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Root exudates associated with the resistance of four chickpea cultivars (*Cicer arietinum*) to two races of *Fusarium oxysporum* f.sp. *ciceri*

P. C. STEVENSON, D. E. PADGHAM *and* M. P. HAWARE*
*Chemical Ecology Resource Centre, Natural Resources Institute, Chatham Maritime, Kent ME4 4TB, UK and *Crop Protection Division, ICRISAT Center, Patancheru P.O., A.P. 502 324, India*

The germination of race 1 spores of *Fusarium oxysporum* f. sp. *ciceri* was significantly inhibited by the root exudate of the wilt-resistant chickpea cvs CPS1 and WR315 compared to untreated spores and spores treated with root exudates from susceptible cultivars. The effect was concentration dependent, such that the exudate from 1 g of root in 2 ml of water almost completely inhibited spore germination, whereas the exudate from 1 g of root in 20 ml water did not do so. The inhibitory effects of the active exudates were negated when the apolar components of the exudates were removed by extraction with ethyl acetate. The root exudates of the susceptible cv. JG62 and the late wilting cv. H208 did not inhibit germination. The hyphal growth of germinated spores was also strongly inhibited by the concentrated exudates of CPS1 and WR315, and diluted exudates were less potent. The highest concentration of the exudate of the susceptible cv. JG62 showed some inhibition of hyphal growth, whereas none of the exudates of H208 were found to contain any antifungal activity. The effect of the exudates on the spores of race 2 was similar to that reported for race 1, except that the water-soluble components of the crude root exudate of WR315 after ethyl acetate extraction were also found to inhibit germination significantly. Overall, the spores of race 2 appeared to be more susceptible to the effects of the exudates. The ethyl acetate fractions of the root exudates of CPS1 and WR315 strongly inhibited germination and hyphal growth of both race 1 and race 2, the effect being concentration dependent. The results suggest that the resistance of chickpeas to vascular wilt depends, at least in part, upon the antifungal activity of the root exudates. Differences in the expression of resistance in the field could depend upon the concentration or rate of production of constitutive antifungal components by the root.

INTRODUCTION

The chickpea, *Cicer arietinum*, is a major source of human and domestic animal food, particularly in the semi-arid tropics where its production is concentrated (Jodha & Subba Rao, 1987). It has been described as the world's third most important pulse crop after beans, (*Phaseolus vulgaris*) and peas (*Pisum sativum*) (Saxena, 1990). India accounts for approximately 75% of world chickpea production (FAO, 1992), highlighting both its importance as a crop and the value of research leading to its improved productivity in India The major constraint in many chickpea regions is Fusarium wilt caused by the root pathogen *Fusarium oxysporum* f.sp. *ciceri* (*F. o. ciceri*) (Nene & Reddy, 1987). The disease is widespread in the Indian subcontinent, north and east Africa, southern Europe and the USA (Haware *et al.*, 1990), so strategies for improved management of *F. o. ciceri* in India could subsequently be applied to other chickpea-growing regions.

The seedborne disease can be controlled by treatment with benomyl (Haware *et al.*, 1978), but the soilborne pathogen is more difficult to control because chlamydospores can persist in the soil in the absence of chickpea for up to 6 years (Haware *et al.*, 1986). Thus, the value of cultural methods of control such as crop rotation is negligible (Haware *et al.*, 1990). Consequently, considerable emphasis has been placed on the use of resistant cultivars (Nene & Haware, 1980; Haware *et al.*, 1992).

Four races of *F. o. ciceri* have been described in India (Haware & Nene, 1982) and two others have been described in Spain (Jiménez-Diaz *et al.*, 1989), based on the different reactions of chickpea cultivars to wilt at different locations. In India only races 1 and 2 are widespread. A

recent study identified 160 germplasm lines of chickpea expressing resistance to race 1 (Haware *et al.*, 1992), and few of these are known to be resistant to race 2 (Haware, unpublished data).

The elucidation of the resistance mechanisms of chickpeas against these races would be of value for understanding and evaluating the variability of the expression of resistance under different field conditions and in different agro-ecological zones. In addition, this information may provide genetic markers for breeding programmes and may facilitate the distinction between different races of *F. o. ciceri*. In preliminary studies, the root exudate of one cv., CPS1, was shown to inhibit spore germination and hyphal growth of *F. o. ciceri* race 1 (Haware & Nene, 1984; Stevenson *et al.*, 1994). In the present study, such a mechanism of resistance was investigated further by evaluating the effects of the root exudates from four chickpea cultivars, each expressing different levels of resistance or susceptibility to *F. o. ciceri* races 1 and 2, on spore germination and hyphal growth of the pathogen.

MATERIALS AND METHODS.

Plant material and preparation of exudates and extracts

The seeds of four cultivars of chickpea which varied in their resistance (Table 1) were sterilized in 2% hypochlorite solution and germinated on filter paper soaked in sterilized water. After 48 h the germinated seeds were placed on a floating polystyrene platform, such that the protruding chickpea radicle projected down through a hole in the platform into 100 ml of sterilized water in an autoclaved 500-ml beaker. The platform was floated on the surface of the water and incubated at 25 ± 3°C under a 12-h light : 12-h dark regime (Haware & Nene, 1984).

Table 1. Four cultivars of chickpea investigated and their reaction to resistance to *Fusarium oxysporum* f. sp. *ciceri* races 1 and 2 in the field (Haware & Nene, 1982)

Cultivar	Race 1	Race 2
WR 315	R[a]	R
CPS1	R	S
H208	LW	S
JG62	S	S

[a] R, resistant; S, susceptible; LW, late wilting.

After 7 days the polystyrene platform and chickpea seedlings were removed and the resulting exudate solution was filtered through sterilized Nalgene filters (0·45 μm) under reduced pressure. The roots from each beaker were weighed so that the exudates from each cultivar could be diluted or concentrated to make exudates equivalent to 1 g root tissue per 10 ml of water. Each exudate was divided into two fractions: A and B. Fraction A was left as a solution of 1 g root tissue per 10 ml of water, and fraction B was divided into two further fractions, C and D. Fraction C was concentrated under vacuum to make a solution equivalent to 1 g root tissue per ml of water. Fraction D was extracted twice in a separating funnel with an equal volume of ethyl acetate. The aqueous fraction was retained and separated into two fractions, E and F, which were adjusted to give solutions equivalent to 1 g root tissue per 10 ml and 1 g root tissue per ml of water, respectively. The two apolar fractions in ethyl acetate were combined to give fraction G, which was evaporated to dryness under reduced pressure and dissolved in methanol to give a solution equivalent to 1 g root tissue per ml. Due to the low water solubility of the components in this fraction, the methanol solution was mixed with an equal volume of water and methanol was then evaporated under reduced pressure. A second volume of water was added and the fraction was evaporated again until the concentration of sample in the solution became equivalent to 1 g root/ml. Control solutions were prepared as described above, using sterilized water incubated for 7 days with the cultivars for root exudate bioassays, and aqueous methanol evaporated to water for bioassays of ethyl acetate extracts of root exudates.

Fusarium culture and bioassay of root exudates

Cultures of *F. o. ciceri* races 1 and 2 were maintained on Potato Dextrose Agar (PDA) at 25°C. Conidia were transferred from these cultures to a Potato Dextrose Broth (PDB), and incubated for 7 days. The spores were then filtered through muslin cloth and used in bioassays. The concentration of spores was measured with a haemocytometer and was found to be approximately $1{\cdot}2 \times 10^6$ and $1{\cdot}4 \times 10^6$ for races 1 and 2, respectively. Aliquots of 100 μl of the spore suspension were mixed with 100 μl of test solution in compartments of an ELISA tray in order to give exudate concentrations equivalent to 0·5 g/ml and 0·05 g/ml. Each treatment

was replicated five times. Dilutions were also made in ELISA tray compartments before adding the spore suspension, and control treatments were set up using 100 μl of water.

After 24-h of incubation, germination was terminated by adding two drops of cotton blue to each ELISA compartment. One drop from each treatment was placed on a slide and viewed under a microscope. Five fields of view were chosen randomly for each of the five replicates for each treatment, and the mean percentage germination and mean hyphal length of germinated spores were calculated. The mean values for percentage germination were arcsine transformed after Gomez and Gomez (1984) for ANOVA, and differences between treatments were tested for significance using Duncan's multiple range test (MRT).

RESULTS

The effect of root exudates on spore germination

The percentage germination of spores of *F. o. ciceri* race 1 was significantly reduced at the highest concentrations of the crude root exudates

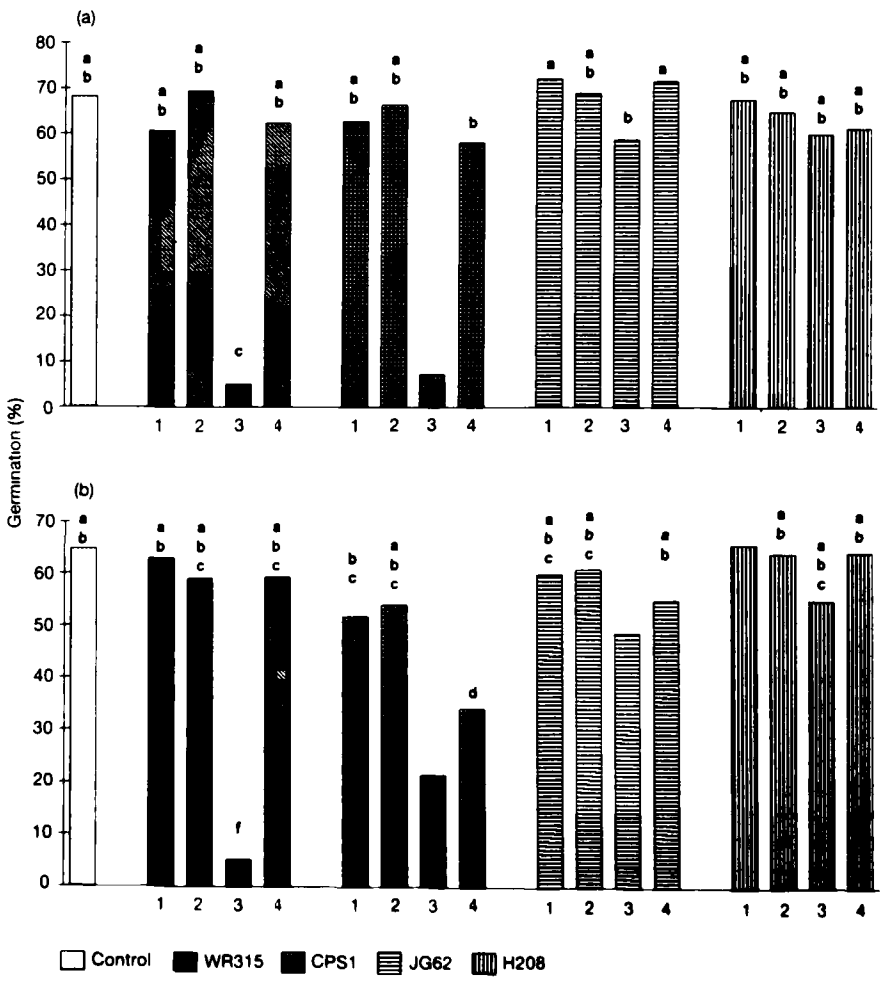

Fig. 1. The effect of crude and ethyl acetate-extracted root exudates of four cultivars of chickpea on the percentage germination of the spores of *F. o. ciceri* races (a) 1 and (b) 2 after 24 h: (1) 1 g per 20 ml of crude root exudate; (2) 1 g per 20 ml of root exudate after ethyl acetate extraction; (3) 1 g per 2 ml of crude root exudate; (4) 1 g per 2 ml of root exudate after ethyl acetate extraction. Bars allocated different letters differ significantly ($P < 0.05$, Duncan's MRT)

of WR315 and CPS1, compared to that of spores in water or treated with the same exudates at lower concentrations. The germination of race 1 spores was not significantly influenced by these exudates after extraction with ethyl acetate, or by exudates from JG62 and H208 before and after extraction (Fig. 1a).

The effect of the same root exudates on the germination of race 2 spores was similar to that described above for race 1, but was less clear. The polar fraction of the CPS1 exudate also inhibited germination of this race, although the rate of inhibition was significantly less than that achieved with the crude exudates of CPS1 and WR315 (Fig. 1b).

The inhibitory effect of the highest concentration of the crude exudate of WR315 on race 2 (5·3 ± 1·46%) was not significantly different from that exerted on race 1 (5·2 ± 0·96%), but the inhibition caused by the equivalent exudate of CPS1 on race 2 (21·5 ± 5·6%) was significantly less than that

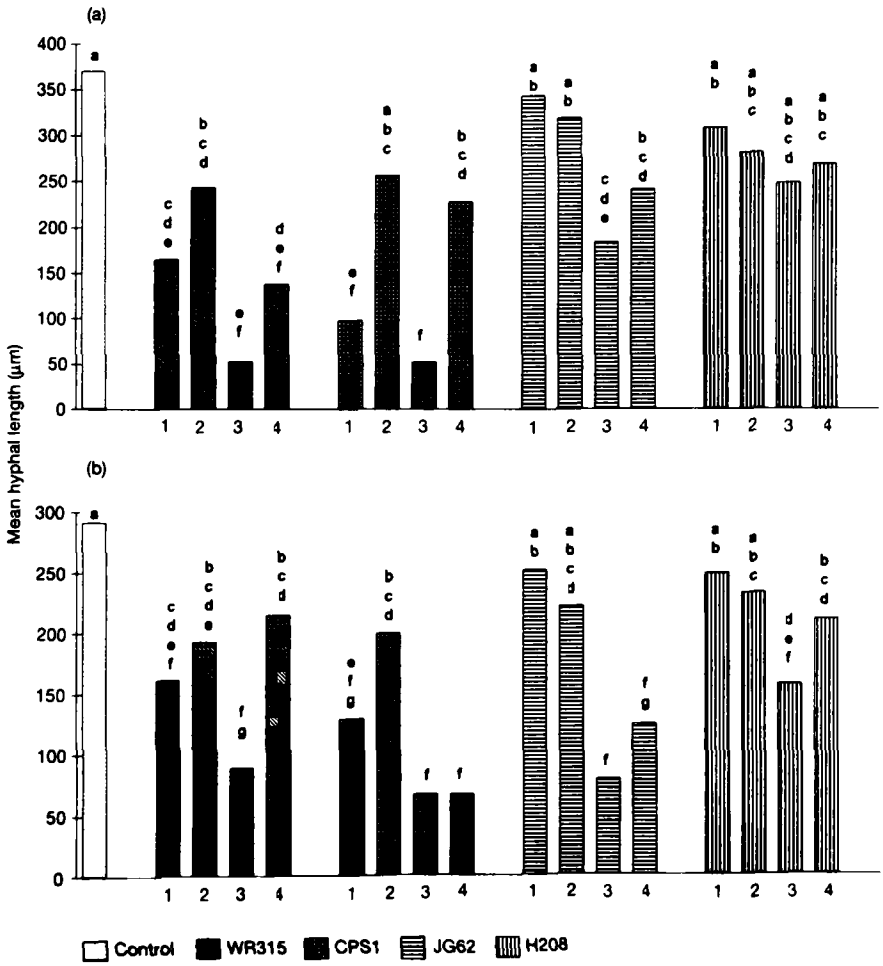

Fig. 2. The effect of crude and ethyl acetate-extracted root exudates of four cultivars of chickpea on the hyphal length of those spores of *F.o. ciceri* races (a) 1 and (b) 2 which had germinated after 24 h (1) 1 g per 20 ml of crude root exudate; (2) 1 g per 20 ml of root exudate after ethyl acetate extraction; (3) 1 g per 2 ml of crude root exudate, (4) 1 g per 2 ml of root exudate after ethyl acetate extraction. Bars allocated different letters differ significantly ($P < 0.05$, Duncan's MRT).

observed on race 1 (7·3 ± 2·14%) (Mann-Whitney test, $P < 0·05$). There was no significant difference in the germination of races 1 and 2 in water, although the overall germination of race 2 spores in the exudates was lower than that observed for race 1.

The effect of root exudates on hyphal growth

There were highly significant differences in hyphal growth between the treated and untreated spores of both race 1 (F ratio = 5.92: $P < 0.001$) and race 2 (F ratio = 10·78; $P < 0·001$) (Figs 2a and 2b). For race 1, the inhibition of hyphal growth by crude root exudates of WR315 and CPS1 was dose dependent. Extraction of these exudates with ethyl acetate reduced the inhibitory effects, but did not remove them completely. The more concentrated exudate from JG62 caused smaller but significant reductions in hyphal growth both before and after extraction with ethyl acetate, but this effect was significantly reduced compared to that of WR315 and CPS1, and the exudate from H208 had no inhibitory effects.

Ethyl acetate extraction partially removed the inhibitory effect of the lower concentration of the CPS1 exudate, but did not alter the inhibitory

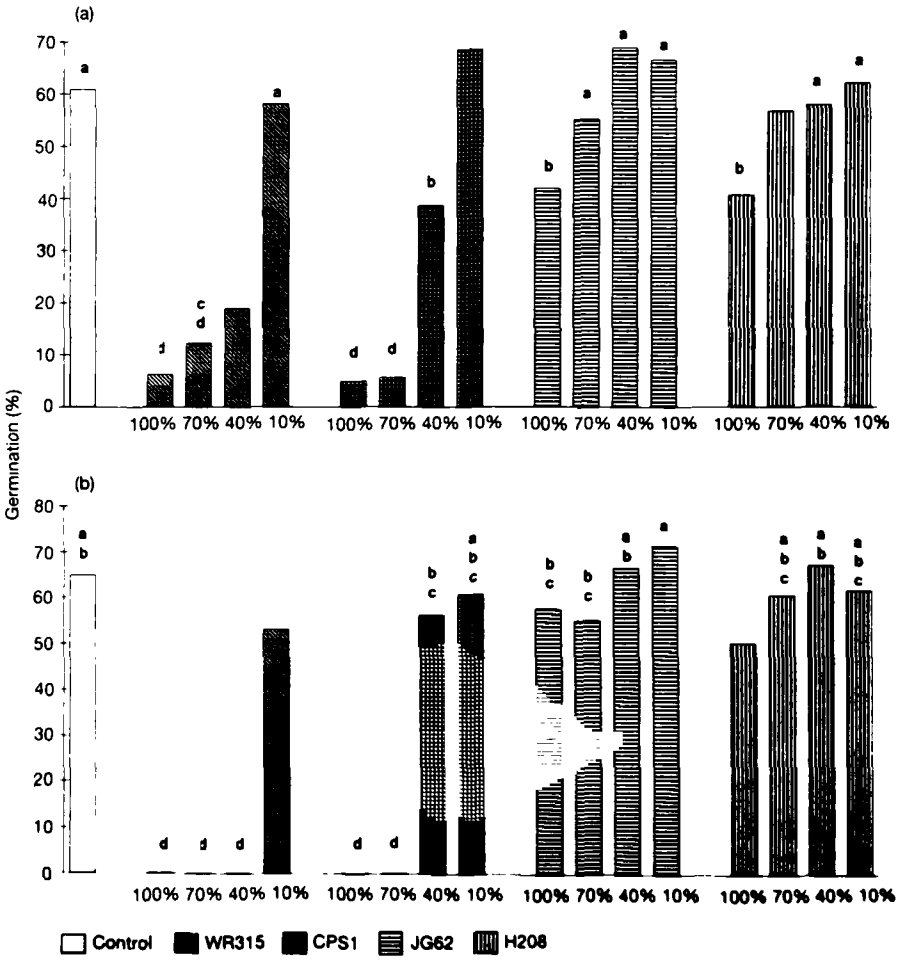

Fig. 3. The effect of different concentrations of a 0·5 g/ml ethyl acetate fraction of root exudates of four cultivars of chickpea on the percentage germination of spores of *F. o. ciceri* races (a) 1 and (b) 2 after 16 h. Bars allocated different letters differ significantly ($P < 0·05$; Duncan's MRT).

effect of the concentrated exudate from CPS1. The more concentrated exudate from JG62 was as effective in inhibiting hyphal growth as the exudates from WR315 and CPS1, but the more dilute exudate had no significant effect. The most concentrated exudate from H208 caused a moderate but significant inhibition of hyphal growth.

The effect of ethyl acetate extracts of root exudates on spore germination

Ethyl acetate extracts of the root exudates from WR315 and CPS1 showed strong, dose-dependent inhibition of germination of race 1 spores (Fig. 3a). The germination of spores in the highest concentration of ethyl acetate extracts of WR315 (mean germination 6·3 ± 2·88%) and CPS1 (5·0 ± 3·15%) was not significantly different from that in crude root exudates of WR315 and CPS1 when tested at equivalent concentrations ($P < 0.05$; Mann–Whitney test), but differed significantly from that of the control (60·9 ± 2·94%) ($P < 0.05$; Duncan's MRT). However, at the lowest concentrations tested (10%), these apolar exudate fractions had no significant effect. In contrast, apolar fractions of JG62 and H208 only caused a significant reduction in germination compared to untreated spores at the highest concentration, but the

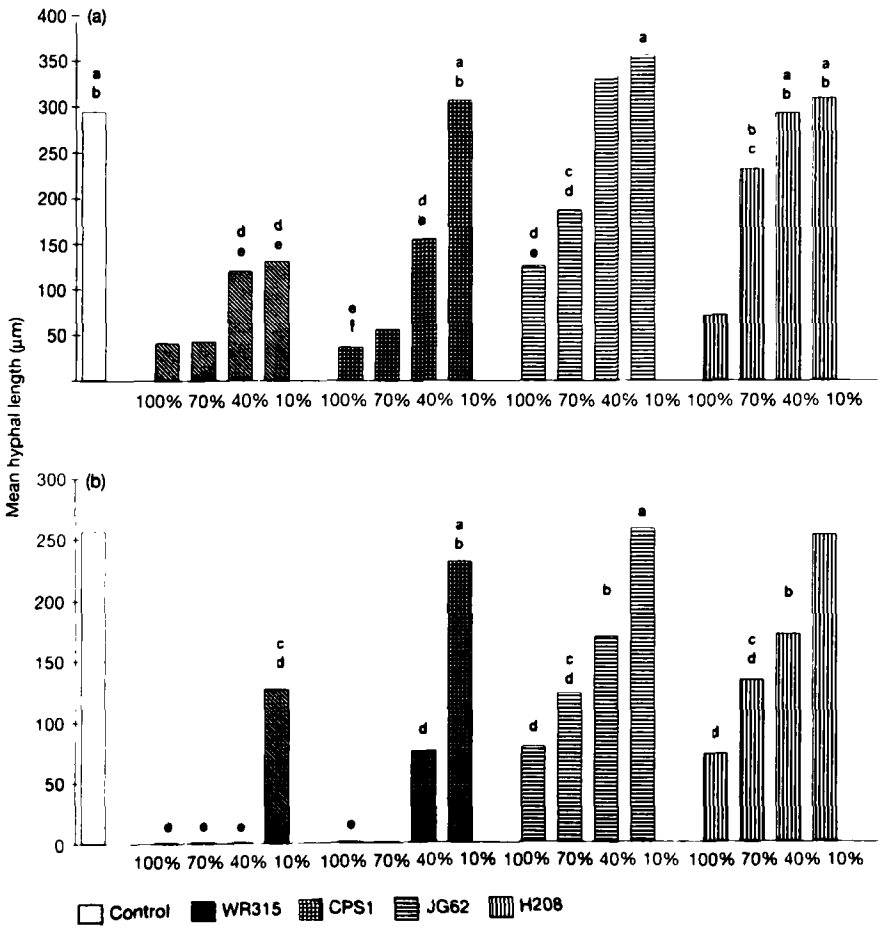

Fig. 4. The effect of different concentrations of a 0·5 g/ml ethyl acetate fraction of root exudates of four cultivars of chickpea on the mean hyphal growth of *F. o. ciceri* spores races (a) 1 and (b) 2 after 16 h. Bars allocated different letters differ significantly ($P < 0.05$; Duncan's MRT).

magnitude of inhibition was much less than that observed with the equivalent extracts from WR315 and CPS1.

The inhibitory effects of the ethyl acetate fractions of exudates from CPS1 and WR315 on the spore germination of race 2 were even greater than those on race 1 (Fig. 3b). None of the spores that were treated with the three higher concentrations of WR315 or the two higher concentrations of ethyl acetate fractions of CPS1 had germinated within 16 h. In fact, race 2 spores did not germinate in 100% (=0·5 g/ml) ethyl acetate extracts, even though they germinated in the crude exudates of 'WR315' and 'CPS1' at a comparable concentration (Fig. 1b). In contrast, the percentage germination of race 2 spores in the 40% dilution of the ethyl acetate extract (= 0·2 g/ml) of CPS1 exudate was not significantly lower than that of the race 2 control, whereas the same test extract did significantly inhibit germination of race 1 spores.

The effect of ethyl acetate extracts of root exudates on hyphal growth

Ethyl acetate fractions of root exudates from all four cultivars of chickpea showed dose-dependent inhibition of hyphal growth of race 1 spores (Fig. 4a). At the highest concentrations, there were no significant differences in inhibition among the four cultivars. However, whereas the extract from WR315 inhibited hyphal growth even at the lowest concentration, no significant inhibition was observed with the equivalent extracts from the other three cultivars. Similar effects on hyphal growth were observed with race 2 (Fig. 4b).

DISCUSSION

The chickpea cv. WR315 is resistant to infection by *F. o. ciceri* races 1 and 2, and cv. CPS1 is resistant to race 1 alone (Haware & Nene, 1982). Thus the presence of antifungal activity in the root exudates of these two cultivars, its absence from the two susceptible cultivars (H208 and JG62) and the removal of antifungal activity by ethyl acetate extraction strongly suggests that the resistance of WR315 and CPS1 is associated with apolar components in their root exudates. Furthermore, the presence of this activity in the absence of root damage or exposure to the pathogen indicates that the antifungal components in the root exudates may be constitutive rather than induced.

Previous studies on the defence mechanisms of chickpeas to pathogenic fungi have concentrated on induced resistance, which is primarily attributed to the production of two pterocarpan phytoalexins, maackiain and medicarpin (Ingham, 1976). Subsequent research demonstrated the importance of phytoalexins and their metabolism in the resistance of chickpeas and other crops to fungal pathogens (e.g. Smith & Ingham, 1981; Clemens *et al.*, 1993). Although no work has shown specifically that the basis of resistance in chickpeas to Fusarium wilt depends on the elicitation of phytoalexin synthesis, a recent study on a similar crop, pigeonpea (*Cajanus cajan*), has shown this to be the case with, amongst others, the phytoalexin cajanol. After artificial inoculation of pigeonpea with spores of *Fusarium udum*, phytoalexin production in the resistant plants was found to be significantly greater than that in susceptible plants (Marley & Hillocks, 1993). A similar study on chickpea would be of considerable value and is currently being undertaken. If the induction of antifungal metabolites by *F. o. ciceri* infection is shown to be an additional effective defence to Fusarium wilt in chickpea, then the combination of an induced and a constitutive mechanism of resistance may be invaluable in combating the potential emergence of *F. o. ciceri* races which can overcome chickpea resistance. The combined integration of different mechanisms into cultivars in ongoing breeding programmes has been stressed as an important contribution to effective, durable resistance (Lamb *et al.*, 1992).

Although CPS1 is susceptible to *F. o. ciceri* race 2, the present study indicates that this cultivar has the potential to inhibit spore germination in the soil by virtue of the activity of components of its root exudate. This result is consistent with an earlier observation that CPS1 could withstand race 2 in a greenhouse environment for at least 3 weeks when other susceptible cultivars (e.g. JG62) were wilting (Stevenson *et al.*, 1994), although it is conceivable that after some time (more than 21 days) CPS1 produces the active components at an insufficient rate in the field and so eventually wilts. The reported differences in the field reactions of CPS1 and WR315 (Haware & Nene, 1982) to races 1 and 2 of the pathogen may be attributable either to qualitative and quantitative differences in active components in the exudates or to the greater sensitivity of race 1

to the active components. The former view is supported by the fact that the effect on spore germination of the concentrated root exudate of CPS1 was significantly reduced in the case of spores of race 2 compared to those of race 1, but that there was no difference in the effect of the exudate of WR315 on the two races. If the latter is the case, then the two races must be physiologically different and race 2 can withstand a greater concentration of the antifungal root components (more than that produced by CPS1), probably because of its greater ability to detoxify antifungal components from the root.

The root exudates from JG62 and H208 did not inhibit spore germination in either race 1 or race 2, but did inhibit their hyphal growth. This suggests either that the components of the root exudate which inhibit hyphal growth are different from those which inhibit germination, or that these two cultivars have the potential to inhibit germination (and thus express resistance to *F. o. ciceri*), but that the rates of production of the antifungal components are too low to be effective in the field. This highlights the importance of identifying the compounds involved in the resistance. If they can be identified and shown to inhibit both germination and hyphal growth, then it may be possible to enhance their production in JG62 through the selection of progeny from breeding programmes with higher levels of the active components. It is also conceivable that genetic manipulation by transformation to enhance the production of resistance components in JG62 (the twin-podded and agriculturally preferred cultivar) might be another way of taking the resistance to the field. These notions, amongst several other strategies to enhance crop resistance to microbial pathogens, were expounded upon in a recent review (Lamb *et al.*, 1992).

From its field reaction to race 1, H208 is described as a 'late wilting' cultivar (Haware & Nene, 1982), suggesting that it withstands infection for a long period and may even produce seed, but eventually succumbs to the disease. Since the root exudate of this cultivar was not active against the pathogen, it is likely that this mechanism is not associated with the disease tolerance of this cultivar.

The current race differentiation (Haware & Nene, 1982; Jiménez-Díaz *et al.*, 1989) of *F. o. ciceri* is valuable but inadequate, because it does not take into account the geographical variability of either the pathogen or the expression of resistance by the plant, both of which may be influenced by local biotic or abiotic factors. Some recent studies have investigated race differentiation in *F. o. ciceri* by comparing protein banding patterns (Suseelendra *et al.*, 1992) and amplified fragment length polymorphisms of genomic DNA (Kelly *et al.*, 1994) of pathotypes with different pathogenic characters. The root exudates exerted different effects on the two races studied in this paper, indicating, as suggested earlier, probable physiological variations under controlled environmental conditions. Knowledge of the compounds associated with the mechanisms of resistance may aid our understanding of why this happens, and could subsequently be useful in differentiating between the existing four races. In addition, such information would aid the evaluation of resistance in the field under different conditions and in different agro-ecological zones.

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