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Phenylalanine Ammonia Lyase Activity in Stem of Pepper *(Capsicum annuum* L.) Infected by *Phytophthora capsici* L.

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ABSTRACT

In this study, PAL activity in stems of pepper (*Capsicum annuum* L.) plants infected by the root rot pathogen *Phytophthora capsici*-22 in a resistant (PM-702) and two susceptible (Kahramanmaraк-Hot (KM-Hot) and Demre-8) cultivar were studied. The response of the PAL activity in the resistant cultivar was faster and higher than in the susceptible cultivars ($p < 0.01$). The increase in production of PAL upon *Phytophthora capsici*-22 were higher in the infected plants compared to the non-infected (control) plants ($p < 0.01$) . An early induction of PAL was observed in the stems of three peppers infected with *P.capsici* within two days.

Keywords: *Capsicum annuum*, defence, pepper, phenylalanine ammonia lyase, *Phytophthora capsici*

1. INTRODUCTION

Phytophthora root rot, caused by *Phytophthora capsici* L., is a devastating disease on peppers. *P. capsici* was first described by Leonian (1922) on chili pepper in New Mexico. In Turkey it was first detected in the surroundings of Kahramanmaras city in 1970, from where it rapidly spread to other parts of the country (Cinar and Bicici 1977). The disease was subsequently reported in many pepper growing areas in the world. Phytophthora root rot causes yield losses up to 100% in pepper fields (Anonymous 2001).

P. capsici is a soilborne pathogenic protist (phylum *Oomycota*) that infects many solanaceous plants. The pathogen has been seen in most of the plant species and the major hosts are red and green peppers, watermelon, cantaloupe, honeydew melon, cucumber, squash, tomato and eggplant (Leonian 1922; Erwin and Ribeiro 1996).

The disease can affect plants at any growth stage, and the damping-off syndrome can kill seedlings within 5 days of infection. The pathogen can also cause crown, leaf and fruit blight, wilting of the whole plant and dark purplish discoloration of the stem. Control methods such as selection of genetically resistant cultivars and planting in well-drained soil are of great importance (Erwin and Ribeiro 1996).

Plants possess a variety of active defense responses, which contribute to resistance against a range of pathogens. Strengthening the endogenous defense capabilities of plants, synthesis of pathogenesis-related (PR) proteins, phytoalexins, accumulation of active oxygen species, rapid alterations in cell walls and enhanced activity of various defense-related enzymes, has been advanced as a promising strategy for crop protection (El Ghaouth et al. 2003).

One of the biochemical mechanisms involved in problems is the production of several secondary plant products originating from or passing through the phenylpropanoid pathway (PPP) . Phenylalanine ammonia-lyase (EC 4.3.1.5., PAL) is a critical enzyme in this pathway, working as a trigger for the production of these secondary compounds.

The PAL enzyme is part of plants defence against many different stresses and an increase of PAL activity in plants under different stresses has been extensively reported (Jones et al. 1984; Dixon 1986; Hahlbrock and Scheel 1989).

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PAL catalyzes the conversion of L-phenylalanine into trans-cinnamate, the initial committed step of the multi-branched PPP in higher plants. As the first step in phenolic metabolism, this is a key biochemical reaction in both plant development and defense (Chang et al. 2008). Branch pathways lead to the synthesis of compounds that have diverse functions in plants, notably in defence, such as cell wall strengthening and repair (e.g. lignin, suberin, phenolics), antimicrobial activity (e.g. furanocoumarin, pterocarpan and isoflavonoid phytoalexins), and as signalling compounds such as salicylic acid (Hammerschmidt 1999).

The differential induction of defence mechanisms in the resistant and susceptible cultivars (Ziouti et al. 1996; El Modafar et al. 2000; El Modafar and El Boustani 2001) may be related to a difference in level of PAL activity, as shown in several hostï pathogen interactions (Corsini and Pavek 1980; Shiraishi et al. 1989; Orczyk et al. 1996; Sharan et al. 1998).

In general, plant metabolism is visibly altered by pathogen attack on host plants. However, the change in plant metabolism is significantly different for susceptible and resistant plants. The studies concerned with obtaining pepper varieties resistant to root rot have necessitated examination and comparison of metabolisms of pepper varieties which has different resistance to *P.capsici*. Unfortunately, the roles of various substances in plants which take part in physiological and biochemical mechanisms and govern the defence against this disease are not fully understood yet (Egea et al. 1999; Requena et al. 2005).

The objectives of this work are to compare the time course of PAL activity induction in stems of resistant (PM-702) and susceptible (KM-Hot and Demre-8) cultivars after inoculation with different concentrations (10^2, 10^3 and 10^4 zoospore mL^{-1}) of *P.capsici* -22.

2. MATERIAL AND METHODS

2.1. Plant Material and Inoculation

Seeds of three *Capsicum annuum* cultivars, two susceptible KM-Hot (Kahramanmaraк-Hot) and Demre-8, and one resistant PM 702 (Criollo de morales = CM 334) were used in this study.

Pepper seeds in good quality were sterilized by soaking into 0.75% sodium hypochloride for 2 minutes and then thoroughly washed with sterile distilled water. The seeds were left to swell in an incubator to germinate at 25 ºC, in continuous dark, for three days. Pepper seeds were sown in a plastic pot (15×75×12 cm) containing a steam-sterilized soil, organic fertilizer (burned manure) and sand mix (1:1:1, v/v/v). In each plastic pot, 75 germination led seeds were sown. The seeds were covered lightly with additional prepared soil, organic fertilizer (burned manure), sand mix and then carefully watered daily until the seeds germinated in greenhouse at 25 ± 2 ºC with 65ï 70% humidity, in day light. The seedlings after emergence (approximately 2-3 leaf stage) were thinned to a single seedling per cell. Of these seedlings left to grow in these conditions, for two months (approximately), the seedlings not showing the required growth capacity were eliminated. 360 seedlings were used for each cultivar of pepper.

P. capsici-22 (obtained from the fungal culture collection of Ankara University, Faculty of Agriculture,, Ankara-Turkey) was grown on V_8 agar plates at 25 ºC in the dark. Zoospores were produced from mycelial (Ward and Stoessl 1974). The zoospores were collected and filtered through Whatman no: 54 to remove sporangial cases and mycelial fragments. The concentration of zoospores was then adjusted to 10^2, 10^3 and 10^4 per milliliter using a hemacytometer (Ward and Stoessl 1974).

Six ï seven leaf stage seedlings of pepper growing in greenhouse conditions for two months were collected; their roots were washed with tap water and disinfected by sodium hypochloride (0.75%) for 1-2 min. Then, they were washed with 1L of sterile distilled water to which 1-2 drops of Tween-20 was added. Five seedlings were brought together in a bunch wrapped by aluminum foil 3-4 cm above the root to level the necks.

Six bunches of plants each with 30 seedlings were put into a sterile glass bottle of 500 mL containing 400 mL of full strength Hoagland solution (Hoagland and Arnon 1950). Four glass bottles (30 seedlings for each bottle) were prepared for control and each zoospore concentration (10^2, 10^3 and 10^4 zoospores mL^{-1}). Accordingly, twelve glass bottles (360 seedlings) were prepared for each pepper cultivar. The plants were left in the plant growth chamber for 3 days under the conditions of 22 ± 3 ºC, 60% humidity and 14 h light period, so that they could accommodate with changing environmental conditions. Three days later, pepper plants at the 6-7 leaf stages were preinoculated with *P. capsici* by immersing the roots in 10^2, 10^3 and 10^4 zoospore mL^{-1} suspensions for 1 h. A control was treated with sterile distilled water. After preinoculation, pepper plants were transferred to hoagland solution under conditions 22 ± 2 ºC, 60-65% humidity, and 14 h light periods. On the 2nd, 4th and 6th days after inoculation, seedlings were randomly taken from the control and infected groups of each cultivar. . When collecting samples, control and infected stems were immediately separated, put into nylon bags, labeled, and stored in -70 ºC deep freezer until analysis.

2.2. Phenylalanine Ammonia Lyase (E.C. 4. 3. 1. 5., PAL) Assay

Both control and infected stems (0.2 g fresh weight) were extracted in 600µl 50 mM Tris-HCl buffer (pH 8.8) containing 1 mM EDTA, 15 mM β-mercaptoethanol, and 50 mM ascorbic acid at 4°C, suggested by Ochoa and Salgado (1992) method. The supernatant was collected and used as PAL enzyme extract.

The assay mixture contained 100µl of extract, 100 mM Tris-HCl buffer (pH 8.8), 0.5 ml of 10 mM L-phenylalanine, and 0.4 ml deionised water. The mixture was incubated for 1 h at 37 ºC and the reaction was

terminated by adding 0.5 ml of 6 M HCl; then sample absorbance was measured at 290 nm. The calibration curve was constructed using cinnamic acid. The blank had the same constituents except that the extract was added after the HCl solution (Ochoa and Gómez 1993).

2.3. Statistical Analysis

All data were analyzed using analysis of variance (ANOVA). Data presented are mean values ± S.E.M (standard error measures) for three replicates. Analysis of variance (three-way ANOVA) was employed to compare the means of three pepper cultivars, and the significance of differences was determined by DUNCAN multiple comparison technique**.** Variance analysis was conducted by using Minitab 15.1 statistical software package, and DUNCAN tests were performed using the software package MSTAT - C statistics. The statistical significance is indicated by appropriate letters within the figure (α = 5%).

3. Results and Discussion

The experiment on the resistance of PM-702 and KM-Hot, Demre-8 to the *P.capsici*-Kahramanmaraк isolate was carried out under controlled conditions.

An early induction of PAL was observed in the stems of all peppers infected with *P.capsici* within two days. The highest PAL activity in non-infected (control) stems was recorded in PM-702 cultivar, and it was significantly higher than KM-Hot and Demre-8 ($p < 0.01$).

When compared to control stems, PAL activity on the 2nd day after inoculation with *P.capsici* -22 was significantly higher ($p < 0.01$) in PM-702 plants, which were inoculated only with 10^2 zoospore mL^{-1}, than in all other treatments. PAL activity in stems of *P.capsici*-treated PM-702 seedlings increased on days 4 and 6 ($p < 0.01$). When compared to control stems, the maximum increase of PAL was observed in the stems of PM-702 seedlings which were infected with 10^3 zoospore mL^{-1} on the 4th and 6th days following the infection ($p < 0.01$) (Fig. 1).

PAL activity in stems of *P. capsici*-treated KM-Hot seedlings increased on days 2, 4, and 6 (Fig. 1). PAL activity on the 2nd day after inoculation with 10^2, 10^3 and 10^4 zoospore mL^{-1} *P.capsici*-22 was significantly higher ($p < 0.01$) in KM-Hot stems. The differences found on the 4th and 6th days were also significant ($p < 0.01$) in three inoculum concentrations.

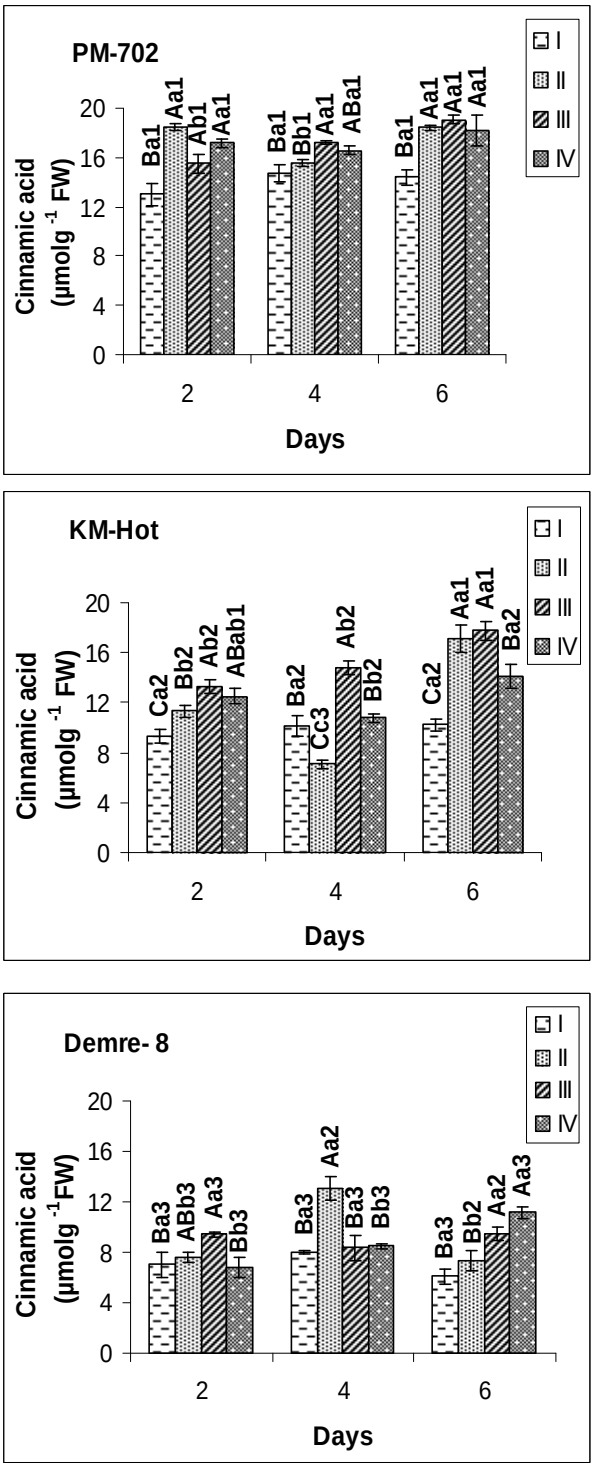

Figure 1. PAL activity in stems of peppers subjected to *P. capsici* at different zoospore mL^{-1} concentrations. (I: Control, II: 10^2 zoospore mL^{-1}, III: 10^3 zoospore mL^{-1}, IV: 10^4 zoospore mL^{-1}). Capital letters represent concentration differences in same cultivar and same day. Lower case letters represent differences in days in same cultivar and same concentration. Numbers represent differences in cultivars in same day and same concentration.

PAL content in the stems of Demre-8 seedlings was significantly lower ($p < 0.01$) than the other two peppers at all times. When compared to control stems, the maximum increase of PAL activity was observed in the stems of Demre-8 seedlings which were infected with 10^2 zoospore mL^{-1}, on the 4^{th} (+ 63.75%) day following the infection ($p < 0.01$). PAL activity on the 6^{th} day after inoculation with 10^2, 10^3 and 10^4 zoospore mL^{-1} *P.capsici*-22 was higher ($p < 0.01$) in Demre-8 stems (Fig. 1).

Plants exhibit natural resistance to disease, which involves inducible defense mechanisms, including accumulation of phytoalexins, deposition of lignin like material, accumulation of cell wall hydroxyproline-rich glycoproteins, and increases in the activity of certain enzymes, such as PAL. PAL is involved in phytoalexin or phenolic compound biosynthesis. This enzyme has been correlated with defence against pathogens in several plants, including tomato (Borden and Higgins 2002), pepper (Jung et al. 2004), and wheat (Mohammi and Kazemi 2002). Resistance to *P.capsici* has been associated with an increase in PAL activity (Mozzetti et al. 1995; Fernandez 1997a ; Fernandez and Liddell 1997b).

The induction of PAL activity is strongly related to the physiological state of the plant tissue. Changes in the environmental conditions such as light, wounding, plant hormone application or pathogen infection stimulate a rapid increase in PAL activity (Dixon and Paiva 1995).

Increased PAL activity has been demonstrated in many plant-pathogen systems. Lummerzheim et al. (1995) showed that there was an increase in PAL during a hypersensitive response of *Arabidopsis thaliana* to *X. axonopodis* pv. *campestris*, and PAL activity in soybean cultivars resistant to *Phytophthora megaspema* f. sp. *qlycinea* increased 7 to 10 fold in six-hour period following inoculation**.** An increase in PAL transcription was also observed in potato tubers infected with *Phytophthora infestans* (Yoshioka et al. 1996; El Modafar et al. 2001) showed that there was an increase in PAL in date palm roots in response to inoculation with *Fusarium oxysporum f. sp. Albedinis.* Umesha (2006) reported an increase in PAL activity in tomato treated with pathogen.

The increased activity of PAL has been assumed to be the key component in local and systemic disease resistance (Kombrink and Somssich 1997). PAL is related to the synthesis of phenolic compounds with deterrent, toxic and antinutritional properties (Apel 1993). Therefore, an increase in the activity of this enzyme indicates the synthesis of plant defense compounds against external agents.

PAL genes can be regulated developmentally, induced by wounding, by low temperatures, by other stress conditions and by pathogen attack (Wu and Lin 2002). PAL induction has been linked to defence responses that involve phenylpropanoids in numerous diseases. Typically, accumulation of PAL activity is more rapid, higher and longer lasting in plant-pathogen interactions (Cui et al. 1996).

In our study, an increase in PAL activity was observed in the stems of peppers treated with *P.capsici*-22 within two days of treatment. The time course experiment showed that PAL activity increased in the stems within 2 days of inoculation, making it one of the earlier responses to infection detected. An early induction of PAL is very important as the biosynthesis of lignin originate from L-phenylalanine. PAL activity is associated with biosynthesis of toxic metabolites, such as phytoalexins, phenols, lignins and salicylic acid in plant defence pathways (Mauch et al. 1996). In addition, induced resistance may also be manifested as release of antifugal compounds in seedlings. PAL accumulation in induced stems may reduce phenylalanine, which is necessary for fungal growth and development (Liu and Rahe 1997). Thus, this work suggested that *P.capsici* disease resistance is based on the systemic activation of natural plant defence mechanisms.

The changes in PAL activity after infection in PM-702 stems were faster and higher than that in KM-Hot and Demre-8. This differential induction of the PAL activity in response to the infection by *P.capsici*-22 could be related to a suppression of the PAL induction in the susceptible cultivars. The suppression of the PAL induction was generally related to extracellular suppressors in the fungal culture filtrate or in the germination fluid of spores (Yamada et al. 1994; Wada et al. 1995).

All these results suggested that induction of PAL by *P.capsici*-22 might result in the activation of defence, but pathogen challenge might cause damage and necrosis which in turn decreases the activation of PAL in pepper stems.

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