POST-INFECTIONAL CHANGES ASSOCIATED WITH THE PROGRESSION OF LEAF SPOT DISEASE IN *WITHANIA SOMNIFERA*

A. Sharma, I. Sharma and P.K. Pati

Department of Biotechnology, Guru Nanak Dev University, Amritsar, India

SUMMARY

Leaf spot caused by *Alternaria alternata* is a prevalent disease of *Withania somnifera*, a high value medicinal plant. The severity of infection is closely related with the progression of the disease. In the present work, various biochemical parameters such as free proline, total chlorophyll, total protein, lipid peroxidation, ion leakage and the activity of some antioxidant enzymes were analyzed to assess the post infectional changes associated with infection. As disease progressed, a decrease of chlorophyll and total protein content was observed. The lowest level of total chlorophyll (16 fold decrease) was observed when the severity of infection was at its peak. A significant increase in free proline content, lipid peroxidation and ion leakage was also observed. Antioxidant enzymes behaved in a differential way. Catalase, ascorbate peroxidase, guaiacol peroxidase and superoxide dismutase specific activities showed a significant increase concomitantly with disease progression. By contrast, glutathione reductase and monodehydroascorbate reductase decreased significantly (2.5 and 6.5 folds, respectively) as disease progressed. Besides superoxide dismutase, the other antioxidant enzymes analysed did not have a significant correlation with the number of spots/cm^2 leaf area. Although the present study suggests a complex pattern of post infectional changes, the information obtained is useful for a better understanding of host pathogen interaction.

Key words: Alternaria alternata, catalase, lipid peroxidation, plant disease, reactive oxygen species, superoxide dismutase.

INTRODUCTION

Withania somnifera is an important medicinal plant in the Indian, Unani and African traditional medicine (Kulkarni *et al.*, 1996; Murthy *et al.*, 2008). It contains many phytochemically active components like withaferins and withanolides (Chatterjee *et al.*, 2010) and is known for its immunomodulatory (Rasool and Varalakshmi, 2006), antistress (Archana and Namasivayan, 1999), cardioprotective (Mohanty *et al.*, 2004), anti-aging (Singh *et al.*, 2008) and anti-tumour (Uma Devi, 1996) properties. It was recently reported that *W. somnifera* leaf extracts selectively kill cancer cells (Widodo *et al.*, 2007).

W. somnifera is prone to attacks by several fungal pathogens (Verma *et al.*, 2007; Maiti *et al.*, 2007; Mahrshi, 1986), viruses (Pathak and Raychoudhuri, 1967), phytoplasmas (Khan *et al.*, 2006; Samad *et al.*, 2006), insects (Kumar *et al.*, 2009; Sharma and Pati, 2011), and nematodes (Sharma and Pandey, 2009). Among fungal diseases, leaf spot caused by *Alternaria alternata* is the most prevalent and responsible for substantial biodeterioration of its pharmaceutically important constituents (Pati *et al.*, 2008).

In this paper, an attempt was made to study the post-infectional changes associated with the leaf spot disease of *W. somnifera*. Various biochemical parameters, including chlorophyll content, total protein content, free proline, lipid peroxidation, ion leakage and changes in the activities of some important antioxidant enzymes were analysed. Although the effects of triadimefon-induced salt tolerance and copper toxicity on the antioxidant system of *W. somnifera* have been described (Jaleel *et al.*, 2008a, 2008b; Khatun *et al.*, 2008), to our knowledge, no studies have been conducted on the biochemical changes associated with the progression of the leaf spot disease.

MATERIAL AND METHODS

Maintenance of healthy and diseased plants *W. somnifera* plants grown in the screenhouse of the Department of Biotechnology, Guru Nanak Dev University, Amritsar (India) (74.82323-74.82332°E, 31.63678-31.63688°N, and 221 meters above sea level) were used in the present work. *A. alternata,* the fungal pathogen isolated from leaf spot-diseased plants (Pati *et al.*, 2008), was deposited in the Microbial Type Culture

Corresponding author: P.K. Pati
Fax: +91.183.2258272
E-mail: pkpati@yahoo.com

Collection and Gene Bank of the Institute of Microbial Technology, Chandigarh (India), under the accession No. MTCC-9617 (http://mtcc.imtech.res.in). This pathogen was inoculated to healthy plants to induce infection, as described by Pati *et al.* (2008). Diseased and healthy plants were maintained separately, under similar conditions of temperature and relative humidity.

Biochemical analysis. Various biochemical analyses were carried out on fourth or fifth fully expanded leaves from the apex from 15 plants, for each disease severity level. Disease severity was rated on a four point scale based on number of spots per leaf and average number of spots per unit leaf area (disease severity level 0, 1, 2 and 3) (Table 1). Samples from all severity levels were homogenized separately and used for various biochemical analysis. Healthy leaves without any spot served as control (Level 0).

Chlorophyll content. The amount of chlorophyll was quantified according to Arnon (1949). One hundred mg of freshly harvested leaves were homogenized in liquid nitrogen. After adding 1.5 ml of 80% acetone, the slurry was centrifuged at 19,000 *g* for 10 min and the absorbance of the supernatant was measured at 645 and 663 nm against 80% acetone as blank. Chlorophyll content was determined by following formulae:

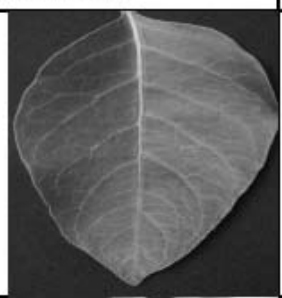
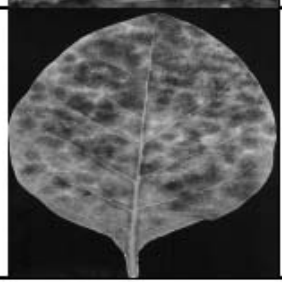
$$\text{Total Chl } (\mu g/ml) = 20.2\ (A_{645}) + 8.02\ (A_{663})$$

$$\text{Chl A} = 12.7\ (A_{663}) - 2.29\ (A_{645})$$

$$\text{Chl B} = 22.9\ (A_{645}) - 4.68\ (A_{663})$$

Free proline content. The free proline content was determined as described (Bates *et al.*, 1973). Five hundred

Table 1. Gradation of sample into four point scale based on severity of infection.

Sample name and disease progression on four point scale		Number of spots/leaf	Spots/cm² leaf area
Healthy (level 0)		-	-
Disease start (level 1)		0.0 to 30.0	0.0 to 1.0
Disease intermediate (level 2)		30.0 to 50.0	1.0 to 1.5
Disease extreme (level 3)		≥50.0	≥1.5

mg of leaf tissues were homogenized in liquid nitrogen, 1.8 ml of 3% sulphosalicylic acid were added and the mixture was centrifuged at 12,000 *g* for 15 min. Two ml of acid ninhydrin and glacial acetic acid were added to 0.8 ml of the supernatant and heated in a water bath at 100°C for 1 h. The reaction was stopped by transferring the tubes into ice. Four ml of toluene were then added and mixed thoroughly by shaking for 15-20 sec. The toluene layer containing the red-coloured proline-ninhydrin product was then separated and warmed to room temperature. Absorbance of the toluene layer was measured at 520 nm. Different known concentrations of free proline were treated in similar manner as standards. Concentrations of free proline in the samples were calculated from the standard curve against their respective absorbance at 520 nm.

Lipid peroxidation. Lipid peroxidation was determined by measuring malondialdehyde (MDA), the end product of polyunsaturated fatty acid oxidation. MDA equivalents/g fresh weight were measured according to Hodges *et al.* (1999) with slight modifications. Leaf tissue (1 g) was homogenized in 80% ethanol and centrifuged at 8,500 *g* for 10 min. One ml aliquots of the supernatant were added to two different test tubes, one containing 1 ml of 20.0% TCA (trichloro acetic acid) (w/v) (-TBA solution) and other containing 0.65% TBA (thiobarbituric acid) (w/v) in 20% TCA (w/v) (+TBA solution). Samples were then mixed vigorously and were heated at 95°C. The reaction was stopped after half an hour by transferring the tubes to ice. Samples were then centrifuged at 12,000 *g* for 10 min and their absorbance measured at 440 nm, 532 nm, and 600 nm. Malondialdehyde equivalents were calculated as follows:

$$\text{(i) } [(\text{Abs } 532_{(+\text{TBA})}) - (\text{Abs } 600_{(+\text{TBA})}) - (\text{Abs } 532_{(-\text{TBA})} - \text{Abs} 600_{(-\text{TBA})})] = \text{A}$$

$$\text{(ii) } [(\text{Abs } 440_{(+\text{TBA})} - \text{Abs } 600_{(+\text{TBA})})\ 0.0571] = \text{B}$$

$$\text{(iii) MDA equivalents (nmol ml}^{-1}) = (\text{A–B}/157000)\ 10^6$$

Ion leakage. Ion leakage, one of the indicators of cell death, was measured following the protocol of Mittler *et al.* (1999). For every measurement, five leaf discs (9 mm diameter) from each severity level were cut with a cork borer and floated, abaxial side up, on 5 ml of distilled water for 3 h at room temperature. Conductivity of the bathing solution was measured after incubation with the Equip-Tronics conductivity meter EQ 661(Equiptronics, India). These values were referred as A. Leaf discs were then returned to the bathing solution, introduced into sealed tubes and incubated at 95°C for 25 min. After incubation, the bathing solution was cooled to room temperature and its conductivity was again measured and referred to as B. For each measurement ion leakage was expressed in terms of percent leakage [(A/B) × 100].

Protein extraction. Leaf tissues from all stages of disease progression (level 0-3) were homogenized in liquid nitrogen in a pre-chilled mortar and pestle into fine powder. One gram leaf powder was then mixed with 3 ml of pre-chilled 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM DTT (Dithiothreitol), 1 mM PMSF (Phenylmethylsulfonyl fluoride), 0.5% (v/v) Triton X-100 and 10% (w/v) PVPP (Polyvinyl Polypyrrolidone) as described by Vyas *et al.* (2007). The homogenate was centrifuged at 13,000 *g* for 20 min at 4°C. The supernatant was further used for analysis of enzyme activities and protein quantification.

Total protein content. Total protein content of healthy and diseased leaves was determined by the method of Bradford (1976) using a protein estimation kit (Bradford macro method, Cat # KT33, India) taking bovine serum albumin (BSA) as standard. Standard curve was plotted between different known concentrations of BSA and their respective absorbance to determine the protein content of leaf samples. In another experiment, the protein content was determined of detached leaves treated with culture filtrate of *A. alternata* and incubated for 24 h at 25°C. Leaves treated with sterile distilled water served as control.

Analysis of antioxidant enzymes. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed spectrophotometerically as described by Nakano and Asada (1981). The 1.0 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) with 0.1 mM EDTA, 0.5 mM ascorbate and 1 mM H_2O_2 (freshly prepared). Reaction was initiated by the addition of 10 µl of enzyme extract. H_2O_2-dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm (ε = 2.8 mM/cm). The reaction was carried out for 3 min at 25°C and enzyme activity was normalized for 1 min. One unit of APX activity is defined as the amount of enzyme that can oxidize 1 µM of ascorbate per minute per gram (min/g) tissue.

Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the initial rate of disappearance of H_2O_2 according to Aebi (1984), with slight modifications. The 1.0 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 (freshly prepared) and 20 µl of enzyme extract. Reaction was carried out at 25°C and was initiated by the addition of enzyme extract and the decrease in H_2O_2 was followed as decline in absorbance at 240 nm for 1 min at the intervals of 6 seconds (ε = 39.4 mM/cm). One unit of enzyme activity is defined as the amount of enzyme decomposing of 1 micromole H_2O_2/min/g tissue.

Glutathione reductase (GR, EC 1.6.4.2) activity was estimated as described by Jahnke *et al.* (1991) and modified after Vyas *et al.* (2007). The 1.0 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM GSSH, 1 mM EDTA and 25 µl of

enzyme extract. To initiate the reaction 0.1 mM NADPH was added and oxidation of NADPH was monitored at 25°C, in terms of decrease in absorbance at 340 nm for 1 min, at 6 sec intervals (ε = 6.22 mM/cm). Unit activity of glutathione reductase is defined as the enzyme catalyzing the reduction of one micromole of GSSG/min/g tissue at pH 7.8 and 25°C.

Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was determined by the method of Fernández-García *et al.* (2004). The 1.0 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2, 9 mM guaiacol and 100 µl enzyme extract. The GPX activity was determined by measuring the absorbance at 436 nm for 1 min at 25°C (ε = 26.6 mM/cm). One unit of GPX activity represents the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol/min/g tissue.

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed as described by Hossain *et al.* (1984). The 1.0 ml reaction mixture contained 50 mM Tris-HCl (pH 7.6) with 2.5 mM ascorbic acid, 0.15 units of ascorbic acid oxidase (Sigma-Aldrich, USA), 50 µl of enzyme extract and 0.1-0.2 mM NADH/NADPH. The reaction was started by adding ascorbic acid oxidase and the decrease in absorbance at 25°C was measured at 340 nm (ε = 6.2 mM/cm) for 1 min. One unit of MDHAR activity is defined as the amount of enzyme required to oxidize one micromole of NADH/min/g tissue.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated using Beyer and Fridovich (1987) method modified after Vyas *et al.* (2007). The 1.0 ml reaction mixture contained 50 mM phosphate buffer pH 7.0 containing 2 µM riboflavin, 100 µM EDTA, 75 µM NBT (Nitro blue tetrazolium chloride) and 13 µM DL-methionine (added at the end) and 16 µl of enzyme extract. After illumination with strong fluorescent light (400 µM/m^2/s) for 10 min, absorbance was measured at 560 nm with a Perkin Elmer Lambda 25 UV-Visible spectrophotometer. The reaction mixture without enzyme extract kept under similar conditions served as blank. SOD activity was determined from the percent inhibition of NBT reduction as compared with illuminated blank showing maximum color change at 25°C and enzyme activity was normalized for 1 min. One unit of enzyme activity is defined as the amount of enzyme required for 50% inhibition of NBT reduction at 25°C.

Statistical analysis. All analyses were performed in triplicate and values were presented as mean ± standard errors. To compare differences in the means, one way analysis of variance (ANOVA) with LSD (least significant difference) at p≤0.05 (5% level of significance) was performed. Student's t-test was used to compare difference of the means in paired sample data. To correlate the various biochemical parameters with disease progression, Pearson's correlation coefficients were calculated with p≤0.05. SPSS software for WINDOWS version 16.0 (SPSS USA) and Microsoft Office Excel 2003 (Microsoft, USA) were used to conduct the statistical analysis.

RESULTS

Quantitative expression of disease progression. Inoculated plants showed initial symptoms of leaf spot disease within 2 weeks. The severity of infection increased gradually. The number of spots per leaf was measured as an indicator of disease severity, which was rated on a four point scale (level 0 to level 3). During disease initiation (level 1) the mean number of spots/leaf was 22.6±3.203, which increased to 41.8±1.855 in intermediately infected leaves (level 2) and further increased to 58.6±6.638 in most severe infection (level 3) 5-6 weeks after onset of symptoms. Spots/cm^2 leaf area were also recorded with each stage of disease development and found increase gradually with disease progression (Table 1). During the initial phase of the disease the number of spots/cm^2 of leaf area was 0.6±0.030, which increased to 1.4 ±0.102 in intermediate disease condition and increased further to 1.9±0.236 in heavily diseased leaves.

Biochemical analysis. It was observed that pathogen invasion and establishment had significant bearing on chlorophyll A, B and total chlorophyll content (Fig. 1a). Compared to healthy leaves, the total chlorophyll content decreased by 219.76 µg/g FW at disease initiation stage (level 1) and then further declined as disease severity level move from level 2 (92.71± 21.29 µg/g FW) to level 3 (27.71±4.52 µg/g FW). Similarly, a significant decrease in chlorophyll B was also monitored along with the progression of disease from level 0 to level 3 (117.59± 20.38 to 39.56±5.92 µg/g FW). A non significant decrease in total protein content was observed at each stage of disease progression, the highest being recorded in healthy leaves (84.26±5.16 µg/g FW) and the lowest in level 3 leaves (73.10±2.02 µg/g FW). On the contrary, detached leaves treated with the culture filtrate of *A. alternata* resulted in significant reduction in protein content compared to the control (Fig. 2). The mean free proline content increased significantly with disease progression (Fig. 1b). It was lowest in healthy leaves (3.29±0.06 µg/g FW) and highest in level 3 leaves (11.66±0.06 µg/g FW). An increasing trend in MDA content was observed with respect to disease development (Fig. 1c). MDA content increased significantly in level 2 (26.480±4.17 nmol/g FW) and level 3 (34.32 ±6.19 nmol/g FW) condition compared to level 0 (6.59±0.51 nmol/g FW).

There was a significant increase in the percent ion leakage from leaf tissues from level 0 to level 3 with dis-

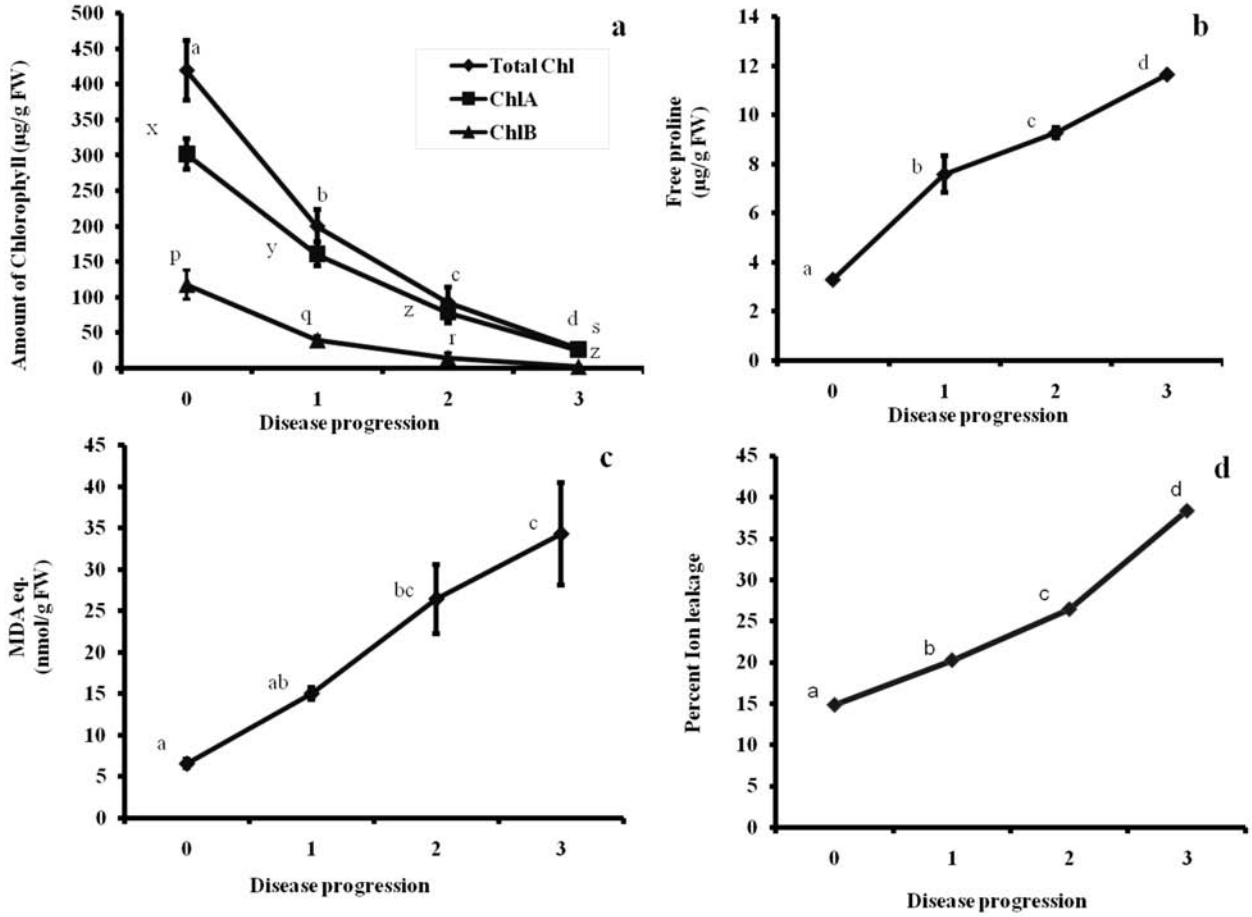


Fig. 1. Effect of leaf spot disease progression on: a. Chlorophyll content; b. Free proline; c. Lipid peroxidation; d. Ion leakage. Values of the same biochemical test with the same letter are not significantly different from each other (LSD, p≤0.05).

ease progression, reaching a maximum (38.37± 0.25) in level 3, where the severity of the infection was the highest (Fig. 1d).

Analysis of antioxidant enzyme. Analysis of the activities of antioxidant enzymes showed a varied response to disease progression (Fig. 3). The activity of antioxidant enzymes, APX, CAT, GPX and SOD exhibited an increasing trend in response to disease whereas the activity of MDHAR and GR decreased. A gradual change in APX activity was monitored with disease progression. It initially dropped in level 1 to shoot up (Fig 3a) in level 3 (0.033±0.01 mol UA/mg protein). A similar trend was also observed when CAT activity was analyzed (Fig 3b). GR activity showed a different trend with sudden significant fall with disease initiation (level 1) and did not differ significantly with further disease progression. There was a *ca.* 2.5 fold decrease in GR activity from level 0 to level 3 (Fig 3c). GPX activity showed a significant increment from level 0 to level 1 (0.027±0.001 mol UA/mg protein). Its activity increased further in level 2 leaves (0.030±0.001 mol UA/mg protein), but this increase was not significant when compared to level 1 (Fig 3d). However, further progression of disease to level 3 (0.022±0.003 mol UA/mg protein), resulted in a significant decrease in its activity than level 2. MDHAR activity showed a significant decline from

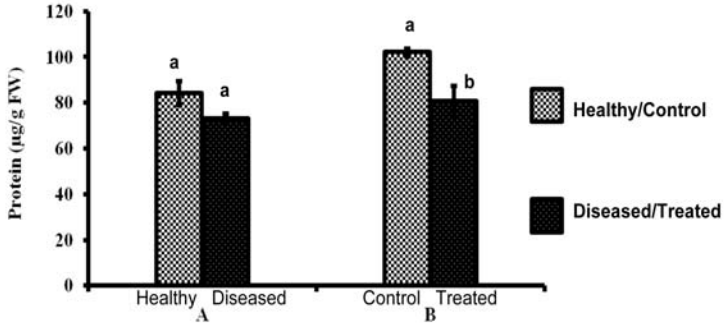


Fig. 2. Analysis of total protein content. A. Comparison of healthy (level 0) and diseased (level 3) leaves infected with *A. alternata*. B. Comparison of detached leaves treated with sterile distilled water and the culture filtrate of *A. alternata*. Values are represented as mean ± SE, n=3. Same letter within the same experiment represents that values are not significantly different from each other (Paired sample Student's t-test, p≤0.05).

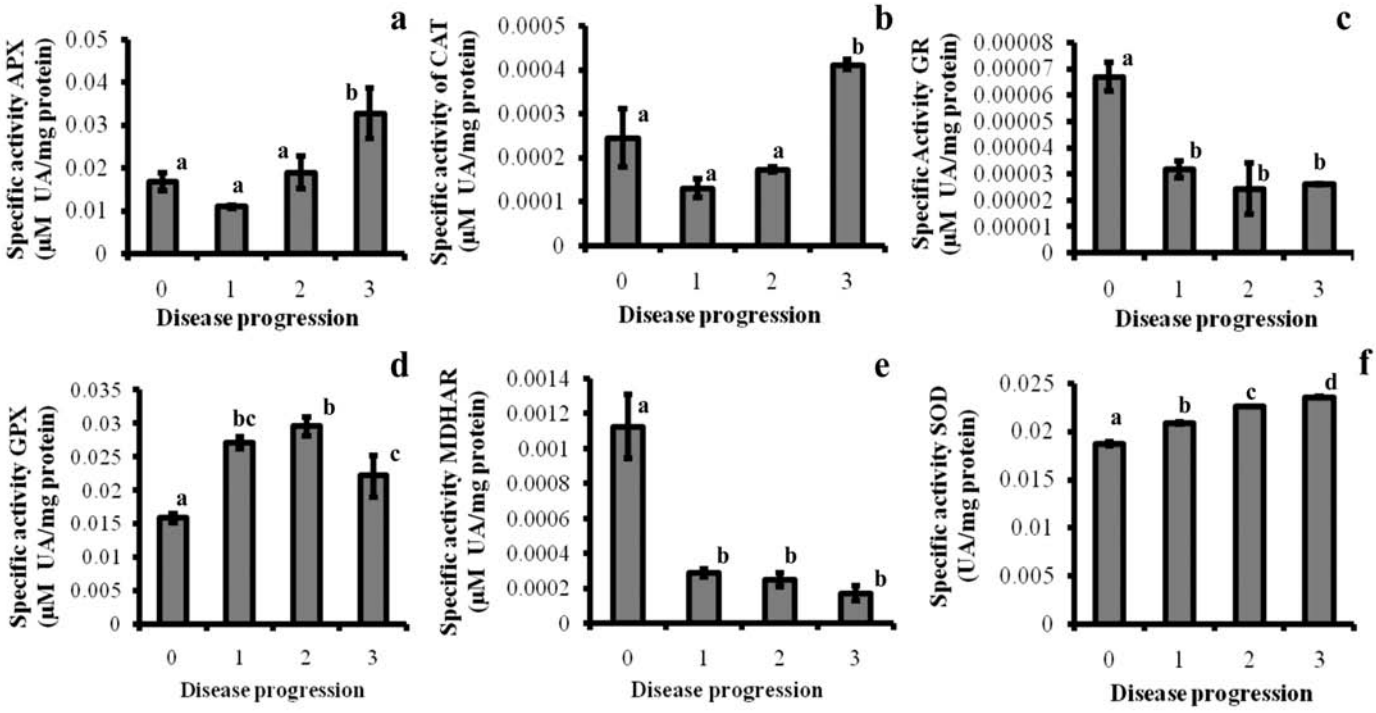


Fig. 3. Effect of disease progression on the activities of antioxidant enzymes. a**.** Ascorbate peroxidase (APX). b**.** Catalase (CAT). c. Glutathione reductase (GR). d. Guaiacol peroxidase (GPX). e. Monodehydroascorbate reductase (MDHAR). f. Superoxide dismutase (SOD). Specific activities of the same antioxidant enzyme with the same letter on the bar graph are not significantly different from each other (LSD, p≦0.05).

healthy to disease initiation stage (level 1). However, no considerable change was observed as the disease progressed (Fig. 3e). SOD activity increased commensurately with disease progression (Fig. 3f) as it was lowest in healthy leaves (0.019± 0.0002 mol UA/mg protein) and highest in level 3 leaves (0.024 ± 0.00001 mol UA/mg protein).

Pearson's correlation analysis of various biochemical parameters and disease progression showed that chlorophyll A, total chlorophyll and total protein tended to decrease with the increase in the number of spots/cm^2 leaf area at p≦0.05 (r values -0.978, -0.966 and -0.996 respectively), whereas the amount of free proline and the MDA and SOD activity tended to increase with the number of spots/cm^2 leaf area at p≦0.05 (r values = 0.973, 1.000 and 0.991, respectively).

DISCUSSION

To monitor the biochemical changes during leaf spot disease of *W. somnifera*, the disease development was rated on a four point scale (0-3) based on severity of infection. Leaf spot disease establishes gradually in foliar tissue of the plant with distinctive changes in chlorophyll composition. Total chlorophyll content was drastically reduced in diseased tissue, with about 16 fold reduction in extremely diseased tissues as compared to healthy tissues. This drop in chloroplast pigments may be attributed to the disorganization of the plastid membrane upon infection as reported by Alwadi and Baka (2001) for *A. solani*.

In the present experiment, a significant increase in the free proline concentration at every stage of disease progression was observed. Proline is known to be involved in reducing stress-induced cellular acidification and maintaining NAD(P)$^+$/NAD(P)H ratios (Hare *et al.*,1997). Its accumulation in response to biotic stress is well documented (Fabro *et al.*, 2004).

The amount of lipid peroxidation was determined as a measure of membrane lipid degradation. MDA content which is a measure of lipid peroxidation increased in response to disease progression. This may be due to the oxidative damage to the membrane lipids by superoxide radical. Similar increase in lipid peroxidation had previously been reported in response to both fungal (Deighton *et al.*, 1999) and bacterial pathogens (Adam *et al.*, 1989; Keppler and Baker, 1989). Host specific AK-toxin I from *A. alternata* is known for ROS (reactive oxygen species) induced lipid peroxidation and plasma membrane modifications in Japanese pear (Shimizu *et al.*, 2006).

Estimation of total protein content of healthy and diseased leaves indicated no significant differences.

However, healthy leaves treated with culture filtrate showed a significant reduction (paired sample student's t-test, $p \leq 0.05$) in protein content (Fig. 2). The reduction of protein in both cases could be ascribed to protein degradation by fungal proteases which are known to be produced by *A. alternata* in culture medium (Patil and Shastri, 1985). The small decrease of protein content in the diseased vis-a-vis the healthy sample may be due to built-up of fungal mycelium in diseased leaf tissue. It is also presumed that the reduction of the protein content could be linked to cell death (Attia *et al.*, 2005). Moreover, pathogen attack often leads to cell death due to disturbed homeostasis (Gilchrist, 1998).

Study of ion leakage is a quick method to monitor cell death resulting from disease incidence (Mittler *et al.*, 1999). In the present investigation, we found that there is a strong positive correlation between the percentage of ion leakage and the number of disease spots/unit leaf area ($r = 0.966$) (Table 2).

Production of ROS such as O_2, $OH^{\cdot}$, H_2O_2, during the so-called oxidative burst, is one of the earliest and most effective defence reactions in plants (Hong *et al.*, 2008), which can be activated by pathogen attack and establishment (Vellosillo *et al.*, 2010). ROS production and its steady state level are tightly regulated by various antioxidant enzymes that work in coordination (Arora *et al.*, 2004). ROS alone and in combination with NO has also been reported to play an important role in plant protection (Delledonne *et al.*, 1998, 2001). Keeping this in mind, we analysed the activities of a number of antioxidant enzymes.

An increase in SOD activity was observed concomitantly with the advancement of disease. A significant positive correlation ($r = 0.991$, at $p \leq 0.05$) was found among number of spots/cm^2 leaf area and specific activity of SOD. A similar trend in SOD activity had also been reported in strawberry leaves infected with *Mycosphaerella fragariae* (Ehsani-Moghaddam *et al.*, 2006). SOD may be helpful in dismutation of superoxide radicals generated during pathogen- induced oxidative burst.

Activity of MDHAR was reduced significantly with disease initiation, and no significant change was further observed with increasing severity of infection. MDHAR activity is responsible for keeping ascorbate in its reduced state. The decreased MDHAR activity we registered suggests that a larger fraction of ascorbate may be in the oxidized form in leaf tissues during pathogen-induced oxidative burst. Likewise, *Botrytis cinerea* infection led to decreased activity of enzymes involved in ascorbate metabolism (MDHAR, DHAR and APX) in the mitochondrial fraction (Kuzniak and Sklodowska, 2004).

Change in APX activity was not significant with disease initiation to intermediate diseased condition in comparison to healthy leaves. However, it increased significantly in extensively diseased leaf tissue. This further supports the fact that the oxidized form of ascorbate may increase in response to *A. alternata* infection.

GR activity in diseased leaves was reduced drastically, indicating a decrease in the reduction of oxidized glutathione in diseased tissue. A similar decrease in GR activity has already been reported in *in vitro*-grown plants of *W. somnifera* exposed to copper toxicity (Khatun *et al.*, 2008). In *Brassica*, it was observed that this enzyme, which has highly conserved disulphide bridge between Cys76 and Cys81 residues, may undergo a breakage due to a host-specific toxin secreted by *Alternaria* spp (Lee *et al.*, 1998). Decrease in GR activity under both biotic and abiotic stress in *W. somnifera* plants indicates that a common mechanism of counteraction may be operative in both kind of stress.

The mechanism of function of CAT in detoxifying harmful H_2O_2 is well understood (Dat *et al.*, 2003). In the present study, CAT activity was monitored at different levels of disease development, showing significant increase in extensively diseased leaves. However, a non significant change was found during the early to moderate infection phase. Increase of CAT activity may be due to the elevated level of H_2O_2 in plant tissue in response to disease.

Disease development in plants is associated with a series of complex biochemical events and their interactions. Understanding the underlying processes is therefore a prerequisite for formulating a strategy for resistance. The present work reports the biochemical changes, including modification of the activity of antioxidant enzymes, taking place during the progression of leaf spot disease, and draws its significance by the fact that *W. somnifera* is a highly valuable medicinal plant whose pharmaceutically important constituents are deteriorated by leaf spot disease. The various parameters studied provide baseline information on the infection process and will facilitate designing suitable strategies for disease resistance.

ACKNOWLEDGEMENTS

Financial assistance from Council for Scientific and Industrial Research (CSIR Grant no. 10-2(5)/2007(ii)-E.U.II), Government of India, New Delhi, India is duly acknowledged.

REFERENCES

Adam A., Farkas T., Somlyai G., Hevesi M., Kiraly Z., 1989. Consequence of $O_2^{\cdot}$ generation during a bacterially induced hypersensitive reaction in tobacco: deterioration of membrane lipids. *Physiological and Molecular Plant Pathology* **34**: 13-26.

Aebi M., 1984. Catalase *in vitro*. *Methods in Enzymology* **105**: 121-126.

Alwadi H.M., Baka Z.A.M., 2001. Microorganisms associated with *Withania somnifera* leaves. *Microbiological Research* **156**: 303-309.

Archana R., Namasivayan A., 1999. Antistressor effect of *Withania somnifera*. *Journal of Ethnopharmacology* **64**: 91-93.

Arnon D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiology* **24**: 1-15.

Arora A., Sairam R.K., Srivastava G.C., 2004. Oxidative stress and antioxidative system in plants. *Current Science* **82**: 1227-1238.

Attia K., Li K., Wei C., He G., Su W., Yang J., 2005. Overexpression of the *OsPDCD5* gene induces programmed cell death in rice. *Journal of Integrative Plant Biology* **47**: 1115-1122.

Bates L.S., Waldern R.P., Teare I.D., 1973. Rapid determination of free proline for water stress studies. *Plant and Soil* **39**: 205-207.

Beyer W.F., Fridovich I., 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in concentrations. *Analytical Biochemistry* **161**: 559-566.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Chatterjee S., Srivastava S., Khalid A., Singh N., Sangwan R.S., Sidhu O.P., Roy R., Khetrapal C.L., Tuli R., 2010. Comprehensive metabolic fingerprinting of *Withania somnifera* leaf and root extracts. *Phytochemistry* **71**: 1085-1094.

Dat J.F., Pellinen R., Beeckman T., Van De Cotte B., Langebartels C., Kangasjärvi J., Inze D., Van Breusegem F., 2003. Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. *The Plant Journal* **33**: 621-632.

Deighton N., Muckenschnabel I., Goodman B.A., Williamson B., 1999. Lipid peroxidation and the oxidative burst associated with infection of *Capsicum annuum* by *Botrytis cinerea*. *The Plant Journal* **20**: 485-492.

Delledonne M., Xia Y., Dixon R.A., Lamb C., 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**: 585-588.

Delledonne M., Zeier J., Marocco A., Lamb C., 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceeding of the National Academy of Sciences USA* **98**: 13454-13459.

Ehsani-Moghaddam B., Charles M.T., Carisse O., Khanizadeh S., 2006. Superoxide dismutase responses in strawberry cultivars to infection by *Mycosphaerella fragariae*. *Journal of Plant Physiology* **163** (Suppl.1): 147-153.

Fabro G., Kovács I., Pavet V., Szabados L., Alvarez M.E., 2004. Proline accumulation and *AtP5CS2* gene activation are induced by plant-pathogen incompatible interactions in *Arabidopsis*. *Molecular Plant-Microbe Interactions* **17**: 343-350.

Fernández-Gracía N., Carvajal M., Olmos E., 2004. Graft union formation in tomato plants: peroxidase and catalase involvement. *Annals of Botany* **93**: 53-60.

Gilchrist D.G., 1998. Programmed cell death in plant disease: the purpose and promise of cellular suicide. *Annual Review of Phytopathology* **36**: 393-414.

Hare P.D., Cress W.A., 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation* **21**: 79-102.

Hodges D.M., DeLong J.M., Forney C.F., Prange R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthrocyanin and other interfering compounds. *Planta* **207**: 604-611.

Hong J.K., Yun B., Kang J., Raja M.U., Kwon E., Sorhagen K., Chu C., Wangand Y., Loake G.J., 2008. Nitric oxide function and signaling in plant disease resistance. *Journal of Experimental Botany* **59**: 147-154.

Hossain M.A., Nakano Y., Asada K., 1984. Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. *Plant and Cell Physiology* **25**: 385-395.

Jahnke L.S., Hull M.R., Long S.P., 1991. Chilling stress and oxygen metabolizing enzymes in *Zea mays* and *Zea diploperennis*. *Plant Cell and Environment* **14**: 97-104.

Jaleel C.A., Gopi R., Manivannan P., Panneerselvam R., 2008a. Exogenous application of triadimefon affects the antioxidant defence system of *Withania somnifera* Dunal. *Pesticide Biochemistry and Physiology* **91**: 170-174.

Jaleel C.A., Lakshmanan G.M.A., Gomathinayagam M., Panneerselvam R., 2008b. Triadimefon induced salt stress tolerance in *Withania somnifera* and its relationship to antioxidant defence system. *South African Journal of Botany* **74**: 126-132.

Keppler L.D., Baker C.J., 1989. O_2^- initiated lipid peroxidation in a bacteria-induced hypersensitive reaction in tobacco cell suspensions. *Phytopathology* **79**: 555-562.

Khan J.A., Shrivastva P., Singh S.K., 2006. Sensitive detection of a phytoplasma associated with little leaf symptoms in *Withania somnifera*. *European Journal of Plant Pathology* **115**: 401-408.

Khatun S., Ali M.B., Hahn E., Paek K., 2008. Copper toxicity in *W. somnifera*: Growth and antioxidant enzymes responses of *in vitro* grown plants. *Environmental and Experimental Botany* **64**: 279-285.

Kulkarni A.A., Thengane S.K., Krishnamurti K.V., 1996. Direct *in vitro* regeneration in leaf explants of *Withania somnifera* (L.) Dunal. *Plant Science* **119**: 163-168.

Kumar A., Singh C.P., Pandey R., 2009. Influence of environmental factors on the population buildup of *Helicoverpa armigera* Hübner and *Epilachna vigintioctopunctata* (Febr.) on Aswagandha (*Withania somnifera* Linn.). *Journal of Entomological Research* **33**: 123-127.

Kuzniak E., Sklodowska M., 2004. The effect of *Botrytis cinerea* infection on the antioxidant profile of mitochondria from tomato leaves. *Journal of Experimental Botany* **55**: 605-612.

Lee H., Jo J., Son D., 1998. Molecular cloning and characterization of the gene encoding glutathione reductase in *Brassica campestris*. *Biochimica et Biophysica Acta* **1395**: 309-314.

Mahrshi R.P., 1986. *Withania somnifera*-a new host for *Myrothecium roridum*. *Indian Journal of Plant Pathology* **16**: 199.

Maiti C.K., Sen S., Paul A.K., Acharya K., 2007. First report of *Alternaria dianthicola* causing leaf blight on *Withania somnifera* from India. *Plant Disease* **91**: 467.

Mittler R., Herr E.H., Orvar B.L., Camp W., Willekens H., Inzé D., Ellis B.E., 1999. Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. *Proceedings of National Academy of Sciences USA* **96**: 14165-14170.

Mohanty I., Arya D.S., Dinda A., Talwar K.K., Joshi S., Gupta S.K., 2004. Mechanisms of cardioprotective effect of *Withania somnifera* in experimentally induced myocardial infarction. *Basic and Clinical Pharmacology and Toxicology* **94**: 184-190.

Murthy H.N., Dijkstra C., Anthony P., White D.A., Davey M.R., Power J.B., Hahn E.J., Paek K.Y., 2008. Establishment of *Withania somnifera* hairy root cultures for the production of withanolide A. *Journal of Integrative Plant Biology* **50**: 975-981.

Nakano Y., Asada K., 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology* **22**: 867-880.

Pathak H.C., Raychoudhuri S.P., 1967. *Withania somnifera* - an additional host of tobacco leaf curl virus. *Science and Culture* **33**: 234-235.

Pati P.K., Sharma M., Salar R.K., Sharma A., Gupta A.P., Singh B., 2008. Studies on leaf spot disease of *Withania somnifera* and its impact on secondary metabolites. *Indian Journal of Microbiology* **48**: 432-437.

Patil M., Shastri N.V., 1985. Purification and properties of proteases produced by *Alternaria alternata* (Fr.) Keissler, regulation of production by fructose. *Journal of Biosciences* **9**: 1-11.

Rasool M., Varalakshmi P., 2006. Immunomodulatory role of *Withania somnifera* root powder on experimental induced inflammation: An *in vivo* and *in vitro* study. *Vascular Pharmacology* **44**: 406-410.

Samad A., Shasany A.K., Gupta S., Ajayakuar P.V., Darokar M.P., Khanuja S.P.S., 2006. First report of a 16SrVI group phytoplasma associated with witches'-broom disease on *Withania somnifera*. *Plant Disease* **90**: 248.

Sharma P., Pandey R., 2009. Biological control of root-knot nematode; *Meloidogyne incognita* in the medicinal plant; *Withania somnifera* and the effect of biocontrol agents on plant growth. *African Journal of Agricultural Research* **4**: 564-567.

Sharma A., Pati P.K., 2011. First record of 28-spotted ladybird beetle *Enosepilachna vigintioctopunctata* (F.) infesting *Withania somnifera* (L.) Dunal in Punjab Province of Northern India. *Pest Technology* (in press).

Shimizu N., Hosogi N., Jiang G.H.S., Inoue K., Park P., 2006. Reactive oxygen species (ROS) generation and ROS-induced lipid peroxidation are associated with plasma membrane modifications in host cells in response to AK-Toxin I from *A. alternata* Japanese pear pathotype. *Journal of General Plant Pathology* **72**: 6-15.

Singh R.H., Narsimhamurthy K., Singh G., 2008. Neuronutrient impact of Ayurvedic Rasayana therapy in brain aging. *Biogerontology* **9**: 369-374.

Uma Devi P., 1996. *Withania somnifera* Dunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. *Indian Journal of Experimental Biology* **34**: 927-932.

Vellosillo T., Vicente J., Kulasekaran S., Hamberg M., Castresana C., 2010. Emerging complexity in reactive oxygen species production and signaling during the response of plants to pathogens. *Plant Physiology* **154**: 444-448.

Verma O.P., Gupta R.B.L., Shivpuri A., 2007. A new host for *Pithomyces chartarum,* the cause of a leaf spot disease on *Withania somnifera. New Disease Reports* **15**: 47.

Vyas D., Kumar S., Ahuja P.S., 2007. Tea (*Camellia sinensis*) clones with shorter periods of winter dormancy exhibit lower accumulation of reactive oxygen species. *Tree Physiology* **27**: 1253-1259.

Widodo N., Kaur K., Shrestha B.G., Takagi Y., Ishii T., Wadhwa R., Kaul S.C., 2007. Selective killing of cancer cells by leaf extract of Ashwagandha: identification of a tumor-inhibitory factor and the first molecular insights to its effect. *Clinical Cancer Research* **13**: 2298-2306.

Received October 25, 2010
Accepted February 15, 2011