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Cultivar resistance to anthracnose disease of cowpea (*Vigna unguiculata* (L.) Walp.) caused by *Colletotrichum destructivum* O'Gara

A.O. Latunde-Dada*, R.J. O'Connell, P. Bowyer and J.A. Lucas**
*IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK; *Present address: College of Plant Science and Crop Production, University of Agriculture, PMB 2240, Abeokuta, Nigeria; **Author for correspondence (Fax: +44-1275-394281; E-mail: john.lucas@bbsrc.ac.uk)*

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Abstract

The infection process of *Colletotrichum destructivum*, a hemibiotrophic anthracnose fungus, was studied by light microscopy in two cowpea (*Vigna unguiculata*) cultivars which differ in disease reaction type. Large, multilobed, intracellular infection vesicles, followed by necrotrophic, radiating, secondary hyphae were produced in tissues of the susceptible cv. IT82E-60. In the resistant cv. TVx 3236, both the production of appressoria and their melanisation were impaired, resulting in reduced penetration. Where penetration occurred, the initially-infected epidermal cells underwent a hypersensitive response, restricting the growth of multilobed vesicles and thereby blocking the destructive necrotrophic phase of disease development. The phytoalexins kievitone and phaseollidin accumulated earlier and more rapidly in stem tissues of the resistant cultivar, associated with the appearance of delimited, necrotic spots on inoculated surfaces. In contrast, delayed and slower accumulation of these compounds occurred in the compatible interaction, together with the development of typical spreading, water-soaked, anthracnose lesions.

Introduction

Colletotrichum destructivum O'Gara was identified recently as the hemibiotrophic fungus causing anthracnose disease of cowpea (*Vigna unguiculata* (L.) Walp.) (Latunde-Dada et al., 1996). This pathogen, originally considered to be a form of *C. lindemuthianum* (Onesirosan and Barker, 1971), is responsible for serious yield losses in cowpea crops cultivated in the humid tropics (Emechebe and Shoyinka, 1985). In susceptible cultivars, water-soaked, spreading anthracnose lesions develop in colonized tissues (Bailey et al., 1990; Latunde-Dada et al., 1996); in contrast, the response of anthracnose-resistant cultivars is characterised by restricted, necrotic flecks on inoculated surfaces (Latunde-Dada, 1990). While the infection process has been described in susceptible hosts (Latunde-Dada et al., 1996), the sequence of events in resistant cowpea cultivars is not known. Cowpea tissues accumulate a number of phytoalexins, including kievitone, phaseollin, phaseollidin, medicarpan and vignafuran in response to fungal and abiotic elicitors (Preston et al., 1975; Patridge and Keen, 1976; Martin and Dewick, 1979; Dixon et al., 1983). However, no information is available on the expression of incompatibility in resistant cowpea cultivars, nor on the possible involvement of these antifungal compounds in interactions between different cowpea cultivars and *C. destructivum*.

The present paper describes the development of *C. destructivum*, and the time-course of phytoalexin accumulation, in a susceptible cowpea cv. IT82E-60, and also in cv. TVx 3236, bred at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria (Anon, 1984) for resistance to anthracnose disease.

Materials and methods

Fungus and inoculum

The isolate of *Colletotrichum destructivum*, LARS 056, used in this study was isolated from a Nigerian cowpea (Bailey et al., 1990). The fungus was cultured in the dark at 25 °C on CM medium (Mathur et al., 1950). Inoculum was obtained from 7-day-old Petri dish cultures by irrigation with sterile, deionized water, and the conidial concentration adjusted by dilution after haemocytometer counts.

Plant material

Seeds of *Vigna unguiculata* cvs. IT82E-60 and TVx 3236 were obtained from IITA, Ibadan, Nigeria. These cultivars have been shown to be susceptible (IT82E-60) and resistant (TVx 3236) to anthracnose in the field in southern Nigeria (Latunde-Dada, 1990). In this study seedlings were raised only from a sub-set of TVx 3236 plants with demonstrably-high resistance ratings. Seeds were sown in 9 cm pots and trays filled with steam-sterilized top-soil, and maintained at 25 °C in a growth cabinet with a 12 h photoperiod (500 µmol PAR m^{-2} s^{-1}) for 10 days.

Inoculation

Intact, unifoliate leaves of 10-day-old seedlings were inoculated with ten 3 µl droplets of spore suspension (1×10^5 conidia ml^{-1}), incubated in a humid propagator (Humex Greenhouse System, New Milton, UK) and examined daily for symptom development. Under these conditions, inoculum droplets remained intact on host surfaces throughout the period of incubation. Control plants were treated with sterile, deionized water. In addition, hypocotyls excised from 10-day-old seedlings were inoculated with *C. destructivum* as described earlier (Latunde-Dada et al., 1996).

Microscopy

Tissue pieces (25 mm^2) were excised from infection loci on inoculated leaves and hypocotyls and decolourized in a 0.15% (w/v) solution of trichloroacetic acid in a 3 : 1 (v/v) mixture of ethanol and chloroform for 12 h. Tissues were then stained in a 0.025% solution of aniline blue in lactophenol for 2 h. Light microscopic examinations were made with a Zeiss Axiophot microscope using Nomarski differential interference contrast. Tissues were also examined for autofluorescence using excitation filter BP 450-495, dichroic mirror FT510 and barrier filter LP 520. Photomicrographs were taken on Kodak Technical Pan film rated at 80 ASA and developed in Diafine (Acufine, Chicago, USA).

Phytoalexin isolation

Seven-day-old seedlings were inoculated by spraying, until run-off, with a 1×10^5 conidia ml^{-1} inoculum of *C. destructivum* and incubated in a humid propagator at 25 °C for 120 h. Control seedlings were sprayed with sterile, deionized water. At harvest stem sections were homogenized with white quartz sand (Aldrich, Dorset, UK) in absolute ethanol in a pestle and mortar. The homogenate was filtered through sintered glass and the filtrate was evaporated to dryness under vacuum. The residue was taken up in water, partitioned in equal volumes with ethyl acetate (3 times), and the organic phase evaporated to dryness under vacuum. The residue was then dissolved in 500 µl ethyl acetate and chromatographed on silica gel plates (Kieselgel $60F_{254}$; Merck, Darmstadt, Germany) developed in ethanol : chloroform (1 : 25 v/v). Antifungal activity was detected on silica gel by spraying plates with a conidial suspension of isolate LARS 056 in liquid CM medium. Sprayed plates were incubated at 25 °C in the dark for 5 days in moist, sealed, trays. Areas of inhibition showed up as white zones against a coloured background on the plates. Areas corresponding to these zones were scraped from fresh plates and the silica eluted in ethanol for spectrophotometry.

The quantification of the phytoalexins kievitone and phaseollidin was based upon their UV absorption maxima in ethanol; kievitone, 294 nm $\log E = 4.17$ (Smith et al., 1973) and phaseollidin, 286 nm $\log E = 3.97$ (Perrin et al., 1972). These phytoalexins were identified by co-chromatography on silica gel with authentic samples kindly supplied by J.A. Bailey (IACR-Long Ashton, UK).

Results

Infection process

Conidia of *C. destructivum* germinated and formed melanized appressoria on the surface of the susceptible cv. IT82E-60 within 12 h of inoculation (Figure 1a).

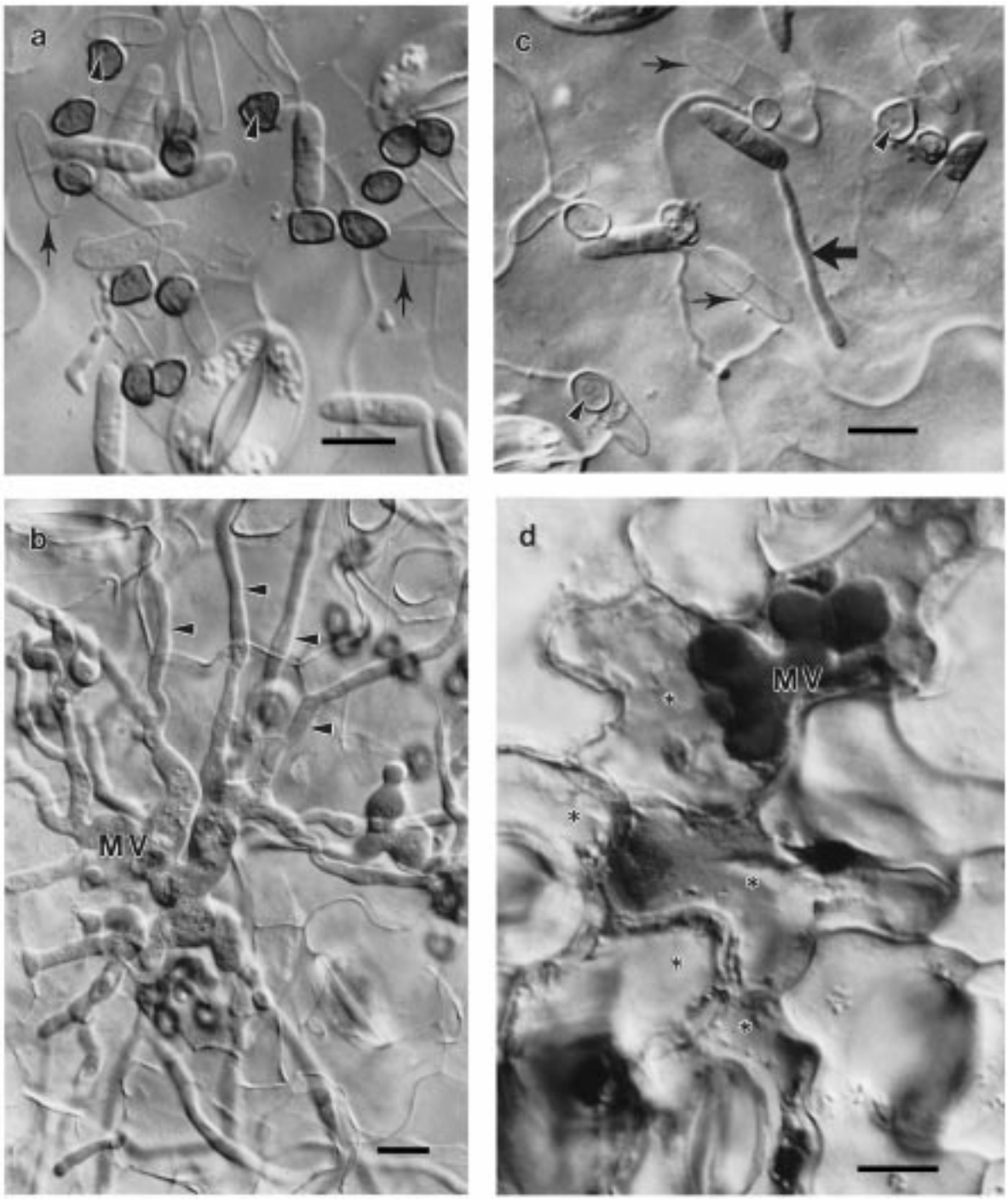

Figure 1. Development of *Colletotrichum destructivum* on leaf tissue of the susceptible cowpea cv. IT82E-60 (a, b) and the resistant cv. TVx 3236 (c, d). Bars = 10 µm. (a) Conidia (arrows) germinating to form normal, melanised appressoria with penetration pores (arrowheads) 18 h after inoculation. (b) Narrow, secondary hyphae (arrowheads) radiating from a large, multilobed infection vesicle (MV) 72 h after inoculation. (c) Conidia (small arrows) germinating to form hyaline appressoria with penetration pores (arrowheads). Large arrows indicate elongated, undifferentiated germ-tubes 18 h after inoculation. (d) Localised hypersensitive response, showing restriction of a small multilobed infection vesicle (MV) within a single necrotic epidermal cell. The penetrated cell, and some adjacent uninfected cells (asterisks), have brown, granulated cytoplasm 72 h after inoculation.

The host cuticle was penetrated directly by 36 h after inoculation, giving rise to an intracellular saccate infection vesicle connected to the appressorium by a long, narrow neck (not illustrated). The infection process in this cultivar progressed through the formation of large, multilobed infection vesicles in epidermal cells by 72 h after inoculation, the development of narrow secondary hyphae radiating from the multilobed vesicles (Figure 1b), initiation of hyphal aggregates, and the eruption of acervuli surrounded by conidia on the surface of infected tissues by 120 h after inoculation (see Latunde-Dada et al., 1996).

A number of differences in this infection process were noted on leaves and hypocotyls of the resistant cv. TVx 3236. About 40% of conidia germinated to form elongated, undifferentiated germtubes without appressoria (Figure 2); these spores became swollen and distorted, measuring 20–37.5 × 6 µm compared to the normal 16.3 ± 1.97 × 4 µm reported for *C. destructivum* LARS 056 (Latunde-Dada et al., 1996). Where appressoria were formed, they had normal morphology and developed penetration pores, but a number of these (about 30%) failed to melanize (Figure 1c) and were unable to penetrate the host. Moreover, penetration of host surfaces occurred at a lower frequency even from those conidia which germinated normally. In consequence, although multilobed vesicles were also formed in epidermal cells of TVx 3236 by 72 h after inoculation, they were significantly less frequent than found in the susceptible cultivar (Figure 3), and tissue colonization was inhibited at the penetration sites (Figure 1d). There was strong autofluorescence from these inhibited vesicles by 96 h after inoculation (not illustrated). In addition, localised browning and cytoplasmic disorganisation resembling a hypersensitive response were observed within infected and surrounding cells (Figure 1d). The multilobed vesicles within hypersensitive cells had brown cytoplasm and failed to develop filamentous secondary hyphae. Hence the fungus was confined to

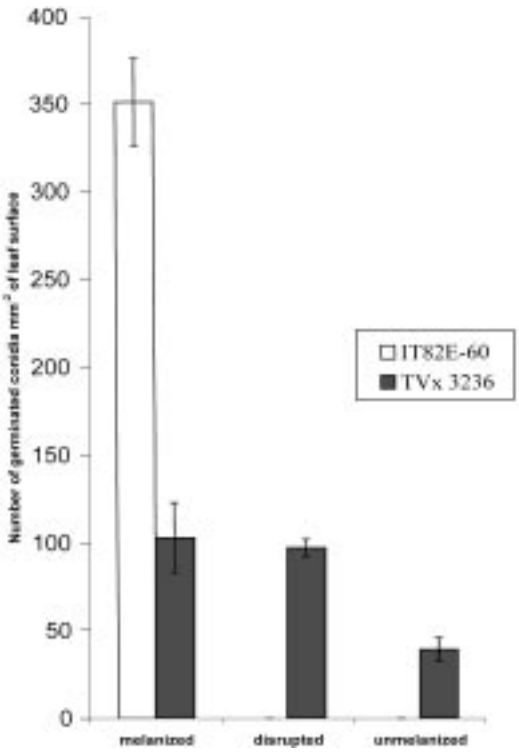

Figure 2. Conidial germination categories on leaf surfaces of the susceptible cv. IT82E-60 and the resistant cv. TVx 3236, 48 h after inoculation with *Colletotrichum destructivum*. Each bar is the mean count ±SE from 30 inoculation droplets.

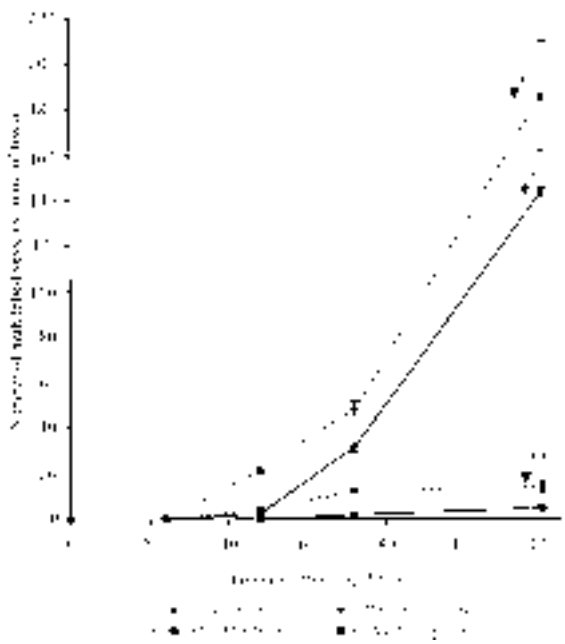

Figure 3. Time course of the formation of intracellular, mutilobed infection vesicles within epidermal cells of the leaves and hypocotyls of cowpea cvs IT82E-60 (susceptible) and TVx 3236 (resistant) inoculated with *Colletotrichum destructivum*. Each value represents the mean of 30 random counts ±SE. Arrows denote the time point at which symptoms were expressed on leaves and hypocotyls; *, spreading, water-soaked lesions on IT82E-60; **, delimited necrotic spots on TVx 3236.

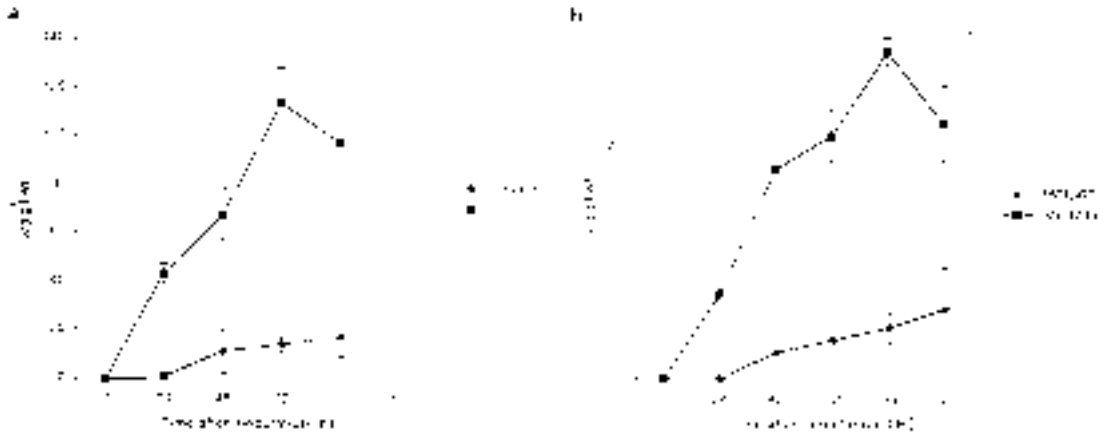

Figure 4. Accumulation of the phytoalexins kievitone (a) and phaseollidin (b) in cowpea cvs IT82E-60 (susceptible) and TVx 3236 (resistant) following inoculation with *Colletotrichum destructivum*. Vertical bars represent the SE.

the first infected epidermal cell. Inhibition of pathogen development in the resistant cv. TVx 3236 culminated in the formation of brown, necrotic spots on the surface of inoculated tissues rather than typical, water-soaked spreading anthracnose lesions.

Phytoalexin accumulation

Two zones of inhibition corresponding to the phytoalexins kievitone ($R_f = 0.11$) and phaseollidin ($R_f = 0.56$) were obtained on bioassay plates after chromatography of extracts from inoculated stem tissues. These phytoalexins were detected within 24 h after inoculation in the resistant cv. TVx 3236, and accumulated thereafter at a more rapid rate and to higher concentrations than in the susceptible cv. IT82E-60 (Figure 4). In the latter, only traces of kievitone and phaseollidin were detected in stem tissues during the first 48 h after inoculation. Thereafter, both phytoalexins accumulated to concentrations between five and ten-fold lower than were detected in the resistant cv. TVx 3236 (Figure 4).

Discussion

The infection process of *C. destructivum* in the susceptible cowpea cv. IT82E-60 comprised an initial, symptomless biotrophic phase, lasting for 72 h, followed by an invasive, necrotrophic colonisation of host tissue which resulted in the development of water-soaked spreading lesions on the host. This hemibiotrophic mode of infection on cowpea has been described in greater detail elsewhere (Latunde-Dada et al., 1996), and closely resembles events observed in the colonization of another host, lucerne (*Medicago sativa* L.) by some isolates of *C. destructivum* (Latunde-Dada et al., 1997).

On cv. TVx 3236, the resistant cultivar used in this study, development of anthracnose disease was impaired at two different stages during infection. On the surface of the resistant host, many germ-tubes failed to differentiate appressoria, and melanization of a proportion of those appressoria that did form was impaired, so that a significant proportion (>55%; Figure 2) of the inoculum lacked the capacity to infect. This disruption of pre-penetration development, has not been previously reported for *Colletotrichum* species and might be due to the presence of constitutive inhibitors in the cuticle of this cultivar, reflecting a type of race non-specific or non-host resistance (Bell, 1981; Heath, 1981). Furthermore, host cuticle was penetrated significantly less frequently in this cultivar even by those appressoria which achieved melanization. The pre-penetration development of the rice blast fungus, *Magnaporthe grisea* has been shown to be inhibited on some resistant rice cultivars (Heath et al., 1990) associated with the presence of toxic materials in leaf diffusates (Pasechnik et al., 1997). While the presence of such compounds was not investigated in this study, naturally-occurring plant coumarins may inhibit melanin biosynthesis in appressoria and hence function as anti-penetrants (Sisler et al., 1984). Melanin biosynthesis alters the permeability and increases the

rigidity of the appressorial wall, increasing turgor and generating the high hydrostatic pressures required to directly penetrate the host (Howard and Valent, 1996). Unmelanized appressoria of *M. grisea* (Howard and Ferrari, 1989) and *Colletotrichum* (Kubo et al., 1985; Kubo and Furusawa, 1991) failed to penetrate the host cuticle.

Where penetration occurred in the resistant cultivar, pathogen development was further restricted by reactions typical of active host defence. Multilobed vesicles were formed (at significantly lower frequencies; Figure 3) but were eventually confined within the initially-penetrated epidermal cell. This inhibition was accompanied by autofluorescence of the multilobed vesicle and browning of cells surrounding the infection site, a response generally associated with hypersensitive cell death (Teasdale et al., 1974; Fernandez and Heath, 1986). Such active resistance in other systems has been shown to involve the rapid transcriptional activation of defence genes and a cascade of responses including biosynthesis of pathogenesis-related proteins, accumulation of hydroxyproline-rich glycoproteins, deposition of lignin and wall-bound phenolics, and the accumulation of phytoalexins (Dixon et al., 1994). In the present study, accumulation of kievitone and phaseollidin was shown to take place earlier and to progress more rapidly in tissues of the resistant cultivar than in the susceptible host. It may be significant that peak concentrations of these phytoalexins in cv. TVx 3236 were attained 72 h after inoculation, coinciding with the stage at which the switch from biotrophic development to necrotrophy normally occurs in the infection process of *C. destructivum* (Latunde-Dada et al., 1996). In this resistant cultivar, the destructive necrotrophic phase was effectively blocked, and symptoms were confined to brown necrotic spots on inoculated organs. Cellular browning and tissue necrosis have been shown to accompany the induction of kievitone accumulation in cowpea hypocotyls (Munn and Drysdale, 1975). In the susceptible cultivar ITE82-60, phytoalexin levels remained low and infection progressed to typical spreading anthracnose lesions followed by reproduction of the pathogen.

In the current work, phytoalexins were analysed in stem tissues alone, and this may have precluded detection of other compounds such as phaseollin and vignafuran, which accumulate preferentially in cowpea leaves (Bailey, 1973; Preston, 1975; Allen, 1983). The accumulation of kievitone and phaseollidin in infected cowpea stems, and their fungitoxicity to *C. lindemuthianum* and other species, have been reported previously (Preston, 1975; Seneviratne and Harborne, 1992). Both compounds were shown to inhibit the growth of *C. destructivum* in bioassays on silica plates in the present study.

Further analysis of the nature and genetic basis of resistance to anthracnose disease in the cowpea cv. TVx 3236 would be of interest, in particular to identify the mechanisms responsible for inhibition of appressorial development. Such a study will also permit comparisons between this inhibition and the defined blocks in melanin biosynthesis identified in known *Colletotrichum* mutants (Takano et al., 1995; Kubo et al., 1996).

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