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Influence of Defoliate Pathotype of *Verticillium dahliae* on Some Physiological and Biochemical Characteristics of Chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitam)

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Abstract

Verticillium dahliae Kleb. is a soilborne pathogen that causes vascular wilt in chrysanthemum plant (*Dendranthema grandiflorum* (Ramat.) Kitam). The objective of the present research was to study the levels of some organic solutes, such as total protein, total soluble sugars, starch and proline, relative water content, RNA level, malondialdehyde and H_2O_2 contents, in the leaves of chrysanthemum inoculated with *V. dahliae*. Changes in these parameters were measured 0, 10, 20, 30 and 40 days after inoculation by spectrophotometric analysis. No changes were detected in relative water content, RNA, and protein levels and a slight decrease was observed in chlorophyll level in infected leaf tissue before the appearance of visible wilt. The decrease in relative water content coincided with a sharp buildup of proline and total soluble sugars in leaves. The leaf starch and protein levels gradually declined in both healthy and infected plants during the time course of the experiment. However, the decrease was more pronounced in infected plants since the third week after inoculation. A high negative correlation was observed between total soluble sugars and starch contents in leaves of diseased plants ($r= 0.764$, $P<0.001$). Changes in malondialdehyde and H_2O_2 concentration occurred in infected plants between 30-40 days after inoculation, while they did not change in the leaves of control plants. These data suggest the posssible role of senescence during the development of *Verticillium* wilt syndrome in chrysanthemum.

Keywords: Antioxidant enzymes, Reactive oxygen species, *Verticillium dahliae*, Wilt.

INTRODUCTION

Plants in nature are constantly challenged by a diverse array of pathogenic microorganisms. In many cases, their protective mechanisms involve an inducible defense system (Dallagnol *et al.*, 2011). The ability of plants to invoke such defense reactions is presumed to be mediated by an initial recognition process that involves detection of certain unique signal molecules of incompatible pathogens by receptor-like molecules in plants, resulting in a cascade of biochemical events that leads to the expression of resistance or susceptibility to a disease (Sapkota *et al.*, 2016).

Chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitam) is one of the most important ornamental plants and its commercial production is often affected by biotic and abiotic stresses (Fan *et al.*, 2016). *Verticillium* wilt, incited by the soil-inhabiting fungus *Verticillium dahliae* Kleb., is a widespread disease that causes vascular wilt in over 160 important crop species worldwide, including pepper, tomato, cotton, alfalfa, cucurbits, eggplant, mint, potato, strawberry, and sunflower (Sanei *et al.*, 2008). *Verticillium* wilt of chrysanthemum is one of the most important diseases affecting chrysanthemum plants (Alexander and Hall, 1974; Singh and Kumar, 2014). Although these organisms live in the soil, they can have a more direct effect on the growth of the root system as contrasted to other soil factors such as water and nutrient stress (Goicoechea *et al.*, 2000; Wheeler and Johnson, 2016). The fungus enters the root through wounds that expose the vascular system or grows between the cells of the apical meristem to gain access to immature xylem elements. Visible symptoms of the syndrome include stunting, epinasty, wilting, foliar chlorosis progressing to necrosis, vascular browning, and leaf abscission (Pomar *et al*., 2004). It has been recognized that *Verticillium* produces several pathogenic metabolites, each of which may exert specific effects on the host (Fradin and Thomma, 2006.).

Water availability is one of the most limiting environmental factors affecting crop productivity. It is a well-known fact that, crop growth is frequently subject to water stress over its lifetime. Stresses imposed during these periods drastically affects crop growth, ultimately leading to a massive loss of yield and quality (Pirzad *et al*., 2011). Most wilt pathogens increase the resistance to water movement along the stem, as a consequence of reduced diameter of the vessel elements by the physical presence of the pathogen itself, their metabolites, or by inducing the formation of gummy substances and tyloses. Such vessel occlusion has been proposed as the primary cause of water stress in *Verticillium* wilted plants (Yang *et al.*, 2010).

The onset of water stress in *Verticillium* wilts may be expected to trigger a series of physiological events analogous to those occurring in drought-affected plants (Land *et al.*, 2017). Under drought stress, osmotic adjustment occurs in plant cells by the accumulation of compatible solutes in the cytosol. In most plants, osmoregulation through the accumulation of solutes, such as proline and total soluble sugars (TSS), has the function of reducing the osmotic potential of the cell in order to maintain cell turgor and growth (Fan *et al.*, 2016). Water deficit in leaves has generally been considered the characteristic and most significant pathologic event in susceptible host plants systemically invaded by vascular wilt fungi. Thus attention has largely been focused on mechanisms inducing the water deficit (Goicoechea *et al*., 2000; Pascual *et al.*, 2010).

Biological stresses cause the accumulation of reactive oxygen species (ROS) including superoxide radicals (O_2), hydroxyl radicals (•HO) and hydrogen peroxide (H_2O_2) in plants (Molassiotis and Fotopoulos, 2011). Antioxidant enzymes, such as guaiacol peroxidase (GPX), seem to play an important role in reducing the harmful effects of ROS (Uarrota *et al.*, 2016). Guaiacol peroxidase is considered as an ROS scavenger because of its tendency to destroy hydrogen peroxide better than catalase (Nayyar and Gupta, 2006). Malondialdehyde (MDA) is a breakdown product of unsaturated fatty acids and is widely used as a parameter for assay of lipid peroxidation (Hajipour and Jabbarzadeh, 2015). It is estimated that more than 75% of the MDA is derived from a-Linoleic acid (Weber *et al.*, 2004). Accumulation of proline is stimulated in response to environmental stresses, osmotic conditions, aging, and other factors. Proline plays an important

role in osmoregulation (Zahed Chakovari *et al.*, 2016). Host-pathogen interactions are presumed to generate signals that activate nuclear genes involved in plant defense responses leading to the induction of stress-related enzymes, differential expression of proteins and release of free amino acids and the associated accumulation of high levels of phenolic compounds. Antimicrobial phytoalexins including sesquiterpenoids, isoflavonoids, coumarins, acetylenic and phenolic compounds also contribute to multilayered plant defense systems (Fradin and Thomma, 2006).

There is limited evidence describing physiological responses during chrysanthemum wilt infection. The aim of this study was to investigate the impact of *V. dahliae* infection on the physiological and antioxidant responses of chrysanthemum.

MATERIALS AND METHODS

Fungal material and pathogen inoculums

Highly virulent isolates of *V. dahliae*, 21 VCG1, were provided from the culture collection of the Plant Pathology Laboratory of the Plant Protection Department, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. Inoculum was prepared from single-spore cultures of isolates and maintained on potato dextrose agar (PDA) slants at 4°C. Inoculations were made with a conidial suspension prepared from 7-day-old cultures grown on potato-dextrose-agar (PDA) slants in Petri dishes at 25±0.5°C in the dark. The cultures were flooded with sterile distilled water and their surface was gently scraped with a sterile scalpel. The resulting suspension was filtered through cheesecloth to remove mycelial fragments. After filtration, the inoculum concentration was adjusted to 4×106 conidia per ml (Schnathorst, 1969).

Plant materials

Chrysanthemum cuttings were planted in 15-cm-diameter pots with sterilized potting mixture (sand:clay 1:1, v:v) and were grown in a greenhouse with 16 h of light at 25-27°C/22°C day/night temperature regime in 4 weeks. For uniform infection, four-week-old plants were inoculated by the stem-injection method at the base with 6 µl of a 4×106 conidia ml-1 suspension in sterile distilled water. Control plants were treated similarly with sterile distilled water. Disease severity on aboveground plant parts was evaluated based on the percentage of foliage affected by chlorotic, necrotic, and wilt (Bejarano-Alcázar *et al*., 1996).

Water status measurement and biochemical determinations

Relative water content (RWC) was used as a measure of the water status of leaf tissue. It was determined according to Turner (1981). Plant dry matter (DM) was determined after drying at 80ºC for 2 days. Leaf soluble protein, total soluble sugars (TSS), starch, and proline were quantified in potassium phosphate buffer (KPB) (50 mM, pH D 7:5) extracts of fresh leaves (0.1 g). These extracts were filtered through four cheesecloth layers and centrifuged at 15,500 rpm for 15 min at 4ºC. The supernatant was collected and stored at 4ºC for protein, TSS, and proline determinations. The pellet was used for starch determination (Jarvis and Walker, 1993). Leaf soluble protein was measured by the protein dye-binding method of Bradford (1976) using bovine serum albumin as standard. TSS was analyzed with the anthrone reagent, and free proline content was estimated using the ninhydrin reagent by Bates *et al*. (1973)'s procedure.

The procedures for estimating RNA content were based on Fletcher and Osborne (1965). Leaf discs were homogenized in a 15-ml hand tissue grinder and extracted 4 times with 80% ethanol. Homogenate designated for RNA analysis was suspended in 2.5 ml of 0.3 N KOH, incubated at 37°C for 16-18 h, cooled in crushed ice, and centrifuged. RNA content of the supernatant was determined by the orcinol method, and total ribonucleotide content was determined by measuring absorbance at 665 nm. Chlorophyll content was determined by measuring absorbance at 645 nm and 663 nm (Pirzad *et al.*, 2011).

Lipid peroxidation in the leaf tissues was determined in terms of malondialdehyde (MDA) content by the thiobarbituric acid (TBA) method as described by Popham and Novacky (1990).

Briefly, 0.2 g of leaf tissue were homogenized in 5 ml of 1% (w: v) trichloroacetic acid (TCA) and then centrifuged at 8000 g for 10 min. One ml of the supernatant was added to 4 ml of 20% TCA that contained 0.5% TBA and the solution was heated for 30 min at 95°C in water bath. The samples were cooled on ice for 5 min and re-centrifuged for 5 min at 8000 g. Absorbance was read at 532 nm and corrected for non-specific absorbance at 600 nm. Malondialdehyde was calculated according to the following formula and extinction coefficient, mM^{-1} Cm^{-1} 155.

$$MDA\ (\mu mol/g\ FW) = [A532\text{-}A600/155] \times 1000$$

Hydrogen peroxide levels were determined according to the Velikova *et al.*, (2000) protocol. 0.1 g fresh weight of leaf tissue was homogenized in 5 ml of 0.1% TCA solution. After centrifugation at 12000 g for 15 min, 0.5 ml of the supernatant was added to the reaction mixture containing 0.5 ml of 10 mM potassium phosphate buffer (pH=7.0) and 1 ml of 1M potassium iodide (KI). The reaction mixture was placed at room temperature in the dark for one hour and then the absorbance was read at 390 nm.

Data analysis

The experiment was carried out based on a randomized complete block design with four replicates. Statistical analyses were carried out using the R software, version 3.3.1. Data for different treatments were directly analyzed using analysis of variance (ANOVA). When significant treatment effects were detected, treatment means were separated using Duncan's Multiple Range test at $P<0.05$.

RESULTS AND DISCUSSION

The first foliar wilting symptoms appeared 24 days after fungal inoculation (Fig. 1), although leaves did not show necrotic areas until day 32. The increase in the percentage of necrosis coincided with the beginning of the defoliation. Non-inoculated controls always remained symptomless.

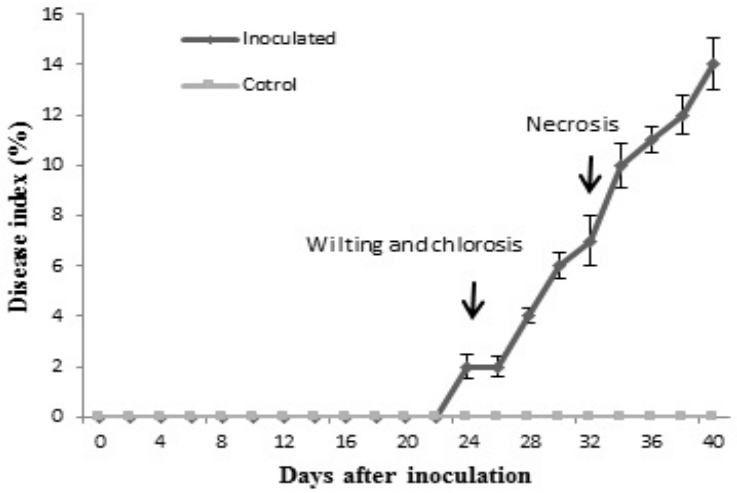

Fig. 1. Disease index in control and inoculated chrysanthemum plants with *V. dahliae*. Standard errors of four replications are shown as the vertical bars.

Relative water content (RWC)

Although the RWC was lower in wilted tissue, there was no difference in the RWC of non-infected leaves and non-wilted infected leaves (Fig. 2). No decrease was detected in the RWC of infected leaf tissue before the appearance of symptoms, but then it was decreased drastically.

Relative water content is considered a measure of plant water status, reflecting the metabolic activity in tissues, and it is used as an index for dehydration tolerance (Jamal *et al.*, 2014). A decrease in the RWC in response to drought stress has been noted in a wide variety of plants (Nayyar and Gupta, 2006). In the present study, RWC was used as a reference parameter for the intensity of water stress induced by *V. dahliae*. RWC remained unchanged during the third week after inoculation (Fig. 2). Therefore, RWC appeared to be more resistant to infection. Higher RWC under drought stress may be related to the fact that the ability of more absorption of water from soil or ability of stomata to reduce the loss of water (Zokaee-Khosroshahi *et al.*, 2014). Wilting is probably due to altered leaf cell permeability caused by a toxin or by cell-wall-degrading enzymes because significant differences were found in RWC at the onset of wilting (Koffler *et al*., 2014).

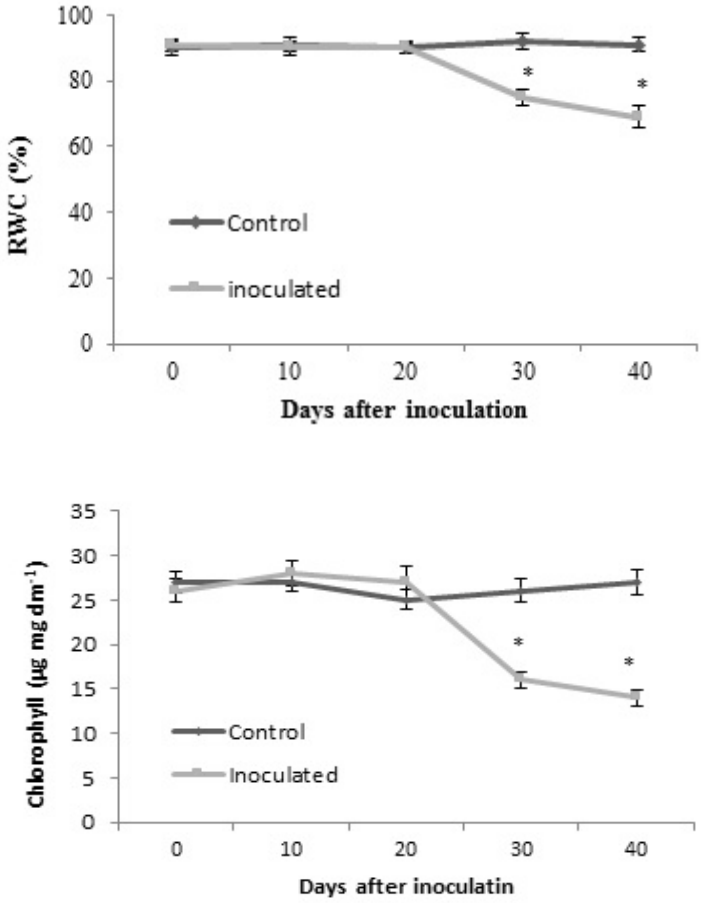

Fig. 2. Changes in relative water (top) and chlorophyll (below) content in leaves of healthy control chrysanthemum plants and in plants inoculated with *V. dahliae*. Bars represent standard deviations of the means. Means followed by an asterisk (*) are significantly different ($P<0.05$) as determined using Duncan's Multiple Range test.

Proline

Proline concentration in foliar tissues of the inoculated plants was increased significantly between days 20 and 40 after inoculation, while they did not change in the leaves of control plants (Table 1). Proline is one of the most common compatible solutes in plants under drought stress. Several possible physiological functions have been ascribed to this amino acid accumulation, such as osmoregulation, a sink for energy and nitrogen, a signal of senescence, and an indicator of drought resistance and/or stress sensor (Fumis and Pedras, 2002). Similar to the findings of Tzeng *et al*. (1985) in cotton and Goicoechea *et al*. (2000) in pepper, higher proline levels were detected in leaves of the *Verticillium*-wilted plants than in those of controls. The sharp increase in proline in leaves of the inoculated plants (Table 1) was coupled with a drastic decrease in RWC (Fig. 2). Therefore, it seems that in inoculated chrysanthemum, accumulation of such osmolyte is related to higher damage caused by fungus attack. Since only proline levels appear to change dramatically in terms of relative importance to overall osmotic adjustment, synthesis of proline may represent the most highly regulated metabolic event during adaptation and as such would appear to be suitable as a parameter to evaluate the degree of adaptation (Irigoyen, *et al.*, 1992; Fumis and Pedras, 2002). A similar behavior was observed by Irigoyen *et al*. (1992) in leaves of alfalfa subjected to drought and Goicoechea *et al*. (2000) in leaves of pepper subjected to *Verticillium* wilt.

Table 1. Effects of *V. dahliae* on some biochemical parameters of leaf tissue of chrysanthemum plants.

Days after inoculation	Soluble protein[a]	Proline	Total soluble sugars	Starch	RNA
Control					
0	123.82 b	69.2 d	102.3 de	99.4 ab	35.02 a
10	118.57 b	53.0 d	105.6 c	98.3 ab	35.37 a
20	118.98 b	74.7 d	100.3 e	99.20 ab	36.50 a
30	127.07 b	61.7 d	106.6 c	99.8 ab	35.97 a
40	125.18 b	57.2 d	102.4 de	97.3 b	34.10 a
Inoculated					
0	123.55 b	55.0 d	104.5 cd	99.35 ab	35.95 a
10	119.09 b	63.2 d	100.2 e	97.7 b	35.00 a
20	124.13 b	89.60 c	104.2 cd	101.3 a	23.17 b
30	185.65 a	119.5 b	201.0 b	78.5 c	15.07 c
40	116.22 b	145.0 a	241.4 a	68.2 d	11.72 c

a Relative water content (%), Soluble protein (mg g dw^{-1}), Proline (µg gfw^{-1}), Total soluble sugars (mg g dw^{-1}), Starch (mg g dw^{-1}) and RNA (µg mg dw^{-1}). Values are mean ± SE (n= 6 plants). Values followed by similar letter(s) are not significantly different ($P<0.05$) as determined using Duncan's Multiple Range Test.

Chlorophyll, RNA and protein

Chlorophyll levels declined slightly in infected leaves before the appearance of wilt symptoms and then it decreased drastically (Fig. 2). No changes occurred in RNA and protein levels in infected leaves before the appearance of wilt symptoms. The RNA and protein levels found in leaves of infected plants were significantly lower than those of controls from the third week after inoculation (Table 1). Generally accepted parameters for the process of senescence are progressive declines in levels of RNA, protein, and chlorophyll (Pascual *et al.*, 2009 and 2010; Fradin and Thomma, 2006). In the present study, levels of these substances were determined in the leaves of infected plants and uninoculated checks. Changes in protein and RNA levels occurred in infected plants only in leaf tissue already expressing wilt symptoms. Thus, the onset of senescence does not appear to be hastened by infection nor does senescence precede the development of wilt. This would suggest that in *Verticillium* wilt of chrysanthemum, water stress as evidenced by wilting is not incidental to senescence.

Starch and total soluble sugars

Accumulation of carbohydrates such as sugars (e.g., glucose, fructose, fructans, and trehalose) and starch occurs under drought stress. The major role played by these carbohydrates in stress mitigation involves osmoprotection, carbon storage, and scavenging of reactive oxygen species (Mahajan and Tuteja, 2005). Such accumulation may result from a greater degree of conversion of plastidic starch into soluble sugars (Dallagnol *et al*., 2011). The present results, as well as earlier results, show that chrysanthemum cells have large capacities for osmotic adjustment when subjected to water stress. In our study, there was a correlation (r= 0.764, P<0.001) between starch and total soluble sugars in leaves of diseased plants (Figure 3).

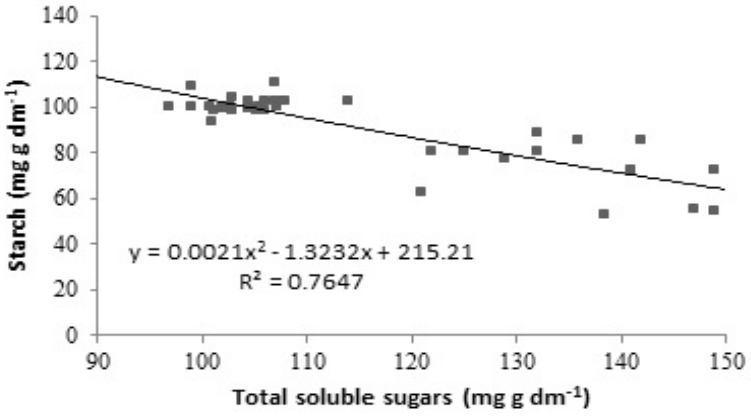

Fig. 3. The relationship between total soluble sugar (TSS) content and starch concentration in leaves of chrysanthemum plants inoculated with *V. dahliae.* Symbols represent the means of six plants (P<0.001).

Malondialdehyde

V. dahliae infection caused a significant increase in malondialdehyde (MDA). MDA concentration in foliar tissues from inoculated plants was increased significantly between 30-40 days after inoculation, while they did not change in the leaves of control plants (Fig. 4). MDA is regarded as a marker of the evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes (Molassiotis and Fotopoulos, 2011). Measuring MDA in plants under biotic (Hasanuzzaman *et al*., 2013) and abiotic stresses (Avis *et al.*, 2007) indicates an increase in their membrane permeability, lipid peroxidation, injury to the cell membrane, and finally a decrease in membrane stability index and organs injury.

It has long been recognized that high levels of free radicals or reactive oxygen species can inflict direct damage to lipids (Mahajan and Tuteja, 2005). Different exogenous stimuli, such as the ionizing radiation, ultraviolet rays, tobacco smoke, pathogen infections, environmental toxins, and exposure to herbicide/insecticides, are sources of *in vivo* reactive oxygen species production. The high levels of post-infectional lipid peroxidation, as indicated by the enhanced production of MDA, could cause an increase in flax membrane permeability that, in turn, may lead to more powdery mildew severity (Mohamed *et al*., 2012). In our study, the increase in MDA showed a positive correlation with the disease severity. This result may suggest that the post-infectional lipid peroxidation plays an important role in determining susceptibility of chrysanthemum to *V. dahliae*.

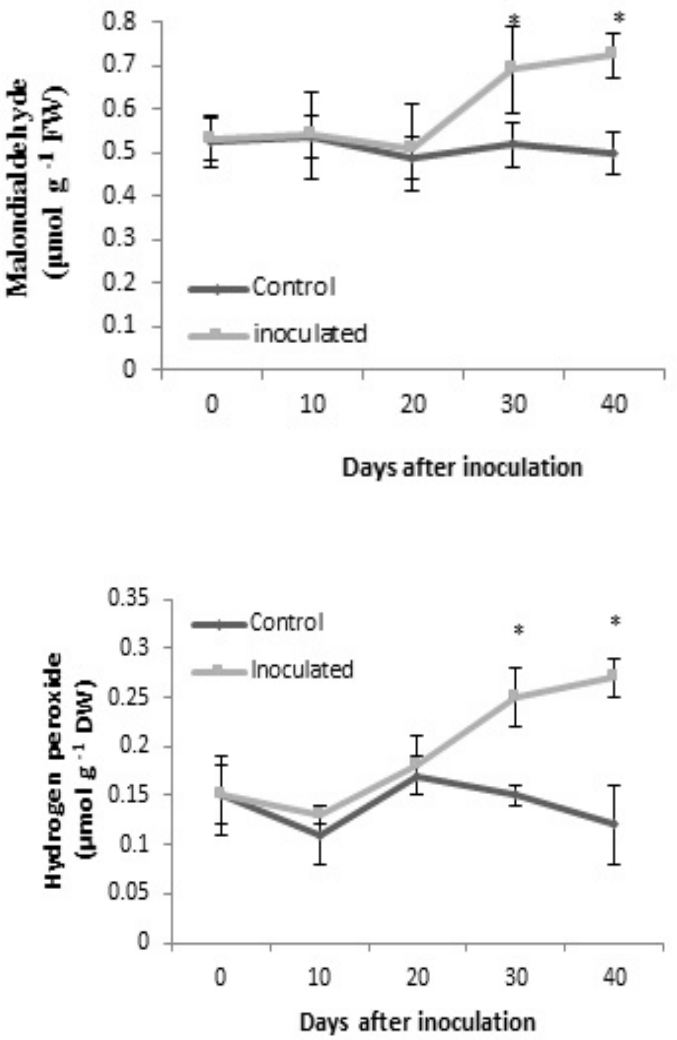

Fig. 4. Changes in relative malondialdehyde (top) and H_2O_2 (below) content in leaves of healthy control chrysanthemum plants and in plants inoculated with *V. dahliae*. Bars represent standard deviations of the means. Means followed by an asterisk (*) are significantly different (P <0.05) as determined using Duncan's Multiple Range Test.

H_2O_2

Verticillium wilt increased hydrogen peroxide significantly. Changes in H_2O_2 concentration occurred in infected plants between 30-40 days after inoculation, while they did not change in the leaves of control plants (Fig. 4). H_2O_2 has a crucial role in the induction of defense responses in plant cells. It has been reported that the interaction of plant cells with pathogens or pathogen-derived elicitors does result in the generation of hydrogen peroxide which is one of the earliest cellular responses to potential pathogens or elicitor molecules. H_2O_2 appears to be a signaling intermediate, resulting in the induction of a variety of defense-related genes and programmed cell death in plant defense (Tripathy and Oelmüller, 2012).

A deep-going understanding of the biochemical alternation underlying plant infection to *V. dahliae* is a major prerequisite for effective control of *Verticillium* wilt. Water deficit is the most important factor limiting crop yield worldwide and plant growth, including biochemical and physiological processes, is affected by water deficit stress. The impacts of water deficit stress depend on the severity and duration of drought as well as the growth stage and genotype of the plant (Ganji Arjenaki *et al*., 2012). Osmotic adjustment in plants has been observed and studied for many years (Pilon *et al*., 2013). Apart from the recognition of osmotic adjustment as an integral part of higher

plant cell growth, a lot of interest has developed in the role of osmotic adjustment in water and salt stress tolerance in recent years (Mensha *et al.*, 2006). It is generally known that, in the absence of various avoidance mechanisms, plant cells which actually experience desiccation to the point of turgor loss must regain turgor through the osmotic adjustment to resume growth (Pirzad *et al.*, 2011).

In summary, changes in proline, TSS, starch and total soluble protein levels, as well as the RNA concentration in leaves of chrysanthemum plants caused by the infection with *V. dahliae* clearly matched the effects of *Verticillium* wilt. Results suggest that the biochemical changes could be sensors of the damage caused by the fungal infection.

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