavenged in the cells. *DHA* dehydroascorbate; *AsA* ascorbate; *DHAR* dehydroascorbate reductase; *GR* glutathione reductase; *GSSG* oxidized glutathione; *GSH* reduced glutathione; *NAD(P)* Nicotinamide adenine dinucleotide phosphate; *NAD(P)H* Nicotinamide adenine dinucleotide phosphate reduced *MDHAR* monodehydroascorbate reductase; *MDHA* monodehydroascorbate; *APX* ascorbate peroxidase; *SOD* superoxide dismutase; *GPX* glutathione peroxidase; *CAT* catalase; H_2O_2 hydrogen peroxide; *PSI* photosystem I (modified after Dizengremel et al. 2008)

Endogenous production of ROS also activates a MAP kinase cascade, which plays crucial roles in plant signal transduction pathways. MAPKs target various effector proteins, which consist of kinases, enzymes or transcription factors (Rodriguez et al. 2010). The transcription factors participate in the expression of genes that are involved in defense pathways, and in primary or secondary metabolism. Ethylene and salicylic acid are produced in plants and together foster the development of lesions and cause cell death (Castagna and Ranieri 2009). When cell death occurs, certain products of lipid peroxidation serve as substrates for synthesis of jasmonic acid. Jasmonic acid acts antagonistically and reduces ethylene-dependent ROS production and the spread of cell death (Kangasjärvi et al. 2005).

The biochemical and physiological performance of plants subjected to O_3-related oxidative stress is greatly affected and such plants display reduced growth and biomass production both of which reduce yield (Morgan et al. 2003;

Ambasth and Agrawal 2003; Biswas et al. 2008; Feng et al. 2008). Yield is of foremost concern, because reduced growth of crop plants translates directly to economic losses. Hence, yield response of plants under O_3 exposure is a major parameter that is routinely studied by plant scientists. A meta analysis of existing data has indicated that soybeans exposed to an average O_3 level of 70 ppb suffer a 24 % reduction in seed yield (Morgan et al. 2003). Moreover, Rai et al. (2007) reported that a wheat cultivar (HUW-234) grown under ambient and elevated O_3 in open top chambers suffered reduced yield. Mishra et al. (2013a) experienced the same results with two other cultivars: HUW-37 and K-9107. De Temmerman et al. (2007) found reduced root yield and altered quality of *Beta vulgaris* (sugar yield, alpha-amino-N, Na and K contents) from O_3 exposure. Intraspecific differences were observed in yield responses of rice (Ariyaphanphitak et al. 2005), wheat (Wahid 2006b; Singh and Agrawal 2009) barley (Wahid 2006a), bean (Flowers et al. 2007), potato (Piikki et al. 2004) and cotton (Zouzoulas et al. 2009) plants under different O_3 exposure regimes (Table 1). In Table 1, we summarize the results of experiments conducted to assess yield responses of different plants exposed to O_3.

3 Protectants Used to Prevent Ozone Toxicity

O_3-induced oxidative stress caused by O_3 has been identified as the major cause for plant yield losses. Hence, many chemical substances have been applied to protect plants against such losses. In recent decades, several researchers have tested various antioxidants in attempts to understand how O_3 injury may be mitigated. The types of chemicals applied have included fungicides, insecticides, growth regulators, natural plant extracts and antioxidants, among others.

Middleton et al. (1953) reported first that O_3 induced injury in pinto bean was reduced when aqueous solution of manganese (maneb) or zinc ethylenebis dithiocarbamate (zineb) was sprayed prior to O_3 fumigation. When sprayed onto poinsettia plants, the fungicides benomyl and diphenylamine (DPA) reduced O_3 injury (Manning et al. 1973a). Similar results were achieved with pinto bean (Pell 1976), potato (Carrasco-Rodriguez et al. 2005) and tobacco (Reinert and Spurr 1972) plants. Gilbert et al. (1975) reported that dust and liquid applications of DPA on apple, and dust application of DPA on bean, muskmelon and petunia also provided protection against O_3 injury. DPA was also used to quantify the effects of ambient oxidants on plants during air quality monitoring in Georgia, U.S.A. (Walker and Barlow 1974). A foliar spray of DPA at 1,000 ppm onto apple and at 1 % in bean, melon, petunia and tobacco reduced O_3 damage by 50 % or more (Lisk 1975). Carrasco-Rodriguez et al. (2005) recorded an increase in fresh biomass and tuber numbers of potato after DPA was applied.

Table 1 A summary of global studies performed to evaluate the effect of tropospheric ozone on yield of plants

Country/ site	Species/cultivar	Experimental setup	Cropping condition	Parameters	O_3 conc.	Yield reductions	Reference
Belgium	*Beta vulgaris* L. Patriot	Open top chamber	Field	Root yield (t fw ha^{-1})	8 h mean, 62 ppb	14 % (2003)	De Temmerman et al. (2007)
				Sugar yield (t ha^{-1})		10.7 % (2004) 14.7 % (2003) 10.9 % (2004)	
China	*Triticum aestivum* L. and *Oryza sativa* L.	Open top chamber	Field	Grain yield $plant^{-1}$	200 ppb	80.5 and 49.1 %	Feng et al. (2003)
					100 ppb	58.6 and 26.1 %	
					50 ppb	10.5 and 8.2 %	
	Ginkgo biloba L.	Open top chamber	Field	Axial shoot and lateral shoot	80 ppb	66 and 47 %	He et al. (2006)
	Ginkgo biloba L.	Open top chamber	Field	Axial shoot and lateral shoot	80 ppb	66 and 47 %	He et al. (2006)
	Oryza sativa L. SY63 WYJ3	Open-air O_3 release system	Field	Grain yield (g per 5 hills)	Ambient (13.8–74.2 ppb, 7 h) Elevated (ambient × 1.5) Ambient (13.8–74.2 ppb, 7 h) Elevated (ambient × 1.5)	20.7 % 6.3 %	Pang et al. (2009)
	Oryza sativa L. Wujing 15 (WJ15), yangdao 6 (YD6), Shanyou 63 (SY63), Liangyoupeijiu (LYP)	O_3-FACE system	Field	Yield $plant^{-1}$ (g)	7 h mean, 56 pp for SY6, 54 ppb for YD6, 56 ppb for WJ15 and 59 ppb for LYP	17.5 % in SY6 and 15.0 % in LYP	Shi et al. (2009)
Egypt	*Gossypium hirsutum* L. Giza 65	Closed chambers	Pots	Number of open balls Seed weight (g)	70 ppb, 10 h day^{-1} for 2 weeks	23 % 15 %	Hassan and Tewfik (2006)
England	*Triticum aestivum* L. Riband	Unenclosed chamber system	Pots	Grain yield (t ha^{-1})	81 ppb, 7 h day^{-1}	13 %	Ollerenshaw and Lyons (1999)
Germany	*Solanum tuberosum* L. Hela	Closed exposure chamber	Pots	Fresh wt of tuber	24 h mean, 65 ppb 110 ppb for 4 h day^{-1}	24 % 11 %	Kollner and Krause (2000)
	Trifolium aestivum L. Nandu	Closed fumigation chamber	Pots	Thousand grain wt (g)	8 h mean for 2 weeks 65 ppb 110 ppb	12.1 % 21.2 %	Meyer et al. (2000)

Greece	*Gossypium hirsutum* L. Romanos and Allegria	Close and environment controlled chamber	Pots	Raw cotton wt. (g) Seed wt. (g)	100 ppb, 7 h day^{-1}	60.5 and 51.5 % 57.3 and 50 %	Zouzoulas et al. (2009)
India	*Triticum aestivum* L. HD 2329 *Brassica campestris* L. Pusa Jaikisan *Vigna radiata* L. Malviya jyoti	Ambient air	Pots	Yield (g $plant^{-1}$)	6 h mean Winter-10–15.4 ppb Summer-9–58.5 ppb	0.5–25.5 % 5.9–29.7 % 34.3–73.4 %	Agrawal et al. (2003)
	Vigna radiata L. Malviya jyoti	Open field	Pots	Seed wt. $plant^{-1}$ (g)	6 h mean, 9.7–58.5	22–79 %	Agrawal et al. (2006)
	Daucus carota L. Pusa kesar	Open top chamber	Field	Root wt. (g)	8 h mean, 38.4 ppb	45.3 %	Tiwari et al. (2006)
	Triticum aestivum L.	Open top chamber	Field	Yield $plant^{-1}$ (g)	8 h mean, 36.4–48 ppb	20.7 %	Rai et al. (2007)
	Oryza sativa L. Saurabh 950 and NDR 97	Open top chamber	Field	Yield (g $plant^{-1}$)	30.5–45.4 ppb	11.5 % 16 %	Rai and Agrawal (2008)
	Brassica campestris L. Kranti	Open top chamber	Field	Yield (g $plant^{-1}$)	41.6–54.2 ppb	16.4 %	Singh et al. (2009a)
	Glycine max L. PK472 Bragg	Open top chamber	Field	Yield $plant^{-1}$ (g)	70 ppb (4 h) 100 ppb (4 h) 70 ppb (4 h) 100 ppb (4 h)	20 % 33.6 % 12 % 30 %	Singh et al. (2010a)
	Triticum aestivum L. cv. HUW 510 Sonalika	Open top chambers	Field	Grain yield (g m^{-2})	12 h mean (45.3 ppb) (50.4 ppb) (55.6 ppb) (45.3 ppb) (50.4 ppb) (55.6 ppb)	20.0 % 37.0 % 46.0 % 11.0 % 25.0 % 38.5 %	Sarkar and Agrawal (2010b)
	Brassica campestris L. Sanjukta and Vardan	Open top chambers	Field	Test weight and oil content	Ambient + 10 ppb (3 h)	12.5 and 47 % 33.4 and 48.5 %	Tripathi and Agrawal (2012)
	Linum usitatissimum L. Padmini and T-397	Open top chambers	field	Weight of seeds $plant^{-1}$ and oil content	Ambient + 10 ppb (3 h)	40.5 % and 46.7 % 42.8 % and 42.5 %	Tripathi and Agrawal (2013)

(continued)

Table 1 (continued)

Country/ site	Species/cultivar	Experimental setup	Cropping condition	Parameters	O_3 conc.	Yield reductions	Reference
	Triticum aestivum L. HUW-37 and K-9107	Open top chambers	Field	Yield (g plant^{-1})	Ambient + 10 ppb (4 h)	First year 39 % and 12.4 % Second year 40.8 % and 14 %	Mishra et al. (2013a)
	Trifolium alexandrium L. Bundel, Wardan, JHB-146, Fahli, Saidi and Mescavi	Open top chambers	Field	Total biomass (g plant^{-1})	Ambient + 10 ppb (6 h)	13.5 % 18.2 % 9.1 % 4.9 % 6.5 % 4.4 %	Chaudhary and Agrawal (2013)
	Zea mays L. HQPM1 and DHM117	Open top chambers	Field	Weight of kernels plant^{-1}	Ambient Ambient + 15 ppb (5 h) Ambient + 30 ppb (5 h)	4.0 % 7.2 % 10.1 % 5.5 % 9.5 % 13.8 %	Singh et al. (2014)
Ireland	*Triticum aestivum* L. Promessa	Open top chamber	Field	Dry wt. of grains plant^{-1} (g)	Ambient + 50 ppb 4 h day,4 days week Ambient + 25 ppb 6 h day, 5 days week Ambient + 25 ppb 6 h day, 5 days week Ambient + 50 ppb 3 h day, 5 days week	53.5 % NS 3.2 % 16.8 %	Finnan et al. (1996)
Italy	*Triticum aestivum* L. Taylor's Horti-cultural, Lingua di Fuoco and Saluggia	Open top chamber	Field	Dry wt of pod (t ha^{-1})	7 h mean, 50 ppb	31.5 % 28.6 % 30.8 %	Schenone and Lorenzini (1992)
	Trifolium repens L. Regal NC-R (O_3 resistant) NC-S (O_3 sensitive)	Ambient field	Pots	Biomass	7 h mean, 49–70 ppb	20–60 % (in comparison to NC-S)	Fumagalli et al. (2003)
	Glycine max L.	Open top chamber	Field	Grain yield (kg m^{-2})	60–70 ppb	47 %	Bou Jaoude et al. (2008)
	Phaseolus vulgaris L. Borlotto Nano Lingua di Fuoco	Open top chamber	Field	Seed wt plant^{-1} (g)	4,675 ppb h (cumulative exposure)	40.6 %	Gerosa et al. (2009)

Malaysia	*Oryza sativa* L. MR 84 and MR 185	Open top chambers	Field	Yield plant^{-1} (g)	8 h mean, 32.5 ppb	3.4 % in MR 84 and 6.3 % in MR 185	Ishii et al. (2004)
Pakistan	*Oryza sativa* L. IRRI-6 and Basmati-385	Open top chamber	Pots	Grain yield plant^{-1} (g)	6 h mean, 35.6 ppb	37 % and 42 %	Wahid et al. (1995)
	Hordeum vulgare L. Haider-93, Haider-91, Jou-87 and Jou-85	Open top chamber	Pots	Seed wt plant^{-1} (g)	8 h mean, 71 ppb	13 %, 30 %, 34 % and 44 %	Wahid (2006a)
	Triticum aestivum L. Inqilab-91, Punjab-96 and Pasban-90	Open top chamber	Pots	Grain yield Plant^{-1} (g)	8 h mean, 72 ppb	18 %, 39 % and 43 %	Wahid (2006b)
	Vigna radiata L. M-28 and 6601	Open top chamber	Ambient Field	Seed yield plant^{-1} (mg)	41–73 ppb	47.1 and 51.1 %	Ahmed (2009)
Saudi Arabia	*Triticum aestivum* L. Giza 68 *Vicia faba* L. Lara *Phaseolus vulgaris* L. Giza 3 *Pisum sativum* L. Perfection	Open field	Pots	Seed yield pot^{-1} (g)	77–166 ppb	9–46 % 13–33 % 20–45 % 3–30 %	Ali et al. (2008)
Spain	*Solanum tuberosum* L. Desiree	Green house	Field	Commercial tuber production	12 h mean, 25.8 ppb 42.5 ppb	53 % 65 %	Calvo et al. (2009)
Sweden	*Solanum tuberosum* L. Bintje	Open top chamber	Field	Tuber Fresh wt (g)	12 h mean, 43 ppb	11.4 %	Persson et al. (2003)
	Solanum tuberosum L. Bintje and Kardal	Open top chamber	Field	Dry mass of tubers Number of tubers	31 and 57 ppb	20 and 30 % (Bintje) 27.8 and 20.4 % (Kardal) 7.7 and 19.2 % (Bintje) 11.4 and 11.4 % (Kardal)	Piikki et al. (2004)
Switzerland	*Triticum aestivum* L. Albis	Open top chamber	Field	Grain yield (t ha^{-1})	Mean 7 h day^{-1} 1989 35.7 ppb 49.5 ppb 62.2 ppb 1990 38.7 ppb 55.6 ppb 71.4 ppb	4.7 % 18.9 % 29.3 % 6.9 % 14.7 % 32.1 %	Fuhrer et al. (1992)

(continued)

Table 1 (continued)

Country/ site	Species/cultivar	Experimental setup	Cropping condition	Parameters	O_3 conc.	Yield reductions	Reference
Thailand	*Oryza sativa* L. Chainat1 Suphanburi 1 Suphanburi 60 Suphanburi 90 Klongluang 1 Pathumthani 1 Gorkor 15 Khowdokmali 105 *Glycine max* L. Sorjor 4 Chaing Mai60	Closed top chamber	Field	% filled seed $year^{-1}$	24.9 ppb	15.9 % 12.3 % 5.6 % 8.2 % 5.7 % 16.6 % 8.2 % 2.7 % 16.3 % 17.7 %	Ariyaphanphitak (2004)
	Oryza sativa L. Klongluang 1, Pathumthani 1, Gorkor 15 and Khowdokmali 105	Closed chamber	Pots	Filled seed ear^{-1}	150 ppb, 7 h day^{-1}	30–78 %	Ariyaphanphitak et al. (2005)
United Kingdom	*Brassica napus* L. Eurol	Controlled environment chambers	Field	Seed yield (t ha^{-1})	77–80 ppb for 49 days	14 %	Ollerenshaw et al. (1999)
USA	*Glycine max* L. Merr. Davis	Open top chamber	Field	Seed yield	104 ppb, 7 h day^{-1}	50 %	Unsworth et al. (1984)
	Glycine max L. Forrest and Essex	Open top chamber	Field	Seed yield (g m^{-2})	62.4 ppb, 7 h day^{-1} 5 days a week	32 % 10 %	Chernikova et al. (2000)
	Glycine max L.	–	Field	Seed weight $plant^{-1}$ (g)	70 ppb	24 %	Morgan et al. (2003)
	Glycine max L. Merr.	Open top chamber	Field and pots	Total seed wt	75 ppb (1999) 67 ppb (2000)	23.9 % (field) 27.2 % (pot) 38.9 % (field) 41.0 % (pot)	Booker and Fiscus (2005)
	Phaseolus vulgaris L. S156, R 123 and R 331	Environmentally controlled field chambers	Pots	Seed yield (g $plant^{-1}$)	60 ppb	77 %, 19 % and 35 %	Flowers et al. (2007)
	Oryza sativa L.	Open top chamber	Field	Seed yield (g)	62 ppb	24 %	Ainsworth (2008)
	Oryza sativa L	Open top chamber	Pots	Seed wt (g m^{-2})	73–77 ppb	17 % (1997) 14 % (1998)	Reid and Fiscus (2008)

NS not significant, *t* tonne, *fw* fresh weight, *ha* hectare

Foliar application of two modern fungicides (azoxystrobin and epoxiconazole) prior to fumigation with injurious doses of O_3 (150–250 ppb; 5days; 7 h/day) reduced visible injury by 50–60 % in spring barley by inducing an antioxidative defense system (Wu and Tiedemann 2002). To prevent O_3 injury benomyl is the most studied benzimidazole derivative fungicide (Manning et al. 1972, 1973a, b, c; Manning and Vardaro 1973a, b). In the 1970s, the discovery that benomyl can protect plants against O_3 injury opened new investigations because carboxin had similar beneficial effects in reducing O_3 injury in plants. However, benomyl was more effective than carboxin, because, to achieve equal protection of benomyl carboxin required a dose that was nearly phytotoxic (Rich et al. 1974; Taylor and Rich 1974; Papple and Ormrod 1977). Hofstra et al. (1978) found that benomyl and carboxin were equally effective in causing yield recovery in navy beans exposed to O_3. Recently, the fungicide 'strobi' conferred the best protective effects on O_3 sensitive clover and tobacco among the studied modern agrochemicals (Blum et al. 2011).

Insecticides have also been applied to achieve protection, although their application has been limited to areas where crop loss does not result from insects. Of ninety chemicals tested, only five showed significant protection from O_3 injury (Koiwai et al. 1974). These five were: 3, 4-methylenedioxyphthaldehyde, benzimidazole, safroxane, xanthone and piperonyl butoxide. Phytohormones such as auxins, cytokinins and abscisic acid (ABA) acted like antioxidants in reducing O_3 injury in plants (Kurchii 2000; Pauls and Thopson 1982; Verbeke et al. 2000). Abscisic acid (ABA), a chemical that induces stomatal closure reduced the O_3 injury in bean (Fletcher et al. 1972). Protective effects from the application of N-6-benzyladenine (BA), gibberellic acid (GA) and indole acetic acid (IAA) against O_3 were observed to occur in radish, and BA was the most effective protectant of the three (Adedipe and Ormrod 1972). Runeckles and Resh (1975) reported that the cytokinins in BA and kinetin stimulated leaf growth and reduced chlorophyll loss, which generally is the most susceptible constituent to O_3 attack.

Ascorbic acid and its salt are effective in minimizing the detrimental effects of O_3 in bean, celery, lettuce, barley, citrus and petunia (Freebairn 1960; Freebairn and Taylor 1960; Dass and Weaver 1968; Lee et al. 1990; Macher and Wasescha 1995). In contrast, Siegel (1962) reported that ascorbic acid failed to provide any appreciable protection to cucumber plants after O_3 exposure. Ozoban, an isomer of ascorbic acid reduced the photosynthetic rate in a low-O_3 environment, but was harmful to chloroplastic plant pigments that were exposed to elevated levels of O_3 (Kuehler and Flagler 1999). Agrawal et al. (2004) studied three wheat cultivars and showed that an ascorbic acid spray improved biochemical parameters and increased biomass and yield, but did not impart any significant change in stomatal conductance. How application of ascorbate induces plant-resistance to O_3 stress is still unclear (Didyk and Blum 2011). Among all protectant types, the performance of

ethylene diurea (EDU) is the best documented, and has been suggested to be the most efficient research tool for analyzing O_3-induced stress in a variety of plants (Manning 1992).

4 Ethylenediurea (EDU) as a Protectant to Prevent Phytotoxicity

The positive effect of ethylenediurea (EDU, N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N′-phenylurea) on plant productivity against ambient O_3 was first reported by Carnahan et al. (1978). It is specific in suppressing O_3 injury and has no effects on peroxylacetylnitrate (PAN) and sulphur dioxide (SO_2)-induced injury (Cathey and Heggestad 1982a; Lee et al. 1992). EDU consists of two urea moieties having an imidazole ring (urea) and a phenylurea ring; these rings are joined by an ethylene group (Fig. 4). An investigation of the relative effectiveness of EDU and constituent amounts of urea and phenylurea in EDU in prevention of O_3 injury was performed by Godzik and Manning (1998). They suggested that urea did not impart protection against O_3 injury, although phenylurea reduced O_3 injury significantly. Urea treatment was clearly not as effective as the treatment with EDU. However, when O_3 exposed plants were treated with 300 ppm EDU or phenylurea, both compounds equally prevented O_3 injury. No published reports addressed the role that the nitrogen present in EDU had in protecting plants from O_3. EDU neither acts as a nutrient, nor shows any pesticidal or plant regulatory effects (Manning 1992). Paoletti et al. (2008) investigated the biochemical leaf responses in EDU infused ash trees and reported that EDU did not contribute nitrogen as a fertilizer (*Fraxinum excelsior*). Lee and Chen (1982) reported cytokinin-like activity of EDU and indicated that EDU retarded the breakdown of chlorophyll, protein and RNA in O_3-sensitive tobacco leaf discs. Whitaker et al. (1990) studied the role of EDU in protecting foliar lipids and concluded that EDU conferred O_3 tolerance by inducing enzyme systems involved in the elimination of activated oxygen species and free radicals.

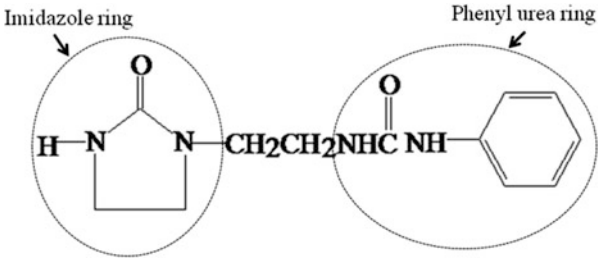

Fig. 4 Structural formula of N-[2-(2-oxo-1-imidazolidinyl) ethyl-]-N′-phenyl urea, (abbreviated as EDU, or ethylene diurea)

4.1 Methods and Timing of EDU Application

In most experiments, EDU has mainly been applied as a soil drench or foliar spray (Table 2). Other EDU application methods have included stem injection and gravitational infusion (Fig. 5), and these have commonly been used in trees and other woody species (Roberts et al. 1987; Ainsworth and Ashmore 1992; Ainsworth et al. 1996; Bortier et al. 2001; Paoletti et al. 2007). Bortier et al. (2001) found that stem injection of EDU in *Populus nigra* was effective in preventing O_3-induced visible injury, accelerated senescence, and led to significant increases in diameter and height of O_3 exposed trees. Ainsworth et al. (1996) reported similar results with two clones of hybrid poplar that showed incremental chlorophyll content increases after EDU treatment. Paoletti et al. (2007) found a significant reduction in O_3-induced foliar injury in ash trees when EDU (300 and 450 ppm) was applied as a gravitational infusion into the trunk. Percent O_3 injury was reduced to 3 % in EDU-infused trees as compared to control plants that manifested 13 % injury on the leaf surface.

Although EDU is usually applied as a soil drench to protect against O_3 injury in plants, the applied EDU may accumulate in soil and produce subsequent toxicity. Some argue therefore that foliar applications are safer than drenching. Moreover, utilizing drenching on a large scale basis, or in large fields, is not always feasible at all stages of plant development, particularly in non-row crops such as hay and broadcasted cereals. In addition, large amounts of EDU are required when used at the field scale.

Surface applications are an alternative application method, but have their own drawbacks, such as being dependent on precipitation to effect the chemical uptake by roots. Feng et al. (2010) performed a meta analysis, which indicated that EDU applied as a soil drench significantly ameliorated plant growth suffering from O_3 stress. In contrast, after EDU was applied as a foliar spray, only a few parameters showed significant positive effects of EDU. Alternative application approaches, like stem injections and gravitational infusion of EDU solution are not possible for delicate plants like vegetables and cereal crops.

Application timing of EDU is critical for achieving maximum protection to plants against O_3 injury. Weidensaul (1980) found that pinto beans were best protected from O_3 injury when EDU was applied 3–7 days prior to O_3 exposure, but afforded no protection to leaves that were not formed at the time of chemical application. McClenahen (1979) reported almost complete protection from O_3 injury in *Fraxinus americana* and *Prunus serotina* that received up to 300 ppb EDU weekly during the seedling stage. Using ^{14}C-EDU (Roberts et al. 1987) and a HPLC technique (Regner-Joosten et al. 1994), Gatta et al. (1997) established that EDU translocation was primarily acropetal, probably via the xylem stream and that EDU remained in the apoplastic region of leaves for more than 10 days, without being redistributed to newly formed leaves. Carnahan et al. (1978) documented that EDU was not translocated to newer or untreated leaves, which suggested that repeated applications were needed to assure continuous protection from O_3 injury.

Table 2 Effects of ethylene diurea (EDU) treatment on shoot and root length, number of leaves and leaf area of plants grown under ozone exposure

Country/ site	Species/ cultivar	EDU application	EDU dose/con.	O_3 con.	Application duration	Shoot length	Root length	Number of leaves	Leaf area	Biomass	Reference
Belgium	*Populus nigra* L. Wolterson	Stem injection	5 mg/ plant	32 ppb (8 h)	14 days	5.3 % (↑)	–	–	–	9 % (↑)	Bortier et al. (2001)
China	*Oryza sativa* L. *Triticum aestivum* L	Foliar spray	150 ppm 300 ppm 450 ppm 150 ppm 300 ppm 450 ppm	Often exceeding 40 ppb	7 days	– – – – – –	– – – – – –	– – – – – –	– – – – – –	NS NS NS NS NS NS	Wang et al. (2007)
Egypt Suburban Rural	*Raphanus sativus* L. *Brassica rapa* L. *Raphanus sativus* L. *Brassica rapa* L.	Soil drench	500 ppm/ 200 mL	54.8 ppb (6 h) 66.9 ppb (6 h)	10 days	– – – –	– – – –	– – – –	– – – –	25 % (↑) 4.2 % (↑) 20.5 % (↑) 13.2 % (↑)	Hassan et al. (1995)
Rural	*Trifolium alexandrinum* L. Messkawy	Soil drench	50 ppm/ 200 mL 100 ppm/ 200 mL 150 ppm/ 200 mL 200 ppm/ 200 mL	88 ppb (8 h day^{-1})	10 days	– – – –	– – – –	– – – –	– – – –	21.4 % (↑) 71.4 % (↑) 92.8 % (↑) 95.2 % (↑)	Hassan et al. (2007)
Urban Peri-urban	*Lycopersicon esculentum* L. Pusa Ruby	Soil drench	400 ppm	88.41 ppb (5 h) 89.53 ppb (5 h)	12 days	21.1 % (↑) 24.3 % (↑)	30 % (↑) 11.4 % (↑)	14.5 % (↑) 15.9 % (↑)	– – –	31.5 % (↑) 33.8 % (↑)	Varshney and Rout (1998)

Peri-urban				89.98 ppb (5 h)		24.8 % (↑)	11.2 % (↑)	16.6 % (↑)		36.6 % (↑)	
Urban	*Triticum aestivum* L HD 2329 HUW 234 HUW 468	Soil drench	500 ppm/ 250 mL	29.2 ppb (6 h)	7 days	7.5 % (↑) 13.7 % (↑) 5.4 % (↑)	15.9 % (↑) 16.7 % (↑) 33.2 % (↑)	13.6 % (↑) 21 % (↑) 16.6 % (↑)	(↑) (↑) (↑)	(↑) (↑) (↑)	Agrawal et al. (2004)
Suburban	*Vigna radiata* L. Malviya Jyoti	Soil drench	500 ppm/ 500 mL	32.6–35.2 ppb (8 h)	7 days	12.3 % (↑)	(↑)	16.8 % (↑)	30 % (↑)	24 % (↑)	Agrawal et al. (2005)
Suburban	*Triticum aestivum* L. M 234 M 533	Soil drench	150 ppm/ 200 mL 300 ppm/ 200 mL 450 ppm/ 200 mL 150 ppm/ 200 mL 300 ppm/ 200 mL 450 ppm/ 200 mL	Often exceeding 40 ppb (8 h)	10 days	– – – – – –	– – – – – –	46.5 % (↑) 63.8 % (↑) 32.7 % (↑) 20.9 % (↑) 22.4 % (↑) 8.9 % (↓)	7.9 % (↑) 8.7 % (↑) 2.6 % (↓) 22 % (↑) 26.6 % (↑) 23 % (↑)	48.1 % (↑) 44.6 % (↑) 62.7 % (↑) 25.5 % (↑) 40.4 % (↑) 60.5 % (↑)	Tiwari et al. (2005)
Suburban	*Beta vulgaris* L. Allgreen	Soil drench	300 ppm/ 200 mL	52.1–73.2 ppb (8 h)	10 days	19.7 % (↑)	–	28 % (↑)	18.3 % (↑)	31.8 % (↑)	Tiwari and Agrawal (2009)

(continued)

Table 2 (continued)

Country/ site	Species/ cultivar	EDU application	EDU dose/con.	O_3 con.	Application duration	Shoot length	Root length	Number of leaves	Leaf area	Biomass	Reference
Suburban	*Triticum aestivum* L HUW234 HUW468 HUW510 PBW343 Sonalika	Soil drench	400 ppm/ 100 mL	35.3–54.2 ppb (8 h)	12 days	12.8 % (↑) 15.2 % (↑) 17.3 % (↑) 9.2 % (↑) 2.3 % (↑)	8 % (↑) 27.2 % (↑) 12.7 % (↑) 12.5 % (↑) 6.3 % (↑)	23.7 % (↑) 41.5 % (↑) 28.5 % (↑) 29.4 % (↑) 14.3 % (↓)	3.3 % (↑) 52 % (↑) 47.9 % (↑) 40.4 % (↑) 28.8 % (↑)	54.5 % (↑) 47.6 % (↑) 31.3 % (↑) 10.9 % (↑) 2.6 % (↑)	Singh and Agrawal (2009)
Rural	*Vigna radiata* L. Malviya Janpriya	Soil drench	400 ppm/ 100 mL	52.9–64.5 ppb (12 h)	10 days	22.8 % (↑)	18.7 % (↑)	(↓)	(↑)	24.9 % (↑)	Singh et al. (2010b)
Pakistan Punjab Post-monsoon Rural roadside Rural Suburban Pre-monsoon Rural roadside Rural Suburban	*Glycine max* L. NARC-1	Soil drench	400 ppm/ 600 mL	48 ppb (6 h) 48 ppb (6 h) 40 ppb (6 h) 70 ppb (6 h) 75 ppb (6 h) 63 ppb (6 h)	10 days	20.7 % (↑) 12.4 % (↑) 8.1 % (↑) 53.9 % (↑) 47.6 % (↑) 29.8 % (↑)	– – – – – –	43.7 % (↑) 30.5 % (↑) 17.7 % (↑) 49.2 % (↑) 37.9 % (↑) 14.8 % (↑)	– – – – – –	– – – – – –	Wahid et al. (2001)

USA	*Pinus taeda* L.	Foliar spray	150 ppm 300 ppm 450 ppm	40–70 ppb	14 days	– (↑) (↑)	– – –	– – –	– – –	– (↑) (↑)	Manning et al. (2003)
	Rudbeckia laciniata L.	Foliar spray	200 ppm 400 ppm 600 ppm	32–38 ppb (12 h)	7 days	– – –	– – –	– – –	– – –	11.9 % (↓) 29.6 % (↓) 22 % (↓)	Szantoi et al. (2009)

(↑) = increase, (↓) = decrease, *NS* not significant

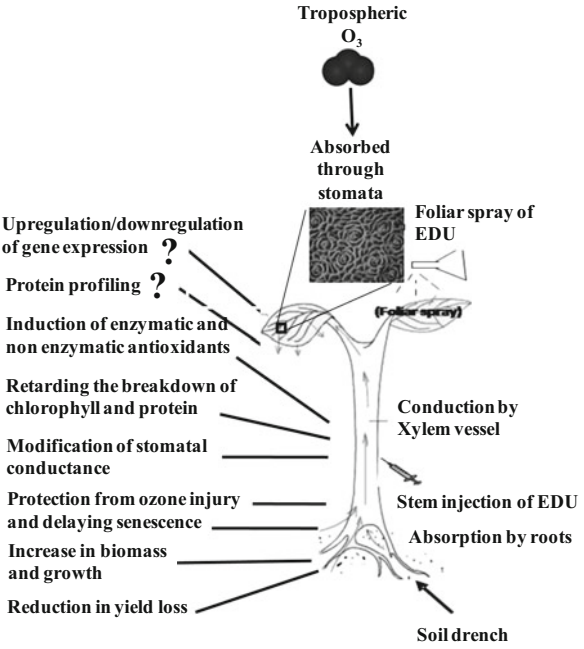

Fig. 5 Flow diagram showing influx of O_3 through stomata and the possible mechanism by which EDU protects plants against O_3 stress

EDU is a systemic antioxidant, and is not redistributed to new tissues; hence, repeated applications are indeed needed to maintain continuous levels of protection (Weidensaul 1980; Regner-Joosten et al. 1994). Depending on plant sensitivity to O_3, Manning et al. (2011) suggested foliar spraying with EDU at weekly or bi-weekly intervals. Paoletti et al. (2009) reviewed the use of EDU on Italian crops and trees and reported that amelioration of visible O_3-induced injury was obtained by regularly applying EDU at 2 or 3 week intervals.

4.2 Application Dose of EDU

To determine the optimal EDU dose that gives the best protection against O_3, without producing side effects, the application rates of EDU must be standardized for each species. Carnahan et al. (1978) was first in trying to standardize the EDU application rates (0–500 ppm) adequate to protect pinto beans from O_3 exposure effects. EDU at 500 ppm was seen as an application rate that optimally protected plants from acute O_3 injury (Carnahan et al. 1978; Weidensaul 1980; Cathey and Heggestad 1982a, b, c). Cathey and Heggestad (1982a, b), in an exposure/response screening trial, confirmed that a foliar spray or soil drench of 500 ppm EDU provided optimal protection for 4 cultivars of petunia and 44 species of herbaceous plants.

EDU has been used as a survey tool for measuring O_3 effects under field conditions. Postiglione and Fagnano (1995) studied ambient O_3 effects on lettuce, subterranean clover, bean and tomato by using EDU. Fumagalli et al. (1997) used EDU (150 ppm) in an ambient O_3 environment, coupled with open-top chambers to study the effects of O_3 on white clover in the Milan region of Italy. In both studies, researchers found that EDU had positive effects in reducing O_3 injury to the respective tested plants.

Manning (1988, 1992, 1995), Kostka-Rick and Manning (1993b, c), Tiwari et al. (2005) and Singh et al. (2010a) performed studies that yielded appropriate dose-responses. Cathey and Heggestad (1982c) observed that a 500 ppm rate of EDU was most appropriate for woody plant protection. Later studies utilized repeatitive (weekly or biweekly) EDU applications to protect plants against chronic O_3 exposures (Clarke et al. 1983, 1990; Hofstra et al. 1983; Bambawale 1986; Heggestad 1988; Brennan et al. 1990; Legassicke and Ormrod 1981; Toivonen et al. 1982). Additional studies utilized variable dosages of EDU (300–500 ppm) to protect plants from acute and chronic O_3 levels, viz., 300 ppm (Hassan 2006), 400 ppm (Wahid et al. 2001; Singh and Agrawal 2009; Singh et al. 2009b) and 500 ppm (Agrawal et al. 2004, 2005). Tiwari et al. (2005) and Wang et al. (2007) conducted dose-response studies on wheat. Results were that ambient O_3-exposed plants treated with 300 ppm EDU, but not at 200 and 400 ppm, displayed various improved growth characteristics. EDU treatment at 450 ppm caused significant increments in growth parameters of Loblolly pine (Manning et al. 2003). In contrast, Szantoi et al. (2009) reported a significant decrease in root and total biomass of *Rudbeckia laciniata* as EDU concentrations increased (200, 400 and 600 ppm), although the percentage of leaf injury was reduced. After performing a meta analysis study, Feng et al. (2010) suggested that EDU applied as a soil drench at a concentration range of 200–400 ppm had the most positive effects on field grown crops.

4.3 Effectiveness of EDU and Its Toxicity

Most EDU studies did not show toxic effects from EDU treatment, when applied at optimal concentrations to address O_3 stress. No protective effects of EDU were reported when it was applied under the following conditions: (i) applied to a O_3-resistant cultivar, (ii) applied on plants grown in O_3-free air; however, an EDU application manifested toxic effects on plants when the EDU concentration was very high. Legassicke and Ormrod (1981) and Foster et al. (1983) found that EDU treatment did not increase plant yield in the O_3 resistant 'New Yorker' tomato cultivar and 'White Rose' potato cultivar. Clarke et al. (1983) observed a similar result in the 'Green Mountain' potato cultivar. Elagoz and Manning (2002) used one sensitive (S156) and another resistant (R123) variety of bean to validate that EDU caused a side-effect on the resistant variety. A significant increase in the above ground biomass (pod and seed weight) of an EDU-treated sensitive variety

was observed. In contrast, significant reductions in the above parameters were observed in R123 (a resistant variety). A similar response trend was observed in sensitive and resistant varieties of tobacco (Godzik and Manning 1998).

No significant yield increases of an O_3-sensitive potato grown in O_3-free air were recorded. In contrast, increasing application frequency resulted in over-dosing and caused side-effects from EDU treatment on root and shoot biomass (Foster et al. 1983; Bisessar and Palmer 1984). No significant differences in chlorophyll content, foliar injury, plant height, pod number and seed yield of soybean were observed in EDU-treated and untreated plants, when O_3 was absent (Greenhalgh et al. 1987). Hassan et al. (1995) also reported insignificant differences in root, shoot, total biomass and the root-shoot ratio in radish and turnip grown in filtered chambers, in the presence or absence of EDU. No significant difference between CF (controlled filtered air)/-EDU and CF (controlled filtered air)/+EDU treatments were observed for the measured parameters (healthy leaf number, injured leaf number, green stem, root and leaf biomass, leaf area index, total nitrogen content in plant parts, total dry biomass, grain yield, seed weight, seed protein, seed oil, etc.) of soybean plants (Ali and Abdel-Fattah 2006). Other literature reports suggested that EDU did not affect plant growth and productivity in the absence of O_3, e.g., for soybean (Kostka-Rick and Manning 1993b) and tobacco (Godzik and Manning 1998).

EDU has been reported to be toxic at high concentrations. In *Nicotiana tabacum*, foliar sprays did not cause visible injury, but soil drench applications at 500, 1,000 and 2,000 ppm caused phytotoxicity in Bel-W3 seedlings (Lee and Chen 1982). Eckardt and Pell (1996) reported complete protection (from a lower EDU dose of 15 ppm) of accelerated foliar senescence that had been induced by O_3-exposure in potato. Higher dosages of EDU (45 and 75 ppm) were associated with leaf curling and marginal necrosis. Szantoi et al. (2009) found a linear reduction in root and total biomass of *R. laciniata* as EDU levels increased. Weidensaul (1980) suggested that higher concentrations of EDU (800–5,000 ppm) applied as foliar sprays are more effective in preventing foliar injury in pinto bean, an O_3 sensitive plant. However, when applied in excess, negative effects on plant physiology were reported by Bennett et al. (1978) and Heagle (1989). Heggestad (1988) reported that weekly applications of a 500 ppm EDU spray on four cultivars of field-grown sweet corn produced phytotoxicity, reduced growth and yield. It is therefore evident that the efficacy of EDU is more apparent in O_3 sensitive cultivars when applied at optimum doses, but only when O_3 stress exists. Resistant varieties gain no benefit from EDU applications. However, low concentrations of EDU that do not affect plant growth can nonetheless provide protection from visible O_3 injury.

5 EDU and Its Modes of Action

In our review of the literature, we sought to better understand the biochemical, growth and metabolic roles of EDU's action and mechanism. In this section we have compiled the results that bear on these points below.

5.1 Effects of EDU on Growth Characteristics and Biomass Accumulation

Plant growth is a complex phenomenon that results from integration and coordination of various physiological and biochemical processes that are genetically controlled and greatly influenced by various environmental factors. Ozone is a strong oxidant that causes many effects in plant species, and in particular cultivars of those species, often at the developmental stage. EDU provides O_3 tolerance by modifying several plant processes, and ultimately protects plants from O_3 damage. Kostka-Rick and Manning (1992) reported that EDU treatment increased root biomass of *Raphanus sativus* (radish) during early growth stages, but imparted no protective effects at later stages. However, EDU treatment did not alter shoot or total biomass of radish. Kostka-Rick and Manning (1993a) reported no growth-related changes in EDU-treated plants of *Phaseolus vulgaris*, whether grown in synthetic substrates or in the field, although symptoms related to EDU toxicity were observed at a high concentration. A general conclusion of all researchers is that EDU treatment reduced or delayed O_3 injury symptoms in plant foliage, and also delayed the senescence process.

In a dose–response study conducted in closed chambers on O_3-exposed *P. vulgaris*, increasing EDU treatment levels (150–300 mg L^{-1}) significantly helped plants to accumulate more stem, leaf and total biomass, in comparison to plants not treated with EDU (Astorino et al. 1995). Brunschon-Harti et al. (1995a) reported reduced growth suppression by O_3 in the form of higher leaf, root and shoot dry weight of EDU-treated plants of *P. vulgaris*. However, Ainsworth et al. (1996) did not find a significant difference in growth characteristics of two clones of hybrid poplar, after EDU was injected in stems at rates of 250 ppm and 1,000 ppm. However, a significant reduction in O_3 injury, decreasing senescence of leaves and an induction in chlorophyll content were observed.

In Table 2, we have summarized studies conducted to assess the impact of O_3 on growth characteristics and biomass accumulation in plants that were subjected to EDU applications. Agrawal et al. (2005) noticed significant increases (viz., 12.3, 16.8, 30 and 24 %, respectively in shoot length, number of leaves, leaf area and total biomass) of EDU-treated (500 ppm) mungbean plants, compared to non-EDU treated plants (Table 2). Singh et al. (2010b) reported that EDU given as soil drench (400 ppm) to mungbean caused significant growth parameter increases, including biomass, but noted a reduction in number of leaves in EDU-treated plants (Table 2). Wang et al. (2007), however, found that EDU concentrations of 150 and 300 ppm had an insignificant effect on the above ground biomass of wheat and rice, although a decline in biomass was observed at the 450 ppm EDU treatment level.

5.2 EDU and Visible Injury

Ozone-induced visible injury on plants includes flecking, the appearance of having a water soaked area, interveinal stippling, chlorosis, bronzing and necrosis, all of

which lead to early senescence of leaves. Young leaves are less susceptible to O_3 injury than are old leaves, because aging leaves contain lower levels of antioxidants than younger ones (Bisessar 1982). This suggests that EDU is more effective during the later stages of plant life than at early stages. Lee et al. (1981) also showed that senescence of red clover leaf discs was delayed when discs were floated on a EDU solution in the dark or under low intensity light. Kostka-Rick and Manning (1992) noted complete O_3 injury protection in EDU-treated radish plants (Table 3).

In Table 3, we provide a summary of experiments conducted to assess the response of O_3 on foliar injury when EDU treatments were used. EDU at 400 ppm completely protected against visible injury from O_3 exposure in soybean plants in Pakistan (Wahid et al. 2001). EDU, used as a low dose soil drench (15 ppm), provided complete protection from accelerated foliar senescence in potato that had been exposed for 11 day to 0.1 μL L^{-1} O_3 for 5 h day^{-1} (Eckardt and Pell 1996). Kostka-Rick and Manning (1993b, c) showed similar foliar injury protection at a low EDU dose (100 mg L^{-1}), although toxicity symptoms occurred at higher dosages (300–800 mg L^{-1}). Postiglione and Fagnano (1995) used three O_3-sensitive plants (subterraneum clover, bean and tomato) and one O_3-tolerant plant (lettuce) to substantiate that EDU protection was not complete, (i.e., visible) injury was observed, though it was delayed and displayed a reduced intensity. Fumagalli et al. (1997) noted that the percent of O_3 injured leaves was significantly reduced in potted *Trifolium repens* plants that had been treated with 150 ppm EDU as a soil drench.

5.3 Role of EDU in Physiology and Photosynthetic Pigments of Plants

The physiological effects of EDU that are linked with its protective properties are still unclear (Blum et al. 2011). Previous studies showed that EDU mitigated O_3 effects through biochemical modifications in plants and not by biophysical changes (Bennett et al. 1978; Lee and Bennett 1982; Hassan et al. 1995). Stomatal conductance in EDU-treated plants that were exposed to ambient O_3 has been measured in only a few studies. In most such studies, the authors have reported no significant EDU effects on stomatal conductance (Bennett et al. 1978; Ainsworth et al. 1996; Hassan et al. 2007). Agrawal and Agrawal (1999) were first to provide evidence that EDU may act against O_3 exposure at the biophysical level by decreasing stomatal conductance. The percent closed stomata were 39.1, 45.7, 55.3 and 66.7, respectively, in control, EDU treated, O_3-exposed and EDU + O_3-exposed snap bean plants. Later, field study results disclosed the action of EDU on plant physiology (see Table 4). Recently, Wahid et al. (2012) found that sesame plants treated with 500 ppm EDU showed an increase of 52 % in stomatal conductance and a 61 % increase in net-photosynthesis rate, compared to non-EDU treated plants. Ozone is known to damage the thylakoid membrane in chloroplasts, which negatively affects

Table 3 Effects of EDU treatment on ozone-induced injury of plant species

Country/site	Species/cultivar	EDU application	EDU dose/conc.	O_3 conc.	Application interval	Injury symptoms	Injury	Reference
Belgium	*Populus nigra* L. cv. Wolterson	Stem injection	0 mg/plant 1 mg/plant 2.5 mg/plant 4 mg/plant 0 mg/plant 1 mg/plant 2.5 mg/plant 4 mg/plant	150–300 ppb for 6 h	–	Injured leaves Injured leaf surface	18 % 3.5 % 1.7 % 0 7.0 % 0.4 % 0.1 % 0	Bortier et al. (2001)
Egypt Suburban Rural	*Raphanus sativus* L. *Brassica rapa* L. *Raphanus sativus* L. *Brassica rapa* L.	Soil drench	500 ppm/200 mL	54.8 ppb (6 h) 66.9 ppb (6 h)	10 days	Chlorotic spots	5 fold (↓) No injury 2 fold (↓) No injury	Hassan et al. (1995)
Suburban Rural	*Solanum tuberosum* L. Kara	Foliar spray	300 ppm/300 mL	78 ppb (10 h) 95.5 ppb (10 h)	10 days	Pinpoint brown dots followed by bronze lesions and necrotic spots	65 % (↓) 82 % (↓)	Hassan (2006)

(continued)

Table 3 (continued)

Country/site	Species/cultivar	EDU application	EDU dose/conc.	O_3 conc.	Application interval	Injury symptoms	Injury	Reference
Rural	*Trifolium alexandrinum* L. Messkawy	Soil drench	0 ppm/200 mL 50 ppm/200 mL 100 ppm/200 mL 150 ppm/200 mL 200 ppm/200 mL	88 ppb (8 h day^{-1})	10 days	Number of injured leaves (flecking)	1.98 % 1.56 % 1.02 % 0.45 % 0.42 %	Hassan et al. (2007)
Germany	*Phaseolus vulgaris* L. Lit	Soil drench	150 ppm/200 mL	–	14 days	Bronzing	Complete protection	Brunschon-Harti et al. (1995a)
Italy	*Populus* L. cv. I-214 and Eridano	Stem injection	0 ppm/0.5 mL 250 ppm/0.5 mL 1,000 ppm/1.0 mL	85 ppb for 8 h day^{-1}	10 days	No injury in I-214 clone Small interveinal small stipples, necrotic area	2.07 % 1.52 % 0.65 %	Ainsworth et al. (1996)
	Fraxinus excelsior L.	Gravitational infusion	0 ppm/13.3 mL/plant 300 ppm/13.3 mL/plant 450 ppm/8.9 mL/plant	150 ppb for 8 h day^{-1}, 21 days	–	Interveinal reddish stipples	13 % 3 % 2 %	Paoletti et al. (2007)

Maryland	*Phaseolus vulgaris* L. Bush Blue Lake 290	Soil drench	0 mg/pot, 100 mL/plant 12.5 mg/pot, 100 mL/plant 25 mg/pot, 100 mL/plant 50 mg/pot, 100 mL/plant	458 ppb	–	Stippling, bifacial necrosis or marginal and tip burn	87 % 75 % 38 % 0 %	Lee and Bennett (1982)
	Trifolium pratense L. cv. Pennscott	–	0 μg/mL 125 μg/mL 250 μg/mL 500 μg/mL	303 ppb for 4 h	–	Stippling, bifacial necrosis or marginal and tip burning	83 % 73 % 22 % Complete protection	Lee et al. (1981)
Ontario	*Arachis hypogaea* L. USDA PI 268661 McRan (Laboratory study) USDA PI 268661 McRan (Field study)	Foliar spray	1 g/L	219 ppb/7 h day^{-1} for 4 d 219 ppb	14 days	White flecks, bronzing. chlorosis only in USDA PI 268661 Foliar bronzing and chlorosis	87.7 % (↓) 75.0 % (↓) 91.4 % (↓) No injury	Ensing et al. (1985)

(continued)

Table 3 (continued)

Country/site	Species/cultivar	EDU application	EDU dose/ conc.	O_3 conc.	Application interval	Injury symptoms	Injury	Reference
USA	*Raphanus sativus* L. cv. Cherry Belle	Soil drench	150 ppm/ 60 mL/ plant	70 ppb/7 h day^{-1}, 5 d	7 days	Leaf margin necrosis and leaf deformation	Complete protection	Kostka-Rick and Manning (1992)
	Phaseolus vulgaris L. Bush Blue Lake 290	Soil drench	0 ppm/ 100 mL 500 ppm/ 100 mL	300 ppb for 3 h	–	Necrosis	45 % 10 %	Lee et al. (1997)
	Rudbeckia laciniata L.	Foliar spray	0 ppm 200 ppm 400 ppm 600 ppm	32–38 ppb (12 h)	7 days	Leaves injured	13.9 % 13.2 % 11.1 % 9.2 %	Szantoi et al. (2009)
Wilmington, DE USA	*Phaseolus vulgaris* L. Pinto 111	Root application	0 mg/ 20 mL 4 mg/ 20 mL	120 ppm for 150 min	–	–	88 % 8 %	Carnahan et al. (1978)

Wooster, OH, USA	*Phaseolus vulgaris* L.	Runoff on leaf surface	0 µg/g 500 µg/g 1,000 µg/g 5,000 µg/g 0 µg/g 500 µg/g 1,000 µg/g 5,000 µg/g 0 µg/g 500 µg/g 1,000 µg/g 5,000 µg/g 0 µg/g 500 µg/g 1,000 µg/g 5,000 µg/g 0 µg/g 500 µg/g 1,000 µg/g 5,000 µg/g 0 µg/g 500 µg/g 1,000 µg/g 5,000 µg/g	0.0 ppm 0.10 ppm 0.25 ppm 0.40 ppm 0.50 ppm 0.75 ppm	– – – – – –	Number of leaf injured (Severe bleaching and bifacial necrosis)	8 8 8 8 8.2 8 8 8 12.4 11.7 10.5 9.9 19.5 16.2 15.2 15.4 26.6 22.8 21.3 14.6 26.3 24.0 23.6 19.2	Weidensaul (1980)

(↓) = reduction

the light quenching capacity of chlorophyll. EDU application improved fluorescence kinetics in O_3-exposed plants of *Beta vulgaris* (Tiwari and Agrawal 2009), *Triticum aestivum* (Singh and Agrawal 2009) and *Trifolium repens* (Singh et al. 2010d) (Table 4). A study on the photosynthetic capacity of ash trees revealed a slight effect of EDU in preventing O_3-induced inactivation of photosynthetic reaction centers (Contran et al. 2009).

Photosynthetic pigments are the basic entities of the plant photosynthetic machinery, and EDU is effective in maintaining high levels of photosynthetic pigments. Lee and Chen (1982) reported that non-EDU-treated leaf discs of tobacco lost more than half of their original chlorophyll after 10 days, whereas discs treated with 1×10^{-3} M EDU lost only 10 % of the initial chlorophyll. Whitaker et al. (1990), however, found that EDU treatment did not alter leaf chlorophyll or carotenoid contents, but a reduction of 14 % was observed when plants were exposed to O_3 alone. Potato plants treated with 45 and 75 mg EDU L^{-1} had higher chlorophyll content, and the chlorophyll was retained for a longer period than in untreated plants (Eckardt and Pell 1996). One main reason that EDU provides protection was that it enhances retention of chlorophyll for longer time (Lee et al. 1997). In Table 5, we summarized results of investigations that have been conducted to evaluate the effects on photosynthetic pigments from O_3 exposure and EDU treatment.

5.4 EDU Protection in Relation to Antioxidants

EDU does not act as an antioxidant itself, but helps to maintain higher levels of cellular antioxidants during O_3 stress in *Phaseolus vulgaris* (Lee et al. 1997). Superoxide dismutase (SOD) activity is normally associated with O_3 tolerance. Lee and Bennett (1982) found a significant induction of SOD and catalase (CAT) activities when snap beans were treated with EDU. Pitcher et al. (1992) refuted the hypothesis that EDU's mode of action works by increasing SOD activity. There was no EDU associated increases in Cu/Zn-SOD or Mn-SOD activities in plants exposed to O_3. Lee et al. (1997) did not observe significant differences in activities of ascorbate peroxidase (APX), guaicol peroxidase (GPX) and SOD in O_3 exposed snap bean leaves that were treated with EDU as compared to untreated plants. However, EDU-treated plants maintained a higher glutathione reductase (GR) activity. Batini et al. (1995) hypothesized that the protection mechanism against O_3 that had been exerted by EDU was caused by the stimulation of only the APX detoxifying system involved in scavenging hydrogen peroxide molecules. Singh et al. (2010b) found more significant reductions in POX and SOD activities in leaves of EDU-treated mungbean plants than in non EDU-treated ones. A compilation of experiments conducted to assess the effects of EDU on antioxidant enzymes under O_3 treatment is given in Table 6.

Among non-enzymatic antioxidants, apoplastic ascorbic acid acts as a 'first line of defense' against O_3 damage (Chameides 1989). Hence, ozone tolerance is

Table 4 Effects of EDU treatment on Fv/Fm ratio, photosynthetic rate and stomatal conductance of plants grown under ozone exposure

Country/ site	Species/cultivar	EDU application	EDU dose/ conc.	O_3 conc.	Application time/ interval	Fv/Fm ratio	Photosynthetic rate	Stomatal conductance	Reference
Egypt Rural	*Raphanus sativus* L. *Brassica rapa* L.	Soil drench	500 ppm/ 200 mL	66.9 ppb (6 h)	10 days	– –	NS NS	NS NS	Hassan et al. (1995)
India	*Phaseolus vulgaris* L. Bush Blue Lake 290	Soil drench	500 ppm/ 100 mL	305 ppb for 3 h	48 h prior to fumigation	–	77.7 % (↑)	27.6 % (↓)	Agrawal and Agrawal (1999)
Urban	*Triticum aestivum* L. HD 2329 HUW 234 HUW 468	Soil drench	500 ppm/ 250 mL	29.2 ppb (6 h)	7 days	– – – –	– – – –	2.8 % (↓) 6.2 % (↓) 14.8 % (↓)	Agrawal et al. (2004)
Suburban	*Beta vulgaris* L. Allgreen	Soil drench	300 ppm/ 200 mL	52.1–73.2 ppb (8 h)	10 days	1.7 % (↑)	–	–	Tiwari and Agrawal (2009)
Suburban	*Triticum aestivum* L. HUW234 HUW468 HUW510 PBW343 Sonalika	Soil drench	400 ppm/ 100 mL	35.3–54.2 ppb (8 h)	12 days	14.5 % (↑) 6.1 % (↑) 5.6 % (↑) NS NS	20.7 % (↑) 27.2 % (↑) 22.1 % (↑) NS NS	16.0 % (↑) 16.9 % (↑) 13.7 % (↑) NS NS	Singh and Agrawal (2009)

(continued)

Table 4 (continued)

Country/ site	Species/cultivar	EDU application	EDU dose/ conc.	O_3 conc.	Application time/ interval	Fv/Fm ratio	Photosynthetic rate	Stomatal conductance	Reference
Rural	*Vigna radiata* L. Malviya Janpriya	Soil drench	400 ppm/ 100 mL	52.9–64.5 ppb (12 h)	10 days	–	31.6 % (↑)	31.2 % (↑)	Singh et al. (2010b)
	Trifolium repens L. Bundel Vardan	Soil drench	150 ppm/ 100 mL 300 ppm/ 100 mL 150 ppm/ 100 mL 300 ppm/ 100 mL	30.3–46.6 ppb (12 h)	10 days	NS NS 5.4 % (↑) 5.4 % (↑)	– – – –	– – – –	Singh et al. (2010d)
Pakistan Suburban	*Sesamum indicum* L. Ts-3 Til 93 S-17	Soil drench	125 ppm/ 100–400 mL 250 ppm/ 100–400 mL 375 ppm/ 100–400 mL 500 ppm/ 100–400 mL	Seasonal mean91 ppb (10 h)	7days	– – – –	NS 25–35 % (↑) 47–52 % (↑) 56–61 % (↑)	NS 13–16 % (↑) 33–39 % (↑) 47–52 % (↑)	Wahid et al. (2012)

(↑) = increase, (↓) = decrease, *NS* not significant, *Fv* variable fluorescence, *Fm* maximum fluorescence

Table 5 Effects of EDU treatment on photosynthetic pigments, protein content, MDA and ascorbic acid contents of plants grown under ozone exposure

Country/ site	Species/ cultivar	EDU application	EDU doses/ conc.	O_3 conc.	Application interval	Chlorophyll content	Carotenoid content	Protein content	MDA content	Ascorbic acid content	Reference
India Urban	*Triticum aestivum* L. HD 2329 HUW 234 HUW 468	Soil drench	500 ppm/ 250 mL	29.2 ppb (6 h)	7 days	(↑) (↑) (↑)	(↑) (↑) (↑)	– – –	– – –	(↑) (↑) (↑)	Agrawal et al. (2004)
Suburban	*Vigna radiata* L. Malviya Jyoti	Soil drench	500 ppm/ 500 mL	32.6–35.2 ppb (8 h)	7 days	12.8 % (↑)	(↑)	9.8 % (↑)	–	13.8 % (↑)	Agrawal et al. (2005)
Suburban	*Beta vulgaris* L. Allgreen	Soil drench	300 ppm/ 200 mL	52.1–73.2 ppb (8 h)	10 days	17.5 % (↑)	28 % (↑)	20.4 % (↑)	37.4 % (↓)	39.8 % (↑)	Tiwari and Agrawal (2009)
Suburban	*Triticum aestivum* L. HUW234 HUW468 HUW510 PBW343 Sonalika	Soil drench	400 ppm/ 100 mL	35.3–54.2 ppb (8 h)	12 days	19.1 % (↑) 22.6 % (↑) 18.9 % (↑) NS NS	22.5 % (↑) 28.3 % (↑) 17.9 % (↑) 26.5 % (↑) 27.8 % (↑)	11.8 % (↑) 14.4 % (↑) 28.15 (↑) 13.9 % (↑) 2.0 % (↑)	13.6 % (↓) 16.2 % (↓) 6.6 % (↓) NS 18.4 % (↓)	NS 17.5 % (↑) 11.5 % (↑) NS NS	Singh and Agrawal (2009)

(continued)

Table 5 (continued)

Country/ site	Species/ cultivar	EDU application	EDU doses/ conc.	O_3 conc.	Application interval	Chlorophyll content	Carotenoid content	Protein content	MDA content	Ascorbic acid content	Reference
Suburban	*Triticum aestivum* L. HUW468	Soil drench	200 ppm/ 100 mL 300 ppm/ 100 mL 400 ppm/ 100 mL 500 ppm/ 100 mL	27.7–59.1 ppb (12 h)	10 days	13.8 % (↑) 11.2 % (↑) 25.2 % (↑) 7.2 % (↑)	29.5 % (↑) 2.9 % (↑) 31.6 % (↑) 14.8 % (↑)	–	–	–	Singh and Agrawal (2010)
Rural	*Vigna radiata* L. Malviya Janpriya	Soil drench	400 ppm/ 100 mL	52.9–64.5 ppb (12 h)	10 days	(↑)	(↑)	(↑)	17.7 % (↓)	60.5 % (↑)	Singh et al. (2010b)
Saudi Arab KSU KFS	*Vicia faba* L. Lara	Soil drench	250 ppm	21.2 ppb 62.7 ppb	12 days	18.6 % (↑) 13.6 % (↑)	14.1 % (↑) 10.7 % (↑)	– –	– –	– –	Al–Qurainy (2008)
USA	*Phaseolus vulgaris* L. Bush Blue Lake 290	Soil drench	500 ppm/ 100 mL	300 ppb for 3 h		144.4 % (↑)	–	–	–	–	Lee et al. (1997)

(↑) = increase, (↓) = decrease, *NS* not significant, *MDA* malondialdehyde

Table 6 Effects of EDU treatment on some antioxidant enzymes of plants grown under ozone exposure

Country/ site	Species/cultivar	EDU application	EDU doses/ conc.	O_3 conc.	Application time/interval	Parameters	Changes	Reference
Germany	*Phaseolus vulgaris* L. Lit	Soil drench	150 ppm/ 200 mL	0.98–31.5 ppb	14 days	POX CAT APX	−0.42 fold +1.47 fold NS	Brunschon-Harti et al. (1995b)
India Suburban	*Triticum aestivum* L. HUW234 HUW468 HUW510 PBW343 Sonalika	Soil drench	400 ppm/ 100 mL	35.3–54.2 ppb (8 h)	12 days	SOD POX APX SOD POX APX SOD POX APX SOD POX APX SOD POX APX	NS +1.64 fold +1.84 fold +1.21 fold +2.32 fold +2.21 fold +1.18 fold +2.10 fold +2.73 fold NS NS NS NS NS NS	Singh et al. (2009b)

(continued)

Table 6 (continued)

Country/ site	Species/cultivar	EDU application	EDU doses/ conc.	O_3 conc.	Application time/interval	Parameters	Changes	Reference
Suburban	*Beta vulgaris* L. Allgreen	Soil drench	300 ppm/ 200 mL	52.1–73.2 ppb	10 days	POX	−0.77 fold	Tiwari and Agrawal (2009)
Rural	*Vigna radiata* L. Malviya Janpriya	Soil drench	400 ppm/ 100 mL	52.9–64.5 ppb (12 h)	10 days	SOD POX	−0.46 fold −0.47 fold	Singh et al. (2010b)
Maryland	*Phaseolus vulgaris* L. Bush Blue Lake 290	Soil drench	25 mg/pot 50 mg/pot 100 mg/pot in 100 mL	457 ppb for 4 h	24 h before O_3 treatment	SOD CAT POX	+1.07 fold +2.31 fold +1.32 Fold +1.17 Fold +2.04 Fold +1.64 fold +1.07 fold +1.97 fold +5.84 fold	Lee and Bennett (1982)

New Jersey	*Phaseolus vulgaris* L. Bush Blue Lake 290	Soil drench	0.3 mg/mL	0.3 ppm for 6 h	24 h before O_3 treatment	SOD	−0.95 fold	Pitcher et al. (1992)
U.S.A.	*Phaseolus vulgaris* L. Bush Blue Lake 290	Soil drench	500 ppm/ 100 mL	300 ppb for 3 h		GR APX GPX CAT SOD	+1.36 fold No change No change NS NS	Lee et al. (1997)

+ = increase, − = decrease

directly correlated to increased apoplastic ascorbic acid in *P. major* population (Zheng et al. 2000), snap bean ecotypes (Burkey 1999; Burkey et al. 2003), soybean cultivars (Robinson and Britz 2000, 2001) and *Sedum album* (Castillo and Greppin 1988). Various studies have revealed that EDU plays a role in maintenance/synthesis of the ascorbic acid pool in plants. Significant increase in the ratio of ascorbic acid (AA)/dehydroascorbic acid (DHA) in EDU-treated plants has been shown by Brunschon-Harti et al. (1995b). However, Gillespie et al. (1998) did not find any significant effect of EDU on the AA and DHA contents of snap bean plants. Singh et al. (2010b) reported a significantly higher content of ascorbic acid in mungbean that had been treated with EDU. Higher ascorbic acid content in foliage is commonly observed to occur after EDU treatment (Table 5).

To understand the EDU-induced O_3 tolerance and the role of glutathione (GSH) in snap bean, Lee et al. (1997) used an HPLC technique to study the effect of EDU. They found that total glutathione and GSH concentrations decreased significantly in O_3 fumigated plants (no EDU + O_3), but GSSG concentrations increased *vis-a-vis* a decrease in the GSH/GSSG ratio, compared to controls (no O_3). Pretreatment with EDU significantly increased the levels of total glutathione and GSH, and reduced the level of GSSG, in comparison to control snap bean plants. Higher concentrations of total glutathione and GSH and a lower GSSG reserve were observed in EDU-treated plants after O_3 exposure (EDU + O_3) vs. control plants (no EDU + O_3). This suggested that EDU-treated plants that were under O_3 stress showed no reduction in glutathione reductase (GR) activity. Lee et al. (1997) suggested that EDU protection against O_3 damage may result from maintenance of GR and GSH levels during O_3 exposure. Hassan (2006) used the same technique to determine the glutathione concentration in potato leaves. EDU-treated plants grown under O_3 exposure had a higher GSH content, but lower GSSG and total glutathione levels, compared to plants exposed to O_3 without EDU treatment. Generally, plants treated with EDU have a higher GSH/GSSG ratio than do the controls.

Polyamines are known as stabilizing factors of biomembranes and macromolecules and are also free radical scavengers (Drolet et al. 1986), either directly or via conjugation with other molecules such as free ferulic and caffeic acids (Didyk and Blum 2011). Bors et al. (1989) suggested that polyamines conjugate with hydroxycinnamic acids and play a role in protecting against the accumulation of O_3-triggered reactive oxygen species. Polyamine content in fully expanded leaves of snap bean treated with EDU was compared with the control (-EDU) leaves before and after O_3 exposure. It was reported that EDU did not alter the polyamine composition of leaves, although O_3 alone (-EDU) induced significant increases in total polyamines (Lee et al. 1992).

5.5 *Effects of EDU on Soluble Protein, MDA (Malondialdehyde) Content and Foliar Lipids*

Brunschon-Harti et al. (1995b) showed that the soluble protein content increased following EDU treatment in snap bean plants, while increasing O_3 dose did not cause any change in total soluble protein in EDU-treated plants. Studies have revealed that an increased O_3 dose increased the MDA content in EDU-treated plants, thus reflecting more damage to plasma membranes. Most study results support the view that the MDA content is increased from EDU treatment, although there are exceptions (Table 5). Whitaker et al. (1990) found that pretreatment with EDU conferred protection against O_3-induced losses of glycerolipids. Leaves of untreated snap bean plants lost approximately 50 % of both the galactolipids (GL) and phospholipids (PL), while EDU-treated plants showed no significant loss of foliar GL and PL. In controls (no EDU), sterylglycosides (SG) showed an incremental increase, while EDU-treated plants showed a small increase in SG.

5.6 *Effects of EDU on Carbohydrates*

Increments in sucrose and other soluble sugars in bean leaves, 48 h after EDU treatment was correlated with development of resistance against O_3 (Lee et al. 1981). Miller et al. (1994) reported higher starch levels in EDU-treated O_3-exposed snap bean plants, whereas foliar starch levels declined under O_3 exposure without EDU treatment, compared to plants exposed to charcoal filtered (CF) air. Singh et al. (2010c) observed that all three test cultivars of blackgram (barkha, shekhar and TU-94-2) showed a significant decrease in reducing sugar content, and a respective increase in total soluble sugar content in seeds of plants treated with EDU. In contrast, starch content increased significantly only in seeds of EDU-treated black gram cultivar TU-94-2 as compared to non-EDU treated ones. Al-Qurainy (2008) studied broad bean plants grown without EDU at O_3 concentrations of 21.2 and 62.7 ppb in ambient air, respectively at King Saud University and King Fahad Street, displayed reduced soluble, insoluble and total leaf carbohydrates as they aged. In contrast, plants treated with EDU at 250 mg L^{-1}showed increments in all forms of carbohydrates with increasing age, compared to non EDU-treated plants.

6 The Role of EDU in Assessing Yield Losses

O_3-related yield losses are a serious and growing concern throughout the world. Different approaches have been utilized to estimate the level of economic losses experienced from O_3 exposure. EDU is a widely used biomonitoring tool that is

commonly utilized to assess plant yield loss from O_3 exposure. EDU successfully protects a wide variety of plants from O_3. EDU has also been used to screen for O_3 sensitive and tolerant/resistant cultivars, and to estimate the extent of damage caused by O_3. Such information is useful for selecting cultivars to grow in areas that experience high O_3 levels. Yield loss assessments are performed by comparing the yield estimates of EDU-treated and untreated plants in O_3 polluted areas of the USA (Ensing et al. 1985; Smith et al. 1987), Asia (Wahid et al. 2001; Agrawal et al. 2004, 2005; Tiwari et al. 2005; Wang et al. 2007; Singh and Agrawal 2009; Singh et al. 2010b, c), Africa (Hassan et al. 1995; Hassan 2006) and Europe (Ribas and Penuelas 2000; Brunschon-Harti et al. 1995a; Pleijel et al. 1999). Yield increments after the application of EDU onto O_3 exposed plants have been reported for onion (Wukasch and Hofstra 1977), navy bean (Hofstra et al. 1978; Temple and Bisessar 1979; Toivonen et al. 1982), tomato (Legassicke and Ormrod 1981), potato (Bisessar 1982; Clarke et al. 1990; Hassan 2006), tobacco (Bisessar and Palmer 1984), watermelon (Fieldhouse 1978), peanut (Ensing et al. 1985), radish (Kostka-Rick and Manning 1992; Hassan et al. 1995), carrot (Tiwari and Agrawal 2010), bush bean (Kostka-Rick and Manning 1993a, c), snap bean (Vandermeiren et al. 1995), mungbean (Agrawal et al. 2005; Singh et al. 2010b), soybean (Wahid et al. 2001), wheat (Agrawal et al. 2004; Tiwari et al. 2005; Wang et al. 2007; Singh and Agrawal 2009) and black gram (Singh et al. 2010c).

Applying EDU has increased the number of seeds per plant and total seed weight per plant for two O_3-sensitive cultivars of soybean, while insignificant effects were observed in the O_3-tolerant lines for these parameters (Damicone 1985). No significant difference in seed size was observed vs. controls in soybean cultivars that were treated with EDU and then exposed to O_3 (Smith et al. 1987). EDU treatment has led to increased seed size in wheat (Agrawal et al. 2004; Singh and Agrawal 2009), and mungbean (Agrawal et al. 2005). Wahid et al. (2001) reported significantly higher seed weight/plant, number of seeds/pod and number of pods/plant for *Glycine max* under EDU treatment at all experimental sites (suburban, rural and rural roadside) in Pakistan that experienced higher O_3 concentrations during pre-monsoon and post-monsoon experiments. Significantly higher tuber weight and number of tubers were reported in EDU-treated potato plants, compared to EDU-untreated plants at both rural and suburban sites in Egypt in areas experiencing high O_3 concentrations (Hassan 2006). In Table 7, we have summarized results of experiments, in which the effect of O_3 on yield was assessed, along with the mitigating effects of having used EDU.

EDU has been widely and successfully used to protect against the effects of O_3, as a research tool to assess plant responses to O_3 stress, and to estimate crop/plant yield losses. The advantages and disadvantages of using EDU for these purposes are stated below:

Table 7 Effects of EDU treatment on yield of plants grown under ozone exposure

Country/ site	Species/cultivar	EDU application	EDU dose/conc.	O_3 conc.	Application interval	Yield	Reference
China	*Triticum aestivum* L. *Oryza sativa* L.	Foliar spray	150 ppm 300 ppm 450 ppm 150 ppm 300 ppm 450 ppm	Frequently exceeding 40 ppb	7 days	3.4 % (↑) 12.7 % (↑) 7.1 % (↑) NS NS NS	Wang et al. (2007)
Egypt Suburban Rural	*Raphanus sativus* L. *Brassica rapa* L. *Raphanus sativus* L. *Brassica rapa* L.	Soil drench	500 ppm/200 mL	54.8 ppb (6 h) 66.9 ppb (6 h)	10 days	33.3 % (↑) 4.3 % (↑) 36.2 % (↑) 20.9 % (↑)	Hassan et al. (1995)
Egypt Suburban Rural	*Solanum tuberosum* L. Kara	Foliar spray	300 ppm/300 mL	78 ppb (10 h) 95.5 ppb (10 h)	10 days	30.5 % (↑) 31.8 % (↑)	Hassan (2006)
Germany	*Phaseolus vulgaris* L. Lit	Soil drench	150 ppm/200 mL	0.98–31.5 ppb	14 days	30–65 % (↑)	Brunschon-Harti et al. (1995a)
India Urban	*Triticum aestivum* L. HD 2329 HUW 234 HUW 468	Soil drench	500 ppm/250 mL	29.2 ppb (6 h)	7 days	22 % (↑) 27 % (↑) 36.3 % (↑)	Agrawal et al. (2004)
Suburban	*Vigna radiata* L. Malviya Jyoti	Soil drench	500 ppm/500 mL	32.6–35.2 ppb (8 h)	7 days	32.2 % (↑)	Agrawal et al. (2005)

(continued)

Table 7 (continued)

Country/ site	Species/cultivar	EDU application	EDU dose/conc.	O_3 conc.	Application interval	Yield	Reference
Suburban	*Triticum aestivum* L. M 234 M 533	Soil drench	150 ppm/200 mL 300 ppm/200 mL 450 ppm/200 mL 150 ppm/200 mL 300 ppm/200 mL 450 ppm/200 mL	Often exceeding 40 ppb (8 h)	10 days	24.8 % (↑) 66.9 % (↑) 66.8 % (↑) 18.8 % (↑) 19.1 % (↑) 20.5 % (↑)	Tiwari et al. (2005)
Suburban	*Triticum aestivum* L. HUW234 HUW468 HUW510 PBW343 Sonalika	Soil drench	400 ppm/100 mL	35.3–54.2 ppb (8 h)	12 days	11.2 % (↑) 25.8 % (↑) 20.5 % (↑) 1.9 % (↑) 10.2 % (↑)	Singh and Agrawal (2009)
Suburban	*Glycine max* L. Pusa 9712 Pusa 9814	Soil drench	400 ppm/200 mL	NFCs NFCs + 20 ppb NFCs NFCs + 20 ppb	10 days	29.8 % (↑) 33.0 % (↑) 28.2 % (↑) 29.0 % (↑)	Singh and Agrawal (2011)

Rural	*Vigna radiata* L. Malviya Janpriya	Soil drench	400 ppm/100 mL	52.9–64.5 ppb (12 h)	10 days	32.3 % (↑)	Singh et al. (2010b)
Suburban	*Vigna mungo* L. cv. Barkha Shekhar TU-94-2	Soil drench	400 ppm/100 mL	41.3–59.9 ppb (12 h)	10 days	36.4 % (↑) 35.6 % (↑) NS	Singh et al. (2010c)
Ontario	*Arachis hypogaea* L. PI 268661	Foliar spray	1,000 ppm	220 ppb (7 h day^{-1}) for 4 d	14 days	24.0 % (↑)	Ensing et al. (1985)
Pakistan Punjab Post-monsoon Rural roadside Rural Suburban Pre-mon-soon Rural roadside Rural Suburban	*Glycine max* L. NARC-1	Soil drench	400 ppm/600 mL	48 ppb (6 h) 48 ppb (6 h) 40 ppb (6 h) 70 ppb (6 h) 75 ppb (6 h) 63 ppb (6 h)	10 days	170 % (↑) 94 % (↑) 47 % (↑) 284.6 % (↑) 181.8 % (↑) 112.8 % (↑)	Wahid et al. (2001)
Spain	*Phaseolus vulgaris* L.	Soil drench	Increasing dose of 100, 150, 200 and 250 ppm/200 mL	10,000 ppb h (AOT40)	12 days	58.4 % (↑)	Ribas and Penuelas (2000)
Sweden	*Raphanus sativus* L. Cherry Belle	Soil drench	200 ppm/100 mL	31 ppb (24 h)	14 days	32 % (↑)	Pleijel et al. (1999)

(↑) = increase, (↓) = decrease, *NS* not significant

6.1 Advantages

1. No chambers or costly investments like FACE (Free Air Carbon dioxide Enrichment) are required. The use of EDU requires only ambient conditions; no modifications of microclimate are needed.
2. Plant numbers and plot sizes can vary according to the requirements of the experiment, which facilitates having many replications.
3. Low technical input is required to utilize EDU, whose utilization is comparatively simple and easy to execute.

6.2 Disadvantages

1. Ozone dose-response studies cannot be performed, unless coupled with OTCs (open top chambers).
2. Ambient O_3 concentrations and environmental variables need careful monitoring.
3. Repeated applications of EDU can cause phytotoxicity, especially under dry soil conditions; hence, detailed plant toxicological studies are needed before starting field experiments.
4. High cost and commercial non availability of EDU has led it to be used mainly in monitoring experiments; the commercial scale use of EDU as a crop protectant has not yet occurred.
5. The use of EDU requires extensive labor and time for application.

7 Summary

Urbanization, industrialization and unsustainable utilization of natural resources have made tropospheric ozone (O_3) one of the world's most significant air pollutants. Past studies reveal that O_3 is a phytotoxic air pollutant that causes or enhances food insecurity across the globe. Plant sensitivity, tolerance and resistance to O_3 involve a wide array of responses that range from growth to the physiological, biochemical and molecular. Although plants have an array of defense systems to combat oxidative stress from O_3 exposure, they still suffer sizable yield reductions. In recent years, the ground-level O_3 concentrations to which crop plants have been exposed have caused yield loses that are economically damaging. Several types of chemicals have been applied or used to mitigate the effects produced by O_3 on plants. These include agrochemicals (fungicides, insecticides, plant growth regulators), natural antioxidants, and others. Such treatments have been effective to one degree to another, in ameliorating O_3-generated stress in plants. Ethylene diurea (EDU) has been the most effective protectant used and has also served as a monitoring agent for assessing plant yield losses from O_3 exposure. In this review,

we summarize the data on how EDU has been used, the treatment methods tested, and application doses found to be both protective and toxic in plants. We have also summarized data that address the nature and modes of action (biophysical and biochemical) of EDU.

In general, the literature discloses that EDU is effective in reducing ozone damage to plants, and indicates that EDU should be more widely used on O_3 sensitive plants as a tool for biomonitoring of O_3 concentrations. Biomonitoring studies that utilize EDU are very useful for rural and remote areas and in developing countries where O_3 monitoring is constrained from unavailability of electricity. The mechanism(s) by which EDU prevents O_3 toxicity in plants is still not completely known. EDU possesses great utility for screening plant sensitivity under field conditions in areas that experience high O_3 concentrations, because EDU prevents O_3 toxicity only in O_3 sensitive plants. Ozone-resistant plants do not respond positively to EDU applications. However, EDU application dose and frequency must be standardized before it can be effectively and widely used for screening O_3 sensitivity in plants. EDU acts primarily by enhancing biochemical plant defense and delaying O_3-induced senescence, thereby reducing chlorophyll loss, and maintaining physiological efficiency and primary metabolites; these actions enhance growth, biomass and yield of plants.

We believe that future studies are needed to better address the EDU dose-response relationship for many plant species, and to screen for new cultivars that can resist O_3 stress. Although some research on the physiological and biochemical mechanisms of action of EDU have been performed, the new 'omics' tools have not been utilized to evaluate EDUs mechanism of action. Such data are needed, as is gene expression and proteome profiling studies on EDU-treated and -untreated plants.

Acknowledgement Authors are thankful to the Head, Department of Botany for facilities and funding agencies Council of Scientific and Industrial Research (CSIR), University Grants Commission (UGC), Department of Science and Technology (DST) and Ministry of Environment and Forests (MOEF), Government of India for providing the financial help. Authors would also like to thank the researchers who helped us indirectly by providing their significant research work on ozone and EDU.

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