Pak. J. Bot., 33(4): 373-382, 2001.

ACCUMULATION OF PHYTOALEXINS AND PHENYLALANINE AMMONIA LYASE IN CHICKPEA AFTER INOCULATION WITH *ASCOCHYTA RABIEI* AND THEIR ROLE IN DEFENCE MECHANISM

NIGHAT SARWAR, FARHAT F. JAMIL AND RIFFAT PARVEEN

Nuclear Institute for Agriculture & Biology (NIAB), P.O.Box 128, Faisalabad, Pakistan.

Abstract

The production of phytoalexins and phenylalanine ammonia-lyase (PAL) was measured in resistant and susceptible varieties of chickpea after inoculation with *Ascochyta rabiei*. The pterocarpan phytoalexins, medicarpin and maackiain were produced in chickpea plant tissues in response to inoculation. Maximum production of both the phytoalexins was obtained 24 hours after inoculation in resistant varieties, whereas it took five to nine days to reach its maximum in susceptible varieties. This accumulation was preceded by a transient rise in activity of PAL, the first enzyme in the biosynthetic pathway of phytoalexins. Maximum PAL activity was observed 12 to 24 hours after inoculation which coincided with the period of most rapid phytoalexin accumulation. These results suggested that rapid production of phytoalexins is the part of defence mechanism in chickpea and PAL has a regulatory role in the biosynthesis of these secondary metabolites.

Introduction

Plant disease resistance involves not only static protection but also active defence mechanism prominent amongst which is the induced accumulation of host- synthesized phytoalexins antibiotics (Lamb *et al.*, 1989; Barz & Mackenbrock 1994; Kuc', 1995). These compounds are accumulated at the site of infection and are considered to be involved in the defence mechanism of plants to potential pathogens (Kuc', 1995). The resistance of chickpea to *Ascochyta rabiei*, the causal fungus of blight disease, is partly explained by increased levels of constituent phenols (Bashir *et al.*, 1995) and possibly by the synthesis of antifungal compounds, phytoalexins which include medicarpin, maackiain, formonentin, dadzein and biochanin A (Jamil *et al.*, 1996).

Phenylalanine ammonia-lyase (PAL) is the first enzyme of phenylpropanoid metabolism in higher plants (Camm & Towers 1977) and it has been suggested to play a significant role in regulating the accumulation of phenolics and phytoalexins in response to infection (Okay *et al.*, 1997; Peltonen *et al.*, 1998). The present study was undertaken to investigate the accumulation of phytoalexins, medicarpin and maackiain and to determine whether change in the activity of PAL is correlated with the production of phytoalexins and their role in plant resistance.

Material and Methods

Plant material and fungal inoculation: Four chickpea varieties (CM72, CM88 resistant and 6153, Pb-1 susceptible) were grown in pots and blight disease was produced at flowering stage of the plant by inoculating with spore suspension of *A. rabiei*. Plant tissues were collected at 0, 1, 2, 3, 5, and 9 days after inoculation with *A. rabiei* from control and inoculated plants of all varieties.

*Department of Chemistry, Bahauddin Zakariya University, Multan, Pakistan.

Extraction of phytoalexins: Plant tissues were weighed (5 g) and immediately added into 80% boiling methanol. After cooling it was homogenized in high speed blender. Homogenate was filtered through Whatman No. 1 filter paper under vacuum. Methanol from the extracts was evaporated on rotary evaporator until aqueous phase was left. The aqueous phase was washed three times with ethyl acetate. The ethyl acetate layer was separated and evaporated to dryness under vacuum using rotary evaporator. The residues were dissolved in methanol and final volume was made as ml/g fresh weight.

Thin layer chromatographic analysis: The above extracts along with standard maackiain (Provided by Dr. Barz of Germany) were subjected to thin layer chromatography (TLC) on silica gel 60 GF_{254} plates (0.5mm thickness). The plates were developed 2 times with n-pentane: Ethyl ether: acetic acid (75: 25: 1 v/v), observed under UV light and sprayed with diazotized p-nitroaniline reagent (Takuma *et al.*, 1997). The Rf values were recorded. The area showing colour reactions were scraped from the unsprayed plates and the phytoalexins in the gel were extracted with methanol. These methanolic extracts were concentrated under vacuum using rotary evaporator and spotted again along with standard maackiain on the silica gel plates. The plates were developed 4 times with chloroform: carbon tetrachloride (3:1 v/v) (Takuma *et al.*, 1997). The developed plates were sprayed with p-nitroaniline reagents and Rf values of separated compounds were recorded. To confirm the identification of medicarpin and maackiain, the extracts were cochromatographed on TLC plates with different solvent systems and by recording their UV spectra.

Quantification of Phytoalexins: The compounds identified as maackiain and medicarpin were eluted from preparative TLC plates and absorbance was recorded at 287 nm on spectrophotometer (CE 1021). Standard curve was prepared using standard maackiain to quantify the concentrations of the compounds (ug/g fresh weight).

Estimation of Enzyme: Plant tissues were collected from control and inoculated plants of two resistant (CM72, CM88) and two susceptible varieties (Pb-1, 6153) of chickpea at 0, 12, 24, 48, 72, 144 and 216 hours after inoculation with *A. rabiei*. Samples were collected in triplicate for enzyme assay and were immediately stored at - 20°C.

Enzyme extraction: One gram of frozen plant tissues were crushed with 4 ml of 0.1 M borate buffer, pH 8.8, in the presence of 0.1g wet polyvinylpolypyrolidone (PVPP) in an ice chilled pestle and mortar placed in ice. The slurry was filtered through cheese-cloth and then centrifuged at 14,000 rpm for 10 min., at 4°C. The supernatant was used for enzyme estimation.

Phenylalanine ammonia-lyase (PAL) was determined by spectrophotometric measurement of the conversion of L-phenylalanine into transcinnamic acid at 290 nm according to Zucker (1965).

Results and Discussion

Identification of phytoalexins: The separated compound on TLC, were eluted with methanol and UV spectrum of each compound was recorded using spectrophotometer

(CARY). Medicarpin and Maackiain were identified by their Rf values (Table 1) after co-chromatography in three solvent systems and their UV spectra (Fig. 1A and 1B).

Table 1. Rf values of seperated compounds co-chromatographed with medicarpin and maackiain in different solvent systems on thin-layer chromatographic plates (0.5 mm thickness) coated with silicagel 60 GF_{254}.

	Solvent system		
Compounds	**I**	**II**	**III**
Medicarpin	0.57	0.27	0.70
Maackiain	0.53	0.21	0.67

Solvent systems: I: Pentane: Diethyl ether: Acetic Acid (75:25:1 v/v).
II: Chloroform: carbon tetrachloride (33:1 v/v).
III: Chloroform: Methanol: (98:2 v/v).

The amount of phytoalexins i.e., medicarpin and maackiain in four varieties of chickpea (CM72, CM88 resistant and 6153, Pb-1 susceptible) at different post inoculation intervals was measured and is presented in Fig. 2 and 3. The resistant varieties of chickpea, after inoculation with *A. rabiei*, accumulated significantly higher amount of phytoalexins as compared to the susceptible varieties. In resistant varieties both pterocarpans, medicarpin and maackiain reached their maximum concentrations in one day after inoculation with *A. rabiei*. Two days after inoculation, the level of both pterocarpans decreased in resistant varieties after reaching its maximum and then remained constant throughout the study period. In susceptible varieties, the production of both phytoalexins slowly increased up to 9 days after inoculation. Production of medicarpin was 5 times higher than the production of maackiain one day after inoculation but afterwards this difference showed a decrease in resistant varieties (Fig. 4A, 4B), while in susceptible varieties the amount of medicarpin was 2 times higher than the amount of maackiain throughout the study period (Fig. 4C, 4D). All the tested varieties produced significantly high amount of phytoalexins as compared to control. Higher amounts of phytoalexins synthesized by resistant varieties shortly after inoculation (24 hours) are supposed to restrict the fungal growth and spread. On the other hand the rapid growth and spread of the fungus in susceptible varieties may be due to slow rate of synthesis and accumulation of phytoalexins, resulting in the restriction of lesions at a later stage. These results also suggest that higher amount of medicarpin may contribute more in host resistance.

The rapid accumulation of phytoalexins in resistant varieties is in agreement with the data from chickpea cell suspension cultures and production of phytoalexins in chickpea (Kessmann & Barz, 1987), sorghum (Nicholson *et al.*, 1987), citrus (Sulistyowatic *et al.*, 1990), cotton (Pierce *et al.*, 1996), alfalfa (Christophe *et al.*, 1997) and sorghum (Lo & Nicholson, 1998). Late production of phytoalexins in susceptible varieties of chickpea is contrary to the finding of Kessmann & Barz (1987) in cell suspension cultures of chickpea where phytoalexins production in both cell suspension cultures derived from susceptible and resistant cultivars reached its maximum at 24 hours after treatment with elicitor. The present observations are similar to the observations obtained from some other crops showing that production of phytoalexins in resistant cultivars was faster than in the susceptible cultivars e.g., in groundnut leaves inoculated with *Cercospora arachidicola, Phaeoisariopsis personata* and *Puccinia arachidis* (Rao *et al.*, 1996); in alfalfa inoculated with *Ascochyta imperfecta* and *Pseudomonas syringae* pv. *pisi* (Takuma *et al.*, 1997; Christophe *et al.*, 1997).

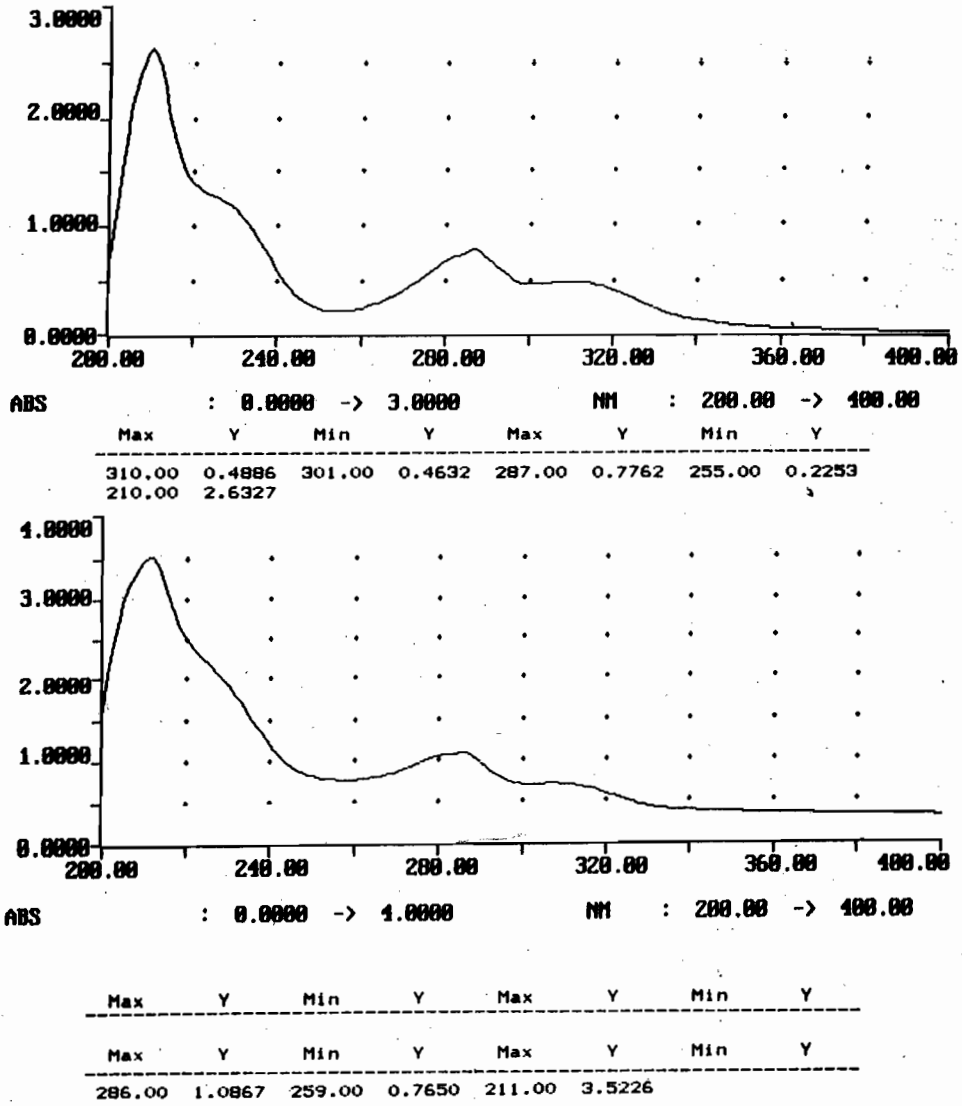

Fig. 1A. UV spectra of compounds, isolated from diseased chickpea plants, matched to Maackiain (top) and Medicarpin (bottom).

Phenylalanine ammonia-lyase (PAL) activity increased within 24 hours after inoculation with *A. rabiei* in both resistant and susceptible varieties of chickpea but increase was more pronounced in resistant varieties than in susceptible ones (Fig. 5). PAL activity was higher in inoculated plants of the resistant varieties than in the inoculated plants of susceptible varieties. The maximum PAL activity occurred 12 to 24 hours after inoculation, which coincided with the most rapid period of phytoalexin accumulation. These results indicate that the production of PAL leading to

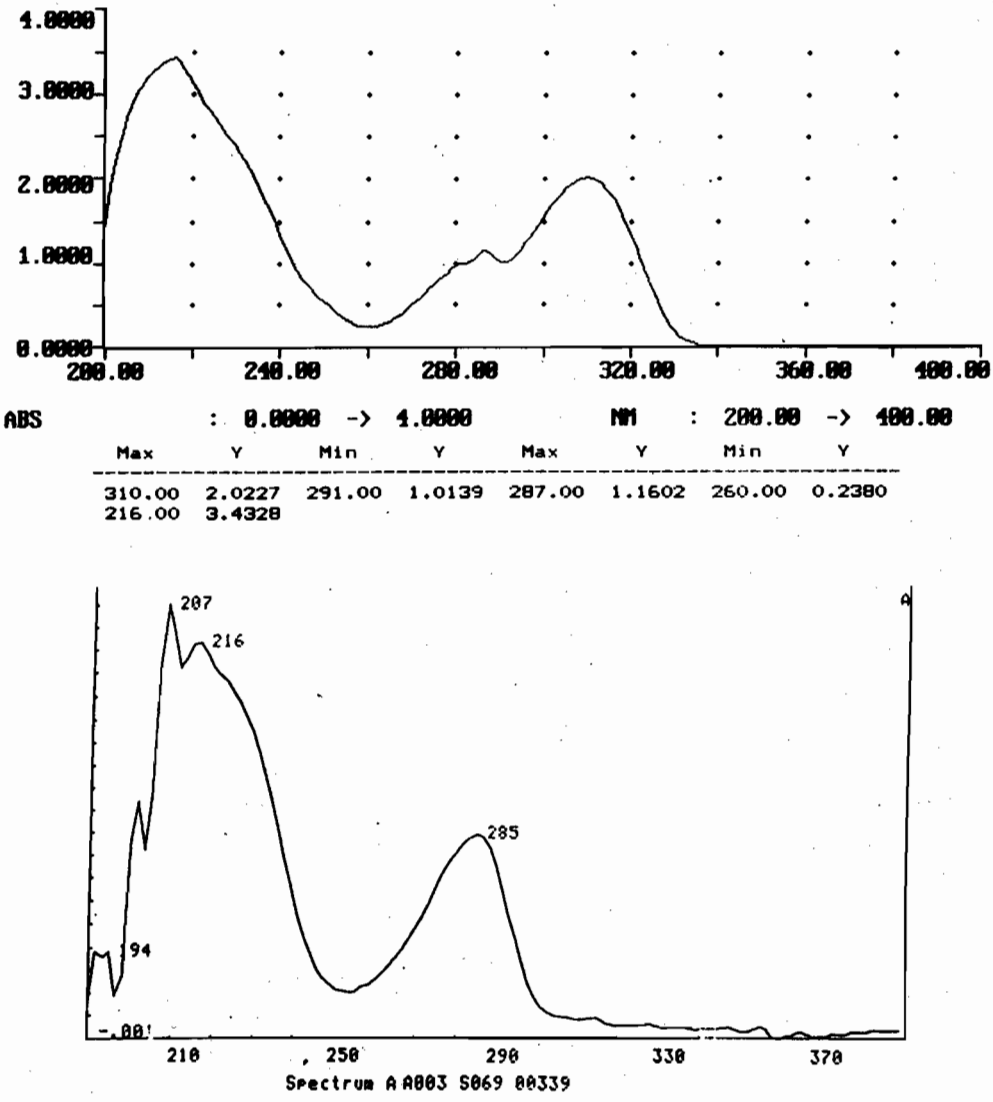

Fig. 1B. UV spectra of Maackiain (top) and Medicarpin (bottom).

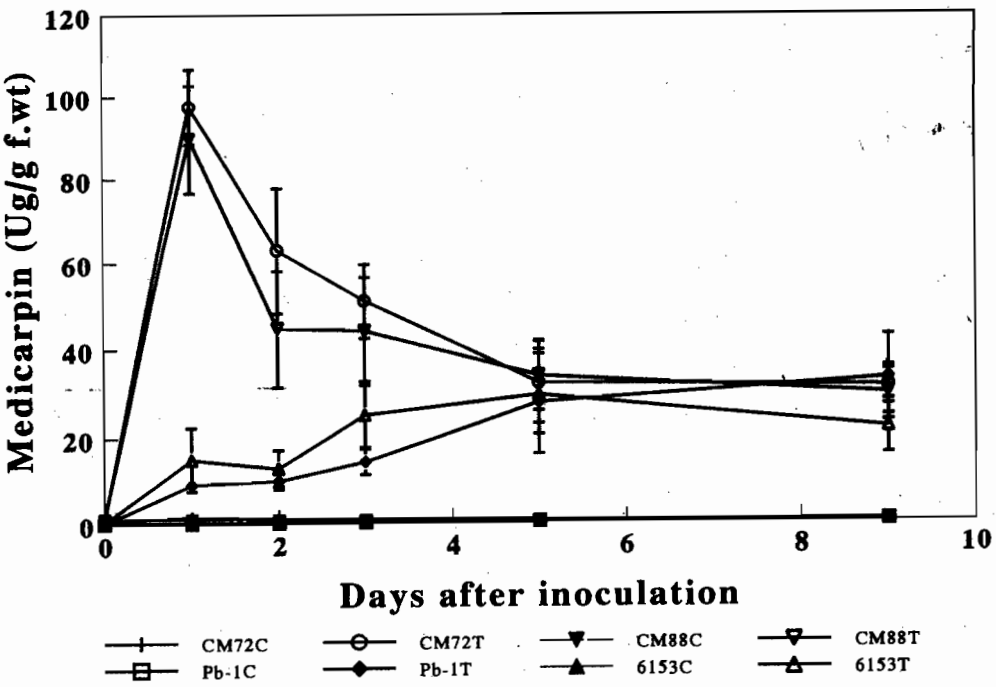

Fig. 2. Medicarpin production in chickpea varieties (CM72, CM 88, Pb-1 and 6153) at different times after inoculation with spore suspension of *Ascochyta rabiei* (T) and in control plants (C).

phenylpropanoid biosynthesis in chickpea is an early defence response to infection. These results are supported by other reports, where the PAL enzyme was rapidly synthesized *de novo* and accumulated in elicitor stimulated bean cultures hypocotyls and leaves (Cramer *et al.*, 1985; Kimpel & Kosuge, 1985; Cosio *et al.*, 1985). High enzyme activity was found in fungal treated cell cultures of carrot (Kurosaki *et al.*, 1986). Similarly a marked increase in mRNAs for PAL was detected in incompatible interaction of soybean to *Phytophthora megasperma* f.sp. *glycina* and *Phaseolus vulgaris* L., to *Colletotrichum lindemuthianum* but not in a compatible interactions (Bell *et al.*, 1986; Esnault *et al.*, 1987). PAL activity was significantly higher in resistant genotypes of many crops (Chakraborty *et al.*, 1993; Koike & Nanbu 1997; Awan *et al.*, 1997, Okey *et al.*, 1997; Peltonen *et al.*, 1998; Glassgen *et al.*, 1998). Increased PAL activity was considered as an indicator of resistance in plants to pathogens (Lawton & Lamb, 1987; Fritzemeier *et al.*, 1987; Jahnen & Hahlbrock 1988. Maher *et al.*, 1994, Zhang *et al.*, 1997). *A. rabiei* has a mechanism for decomposing constitutive isoflavone (Kraft & Barz, 1985) and phytoalexins; medicarpin and maackiain (Kraft & Barz, 1987; Hohl *et al.*, 1989; Tenhaken *et al.*, 1991). It has been reported that suppressor from *A. rabiei* inhibited the accumulation of both the phytoalexins and isoflavones with their conjugates in sliced cotyledons of chickpea (Kessmann & Barz, 1986). It suggests that the accumulation of isoflavones and phytoalexins may be inhibited or degraded more rapidly in susceptible varieties of chickpea than resistant ones. In susceptible plants the products of defence mechanism might be broken down by the fungus. It is concluded that PAL is first regulatory enzyme in phytoalexin accumulation and phytoalexins are a component of defence mechanism in chickpea against *Ascochyta* blight.

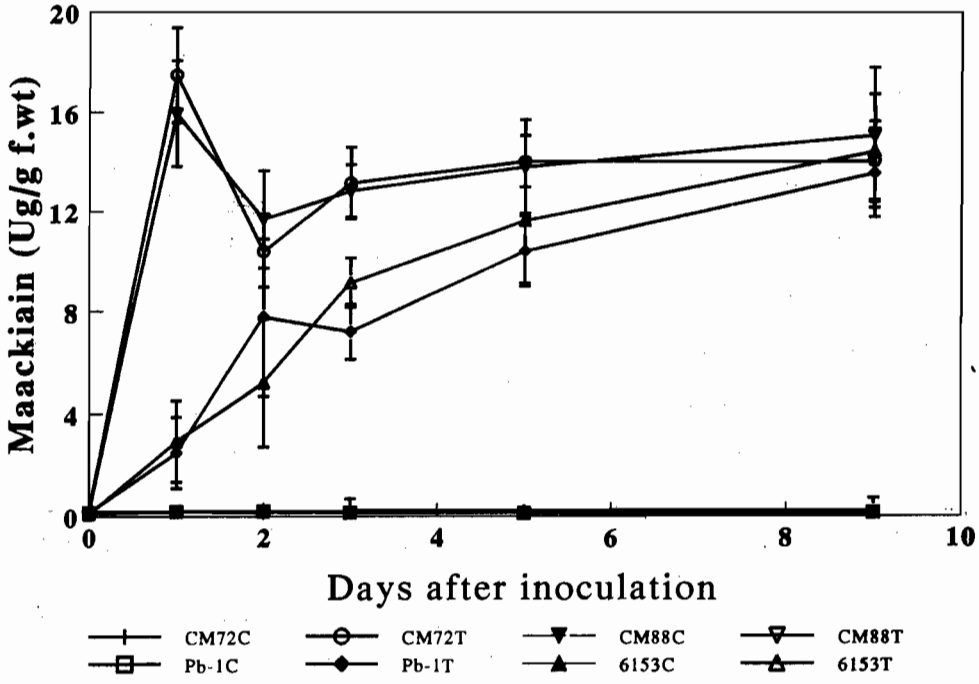

Fig. 3. Maackiain production in chickpea varieties (CM72, CM88, Pb-1 and 6153) at different times after inoculation with spore suspension of *Ascochyta rabiei* (T) and in control plants (C).

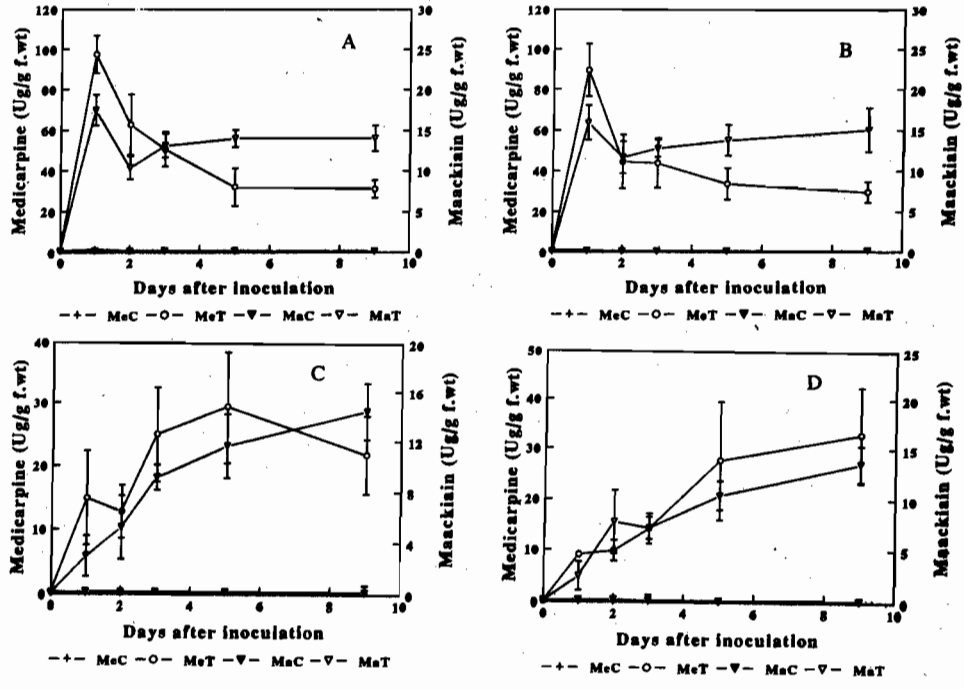

Fig. 4. Accumulation of the medicarpin (Me) and Maackiain (Ma) in control (C) and inoculated (T) plants of chickpea varieties CM72 (A); CM88 (B), 6153 (C) and Pb-1 (D). The plants were inoculated with *Ascochyta rabiei* at flowering stage.

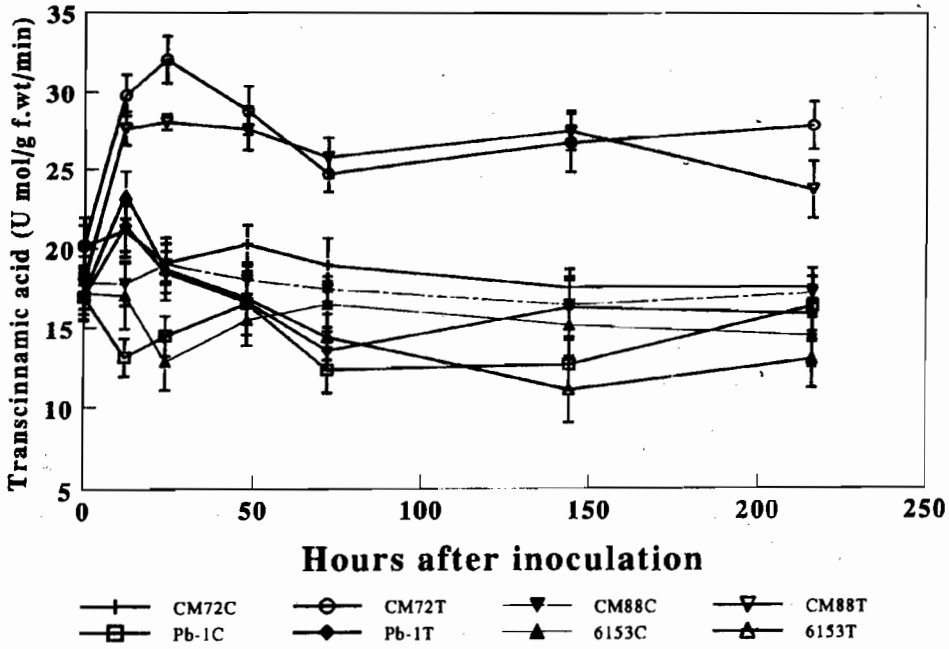

Fig. 5. Activity of phenylalanine ammonia lyase in extracts from control (C) and inoculated (T) plants of chickpea varieties CM72, CM88, (resistant) Pb-1 and 6153 (susceptible) at different times after inoculation with spore suspension of *Ascochyta rabiei*.

References

Awan, M.F., U. Maciejewska and B. Weilgat. 1997. Differential responses of potato cell suspension to a culture filterate of *Phytophthora infestants*. *Zeitschrift fur Naturforschung section C. J. Biosciences*, 52: 333-338.

Barz, W. and U. Mackenbrock. 1994. Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (*Cicer-arietinum*) cell suspension cultures. *Plant cell tissue and organ culture*, 38: 199-211.

Bashir, N., F.F. Jamil and I. Haq. 1995. Resistance of chickpea to *Ascochyta rabiei*: changes in host phenolics and their antifungal activity. In: *Proc. COMSTECH-NIAB INT. Workshop on Agroclimatology, Pest and Diseases and their Control*. (Eds.): F.F. Jamil and S. H. M. Naqvi, COMSTECH Secretariat, Islamabad, Pakistan. PP. 166-175.

Bell, C.N., T.S. Ryder, V.P.M. Wingate, J.A. Bailey and C.J. Lamb. 1986. Differential accumulation of plant defence gene transcripts in a compatible and an incompatible plant-pathogen interaction. *Molecular and Cellular Biology*, 6: 1615-1623.

Camm, E.L. and G.H.N. Towers. 1977. Phenylalanine ammonia-lyase. In: *Progress in Phytochemistry*, vol. 4. (Eds.): L. Reinhold, J.B. Harborne and T. Swain, Pergamon Press, New York. pp. 169-188.

Chakraborty, U., B.N. Chakraborty and M. Kapoor. 1993. Changes in the levels of peroxidase and phenylalanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptoshaeria maculans*. *Folia Microbiologia*, 38: 491-496.

Christophe, S., J. Zuanazzi, J. El-Turk, J. Leymaie, C. Breda, D. Buffard, I.D. Kozak, P. Ratet, P. Husson, A. Kondorosi and R. Esnault. 1997. Gene expression is not systemically linked to phytoalexin production during alfalfa leaf interaction with pathogenic bacteria. *Mol. Plant-Microbe Inter.*, 10: 257-267.

Cosio, E.G., G. Weissenbock and J.W. McClure. 1985. Acifluorfen-induced isoflavonoids and enzymes of their biosynthesis in mature soybean leaves. *Plant Physiol.*, 78: 14-19.

Cramer, C.L., J.N. Bell, T.B. Ryder, J.A. Bailey, W. Schuch, G.P. Bolwell, M.P. Robbins, R.A. Dixon and C.J. Lamb. 1985. Co-ordinated synthesis of phytoalexin biosynthetic enzymes in biologically-stressed cells of bean (*Phaseolus vulgaris* L.). *The Embo J.*, 4: 285-289.

Esnault, R., R. N. Chibbbar, D. Lee and R. R. VanHuystee. 1987. Early differences in production of mRNAs for phenylalanine ammonia-lyase and chalcone synthase in resistant and susceptible cultivars of soybean inoculated with *Phytophthora megasperma* f. sp. *glycinea*. *Physiol. Mol. Plant Pathol.*, 30: 293-297.

Fritzemeier, K.H., C. Cretin, E. Kombrink, F. Rohwer and J. Taylor. 1987. Transient induction of phenylalanine ammonia-lyase and 4-coumarate: CoA ligase mRNA in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. *Plant Physiol.*, 85: 34-41.

Glassgen, W.E., A. Rose, J. Madlung, W. Kock, J. Gleitz and H.V. Seitz. 1998. Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet-light and fungal elicitors. *Planta*, 204: 490-498.

Hohl, B., M. Arnemann, L. Schwenen, D. Stock, G. Bringmann, J. Jansen and W. Barz. 1989. Degradation of the pterocarpan phytoalexin maackiain by *Ascochyta rabiei*. *Z. Naturforsch Sect. C. Biosci.*, 44: 771-776.

Jahnen, W. and K. Hahlbrock. 1988. Cellular localization of non-host resistance reactions of parsley (*Petroselinum crispum*) to fungal infection. *Planta (Berl)*, 173: 197-204.

Jamil, F.F. , N. Bashir, I. Haq, J. Rath and W. Barz. 1996. Accumulation of phytoalexins and other defence - related phenolics in chickpea cultivars during infection with *Ascochyta rabiei*. *Pak. J. Phytopathol.*, 8: 1-4.

Kessmann, H. and W. Barz. 1986. Elicitation and suppression of phytoalexin and isoflavone accumulation in cotyledons of *Cicer arietinum* L., as caused by wounding and by polymeric components from the fungus *Ascochyta rabiei*. *J. Phytopathology*, 117: 321-335.

Kessmann, H. and W. Barz. 1987. Accumulation of isoflavones and pterocarpan phytoalexin in cell suspension cultures of different cultivars of chickpea (*Cicer arietinum* L.). *Plant Cell Report*, 6: 55-59.

Kimpel, J.A. and T. Kosuge. 1985. Metabolic regulation during glyceollin biosynthesis in green soybean hypocotyls. *Plant Physiol.*, 77: 1-7.

Koike, M. and K. Nanbu. 1997. Phenylalanine ammonia-lyase activity in alfalfa suspension cultures treated with conidia and elicitors of *Verticillium albo-atrum*. *Biologia Plantarum* (Prague), 39: 349-353.

Kraft, B. and W. Barz. 1985. Degradation of the isoflavone biochanin A and it's glucoside conjugates by *Ascochyta rabiei*. *Appl. Environ. Microbiol.*, 50: 45-58.

Kuc', J. 1995. Phytoalexins, stress metabolism, and disease resistance in plants. *Ann. Rev. Phytopathol.*, 33: 275-297.

Kurosaki, F., N. Tashiro and A. Nishi. 1986. Induction of chitinase and phenylalanine ammonia-lyase in cultured carrot cells treated with fungal mycelial walls. *Plant Cell Physiol.*, 27: 1587-1591.

Lamb, C.J., M.A. Lawton, M. Dron and R.A. Dixon. 1989. Signals and transduction mechanism for activation of plant defences against microbial attack. *Cell*, 56: 215-244.

Lawton, M.A. and C.J. Lamb. 1987. Transcriptional activation of plant defence genes by fungal elicitor, wounding, and infection. *Mol. Cell. Biol.*, 7: 335-341.

Lo, S. C. C. and R. L. Nicholson. 1998. Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls: Implication for a compensatory role in the defence response. *Plant Physiol.*, 116: 979-989.

Maher, E.A., N.J. Bate, W.T. Ni, Y. Elkind, R.A. Dixon and C.J. Lamb. 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proc. Natl. Acad. Sci. USA*, 91: 7802-7806.

Nicholson, R.L., S.S. Kolipara, J.R. Vincent, P.C. Lyons and G. Cadena, Gomez. 1987. Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and non-pathogenic fungi. *Proc. Natl. Acad. Sci. USA*, 84: 5520-5524.

Okey, E.N., E.J. Duncan, G. Sirju-charran and T.N. Sreenivasan. 1997. *Phytophthora* canker resistance in cacao: Role of peroxidase, polyphenoloxidase and phenylalanine ammonia-lyase. *J. Phytopathol.* (Berlin), 145: 295-299.

Peltonen, S., L. Mannonen, R. Karjalainen. 1998. Elicitor-induced changes of phenylalanine ammonia-lyase activity in barley cell suspension cultures. *Plant Cell Tiss. Org. Culture*, 50: 185-193.

Pierce, M.L., E.C. Cover, P.E. Richardson, V.E. Scholes and M. Essenberg. 1996. Adequacy of cellular phytoalexin concentrations in hypersensitively responding cotton leaves. *Physiol. Mol. Plant Pathol.*, 48: 305-324.

Rao, P.V.S., K.D.R. Wadla and R.N. Strange. 1996. Biotic and abiotic elicitation of phytoalexins in leaves of ground nut (*Arachis hypogaea* L.). *Physiol. Mol.Plant Pathol.*, 49: 343-357.

Sulistyowati, L., P.J. Keane and J.W. Anderson. 1990. Accumulation of the phytoalexin, 6-7-dimethoxycoumarin, in roots and stems of citrus seedlings following inoculation with *Phytophthora citrophthora*. *Physiol. Mol. Plant Pathol.*, 37: 451-461.

Takuma, T., E. Miyagawa, E. Okamoto, K.I. Obnishi, H. Shimizu, K. Asano and Y. Matsui. 1997. Phytoalexin produced by alfalfa and their role in prevention of spring black stem and leaf spot by *Ascochyta imperfecta*. *J. Rakumo Garuen University, Natural Science*, 21: 173-183.

Tenhaken, R., Ch.H. Salmen and W. Barz. 1991. Purification and characterization of pterocarpan hydroxylase, a flavoprotein monooxygenase from the fungus *Ascochyta rabiei* involved in pterocarpan phytoalexin metabolism. *Arch. Microbiol.*, 155: 353-359.

Tenhaken, R. and W. Barz. 1991. Characterization of pectic enzymes from the chickpea pathogen *Ascochyta rabiei*. *Z. Naturforsch*, 46C: 51 - 57.

Zhang, I., M.P. Robbins, I.I.W. Carver and R.J. Zeyen. 1997. Induction of phenylpropanoid gene transcripts in oat attacked by *Erysiphe graminis* at 20°C and 10°C. *Physiol. Mol. Plant Pathol.*, 51: 15-33.

Zucker, M. 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.*, 40: 779-784.

(Received for publication 6 November 2000)