PRODUCTION OF SOLANAPYRONE A BY ALGERIAN ISOLATES OF *ASCOCHYTA RABIEI* AS THE CAUSE OF THE TOXICITY OF CULTURE FILTRATES TO CHICKPEA (*CICER ARIETINUM*) SEEDS AND SEEDLINGS

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SUMMARY

Cultures, designated Ag1, Ag2 and Ag3, of a fungus resembling *Ascochyta rabiei*, *Didymella rabiei* (teleomorph), were isolated from blighted chickpea plants growing in three regions of Algeria. The isolates were shown to be the cause of the disease by fulfilling Koch's postulates and were identified as *A. rabiei* by sequencing ribosomal DNA. When grown on a defined liquid medium, consisting of Czapek Dox nutrients and five cations, the filtrates inhibited germination of chickpea seed, and elongation of hypocotyls and radicles of seedlings. All three isolates produced the phytotoxin solanapyrone A in culture and maximal concentrations in the culture filtrates, recorded after incubation for 14 days were 15.1 ± 1.29 µg/ml, 8.4 ± 1.19 µg/ml and 7.4 ± 0.85 mg/ml for Ag 1 Ag2 and Ag3, respectively. ED_{50} values were 7.15 ± 1.77, 5.87 ± 1.40 and 3.60 ± 1.47 µg solanapyrone A/ml for inhibition of germination, hypocotyl elongation and radicle elongation, respectively. Concentrations of solanapyrone A in dilutions of culture filtrates that caused 50% inhibition of these three parameters were sufficient to explain their inhibitory effects in all cases except the inhibition of germination and hypocotyl elongation by filtrates of Ag2 and Ag3. Here they were only 65% and 58% of the amount required to cause the inhibition of germination, respectively and 60% and 63% of the amount required to inhibit hypocotyl elongation, respectively, suggesting that other factors may be involved.

Key words: *Ascochyta rabiei*, *Cicer arietinum*, *Didymella rabiei*, phytotoxins, seed viability, solanapyrone A.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important grain legume in the world after common bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) (Tekeoglu *et al.*, 2000). The seed is a major source of dietary protein for humans in some areas such as India and Pakistan (Singh, 1997) and the crop contributes to soil fertility by fixing nitrogen in symbiosis with *Rhizobium* (Gan *et al.*, 2006). World production of chickpea was 8.6 million tonnes in 2004 and 9.2 million tonnes in 2005 (FAOSTAT, 2005). Although India is the major producer with 5.7 and 6.0 million tonnes in 2004 and 2005, respectively, significant production is also present in Pakistan and Turkey as well as North African and European countries with Mediterranean seaboards (FAOSTAT, 2005).

Blight, caused by the fungus *Ascochyta rabiei* (anamorph), *Didymella rabiei* (teleomorph), is the most serious disease of chickpea in many parts of the world, especially in Western Asia, the North West region of India and Pakistan and North Africa, including Algeria (Nene, 1982; Bouznad *et al.*, 1996). In cool and wet conditions losses may be total (Kimber *et al.*, 2006).

A. rabiei is seed-borne and may remain viable in the seed for more than a year (Kaiser, 1987) but causes rapid deterioration of viability (Ahmad *et al.*, 2006). If sown, infected seed gives rise to diseased seedlings (Kimber *et al.*, 2006) and provides the initial inoculum for the new crop (Iqbal *et al.*, 2002). Infected seed is often the means by which the disease is introduced into countries or regions that had previously been free of it such as Australia (Cother, 1977), Canada (Morrall and McKenzie, 1974), Iran (Kaiser, 1972) and the USA (Kaiser and Muehlbauer, 1984).

All aerial parts of the plant are attacked by the pathogen, giving rise to symptoms consisting of epinasty of petioles and young branches and water-soaked lesions which become necrotic. When stems and petioles are girdled by necrotic lesions they often break (Haware *et al.*, 1986; Alam *et al.*, 1989; Hamid and Strange, 2000). These symptoms are consistent with toxin production by the pathogen, causing dysfunction of the host's membranes, leading in turn to loss of the turgor required for the support of these structures. Culture filtrates of the fungus kill isolated cells derived from chickpea leaflets, leading to the isolation of three phytotoxic compounds and their identification as solanapyrones A, B and C (Alam *et al.*, 1989; Chen *et al.*, 1991, Höhl *et al.*, 1991; Benning and

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Barz, 1995). These compounds were originally identified in culture filtrates of *Alternaria solani*, the cause of early blight of potatoes (Ichihara, *et al.*, 1983).

The present experiments were therefore performed in order to ascertain if blight symptoms found in chickpeas growing in Algeria could be attributed unequivocally to *A. rabiei,* to test if culture filtrates of the isolates were inhibitory to seed germination and growth of seedlings and to establish whether or not any inhibitory effects could be attributed to the solanapyrone toxins.

MATERIALS AND METHODS

Isolation of fungi, proof of Koch's postulates, identification and storage. Fungal cultures designated Ag1, Ag2 and Ag3 were isolated from chickpea debris left in fields near the Algerian towns of Sétif, Guelma and Oued Smar, respectively. The infected debris were washed in water, surface sterilized in 2% sodium hypochlorite, washed twice in sterile distilled water and plated on PDA. Colonies were sub-cultured onto further plates of PDA and the pycnidiospores produced harvested in sterile distilled water by agitation. The resulting spore suspensions were used to spray chickpea plants (cv Rabat-9). When symptoms appeared the fungus was re-isolated, purified by single spore isolation and the morphology of cultures derived from them compared with the original isolates by conventional microscopy and by sequencing rDNA, essentially according to the protocol of El-Kassas *et al*. (2005).

For storage, single spore cultures were propagated on sterilized chickpea seed by the method of Alam *et al*. (1987). Pycnidiospores from these cultures were washed three times by centrifugation in sterile distilled water and made up to 10^7 ml^{-1} in sterile 10% glycerol before storage at -80ºC or in liquid nitrogen (Alam *et al*., 1987).

Toxin production. Fungal isolates were grown on 30 ml Czapek Dox liquid medium supplemented with cations (CDCLM) in triplicate according to the protocol of Bahti and Strange (2004). Flasks were inoculated with 30 µl of a suspension of pycnidiospores (10^7 ml^{-1}) which had been stored in 10% glycerol at low temperature and incubated without shaking at 20°C for 14, 16 and 18 days. Mycelium was removed by filtration through four layers of muslin cloth and spores were removed from the filtrate by centrifugation at *ca.* 10,000 *g* for 20 min. Supernatants were passed through end-capped Isolute cartridges (1 g: C18: International Sorbent Technology, Duffryn Industrial Estate, Ystrad Mynach, Hengoed, Glamorgan, UK), which had been conditioned with 5 ml methanol, followed by 5 ml distilled water. After passing the sample through the cartridge, the remaining non-adsorbed material was washed through the cartridge with distilled water (5 ml) and toxins were eluted in 2 ml acetonitrile (HPLC grade) (Hamid and Strange, 2000). Production of toxin in bulk was achieved by growing a Turkish isolate of *A. rabiei*, Tk21, obtained from the University of Ankara, on CDCLM. The filtrates from 33 flasks (30 ml of medium/flask) were pooled and, after centrifugation and reducing the pH to 3.0 with H_2SO_4, partitioned against ethyl acetate. Pure samples of toxin were obtained by flash chromatography of the ethyl acetate extract on silica gel and dissolved in methanol according to the method of Bahti and Strange (2004).

Analytical High Performance Liquid Chromatography (HPLC). Toxin samples dissolved in acetonitrile were separated on a Philips HPLC equipped with a diode array detector essentially according to Hamid and Strange (2000) except that the solvent system consisted of water 60%, methanol 20.1%, tetrahydrofuran 18.1% and acetonitrile 1.8% (v/v/v/v) which was pumped at a flow rate of 1 ml/min^{-1} (Bahti and Strange, 2004). The stationary phase was an ODS column (Spherisob ODS 2; 150 × 4.5 mm diam; Jones Chromatography, Glamorgan, UK) which was protected by a guard column (20 × 4.6 mm diam) of the same material. The solanapyrone toxins were recognized by their retention times and UV spectra which were compared with those of authentic samples by superimposition. Solanapyrone A was quantified by extraction of chromatograms at 327 nm, the λmax of the compound, from the three-dimensional chromascans and comparison of peak areas with standards (Hamid and Strange, 2000).

Preparation of a solution of solanapyrone A for bioassays. To test the effects of solanapyrone A on seed germination and the elongation of hypocotyls and radicles, the methanol solution of solanapyrone A from bulk preparations was diluted 1:9 with distilled water, giving a concentration of 18.6 µg ml^{-1}.

Seed germination tests. Chickpea seeds (cv Rabat-9) were surface sterilized in a solution of 2% sodium hypochlorite for 2 min, rinsed in sterile distilled water and placed on filter paper discs (Whatman No. 1; 7 cm diam.) in Petri plates (16 per 90 mm plate). They were treated in quadruplicate with 5 ml of culture filtrates or their aqueous dilutions (100%, 50%, 25% and 5% culture filtrate; four Petri plates per dilution). Controls contained water alone. The contents of the Petri plates were kept moist by the periodic addition of water and results were recorded after incubation for 5 days. A similar approach was adopted for assaying solanapyrone A but here the concentrations tested were 18.6, 9.3, 4.65 and 0.93 µg ml^{-1}. The percent reduction in seed germination compared with controls was calculated according to Haider *et al.* (1986) as follows:

$$\frac{\text{Percent germination in controls} - \text{percent germination in tests}}{\text{Percent germination in controls}} \times 100$$

Results were converted to prohibit values (Finney, 1971) and plotted against the $\log_2$ of the dilution factor in order to determine the dilution required to inhibit germination by 50%.

Hypocotyl and radicle elongation test. The effects on hypocotyl and radicle elongation of culture filtrates were determined in quadruplicate using the same dilution series of solanapyrone A and culture filtrates as those for inhibition of seed germination. Five seedlings with hypocotyl lengths of 10 mm were placed in Petri plates (9 cm) on filter paper (Whatman No. 1; 7 cm diam) containing the dilution series or water for controls (5 ml; four Petri plates per dilution or control). Similarly, five seedlings with radicle lengths of 10 mm were treated in the same way. The seedlings were incubated at 25°C under a photoperiod of 12 h with illumination from sodium high pressure lamps giving 300 µEinstein per m^2 for 5 days. Percent inhibition was calculated according to Haider *et al*. (1986) as follows:

$$\frac{\text{Length in controls} - \text{Length in tests}}{\text{Length in controls}} \times 100$$

Results were plotted against the $\log_2$ of the dilution factor in order to determine the dilution required to inhibit elongation by 50%.

Statistical analysis. The variance data were statistically analysed using one-way ANOVA, Student-Newman-Keuls for the seed germination, radicle and hypocotyl tests and the Tukey Test for the production of solanapyrone A.

RESULTS

Confirmation of Koch's postulates and identity of the pathogen. Symptoms consisting of epinasty of petioles and young branches and development of water-soaked lesions, which became necrotic, developed on artificially inoculated plants and were similar to those of plants naturally infected by the fungus. Morphology of the original isolates of the fungus and the cultures obtained from the artificially inoculated plants were identical and accorded with the description given by Haware *et al.* (1986) for *A. rabiei*. The identity of the fungus was confirmed by sequencing the rDNA of two of the isolates, Ag1 and Ag2. The sequence for Ag1 gave a perfect match for 448 bases of a reference sample AR 738 in Genbank and essentially the same result for 454 bases of Ag2, although here there were four bases in which the assignment by the sequencer was in some doubt owing to low signal (GenBank accession number DQ383950). Isolate Ag3 was not sequenced.

Production of solanapyrone A. The three isolates grew well on Czapek Dox medium supplemented with cations. Only solanapyrone A and sometimes a trace of solanapyrone C were produced. The highest mean concentrations of solanapyrone A on the basis of an extinction coefficient of 9,400 at 327 nm (Ichihara *et al.*, 1983) were found in 14-day-old cultures, the earliest sampling date, and declined thereafter. They were 15.1 ± 1.29 µg ml^{-1}, 8.4 ± 1.19 µg ml^{-1} and 7.4 ± 0.85 µg ml^{-1} for isolates Ag1, Ag2 and Ag3, respectively (Fig. 1).

Separation and purification of solanapyrone A from cultures of isolate Tk21 by flash chromatography. Solanapyrone A was obtained in higher yields from the Turkish isolate Tk21 of *A. rabiei* - 37.2 µg/ml^{-1} culture filtrate - than the Algerian isolates and was eluted in dichloromethane, cyclohexane, ethyl acetate (3:3:1) on flash chromatography. Examination of the combined fractions containing solanapyrone A, as determined by their UV spectra, using analytical HPLC and diode array detection, showed that only this compound was present and that its spectrum between 230 and 400 nm was a 99.52% match with that of an authentic sample of the toxin.

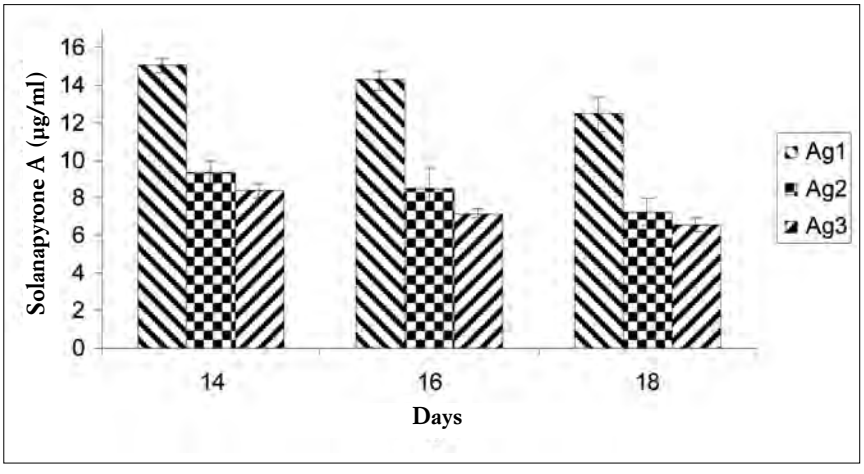

Fig. 1. Variation in solanapyrone A production by three Algerian isolates of *A. rabiei*, Ag1, Ag2 and Ag3 grown on a medium consisting of Czapek-Dox nutrients supplemented with five cations. Bars are standard deviations.

Inhibition of seed germination and elongation of hypocotyls and radicles by solanapyrone A and culture filtrates of *A. rabiei*. Solanapyrone A and culture filtrates of the three Algerian isolates of *A. rabiei* inhibited the germination of chickpea seeds in which the percentage germination of controls was 87.11 ± 2.21. Probit percent inhibition was proportional to the $\log_2$ dilution factor of both solanapyrone A and culture filtrates of the three isolates.

Hypocotyls and radicles grew 22.3 ± 1.98 mm and 35.95 ± 2.41 mm, respectively, during the course of the experiment but growth was inhibited by solanapyrone A and culture filtrates of the Algerian isolates of *A. rabiei*. Inhibition was proportional to the $\log_2$ dilution factor for solanapyrone A and culture filtrates of the three isolates.

The concentration of solanapyrone A in the diluted culture filtrate of Ag1 that caused 50% inhibition of germination, 6.49 ± 1.22 μg ml^{-1}, was sufficient to explain the observed inhibition, there being no significant difference between these values and the concentration of the pure compound, 7.15 ± 1.77 μg ml^{-1}, causing this effect. In contrast, the concentrations in dilutions of filtrates of Ag2 and Ag3 that caused 50% inhibition of germination were only 65% and 58%, respectively, of that required to explain the inhibition. With Ag2, the mean of 4.62 ± 1.25 μg ml^{-1} was not significantly different from that of Ag1 (6.49 ± 1.22 μg ml^{-1}) but was significantly different from that of solanapyrone A (7.15 ± 1.77 μg ml^{-1}), whereas the figure for Ag3, 4.13 ± 0.50 μg ml^{-1}, was significantly different from both Ag1 and solanapyrone A (Fig. 2A).

Similarly, the concentrations of solanapyrone A in filtrates of Ag1, 6.10 ± 0.54 μg ml^{-1} was sufficient to explain the inhibition of hypocotyl elongation, there being no significant difference between these values and the concentration of the pure compound, 5.87±1.40 μg ml^{-1}, causing 50% inhibition. In contrast, the concentration of solanapyrone A in filtrates of Ag2 and Ag3 was only 60% and 63%, respectively, of that required to explain the inhibition (Fig. 2B). Radicle elongation was more sensitive to inhibition by solanapyrone A than germination or hypocotyl elongation with 50% inhibition being recorded for concentrations of 3.60±1.47 μg ml^{-1}. There was no significant difference among these values for the pure compound and the concentrations of solanapyrone A in the dilutions of the culture filtrates of the three isolates of *A. rabiei* that also inhibited radicle elongation by 50%. However, the standard deviations in this test were high (Fig. 2C).

DISCUSSION

In this study, three Algerian fungal isolates from diseased chickpea were confirmed as pathogens by Koch's postulates and were identified as *A. rabiei* by morphology and sequencing ribosomal DNA. Furthermore all three isolates produced solanapyrone A and occasionally a trace of solanapyrone C. The finding of solanapyrone compounds in cultural filtrates of the fungus confirms the observation that all reliably identified isolates of *A. rabiei* produce these compounds although the amount of each varies according to isolate, media and incubation conditions (Alam *et al.*, 1989; Chen *et al.*, 1991, Höhl *et al.*, 1991; Benning and Barz, 1995). One exception was an isolate that produced cytochalasin D (Latif *et al.*, 1993) but no sequence data for the organism was provided. Possibly it was a species of *Phoma,* a known cause of disease in chickpea (Haware and Nene, 1981), some species of which produce cytochalasins (Evidente *et al.*, 2003).

The universality of solanapyrone production by *A. rabiei* argues strongly for the importance of the toxins to the fungus and, owing to their effects on the host, it seems likely that they contribute to virulence or may even be necessary for pathogenicity. However, further evidence for this awaits the development of mutants deficient in toxin production and demonstration that their ability to cause disease in chickpea is impaired (Mogensen *et al.*, 2006; White and Chen, 2006).

Infection of chickpea seed by *A. rabiei* adversely affects viability (Ahmad *et al.*, 2006), with germination rates ranging from 8% to 80% (Kaiser and Hannan, 1988; Takao *et al.*, 2001). As all confirmed isolates of *A. rabiei* invariably produce the solanapyrone toxins, it was of interest to determine if the toxins were able to compromise seed germination and the early stages of seedling development. In the case of isolate Ag1, inhibition caused by culture filtrates was explicable in terms of their concentrations of solanapyrone A. However, for isolates Ag2 and Ag3, the concentrations of solanapyrone A in dilutions causing 50% inhibition of germination were significantly less than those of the pure compound. Similar results were obtained for the inhibition of elongation of hypocotyls, suggesting that other factor(s) in the culture filtrates of isolates Ag2 and Ag3 were involved. One possibility could be the proteinaceous toxin described by Chen and Strange (1994). Radicles were more sensitive than germination or hypocotyl elongation to solanapyrone A but variation in radicle elongation was greater than that of hypocotyl elongation. No significant differences were found among the ED_{50} values for solanapyrone A and the concentrations of the compound causing inhibition in culture filtrates of the three isolates of the fungus in this test.

To our knowledge there has been only one other study of the effect of solanapyrone toxins on the growth of chickpea (Kaur, 1995). In this work a 250 μM (= 75.5 μg ml^{-1}) solution of solanapyrone A was reported to inhibit the root growth of seedlings of cultivar Pusa 209 by 50%. This is considerably higher than the figure of 3.60 ± 1.47 μg solanapyrone A/ml^{-1} reported in the present investigation. Possibly the difference may be attri-

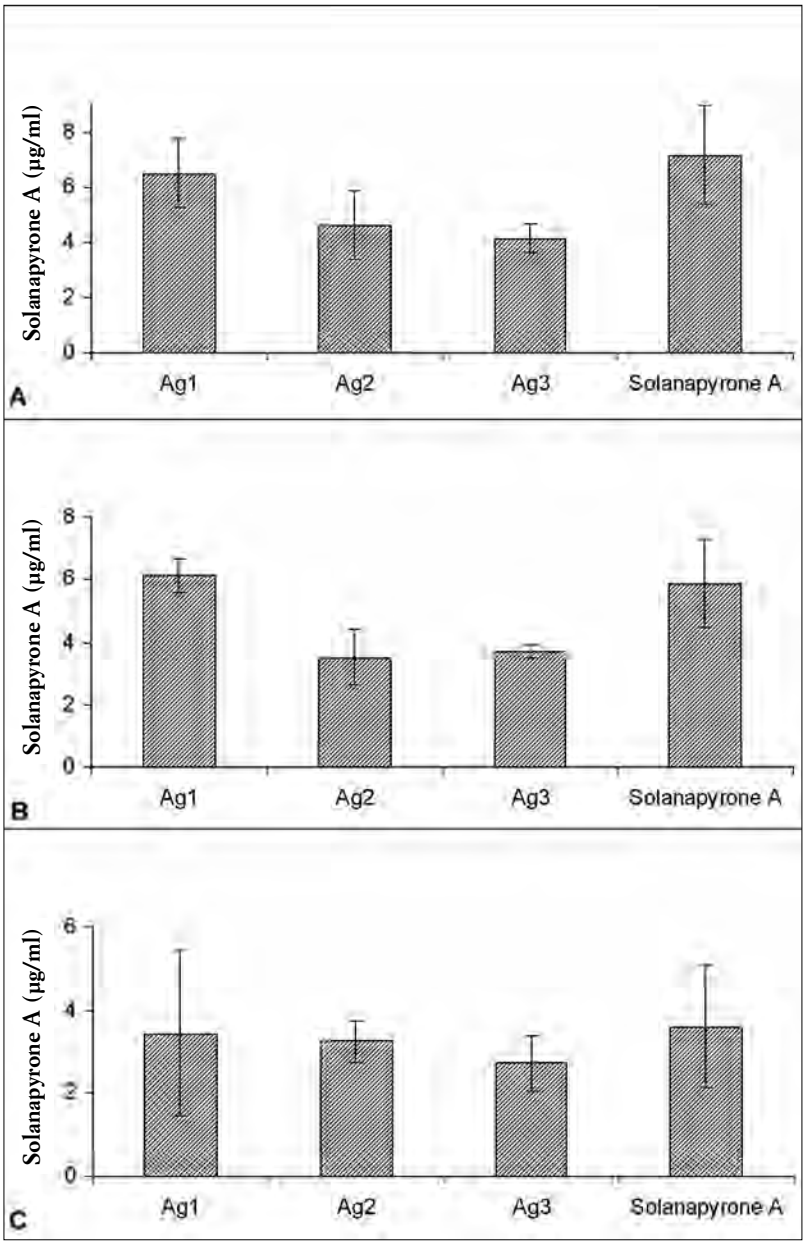

Fig. 2. Concentrations of solanapyrone A in culture filtrates of the Algerian isolates Ag1, Ag2 and Ag3 of *A. rabiei* in the dilutions required to inhibit germination of chickpea seeds (**A**), elongation of chickpea hypocotyls (**B**) and elongation of chickpea radicles (**C**) by 50% compared with the concentration of the pure toxin required to give the same effect. Bars are standard deviations.

buted to the different cultivars used. In the present experiments radicle elongation was the most sensitive tissue but only one cultivar was used. It would be interesting to determine the extent of variation in sensitivity among cultivars of chickpea to solanapyrone A and determine its relation to susceptibility of whole plants to the disease. A close correlation would indicate that the radicle test could be used as a preliminary screen for susceptibility of chickpea genotypes to Ascochyta blight.

In conclusion, we have shown that *A. rabiei* causes chickpea blight in three locations in Algeria, and it seems possible that solanapyrone toxins play important roles in the loss of viability of infected chickpea seed and seedling mortality. Although demonstration of sufficient concentrations of solanapyrone A in infected seeds to cause these effects would be desirable, this is unlikely to be feasible owing to the lability of the toxin (Hamid and Strange, 2000; Bahti and Strange, 2004). Production of mutants deficient in toxin production may provide better evidence (Mogensen *et al.*, 2006; White and Chen, 2006).

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