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BIOCHEMISTRY OF RESISTANCE IN CHICKPEA AGAINST WILT DISEASE CAUSED BY *FUSARIUM OXYSPORUM* F. SP. *CICERIS*

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Abstract

Chickpea lines Flip 90-131C, Flip 96-152C, Flip 96-153C, Flip 96-155C, Flip 96-158C and ICCV 95503 were found highly resistant (0% incidence) to wilt disease whereas Flip 85-29C, Flip 85-30C and Flip 96-154C (16-17% incidence) were resistant to wilt disease. The chickpea lines Flip 85-29C, Flip 89-14C, Flip 90-2C, Flip 92-148C and UC 27 were found resistant when screened against culture filtrate of the same isolate, while the lines Flip 90-74C, Flip 96-153, Flip 96-155C, Flip 96-157C, ICCV 95503 and UC 15 were tolerant. The two methods of screening did not show complete correlation.

Total phenols in the uninoculated roots of resistant/susceptible test lines did not show any correlation with the wilt resistance because the susceptible lines produced higher phenolic contents as compared to the resistant lines. The uninoculated roots of resistant chickpea lines produced antifungal compounds whereas the susceptible line did not produce any active compounds.

The inoculated roots of both resistant and susceptible lines produced higher antifungal activity as compared to uninoculated ones. The resistant chickpea lines produced an additional antifungal compound at Rf value 0.79 which was absent in susceptible lines, which might have a role in imparting resistance against wilt disease. The methanol extract of the stem produced one inhibitory zone at Rf value 0.11.

Introduction

Among the many fungal diseases, chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is the most devastating disease resulting 10-50% crop loss every year in Pakistan (Hanif *et al*., 1999). The fungus is seed borne as well as soil borne in nature; it is impracticable to control the disease by using fungicides and through crop rotation. Use of resistant varieties is the best way to combat the disease. For this purpose it is necessary to have a full knowledge about the fungus, disease and mode of resistance in host.

Disease resistance is a multicomponent phenomenon. It includes preformed barriers and antimicrobial compounds, often in external tissues and a response phase mechanism. Following infection by pathogens several higher plants rapidly synthesize antibiotic compounds termed as "phytoalexins" (Ingahm, 1982), which are believed to play a significant role in the defense of higher plants (Van Etten *et al*., 1982; Mansfield, 1982, Hahn *et al*., 1985). Chickpea produced a phytoalexin 'cicerin' when spore suspension of *Ascochyta rabiei* was incubated in the seed cavities of detached pods (Kunzuru & Sinha 1966). Koster *et al*., (1983) reported that isoflavones occur mainly as isoflavone 7-O, glucoside, 6-malonoate in chickpea and other legumes.

Phytochemical studies of wild species of *Cicer* have shown that both roots and foliage express a phytoalexin response dominated by the pterocarpan isoflavonoid maackiain. Medicarpin is also produced in low concentrations in some species. Maackiain and

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medicarpin were shown to exhibit potent anti-fungal activity towards *Fusarium* spores at natural concentrations by inhibiting their germination and the hyphal growth of those spores which did germinate. In addition high constitutive levels of maackiain in the roots and increased production in the presence of the pathogen were both strongly associated with resistance Furthermore, maackiain occurs at very high concentrations as the glucoside and malonylglucoside in the roots of some wild species of *Cicer* (Steveson *et al*., 1994) but these substituted derivatives are not antifungal. Fungal invasion of the roots of cultivated species *(Cicer arietinim* L.) elicits the production of maackiain. This is a three-step reduction process from the isoflavonoid formononetin (Herbert, 1989). Formononetin, the indirect source of maackiain in cultivated chickpeas, occurs at high concentrations as the aglycone, glucoside and malonyl glueoside. In wild species, however, the source of maackiain appears to be its glucoside and malonylglucoside. These derivatives are more readily available to the plant through a single glycosylation and the storage of maackiain glycosides may be a valuable character in the development of resistant varieties.

Accumulation of isoflavone glucosides has been reported in chickpea during infection with *Ascochyta rabiei* (Weigand *et al*., 1986). Accumulation of such antifungal compounds appears to be an important trait of a resistant plant, (Tani & mayama 1982, Kuc & Rush 1985). Although much work has been done to identify the antifungal compounds in the stem of chickpea against blight disease, but little information is available about the antifungal compounds produced in the roots of chickpea against wilt disease. The objectives of the present studies were to identify the antifungal compound(s) in root of chickpea and the involvement of these compound(s) in wilt resistance.

Materials and Methods

Chickpea material: A total of 40 advanced chickpea lines (Kabuli type) were obtained from ICARDA, Aleppo, Syria.

Fungus: A virulent strain of *Fusarium oxysporum* f. sp. *ciceris* 2012 isolated from the diseased chickpea samples from Rangpur, (Thal, Punjab, Pakistan) during a survey in 2000, was used in this study.

Screening of chickpea lines: Chickpea lines were tested for wilt resistance in pots by standard method of Nene *et al*., (1981) against FOC isolate 2012. Aug-424/ILC-1929 (susceptible) and CM 98 (resistant) were used as checks. Four seeds of the test lines and both checks were sown in each pot in three replications. The observations were made 15 to 30 days after germination. The resistance/ susceptibility of the test lines was determined by using the rating scale of Iqbal *et al*., (1993) as follows: 0-10% mortality = highly resistant, 11-20% = resistant, 21-30% = moderately resistant (tolerant), 31-50% = susceptible and 51-100% = highly susceptible.

The chickpea lines were also screened against spore free culture filtrates of the isolate 2012 by the method of Bajwa *et al*., (2000) and the rating scale was as follows: 0-0.5= highly resistant, 0.6-1.0= resistant, 1.1-1.9= tolerant and 1.6-3.0= susceptible, where 0= healthy, 1= burning or yellowing of leaves, 2= drooping and 3= wilting.

Estimation of total phenols: Two resistant test lines and four susceptible check lines were sown in small plastic pots (4"x4") containing autoclaved soil. After 10 days of germination total phenols in the roots of the check lines were estimated using Folin Ciocalteu reagent by the procedure given by Simson & Ross (1971).

Table 1. Reaction of chickpea genotypes to *Fusarium* wilt at pot experiment.

Disease reaction	Wilt incidence %	Genotypes
Highly resistant	0	Flip 90-131C, Flip 96-152C, Flip 96-153C, Flip 96-155C, Flip96-158C, ICCV 95503
Resistant	11-20	Flip 85-29C, Flip 85-30C, Flip 96-154C
Susceptible to highly susceptible	31-100	Flip 85-7C, Flip 88-1C, Flip 89-14C, Flip 89-3C,Flip 89-126C,Flip 90-2C, Flip 90-74C, Flip 90-144C, Flip 90-155C, Flip 90-181C, Flip 91-20C, Flip 91-217C, Flip 92-16C, Flip 92- 48C, Flip 92-49C, Flip 92-75C, Flip 92-104C, Flip 92-113C, Flip92-139C, Flip92-148C, Flip92-171C, Flip 93- 22C, Flip 93-23C, Flip93-28C, Flip93-50C, Flip93-52C, Flip 93-226C, Flip 96-157C, ICCV 95506, UC 15.

One test line UC 27 had poor germination.

Detection of antifungal compounds: Fresh roots/ leaves of chickpea (0.1 g) were removed from 12 days old chickpea lines (resistant and susceptible lines). The roots were grinded with pestle and mortar in 5.0 ml of 80% acidified methanol (0.1% HCl) and the material was filtered through buchner funnel. The solvent was evaporated at room temperature and finally dissolved in 0.5 ml of methanol. The methanol extract of each sample (50 µl) were spotted on thin layer chromatographic (TLC) plate (0.5 mm thick silica gel 60 GF_{254} plates). The plates were developed separately in solvent systems containing chloroform-methanol (97:3) and benzene: nitro methane: acetic acid (75:25:2). The developed TLC plates were bioautographed against the test fungus *Cladosporium cucumerinum* as described by Sibtain *et al*., (2002).

Results and Discussion

The susceptible checks (Aug-424/ILC-1929) completely wilted at 15 days of germination and the resistant check (CM 98) wilted at 25-27 days of germination. The lines Flip 90-131C, Flip 96-152C, Flip 96-153C, Flip 96-155C, Flip 96-158C and ICCV 95503 showed 0% wilt incidence up to 30 days of germination and were considered highly resistant (Table 1). The test lines Flip 85-29C, Flip 85-30C and Flip 96-154C showed 16-17% wilt incidence were considered resistant (Table 1). Rests of the lines were found susceptible to highly susceptible to wilt disease.

The chickpea lines Flip 85-29C, Flip 89-14C, Flip 90-2C, Flip 92-148C and UC 27 were found resistant when screened against culture filtrate of the same isolate, while the lines Flip 90-74C, Flip 96-153, Flip 96-155C, Flip 96-157C, ICCV 95503 and UC 15 were found tolerant. Rest of the lines were considered susceptible to highly susceptible (Table 2). The sources of resistance to *Fusarium* wilt are not uncommon and a number of workers have reported a high level of resistance against the disease (Bajwa *et al*., 2000; Yu & Su 1997; Sibtain *et al*., 2001). The resistant lines identified in the present studies can be used as a source for wilt resistance in the chickpea breeding program.

Table 2. Reaction of chickpea genotypes to culture filtrates of *Fusarium oxysporum* f. sp. *ciceris*

Disease reaction	Rating	Genotypes
Resistant	0-0.5	Flip 85-29C, Flip 89-14C, Flip 90-2C, Flip 92-148C, UC 27
Tolerant	1.1-1.9	Flip 90-74C, Flip 96-153, Flip 96-155C, Flip 96-157C, ICCV 95503 and UC 15
Susceptible	1.9-3.0	Flip 85-30C, Flip 85-7C, Flip 88-1C, Flip 89-14C, Flip 89-73C, Flip 89-126C, Flip 90131C, Flip 90-144C, Flip 90-155C, Flip 90-181C, Flip 91-20C, Flip 91-217C, Flip 92-16C, Flip 92- 48C, Flip 92-49C, Flip 92-75C, Flip 92-104C, Flip 92-113C, Flip 92-139C, Flip92-171C, Flip 93-22C, Flip 93-23C, Flip93-28C, Flip93-50C, Flip93-52C, Flip 93-226C, Flip 96-152C, Flip 96-153C, Flip 96-154C, Flip 96-155C, Flip 96-158C, ICCV 95506.

Table 3. Estimation of phenols in roots of resistant and susceptible chickpea lines.

S. No.	Test lines		Total phenols Mg/g fresh wt. of roots
1.	Flip 90-2C	(Susceptible)	0.675
2.	Flip 93-28C	(Susceptible)	0.774
3.	Flip 90-155C	(Susceptible)	0.715
4.	Flip 96-153C	(Resistant)	0.673
5.	Flip 96-155C	(Susceptible)	0.59
6.	ILC 1929	(Susceptible)	0.51

Most of the susceptible lines and resistant/ tolerant lines viz., Flip 89-29C, Flip 96-153C, Flip 96-155C and ICCV 95503 showed a correlation between the two methods of screening. Among these the resistant/ tolerant lines might have better wilt resistance mechanism operating in their roots. The lines Flip 85-30C, Flip 90-131C, Flip 96-152C, Flip 96-154C and Flip 96-158C which were considered resistant/ tolerant in pot method were found susceptible in culture filtrate screening method, did not show correlation between the two methods of screening. These lines might have a separate wilt resistance mechanisms operating in them. Many workers have reported a correlation between these two methods of screening (Bajwa *et al*., 2000; Sibtain *et al*., 2001) but our results did not show complete correlation indicating that resistance in chickpea might be controlled by various genes. The reports also revealed that various genes are involved in chickpea resistance and early or late wilting depends upon how many genes are present in the host cultivars (Upadhyaya *et al*., 1983). Our results suggested that screening of chickpea materials against culture filtrates of FOC is not enough to judge the resistance however this method could be used to investigate those lines in which phytotoxins degrading mechanism is being operated and is only useful in those plant-pathogen systems where phytotoxins are the only pathogenecity/ virulence factor. Artes Perez & Tena (1990) have reported that *Fusarium oxysporum* f. sp. *ciceris* produce multiple forms of pectic enzymes, and production of PL and PG activities were markedly different in race 0 and 5-Endo-PG enzymes were found relevant for pathogenesis in producing the yellowing syndrome (Artes Perez & Tena, 1990). So in the chickpea system the method of screening through culture filtrate of FOC is not useful but it can be rather misleading.

Total phenols in the healthy (uninoculated) roots of resistant/susceptible test lines did not show any correlation with the wilt resistance (Table 3). The susceptible lines Flip 90-2C,

Flip 93-28C and Flip 90-155C produced higher phenolic contents as compared to the resistant lines Flip 96-153C and Flip 96-155C. The results are in agreement with Sahi *et al*., (2000), who reported that total phenolic contents were higher in susceptible lentil lines. The difference in the production of certain highly active phenolic compound(s) prior to fungal invasion or phytoalexin production after invasion might be the factor, operating resistance in chickpea and not depends upon the total phenolic contents. The bioautography of the methanol extract of the inoculated/ uninoculated roots of chickpea lines on TLC revealed that uninoculated roots of resistant chickpea lines (Flip 96-155C and ICCV 95503) were producing inhibitory zones whereas no inhibitory zones were produced by the susceptible line Flip 93-28C (Fig. 1). The line Flip 96-155C produced one antifungal compound at Rf value 0.85, while the line ICCV 95503 produced one inhibitory zone at Rf value 0.79 in chloroform: methanol (97:3) solvent system. This confirmed that phenolic contents already present in chickpea tissues prior to pathogenic attack are not important for imparting resistance so could not be the criteria to evaluate resistance.

The inoculated roots of both resistant and susceptible lines produced higher antifungal activity as compared to uninoculated ones showing that antifungal activity increased after pathogen attack. There are reports which describe that phenolic contents /antifungal compounds increased in plant tissues after inoculation of pathogen (Sahi *et al*., 2000 and Jamil *et al*., 1996), which are termed as phytoalexins (Van Etten *et al*., 1982). The resistant chickpea lines (Flip 96-153C and Flip 96-155C) produced two antifungal compounds (Fig. 2) at Rf values 0.85 and 0.79, and the susceptible chickpea line Flip 93-52C produced three inhibitory spots at Rf values 0.85, 0.74 and 0.64 whereas the susceptible line Flip 93-28C was producing two inhibition zones at Rf values 0.74 and 0.64. The antifungal compound at Rf value 0.79 was additionally produced by the resistant chickpea lines and was absent in susceptible lines while the susceptible lines produced a different antifungal compound at Rf value 0.74 which was not produced by the resistant lines. The compound produced by the resistant lines at Rf value 0.79 might be the most potent antifungal compound as compared to the other compounds especially the one produced by the susceptible lines at Rf 0.74, so it must have a role in imparting resistance against wilt disease because only wilt resistant lines produced this compound.

The methanol extract of the stem of lines Flip 93-52C (susceptible) and Flip 96-153C, Flip 96-155C, ICCV 95503 (resistant) produced one inhibitory zone (Fig. 3) at Rf value 0.11 in solvent system benzene: nitro methane: acetic acid (75:25:2) and 0.43 in solvent system chloroform: methanol (97:3), while no antifungal zone was shown by the line Flip 93-128C (susceptible). A correlation was found between the antifungal activity produced in stem and wilt resistance in chickpea lines except the line Flip 93-52C (susceptible), this line might be resistant to pathogen attacking the aerial parts (leaves and stem) especially blight disease, which needs further confirmation.

The antifungal compounds produced blue color after spraying with Folin reagent, which confirmed that the compounds were phenols in nature. Further work is in progress to purify and identify the structure of these antifungal compounds.

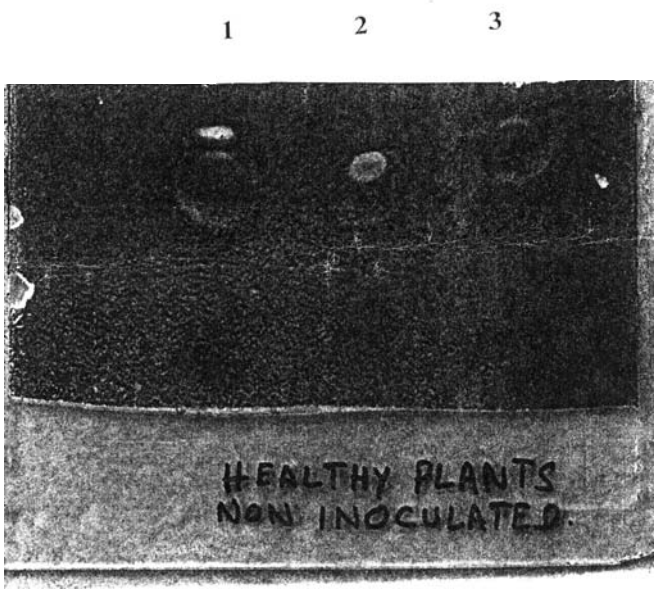

Fig. 1. Antifungal zones produced by methanol extract of uninoculated roots of chickpea
1) Flip 96-155C and 2) ICCV 95503 Resistant, 3) Flip 93 -28C Susceptible

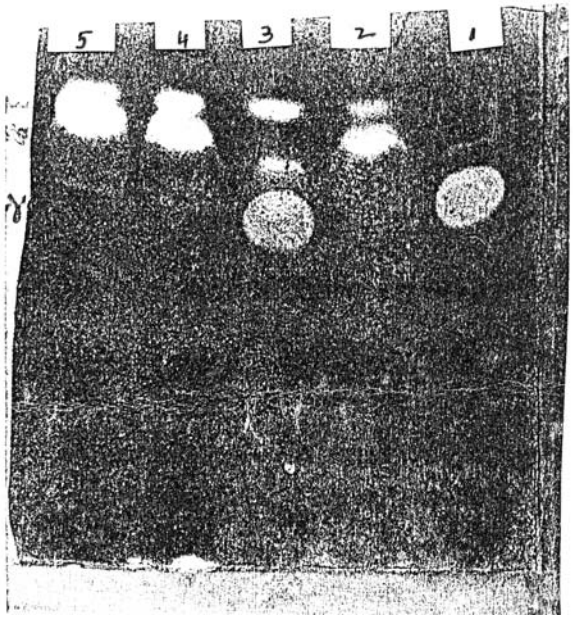

Fig. 2. Antifungal zones of methanol extract of inoculated roots of chickpea
2) Flip 96-155C, 4) ICCV 95503 and 5) Flip 96-153C Resistant, 1) Flip 93-28C and 3) Flip 93-52C Susceptible

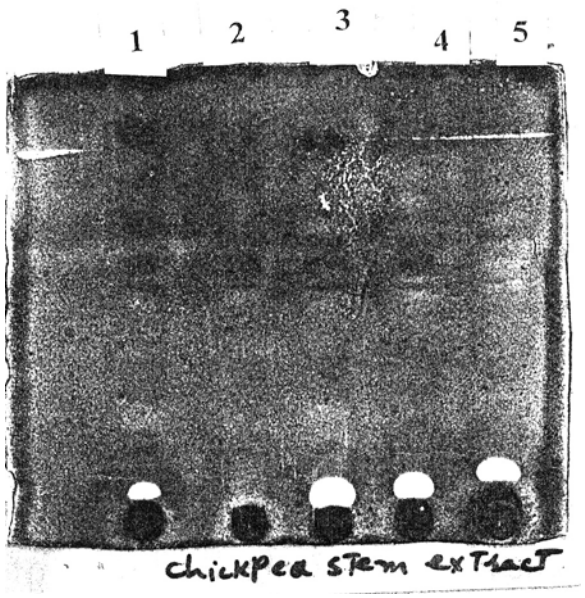

Fig. 3. Antifungal band of methanol extract of chickpea stem
1) Flip 96-155C, 3) ICCV 95503 and 5) Flip 96-153C Resistant, 2) Flip 93-28C and 3) Flip 93-52C Susceptible

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