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Production of Phytotoxins by *Ascochyta pisi* Lib., the Causal Agent of Leaf Spot Disease of Pea

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ABSTRACT

Ascochyta pisi lib. is one of the causal agents of an important disease of pea (*Pisum sativum* L.) known as leaf spot. *A. pisi* lib. isolated from scattered cultivated fields in Egypt was found to produce toxic metabolites when cultured on autoclaved wheat kernels. Two main phytotoxic compounds namely, product I (PRI) and product II (PRII) were isolated by the exhaustive extraction of dried mycelia grown on solid media involving two successive steps using two different organic solvents. When tested on host (pea) and on non-host plants (tomato and tobacco), both isolated compounds proved to be phytotoxic. PRI and PRII lacked the antifungal activity when assayed on media using discs already seeded with the test fungi. Towards the larvae of *Artemia salina*, only PRI was weakly toxic at high concentration whilst PRII showed no zootoxicity.

Key Words: *Ascochyta pisi* Lib.*;* Leaf spot; Plant pathogenesis; Phytotoxins

INTRODUCTION

Some species of *Ascochyta* are now described as causal agents of foliar and pod diseases of leguminous and ornamental plants. *A. pisi* Lib., *A. pinodes* Vestergr (*Mycosphaerella pinodes* Berk & Blox) and *A. pinodella* Jones are associated with a disease complex of peas known as *Ascochyta* blight (Horst, 1979; Sherf & Macnab, 1986). The disease is characterized by wilting of the plant, cessation of growth and death of leaves, without rotting. The three pathogens occur worldwide and can induce the disease to many plants and cause significant losses especially when cropping system and environmental conditions favour their development. Sherf and Macnab (1986) reported that these fungi are carried through infected seeds and over winter in the plant debris. These can attack other hosts such as sweet peas, numerous annual and perennial vetches (*Vicia* spp). *A. Pisi* causes leaf, stem and pod spots in peas but without foot rot. Leaf spots are small, dark greyish circular lesions with brown margins but as they develop, they become slightly sunken having dark grey centres with black pycnidia. In addition, pod spots are similar to those produced on the leaves (Horst, 1979). As this disease may result in significant reduction in both pea yield and quality, the present investigation was conducted to study the possible role of compounds produced in cultures of *A. pisi* in developing symptoms of leaf spot disease of pea.

This work describes the field observations of leaf spot disease in cultivated pea and the isolation of the causal fungal pathogen. Production, purification and biological characterization of the main phytotoxic compounds produced by *A. pisi*, was carried out to assess the possible role of the two isolated phytotoxins in the process of pathogenesis.

MATERIALS AND METHODS

Isolation of *A. Pisi.* Severely naturally infected leaves and pods of cultivates peas (*Pisum sativum* L.), were collected at random from scattered cultivated fields near Diarab Negm in El-Sharkia province (nearly 100 Kilometres north of Cairo). Sections from leaves and pods were surface disinfected for 3 min in 0.5% sodium hypochlorite. They were rinsed several times in sterile distilled water before being placed in a sterile moist chamber for a week at 27°C. Fungal growth from the infected pieces of leaves and pods was cultured on wheat bran agar medium supplemented with antibacterial (Chloramphenicol). The plates were then incubated in the dark at 27°C for 10 days. The growing colonies of *A. pisi* were examined and identified according to the morphological characteristics of cultures grown on kzapecks, potato dextrose agar (PDA) and sabouraud's media with the help of available literature (Jones, 1927; Satter, 1933; Hare & Walker, 1944; Mande, 1966). Isolates of *A. pisi* were transferred to wheat bran agar and stored at 4°C to maintain viability. *A. pisi* was re-cultured every four weeks.

Production, extraction and purification of *A. pisi* phytotoxins, product I (PRI) and product II (PRII). Erlenmeyer flasks (500 mL cap.) containing 300 g wheat kernels, brought to 45% moisture and sterilized for 20 min at 121°C, were inoculated with agar plugs containing actively growing *A. pisi* mycelium. these were incubated at 27° for two weeks. After harvesting, the wheat was dried in a drier chamber and finely ground using a mill. The dried and ground material (45 g) was extracted with 120 mL methanol/water (55/45, v/v, 1% sodium chloride) and filtered. The culture filtrate (50 mL) thus obtained was

defatted with *n*-hexane and extracted with methylene chloride (4×50 mL). All extracts were combined, dried (sodium thiosulphate) and evaporated under reduced pressure to give a pale brown oily residue (218 mg). This oily residue was fractionated by silica gel chromatography (Merck, Kieselgel, 60, 0.063-0.2 mm) and eluted with chloroform:*iso*-propanol (85/15, v/v) which yielded 12 homogenous fractions (Fig. 1). Purification of fractions 10 and 11, which showed characteristic phytotoxicity, on silica gel plates (Merck, Kieselgel, 60, F_{254}, 0.50 mm) using the same eluent system but in different concentration (9/1, v/v) gave the two main compounds [having the R*f* values 0.369 and 0.378, respectively] and which were visualised by spraying with 10% sulphuric acid in methanol then by 5% phosphomolybdic acid in methanol and by heating at 110°C for at least 5 min. The two phytotoxins were designated as PRI and PRII.

Phytotoxicity of isolated compounds on host and non-host plants. Phytotoxicity was assayed using the two isolated compounds in mixture on detached leaves and pods of pea and on detached leaves of tobacco (*Nicotiana tabacum* L.). The assay was carried out by placing around 10 µL droplet from the solution of the two toxins (2 mg/mL methanol) on previously scratched leaves and pods using a hypodermic needle fitted to a syringe. The inoculated leaves and pods were incubated in a moist chamber at 27°C for 48-72 h and the development of necrotic lesions were observed. Phytotoxicity on non-host plant was also measured by using 4-week-old tomato plants (*Lycopersicon lycopersium* Karsf.). The stems were cut then immersed into glass test tubes containing solution of: organic extract (1 mg mL^{-1} methanol), each of the two isolated compounds (2 mg mL^{-1}) in comparison to the control (2% methanol/water). The test tubes were all incubated at 27°C for 48. Afterwards, cuttings were transferred to other test tubes containing distilled water and wilt symptoms were recorded. All experiments were repeated twice.

***In-vitro* fungal activity of PRI and PRII.** Known weight (2 mg) of the two isolated compounds, PRI and PRII was dissolved in 1 mL of methanol. Antibiotic assay discs (8 mm in diameter Whatman 3 Mm paper) were wetted with 20 µL of each the prepared toxin solution and allowed to evaporate. The treated discs were place on PDA plates seeded with the test fungi (*Aspergillus flavus*, *Fusarium solani*, *Penecillium notatum* and *Geotrichum candidum*). Control discs were treated with 20 µL of methanol which was allowed to evaporate before placing on PDA plates, already seeded with the same test fungi. All plates were incubated at 27°C for 48 h and growth inhibition zones were observed daily. Two replicate plates were used for the experiment.

Fig. 1. Silica gel plates pattern of the 12 fractions obtained from column chromatography eluted with chloroform:*iso*-propanol (85:15) and detected by 10% sulphuric and 5% phosphomolybdic acids

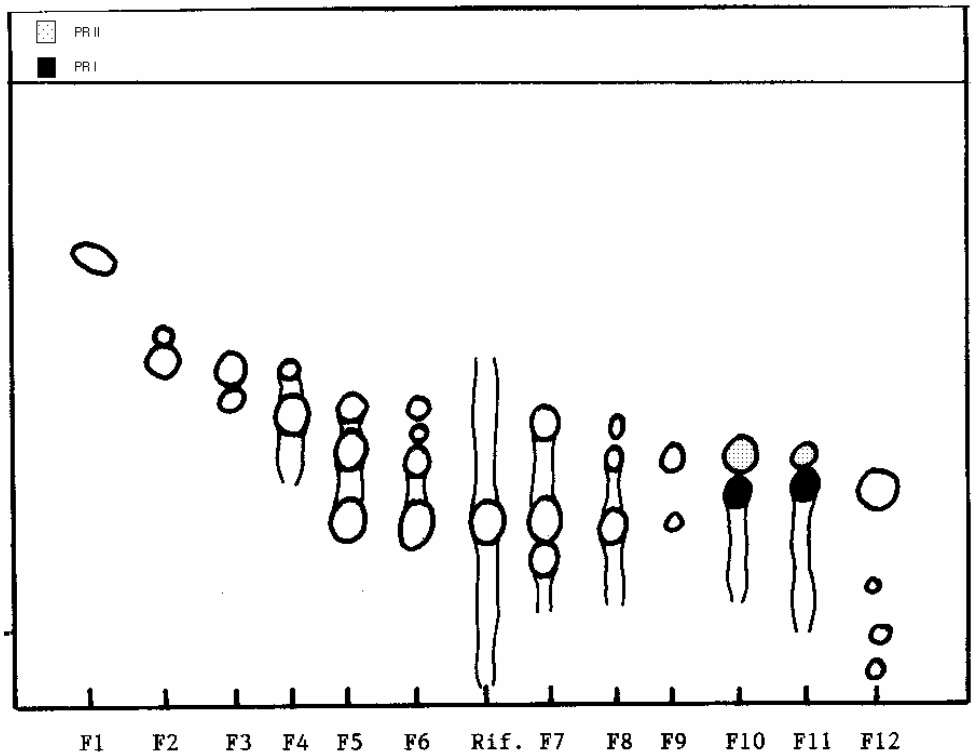


Fig. 2. Phytotoxic effect of a mixture of PRI and PRII on pods of pea

Fig. 3. Phytotoxic effect of a mixture of PRI and PRII on tobacco leaf

Activity of PRI and PRII on larvae of *Artemia salina.* Both isolated compounds were tested at concentrations of 15 μg mL^{-1} and up to 150 μg mL^{-1} on the larvae of brine shrimps (*Artemia salina*) in artificial sea water prepared as follows (g/1 L Water): Sodium (10.7); Potassium (0.39); Calcium (0.42); Magnesium (1.31); Chlorine (19.3); Sulfate (2.69); Carbonate (0.073); total salinity (3.49%). The sedimentation of the tested larvae was recorded.

RESULTS AND DISCUSSION

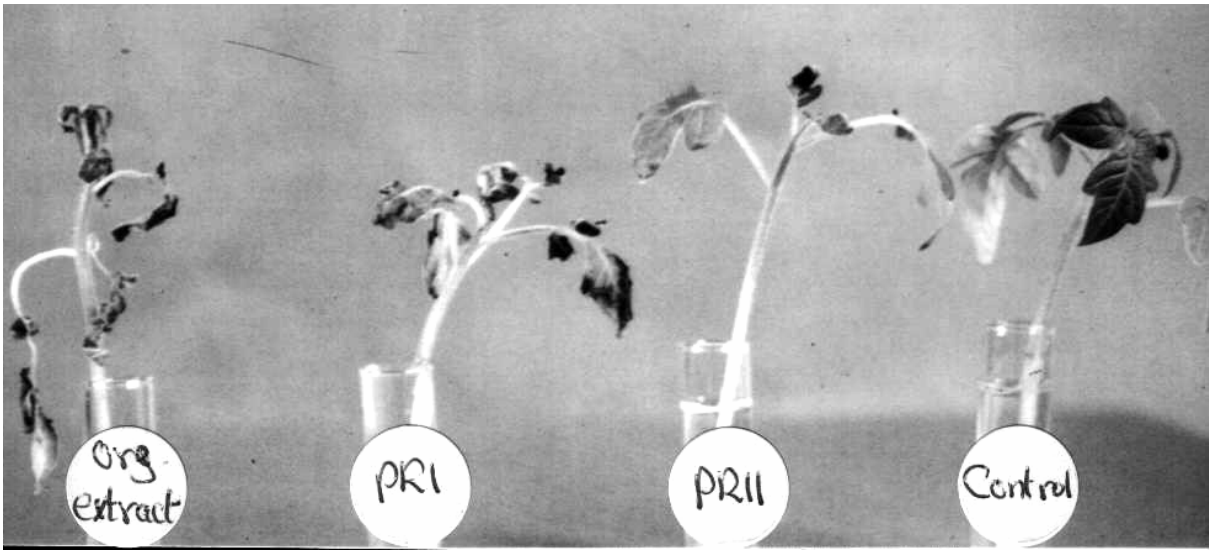
Peas are subject to a number of diseases, of which several may cause serious crop damage and loss, the annual losses from diseases can scarcely be estimated since they vary from year to year depending on environmental conditions. Heavy losses due to foliar and pod diseases may also occur due to the pathogen *Ascochyta* (Wallen, 1974). Toxins isolated from some species of this genus have been previously described by several workers (Capasso *et al*., 1988; Le Poivre, 1988; Evidente *et al*., 1993; Sarvjeet, 1995). Evidence for the involvement of phytotoxins in the pathogenicity of *A. pisi*, isolated from infected leaves and pods of pea and associated with the leaf spot disease of pea, has come from studies on the morphological observations of the infected plant and by experiments carried out on the two

Table I. Phytotoxicity of organic extract and the two isolated compounds on tomato cuttings

Tested compound	Concentration	Phytotoxicity[#]
Organic extract	1 mg/ml	4
PRI	2 mg/ml	2-3
PRII	2 mg/ml	1
Control	2% (methanol/water)	0

[#] Wilt symptoms were evaluated after 48 h and rated on a scale of 0-4 where 0 = no symptoms and 4 = 100% wilt

Fig. 4. Phytotoxic effect of organic extract, PRI and PRII in comparison to the control on tomato cuttings



compounds isolated from cultures of the isolated fungal pathogen.

The phytotoxic organic residue obtained from the extraction process of sterilised wheat kernels inoculated with *A. pisi*, when purified on silica gel column chromatography and then on silica gel plates using two different concentrations from the same eluent system (chloroform:*iso*-propanol) yielded the two compounds designated as PRI and PRII. These two compounds when assayed together on leaves and pods (Fig. 2) of pea and on leaves of tobacco (Fig. 3), caused the appearance of necrotic lesions 48 h after incubation of test plants at 27°C. Such lesions were found to enlarge when left for further 72 h on a laboratory bench. When the two compounds were assayed separately in comparison to the initial organic extract and to the control on tomato cuttings, they all caused wilt disease (Fig. 4) but with varying degree (Table I).

None of the two tested compounds showed activity when assayed on the three test fungi even at concentrations up to 60 μg $disc^{-1}$. Whereas, against the larvae of *Artemia salina*, only PRI showed a weak zootoxicity when tested at high concentration (150 μg mL^{-1}) while PRII was completely inactive.

CONCLUSION

In conclusion, the two isolated phytotoxic compounds PRI and PRII, produced symptoms when tested on host plant comparable with those caused by the pathogen in the naturally infected field. This suggests that the two compounds could have an essential role in disease development causing at least part of the recorded symptoms.

The two studied compounds proved to be non-host specific since they caused the appearance of the same symptoms when assayed on tobacco leaves and induced wilting when assayed on tomato cuttings. The determination of the role played by the two isolated phytotoxins in disease development and establishing new facts in host pathogen interaction may help in reducing the impact of the studied fungal pathogen.

Apart from their obvious importance in both fields, agriculture and plant pathology, phytotoxins are considered now as useful tools for gaining a clear understanding on the mode of action of many biologically active compounds (Goodman *et al*., 1986; Graniti, 1991).

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