European Journal of Plant Pathology **106:** 19–25, 2000.

Verticillium dahliae modifies the concentrations of proline, soluble sugars, starch, soluble protein and abscisic acid in pepper plants

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Accepted 14 October 1999

Key words: Capsicum annuum, organic solutes, vascular wilt, water status

Abstract

The objective of this research was to study the levels of some organic solutes, such as total protein, total soluble sugars, starch and proline in leaves, as well as abscisic acid concentration in xylem of pepper plants inoculated with *Verticillium dahliae* Kleb. Healthy and inoculated plants were always kept well watered. Measurements were made at time intervals after inoculation. Leaf water potential rapidly decreased as a consequence of fungal infection. However, relative water content in leaves only changed significantly from day 20 after inoculation, and such decreases coincided with a sharp build up of proline and total soluble sugars in leaves. Starch and protein levels, as well as abscisic acid concentration in xylem, declined in healthy and inoculated peppers as they became older. However, such decreases were more pronounced in infected plants, especially soon after inoculation. Results suggest that proline and total soluble sugars accumulation could be sensors of the damage caused by the fungal infection.

Abbreviations: ABA – abscisic acid; DM – dry matter; RWC – relative water content; TSS – total soluble sugars; Ψ – leaf water potential.

Introduction

Verticillium dahliae Kleb. is a systemic pathogen that causes vascular wilt disease in several plant species, including tomato, potato, cotton and pepper (Pegg, 1989). 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. The onset of water stress in infected plants may be expected to set in motion a series of physiological events analogous to those occurring in droughted plants (Slatyer, 1967; Aguirreolea et al., 1995). In general, wilt disease is the result of restricted water movement but is also caused by complex interactions between toxins, enzymes and hormones (Cooper and Wood, 1980).

Drought is one of the most crucial environmental stresses for the productivity of crops. An important adaptation of plants to water stress is the increase in the concentration of intracellular solutes, such as proline and total soluble sugars (TSS), which facilitate the maintenance of cell pressure potential. Moreover, abscisic acid (ABA) usually increases in plants during drought and it could be involved in the root-to-shoot signalling of drying soil (Davies et al., 1994).

One of the main visible symptoms of the disease caused by *V. dahliae* is the foliar wilting and drying in affected plants. Thus, the first objective of this research was to study the levels of solutes such as total soluble proteins, proline, TSS and starch in leaves of inoculated pepper plants, as well as the ABA concentration in the xylem sap. The second aim was to analyze the suitability of proline and TSS accumulation in leaves as an index of fungal infection.

Materials and methods

Biological material, growth conditions and experimental design

Sixty plants of *Capsicum annuum* cv. Piquillo were grown from surface-disinfected seeds in 1.5 l capacity pots (one per pot) containing vermiculite substrate in a glasshouse with a day/night regime of 25/15 °C and 14-h photoperiod. Plants were fertilized (Cenoz et al., 1997). Two treatments were studied: non-inoculated healthy controls and infected plants. When two-months-old, 30 plants were inoculated by adding a suspension of 5×10^7 conidia of *V. dahliae* Kleb. in the substrate of each pot (Hoyos et al., 1993). The fungus was isolated from pepper and maintained on potato dextrose agar (PDA). Thirty other plants were kept as non-inoculated controls. Both control and infected plants were always well-watered. One week prior to measurements plants were transferred to a controlled environmental chamber with a day/night regime of 25/15 °C and 60/80% relative humidity. A photosynthetically active radiation (PAR) of 310 μmol of photons $m^{-2}s^{-1}$ (400–700 nm) on the upper leaves was provided by fluorescent tubes Sylvania, L 36 W/20 and incandescent bulbs Philips softone 60 W for a daily photoperiod of 14 h. Plant harvests took place 0, 7, 21, 28 and 33 days after fungal inoculation. In order to test if the fungus had progressed from root to shoot, surface-disinfected cross-stem sections were cut at a distance of 4 cm from the collar and plated on PDA for *Verticillium* isolation after every harvest. Plates were incubated in the light at 20 °C for 6–8 days and colonies of *V. dahliae* were identified under the microscope. The disease severity was non-destructively estimated (Cenoz et al., 1997), and disease index was calculated as the summatory of wilted, chlorotic and necrotic leaves related to the total leaves per plant, expressed as percentage.

Statistics

The experiment was carried out twice. Mean ± SE were calculated and their differences tested for significance using a LSD technique with Student's *t*-test. The number of collected samples for each determination was chosen according to standard errors (SE) observed in a preliminary experiment. The lowest number of samples ($n = 3$) was used when SE were equal or lower than 10%. Data are mean ± SE ($n = 3$) for plant growth parameters (Table 1), water status measurements (Figure 2) and proline determinations (Figure 3b). Values are mean ± SE ($n = 6$) for soluble protein (Figure 3a), total soluble sugars (TSS) (Figure 3c), starch (Figure 3d) and ABA (Table 2) analysis. For determining the disease index, six to ten plants were used. The correlation functions between parameters were calculated using the CricketGraph 1.3.1. program.

Water status measurement and plant growth parameters

Midday leaf water potential (Ψ) was determined with a pressure chamber (Scholander et al., 1965), and relative water content (RWC) was estimated by a modification of Weatherley's method (1950), both parameters on the youngest fully-mature leaves. Plant dry matter (DM) was determined after drying at 80 °C for 2 days, and leaf areas were measured using an automatic leaf area meter (Li-Cor, LI-3000 model).

Chemical and biochemical determinations

Plants were immediately defoliated following measurement of bulk water content for chemical and biochemical determinations. Measurements were made in physiologically comparable leaves to those used for the Ψ determination. Leaf soluble protein, total soluble sugars (TSS), starch and proline were quantified in potassium phosphate buffer (KPB) (50 mM, pH = 7.5) extracts of fresh leaves (0.1 g). These extracts were filtered through four cheese cloth layers and centrifuged at 15,500 rpm for 15 min at 4 °C. The supernatant was collected and stored at 4 °C 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 were analysed with the anthrone reagent in a Bausch and Lomb Spectrophotometer (Yemm and Willis, 1954). Free proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrine reaction (Irigoyen et al., 1992). Xylem sap was collected from the whole pepper shoot by using the pressure chamber. Abscisic acid (ABA) analysis were performed by HPLC (Goicoechea et al., 1997).

Results

The first foliar wilting symptoms appeared 18 days after fungal inoculation (Figure 1), although leaves did not show necrotic areas until day 26. The increase in the percentage of necrosis coincided with the beginning of the defoliation. Non-inoculated controls always remained symptomless.

Shoot height (Table 1) was similar in healthy and inoculated plants during the entire course of the experiment. Plant dry matter was reduced in inoculated plants 3 weeks after inoculation, and such decreases were concomitant with a significant loss of total leaf area due to defoliation (Table 1).

Leaf water potential (Ψ) was greatly influenced by inoculation. Infected plants showed significantly lower Ψ than healthy ones from the first week after inoculation and it gradually decreased until the end of the experiment (Figure 2a). By contrast, relative water content (RWC) in leaves from diseased plants (Figure 2b) remained unchanged for almost 1 month after inoculation and, then, it decreased drastically.

Total soluble protein concentration in leaves was reduced as plants became older, in both healthy controls

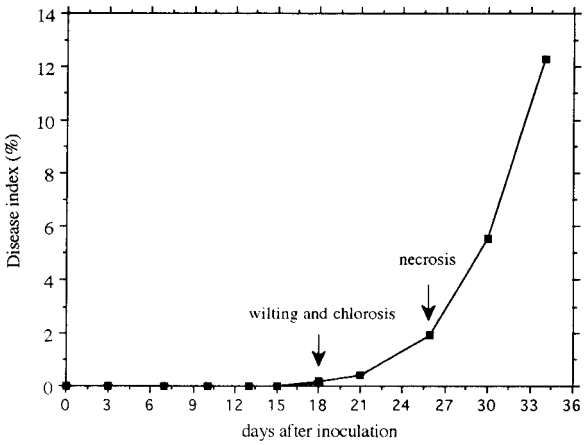

Figure 1. Disease index in inoculated pepper plants. Each point represents the mean of 6–10 plants. Standard Errors lower than 10% were not represented. Comparison between means were made with the Student's *t*-test.

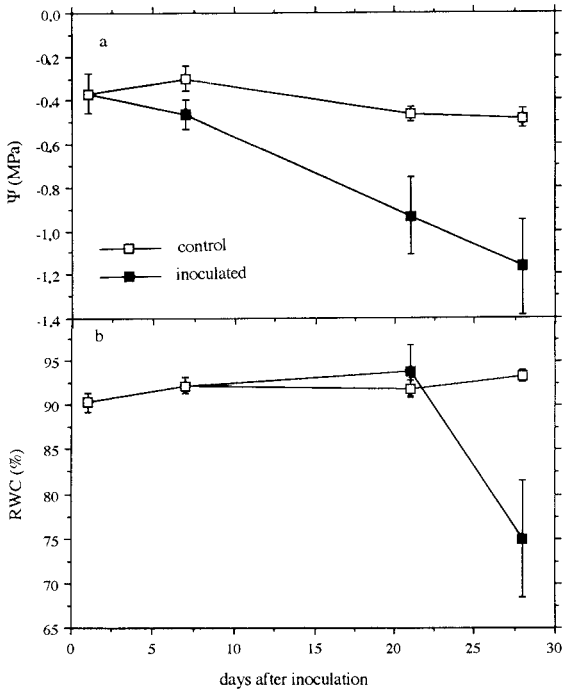

Figure 2. Water potential, Ψ (a) and relative water content, RWC (b) in leaves of healthy control pepper plants (□) and in plants inoculated with *V. dahliae* (■). Symbols represent the mean of 3 plants. The bars indicate standard error (SE) of the mean; SEs lower than 10% were not represented. Comparison between means were made with the Student's *t*-test.

Table 1. Shoot height, plant dry matter and total leaf area per plant in pepper healthy controls and in plants inoculated with *V. dahliae*. Values are mean ± SE ($n = 3$ plants). Comparison between means were made with Student's *t*-test within each column. Values followed by a common letter are not significantly different ($P < 0.05$)

Days after inoculations	Healthy controls			Inoculated plants		
	Shoot height (cm)	Plant dry matter (g)	Total leaf area (cm^2)	Shoot height (cm)	Plant dry matter (g)	Total leaf area (cm^2)
0	15.00 ± 1.53a	5.65 ± 0.23a	709.84 ± 37.62a	15.00 ± 1.53a	5.65 ± 0.23a	709.84 ± 37.62a
7	21.33 ± 0.88a	6.26 ± 0.69ab	771.12 ± 57.48a	20.00 ± 0.58a	6.81 ± 0.80ab	763.27 ± 54.09a
21	22.33 ± 1.76a	8.60 ± 0.16b	859.12 ± 57.33a	21.83 ± 0.60a	7.58 ± 1.11b	777.39 ± 80.37a
28	21.83 ± 0.67a	7.19 ± 0.28b	833.48 ± 142.49a	21.17 ± 0.73a	6.20 ± 0.11ab	500.12 ± 50.90b
33	19.33 ± 1.20a	6.98 ± 0.55ab	807.83 ± 227.64a	20.33 ± 0.33a	5.99 ± 0.35ab	352.87 ± 77.38c

and inoculated plants (Figure 3a). This parameter was very sensitive to infection and the protein levels found in leaves of infected plants were significantly lower than those of controls from the first week after inoculation. However, protein content measured at the end of the experiment did not differ between the two groups of plants. Proline concentration in foliar tissues from inoculated plants (Figure 3b) increased significantly between days 21 and 28 after inoculation,

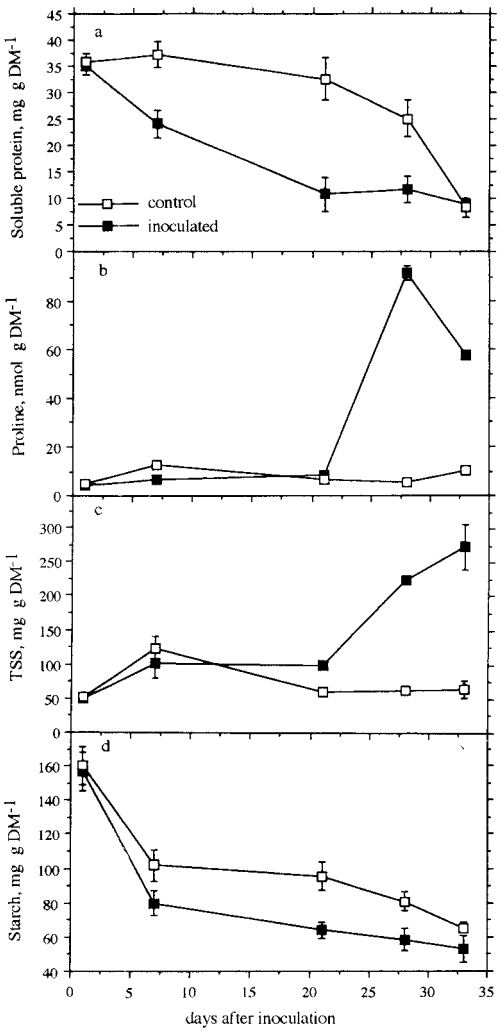

Figure 3. Soluble protein (a), proline (b), total soluble sugars, TSS (c) and starch (d) in leaves of healthy control pepper plants (□) and in plants inoculated with *V. dahliae* (■). Symbols represent the mean of 3 plants for proline and the mean of 5–6 plants for soluble protein, TSS and starch determinations. Otherwise as for Figure 2.

while they did not change in the leaves of control plants. Simultaneously, a significant buildup was observed in TSS levels in leaves of infected plants (Figure 3c). As found for changes in protein content, the leaf starch concentration gradually declined in both healthy and infected plants during the time course of the experiment (Figure 3d). However, the decrease was more pronounced in infected plants from the first week after inoculation. Figure 4 shows the high and negative correlation between total soluble sugars and starch contents in leaves of diseased plants ($r = 0.871$, $P < 0.05$).

The ABA concentrations detected in the xylem sap of plants are shown in Table 2. In infected plants, a dramatic decrease of this phytohormone occurred by the first week after inoculation, whereas ABA levels in the xylem of control plants remained unchanged until they were 80 days old (third harvest). ABA concentrations in the xylem decreased significantly in plants from both treatments as they became older; however, at the end

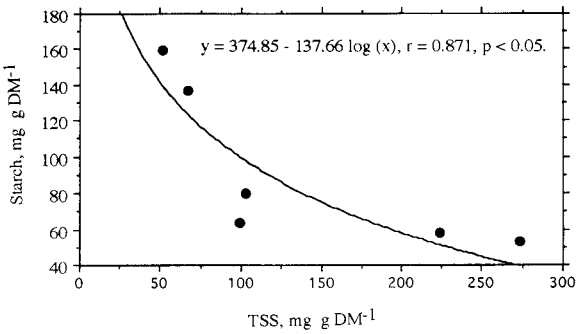

Figure 4. Relationship between total soluble sugar (TSS) content and starch concentration in leaves of pepper plants inoculated with *V. dahliae*. Symbols represent the mean of 6 plants.

Table 2. Abscisic acid (ABA) concentration in the xylem sap (pmol cm^{-3}) of pepper healthy controls and plants inoculated with *V. dahliae*. Values are mean ± SE ($n = 6$ plants). Comparison between means were made with Student's *t*-test within each column. Values followed by a common letter are not significantly different ($P < 0.05$)

Days after inoculation	Treatment	
	Healthy controls	Inoculated plants
0	593.7 ± 101.3a	593.7 ± 101.3a
7	511.0 ± 115.1a	204.4 ± 17.0b
21	428.4 ± 64.2a	212.0 ± 36.9b
28	85.1 ± 3.4cd	93.4 ± 4.8c
33	45.4 ± 4.9e	69.0 ± 4.9d

of the experiment, ABA levels were higher in infected than in control plants.

Discussion

Visible symptoms of the disease were not evident until day 18 after inoculation (Figure 1). Moreover, the development of fungal colonies from cross-stem sections only occurred from day 21. By contrast, the leaf water potential (Figure 2a), the total protein in leaves (Figure 3a) and the ABA concentration (Table 2) in the xylem sap of inoculated plants were significantly different than those measured in controls on day 7 after inoculation. In agreement with the findings of other authors working with *V. albo-atrum* (Lorenzini et al., 1997), our results suggest that *V. dahliae* may release toxic substances that alter the physiology of the plant host even when the fungus is still localized in the roots.

Data in Table 1 show that shoot plant height was similar in both inoculated and healthy controls, which is in contrast with findings for cotton (Tzeng et al., 1985). However, fungus infection in this last work took place in the early stages of plant development. In our study, the total DM of inoculated plants was significantly lower than that of controls only at the end of the experiment. This fact can be explained by the smaller total leaf area measured in the inoculated plants due to defoliation (Table 1).

Leaf water potential (Ψ) of inoculated plants decreased significantly from the day of inoculation until the end of the experiment (Figure 2a). Similar behaviour of leaf water potential was observed in pepper inoculated with *Phytophthora capsici* (Aguirreolea et al., 1995). On the other hand, relative water content (RWC) remained unchanged during the first month after inoculation (Figure 2b). In other words, Ψ appeared to be more sensitive to infection than RWC. As has been widely demonstrated, most wilt pathogens increase the resistance to water movement as a consequence of reduced diameter of the conductive elements. Such leaf Ψ decrease could be a mechanism developed by infected plants in order to achieve the required tension to let water move from soil to shoot. The relationship between RWC and Ψ has sometimes been used to quantify the dehydration tolerance of tissues (Sánchez-Díaz and Kramer, 1971). It has been suggested that tissues able to retain a high RWC as Ψ declines are more tolerant to dehydration. Also, maintenance of RWC at any given Ψ may reflect a greater rigidity of the cell walls and their ability to withstand mechanical collapse as water is being lost from the tissue (Bennet-Clark, 1959).

The marked decline in leaf RWC in infected plants coincided with a drastic increase in free proline (Figure 3b) and TSS (Figure 3c). A similar behaviour was observed by Irigoyen et al. (1992) in leaves of alfalfa subjected to drought. Soluble sugars, especially fructose and glucose in higher plants can accumulate in tissues as a consequence of different types of stress (Zhang and Archbold, 1993), and can contribute to regulate cellular turgidity (Moore and Cosgrove, 1991). Such accumulation may result from a greater degree of conversion of plastidic starch into soluble sugars (Geigenberger et al., 1997). In our study, there was a correlation ($r = 0.871$, $P < 0.05$) between starch and total soluble sugars in leaves of diseased plants (Figure 4). Moreover, results in Figure 3d agree with those of Geigenberger et al. (1997), who indicated that the rapid conversion of starch to sucrose may also be associated with inhibition of starch synthesis under stress conditions.

Similar to the findings of Tzeng and DeVay (1985) in cotton, higher proline levels were detected in leaves from inoculated plants than in those from controls. 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 (Aspinall and Paleg, 1981). As explained above, the sharp increase in proline in leaves from inoculated plants (Figure 3b) was coupled with a drastic decrease in RWC (Figure 2b). Therefore, it seems that, in inoculated pepper, accumulation of such osmolite is related to higher damage caused by fungus attack. It is interesting to note that the proline peak (Figure 3b) measured in diseased plants was not dependent upon protein hydrolysis (Figure 3a). Moreover, proline and TSS increases occurred simultaneously, suggesting that proline and sugar accumulation may be related. Our findings agree with reports of Irigoyen et al. (1992) in alfalfa under water deficit.

Abscisic acid has been reported to be involved in controlling resistance or susceptibility of plants to some fungal diseases, and in altering the severity of symptoms (Fraser, 1991). Results in Table 2 show that infection affected ABA concentration in xylem sap. Such concentrations were clearly lower in infected plants than in control ones in the first 20 days after inoculation. It is well known that water stressed plants

usually increase ABA levels (Zhang and Davies, 1989), including pepper (Ismail and Davies, 1997). However, the fact that *Verticillium*-wilted plants were well watered and, consequently, their roots remained wet could explain why, in the early stages of the infection, they did not show an increase in xylematic ABA. By contrast, 1 month after inoculation ABA levels in xylem of *Verticillium*-inoculated plants were higher than that of healthy controls, and this fact was associated with a strong defoliation of diseased plants (Table 1). Likewise, ABA in *Fusarium*-infected tomato plants did not build up until the expressive phase of the disease (Beckman, 1987). Literature on the role of ABA in host–pathogen interactions is controversial. This phytohormone inhibited the development of infection in susceptible varieties of wheat by *Puccinia recondita* (Levin, 1984). Similarly, Dunn et al. (1990) observed that low ABA levels were correlated with increased symptom severity in *Phaseolus vulgaris* inoculated with *Colletotrichum lindemuthianum*. On the other hand, ABA has been shown to increase susceptibility of potato infected with *Fusarium roseum* (Hammerschmidt, 1984) and soybean with *Phytophthora megasperma* (Ward et al., 1989).

In summary, results indicate that *V. dahliae* modifies the proline, TSS, starch and total soluble protein levels in leaves, as well as the ABA concentration in xylem of pepper plants. Results suggest that proline and TSS accumulation could be sensors of the damage caused by the fungal infection.

Acknowledgements

This work has been partially supported by the group Inabonos-Roullier. The authors wish to thank Mr. Amadeo Urdiáin for technical assistance.

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