Defence responses of chilli fruits to *Colletotrichum capsici* and *Alternaria alternata*

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Abstract

The induction of defence compounds and enzymes involved in the phenylpropanoid pathway were studied in the ripe and green chilli fruits inoculated with *Colletotrichum capsici* and *Alternaria alternata*. Total phenols and the activity of phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO) and catalase (CAT) increased in the inoculated ripe and green chilli fruits compared to the corresponding healthy fruits. Total phenols and the activities of the enzymes were at the maximum 2 - 3 d after inoculation and thereafter declined sharply in ripe chilli fruits, whereas slowly in green chilli fruits. In comparison with ripe chilli fruits, green chilli fruits were more resistant as they showed higher accumulation of total phenols and also higher activities of enzymes.

Additional key words: *Capsicum annuum*, defence enzymes, phenols.

Introduction

Chilli (*Capsicum annuum* L.) is an important vegetable crop grown throughout the world. An alkaloid, capsaicin is extracted from chilli, which has medicinal value. In recent years the fruit rot of chilli caused by *Colletotrichum capsici* (Syd.) Butler and Bisby and *Alternaria alternata* (Fr.) Keissler has become a severe one. The ripe chilli fruits were more susceptible to these pathogens than green chilli fruits (Prakasam 1983). Plants are able to defend themselves successfully with a complex set of preformed structures and inducible reactions. The inducible reactions require the perception of either plant-derived (endogenous) or pathogen derived (exogenous) signal molecules. These so-called elicitors are diverse chemically and include proteins, peptides, glycoproteins, lipids and oligosaccharides. Elicitors trigger plant defence responses that are part of the basic or non-host resistance of plants (Nürnberger 1999). The structural and cultivar specificity of elicitors and their ability to trigger plant defence responses at very low concentrations strongly suggest the existence of a receptor at the plasma membrane and a downstream signal transduction cascade (Ebel and Cosio 1994). The defence response is often associated with localized hypersensitive cell reaction (Mittler *et al*. 1997) and the *de novo* formation of phytoalexins (Hammond-Kosack and Jones 1996). The reinforcement of cell wall constituents is also part of the defence response (Bruce and West 1989). Various signalling molecules mediate induction of pathogenesis-related (PR) proteins gene expression. These mechanisms against a pathogen fail when the plant is infected by a virulent pathogen, which avoids triggering or suppresses resistance reactions or evades the effects of activated defences. The defence gene products include peroxidase (PO), polyphenol oxidase (PPO) that catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexin and phenolics biosynthesis. Some PR proteins such as chitinases (PR-3; Legrand *et al*. 1987) and β-1,3 glucanase (PR-2; Kauffmann *et al*. 1987) have potential antifungal activity which degrade the fungal cell wall and cause lysis of fungal cells. Chitin and glucan oligomers released during degradation of fungal cell wall act as elicitors that elicit various defence mechanisms in the plants (Frindlender *et al*. 1993). Constitutive, high expression of these defence enzymes in crop plants can enhance resistance to a pathogen (Broglie *et al*. 1991). When a pathogen lands on a host surface, it activates the host's defence mechanism(s) probably by releasing elicitors from its cell walls. Plants are endowed with defence genes, which are quiescent in healthy plants,

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Abbreviations: CAT - catalase; PAL - phenylalanine ammonia lyase; PO - peroxidase; PPO - polyphenol oxidase.
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but, when activated by various factors, they induce systemic disease resistance. Defence enzymes that are induced in response to invasion by a pathogen have greater antifungal activity to the pathogen in suppressing symptoms. Induction of defence enzymes makes the plant resistant to pathogen invasion (Van Loon *et al*. 1998). Expression of these proteins is correlated with the development of systemic acquired resistance in plants (Ryals *et al*. 1996). Thus, if defence mechanisms are triggered quickly and maintained enhanced level due to infection by a plant pathogen, disease severity can be reduced. In the present study an attempt was made to compare and quantitatively analyze the induction of defence compounds and enzymes in green and ripe chilli fruits inoculated with *C. capsici* and *A. alternata.*

Materials and methods

Chilli (*Capsicum annuum* L.) cultivar K2 was obtained from the Tamil Nadu Agricultural University, Coimbatore, India. The fruit rot pathogens *Colletotrichum capsici* (Syd.) Butler and Bisby and *Alternaria alternata* (Fr.) Keissler were isolated on potato dextrose agar (PDA) medium from the diseased specimen showing the typical symptoms. The infected portion of the fruit was cut into small bits, surface sterilized in 1 % $HgCl_2$ for 30 s, washed in repeatedly by sterile distilled water and plated onto PDA medium in Petri dishes. The plates were incubated at room temperature (28 ± 2 ºC) for 5 d and observed for the fungal growth. The fungus was purified by single spore isolation technique (Riker and Riker 1936). Pathogenicity was tested and a highly virulent isolate was selected and used throughout the experiment.

The spore suspension was prepared by adding 20 cm^3 of sterile distilled water to the Petri plates containing 10-d-old cultures of *C. capsici* and *A. alternata* and this was mixed well and filtered through muslin cloth. The spore suspension (5×10^5 cm^{-3}) was used for inoculation.

Induction of defence genes in chilli cultivar K2 against fruit rot fungi was studied in greenhouse grown chilli seedlings (35-d-old) planted in earthen pots filled with sterilized soil (2 seedlings per pot). Fifty days after planting, chilli plants containing both green and ripe fruits were inoculated with *C. capsici* and *A. alternata* (separately and in combination) and the fruits not inoculated with the pathogens were kept as control. All the treatments were replicated five times in factorial completely randomized design (CRD).

Fruits (green and ripe) were collected from treated chilli plants at various time intervals (1, 2, 3, 4, and 5 d after inoculation) and washed in running tap water and used for biochemical analysis.

The content of the total phenols present in the fruits was estimated following the procedure of Bray and Thorpe (1954). Fresh fruit samples of 0.5 g(f.m.) were blended with 10 cm^3 of 80 % ethanol at 50 ºC for 30 min. Then the extract was first filtered through cheese cloth and centrifuged at 15 000 *g* for 10 min. The volume was made up to 10 cm^3 with ethanol. An aliquot of 1 cm^3 was taken in a series of boiling tubes and made up to 3 cm^3 with distilled water. To this 1 cm^3 of Folin-Ciocalteu reagent and 2 cm^3 of 20 % sodium carbonate were added. The tubes were heated for 1 min in a boiling water bath and cooled in running water. The solution was diluted to 10 cm^3 with distilled water and the intensity of the blue colour was measured at 660 nm in a spectrophotometer against a blank. Three replications were measured and catechol was used for calibration.

For analysis of PAL, 1 g sample was homogenized in 3 cm^3 of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinyl pyrrolidone. The homogenate was centrifuged at 15 000 *g* for 15 min and the supernatant was used as enzyme source. Sample containing 0.4 cm^3 of enzyme extract was incubated for 30 min at 30 ºC with 0.5 cm^3 of 0.1 M borate buffer, pH 8.8 and 0.5 cm^3 of 12 mM L-phenylalanine prepared in the same buffer. The reaction was stopped by the addition of 0.5 cm^3 of 1 M trichloroacetic acid and the absorbance was measured at 290 nm. A control was run by addition of trichloroacetic acid before the addition of phenylalanine in the above set of reaction. A standard graph was prepared using L-phenylalanine (Dickerson *et al*. 1984).

Fruit samples (1 g) were homogenized in 2 cm^3 of 0.1 M phosphate buffer, pH 7.0 at 4 ºC. The homogenate was centrifuged at 15 000 *g* at 4 ºC for 15 min and the supernatant was used as enzyme source.

The reaction mixture for PO assay consisted of 0.1 cm^3 of enzyme source, 1 cm^3 of 0.001 M pyrogallol in 0.05 M sodium phosphate buffer at pH 6.5, 1.8 cm^3 of distilled water and 0.1 cm^3 of 2 % hydrogen peroxide (Hampton 1963). The changes in absorbance at 30 s intervals for 3 min were recorded in spectrophotometer *Spectronic 20* (*Thermo Spectronic*, USA).

The reaction mixture for PPO assay consisted of 4.5 cm^3 of distilled water, 1 cm^3 of 0.1 M catechol and 0.5 cm^3 of enzyme source (Matta and Dimond 1963). The changes in absorbance at 495 nm at 30 s intervals for 3 min were recorded in *Spectronic 20*.

The catalase activity was estimated following the procedure described by Dekock *et al.* (1960). The sample (500 mg) was homogenized in 10 cm^3 of ice cold 0.067 M phosphate buffer (pH 7.0) and centrifuged at 15 000 *g* at 4 ºC for 15 min. The supernatant was used as enzyme source. The reaction mixture consisted of 3 cm^3 of H_2O_2, phosphate buffer and 0.03 cm^3 of enzyme extract. The time required for decrease in absorbance from 0.45 to 0.40 was noted.

To study the expression pattern of different isoforms of PO in different treatments, native anionic polyacrylamide gel electrophoresis (resolving gel of 8 % acrylamide and stacking gel of 4 % acrylamide) was

carried out. After electrophoresis, the gels were stained with the solution containing 0.15 cm^3 benzidine in 6 cm^3 NH_4Cl for 30 min in dark (Sindhu *et al.* 1984).

The PPO is extracted by homogenizing 1 g of tissue in 0.1 M potassium phosphate buffer (pH 7.0). The homogenate is centrifuged at 15 000 *g* for 15 min at 4 °C and the supernatant is used as the enzyme source. After native electrophoresis, the gel was equilibrated for 30 min in 0.1 % *p*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by addition of 10 mM catechol in the same buffer (Jayaraman *et al.* 1987).

The data were statistically analyzed using the *IRRISTAT* version *92-a* developed by Biometrics Unit of International Rice Research Institute, the Philippines.

Results

Total phenols increased in ripe and green chilli fruits in response to inoculation with *C. capsici* and *A. alternata* as compared to uninoculated ones. The increase in total phenols was observed on the very next day after inoculation with the pathogens. The increasing trend continued up to 3rd day after inoculation and thereafter the content started to decrease. In ripe and green chilli fruits, the maximum total phenols contents of 0.40 and 0.48 mg g^{-1}, respectively, were found in *A. alternata* inoculated fruits on 3rd day after inoculation. Green chilli fruits had always higher content of total phenols than the corresponding ripe chilli fruits. Though the phenol content decreased from 4th day after inoculation, the content was always higher in the inoculated ripe and green chilli fruits than the uninoculated ones (Fig. 1*A*,*B*).

In the ripe chilli fruits, the PAL activity increased from the 1st day after inoculation and reached the maximum on 2nd day in case of combined inoculation

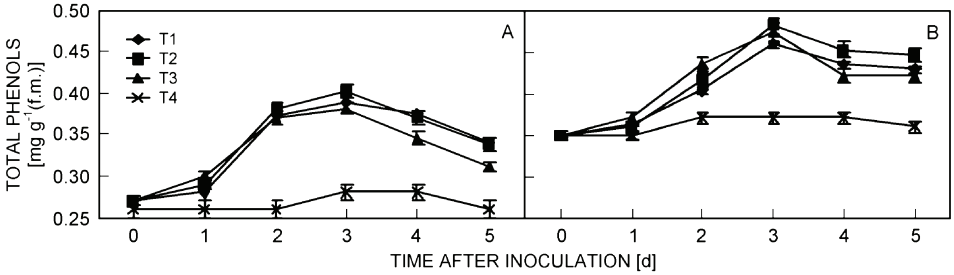

Fig. 1. Changes in total phenols in chilli fruits inoculated with *C. capsici* and *A. alternata*. *A* - ripe chilli fruits, *B* - green chilli fruits; T1 - inoculated with *C. capsici*, T2 - inoculated with *A. alternata*, T3 - combined inoculation, T4 - uninoculated control; *error bars* indicated SD.

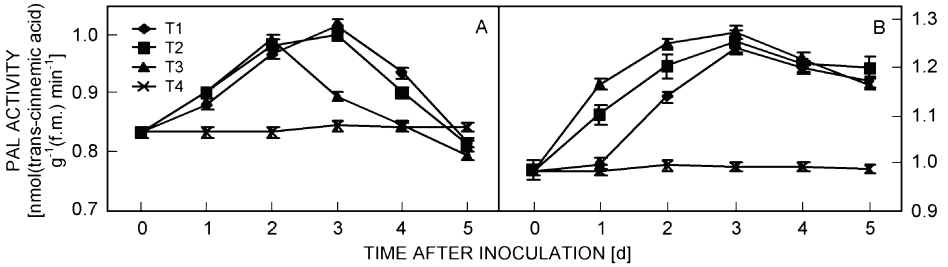

Fig. 2. Changes in phenylalanine ammonia lyase in chilli fruits inoculated with *C. capsici* and *A. alternata* (for detail see Fig. 1).

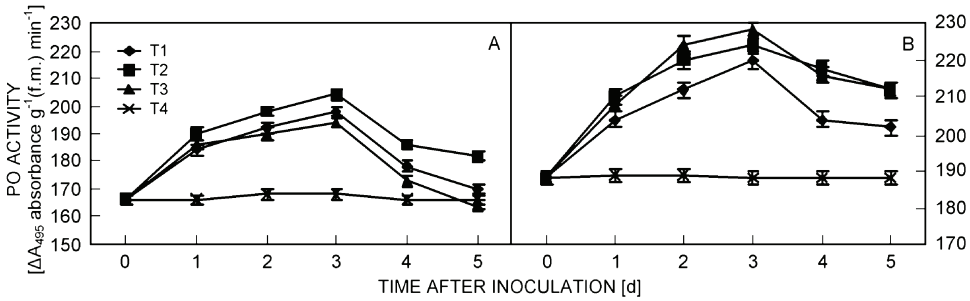

Fig. 3. Changes in peroxidase in chilli fruits inoculated with *C. capsici* and *A. alternata* (for detail see Fig. 1).

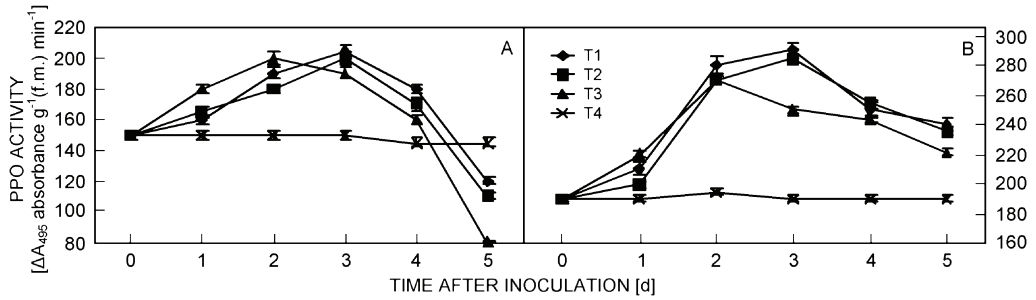

Fig. 4. Changes in polyphenol oxidase in chilli fruits inoculated with *C. capsici* and *A. alternata* (for detail see Fig. 1).

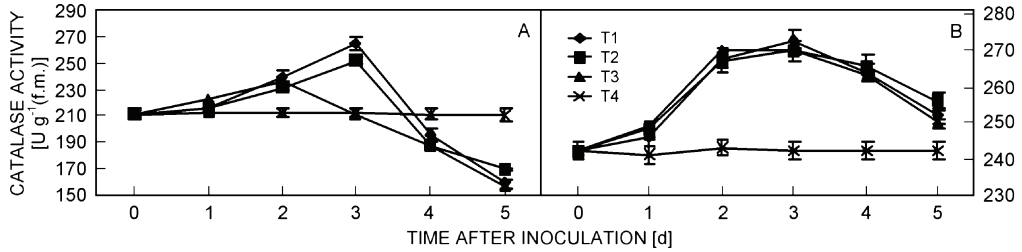

Fig. 5. Changes in catalase in chilli fruits inoculated with *C. capsici* and *A. alternata* (for detail see Fig. 1).

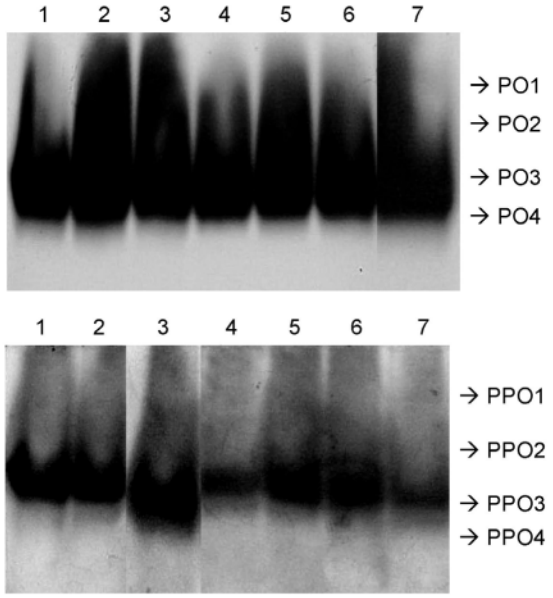

Fig. 6. Native PAGE analysis of PO (*above*) and PPO (*below*) isoforms in chilli fruits. *Lane 1* - green chilli fruits inoculated with *C. capsici*, *lane 2* - green chilli fruits inoculated with *A. alternata*, *lane 3* - green chilli fruits inoculated with *C. capsici* and *A. alternata*, *lane 4* - red chilli fruits inoculated with *C. capsici*, *lane 5* - red chilli fruits inoculated with *A. alternata*, *lane 6* - chilli fruits inoculated with *C. capsici* and *A. alternata*, *lane 7* - uninoculated control.

(inoculated with both the pathogens) and on 3rd day in fruits inoculated with either *C. capsici* or *A. alternata* alone. In green chilli fruits the activity increased gradually, reached the maximum on 3rd day and thereafter decreased. The PAL activity was higher in green than in ripe chilli fruits. In uninoculated ripe and green chilli fruits, there was no significant difference in enzyme activity of controls during the experimental period (Fig. 2*A*,*B*).

In general, there was marked increase in the PO activity in the inoculated ripe and green chilli fruits compared to the corresponding uninoculated healthy fruits. In ripe chilli fruits, PO activity increased from the 1st day after inoculation and reached the maximum on 3rd day and thereafter the activity decreased. However, even on 5th day after inoculation, the activity was higher than the initial level. In green chilli fruits also inoculation with the pathogens increased the activity of PO and the activity reached the maximum on 3rd day and thereafter almost the same level was maintained throughout the experimental period. Green chilli fruits had higher PO activity than ripe chilli fruits. In the uninoculated ripe and green chilli fruits, there was no significant difference in the enzyme activity during the experimental period (Fig. 3*A*,*B*).

PPO activity increased in ripe and green chilli fruits inoculated with *C. capsici* and *A. alternata* separately and also in combined inoculation. In ripe and green chilli fruits, PPO reached the maximum on 2nd or 3rd day and thereafter declined. In comparison with ripe chilli fruits, green chilli fruits had higher enzyme activity. The uninoculated ripe and green chilli fruits, showed no changes in PPO activity during the experimental period (Fig. 4*A*,*B*).

The inoculated ripe and green chilli fruits showed increased catalase activity than the uninoculated healthy fruits. In ripe and green chilli fruits, the enzyme activity gradually increased and reached the maximum on 3rd day after inoculation and then the activity decreased. Green chilli fruits had higher CAT activity than ripe chilli fruits (Fig. 5*A*,*B*).

Native gel electrophoretic separation of enzyme

extract from *C. capsici* and *A. alternata* inoculated ripe and green chilli fruits showed different PO and PPO patterns. The green chilli fruits showed higher induction of different isoforms of PO and PPO compared to pathogen inoculated ripe chilli fruits. The expression of the isoforms was always less in uninoculated fruits (Fig. 6).

Discussion

Phenolics, are in general fungitoxic and increase the mechanical strength of the host cell wall. Lignin is a phenolic polymer which is difficult to be breached by pathogens and has been implicated in plant defence against pests and diseases (Nicholson and Hammerschmidt 1992). The role of phenolic substances has been reported in several host-pathogen interactions (Glazener 1982, Benhamou *et al*. 1996, Ramamoorthy and Samiyappan 2001). The present study revealed that green chilli fruits had higher content of total phenols than ripe chilli fruits. The decrease in phenolic compounds in chilli as the fruit ripens has been reported by Prakasam (1983) and Jeyalakshmi (1996). High content of phenolics in green chilli fruits may be one of the reasons for the less ability of *C. capsici* and *A. alternata* to infect green chilli fruits.

The total phenol content increased in ripe as well as green chilli fruits in response to inoculation with *C. capsici* or *A. alternata.* The increase was very rapid and much more conspicuous in green chilli fruits than in ripe chilli fruits. One of the immediate host responses to infection is the accumulation of phenolics around the infection site (Vidhyasekaran 1997, M'Piga *et al*. 1997). Jeyalakshmi (1996) and Bharathi *et al*. (2004) also reported the increased total phenolic content in the diseased chilli fruits as compared to the healthy ones. More rapid accumulation of phenolics in incompatible host-pathogen combinations than in compatible host-pathogen is a universal phenomenon (Matta *et al*. 1969, Azad 1991, Bharathi *et al*. 2004).

The phenolic content decreased from 4th day after inoculation and this decrease was more pronounced in ripe than in green chilli fruits. The decrease in phenolic content coincided with the necrosis of cells in the inoculated tissues. The decrease in phenolic content can be attributed to oxidative polymerization of phenolics into melanin in necrotic tissues or incorporation of phenols into lignin (Thompson 1964).

In general, the activity of the enzymes PAL, PO, PPO and CAT was higher in inoculated fruits compared to the corresponding healthy fruits. The activity of these enzymes increased in the initial stages of the disease and decreased as the disease progressed coinciding with necrosis of tissues.

PAL catalyzes the conversion of phenylalanine to trans-cinnamic acid, the first step of phenylpropanoid pathway from which other phenolics, phytoalexins and lignin are synthesized (Daafy *et al*. 1997, Ramanathan *et al*. 2000). PAL activity could be induced during plant-pathogen interactions (Ramanathan *et al.* 2000, Bharathi *et al*. 2004, Radjacommare 2000, Kavino *et al*. 2008, Sullivan 2009). In the present study, though PAL activity increased both in ripe and green chilli fruits in response to inoculation with the fruit rot pathogens, the increase was much more in green than in ripe chilli fruits. Higher increase in PAL activity in inoculated incompatible host than in susceptible one has been reported (Moesta and Grisebach 1982, Vidhyasekaran 1997).

PO is a key enzyme in the biosynthesis of lignin (Bruce and West 1989). Peroxidases have been implicated in a number of physiological functions that may contribute to resistance including cross linking of extensin monomers (Everdeen *et al*. 1988) and lignification (Walter 1992) and they are also associated with deposition of phenolic compounds into plant cell walls during resistance interactions (Graham and Graham 1991). Another oxidative enzyme PPO catalyze the oxidation of monophenolic and *o*-diphenolic compounds. Jennings *et al.* (1969) reported PPO and PO as terminal oxidase in infected plant tissue and the fungi toxins might activate these enzymes. Ophiobolin, a toxin produced by *Cochliobolus miyabeanus* has been reported to activate PPO of rice leaves (Nakamura and Oku 1960). In the present study, increased activity of PO and PPO was recorded both in ripe and green chilli fruits inoculated with *C. capsici* and *A. alternata*. Bharathi *et al.* (2004) also reported sharp increase in PO and PPO activity due to infestation by anthracnose pathogen in chilli plants.

An increase of CAT activity in the inoculated fruit suggested an increased H_2O_2 content in the host tissues. The increased CAT activity eliminated the harmful effect of H_2O_2 accumulation (Gangopadhyay *et al*. 1996).

The present study confirmed earlier and higher accumulation of phenolics, PAL, PO, PPO and CAT activities in green than ripe chilli fruits in response to invasion by *C. capsici* and *A. alternata* which contributed to higher resistance in green chilli fruits against *C. capsici* and *A. alternata*.

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