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REVIEW ARTICLE

Ethylenediurea as a potential tool in evaluating ozone phytotoxicity: a review study on physiological, biochemical and morphological responses of plants

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Abstract Present-day climate change scenario has intensified the problem of continuously increasing ground-level ozone (O_3), which is responsible for causing deleterious effects on growth and development of plants. Studies involving use of ethylenediurea (EDU), a chemical with antiozonant properties, have given some promising results in evaluating O_3 injury in plants. The use of EDU is especially advantageous in developing countries which face a more severe problem of ground-level O_3, and technical O_3-induced yield loss assessment techniques like open-top chambers cannot be used. Recent studies have detected a hormetic response of EDU on plants; i.e. treatment with higher EDU concentrations may or may not show any adverse effect on plants depending upon the experimental conditions. Although the mode of action of EDU is still debated, it is confirmed that EDU remains confined in the apoplastic regions. Certain studies indicate that EDU significantly affects the electron transport chain and has positive impact on the antioxidant defence machinery of the plants. However, the mechanism of protecting the yield of plants without significantly affecting photosynthesis is still questionable. This review discusses in details the probable mode of action of EDU on the basis of available data along with the impact of EDU on physiological, biochemical, growth and yield response of plants under O_3 stress. Data regarding the effect of EDU on plant 'omics' is highly insufficient and can form an important aspect of future EDU research.

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Introduction

The evidence of the phytotoxic nature of tropospheric O_3 was given by Middleton (1956) and Richards et al. (1958) who observed O_3 injury symptoms on different crop plants. Since then, tropospheric O_3 has become an issue of serious concern as far as the productivities of agricultural, horticultural and forest ecosystems are concerned. In the last few years, concentration of O_3 in the troposphere has increased at a surprisingly higher rate due to the anthropogenic activities and global climate change scenarios (Ebojie et al. 2016; Cooper et al. 2014; Parrish et al. 2012). Nowadays, as the environmentalists suggest, tropospheric O_3 is the most phytotoxic air pollutant in most parts of the world causing significant damage to both agricultural and natural species (Tiwari and Agrawal 2010; Singh et al. (2010a); Booker et al. 2009; Mills et al. 2007; Wittig et al. 2009; Serengil et al. 2011). Background O_3 concentrations have increased from 10 ppb before industrial revolution (Volz and Kley 1988) to present global mean of approximately 50 ppb (8 h summer seasonal average) (Ebojie et al. 2016; Ziemke et al. 2011; The Royal Society 2008) and are expected to increase further at an annual rate of 0.3 ppb per year (Wilkinson et al. 2012). The increase in the O_3 concentration is more severe in East and South Asian regions experiencing favourable climatic conditions for O_3 formation (Feng et al. 2015; Lee et al. 2015; Tiwari et al. 2008; Permadi and Oanh 2008). Air monitoring studies suggest that mean monthly O_3 concentration of 50 ppb is of common occurrence in several parts of Asian regions, especially during the growing season of important agricultural crops (EANET 2006). Global photochemical models predict that under current

legislation emission scenario, parts of Asia will experience significant increase in O_3 concentration by 2030 (Deneter et al. 2006). This will result in significant yield reduction of 5–20% for several important crops (Emberson et al. 2009). Different individual monitoring studies have also shown high O_3 concentrations in Asian countries like India (Tiwari et al. 2008; Debaje and Kakade 2008), China and Japan (Feng et al. 2015; Lee et al. 2015), Jakarta (Permadi and Oanh 2008) and Hong Kong (Wang et al. 2009). Model-based studies conducted in India also suggest higher increments in O_3 concentrations in the coming years (Mittal et al. 2007; Roy et al. 2009; Kulkarni et al. 2010). The Indo-Gangetic Plain, which is a major productive area, is highly prone to O_3-induced risk as a result of extreme industrialization, urbanization, land use changes and intense agricultural uses (reviewed in Oksanen et al. 2013). The regional chemistry transport model (REMO-CTM) predicts higher O_3 precursors (CO, NO_x) and O_3 concentrations over the Indo-Gangetic Plains compared to other parts of India. Due to the predicted high O_3 concentrations, the relative crop production losses are inevitable which are estimated to vary from 10 to 48% for wheat, 5–28% for rice, 8–3% for soybeans and 27–52% for beans (reviewed in Oksanen et al. 2013).

The O_3 precursors are carried to far off places by the prevailing winds; as such, the problem of increasing O_3 concentration in troposphere has assumed a global significance. Doherty (2015) reported that O_3 along with its precursors is transported over the Pacific Ocean and reaches North America. Certain recent modelling studies suggest that global O_3 concentrations will increase during the early part of the twenty-first century as a result of increasing precursor emissions especially in Northern mid latitudes, with western North America being particularly sensitive to rising Asian emissions (Cooper et al. 2014).

In view of the increasing O_3 concentrations and their adverse effects on plant metabolism and productivity, several efforts are being taken to assess the O_3 injury in plants and to develop techniques to protect the plants against high ambient O_3 in the coming years. The most convenient method is the use of open-top chambers (Heagle 1989). O_3 can be introduced in these chambers at regulated concentrations. As such, these chambers are quite useful for conducting dose-response studies. However, high cost of construction and maintenance of these chambers and a continuous supply of electricity restrict its use to developed countries. Moreover, the controlled environmental conditions maintained inside the chambers, at times, overestimate the effects of O_3 due to chamber effect (Manning and Krupa 1992; Colls 2002). Therefore, the comparison of ambient conditions to that of the controlled environmental conditions inside the OTCs requires considerable rationalization. To avoid the chamber effect, different chemicals, collectively termed as ozone protectants, have been used from time to time for assessing O_3 injury in plants (Xin et al. 2016; Yuan et al. 2015; Agathokleous et al. 2014; Saitanis et al. 2015; Tiwari and Agrawal 2010).

O_3 protectants can be grouped as pesticides including fungicides, insecticides and herbicides, plant growth regulators, mechanical barriers and antioxidants such as ethylenediurea (EDU). Several studies suggest that the application of these chemical protectants against O_3 might be a reliable means to assess O_3 effects on crops under field conditions (Agathokleous et al. 2016b; Chaudhary and Agrawal. 2014; Manning and Krupa 1992, Manning 2000). Freebairn and Taylor (1960) were the first to use metabolic effectors like vitamin C to protect the plants from O_3 injury. Since then, a large number of chemicals are being used singly or in combination to assess their effectiveness in protecting the plants against O_3 injury (Table 1). While several chemicals that were used conveyed protection to the plants to different degrees against O_3 injury, many were ineffective and had unacceptable side effects, thus leaving them less useful in the field conditions. For example, though fungicide benomyl was found to be effective in controlling O_3 injury in a number of plants (Table 1), however, it is difficult to separate its fungicidal properties from its antiozonant properties in field conditions. Ascorbic acid and its salts have been used with success in reducing O_3 injury in a number of plants (Freebairn and Taylor 1960; Lee et al. 1990). Ozoban, an isomer of ascorbic acid, is marketed by Pfizer Chemical Company as an antioxidant spray to reduce yield losses due to O_3. However, field results with Ozoban on grapes in Riverside, CA, yielded mixed results with no consistent results on fruit yield (Flagler et al. 1994). Kuehler and Flagler (1999) showed that Ozoban reduced photosynthetic rates in loblolly pine in low O_3 environment but appeared to be harmful to chloroplast in plants exposed to elevated O_3 environment. Lee et al. (1990) tested the efficacy of a number of antioxidants in protecting bean leaves from O_3 injury and found that EDU was most effective in rendering protection from O_3 injury. The present review emphasizes upon the role of EDU as an effective research tool in evaluating O_3 injury in plants. Based on the available information, we have also tried to explore the mechanism of EDU action on plants. In addition to this, the present review also summarizes the different chemicals that have been used from time to time to protect the plants from O_3 injury.

Ethylenediurea

Ethylenediurea (EDU), chemical name *N*-[2-(2-oxo-1-imidazolidinyl)ethyl]-*N*′-phenylurea, is an antiozonant that protects plant tissues from oxidative injury and early senescence caused by O_3. It was developed by DuPont Chemical Company in 1970 and was first used by Carnahan et al. (1978) to protect snap bean plants (cv. Pinto 111) from O_3 injury. Since then, EDU has been extensively used as an

Table 1 Different chemicals used as plant protectants against ozone injury

S. no.	Chemicals	Type of protectants	Plant species	Reference
1.	Maneb, zineb (ethylene bisdithiocarbamates)	Fungicides	Bean, tobacco	Tomlinson and Rich (1973b); Kitano et al. (1975); Lee et al. (1990)
2.	Benomyl (methyl-1-butyl-carbamyl-2-benzimidazole-carbamate)	Fungicide	Bean, grapevine, tobacco, poinsettia, clover	Reinert and Spurr (1972); Kender et al. (1973); Manning et al. (1973a, b, c); Lee et al. (1990); Hassan et al. (2007)
3.	NC 2983 (5,6-dichloro-2-trifluoromethyl-benzimidazole)	Fungicide	Bean	Seem et al. (1972)
4.	Carboxin (5,6-dichloro-2-methyl-1,4-oxathiin-3-carboxanilide)	Fungicide	Azalea, bean	Moyer et al. (1974b); Hofstra et al. (1978)
5.	Chlorothalonil	Fungicide	Potato	Hassan 2006
6.	Topaz	Fungicide	Clover	Blum and Didyk (2007)
7.	Calixin (*N*-tridecyl-2,6-dimethyl)	Fungicide	Bean	Seem et al. (1972)
8.	Thiophanatemethyl analogue (1,2-bis (3-ethyl and its ethoxycarbonyl) benzene and 1,2-bis(3-methoxycarbonyl-2-thioureido)	Fungicide	Bean, marigold	Seem et al. (1973); Klingaman and Link (1975)
9.	Dodine (dodecylguanadine acetate)	Fungicide	Tobacco	Reinert and Spurr (1972)
10.	Triadimefon (1-(4-chlorophenoxy)3,3 dimethyl-1-([H-], 2,4-triazol-1-yl)-2-butanone)	Fungicide	Bean	Fletcher and Hofstra (1985)
11.	Diphenamid (*N*,*N*-dimethyl-2,2-diphenyl acetamide)	Herbicide	Tobacco	Sung and Moore (1979)
12.	Isopropalin (2,6-dinitro-*N*,*N*-dipropyl cumidine)	Herbicide	Tobacco	Sung and Moore (1979)
13.	Pebulate (S-propyl butylethylthiocarbamate)	Herbicide	Tobacco	Sung and Moore (1979)
14.	Atrazine (2-chloro-4 ethylamino-6-isopropyl-amino-s-triazine)	Herbicide	Bean	Seem et al. (1972, 1973)
15.	Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate)	Insecticide	Bean	Seem et al. (1972, 1973)
16.	Gibberellic acid (GA)	Hormone (growth regulator)	Radish, bean	Adedipe and Ormrod (1972); Seem et al. (1973)
17.	Indole-3 acetic acid (IAA)	Hormone (growth regulator)	Radish, bean	Adedipe and Ormrod (1972); Seem et al. (1973)
18.	Cytokinins (*N*-6-benzyladine)	Hormone (antisenescence agent, growth regulator)	Radish, bean, marigold	Adedipe and Ormrod (1972); Tomlinson and Rich (1973a)
19.	Abscisic acid (ABA)	Hormone (growth regulator)	Radish, bean	Fletcher et al. (1972)
20.	Polyamines	Antisenescence agents	Tomato, tobacco	Ormrod and Beckerson (1986); Bors et al. (1989)
21.	Peroxidase	Enzyme	Bean, tobacco	Larkin (1973)
22.	Folicote (paraffinic hydrocarbon waxes)	Antitranspirant	Bean, marigold	Knapp and Fieldhouse (1970); Klingaman and link (1975)
22.	Wilt purf	Antitranspirant	Apple	Elfving et al. (1976)
23.	Charcoal, diatomaceous earth, clay	Dusts	Tobacco, bean	Bertinuson et al. (1961)
24.	Ascorbic acid	Antioxidant	Bean, wheat	Dass and Weaver (1968) Lee et al. (1990); Agrawal et al. (2004)
25.	Sodium erythorbate	Antioxidant	Pine	Manning et al. (2003a, b); Flagler et al. (1994)
26.	Diphenylamine (DPA)	Antioxidant	Potato	Carrasco-Rodriguez et al. (2005)
27.	Metal-quinolinol (manganous 1,2-napthoqinone-2-oxime)	Antioxidant	Tomato	Rich and Taylor (1960)
28.	Nickle-*N*-dibutyldithiocarbomate (NBC)	Antioxidant	Bean	Dass and Weaver (1968)
29.	Phenylurea	Antioxidant	Bean	Tomlinson and Rich (1974)
30.	Glutathione	Antioxidant	Soybean	Lee et al. (1990)
31.	Butylhydroxyltoluene (BHT)	Antioxidant	Soybean	Lee et al. (1990)
32.	Tertiary butylhydroquinone (TBHQ)	Antioxidant	Soybean	Lee et al. (1990)
33.	6-Benzylaminoputine (6-BAP)	Antioxidant	Soybean	Lee et al. (1990)

antiozonant with successful results in a majority of cases (Paoletti et al. 2008; Szantoi et al. 2009; Manning et al. 2003a, b; Bortier et al. 2001; Kostka-Rick and Manning 1992). EDU has been used in the last few years in experiments conducted to assess the effectiveness of this chemical in tropical conditions (Varshney and Rout 1998; Tiwari et al. 2005; Tiwari and Agrawal 2009, 2010; Singh and Agrawal 2009, 2011a, b). The important characteristics of EDU as an effective antioxidant in protecting plants against O_3 injury are as follows:

1. EDU is specific for suppression of O_3 injury only and is not effective against PAN or SO_2 injury (Agathokleous et al. 2015; Cathey and Heggestad 1982; Lee et al. 1992).
2. The role of nitrogen (N) component of EDU is still debated. Earlier studies indicated that N in EDU does not have any fertilization effect; however, a recent study suggested that EDU at higher concentration increased the foliar N content of the plants (Agathokleous et al. 2016c)
3. It is systemic in nature, and repeated applications at regular intervals are required to ensure plant protection from O_3 injury (Manning et al. 2011; Gatta et al. 1997).
4. The degree of protection inferred by EDU depends on the inherent sensitivity of the crop, EDU dose and the amount of injury acceptable (Agathokleous et al. 2016a; Feng et al. 2010a, b; Tiwari et al. 2005).
5. EDU treatment is known to be cultivar specific (Pandey et al. 2014a, b; Singh et al. 2009; Singh and Agrawal 2011a).

EDU is a non-commercial compound whose detailed synthesis has never been reported. EDU consists of urea (U) and phenylurea (PU) components (total nitrogen 21.89%). U constitutes 23.46% and PU 53.18% of the molecular weight of EDU. It is a nitrogen-containing compound, with 10.5% nitrogen coming from urea and 10.94% coming from phenylurea components. The role of nitrogen in EDU is dubious, and there have been speculations if N has a fertilization effect on the plant performance (Manning et al. 2011). Godzik and Manning (1998) studied the effect of EDU (300 mg L^{-1}) and the two components of EDU (U and PU), separately on the two cultivars of *Nicotiana tabacum*, Bel W3 (O_3 sensitive) and Bel-B (O_3 resistant), before exposing them to O_3. It was observed that the urea (70 mg L^{-1})-treated plants experienced greater foliar injury than phenylurea (159 mg L^{-1})-treated ones. Other experiments have also shown that EDU did not significantly change the N content of ash leaves (Paoletti et al. 2007b) or crop plant (Feng et al. 2010a, b) exposed to ambient O_3. Manning et al. (2011), however, reported that foliar application of 300 mg L^{-1} EDU to O_3-sensitive snap bean (*Phaseolus vulgaris* L. var. 156) resulted in a slight increase in N and ^{15}N contents, a day after the application followed by a decline. Kuehler and Flagler (1999) also reported an increase in foliar N content of *Pinus taeda* L, treated with biweekly spray applications of 300 mg L^{-1} EDU, during later part of the study. In a recent study, Agathokleous et al. (2016c) have reported a significant increase in the foliar N content of the willow tree (*Salix sachalinensis* Fr. Schm) grown in a nutrient-poor soil and treated with very high (800 and 1600 mg L^{-1}) doses of EDU at 9-day interval. The accumulation of N in the leaves of EDU-treated plants led to a higher leaf dry mass and lower specific leaf area (SLA), a characteristic effect of N (Agathokleous et al. 2016c). These results point towards the need of further investigations to confirm whether EDU acts as a source of N to the treated plants.

The protective nature of EDU persists for a relatively short period after application, and to ensure full protection, EDU has to be applied repeatedly at regular intervals (Gatta et al. 1997). An analysis of EDU concentration in protoplast and intercellular washing fluid showed that EDU did not enter the cells but was retained in the apoplast (Pasqualini et al. 2016). Similar results were also recorded by Regner-Joosten et al. (1994) and Gatta et al. (1997) who studied uptake, partitioning and persistence of EDU in plants and observed the translocation of EDU to leaf apoplast where it persists for 8–21 days depending upon the experimental conditions (Pasqualini et al. 2016) and then degrades slowly (Gatta et al. 1997). Pasqualini et al. (2016) studied the variation in the concentration of EDU accumulated in the apoplastic space of leaves of O_3-sensitive Bel W3 cultivar of tobacco treated with EDU either as foliar spray or as soil drench. The overall result of the experiment suggested that both foliar spray and the soil drench application have the potential to accumulate EDU in the leaves over a period of 21 days (Pasqualini et al. 2016). In case of soil drench, concentration of EDU in the foliar apoplast remains constant up to 7 days after treatment and then increased significantly between 10 and 18 days with respect to day 1. This behaviour of EDU accumulation can be attributed to the adsorption of EDU by soil organic matter particles (Manning et al. 2011; Agathokleous et al. 2015) and gradual resolubilization by irrigation water (Pasqualini et al. 2016). However, retention time of EDU is just 7 days when soil is lacking in organic matter content (Agathokleous et al. 2016c). In case of foliar spray, higher amount of EDU in leaf apoplast at day 21 (as compared to day 1) can be explained by the dry deposition of EDU on the foliar surface followed by its rewetting due to high humidity which promoted the gradual uptake of EDU (Pasqualini et al. 2016). However, if the foliar surface is washed after 1 h of EDU application, EDU concentration decreases by day 3 of EDU application (Pasqualini et al. 2016). Further, no mobilization of the chemical from old to new leaves has been detected; therefore, repeated EDU applications become necessary to protect the newly emerging leaves (Paoletti et al. 2009; Weidensaul 1980).

Feng et al. (2010a, b) reported that EDU applied at a concentration of 200–400 mg L^{-1} has the highest positive effect on the crops grown in field. However, cases are reported where EDU concentration as low as 150 mg L^{-1} was found to be effective (Tiwari and Agrawal 2010). Agathokleous et al. (2016a), for the first time, evaluated the phytotoxicity potential of EDU by using a series of EDU treatments on *Lemna minor* L. The study depicted a hermetic effect of EDU, thus stimulating plant performance at lower EDU concentration, while inhibiting it at higher concentration. EDU concentration of 296 mg L^{-1} (~300 mg L^{-1}) was established a no-observed-toxic-effect concentration (NOEL), which falls in the EDU concentration range approved by Feng et al. (2010a, b) for protection of plants against O_3 injury. The higher EDU concentration greater than 593 mg L^{-1} (~600 mg L^{-1}) leads to an inhibitory effect on the growth and development of *L. minor* L. Agathokleous et al. (2016d) reported that higher concentration of EDU (>593 mg L^{-1}) resulted in approximately 10% higher fresh mass/dry mass (FM/DM) ratio, which indicated the plant's preference for rapid production of biomass rather than an efficient conservation of nutrients. However, *S. sachalinensis* L. Schm did not show any inhibitory effect of EDU concentration as high as 1600 mg L^{-1}; these higher EDU doses are though not recommended under uncontrolled conditions (Agathokleous et al. 2016c).

The mode of action of EDU is still remains disputed and unconfirmed. Unlike other antioxidants, EDU does not contain olefinic double bond; therefore, it is dubious to have any role in direct scavenging of O_3. O_3 injury depends upon the balance of O_3 uptake through stomatal flux and O_3 detoxification inside leaf mesophyll (Matyssek et al. 2007). O_3 is well known to induce a biphasic ROS burst in leaves, which is kept in control by several enzymatic and non-enzymatic antioxidant systems (Castagna and Ranieri 2009). It has been suggested that the maintenance of the activities of antioxidant enzymes, especially ascorbate peroxidase and high levels of ascorbic acid, was mainly responsible for the protective effect of EDU (Feng et al. 2010a, b). Higher ascorbate peroxidase activity indicates a better ROS scavenging capacity of the EDU-treated plants (Paoletti et al. 2008). Several workers have reported that the protective effects of EDU on plants were correlated with the increments in total ascorbic acid contents of their foliage (Pandey et al. 2014a, b; Brunschon-Harti et al. 1995b; Tiwari and Agrawal 2009, 2010). Recent studies further indicate that O_3-induced effects in plants may not be caused by O_3 itself, but by its secondary toxicants formed due to spontaneous aqueous decomposition of O_3 in the apoplast (Halliwell and Gutteridge 2007). However, much research needs to be done to quantify the toxic by-products potentially scavenged by EDU.

The role of EDU as a protective agent against O_3 injury certainly involves some biochemical aspects of the plant metabolism with antioxidants playing an important role. However, the effect of EDU on biophysical aspects is still debated. Increments in stomatal conductance of EDU-treated plants were reported in O_3-sensitive cultivars of tropical wheat (Singh et al. 2009). However, Agrawal and Agrawal (1999) showed that *P. vulgaris* treated with EDU prior to O_3 exposure exhibited greater number of closed stomata as observed by low-temperature scanning microscopy. In another study, increased ascorbate peroxidase activity with decreased stomatal conductance was recorded in ash trees treated with 450-ppm EDU treatment, which suggested that both biochemical and biophysical processes may be modified under EDU treatment (Manning et al. 2011; Paoletti et al. 2008). Contrary to these results, a recent meta-analytical study of EDU on one crop and two tree species has suggested that the direct effect of EDU on stomata was negligible (Feng et al. 2010a, b). This result is consistent with no EDU effect on photosynthesis, as reported in a few studies (Ainsworth et al. 1996; Kuehler and Flagler 1999; Paoletti et al. 2008), as stomatal conductance and photosynthesis are usually coupled. There are, however, pieces of evidence which suggest an increased photosynthetic rate in plants upon EDU application (Feng et al. 2015). According to Manning et al. (2011), the ameliorative effect of EDU on stomatal gas exchange is indirect and is due to the beneficial effect of EDU on O_3-induced ROS generation. Modifications in chlorophyll fluorescence parameters in plants upon EDU treatment are also reported, which indicate that photosystem II (PS II) also plays a significant role in deciding the mode of action of EDU (Feng et al. 2015; Yuan et al. 2015; Agathokleous et al. 2016a).

Recent studies have focused upon the mechanism of EDU action through up/downregulation of genes responsible for transcription of major antioxidants in plants. Paoletti et al. (2014) have shown that EDU may halt the O_3-induced ROS generation within 24 h of exposure and induces a downstream cascade mechanism leading to variation in gene expression of different antioxidant enzymes. It was shown that EDU reduced the accumulation of H_2O_2 in O_3-exposed leaver of *P. vulgaris* cv. S156 so that its level was similar to non-fumigated leaves (Paoletti et al. 2014). This observation clearly indicates that EDU may indirectly influence the Halliway-Asada pathway wherein apoplastic ascorbate peroxidase and symplastic glutathione reductase coordinate together to decompose H_2O_2 formed under O_3 stress (Fig. 1).

Mode of application of EDU in plants

EDU is applied to the plants in different manners. The important and frequently used techniques of EDU application are as follows:

1. Soil drench: This is a more common technique of EDU application in plants. The required concentration of EDU

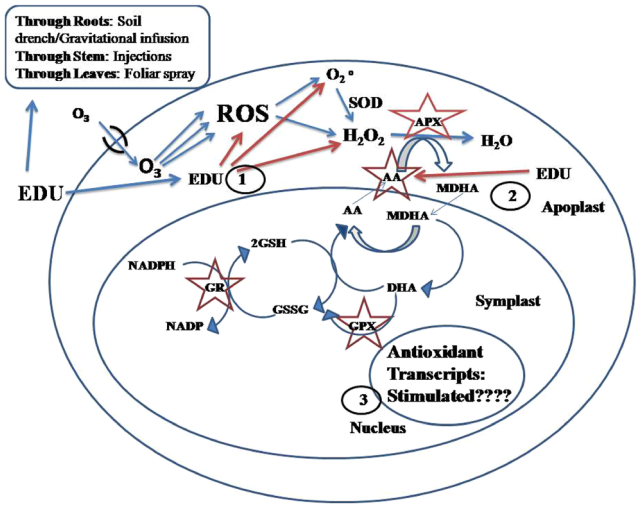

Fig. 1 Possible mechanism through which EDU protects the plants: *1* Direct scavenging of O_3-generated ROS. *2* Maintenance of activities and levels of enzymatic and non-enzymatic antioxidants scavenging the ROS. *3* Effect on transcription of genes encoding the components of antioxidant defence system of plants still needs more experimentation. *O_2* superoxide anion, *H_2O_2* hydrogen peroxide, *AA* ascorbic acid, *APX* ascorbate peroxidase, *MDHA* monohydroascorbate, *DHA* dihydroascorbate, *GSH* reduced glutathione, *GSSH* oxidized glutathione, *GR* glutathione reductase, *GPX* glutathione peroxidise, *NAD(P)* nicotinamide adenine dinucleotide phosphate, *NAD(P)H* nicotinamide adenine dinucleotide phosphate reduced

solution is prepared in warm water and is carefully applied to the soil around the roots of the plants avoiding the foliage to be wet. In this technique, EDU is taken up by the roots and is then carried upwards and accumulates in the substomatal cavity. This technique has been followed up by workers like Agathokleous et al. (2016c), Tiwari et al. (2005; Tiwari and Agrawal 2009, 2010), Singh and Agrawal (2009) and Singh and Agrawal (2011a, b). While Musselman et al. (1978) found this technique to be ineffective, Cathey and Heggestad (1982) found drenches to be effective. Clarke et al. (1978) found that EDU application as soil drench was highly effective in preventing foliar injury.

2. Foliar spray: EDU solution in this case is hand sprayed on complete plant foliage until the plant becomes visibly saturated. In this technique, EDU directly enters the substomatal spaces of plants via stomata. This mode of EDU application is used in large-scale field studies, where it is not feasible to apply EDU as soil drenches as it would require large volumes of solution. Foliar spray has been used by several workers with significant positive results (Paoletti et al. 2011; Szantoi et al. 2007, 2009; Wang et al. 2007; Hassan 2006; Elagoz and Manning 2005; Manning et al. 2003a, b; Brunschon-Harti et al. 1995a, 1995b).
3. Gravitational infusion: EDU has a great potential in the assessment of effects of O_3 on trees (Manning 2005); the application to adult trees is difficult. Foliar spray and soil drench would require a prohibitive amount of EDU to treat large trees. Therefore, some other more convenient techniques were developed for treatment of EDU in trees. Ainsworth et al. (1996) tested EDU low-pressure injections into stem of small trees (*Populus eumericana*). Bortier et al. (2001) in their experiment with *Populus nigra* applied EDU by injection technique described by Gregory et al. (1971). The solution was pipetted into serum vial caps fixed to the base of the stem and taken up by the plants by wounding the stem with a needle (Bortier et al. 2001). This technique was found to be effective in introducing EDU in woody tissues and protecting adult *Fraxinus excelsior* against visible O_3 injury (Paoletti et al. 2007a). In order to get an even distribution of EDU inside the crown, two 2-cm-long holes on opposite sides were made at breast height by using a drill equipped with a 5-mm point. A 1-mm pipette tip was inserted into the holes and connected to a commercially available infusion bag containing the EDU solution required for each tree. The bags were hung on the trees at least 1 m above the hole. To prevent the overflow of the solution outside the holes, flow was controlled by a Hoffman clamp. After a week, the bags were removed and the holes were sterilized with 10 g L^{-1} Bordeux mixture and closed with Lac Balsam. The holes for the next infusion were drilled 3–4 cm above the previous ones (Paoletti et al. 2007a).

Foliar spray and soil drench are two main modes of EDU application (Paoletti et al. 2009; Agathokleous et al. 2015). However, both the techniques have a few drawbacks. In the soil drench technique, oil influences the effectiveness of EDU (Manning et al. 2011; Pasqualini et al. 2016), whereas foliar spray seems to be technically difficult in case of large trees (Paoletti et al. 2009). Agathokleous et al. (2016d) assessed the effectiveness of the two application modes of EDU by studying the difference in response of *S. sachalinensis* L. Scmh to EDU applied as foliar spray and soil drench. The EDU concentration used was 200–400 mg L^{-1}, the usually applied range for protection against O_3 phytotoxicity (Feng et al. 2010a, b). The EDU provided as soil drench protected the plants against O_3-induced senescence but did not protect the other growth variables of the plant. However, when applied as foliar spray, the same amount of EDU was able to provide partial protection to the plant growth variables in addition to delaying O_3-induced senescence (Agathokleous et al. 2016d). This experiment clearly showed that the amount of EDU used in each application was more when given as soil drench (250 mL) while less when applied as foliar spray (117 mL during initial applications and 88 mL during later ones). Thus, EDU needed for foliar spray was 2.3 times lower than that needed for soil drench at the final EDU application, indicating that foliar spray was a more

economical and more effective method of EDU application (Agathokleous et al. 2016d). Application of EDU as foliar spray is more suitable for small-sized plants with lesser leaf area as it is not only economical but also minimizes the influence of soil, which may cause some errors in the results. However, in the adult tree with large leaf area, soil drench is a more preferred mode of EDU application (Paoletti et al. 2011).

Effect of EDU on plants

The effects of O_3 on different features of plant metabolism are well cited in literature (Oksanen et al. 2013). EDU applied to the plants growing under ozone stress affects the physiological and biochemical characteristics, growth and biomass allocation pattern and yield. Figure 2 shows how EDU affects the O_3-induced variations in plant metabolism. The effect of EDU upon the O_3-induced variations helps us to predict the mechanism of protective action of EDU upon O_3 injury.

Effect of EDU on biochemical processes

Effect on membrane permeability

O_3 is known to affect the membrane permeability through peroxidation of lipids present in the membranes (Calatayud et al. 2003). After penetrating the stomata, O_3 is known to react with membrane and cellular water forming oxyradicals (Apel and Hirt 2004). Any double bonds in hydrocarbons are likely to be highly sensitive to chain breaking and cross-linking reactions initiated by O_3 (Wellburn 1988). The hydrated form of O_3 may form hydrogen peroxide and aldehydes by ozonolysis, resulting in the damage of membrane lipids partly via lipid peroxidation (Heath 1975). Several studies have shown that EDU treatment reduces the level of lipid peroxidation in plants as compared to the non-EDU-treated ones (Tiwari and Agrawal 2009; Singh et al. 2010b). EDU protects plant tissues against lipid peroxidation by increasing the levels of enzymes involved in the elimination of O_3-generated ROS and free radicals (Upadhyaya et al. 1985).

A detailed study was carried out by Whitaker et al. (1990) to study the behaviour of different membrane components of snap bean plants upon O_3 exposure and EDU treatment. In this experiment, one set of snap bean plants were treated with EDU without any O_3 fumigation. Another set of snap bean plants were fumigated with 40 ppb O_3 for 3 h prior to EDU treatment. In both the cases, the non-EDU-treated plants were taken as control. This study gave some very interesting results. Immediately after O_3 exposure, leaves of untreated control plants lost both galactolipids (GL) and phospholipids (PL), whereas no significant loss occurred in the leaves of EDU-treated plants (Whitaker et al. 1990) (Fig. 3). These results also showed that the protective effects of EDU were more prominent in GL as compared to PL (Whitaker et al. 1990). This can be attributed to the fact that peroxidation of unsaturated fatty acids does not play a major role in O_3-induced degradation of membrane PL (Heath 1988). Similar observation was also reported in a recent meta-analytical review of EDU effects on crop and tree species (Feng et al. 2010a, b). Rotham and Lenard (1977) suggested that O_3-induced peroxidation of PL may have been selective; i.e. specific classes or molecular species were peroxidized more rapidly than others.

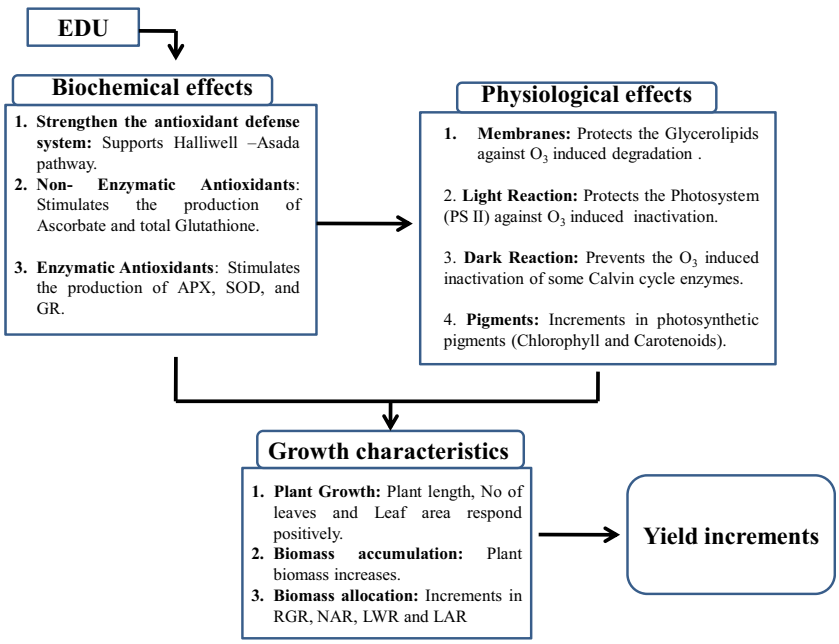

Fig. 2 Effect of EDU on physiological, biochemical and morphological characteristics and yield of plants

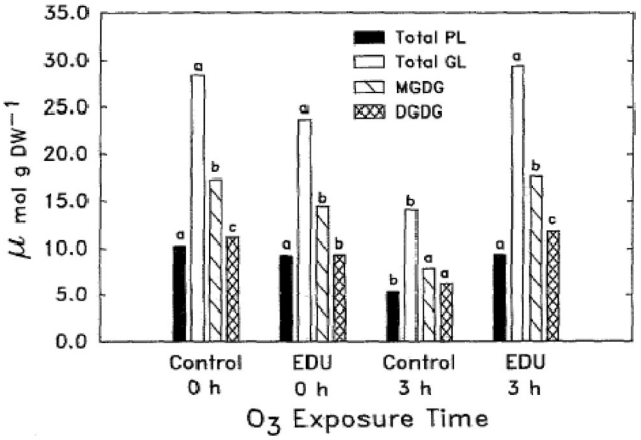

Fig. 3 Effect of EDU treatment and ozone exposure on the levels of glycerolipids in the first fully expanded trifoliate leaf of snap bean plants. Values of total PL, total GL, MGDG and DGDG are expressed in μmol $(g\ DW)^{-1}$ of leaf tissue (Source: Whitaker et al. (1990)

The ratio of MGDG to DGDG also declined in the leaves of O_3-fumigated control plants indicating more rapid loss of MGDG.

In the same experiment, Whitaker et al. (1990) showed that the total membrane sterol (TMS) did not show any significant changes upon O_3 or EDU treatment. However, O_3 fumigation induced changes in the levels of individual steryl lipids such as steryl glycosides (SG), acylated steryl glycosides (ASG) and free sterol (FS) in both EDU-treated and non-EDU-treated plants (Table 2).

These values suggest that large increase in ASG and SG in O_3 was offset by a large decline in FS during O_3 exposure. However, EDU treatment was able to significantly restore the level of FS that declined during O_3 exposure (Whitaker et al. 1990). A 3-h exposure of 25 ppb of O_3 on pinto bean did not cause any change in the total steryl lipids but caused a decline in FS with a concomitant increase in ASG and SG (Tomlinson and Rich 1971). It was suggested that O_3-induced acylation of SG may occur at the expense of leaf glycerolipids and that O_3-induced increase in electrolyte leakage may result from the altered steryl lipid composition (Tomlinson and Rich 1971).

Table 2 Effect of EDU pre-treatment on the steryl lipid sterol composition of snap bean leaves

Sterol moiety	No O_3 exposure					
	Untreated control			EDU treated		
	FS	ASG	SG	FS	ASG	SG
Cholesterol	0.7	1.0	0.5	0.7	0.8	0.9
Campesterol	6.7	4.8	5.1	6.6	4.7	4.9
Stigmasterol	32.4	39.0	35.0	33.4	37.8	33.7
Sitosterol	51.2	52.8	54.4	50.4	53.7	56.2
Others	9.1	2.4	5.0	8.9	3.0	4.3

Values are expressed as weight percent of each sterol in steryl lipid and represent the mean (Source: Whitaker et al. (1990)

One explanation for the dramatic increase in ASG and SG at the expense of FS during O_3 exposure is that the degradation of glycerolipids results in activation of enzymes involved in sterol glycosylation and acylation. Nouchi and Toyama (1988) observed that O_3 exposure affected the lipid metabolic processes in morning glory during the initial exposure stages. Metabolic decomposition of glycolipids, increased amounts of triacylglycerol (TG) being synthesized from acyl moieties of monogalactosylglycerol (MGDG) and increased amounts of free fatty acid, produced by decomposition of MGDG, were observed in O_3-exposed plants (Sakaki et al. 1990a, b, c, 2008). Francini et al. (2007) studied the metabolic response of two clover clones (NC-S and NC-R) at 200 ppb O_3 for 5 h in the form of a square wave and observed that NC-S exhibited a significant increase in membrane permeability, whereas no alteration was observed in NC-R. Barth and Conklin (2003) reported that the recessive O_3-sensitive *Arabidopsis* mutant *lcd-1* exhibited an increased amount of the lipid peroxidation product malondialdehyde (MDA) in leaves, compared to the wild type, when exposed to O_3.

Significant reductions in lipid peroxidation were recorded in palak (Tiwari and Agrawal 2009), carrot (Tiwari and Agrawal 2010) and mung bean (Singh et al. 2010b).

Effect on antioxidative system

Ozone dissolves in the intercellular spaces and gives rise to a series of potentially damaging ROS, triggering an antioxidant response (Baier et al. 2005; Junqua et al. 2000). These oxidative processes also produce H_2O_2 in the humid environment of the leaf interior. H_2O_2 is one of the most important O_3-derived oxidants and may lead to the formation of other ROS such as superoxide ($\cdot O_2$) and hydroxyl ($\cdot OH$) radicals (Langebartels et al. 2002). The ROS oxidize cellular constituents such as lipids, proteins, sulfhydryl groups and nucleic acids and can initiate radical chain reactions (Kangasjarvi et al. 1994). Several antioxidants like ascorbic acid and glutathione and antioxidative enzymes such as ascorbate peroxidase (APX), superoxide dismutase (SOD) and catalase (CAT) protect the plants from the injurious oxidative effects of O_3. Studies have shown that the beneficial effects of EDU in plants grown under O_3 stress can be attributed to the fact that EDU brings about changes in the level of apoplastic antioxidants in plants so as to increase the efficiency ROS scavenging enzymes (Brunschon-Harti et al. 1995b; Tiwari and Agrawal 2009; Pandey et al. 2014a, b). EDU induces increased activities of ascorbate-glutathione cycle enzymes together with other enzymes (SOD and CAT), which helped in the detoxification of ROS in EDU treatments in two cultivars (Kranti and Peela sona) of mustard (*Brassica rapa* syn. *B. campestris*) (Pandey et al. 2014a, b). The same study has shown that the response of antioxidant pool towards EDU varied with cultivars and developmental stages. In sensitive variety (Peela sona), the most

active enzymatic scavenging occurred at the vegetative stage, whereas the resistant variety (Kranti) maintained active enzymatic scavenging throughout the study (Pandey et al. 2014a, b).

Ascorbate-dependent peroxidase (APX) is an important oxidative enzyme oxidizing ascorbate (ASA) to dehydroascorbate (dehydroascorbic acid (DHA)) while reducing H_2O_2 to water (Asada 1992). The ratio of both these forms, ASA and DHA, can be used as an indicator of oxidative stress in plants. *P. vulgaris* L. cv. Lit. showed increments in total ascorbic acid pool and the amount of ascorbic acid in its reduced form upon 150-ppm EDU treatment (Brunschon-Harti et al. 1995b). This result suggests that EDU-treated plants were more resistant against O_3 damage as compared to non-EDU treated plants. It was proposed that EDU might induce the synthesis of ascorbic acid which itself is a very potent antioxidant. Further, a decreased ratio of ASA to DHA in non-EDU-treated plants suggests a poor efficiency of defence mechanism due to severe oxidant disruption, therefore indicating a more senescent state of non-EDU-treated plants as compared to EDU-treated plants (Brunschon-Harti et al. 1995b). Paoletti et al. (2008) compared O_3-sensitive symptomatic and O_3-tolerant asymptomatic trees (*F. excelsior*) upon treatment with 450 ppm EDU and observed that total ASA increased only in tolerant plants. Paoletti et al. (2008) measured ascorbate (ASA) and its oxidized form, dehydroascorbic acid (DHA), in both leaf apoplast and symplast and reported that EDU improved both symplastic ASA and DHA in O_3-tolerant plants; however, the increase was not significant. The apoplastic DHA was reduced upon EDU treatment. However, these have any significant effect upon the redox ratio ASA/total ASA. The ratio of apoplastic DHA to symplastic ASA was significantly lower in O_3-tolerant plants, suggesting that the efficiency of DHA translocation into cytosol is a mechanism to increase tolerance and is stimulated by EDU. The apoplastic ASA system is kept in reduced state by an efficient exchange of apoplastic DHA for symplastic ASA, as DHA cannot be reduced in apoplast (Mittler et al. 2004). Significant increments in the total ascorbate contents upon EDU treatment were also reported in wheat (Singh et al. 2009), mung bean (Singh et al. 2010b), palak (Tiwari and Agrawal 2009) and carrot (Tiwari and Agrawal 2010).

SOD in the leaves is the primary scavenger of superoxide radicals generated both as a by-product of normal physiological activities and exposure to stress conditions such as O_3 pollution. Lee and Bennett (1982) published experimental results indicating that EDU-induced O_3 tolerance in snap beans is correlated with significant increases in SOD and CAT activities in leaf tissues. It was also observed that young leaves which were more O_3 tolerant had a higher SOD activity than older leaves which were less O_3 tolerant (Lee and Bennett 1982). However, no significant positive correlation was observed between O_3 tolerance and SOD activity in bean cultivars with differing O_3 tolerance (Mckersie et al. 1982). Pitcher et al. (1992) reported lower SOD activity in the leaf extracts of *P. vulgaris* L. cv. Bush Blue Lake 290 at 300-ppm EDU treatment at 0.3-ppm O_3 exposure for 6 h under greenhouse conditions. Beside SOD, certain other enzymes such as CAT and peroxidase (POD) responsible for scavenging O_3-generated ROS are also affected by EDU treatment (Brunschon-Harti et al. 1995b).

Brunschon-Harti et al. (1995b) exposed *P. vulgaris* L. var. Lit to different O_3 concentrations and 100-ppm EDU treatment. It was observed that POD activity decreased significantly only in EDU-treated plants exposed to high O_3 concentration (twice ambient O_3) as compared to non-EDU-treated plants exposed to same O_3 dose. However, no significant variation was recorded in EDU-treated plants exposed to lower O_3 concentration. Contrary to this observation, CAT activity increased significantly in EDU-treated plants exposed to low O_3 concentration as compared to non-EDU-treated ones (Brunschon-Harti et al. 1995b).

Significant reduction in POD and SOD activities was recorded in *Vigna radiata* treated with 400 ppm EDU as compared to non-EDU-treated control plants under mean monthly ambient O_3 concentrations varying between 52.9 and 64.5 ppb (Singh et al. 2010b). Reduction in POD activity of EDU-treated plants was also recorded in several other studies (Tiwari and Agrawal 2009, 2010). POD catalyzes the reduction of H_2O_2 and is recognized as one of the most efficient ROS scavenging systems (Foyer et al. 1994). Reduction in POD activity upon EDU treatment indicates the protective nature of EDU against O_3 in scavenging ROS, thus preventing H_2O_2 formation.

In a recent study by Paoletti et al. (2014), it was found that in snap bean plants (*P. vulgaris* L. S156) exposed to O_3 with no pre-EDU treatment, there was a significant increase in activity and transcript of CAT, GPX, APX and GR within 24–96 h after the beginning of exposure. However, under O_3 + EDU combined treatment, no stimulation in the antioxidant activity and transcripts was observed (Paoletti et al. 2014). Based on this experiment, it was concluded that EDU was able to halt the O_3-induced ROS generation within 24 h from the exposure, which prevented the downstream cascade mechanisms stimulating the detoxification mechanisms of the cell. This result suggests that the EDU primary protection mechanism involves mitigation of O_3-induced ROS generation prior to effects on antioxidant enzymes (Paoletti et al. 2014). The discrepancies in the responses of the antioxidative machinery of different plants upon EDU treatment suggest that more research is required to understand the role of enzymatic and non-enzymatic defence mechanisms, especially the ascorbate-glutathione cycle operating in plants, in regulating EDU-induced responses.

Effect on physiological characteristics

O_3 exposure negatively affects one or the several of the electron transport processes occurring in the thylakoid membranes of the chloroplasts. The oxidative stress caused by the excessive oxidation energy via high photon capture by leaves could enhance the negative effects of O_3 on photosynthetic apparatus (Calatayud et al. 2002, 2003; Castagna et al. 2001). As discussed earlier, pre-treatment with EDU, however, conferred protection against O_3-induced losses of glycerolipids and chlorophyll (Whitaker et al. 1990; Feng et al. 2010a, b). Studies have shown that EDU helped in reducing the O_3-induced inactivation of reaction centres in PS II (Contran et al. 2009). Lowering of Fv suggests decrement in the light quenching capacity of chlorophyll molecules due to thylakoid damage (He et al. 1994). Fv/Fm ratio in the unstressed leaves ranges between 0.78 and 0.85. Under O_3 stress, this value of Fv/Fm ratio shows a significant decline. EDU treatment maintains the Fv/Fm ratio which depicts that efficiency of PS II to reduce the primary acceptor Q_A, which was negatively influenced by O_3, was resumed by EDU treatment (Tiwari and Agrawal 2009). Singh et al. (2009) observed a reduction in Fv/Fm ratio along with an increase in F_o of non-EDU-treated wheat cultivars, HUW 468 and HUW 510, suggesting certain alterations induced in the electron transport chain due to O_3 stress. However, reduction in Fv/Fm ratio of non-EDU-treated plants is not always accompanied by significant variation in F_o (Agathokleous et al. 2016c; Tiwari and Agrawal 2009). Non-significant variation in F_o upon EDU treatment suggests that O_3 did not induce modification at the antennae pigment level or in the efficiency of excitation trapping at the active centres of PS II (Tiwari and Agrawal 2009).

The positive influence of EDU treatment to light reactions is carried over to the dark reactions, wherein the EDU-treated plants showed increments in carbon fixation ability as depicted by the increased photosynthetic rates in the plants (Singh et al. 2009, 2010b). EDU prevented the inactivation of some enzymes of Calvin cycle, particularly stromal fructose 1,6-bisphosphate phosphatase, occurring immediately after an O_3 spike (Valenti et al. 1995). EDU is also known to provide a certain degree of protection to ribulose 1,5-bisphospate carboxylase oxygenase (Eckardt and Pell 1996). Higher photosynthetic pigments in most of the cases also support a positive action of EDU on photosynthesis (Agrawal et al. 2005; Tiwari and Agrawal 2009, 2010; Singh et al. 2010b; Rai et al. 2015). However, most of the works suggest a non-significant effect of EDU on photosynthesis (Kuehler and Flagler 1999; Paoletti et al. 2008, Feng et al. 2010a, b). It is a matter of interest for the researchers to investigate how EDU-treated plants grow better even without a significant effect on photosynthesis. It has been hypothesised that EDU effects on antioxidant pool contribute to less significant O_3 injury due to which smaller carbon is used for repairing O_3 injury and a major proportion can be utilized for detoxification and growth (Paoletti et al. 2009).

The effects of EDU on stomatal conductance have been studied in a few plants exposed to ambient O_3, and most of them reported no significant effect of EDU (Ainsworth et al. 1996; Kuehler and Flagler 1999; Hassan et al. 2007). A recent meta-analytical review of EDU effects on plants also suggests that direct EDU effect on stomata was negligible (Feng et al. 2010a, b). Yuan et al. (2015) worked on two sensitive and tolerant genotypes of snap bean and observed that though the sensitive genotypes showed increments, tolerant ones showed a reduction in stomatal conductance upon EDU treatment. Reduced stomatal conductance could have attributed to higher tolerance in the genotypes. Lower stomatal conductance is related to O_3 avoidance, and high antioxidant capacity may support the plant to cope up with oxidative stress induced by O_3 (Dizengremel et al. 2013). An EDU-induced decrease in stomatal conductance was reported in O_3-tolerant cultivars of tropical wheat (Singh et al. 2009) and in adult ash trees at the end of the growing season (Paoletti et al. 2008). This aspect of EDU action, however, requires more research due to the complicated nature of O_3 action on stomata, which may interfere with the possible EDU effects on stomatal conductance.

Effect on growth and morphological parameters

The protective nature of EDU that is clearly evident in the previous discussion positively affects the growth characteristics and biomass accumulation in plants. Several studies have shown that EDU improved various morphological parameters of plants growing at high O_3 concentrations (Singh and Agrawal 2009; Tiwari and Agrawal 2010; Wang et al. 2007; Tiwari et al. 2005; Agrawal et al. 2005). Agrawal et al. (2003) reported that 400 ppm EDU significantly increased the height of mung bean plants at a suburban field site experiencing 6-h mean O_3 concentration of 42 ppb. Significant increments in shoot length of mung bean plants were also reported by Agrawal et al. (2005) at 500-ppm EDU treatment given to the plants growing at mean O_3 concentration of 34 ppb and by Singh and Agrawal (2009) at 400-ppm EDU treatment in plants growing at 12-h O_3 concentration in the range 52.9–64.5 ppb. Tiwari et al. (2005) also reported significant increase in plant height at 300-ppm EDU concentration in wheat cv. M533. Increments in shoot length were also observed in *F. excelsior* L. due to application of 450-ppm EDU treatment given through the gravitational infusion technique, but this increase was not significant (Paoletti et al. 2008). Other growth characteristics were observed such as number of leaves, tillers and leaf area in the four wheat cultivars (cv. HUW 234, HUW 468, HUW 510 and PBW 343) as observed

by Singh and Agrawal (2009) at 400-ppm EDU treatment, with mean O_3 concentrations varying between 35.3 and 54.2 ppb. Under similar experimental conditions, *Triticum aestivum* cv. Sonalika did not show any significant response to EDU treatment as far as the morphological characteristics were concerned (Singh and Agrawal 2009). Agrawal et al. (2004) also observed significant increments in the number of leaves due to 500-ppm EDU treatment in three wheat cultivars. However, Tiwari et al. (2005) did not show any significant effect of EDU treatment on the leaf number of two wheat cultivars. Increments in leaf area were also observed in soybean due to EDU treatment (Wahid et al. 2001). Szantoi et al. (2009) reported that EDU treatment led to significant reduction in O_3-induced leaf injury in *Echinacea purpurea* (purple coneflower) plants growing at 12-h O_3 concentration of 73 ppb. The most positive recovery of leaf injury was found in plants treated with 600 ppm EDU as compared to non-EDU-treated plants (Szantoi et al. 2009).

O_3 concentration in the range of 50–100 ppb has been shown to influence carbon partitioning between root and shoots in plants (Cooley and Manning 1987). EDU is known to adjust the hazardous effects of O_3 on biomass accumulation in different plants (Tiwari et al. 2005; Agrawal et al. 2005). Brunschon-Harti et al. 1995a studied the response of *P. vulgaris* L. var. Lit. to 150-ppm EDU treatment under field conditions and found that EDU treatment was highly significant for leaf, root and shoot dry weight. In the same experiment, O_3 treatment caused a dose-dependent decrease of root biomass in both EDU-treated and non-treated plants. Significant O_3 × EDU interactions for root weight indicated that EDU reduced the root growth suppression caused by O_3 (Brunschon-Harti et al. 1995a). Hassan et al. (1995) also assessed the effectiveness of EDU in mitigation of toxic effects of O_3 by studying the response of local varieties of radish (*Raphnus sativus* L.) and turnip (*B. rapa* L.) at two sites of Northern Egypt. The site with high O_3 concentration (Abbis) showed more positive response of the two crops to 500-ppm EDU treatment in terms of their root and shoot biomass as compared to the plants growing at the site with low O_3 concentration (Alexandria) under similar EDU treatment (Hassan et al. 1995).

Szantoi et al. (2009) recorded some interesting results from their experiments in cutleaf coneflower grown in charcoal-filtered (CF), non-filtered (NF) and twice ambient O_3. All the plants were treated with different doses of EDU (0, 200, 400 or 600 ppm). Reduction in plant biomass upon O_3 exposure is well documented, and the results of this experiment also followed the same trend. Reductions of 34 and 47% were recorded in total biomass of the plants grown in twice ambient O_3 as compared to those grown in CF and NF chambers (Szantoi et al. 2009). However, a significant linear trend of decreasing total and root biomass was observed with increasing EDU levels. Significant reductions of 10.5, 32.9 and 22.8% in root biomass and 11.9, 29.59 and 21.9% in total biomass at 200-, 400- and 600-ppm EDU concentration were reported (Szantoi et al. 2009). However, no significant effect of EDU treatment was observed on foliage dry weight (Szantoi et al. 2009). A meta-analytical study showed that EDU application significantly increased total plant biomass and increments in root biomass (20%) were more than the shoot biomass (6.7%) (Feng et al. 2010a, b). This study further showed that the increase in above ground biomass was due to combined increase in the plant height (8%) and leaf biomass (19%) (Feng et al. 2010a, b).

Phil et al. (1995) found that the growth response of three clover species to ambient air upon EDU treatment in southwest Sweden depended upon the O_3 sensitivity of the species; the higher the sensitivity to O_3, the more the growth in EDU-treated plants was favoured over that in untreated plants.

The biomass allocation pattern of the plants also shows positive response of EDU treatment (Tiwari and Agrawal 2009; Singh and Agrawal 2009). Increment in growth indices like relative growth rate (RGR) and net assimilation rate (NAR) indicates that EDU treatment in plants leads to accumulation of more biomass, thus helping the plants to overcome the negative effects of O_3. Higher leaf weight ratio (LWR) represents more partitioning of photosynthate in leaf growth, which leads to higher available leaf area ratio (LAR) in EDU-treated plants as compared to non-EDU-treated ones. Miller et al. (1994) reported that EDU affected biomass partitioning in *P. vulgaris*, causing an increase in leaf biomass at expense of pod biomass. Singh et al. (2010b) showed significant increments in LWR and LAR of EDU-treated mung bean plants as compared to non-EDU-treated ones. EDU-treated plants showed a higher root shoot ratio (RSR) as compared to non-EDU-treated ones, which suggest a protective role of EDU on biomass allocation to roots, which is known to be hampered under O_3 stress (Tiwari and Agrawal 2009). Higher RSR in EDU-treated plants was recorded by Agrawal et al. (2005) in *V. radiata*.

Effect of EDU on yield responses

The protective nature of EDU was reflected in the responses of yield and yield characteristics, which increased significantly in EDU-treated plants as compared to non-EDU-treated ones. Singh and Agrawal (2009) observed that EDU treatment of 400 ppm significantly increased the yield of HUW510 and HUW468 under ambient O_3 concentrations varying between 34.2 and 54.2 ppb. Similar findings were also reported for mung bean (Agrawal et al. 2005), wheat (Tiwari et al. 2005; Agrawal et al. 2004) and soybean (Wahid et al. 2001) (Table 3). Singh et al. (2010b) recorded a positive influence of EDU treatment on mung bean (*V. radiata* L. var. Malviya Janpriya). Weight of seed plant^{-1} and weight of pods plant^{-1} increased significantly by 32.28 and 30.32%, respectively, in EDU-treated plants as compared to non-EDU-treated ones

Table 3 Yield response of plants upon EDU treatment

Plant	Species	O_3 concentration	EDU dose (ppm)	% Change in yield	Reference
Triticum aestivum L.	HUW 234	35.3–54.2 ppb (mean monthly)	400	(+) 8.6	Singh and Agrawal (2009)
	HUW 468			(+) 25.6	
	HUW 510			(+) 20.4	
	PBW 343			(+) 24	
	Sonalika			(+) 1.9	
	Yangmei 185	–	150	(+) 3.4	Wang et al. (2007)
			300	(+) 12.7	
			450	(+) 7.1	
	Malviya 234	–	150	(+) 24.8	Tiwari et al. (2005)
			300	(+) 66.9	
			450	(+) 66.8	
	Malviya 533	–	150	(+) 18.8	Tiwari et al. (2005)
			300	(+) 19.1	
			450	(+) 20.5	
	HUW 234	29.2 ppb (average)	500	(+) 27	Agrawal et al. (2004)
	HUW 468			(+) 22	
	HUW 2329			(+) 36.3	
Oryza sativa L.	Jiahua 2	–	150	(+) 0.15 (NS)	Wang et al. (2007)
			300	(+) 3.8 (NS)	
			450	(+) 3.4 (NS)	
Phaseolus vulgaris L.	S156	41–59 ppb (average hourly)	300	(+) 71	Elagoz and Manning (2005)
	R123			(−) 38	
	BBL-290			(−) 5 NS	
	BBL-274			(+) 3 NS	
				(Seed dry weight)	
	cv. Lit.	14.98 ppb	150	(+) 65	Brunschon-Harti et al. (1995a, 1995b)
	cv. Lit	31.56 ppb		(+) 31	
Vigna radiate L.	Malviya Janpriya	52.9–64.5 ppb (mean monthly)	400	(+) 32.28	Singh et al. (2010b)
	Malviya Jyoti	32.64–35.19 ppb (mean monthly)	500	(+) 32.2	Agrawal et al. (2005)
	cv. Pratikshya	29.3–39.1 ppb (mean monthly)	400	(+) 24	Kharel and Amgain (2010)
	Azad-1	41.3–59.9 ppb	400	(+) 44.4	Singh and Agrawal (2011b)
	BHU-1			(+) 40.9	
Glycine max L.	NARC-I	40 ppb	400	(+) 96.96	Wahid et al. (2001)
		48 ppb		(+) 94.3	
		63 ppb		(+) 112.82	
		75 ppb		(+) 181.81	
		70 ppb		(+) 284.61	
	Pusa 9814	50.5–58.9 ppb (mean monthly)	400	(+) 28.2	Singh and Agrawal (2011a)

Table 3 (continued)

Plant	Species	O_3 concentration	EDU dose (ppm)	% Change in yield	Reference
	Pusa 9712			(+) 29.8	
Trifolium alexandrinum L.	cv. Messkawy	88 ppb (8 h average)	50	(+) 14.28	Hassan et al. (2007)
			100	(+) 62.85	
			150	(+) 74.28	
			200	(+) 74.28	
Trifolium repens L.	cv. Menna	3000 ppb.h (AOT40)	150	(−)17.3 (EDU Biomass ratio)	Ball et al. (1998)
	cv. Vardan	30.3–46.6 ppb (12 h mean)	300	(+) 46.2	Singh et al. (2010c)
	cv. Bundel			(+) 21	
Trifolium subterraneum L.	cv. Geraldton	51 ppb (7 h mean)	150	(+) 13	Tonneijk and van Dijk (1997)
Arachis hypogaea L.	cv. P1268661	43 ppb (7 h, 4 days)	2 g of 50% EDU	(+) 24	Ensing et al. (1985)
	cv. McRan			NS	
Daucus carota L.	Pusa Kesar	36.1 ppb (average)	150	(+) 22.8	Tiwari and Agrawal (2010)
			300	NS	
			450	NS	
Beta vulgaris L.	Allgreen	52–73 ppb (mean monthly)	30	(+) 28.9	Tiwari and Agrawal (2009)
Solanum tuberosum L.	cv. Kara	81 ppb	300	(+) 35	Hassan (2006)
Raphanus sativus L.	cv. Cherry Belle	36 ppb (7 h mean)	200	(+) 32	Pleijel et al. (1999)
Raphanus sativus L.	Local Egyptian variety	54.8 ppb (6 h mean)	500	(+) 33.3	Hassan et al. (1995)
		66.9 ppb (6 h mean)		(+) 36.2	
Brassica rapa L.		54.8 ppb (6 h mean)	500	NS	Hassan et al. (1995)
		66.9 ppb (6 h mean)		(+) 20.9	
Glycine max L.	cv. JS 335	42 ppb (12 h mean)	400	(+) 26.7	Rai et al. (2015)
Phaseolus vulgaris L.	cv. S156	71.3 ppb (8 h mean)	450	(+) 55	Yuan et al. (2015)
	cv. DDW			(+) 46	
	cv. R123			NS	
	cv. NX816			NS	

grown at mean monthly O_3 concentrations varying between 52.9 and 64.5 ppb during the growth period (Singh et al. 2010b). Several studies have shown that harvest index of mung bean increased significantly by 11.2% (Singh et al. 2010b) and 14% (Agrawal et al. 2003) at 400-ppm EDU treatment and 16.9% (Agrawal et al. 2005) at 500-ppm EDU treatment as compared to non-EDU-treated test plants. Agrawal et al. (2005) treated *V. radiata* L var. Malviya Jyoti with 500-ppm EDU treatment at mean monthly O_3 concentrations varying between 32.64 and 35.19 ppb during the growth period and reported significant increments in the number of pods and seeds pod^{-1} and test weight, which contributed towards higher harvest index (16.9%) and seed yield (32.2%) in EDU-treated plants as compared to non-EDU-treated ones. EDU applications of 300 ppm in areas experiencing more than 50 ppb O_3 significantly increased yield, number of seeds $plant^{-1}$ and harvest index of rice (Wang et al. 2007).

Several studies have proved that more sensitive cultivars showed more positive response to EDU treatments. Singh and Agrawal (2009) treated five wheat cultivars (HUW468, HUW510, HUW234, Sonalika and PBW343) with 400-ppm EDU treatment and found that the most sensitive cultivar HUW468 showed maximum yield increments upon EDU treatments as compared to other cultivars used in the experiment. Greater O_3 sensitivity of wheat cultivar HUW468 in terms of yield as compared to other cultivars (HUW234 and HD2329) has been shown by Agrawal et al. (2004). Singh and Agrawal (2009) recorded significant reductions in harvest index for cv. HUW468 due to EDU treatments but not for other cultivars, suggesting that more photoassimilates were translocated to reproductive parts than to vegetative parts in cv. HUW468. Cultivar-specific sensitivity in plants due to O_3 stress was also reported by Wang et al. (2007).

Tiwari et al. (2005) studied the dose-response of two wheat cultivars (M234 and M533) upon EDU treatments of 150, 300 and 450 ppm. Yield (seed weight $plant^{-1}$) increased by 18.8, 19.1 and 20.5% in M533 and 24.8, 66.9 and 66.8% in M234 at 150-, 300- and 450-ppm EDU treatments, respectively, as compared to non-EDU-treated plants (Tiwari et al. 2005). This study indicated that in M533, yield increments did not vary between EDU treatments, suggesting that 150 ppm EDU is sufficient to protect the plants against O_3 injury (Fig. 4). However, in case of M234, the highest increment in yield was observed at 300- and 450-ppm EDU treatments (Fig. 4) (Tiwari et al. 2005). In the same experiment, harvest index did not vary significantly upon EDU treatments in M533 but was significantly higher in M234 at 300- and 450-ppm EDU treatments. These observations suggest that M234, which is a sensitive variety, showed a positive response at higher EDU concentrations.

Meta-analysis studies done by Feng et al. (2010a, b) using 35 crop species showed a 15% increase in the crop yield due to EDU treatment. The higher yield in EDU-treated plants was

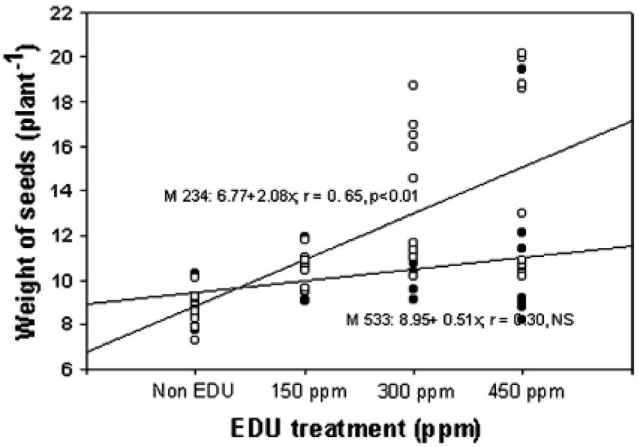

Fig. 4 Correlation coefficient (r) and regression equations between EDU concentrations and weight of seeds $plant^{-1}$ (yield) of two wheat cultivars (Source: Tiwari et al. 2005)

largely attributed to significant increase in ear/pod/tuber number $plant^{-1}$, although grain/seed number per ear/pod and individual grain/seed/tuber weight was also increased (Feng et al. 2010a, b). Feng et al. (2010a, b) further observed that response of EDU treatment varied with species. In *P. vulgaris*, only seed weight increased significantly (3.8%) after EDU application; for *G. max*, EDU increased pod number and single seed weight significantly by 61 and 11.3%, respectively; and for *T. aestivum*, significant positive effect (20.8%) was observed only in ear number $plant^{-1}$ (Feng et al. 2010a, b). The effects of EDU treatments on yield parameters of plants are clearly depicted in Table 3.

Effect of EDU on plant senescence

It is well known that O_3 induces accelerated foliar senescence. In normal cases, decline in Rubisco synthesis, followed by reduction in Rubisco protein contents, is some of the important events that mark the onset of senescence in plants (Davies and Gen 2012). O_3-induced accelerated foliar senescence is marked by premature decline in Rubisco protein and messenger RNA (mRNA) levels (Eneydi et al. 1992; Reddy et al. 1993; Pell et al. 1994). Reduction in lipid peroxidation of EDU-treated plants confirms the anti senescence property of EDU (Whitaker et al. 1990). It has been proposed by some researchers that nitrogen in EDU molecule prevents the O_3-induced senescence. However, this is just based upon speculations and there is no experimental proof of it. Recent experiment on *S. sachalinensis* L. Schm have shown that high EDU application (800 and 1600 mg L^{-1}) lead to an increase in foliar N content but there are no pieces of evidence to predict that this increase was responsible for the observable positive effects of EDU (Agathokleous et al. 2016c).

Several reports have attributed 'antisenescent' properties to EDU. Lee et al. (1981) showed that the senescence of red clover leaf discs was delayed when kept in EDU solution. The delayed senescence was characterized by retention of protein, chlorophyll and RNA. Lee and Chen (1982) showed that EDU behaved like cytokinin in retarding chlorophyll degradation, sustaining protein and RNA synthesis and stimulating cell proliferation in tobacco callus tissues.

Eckardt and Pell (1996) examined the antiozonant activity of EDU on O_3-sensitive potato cultivar (*Solanum tuberosum* L. cv. Norland). A dose-response experiment showed that an EDU concentration of 150 mg L^{-1} given as soil drench provided complete protection from accelerated foliar senescence induced by exposure to 0.1 μL L^{-1} O_3 for 5 h day^{-1} for 11 days. It was observed that O_3 exposure in absence of EDU resulted in accelerated foliar senescence characterized by early declines in net photosynthesis and Rubisco quality in O_3-treated plants, whereas complete protection against symptoms of accelerated senescence was recorded in EDU-treated potato plants (Eckardt and Pell 1996). O_3-treated plants showed a non-significant effect on rbcS mRNA levels whereas rbcL mRNA levels of O_3-treated plants were significantly reduced (Eckardt and Pell 1996). However, Reddy et al. (1993) found that rbcS mRNA levels were more sensitive than rbcL to chronic and acute O_3 doses. Glick et al. (1995) also reported a significant drop in rbcS mRNA in Norland potatoes exposed to 0.08 μL L^{-1} O_3 after 3 days of O_3 exposure. Kostka-Rick and Manning (1992) also reported delayed foliar senescence of radish (*R. sativus*) treated with EDU. The reviews by Paoletti et al. (2009) and Singh et al. (2015) suggested that EDU delays O_3-induced accelerated senescence and it has also been confirmed by Agathokleous et al. (2016d).

Singh and Agrawal (2009) reported delayed senescence in five wheat cultivars upon 400-ppm EDU treatment, when grown in ambient O_3 concentrations ranging between 35.3 and 54.2 ppb. It was observed that the number of standing dead was fewer in EDU-treated plants as compared to the non-EDU-treated ones (Singh and Agrawal 2009). Antisenescent properties of EDU were also confirmed by Tiwari and Agrawal (2009) in palak (*Beta vulgaris*) plants treated with 300 ppm EDU, which showed delayed senescence as compared to non-EDU-treated plants. Bortier et al. (2001) observed that leaves at the base of the stem of *Populus niger* shed faster in non-EDU-treated plants as compared to plants repeatedly injected with EDU solution (5 mg $plant^{-1}$) at 14-day interval. Tiwari et al. (2005) observed that senescence was delayed by 7 days upon EDU treatment in two wheat cultivars as compared to non-EDU-treated plants. Higher amounts of photosynthetic pigments were recorded in EDU-treated plants as compared to non-EDU-treated ones, which indicate delayed senescence in EDU-treated plants (Tiwari et al. 2005; Tiwari and Agrawal 2009; Singh and Agrawal 2011a, b; Tiwari and Agrawal 2010). EDU treatment helped to retain chlorophyll content by reducing oxidative stress and delaying senescence. An increase in carotenoid contents under EDU application also supports the antisenescent property of EDU as carotenoids play an important role in protecting chlorophyll from photooxidative damage.

Effect of EDU on trees

EDU is known to suppress acute and chronic O_3 injury on a wide range of plants, without appreciable effects of its own (Brunschon-Harti et al. 1995a, 1995b; Godzik and Manning 1998; Bortier et al. 2001; Ainsworth et al. 1996). Long and Davis (1991) used foliar sprays of EDU (1000 ppm) over 4-year period to protect ground black cherry (*Prunus serotina*) seedlings from O_3 injury. Ambient O_3 caused reductions in tree height and basal diameters and caused 47% reduction in above ground leafless biomass (Long and Davis 1991). Bortier et al. (2001) injected EDU in the stems of black poplar (*P. nigra*) trees and found positive results of EDU treatments. Ainsworth et al. (1996) found for poplar varieties (*Populus deltoides* × *maximowiczii* and *P. deltoides eumericana*) that treatment with EDU provided significant protection against visible injury, but they found no effect on growth suggesting that EDU was incapable of protecting trees against long-term damage. However, studies on *P. serotina* (Long and Davis 1991), *Gledistsia triacanthos* (Roberts et al. 1985) and *P. nigra* (Bortier et al. 2001) have demonstrated that EDU can provide protection against visible injury as well as against growth reductions in these woody species. Depending upon species, cultivar, age, environmental and O_3 conditions, different EDU doses and frequencies may be necessary to provide full protection (Paoletti et al. 2008). In contrast with Ainsworth et al. (1996), Paoletti et al. (2007b, 2008) found that EDU stimulated shoot growth and leaf number in both symptomatic (O_3-sensitive) and asymptomatic (O_3-resistant) ash trees (*F. excelsior*) exposed to elevated ambient O_3 concentrations (32.5 ppm h AOT40). Manning et al. (2003a, b) observed that EDU did not prevent O_3 injury in O_3-sensitive loblolly pine seedlings (*Pinus tadea*); however, the growth parameters showed positive response to EDU (300 and 450 ppm) treatments. No effects of EDU treatment on visible foliar injury have also been observed in pine seedlings by other workers (Flagler and Toups 1992; Flagler et al. 1994; Kuehler and Flagler 1999). The reason for this lack of protection from O_3 injury symptoms is not clear. Visible O_3 injury symptoms are not necessarily correlated with the adverse effects on tree growth (Chappelka and Chevone 1992).

EDU treatment also affects the physiological and biochemical characteristics of trees grown at ambient as well as high O_3 concentrations. In agreement with Ainsworth et al. (1996), Paoletti et al. (2008) found that EDU induced no change in

carbon gain and photosynthetic capacity. A few studies have reported no significant effects of EDU treatments on stomatal conductance (Ainsworth et al. 1996; Kuehler and Flagler 1999). Paoletti et al. (2008) recorded reductions in stomatal conductance in EDU-treated *F. excelsior* trees. However, the reduced stomatal conductance did not translate to a significant increase in leaf water potential (Paoletti et al. 2008). The asymptomatic ash trees (*F. excelsior*) also exhibited major ascorbate peroxidise (APX) activity, which was probably sustained by greater ascorbate availability (Paoletti et al. 2008). Recent studies done by Agathokleous et al. (2016a, 2016c, 2016d) using *S. sachalinensis* L. Schm as the model plant have been helpful in partially solving a plethora of questions related to EDU application techniques and frequency along with its mode of action.

Conclusion

The results of different experiments done worldwide clearly indicate the protective nature of EDU towards O_3 injury in plants. However, the protection appears to be incomplete as the loss in productivity of EDU-treated plants was reduced but not avoided. EDU can effectively be used as an important biomonitoring tool in assessing O_3 injury in plants. This review discusses for the first time the advantages of different EDU application techniques and their preferences over one another, NOEL concentration of EDU treatment and the significance of the hormetic response of EDU-treated plants. The present review can be summarized as follows:

1. EDU remains confined in the foliar apoplast where it persists for about 8 days and then slowly degrades; the degradation mechanisms of EDU are still unknown.
2. The effectiveness of EDU depends upon its mode of application, foliar spray technique being more effective than the soil drench technique; the amount of EDU sufficient for the protective action is less in case of foliar spray than soil drench.
3. The persistence of EDU in the soil depends upon the soil properties. Soil rich in organic content has the ability to retain EDU for a longer duration due to the adsorption of EDU to organic matter particles and gradual resolubilization by irrigation water. In soil which is devoid of organic content, retention time of EDU in soil is minimum and EDU is quickly transported via xylem to the leaves.
4. EDU concentration in the range 200–400 mg L^{-1} is found to be sufficient to provide the required protection to the plants from O_3 injury under field conditions. To the best of our knowledge, in most of the experiments conducted so far, no-observed-toxic-effect concentration (NOEL) of EDU lied within this range. However, effectual EDU treatments with higher and lower concentrations are also reported.
5. The plants depicted a hermetic response upon EDU treatment which indicates biological plasticity; however, the actual mode of action is still debated.
6. EDU is more effective at lower concentration. Higher concentrations may or may not show inhibitory effect but are usually not recommended.
7. EDU-induced tolerance to O_3 can be attributed to its ability of maintaining higher levels of photosynthetic pigments and ascorbic acid leading to significantly higher yields. Recent studies have shown that decreased stomatal conductance and increased APX activity observed in EDU-treated plants provided protection against O_3-induced ROS and lead to a limited production of H_2O_2 and lesser visible leaf injury symptoms.

EDU can be successfully used as a research tool for assessing the phytotoxic effects of O_3 on agricultural and non-agricultural crops and trees. However, more experimentation needs to be done before recommending the use of EDU at commercial level. The future studies should focus more precisely upon EDU mode of action as evaluated through the '-omics' tool, which is a much less explored area as far as EDU-related studies are concerned. The gene expression and protein profiling studies can help us in getting better insight into the actual mechanism of providing partial protection to plants against O_3 injury. The use of EDU for assessing O_3 impacts on plants is especially beneficial in developing countries that do not have efficient O_3 monitoring techniques. In view of the future perspectives, more EDU-related studies are required in the countries experiencing a tropical climatic condition which is favourable for O_3 formation.

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