Turk J Bot
31 (2007) 135-141

Research Article

Effect of Ozone on Induction of Resistance in *Rhinacanthus nasutus* (L.) Kurz. against Acute Ozone Exposure

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Received: 10.05.2006
Accepted: 11.12.2006

Abstract: Studies were undertaken for induction of resistance against acute ozone exposure in *Rhinacanthus nasutus* (L.) Kurz. plants using ozone. Callus induced from *Rhinacanthus nasutus* leaf explants on Murashige & Skoog's (MS) medium supplemented with 3.40 µM of 2,4-dichlorophenoxy acetic acid (2,4-D) were treated with different concentrations of ozone (T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2), T_3 = 2.0 µmol mol^{-1} (±0.2)), and for the control (C) filtered air was supplied. Regeneration of shoots was obtained by culturing ozone-treated calli on MS medium supplemented with 5.57 µM of kinetin (KIN) and 1.30 µM of gibberellic acid (GA3). The frequency of regeneration of shoots from the callus was T_1 = 60%, T_2 = 49%, T_3 = 32%, but for the control 81% regeneration was obtained. Regenerated shoots were rooted in half-strength MS medium containing 4.92 µM of indole-3 butyric acid (IBA) and successfully acclimatised. The seedlings regenerated from ozone treated calli are referred to as T_1, T_2 and T_3 seedlings and the seedlings regenerated from filtered air-treated callus are referred to as control seedlings. T_1 seedlings hold remarkably more total soluble phenol content than T_2 and T_3 compared to the control seedlings. T_1 seedlings developed more resistance to withstand acute ozone exposure by increased phenylalanine ammonia-lyase activity and possessed more chlorophyll pigments and decreased H_2O_2 content relative to T_2 and T_3 seedlings compared to control seedlings.

Key Words: Callus, chlorophyll, soluble phenol, phenylalanine ammonia-lyase (PAL)

Introduction

The biological effects of ozone on plants have been studied for more than 50 years (Heggestad, 1991; Davison & Reiling, 1995). 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). 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). When the ozone concentration in the atmosphere increases, visible foliar injury will be observed in sensitive vegetation (Chappelka & Samuelson, 1998; Hales, 2003).

Plants have evolved a complex of defence response mechanisms to respond to 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 (Kangasjärvi et al., 1994). 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 derives 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).

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Based on these observations, a study was undertaken to determine resistance against ozone in *Rhinacanthus nasutus* (L.) Kurz. (Acanthaceae), an important medicinal plant, widely distributed in some parts of the sub-continent of India, China and South-East Asia. This plant is reported to possess anticancer (Punturee et al., 2004), antifungal (Sattar et al., 2004) and antiviral (Sendl et al., 1996) properties. An attempt was made to detect changes in total soluble phenol content in the callus and the plant developed from ozone-treated callus 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 in vitro propagated *Rhinacanthus nasutus* plant against acute ozone exposure by applying mild concentrations of ozone to the callus.

Materials and Methods

Development of callus and ozone generation:

Healthy young leaves of *Rhinacanthus nasutus* were collected from well-established mature herb from the fields of Madras University Botany Field Research Laboratory (MUFRL) at Maduravoyal. Leaf explants were surface sterilised and cultured on modified MS basal medium (Murashige & Skoog, 1962) containing 3.40 µM of 2,4-dichloro phenoxy acetic acid (2,4-D) for callusing. For each experiment 15 replicates were used and all the experiments were repeated at least 3 times.

Ozone gas was generated by passing dry oxygen gas through a corona discharge type ozone generator (V can Network model M221) supplied with dry oxygen and the output was estimated with an O_3 analyser (BMT 961). The stability of the ozone concentration in the fumigation chamber during the ozone exposure period was monitored carefully and estimated by UV photometric ozone analyser (Thermo Environmental Instruments, Franklin, MA, USA).

Ozone treatments:

Thirty-day-old well-developed *Rhinacanthus nasutus* calli were treated with different concentrations of ozone T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2) and T_3 = 2.0 µmol mol^{-1} (±0.2) for 5 min a day repeatedly for 7 days. All the treatments (T_1, T_2, T_3 and C) were done in sterilised closed chambers using the method described previously (Sgarbi et al., 2003). The callus was left undisturbed and incubated at 25 ± 2 °C under 16-h photoperiod at a light intensity of 40 µE m^{-2} s^{-1} (cool-white fluorescent tubes). For each treatment, 15 replicates were made and each experiment was repeated at least 3 times.

Growing conditions and ozone exposure:

The ozone-treated calli were transferred to a medium containing 5.57 µM of kinetin (KIN) and 1.30 µM of gibberellic acid (GA_3) for shooting. The developed shoots were transferred to a rooting medium containing 4.92 µM of indole-3 butyric acid (IBA). Rooted seedlings from which the agar-based medium was removed under running tap water were individually transferred to 10-cm plastic pots containing soil and peat moss in a 1:1 ratio. In order to prevent fungal infection, seedlings were watered with 0.5 g/l bavistin solution, after which each pot was covered with a plastic bag. The pots were maintained under controlled environmental conditions (25 ± 2 °C less than 16-h photoperiod at a light intensity of 40 µE m^{-2} s^{-1}) and the plastic bags were progressively opened over a 2-week period.

The grown seedlings were treated with acute ozone exposure in the open top chambers (Heagle et al., 1989) (122 cm in height x 122 cm in diam.). All the chambers were fitted with frustums to remove excess water. T_1, T_2 T_3 and control pots were arranged centrally in the chambers and subjected to a 100 ppb concentration of ozone at a flow rate of 1 l/min, 30 min a day for 7 days. Seedlings were harvested after the acute ozone exposure for the analysis of the effect of acute ozone exposure on the seedlings.

Chlorophyll Extraction and Assay:

Samples consisting of 2.5-cm^2 leaf disks were frozen in liquid nitrogen and stored at –80 °C. Chlorophyll was extracted from leaf tissue using dimethylformamide in the dark for 48 h at 4 °C. The extract was then assayed for chlorophyll (Chl) using the spectrophotometric procedure described by Wellburn & Chitenthaler (1984).

H_2O_2 Measurements:

H_2O_2 levels in the leaves were determined as described by Creissen et al. (1999).

Extraction and Estimation of Phenol:

The method reported by Swain & Hillis (1959) was used for the extraction and quantification of total soluble phenolics. Fresh calli (1 g) and seedling leaves (1 g) were extracted separately in 80% methanol for 90 min at 80 ºC. The extracts were centrifuged at 14,000 g for 15 min, and 100 µl of the extract was diluted to 1 ml with water and mixed with 0.5 ml of 2.0 M Folin-Ciocalteu's reagent and 0.5 ml of 1 M Na_2CO_3. After 1 h, absorbance of the sample solution was measured at 725 nm using a T 11 7 spectrophotometer (Systronics, India). Concentration of total soluble phenolics in the extracts was calculated from a standard curve prepared with gallic acid.

Phenylalanine ammonia-lyase Activity (PAL):

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). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30 ºC. In the reference cell, 0.4 ml of enzyme extract was taken along with 1 ml of borate buffer. The amount of trans-cinnamic acid synthesised was calculated as described by Dickerson et al. (1984). Enzyme activity was expressed on a fresh weight basis (n mole of trans-cinnamic acid $min^{-1}\ g^{-1}$).

Results and Discussion

Green morphogenic calli were induced from *Rhinacanthus nasutus* leaf explants within 2 weeks of culture on MS medium supplemented with 3.40 µM of 2,4-D. When 30-day-old callus was exposed to different concentrations of ozone (T_1, T_2 and T_3) fresh and dry weight of the callus decreased as the concentration of ozone increased (Table 1). The reduced callus growth may be due to the biological effects of O_3 on plants (Heggestad, 1991; Davison & Reiling, 1995). A number of studies have involved the phytotoxicity of ozone and its effects on the primary and secondary metabolism of plants (Kangasjärvi et al., 1994; Sandermann et al., 1998). Regeneration of multiple shoots was noted in all treatments within 2 weeks following transfer to the MS medium containing 5.57 µM KIN and 1.30 µM GA_3. Eighty-one percentages of the shoots were regenerated successfully in the control (treated with filtered air), whereas in T_1, T_2 and T_3 treatments the frequency of regeneration of shoots was about 60%, 49% and 32%, respectively (Table 2). Regenerated shoots were rooted in half-strength MS medium supplemented with 4.92 µM IBA and successfully acclimatised. The seedlings regenerated from ozone-treated callus are referred to as T_1, T_2 and T_3 seedlings and the plants regenerated from filtered air-treated callus are referred to as control seedlings. Survivability rates of the seedlings (C, T_1, T_2 and T_3) in the greenhouse were 85%, 82%, 72% and 65%, respectively (Table 2).

Table 1. Fresh and dry weights of callus (*Rhinacanthus nasutus*) after different treatments of ozone.

Period	Control		T_1		T_2		T_3	
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
60 days	12.43 (± 1.82)	4.02 (± 0.51)	9.08 (± 1.43)	2.92 (± 0.44)	7.16 (± 1.43)	2.36 (± 0.62)	5.18 (± 1.58)	1.72 (± 0.40)
90 days	28.74 (± 6.42)	9.21 (± 1.36)	24.36 (± 2.64)	7.04 (± 1.02)	18.32 (± 2.48)	5.93 (± 0.92)	14.16 (± 2.24)	4.12 (± 0.74)

Each value is mean ± SD of 3 experiments with 15 replicates each, where T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2), T_3 = 2.0 µmol mol^{-1} (±0.2), Control = filtered air.

Table 2. Effect of ozone treatment on frequency of regeneration and survivability of *Rhinacanthus nasutus*.

Treatments	Frequency of regeneration[1]	Frequency of survivability[2] in %.
C	81.31 (± 3.23)	85.40 (± 4.22)
T_1	60.22 (± 4.03)	82.12 (± 3.45)
T_2	48.92 (± 1.22)	75.46 (± 2.83)
T_3	32.33 (± 2.43)	62.12 (± 2.61)

Values are mean ± SD of 3 experiments with 15 replicates each, where T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2), T_3 = 2.0 µmol mol^{-1} (±0.2), C = filtered air.

[1] = the plants after ozone treatment. [2] = plants after acclimatisation.

Callus after the ozone treatment produces signal substances, phytoalexins, antioxidant compounds and synthesis of substances involved in the formation of the cellular barriers (Schraudner et al., 1996; Sandermann et al., 1998). There is an interaction between the O_3 exposures to callus and the innate resistance to the seedlings developed because ozone or its oxidation by-products (i.e. free radicals) migrates into the cellular organelles; thereby it influences the metabolic process and leads to significant alterations in biochemical and physiological processes. When ozone penetrates into the intracellular spaces, it is rapidly decomposed, creating reactive oxygen species (ROS) and giving rise to the oxidative burst (Bolewell, 1996). It is generally held that this oxidative process induces several defence reactions in the seedlings. The action of ozone as well as the reaction of the callus may vary depending upon the genetic background, growth conditions, time duration and accumulated ozone dose applied. All the seedlings (T_1, T_2, T_3 and C) were examined for their ability to withstand acute ozone exposure by open top chamber study.

Following acute ozone exposure, the extent to which seedlings were injured by acute ozone exposure was analysed using chlorophyll content. Wallin et al. (1990) and Skärby et al. (1995) reported that exposure to ozone resulted in a reduction in chlorophyll content that indicates the severity of injury caused by ozone. Twenty-four hours after acute ozone exposure, the level of Chl *a* was higher in T1 = 11%, T_2 = 6% and T_3 = 1% compared to control leaves (Figure 1). Likewise, the level of Chl *b* content was also higher among the 3 lines (T_1 = 8%, T_2 = 6%, T_3 = 4%) compared to control leaves (Figure 1).

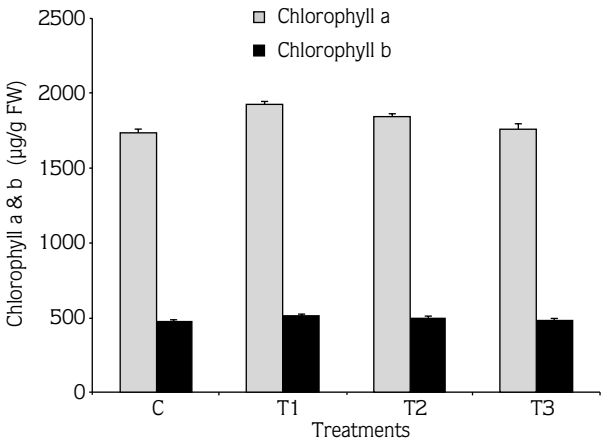

Figure 1. Chlorophyll *a* & *b* content in the first fully expanded leaves of C, T_1, T_2 and T_3 *Rhinacanthus nasutus* seedlings 24 h after acute ozone exposure.
Each bar is mean of 15 replicate samples (± SE), where T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2), T_3 = 2.0 µmol mol^{-1} (±0.2), C = filtered air.

To determine whether the foliar level of H_2O_2 increased following acute exposure to ozone, the amount of H_2O_2 was measured in leaves of T_1, T_2, T_3 and control seedlings. Twenty-four hours after acute ozone exposure, the foliar level of H_2O_2 was significantly lower in T_1 leaves (55%), followed by T_2 (33%) and T_3 (11%) leaves relative to control leaves (Figure 2). These results reveal that T_1 leaves are consistent with an increased ability to detoxify invading ozone.

Ozone stress or injury to plants can stimulate the production of phenolic compounds (Sgarbi et al., 2003), including lignin and suberin (Rhodes & Wooltorton, 1978). Phenolic acids are involved in phytoalexin accumulation, biosynthesis of lignin and formation of structural barriers, which play a major role in resistance to stress. Several associations have been reported between phenolics and the resistance of plants to pathogens (Panda & Khush, 1995). In the present investigation, it is evident from Figure 3 that total soluble phenol content was enhanced up to 3-fold in T_1, 2-fold in T_2 and 1-fold in T_3 calli compared to control calli. Similarly, 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. Due to the increased phenol content, seedlings developed from the ozone-treated calli possess elevated phenol content (T_1 = 3-fold, T_2 = 2-fold, T_3 = 1-fold) compared to control seedlings (Figure 3). 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 stress (Boudet et al., 1995). Rohde (1972) indicated the possible role of preformed simple phenols in the incompatible host and parasite interactions.

Rapid increases in transcript levels for phenylalanine ammonia-lyase in response to ozone have been observed in parsley (Eckey-Kaltenbach et al., 1994), soybean (Tingey et al., 1975) and tobacco (Bahl et al., 1995). Phenylalanine ammonia-lyase levels in T_1, T_2 and T_3 seedlings are relatively high compared to control seedlings. T_1 seedlings demonstrates 51% increased activity, but T_2 manifests only 37% and T_3 shows 14% increased PAL activity compared to control seedlings (Figure 4) 24 h after acute ozone exposure. T_1 seedlings possess a greater defence response in nature against stress conditions than T_2 and T_3 seedlings. PAL activity is essential for the synthesis of all the protective substances induced in plants against stresses (Pascholati et al., 1986). PAL increases when the callus is exposed to ozone (Sgarbi et al., 2003). 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 supplying carbon skeletons for secondary products (Ramanathan et al., 2000). The observed increase in PAL activity in ozone-treated plants was presumably related to the lignification

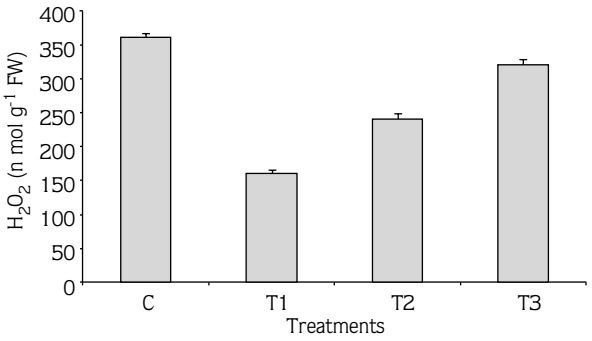

Figure 2. H_2O_2 content in the leaves of C, T_1, T_2 and T_3 *Rhinacanthus nasutus* seedlings 24 h after acute ozone exposure.
Each bar is mean of 15 replicate samples (± SE), where T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2), T_3 = 2.0 µmol mol^{-1} (±0.2), C = filtered air.

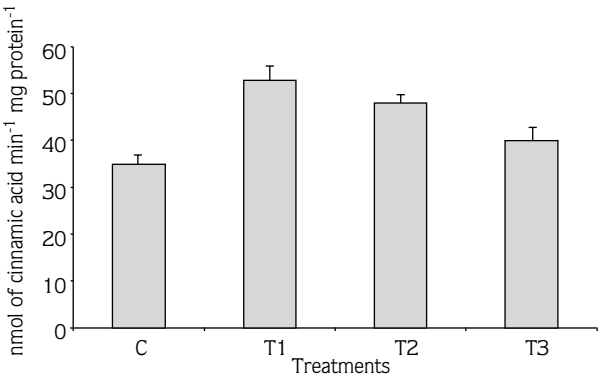

Figure 3. Total soluble phenolic content in the callus and leaves of C, T_1, T_2 and T_3 *Rhinacanthus nasutus* seedlings.
Each bar is mean of 15 replicate samples (± SE), where T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2), T_3 = 2.0 µmol mol^{-1} (±0.2), C = filtered air.

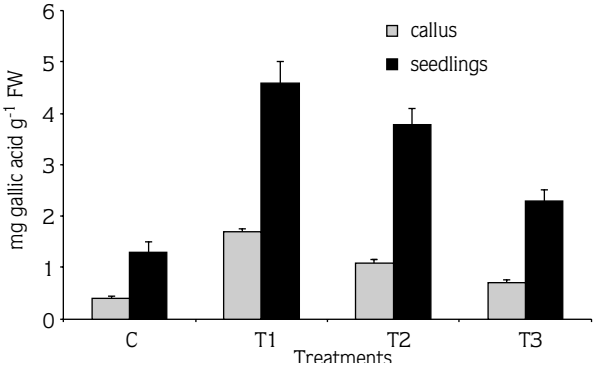

Figure 4. Effect of ozone on phenylalanine ammonia lyase (PAL) activity in *Rhinacanthus nasutus* seedlings 24 h after acute ozone exposure.
Each bar is mean of 15 replicate samples (± SE). T_1 = 1.0 µmol mol^{-1} (±0.2), T_2 = 1.5 µmol mol^{-1} (±0.2), T_3 = 2.0 µmol mol^{-1} (±0.2), C = filtered air.

process. Cell wall lignifications might be caused by an induction by ozone on plants normally induced as a defence (Sandermann, 1996). It is evident from the present investigation that callus treated with mild ozone (T_1) activate at least some components of ozone resistance in seedlings to detoxify the invading ozone.

Acknowledgements

We thank Prof. N. Anand. D.Sc, Director, CAS in Botany, University of Madras, for providing us with the laboratory facilities.

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