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Application of ozone on induction of resistance in *Vigna unguiculata* cv. Co 6, against *Fusarium wilt*

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Abstract
The efficacy of ozone in controlling *Fusarium oxysporum*, the casual agent of wilt disease, was evaluated in *Vigna unguiculata* (cowpea). Different concentrations of ozone (T1, T2, T3, T4 and T5) were passed to the *Vigna unguiculata* seedlings among which T_3 treatment reduced *Fusarium wilt* more effectively than other treatments by enhancing growth promotion along with the activation of the defense-related enzymes than other treatments. T3 treatment provokes enhanced biomass production along with increased activity of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia-lyase than other treatments relative to control seedlings. Moreover, this is the first report of ozone protecting seedlings against *Fusarium wilt* of *Vigna unguiculata* plants.

Keywords: *β-1,3-glucanase, chitinase,* Fusarium oxysporum*, peroxidase, phenol, phenylalanine ammonia-lyase*

Introduction

Fusarium oxysporum has a long history because available control methods are inefficient and difficult to apply (Alabouvette et al. 1998). Even the biocontrol agents has provided inconsistent results against *Fusarium oxysporum* (Weller & Thomashow 1994). An alternative (1) approach to combat the disease is to induce the natural defense systems in plants.

Different biocontrol agents, avirulent pathogens and abiotic elicitors like ozone (O_3) activate an array of inducible defence reactions in plants, including the synthesis of lignin and low molecular weight substances called phytoalexins, pathogenesis-related (PR) proteins and hydroxyproline-rich glycoproteins. Induced systemic resistance (ISR) activates multiple defense mechanisms that include increased activity of PR proteins like chitinase, β-1,3-glucanase and peroxidase (Maurhofer et al. 1994; Xue et al. 1998), and also the accumulation of phytoalexins (Van Peer & Schippers 1992). O_3 is considered to be the most important phyto-toxic gas in the tropospheric atmosphere (Berlett et al. 1996), since it reduces photosynthesis, growth, and enhances premature senescence in plants at concentrations not much in excess of maximum natural levels (Mudd et al. 1997; Hemmingsen et al. 1999).

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Many plants react to ozone-induced stress with a variety of active morphological and ultrastructural responses. These responses include: specific pectanaceous cell wall protrusions, phenolic cell wall incrustations, tonoplast vesicles, and homogenous, condensed or precipitated phenolic material in the vacuoles (Günthardt – Goerg et al. 1997, 1999, 2000). Even the accumulation of phenols, production of antimicrobial phytoalexins, and the reinforcement of cell walls at invasion sites through synthesis and deposition of hydroxylproline-rich glycoproteins, callose and lignin compounds contribute to multilayered plant defence systems (Keen 1992).

Chitinase and β-1,3-glucanases are a structurally and functionally diverse group of hydrolytic enzymes involved in defense reactions of plants against pathogens (Jackson & Taylor 1996). Peroxidase and phenylalanine ammonia-lyase (PAL) are the key enzymes involved in phenyl-propanoid metabolism. O_3 is considered to evoke the plant defence systems (Kangasjärvi et al. 1994). Among the PR proteins, β-1,3-glucanases (PR-2) and chitinases (PR-3) possess *in vitro* (Mauch et al. 1988) and in vivo (Zhu et al. 1994) antifungal activity. Peroxidase and phenylalanine ammonia lyase are key components in the induction of local and systemic disease resistance (Kombrink & Somssich 1995). Peroxidase is involved in crosslinking of extensin molecules to form lignin (Brisson et al. 1994), which strengthens the plant cell wall against fungal invasion. Phenylalanine ammonia lyase is associated with biosynthesis of phytoalexins, phenols, lignins and salicylic acid (Mauch-Mani & Slusarenko 1996). Rapid activation of these enzymes results in protection of plants against fungal pathogens (Podile & Laxmi 1998).

Plants treated with O_3 often respond with increased levels of defense related enzymes. Thus, the main purpose of this study was to determine a novel method to suppress the severity of *Fusarium wilt* using O_3.

Materials and methods

Preparation of inoculum of F. oxysporum

F. oxysporum Fo 1004, race 1 was used in this study which was re-isolated from infected *Vigna unguiculata* plants to check its infectivity. Then the re-isolated culture was cultured in aerated 2% malt exract (Hi Media, India) medium at 22°C. After 14 days of growth, cultures were filtered through sterile glass wool to remove mycelial mats. Microconidia were harvested by centrifugation at 8000*g* for 20 min, resuspended in 10 mM $MgSO_4$, and mixed through a peat-sand (1:1, vol/vol) mixture to a density of 3×10^4 CFU/g. Infested peat was incubated in polyethylene bags for 3–5 days at 20°C before use in the potting soil to allow colonisation of the peat by the pathogen. The *F. oxysporum* infested peat, autoclaved soil was mixed in order to obtain final densities of 10^4 conidia per g of soil of *F. oxysporum*.

Ozone generation

O_3 gas was generated by passing dry oxygen gas through a corona discharge type O_3 generator (V can Network model M221, India). The dry oxygen supply and O_3 production were estimated with an O_3 analyser (BMT 961).

Open top chambers

Open top chambers (OTCs) were employed for the treatments. O_3 treatments in the OTCs (122 cm in height $\times$ 122 cm in diameter) was given to the seedlings after the emergence of 3rd and 4th leaves on the CAS in Botany Glass House, University of Madras, Chennai (Heagle et al. 1989; Chappelka et al. 1990) for two weeks. All the chambers were fitted with frustrums to remove excess of water. Control OTC was passed with non-filtered air (500 ml/min) (Cl-NF), other chambers were treated with O_3 twice a day. The non-filtered air contained approximately 40 – 60 ppb (parts per billion) of ambient O_3 (Senthilkumar 2005). Three pots were arranged centrally to the chamber and watered manually.

Ozone treatments

O_3 treatments in the OTCs were done twice a day, T_1 was supplied with 10 ppm (parts per million) of O_3 stress, T_2 treatment was supplied with 20 ppm, T_3 treatment was supplied with 30 ppm, T_4 with 40 ppm of O_3 and T_5 treatment was supplied with 50 ppm. The exposure of seedlings to O_3 lasted for only 2 minutes to avoid "physical ozone injury" to the seedlings.

Growth measurements, pigment and phenolic content analysis

After 10 days of O_3 exposure, plantlets were harvested for analyses. Growth variables such as shoot elongation, root length, fresh weight and dry weight were analysed immediately after harvest. For dry-weight measurements, samples were dried at 70°C for 48 h. The chlorophyll a, b and total were estimated according to the method of Aronon (1949); phenol content was measured by Swain and Hillis (1959).

Assay of induced enzymes

Tissue collection. The leaf portions of *Vigna unguiculata* plants treated with O_3 and control were collected after the 10 days O_3 exposure. Collected leaf portions were quickly frozen in liquid nitrogen and stored −70°C.

Assay of chitinase, β-1,3-glucanase, peroxidase, and phenylalanine ammonia-lyase

One gram of sample was ground using a chilled pestle and mortar with 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged at 10 000 rpm for 20 min. The supernatant was used as a crude enzyme extract for assaying chitinase activity. Instead of sodium citrate buffer, 0.05 M sodium acetate buffer (pH 5.0), 0.1 M phosphate buffer (pH 7.0) and 0.1 M sodium borate buffer (pH 7.0) at 4°C were used for extraction of β-1,3-glucanase, peroxidase and phenylalanine ammonia-lyase, respectively.

The changes in the chitinase and peroxidase activities were determined by colorimetric assays described by Boller and Mauch (1988) and Hammerschmidt et al. (1982), respectively, β-1,3-glucanaseactivity was assayed by the laminarin-dinitrosalicylic acid method (Pan et al. 1991). 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 amount of *trans*-cinnamic acid synthesised was calculated using its extinction coefficient of 9630 M^{-1}.

Induction of fusarium wilt

After O_3 treatment, the seedlings were transferred to the soil containing *F. oxysporum* conidia to induce *Fusarium wilt*.

Growing conditions

Pots were maintained under controlled environmental conditions (25 $\pm$ 2°C under 16 h photoperiod at a light intensity of 40 μE/m/s). Subsequently, plants were checked for the wilt symptoms. A minimum of 25–30 seedlings was grown for each treatment combination and each experiment was repeated four times.

Statistical analysis

Statistical analysis of the experiment data was performed using Agdata and Agres (ANOVA) package for researchers version 7.01 Pascal International Software (1994), and the treatment means were compared using least significant difference (LSD). All the values are represented in tables as means $\pm$ 95% confidence limits, and percentage values were subjected to arc sine transformation.

Results and discussion

The effect of O_3 on *Vigna unguiculata* seedlings by least contact generate fungal resistance through poorly understood mechanisms, which can be effectively used in the management of disease resistant in plants. The effective dosage of O_3 varies considerably for each plant species, hence the protective concentration of O_3 to *Vigna unguiculata* was investigated. In the present study, the results showed that the O_3 concentration at T_3 treatment inhibited the *Fusarium wilt*; it has also induced the defense-related enzyme activity, which was evidenced by the lower number of seedlings affected by *Fusarium wilt* in this treatment. Among the five treatments of O_3 exposure the shoot length increased up to 3.2% in T_1, T_2 about 8.1%, T_3 had the maximum length of 8.3%, T_4 of 5.4% and in T_5 4.9% of the control. Root length decreased in T_1 by 1.7%, T_2 by 0.7%, but in T_3 it increased to about 20%, T_4 also increased to 3.7% and in T_5 it reduced to 15.3% compared to control. Fresh weight of the seedlings decreased in T_1 about 4.1%, in T_2 it increased about 14.6%, T_3 attained the highest assessment of 77.0%, T_4 had an increase of 58.2% and T_5 also increased to 24.8% compared to control. The dry weight of the plants in T_1 decreased to 1.1%, T_2 also decreased to 24.1%, the maximum assessment was in T_3 an increase of about 20.7%, T_4 also had an increase of 15.1% and in T_5 the increase value was similar to that of T_4 which is 15.7% greater than control. O_3 stress caused morphological changes by means of an increase in the biomass (shoot, root length, fresh and dry weight) in T_3 treatment; the oxidative stress at minimum time duration makes increases the shoot length compared to the control (Table I).

Table I. Effect of O_3 treatments on morphological changes in *Vigna unguiculata* seedlings.

Treatments	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Cl-NF	10.60^{d}	6.95^{c}	1.410^{d}	0.178^{c}
T_1	10.94^{c}	6.83^{c}	1.351^{e}	0.176^{c}
T_2	11.46^{a}	6.90^{c}	1.616^{c}	0.135^{d}
T_3	11.48^{a}	8.38^{a}	2.496^{a}	0.215^{a}
T_4	11.18^{b}	7.21^{b}	2.231^{b}	0.205^{b}
T_5	11.12^{b}	5.88^{d}	1.761^{c}	0.206^{b}
LSD ($p = 0.05$)	0.1843	0.1191	0.0451	0.0028

Data are based on the means of four replicates. Data followed by the same letter in a column are not significant different from each other according to least significant difference (LSD). Transformed values are in parentheses.

The stimulated biomass increase was observed at an O_3 concentration of T_3 and T_4 treatments but even at these treatments, photosynthetic pigments reduced (Table II). By comparison to that of the photosynthetic pigments of O_3 treated with control chlorophyll A got reduced in T_1 to 13.7%, in T_2 it also got reduced to 45.2%, in T_3 the reduction was minimum to match up to all other treatments of 12.4%, in T_4 it was reduced to 17.8% and in T_5 it reduced to 43.9%. Chlorophyll B got reduced to 24.2% in T_1, in T_2 the reduction was 54.8%, in T_3 the reduction was 0.85%, in T_4 it was about 8% and in T_5 the reduction was 38.7% compared to control. The chlorophyll content was higher in Cl-NF, next to this comes T_3 treatment, but the chlorophyll content was affected severely in T_2 treatment compared to T_4 and T_5 treatments. Interestingly, similar results of O_3 affecting the chloroplast have been reported (Gross et al. 1969; Hill & Littlefield 1969; Mudd et al. 1971; Nobel & Wang 1973; Coulson & Heath 1974).

There was a steady increase in defense related enzyme activity in O_3 treated plants compared to control. The phenol concentration also increased up to 4.1% in T_1, in T_2 it increased about by 14.5%, in T_3 the raise was 29.1%, and in T_4 and T_5 treatments it was about a 25% increase compared to control. The phenolic content increased in the T_3 treatment compared to T_4 and T_5 (Table II). Among the treatments the T_3 treatment showed the higher PAL enzyme activity where as T_4 treatment shows the higher enzyme activity in chitinase, peroxidase and β-1,3-glucanase. Among all the treatments there was an increase in enzyme activity in all the O_3 treatments and T_4 treatment was the most effective for increasing enzyme concentration. The defense-related enzyme chitinase increased up to 6.6% in T_1, 12.9% in T_2, 40% in T_3, 40.3% in T_4 treatment and in T_5 treatment it showed about 31.7% increase relative to control (Figure 1a). β-1,3-glucanase also increased in all the treatments as in T_1 to 44%, in T_2 about 66%, in T_3 142%, in T_4 about 152.4% and in T_5 about 150.6% greater than control (Figure 1b). Peroxidase also increased in O_3 treated seedlings, as in T_1 it increased by up to 14.6%, in T_2 about 19.2%, in T_3 26.1%, in T_4 27.5% and in T_5 it was 25% greater than control (Figure 1c). Finally, PAL activity also increased in all the treatments; in T_1 it increased to 3%, in T_2 about 10.6%, in T_3 30.8%, in T_4 27.3% and in T_5 about 22.8% greater than control (Figure 1d).

In time course experiments, a maximum survivability of plants against *Fusarium wilt* was increased to 144.7% in T_3 treatment, 80% in T_4 treatment, 43.5% in T_1 treatment, 37% in T_2 treatment than control but it reduced in T_5 treatment to 16.7% (Table III). The protective effect of O_3 on the plants in T_3 treatment may be due to the enhancement of biomass and defense-related enzymes. In our previous work we successfully demonstrated that O_3 induced the defence responses by increasing the defence-related enzyme activities in tomato against cucumber mosaic virus (Sudhakar et al. 2007). These four defence-related enzymes are

Table II. Effect of O_3 treatments on biochemical changes in *Vigna unguiculata* seedlings.

Treatments	Chlorophyll a mg g^{-1}	Chlorophyll b mg g^{-1}	Total Phenol μm/g of FW of leaf tissue
Cl-NF	4.66^a	2.35^a	480^e
T_1	4.02^c	1.78^d	500^d
T_2	2.55^f	1.06^f	550^c
T_3	4.08^b	2.33^b	620^a
T_4	3.83^d	2.16^c	600^b
T_5	2.61^e	1.44^e	600^b
LSD ($p = 0.05$)	0.0113	0.0101	6.4275

Data are based on the means of four replicates. Data followed by the same letter in a column are not significant different from each other according to least significant difference (LSD). Transformed values are in parentheses.

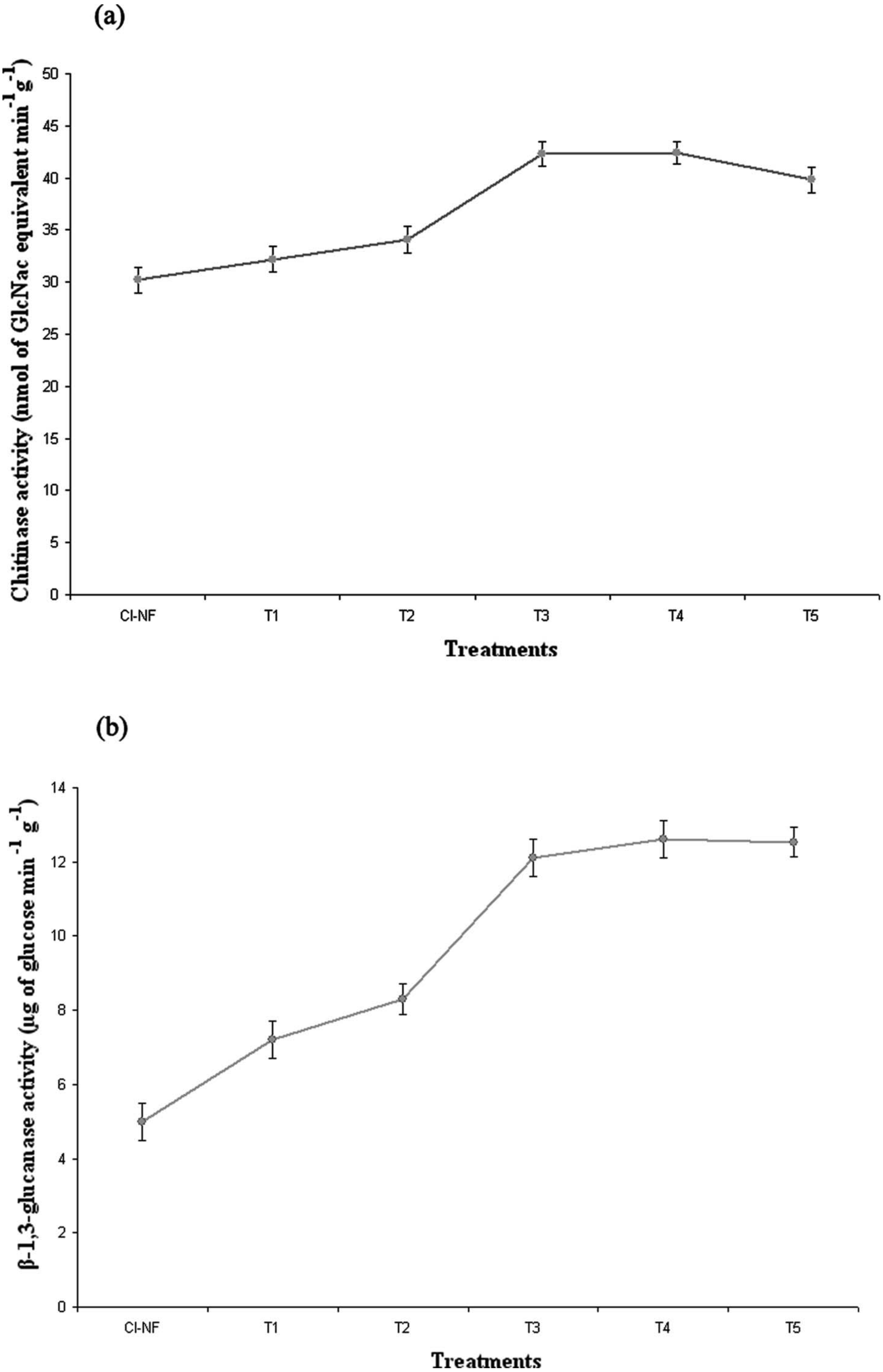

Figure 1. Changes in the activities of defence-related enzymes of *Vigna unguiculata* upon application of O_3: (a) chitinase, (b) β-1,3-glucanase, (c) peroxidase and (d) phenylalanine ammonia lyase. Data points are the mean of four replications in three sets of the experiment.

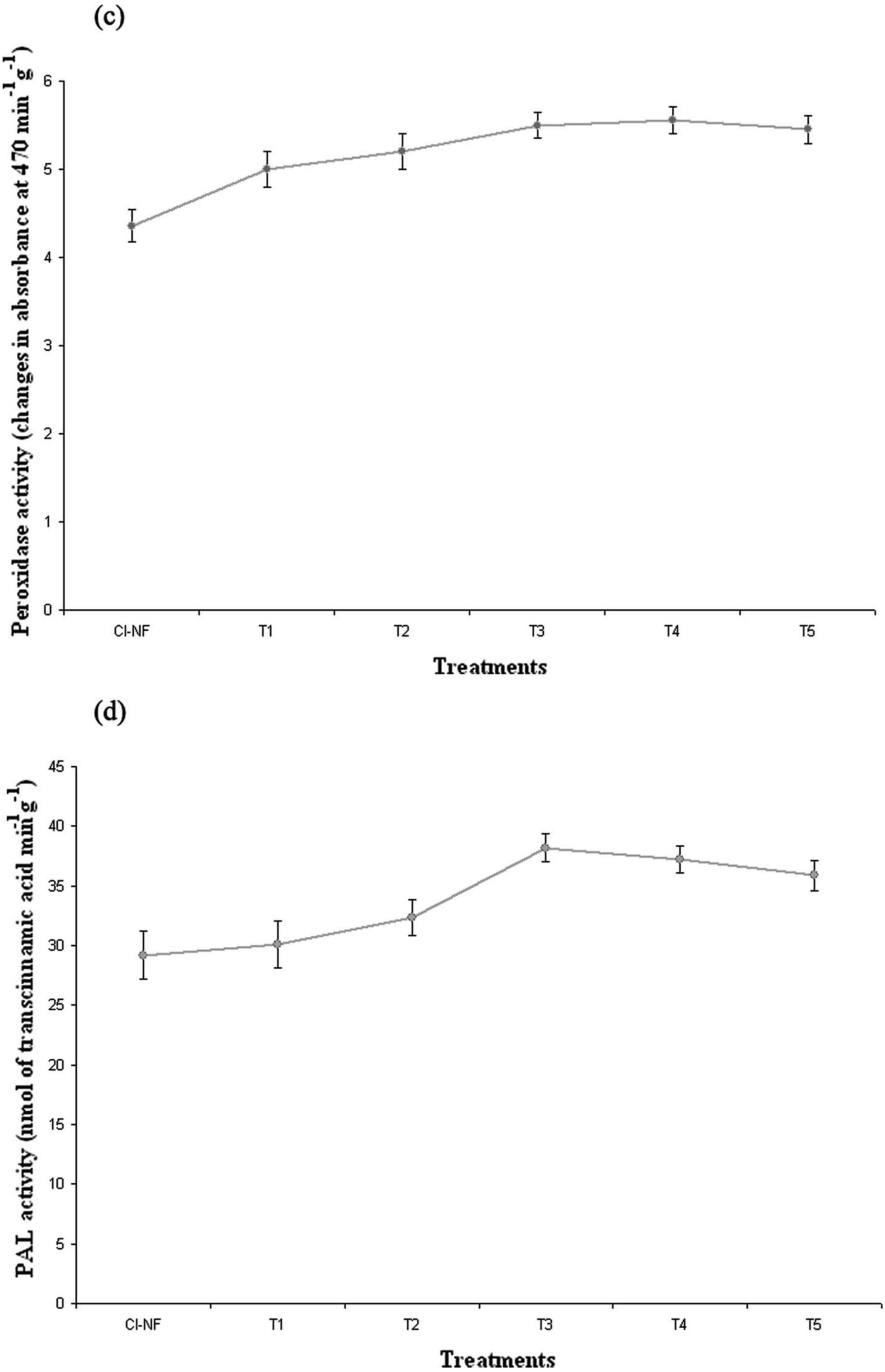

Figure 1. (*Continued*).

Table III. Survival of *Vigna unguiculata* plantlets in the infested soil with *Fusarium oxysporum*.

Treatments	Mean survivability level of plantlets
Cl-NF	12.26[d]
T_1	17.60[c]
T_2	16.80[c]
T_3	30.01[a]
T_4	22.08[b]
T_5	10.21[e]
LSD ($p = 0.05$)	0.9264

Data are based on the means of four replicates. Data followed by the same letter in a column are not significant different from each other according to least significant difference (LSD). Transformed values are in parentheses.

known to protect the plants from fungal attack either by direct inhibition of the pathogen or indirect activation of host defence responses including cell wall thickening (Zhu et al. 1994; Kombrink & Somssich 1995). Previous studies have suggested that the O_3 stimulates phenolic metabolism and biosynthesis of lignin or other substances partly derived from coniferyl alchol (Galliano et al. 1993; Kangasjärvi et al. 1994). The deposition of lignin and related phenolic products in cell walls increases their mechanical strength, decreases apoplastic solute conductance and permeability to water, and, in some cases, alters susceptibility to pathogens (Nicholson & Hammerschmidt 1992; Boudet et al. 1995), thus the increase in the phenolic content in O_3 treatments may be the reason behind the reduction in the *Fusarium wilt* compared to the control. PAL activity is essential for the synthesis of all protective substances induced in plant against biotic and a biotic stresses. PAL gets increased in when the callus are exposed to O_3 (Sgarbi et al. 2003).This also indicates that the increased content of PAL in T_3 treatment may be essential for reduction of the *Fusarium wilt*. Moreover, our findings indicate that in T_4 treatment there was an increase in chitinase, β-1,3-glucanase, and peroxidase enzymes which appears to be less effective than T_3 treatment in preventing the *Fusarium wilt*. The reason behind this protection in T_3 treatment than T_4 treatment against *Fusarium wilt* by O_3 is due to the assumption that an increase in the biomass helps to protect the plants.

Conclusions

This work gains importance for nursery growers to protect plants by pretreatment with O_3 which then develop resistance in the plants against *Fusarium wilt*. The observations of the present study are in agreement to promote healthy seedlings by induction of systemic resistance in *Vigna unguiculata* by O_3 treatment as elicitor. This warrants further research to examine the potential of O_3 to protect plants against fungal pathogens.

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