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A preliminary study on the effects of ozone on induction of resistance in Cicer arietinum and Trigonella foenum against acute ozone exposure

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Abstract: *The potential for ozone to damage vegetation has been known for over 30 years, but it is only over the last decade that its impacts have become of concern in India and other countries. It is now clearly established that ozone, at the ambient concentrations can cause a range of effects including visible leaf injury, growth and yield reductions, and altered sensitivity to biotic and abiotic stresses. Research in recent years has advanced our understanding of the mechanisms underlying ozone effects on agricultural crops, and to a lesser extent on trees and native plant species. A study was conducted to evaluate the consequence of application of ozone for a diminutive period repeatedly on germinated seedlings of Cicer arietinum and Trigonella foenum. The seedlings were exposed to different concentrations of ozone (5 ppm and 10 ppm) for different time intervals (5 min, 10 min, 15 min and 20 min) per day for three days. Ozone exposed and untreated (control) plants were used for biochemical analysis. These include chlorophyll content, total phenol content and phenylalanine ammonia-lyase assay (PAL). This study has revealed that increase in the ozone concentration and exposure time enhances the production of soluble phenol contents, PAL activity and reduces the chlorophyll content compared to control, seedlings.*

Key words: *Chlorophyll, Ozone, Phenylalanine ammonia-lyase (PAL), Soluble phenol*

I. Introduction

Surface or tropospheric ozone is a phytotoxic air pollutant that causes more damage to vegetation worldwide than all other air pollutants combined (Ashmore & Bell, 1991). The biological effects of ozone on plants have been studied for more than 50 years (Heggestad, 1991; Davison & Reiling, 1995). Chemical characteristics that are at the basis of its behavior are: high oxidizing power, diffusion coefficient similar to the one of CO_2 (and consequently a certain facility to penetrate the plant tissues), solubility in water 10 times higher than CO_2 and tendency to react with water in sub-basic environment (Izuta, 2006). Ozone can cause foliar injury, changes in crop quality, and reductions in plant growth and productivity (Schenone et al., 1992, Heagle et al., 1998). With elevated levels of ozone, changes such as reduced stomatal conductance, rates of photosynthesis (Inclán et al., 1998) and pigment concentrations (Alonso et al., 2001) have also been reported. Consequently, many of the world's most productive agricultural and forested regions are currently exposed to harmful elevated levels of ozone (Chameides et al., 1994).

Plants have evolved a complex of defense response mechanisms to respond various environmental stresses from morphological, biochemical and physiological changes triggered by ozone. Molecular and biochemical studies have suggested that the air pollutant ozone also stimulates phenol metabolism and biosynthesis of lignin or substances partly derived from coniferyl alcohol (Galliano et al., 1993; Kangasjarvi et al., 1994). The biosynthesis of lignin and related products occurs through a sequence of reaction from intermediate metabolism through the shikimate pathway to the common phenyl propanoid pathway and the specific lignin pathway (Boudet et al., 1995). Ozone acts as a cross inducer and activate both pathways at the metabolite, enzyme and transcript levels to produce elicitor and the pathogenesis-related proteins (Eckey-Kaltenbach et al., 1994; Karenlampi et al., 1994).

Plants have several lines of defense against invading pathogens including preformed barriers and induced responses. Systemic acquired resistance is characteristically associated with accumulation of salicylic acid, enhanced expression of pathogenesis-related proteins and activation of phenylpropanoid pathway, leading to the synthesis of higher phenolic compounds. Phenolics have been associated extensively with the defense of plants against microbes, insects and other herbivores (Metraux and Raskin, 1993). A number of phenols are regarded as preinfection inhibitors, providing the plant with a certain degree of basic resistance against pathogenic micro-organisms. Phenol metabolism and cell wall lignification are thus involved in, and have consequences for, a number of cellular, whole plant and ecological processes, that might even provide plants, the immunity against destructive agents (Asai et al., 2002).

Salicylic acid is assumed to be the systemic signal molecule that induces synthesis of pathogenesis-related proteins and/or other components of systemic acquired resistance (Malamy et al., 1990; Yalpani et al., 1994). Salicylic acid activates resistance mechanisms such as phytoalexin production, proteinase inhibitors, cell wall strengthening and lignification.

Ozone stress or injury to plants can stimulate the production of phenolic compounds (Sgarbi et al., 2003), including lignin and suberin (Rhodes & Wooltorton, 1978). Ozone increases the salicylic acid level by participating in the regulation of ozone-induced phenylalanine ammonia-lyase (PAL) expression. Early studies showed that in higher plants salicylic acid derived from the shikimate-phenylpropanoid pathway (Hahlbrock & Scheel, 1989). PAL catalyses the deamination of phenylalanine to produce transcinnamic acid, the first step in controlling the rate of phenylpropanoid metabolism (Koukol & Conn, 1961). The production of phenylpropanoid compounds is important in plant development, plant-microbe signaling and plant defense (Hahlbrock and Scheel, 1989).

Hence O_3 revealed itself as an important instrument for the study of plant responses to biotic and abiotic stress, and a valid alternative to more expensive and complicated treatments for the induction of resistance to several pathogens and abiotic stress, without presenting moreover particular problems in terms of environmental impact (Sudhakar et al., 2007a, b; Nagendra-Prasad et al., 2007).

Based on these observations, a study was undertaken for stimulating systemic acquired resistance in Trigonella foenum and Cicer arietinum. Trigonella foenum is commonly known as methi is extensively cultivated in India for use a nutritious green leafy vegetable, food flavourants, and pharmaceuticals. It also has a long history of use for the treatment of reproductive disorders, to induce labor and to reduce menstrual pain. Recent studies have shown that methi helps lower blood glucose and cholestrol levels, and may be an effective treatment for both type 1 and 2 diabetes. This crop is attacked by several pathogens causing powdery mildew, root rot, rust, collar rot and viral diseases and seed mycoflora affecting the seed quality and viability.

Chickpea (Cicer arietinum) is the 4th largest grain-legume crop in the world and it is an important pulse crop grown and consumed all over the world. Chick pea is a good source of carbohydrates and protein, and protein quality is considered to be better than other pulses. Chickpea consumption is reported to have some physiologic benefits that may reduce the risk of chronic diseases and optimize health. Recent reports of the importance of chickpea consumption in relation to health are Cardiovascular Disease (CVD), Coronary Heart Disease (CHD) and Cholesterol Control. Several diseases reported to affect chick peas. The important diseases are wilt, sclerotinia blight, grey mold, rust, Ascochyta blight and powdery mildew. An attempt was made to detect changes in total soluble phenol content in the ozone-treated plants and activity of the enzyme known to be involved with systemic acquired resistance, i.e. PAL. The aim of the present study was to develop systemic resistance in the Trigonella foenum and Cicer arietinum plants against acute ozone exposure and induction of disease resistance mechanism by applying mild concentrations of ozone.

II. Materials and methods

Plant material

Chickpea (Cicer arietinum) and fenugreek (Trigonella foenum) seeds were obtained from Ratanshi seeds and hortitech, Byculla, Mumbai. Healthy plants were obtained from the seeds after sowing them in plastic trays filled with fertile soil. The plants were watered regularly around 11 am and maintained under natural environmental conditions.

Ozone treatment

Seven day old chickpea and methi seedlings were treated with different concentrations of ozone (5 ppm and 10 ppm) for different time period (5 mins, 10mins, 15mins and 20 mins) per day for 3 days. Ozone was obtained using the corona discharge plasma ozone generator available in the 'Pillai Institute of Information Technology and Research (PIIT)', New Panvel, Navi Mumbai. The seedlings were further allowed to grow for five more days after ozone exposure. Fifteen day old ozone exposed and control plants were taken for biochemical analysis.

Biochemical analysis

Determination of total phenolic content

Total soluble phenolics were extracted and quantified using the procedure described by Swain and Hillis (1959). Fresh leaves (1g) were extracted in 80% methanol for 90 min using mortar and pestle. The extract was centrifuged at 1400 g for 15 mins. The supernatant was diluted to 1 ml with distilled water and mixed with 0.5ml of 2.0 M folin ciolalteuo's reagent and 0.5 ml of 1 M Na_2CO_3.After 1 hr, absorbance of the sample solution was measured at 725nm using spectrophotometer. Concentration of total soluble phenolics in extract was calculated from a standard curve prepared with gallic acid.

Determination of Phenylalanine Ammonia Lyase (PAL) activity

PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson et al, (1984). The sample tissue (5gm) was homogenized in 3ml of 50mM sodium acetate buffer (pH 5.2).The homogenized extract was centrifuged at 14000g for 20 mins. Here after centrifugation supernatant obtained is called plant extract. For PAL analysis, 100 µl of plant extract + 50µl of L-phenylalanine (6µM) + 850µl of tris HCl 50mM (pH 8.4) buffer solution was used. The reactive mixture was placed in a boiling water bath at 37^0c for 70 mins. The amount of transcinnamic acid formed by phenylalanine was measured on spectrophotometer at 290nm and the result expressed as PAL activity unit (pK at) per mg of protein or n mole of cinnamic acid min^{-1} mg $protein^{-1}$ contained in each sample.

Determination of Chlorophyll content

The chlorophyll level in the foliar extracts was tested by using the spectrophotometric procedure Wellburn, A.R and Lichtenthaler, H. 1984. Samples of approximately 0.2g of leaves were taken. The leaf tissues were homogenized in 5 ml of 80% acetone at 4^0c by using mortar and pestle. The crude extract was centrifuged at 2500 rpm for 5 minutes. After centrifugation the pellet was discarded and supernatant was used to measure the absorbance at 663nm and 645nm which are major absorption peaks of chlorophyll a and b respectively.

Chlorophyll content (in mg/l) in both the plants was estimated by using Arnon equation:

$C = 20.2\ A_{645} + 8.02\ A_{663}$

Where, C= chlorophyll content in mg/litre, A645 = absorbance of chlorophyll b at 645nm, A_{663} = absorbance of chlorophyll a at 663nm.

III. Results and Discussion

Necrotic spots were observed in the ozone exposed plants of both the varieties i.e. in Cicer arietinum and Trigonella foenum. Such spots appeared mainly on the mature leaves and were greater on the plants exposed to O_3 concentration for 15 min and 20 min. This indicates that cereal plants can tolerate lower concentration of O_3. The ozone exposure time and concentration determines the extent of damage done to the plants.

Effect of ozone on Total phenolics content in Cicer arietinum and Trigonella foenum

The total phenolic content in both ozone exposed plants and control plants was determined using the procedure described by Swain and Hllis (1959). O_3 exposed plants showed significant increase in the total phenolic content with increase in ozone concentration (5 ppm and 10 ppm) and exposure time (5 min, 10 min, 15 min, 20 min).The phenolic content enhanced 3 to 4 folds in both Cicer arietinum and Trigonella foenum exposed to 5 ppm O_3 concentration. Seedlings exposed to 10 ppm O_3 concentration showed 4 and 5 fold increase in the total phenolic content C. arietinum and T. foenum respectively as compared to the control plants (Fig.1 and 2).

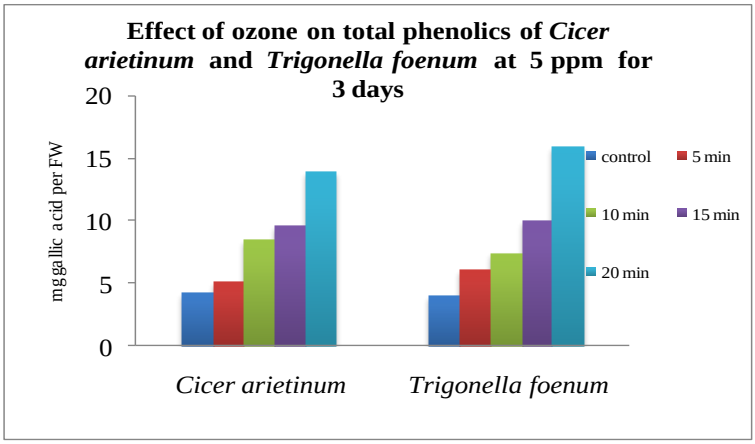

Figure 1. Total phenolic content of C. arietinum and T. foenum: Each bar represents a mean of 5 replicate samples. The total phenolic content is found to be increased in both C.arietinum and T. foenum as compared to the control plants. The phenolic content is maximum in plants exposed to ozone for longer period of time i.e. 20 mins**.**

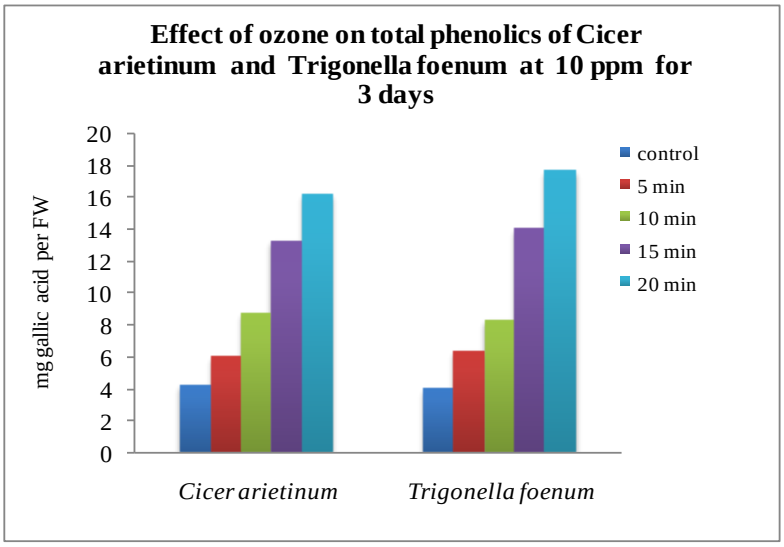

Figure 2. Total phenolic content of Cicer C. arietinum and T. foenum: Each bar represent a mean of 5 replicate samples. The total phenolic content is found to be increased in both C. arietinum and T. foenum as compared to the control plants. The phenolic content is maximum in plants exposed to ozone for longer period of time i.e. 20 mins.

Several associations have been reported between phenolics and the resistance of plants to pathogen (Panda and Khush, 1995). Phenolic acids are involved in phytoalexin accumulation, biosynthesis of lignin and formation of structural barriers, which play a major role in resistance against the pathogen. Ozone stress or injury to plants can stimulate the production of phenolic compounds (Sgarbi et al., 2003) including lignin and suberin (Rhodes and Wooltorton, 1978). Zobel et al. (2003) reported that the concentration of phenolic compounds was significantly higher in leaves collected in a polluted environment compared to those of plants growing in a cleaner one. Moderate levels of ozone concentration increased the total phenol content in rice plants. The content of the total phenolics in red clover leaves was increased by ozone exposure by 5–12% in red clover leaves. Increased accumulation of phenolic compounds, especially flavonoids in leaves of forest trees in response to ozone exposure has been reported in numerous studies. Further, increased levels of transcription of genes involved in flavonoid biosynthesis was found in ozone resistant leguminous cultivars (Puckette et al., 2008), suggesting that a large number of transcription factors and signaling genes regulated differently enable resistant plants to adapt rapidly to ozone stress.

Due to the increased phenol content, plants possess more defense response in nature against the pathogen invasion. Rohde (1972) indicated the possible role of preformed simple phenols in the incompatible host and parasite interactions. Ramanathan et al. (2000) reported that when tomato plants were treated with ozone, marked accumulation of phenols was observed and it resulted in suppression of Fusarium wilt in tomato. Cell wall lignifications might be caused by an induction by ozone on plants normally induced against pathogens defense (Sandermann, 1996). The deposition of lignin and related phenol ducts in cell walls increases their mechanical strength, decreases apoplastic solute conductance and permeability to water and in some cases alters susceptibility to pathogens (Boudet et al., 1995)

Previous studies have shown that there is a significant overlap in the pattern of gene expression observed in ozone-treated plants and plants exhibiting a disease resistance response, suggesting that there may be some commonality between the signal transduction pathways for disease resistance and ozone (Ernst et al., 1992; Eckey-Kaltenbach et al., 1994). Early study has shown that tobacco plants treated with either ozone or UV light accumulated salicylic acid and exhibited increased resistance to infection with Tobacco mosaic virus (Yalpani et al., 1994). Ozone, a major photochemical oxidant, induces salicylic acid synthesis, which is the hallmark signal for systemic acquired resistance (Keen, 1992). Since salicylic acid has been shown to be a critical factor for the induction of systemic acquired resistance and may also modulate HR, it is possible that salicylic acid is a common link between the various stress-activated pathways.

Effect of ozone on Phenylalanine Ammonia-Lyase (PAL) activity in Cicer arietinum and Trigonella foenum

The PAL activity was measured in both the ozone exposed and control plants by direct spectroscopic method. It was seen that the PAL levels increases with the increase in the ozone concentration (5 ppm and 10 ppm) and exposure time (5 min, 10 min, 15 min and 20 min) in both Cicer arietinum and Trigonella foenum as compared to the control plants. The PAL activity increases up to 5 folds in plants exposed to 5 ppm O_3 concentration and 6 folds in those exposed to 10 ppm O_3 concentration (Fig.3 and 4). Rapid increase in transcript levels for phenylalanine ammonia lyase in response to ozone has been observed in parsley (Eckey-kaltenbach et al 1994), soybean (Tingey et al 1975) and tobacco (Bahl et al., 1995).

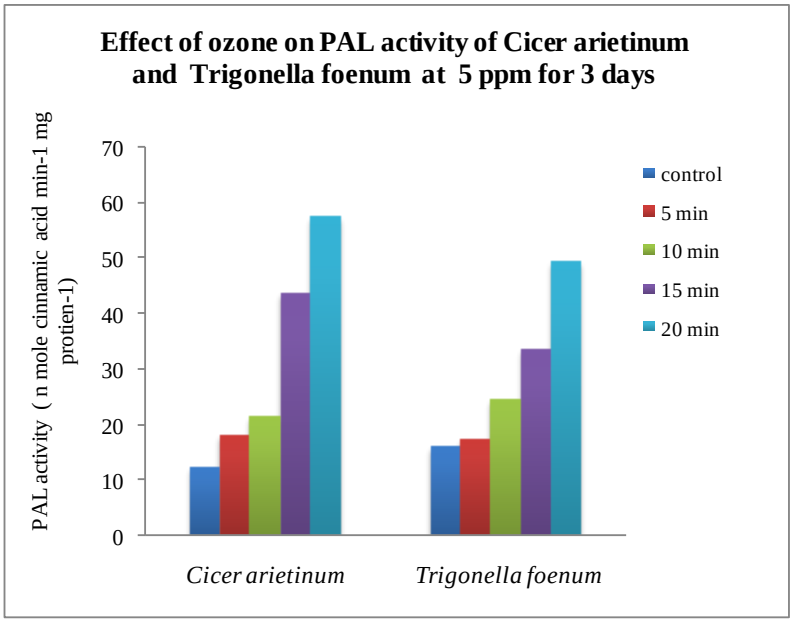

Figure 3. Foliar levels of phenylalanine ammonia lyase activity in C. arietinum and T. foenum: Each bar represents mean of 5 replicate samples. PAL activity increases with the increase in ozone exposure time. Plants exposed to ozone for 20 mins showed maximum PAL activity.

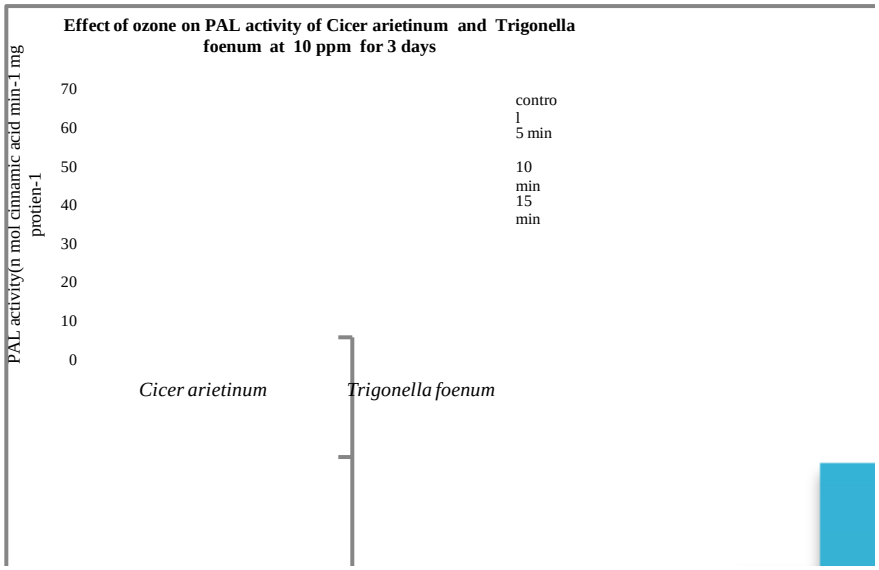

Figure 4. Foliar levels of phenylalanine ammonia lyase activity in C. arietinum and T. foenum Each bar represents mean of 5 replicate samples. PAL activity increases with the increase in ozone exposure time. Plants exposed to ozone for 20 mins showed the maximum PAL activity.

PAL activity is essential for the synthesis of all the protective substances induced in plants against stresses (Pascholati et al., 1986). PAL is an extremely sensitive indicator of stress conditions (Tuomainen et al., 1996) and ozone treatment elevates the level of flux through the phenylpropanoid pathway, thereby supply carbon skeletons for secondary products (Ramanathan et al., 2000). PAL activity is found to be increased in tobacco, bean and poplar leaves after acute exposure to ozone. PAL increases when plants are exposed to ozone (Sgarbi et.al. 2003). The observed increase in PAL activity in ozone treated plants was presumably related to lignifications process. Cell wall lignifications might be caused by an induction by ozone on plants normally induced as a defense (Sandermann, 1996).

Ozone treatment has been shown to increase the activities of phenylalanine ammonia lyase, chinnamyl alchohol and chalcone synthase enzymes controlling respectively the phenylpropanoid, flavonoid and lignin biosynthesis pathways. These pathways play a significant role in plant defense responses because they synthesize many potentially protective compounds including flavonoids (UV protectants and phytoalexins), furanocoumarins (phytoalexins) and lignin. Their induction under environmental stress conditions such as wounding, pathogen attack, UV light and ozone is well characterized at both the biochemical and gene levels (Sandermann 1996).

The results presented in this study confirm the non-specificity of the protection afforded by ozone at concentrations of 5 ppm and 10 ppm. It is evident from the present experiment that plants treated with mild concentrations of ozone activate at least some components of ozone resistance in them to detoxify the invading ozone.

Effect of ozone on Chlorophyll content in Cicer arietinum and Trigonella foenum

Following ozone exposure, the extent to which seedlings were injured by ozone exposure was analyzed using chlorophyll content. Wallin et al (1990) and Skarby et al. (1995) reported that the exposure to ozone resulted in a reduction in chlorophyll content that indicates the severity of injury caused by ozone. The chlorophyll content was determined using the procedure described by Wellburn, A.R and Lichtenthaler H. 1984.

The ozone exposed plants showed significant decrease in the chlorophyll content. With the increase in ozone concentration and exposure time, significant chlorophyll reduction was observed in both Cicer arietinum and Trigonella foenum plants (Fig. 5 and 6). It was seen that the chlorophyll content reduced up to 5 folds in seedlings exposed to 5 ppm O_3 concentration for 20 minutes while those exposed to O_3 concentration of 10 ppm for 20 minutes showed 6 fold reductions as compared to the control plants. The intensity of visible injury was correlated with a decrease in the chlorophyll content of leaves suggesting that, in addition to necrotic spots, plants were damaged via chlorophyll diminution over the whole leaf area as a result of ozone exposure. Reich (1983) reported that exposure of plants to ozone results in a reduction in chlorophyll content.

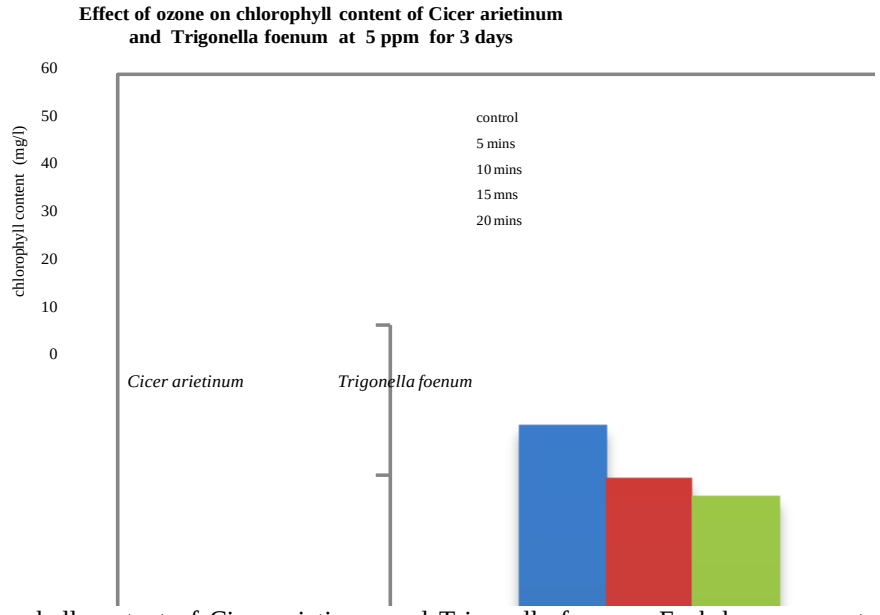

Figure 5. The chlorophyll content of Cicer arietinum and Trigonella foenum: Each bar represents mean of 5 replicate samples. Chlorophyll content is highest in the control plants. Significant decrease is seen with increase in the exposure time with constant O_3 concentration of 5 ppm. Chlorophyll content is lowest in plants exposed to ozone for longer period i.e for 20 min.

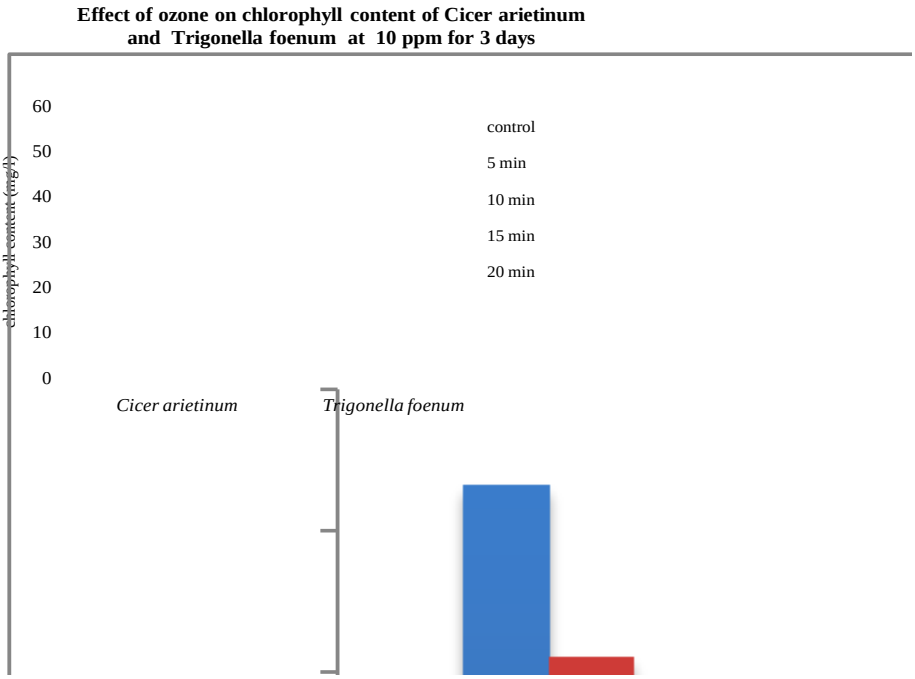

Figure 6. The chlorophyll content of Cicer arietinum and Trigonella foenum: Each bar represents mean of 5 replicate samples. Chlorophyll content is highest in the control plants. Significant decrease is seen with increase in the exposure time with constant O_3 concentration of 10 ppm. Chlorophyll content is lowest in plants exposed to ozone for longer period i.e. for 20 mins.

When a plant is attacked by O_3, it puts in action a series of metabolic responses that can result in either induction of damage or resistance (Sudhakar et.al., 2007, Nagendra Prasad 2007). O_3 enters the plant through the stomata, diffuses in the apoplast and once there is rapidly decomposed, giving hydroxyl radical, superoxide, hydrogen peroxide and other reactive oxygen species (ROS) and giving rise to the oxidative burst (Health and Taylor 1997, Bolewell, 1996). It is generally held that this oxidative process induces several defense reactions in the seedlings. Chloroplasts are the most important targets of O_3 exposure: characteristic symptoms are represented by alterations in their size and functionality, and in the composition of the stroma. Both O_3 fumigations and natural tropospheric concentrations induced significant size reductions in the chloroplasts of needles of Scots pine and Norway spruce, and an increase in the electron density of the stroma, especially on the upper side of the leaves (Kivimaenpaa et al., 2005).

As can be seen, a problem for plants exposed to chronic O_3 concentrations is to reduce the photosynthetic rate, tune it with the changed metabolic demands, and put into action different alternative strategies for reducing equivalents. Another possible mechanism of the photosynthetic response can in fact involve more precocious steps, such as the inhibition of PS-II, with no alterations in quenching parameters (Guidi et al 2001). Ranieri et al (2001) showed in Poplar how chronic fumigation with O_3 can induce alterations in thylakoid functionality and composition; the activity of both the photosystems (PS-II and PS-I) was significantly reduced, and so was the concentration of all the polypeptides analyzed. This provides evidence of the fact that, at a chronic level, O_3 generally inhibits the activity of the electron transport chain by lowering the PS protein and pigment content, and all of these are strategies to reduce the rate of photosynthetic activity to face the adverse conditions.

Some similarities exist between the effects caused on the photosynthetic process by chronic O_3 exposure and fungal pathogens. Carter and Knapp (2001) analyzed a large amount of published and unpublished data to show that, among others stressors, fungal pathogens and O_3 cause alterations in the optical properties of leaves at almost the same wavelengths. The results shown provide evidence of an interaction between the primary metabolism and the possible responses to O_3 stress, causing changes in the cell biochemistry, structure of chloroplasts and composition of their proteins, levels of reducing substances (such as nicotinamide adenine dinucleotide [NADH] and other reduced equivalents) and the redox balance of glutathione and ascorbate in the stroma. These changes can affect plant productivity, shift certain metabolic pathways (e.g. the shikimate way, Schmid and Amrhein 1995) and alter the capacity of the plant to react to future biotic and abiotic stresses.

IV. Conclusion

Elevated ozone has markedly increased the concentrations of total soluble phenolic content and PAL in Cicer arietinum and Trigonella foenum. In conclusion, O_3 is an important instrument for the study of plant responses to biotic and abiotic stress, and a valid alternative to more expensive and complicated treatments for the induction of resistance to several pathogens, with no particular environmental impact. However, this subject has not been studied deeply enough yet, since each plant can show a different set of responses to different applications of O_3; moreover, the constant increase in tropospheric O_3 in several parts of the world is causing a huge change on a global scale. The two-way activity of O_3, which is capable of both predisposing plants to the attack of viruses, pathogens and insects, and inducing resistance to these same factors, depending on factors such as the kind of plant and the nature of the exposure, makes us realize how the medium- and long term effects of this phenomenon are not easily predictable.

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