Phytoalexin accumulation in the roots of chickpea (*Cicer arietinum* L.) seedlings associated with resistance to fusarium wilt (*Fusarium oxysporum* f.sp. *ciceri*)

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Four cultivars of chickpea which differed in their reaction to *Fusarium oxysporum* f.sp. *ciceri*, the pathogen causing wilt, were grown in wilt infested soil. The root xylem of plants showing wilt symptoms was heavily occluded by hyphae. Hyphal occlusion of stem xylem was also recorded up to the fifth internode. The roots of resistant cultivars were penetrated but hyphal growth was very slow. No localized cell death (hypersensitivity) or gross structural changes (lignification) were observed in the vicinity of invading hyphae in resistant cultivars, suggesting that the resistance was dependent upon chemical rather than physical mechanisms. The concentration of the pterocarpans medicarpin and maackiain in the roots increased in the presence of two races of the pathogen. Both pre- and post-induction concentrations of pterocarpans were significantly greater in wilt-resistant cultivars indicating an association between phytoalexin induction and resistance. Medicarpin and maackiain showed antifungal activity to *F. oxysporum* f.sp *ciceri* at similar concentrations to those recorded in wilt-resistant chickpea roots. No significant difference in the sensitivity of races 1 and 2 to the antifungal activity of the pterocarpans was detected, but the accumulation of phytoalexins in response to the more virulent pathotype race 2 was lower in all cultivars than those produced in response to race 1. We conclude that they are fundamental components of the resistance mechanism of chickpeas to wilt. © 1997 Academic Press Limited

INTRODUCTION

Chickpea, *Cicer arietinum* L., is a major source of human and domestic animal food, particularly in the semi-arid tropics where its production is concentrated [7]. It has been described as the world's third most important pulse crop after dry beans (*Phaseolus vulgaris* L.) and dry peas (*Pisum sativum* L.) [13]. One of the major constraints to chickpea production is fusarium wilt, caused by *Fusarium oxysporum* f.sp. *ciceri* (Padwick) [11]. Four races of *F. oxysporum* f.sp. *ciceri* have been described from India, but only races 1 and 2 are widespread [5] and these have been the focus of attention in this and previous studies [14, 15]. Emphasis has been placed on the identification of resistance because other forms of disease management are inappropriate [4, 12].

Identification of the mechanisms of resistance to *F. oxysporum* f.sp. *ciceri* in chickpeas will be of value in understanding and evaluating the variability of the expression of resistance under different field conditions and in different agro-ecological zones. In

Abbreviations used in text: PDA, potato dextrose agar; PDB, potato dextrose broth.

addition, this information may provide genetic markers for breeding programmes and may facilitate distinguishing between different races of *F. oxysporum* f.sp. *ciceri* [*8, 17*].

The root exudates of four cultivars of chickpea which varied in their resistance to two races of *F. oxysporum* f.sp. *ciceri* were antifungal [*14, 15*]. It was concluded that the presence of higher concentrations of medicarpin and maackiain as constitutive antifungal compounds in the exudates of resistant varieties was at least partly responsible for resistance to fusarium wilt. This report focuses on the induction of antifungal phytoalexins by *F. oxysporum* f.sp. *ciceri* and the process of infection by invading hyphae.

MATERIALS AND METHODS

Fungal material

Freeze-dried samples of race 1 (IMI 361496) and race 2 (IMI 361497) of *F. oxysporum* f.sp. *ciceri* were obtained from the collection at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). Cultures were maintained on potato dextrose agar (PDA) at 25 °C and spores were transferred from this culture to potato dextrose broth (PDB) for inoculation of soil and roots for biological activity testing.

Plant material

Four chickpea cultivars which differed in their reaction to the wilt pathogen *F. oxysporum* f.sp. *ciceri* were used for histological studies of the infection process and for comparative phytochemical analysis. JG62 (ICC4951) and H208 (ICC4954) were used as susceptible controls. Both are susceptible to races 1 and 2, although H208 is considered to be more tolerant of race 1 (late wilting). The two resistant cultivars were CPS1 (ICC11323), which is only resistant to race 1, and WR315 (ICC 11322), which is resistant to both races 1 and 2.

Histological analyses of the infection process

Seedlings of each of the four cultivars, up to 21 days old, were collected from wilt-infested soil at ICRISAT, and cleared in methanol. Additionally, seeds were surface sterilized, germinated for 2 or 4 days and the roots dip-inoculated with a suspension of washed spores from *F. oxysporum* f.sp. *ciceri* race 1 or 2. These seedlings were incubated on water agar. Control seedlings were dipped in sterile distilled water only. Roots from 4-day-old seedlings were also wounded with a sterile scalpel immediately prior to dipping in a spore suspension. Samples were collected daily and placed in methanol (up to 4 radicles 5 ml^{-1} methanol).

Transverse and longitudinal sections were made from the upper and middle regions of the roots. Tissue squashes were prepared from the root tips. This material was stained with cotton blue in lactophenol, or with 0·05% (w/v) toluidine blue and observed under the microscope. Where a significant fungal presence was observed in the root, the shoot was also sectioned and stained.

Effect of medicarpin and maackiain on germination and germ tube growth of F. oxysporum f.sp. ciceri

Cultures of races 1 and 2 of *F. oxysporum* f.sp. *ciceri* were grown in PDB at 25 °C. After 7 days, the spores were filtered through muslin and washed in sterilized water through

a 3 µm Millipore filter to remove auto-inhibitors. The concentration of the spores was measured with a haemocytometer and diluted to 1.0×10^6 spores ml^{-1}. Spore suspensions were pipetted in 95 µl samples into the wells of an ELISA tray. The pterocarpan isoflavanoids medicarpin and maackiain, which are insoluble in water, were dissolved in methanol and added to the spore suspension as 5 µl samples [10]. Each compound was tested at concentrations ranging from 0 to 250 µg ml^{-1}. Each treatment was replicated five times. Control treatments comprised 95 µl spore suspensions with 5 µl of methanol.

After 12 h, the bioassay was terminated by adding two drops of cotton blue in lactophenol to each well and mixing the contents thoroughly. One drop of approximately 50 µl of each replicate, per treatment, was transferred onto a slide and viewed under a microscope. Five fields of view were chosen randomly for each of the five replicates of each treatment, and the mean percentage germination and mean germ tube length (of those spores which had germinated) were evaluated.

Growth of chickpea seedlings for chemical analysis

Seeds of the same four cultivars of chickpea showing differences in their resistance to fusarium wilt [15] were sown in to sterile soil or soil inoculated with either race 1 or race 2 of *F. oxysporum* f.sp. *ciceri*, with five seedlings per pot and five pots per treatment. Soil was inoculated using a chickpea flour/sand inoculum consisting of a sand:chickpea flour:water (90 g:10 g:20 ml) mixture inoculated using a wire loop which had been dipped into the PDB culture and immediately transferred to the sand and flour mixture. The inoculum was allowed to grow for 14 days at 23 ± 5 °C, shaking every 3 days to break up the sand. Five hundred g of sterilized soil was mixed with 500 g of sterile sand and inoculated with 50 g of the inoculum. Seedlings were collected at 2, 3, 4, 5 and 6 days after sowing.

Artificial inoculation of 7-day-old chickpea plants for phytochemical analysis and disease assessment

Chickpea seeds were germinated under greenhouse conditions in sterile sand which had been thoroughly watered. After 7 days the plants were removed and the roots washed in sterile water. Roots were dipped for 20 s in a muslin-filtered PDB culture of either *F. oxysporum* f.sp. *ciceri* race 1 or race 2, diluted to approximately 1.0×10^6 spores ml^{-1}, or in sterile PDB. They were wrapped in damp tissue paper in low, wide-rimmed beakers and maintained at 25 °C for a further 5 days. The filter paper was inspected daily and kept moist. Twenty plants of each cultivar were replanted in soil and were allowed to grow for a further 14 days to evaluate the development of wilt symptoms.

Preparation and analysis of root extracts

Roots were washed, cut at the point of seed attachment, weighed and macerated in methanol with a mortar and pestle. After extraction at room temperature overnight (approximately 50 ml methanol g^{-1} root) the slurry was filtered through Whatman No. 1 filter paper. The filtrate was evaporated to dryness under reduced pressure, re-

dissolved in a small quantity of methanol, transferred through Millipore 0·45 μm filters to autosampler vials and dried under a continuous flow of nitrogen. Methanol was added to each sample to give the equivalent of 0·5 g fresh weight of plant material ml^{-1}. Samples were analysed by HPLC using a Waters LC 600 pump, Waters 717 autosampler and a Waters 996 photodiode array detector. Samples were injected onto a Spherisorb ODS column (25 cm × 4·6 mm i.d.; 5 μm particle size) and were separated using a 2 % acetic acid in water (A) and 2 % acetic acid in acetonitrile (B) gradient programme: time = 0 min, A = 50 %; time = 20 min, A = 40 %: using a convex gradient (Waters LC 600 pump curve 4). Peaks were identified and quantified by co-chromatographic comparison of retention times and u.v. spectra with genuine standards [16] (Plantech UK Ltd).

RESULTS

Histological analyses of the infection process

Transverse and longitudinal sections of wilted plants from ICRISAT revealed many hyphae within the xylem vessels of the chickpea roots (Fig. 1a). All wilting plants (JG62 exposed to race 1 or race 2 of *F. oxysporum* f.sp. *ciceri*) showed hyphae in the xylem. In severe cases, the surrounding tissues were also invaded. In some of the more severely wilted plants, a large proportion of the xylem vessels in the stem were also partially or fully occluded by hyphae, up to five internodes above the point of seed attachment (Fig. 1b). No other stem tissues showed signs of invasion. Hyphal material was not observed in the root xylem of non-wilted plants.

In artificially inoculated seedlings of JG62, WR315 and CPS1, sectioning revealed that, where infection occurred, the first signs of hyphal penetration of the root were visible at between 5 and 10 days after inoculation. Despite the presence of hyphae over much of the root surface, penetration was almost always effected in the first 5 or 10 mm below the point of seed attachment. This region contains a number of pores which were often, although not exclusively, the point of entry for the pathogen (Fig. 1c).

Inside the root, hyphae spread intercellularly and no obvious structural changes in the host's cells were observed (Fig. 1d). Where an infection was well established, it could be seen that the pathogen exhibited a characteristic growth pattern. In the outer third of the cortex, hyphae grew predominantly parallel to the root axis, while deeper penetration of the root was effected by hyphae growing in the transverse plane towards the central stele. Degradation of the outer cortical cells was often associated with hyphal proliferation in this region. Wounding was used to encourage hyphal invasion of the root. However, infection was rare at wound sites despite the presence of spores and hyphae in and around the wound.

The effect of medicarpin and maackiain on spore germination and germ tube growth

The germination of spores from both races 1 and 2 was inhibited by the presence of either medicarpin or maackiain (Fig. 2). The inhibitory effect of medicarpin was significantly greater than that of maackiain at 66, 125 and 250 μg ml^{-1}. For example, the percentage germination of race 1 spores treated with 250 μg ml^{-1} medicarpin was approximately 10 % compared to nearly 40 % when treated with the same concentration of maackiain (Fig. 2). The ED_{50} values for medicarpin and maackiain
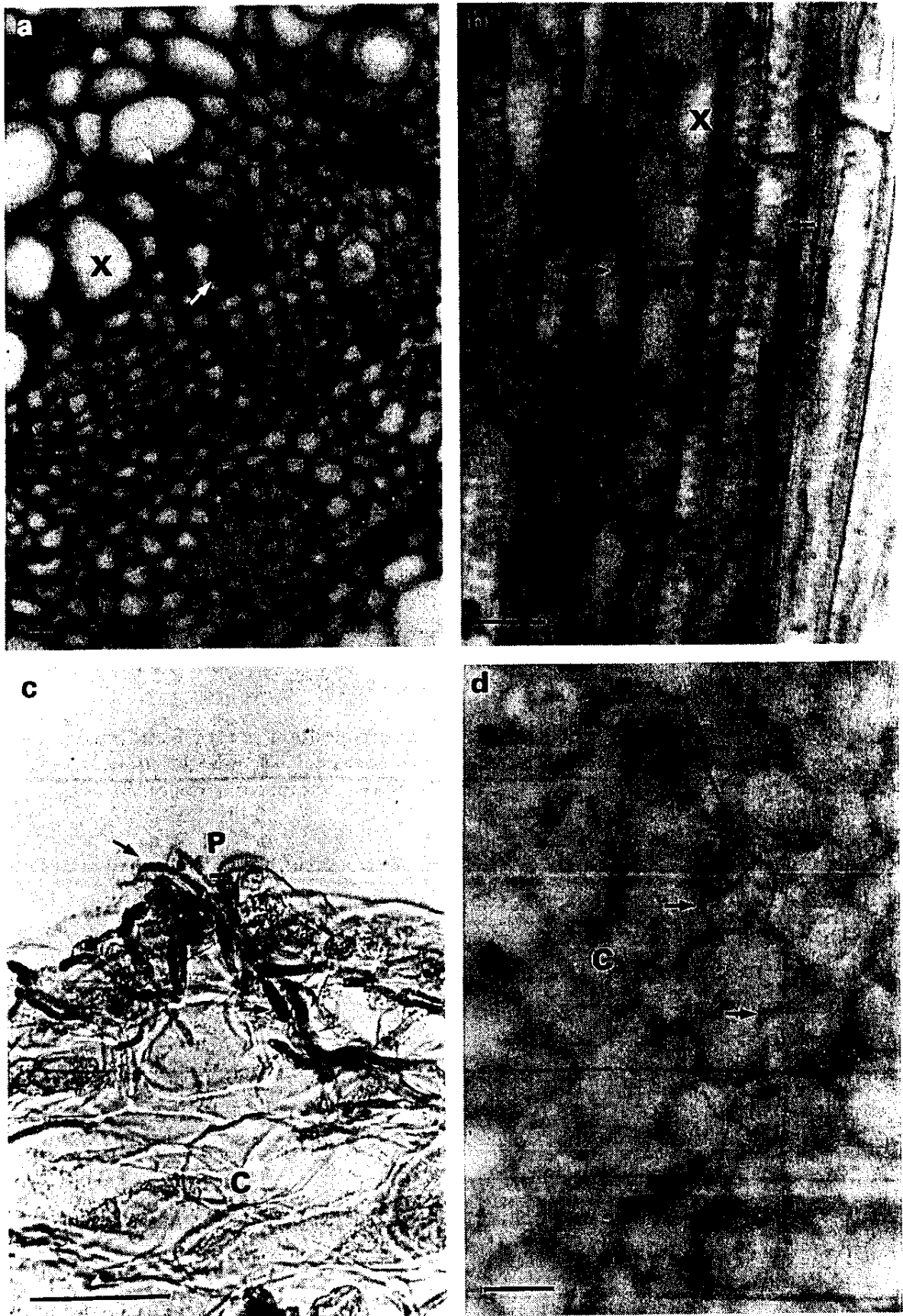
FIG. 1. Presence of *F. oxysporum* f.sp. *ciceri* hyphae within the tissues of chickpea roots and stem. Hyphae (arrows) are visible in the lumen of xylem vessels (X) of the root (a) and of the stem (b) five internodes above the point of seed attachment. Penetration of the upper root through a surface pore (P) is shown in panel (c). Progression of the hyphae through the root cortex (C) did not induce obvious structural changes in the adjacent cells (d). Scale bar is equivalent to 40 μm.

were approximately 80 µg ml^{-1} and 160 µg ml^{-1} respectively. No significant difference in effect for either medicarpin or maackiain was recorded between the two races ($P >$ 0·05; Mann-Whitney).
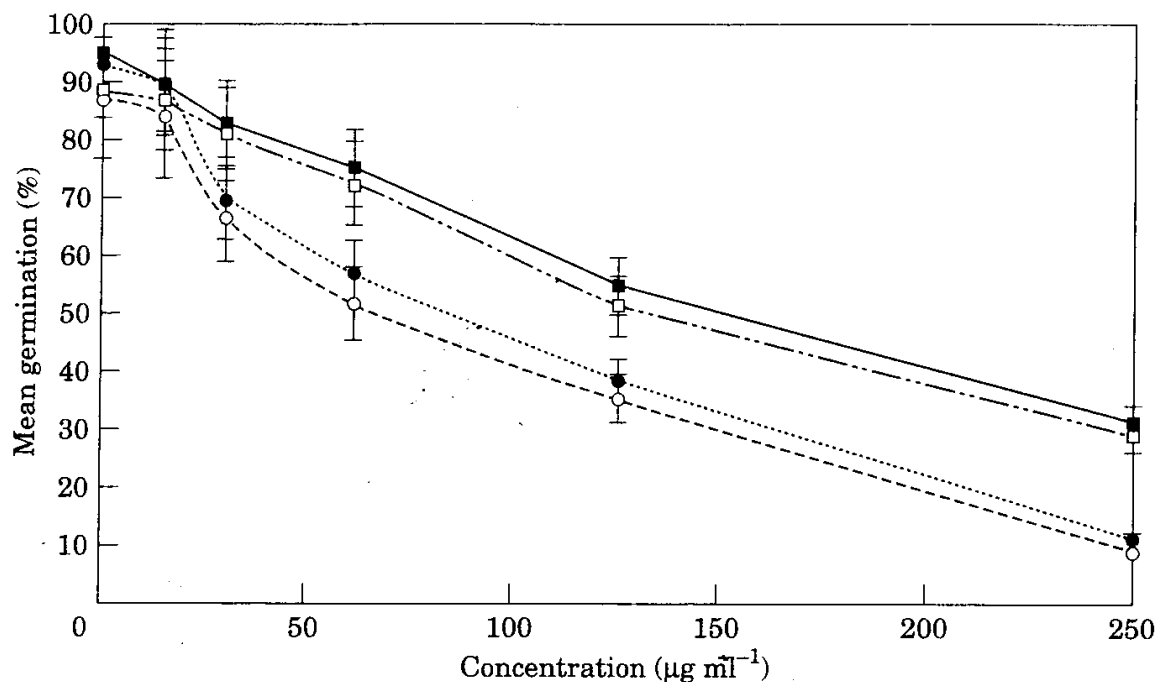

Fig. 2. Effect of maackiain and medicarpin on the germination of race 1 and race 2 spores of *F. oxysporum* f.sp. *ciceri* after 24 h (■) maackiain vs. race 1; (□) maackiain vs. race 2; (●) medicarpin vs. race 1; (○) medicarpin vs. race 2). Bars = standard errors of the mean.

Medicarpin and maackiain both inhibited germ tube growth of those spores which did germinate. The concentrations of medicarpin and maackiain required to cause a 50% reduction in germ tube length were both approximately 15 µg ml^{-1}. For both compounds the maximum inhibition of germ tube growth was recorded at concentrations as low as 66 µg ml^{-1}.

Accumulation of medicarpin and maackiain in roots of chickpeas grown in inoculated soil

Because the antifungal effects of medicarpin and maackiain were similar in magnitude and the concentrations of these compounds in roots of plants grown in inoculated soil were low, for cv. comparisons, the concentrations of medicarpin and maackiain were combined to give the levels of total pterocarpan in the roots. A gradual increase in the concentration of pterocarpans was recorded in all cultivars grown in sterile soil over the first 6 days after sowing (Fig. 3). After 2 days, the concentration was approximately 1 µg g^{-1} root, whereas after 6 days the concentration was between 12 and 23 µg g^{-1} for each of the four cultivars (Fig. 3a). The concentration of pterocarpans in the roots of variety JG62 was significantly lower than other cultivars by day 3 and the highest concentration was recorded in the roots of CPS1 after 6 days (Fig. 3a) ($P < 0·05$; Mann-Whitney).

The concentration of total pterocarpans in roots of plants grown in soil inoculated with race 1 also increased over the first 6 days after sowing and was consistently greater than that recorded in roots from sterile soil (Fig. 3b). After 6 days in sterile soil, roots of the resistant varieties CPS1 and WR315 contained approximately 20 µg total

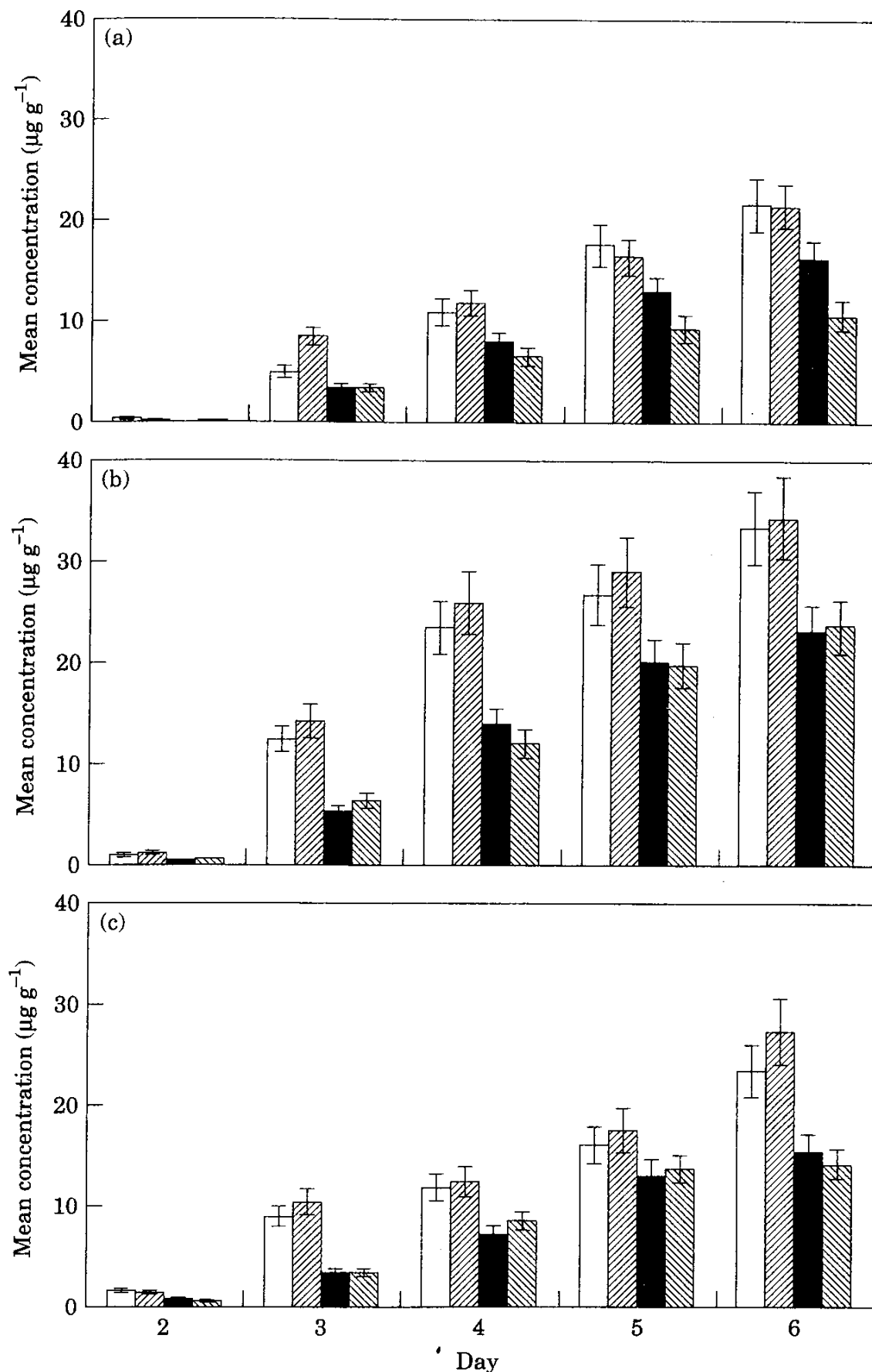Fig. 3. Total pterocarpan (medicarpin and maackiain) concentration in roots of CPS1 (□), WR315 (▨), H208 (■) and JG62 (▨) from (a) sterile soil, from (b) *F. oxysporum* f.sp. *ciceri* race 1-inoculated soil and (c) *F. oxysporum* f.sp. *ciceri* race 2-inoculated soil. Bars = standard errors of the mean.
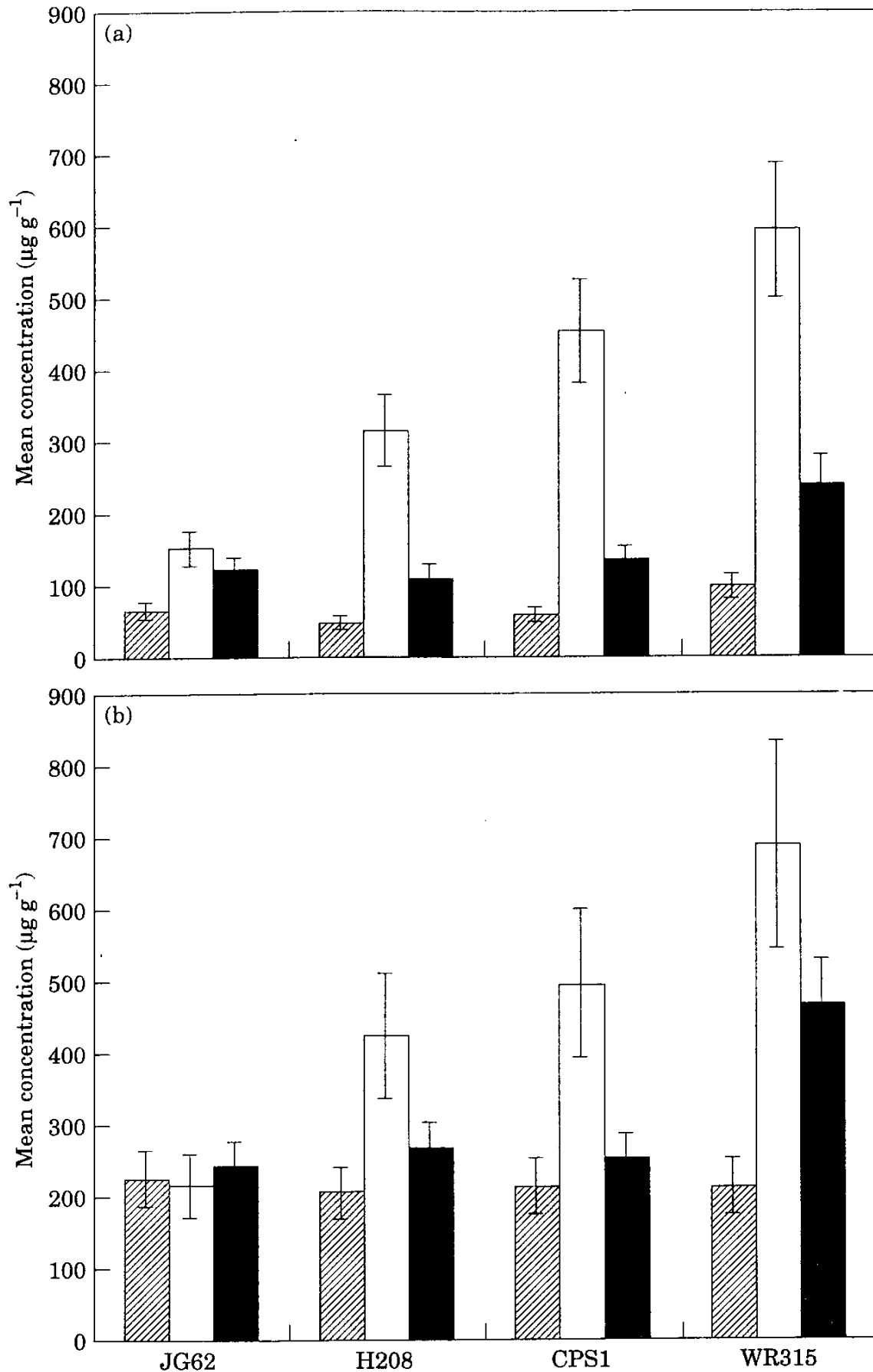
Fig. 4. Concentration of (a) medicarpin and (b) maackiain in roots of four cultivars of chickpea 5 days after root dipping in race 1 (□) or race 2 (■) spores from *F. oxysporum* f.sp. *ciceri* culture in PDB or in sterile PDB (▨). Bars = standard errors of the mean.

pterocarpan g^{-1} root. In contrast, roots from the same cultivars grown in soil inoculated with race 1 contained approximately 35 μg g^{-1} roots. Furthermore, the concentrations of total pterocarpan in the roots of the susceptible cv. JG62 and the late wilting cv. H208, whilst higher than when grown in sterile soil, were considerably lower than those of the resistant cvs, when grown in the presence of the fungus. The same trend of differences between the cvs was also recorded for roots grown in soil inoculated with race 2, although the amount of the pterocarpans was not significantly greater than that recorded for roots grown in sterile soil (Fig 3c) ($P > 0.05$; Mann-Whitney).

Accumulation of medicarpin and maackiain in artificially inoculated roots
The constitutive (i.e. pre-induction) levels of the pterocarpans did not differ significantly between cultivars, with the exception of the concentration of medicarpin in the roots of WR315, which contained more than 100 μg g^{-1}. The amounts of pterocarpans in inoculated roots were greater than the constitutive amounts in all cultivars, except for the level of maackiain in the race 1- and race 2-inoculated roots of the highly susceptible cv. JG62 (Fig. 4). This induction of phytoalexins by *F. oxysporum* f.sp. *ciceri*, however, showed considerable variation among the cultivars. WR315 showed a very high level of induction, especially after inoculation with race 1, where the induced level of medicarpin was more than 10 times higher than the constitutive level. Furthermore, the induced level of maackiain was five times higher than the constitutive level (Fig. 4). The level of elicitation of phytoalexins in roots exposed to race 2, compared to those exposed to race 1, was considerably lower, which reflects the result recorded for plants grown in inoculated soil in the previous experiment.

Wilting in artificially inoculated plants
JG62 plants were showing signs of wilt at 12 days after inoculation and replanting and many were heavily wilted. None of the other cvs showed signs of wilt after this time interval. In the field experiment, where only race 1 could be evaluated, all JG62 plants and 42% of H208 plants were wilting after 21 days. None of the race 1-resistant plants of WR315 or CPS1 showed any signs of wilt.

DISCUSSION

Histological considerations
All the soil-grown or artificially inoculated plants from ICRISAT that showed signs of wilting were found to have hyphae within the root xylem. In severe cases, a number of xylem vessels in the stem were also occluded up to the fifth internode above the point of seed attachment (Fig. 1b). Thus, occlusion of the vascular tissue by hyphae may be directly responsible for wilt symptoms. This is in agreement with other studies on fusarium wilts [2], although the latter relied on a method of artificial inoculation which exposed severed mature xylem vessels to the pathogen. Our histological studies of the early stages of infection of chickpea roots by *F. oxysporum* f.sp. *ciceri* showed that the preferred site for infection was the upper radicle, no more than 10 mm below the point of seed attachment. This region appears to be more vulnerable because the epidermal cell walls are thinner than in other parts of the root and there are a greater number

of pores. However, this anatomy was found in all of the cultivars examined, adding support to the notion that differences in susceptibility to infection by *F. oxysporum* f.sp. *ciceri* were not due to structural features and were thus more likely to be biochemical in nature. In a few instances the resistant cultivar, WR315, showed a limited amount of infection by race 2 in this vulnerable region, but these incursions did not develop beyond a few cells from the site of penetration. In contrast, roots from the susceptible cultivar JG62 showed well-developed infection, with penetration of the cortex almost to the endodermis. No localized rapid cell death or gross structural changes were observed in the infected regions of the resistant roots. Again this suggests that, for resistance, chickpea roots depend upon biochemical rather than mechanical defences.

The preferred site of infection of wounded seedlings was also via pores in the upper 10 mm of the root rather than wound sites themselves, even though both spores and hyphae could be seen in and around the damaged tissue. This suggests that, while wounding does not trigger a defensive reaction capable of preventing *Fusarium* infection elsewhere in the root, an effective resistance response is triggered at the wound site.

Differences in pterocarpan accumulation in the chickpea cultivars

The antifungal activity of medicarpin and maackiain is well known [6], but they are not known to be universally active [3]. Thus, it was essential to show that they have antifungal properties towards *F. oxysporum* f.sp. *ciceri* before any conclusions could be drawn about their role in the resistance of chickpea to this pathogen. The concentration of pterocarpans required to inhibit germination was comparable to that reported for other pterocarpans against other *Fusarium* species. For example, 100 % inhibition of *Fusarium udum*, the causal agent of fungal wilt in pigeon pea, was obtained with 300 μg ml^{-1} of cajanol, although high levels of inhibition were also recorded for comparatively lower concentrations [10]. However, ED_{50} values against *Helmintho-sporium carbonarum* were less than 40 μg ml^{-1} medicarpin [6].

This study also showed that *F. oxysporum* f.sp. *ciceri*, whether present in the soil or artificially applied to chickpea roots, induces an increase in the concentration of medicarpin and maackiain in the roots. Whilst other studies have shown this response to occur in chickpea plants as a whole, none have done so with roots or with *F. oxysporum* f.sp. *ciceri* challenged chickpea plants. Furthermore, previous studies have concentrated only on stems, leaves and cell cultures, and in all of these cases have shown phytoalexin induction through either artificial inoculation at a site of damage [6] or through elicitor-challenged cell cultures [9]. Our histological study has shown that hyphal invasion did not occur at sites of damage, which suggests that the damage itself induces defence responses. This local variation demonstrates the importance of evaluating variations in the phytoalexin biosynthesis by natural means of infection. This is the first study which shows that the presence of *F. oxysporum* f.sp. *ciceri* in undamaged roots caused the elicitation of medicarpin and maackiain biosynthesis in roots.

The potential of different chickpea cvs to biosynthesize phytoalexins was shown to be variable and the examples investigated in this study show a strong association between high induction of phytoalexins in roots and resistance to *F. oxysporum* f.sp. *ciceri*. A similar association between higher levels of phytoalexin synthesis and resistance to *Ascochyta rabiei* was reported in chickpea cell cultures [1]. If one considers that the

pterocarpans medicarpin and maackiain are potent inhibitors of spore germination of *F. oxysporum* f.sp. *ciceri* and that they are induced, by inoculation with *F. oxysporum* f.sp. *ciceri*, to concentrations capable of causing such inhibition, it is reasonable to conclude that the pterocarpans make a significant contribution to chickpea resistance to *Fusarium*.

When the roots were saturated with spores by root-dipping, the variation between the cultivars in terms of recorded levels of phytoalexins was more pronounced, with the data showing that phytoalexin production in WR315, the cv. expressing resistance to both *Fusarium* races, was much greater than in the susceptible cvs. The concentration of pterocarpans in these roots even when measured on a whole root basis was of a magnitude likely to have an impact on fungal growth. Since the level of induced medicarpin and maackiain in race 2 challenged roots of WR315 was high compared to the other three cvs, which were reported to be susceptible to race 2 [5], it is likely that this race-dependent reaction is associated with the phytoalexin induction potential of the cultivars.

Differences in pterocarpan accumulation response to F. oxysporum *f.sp.* ciceri *races*
The lower induction of pterocarpans by spores of race 2 compared to spores of race 1 is also important. Race 2 is considered to be more virulent, as there are fewer cvs of chickpea which express resistance to this race than to race 1. In both experiments investigating the levels of phytoalexin induction, the elicitation by race 2 was consistently much lower than that by race 1 in all cultivars. The reason this occurs is not presently known, but may provide important information to show why race 2 is more virulent.

Because race 2 of *F. oxysporum* f.sp. *ciceri* induces a lower level of pterocarpan biosynthesis than does race 1, despite artificial inoculation under identical conditions, it would appear that there has to be a difference in elicitation. Exposure to race 2 does induce some increase in pterocarpan production, suggesting that some recognition of race 2-derived elicitors occurs. That induction is lower for race 2 than for race 1, under identical inoculation conditions, may be attributable to lower elicitor production by race 2. Another explanation, however, would be that *F. oxysporum* f.sp. *ciceri* races produce more than one elicitor, but fewer of the race 2 elicitors are recognizable to the host plants than the race 1 elicitors. The phytoalexin responses of the roots exposed to race 2 were similar to, although lower than, those of roots exposed to race 1, which suggests that the variation occurs in the pathogen alone rather than in an ability of the plant to maintain a high level of phytoalexin biosynthesis. WR315 is reported to be resistant to race 2, whilst the other three cultivars used in this study are reported to be susceptible to race 2 [5]. The differences in the phytoalexin response would explain this field variation, because the level of pterocarpans in the roots of WR315 were significantly higher than in roots from the other cultivars.

The results indicate that roots of resistant chickpea cultivars are protected biochemically against infection by the wilt pathogen *F. oxysporum* f.sp. *ciceri*; that the production of the pterocarpans medicarpin and maackiain is induced in chickpea roots by exposure to *F. oxysporum* f.sp. *ciceri*, with greater induction occurring in resistant cultivars than in susceptible cultivars; that medicarpin and maackiain are capable of inhibiting the germination and hyphal growth of *F. oxysporum* f.sp. *ciceri* spores from

both race 1 and race 2; that infected wilting plants suffer considerable vascular occlusion through fungal invasion of the xylem vessels, both above and below ground; and that the difference in pathogenicity between races 1 and 2 appears to be due, at least partly, to a difference in their capacity to induce pterocarpan biosynthesis.

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