Biochemical Analysis of Chickpea Protection Against Fusarium Wilt Afforded by Two Rhizobium Isolates

A. Arfaoui, B. Siifi, M. El Hassni, I. El Hadrami, A. Boudabbous and M. Cherif
1Laboratoire des Légumineuses à Graines, Institut National de la Recherche Agronomique de Tunisie
2Laboratoire de Microbiologie, Faculté des Sciences de Tunis
3Laboratoire de Physiologie végétale, Faculté des Sciences Semlalia, B. P. 2390, 40000, Marrakech, Maroc
4Laboratoire de Phytopathologie, Institut National Agronomique de Tunisie, Cité Mahragène 1002 Tunis, Tunisie

Abstract: Germinated seeds of two chickpea cultivars ILC482 and INRAT87/1, respectively susceptible and moderately resistant to Fusarium oxysporum f. sp. ciceris (Foc), were preincubated with a suspension of two Rhizobium isolates PchDMS and Pch43. Three days later, the seedlings were challenged by root dip with a conidial suspension of Foc race 0. The two Rhizobium isolates protected chickpea plants from F. oxysporum infection; the best protection has been obtained by PchDMS for the two cultivars. For the susceptible cultivar, mortality was 12.5 and 33.33% for treated plants, respectively with PchDMS and Pch43 as compared to the 79.16% in the inoculated control with Foc only. For the INRAT87/1 Cv. mortality was 8.33% and 12.5% for treated plants, respectively with PchDMS and Pch43 as compared to the 54.16% in the control inoculated treatment. The two Rhizobium isolates stimulated the peroxidases and polyphenoloxidases activities and induced the accumulation of phenolic compounds. The maximum of peroxidases activities in plant roots were reached 24 h after challenging. However, the higher activity of polyphenoloxidases and the higher level of the phenolic compounds were recorded 72 h after Foc inoculation. Comparing the two strains, PchDMS was more effective in inducing enzymes and phenolic compounds and highest levels were recorded in INRAT87/1 cultivar.

Key words: Chickpea, Fusarium oxysporum, biological control, peroxidases, polyphenoloxidases, phenolics

INTRODUCTION

Chickpea (Cicer arietinum L.) is one of the most important legumes grown worldwide, especially in dry areas of the Indian subcontinent[1]. Fusarium wilt, caused by Fusarium oxysporum f. sp. ciceris (Foc), is a major constraint to chickpea culture[2]. Annual chickpea yield losses implicating Fusarium wilt vary from 10 to 15%[3], but the disease can completely destroy the crop under unfavorable conditions[4,5].

The use of resistant cultivars[6] for the chickpea Fusarium wilt management may be enhanced by means of biological control using either bacterial and/or fungal antagonists.

Biological control by nonhost Fusarium oxysporum isolates and incompatible races of the same forma specialis is a promising strategy for management of Fusarium wilt diseases[7-9].

Hervas et al.[10] showed that prior inoculation of germinated chickpea seeds with either incompatible Foc races or non host F. oxysporum isolates can suppress Fusarium wilt caused by the highly virulent Foc race 5.

Currently Rhizobium spp. had received considerable attention in the biocontrol of many fungal diseases. Among the Rhizobium group, R. leguminosarum, R. meliloti, R. japonicum, were used successfully against different fungal pathogen belonging to the genera of Macrophomina, Rhizoctonia and Fusarium[11].

Different mechanisms may be involved in the biological control of fungal diseases by Rhizobium. These mechanisms including competition for nutrients or iron[12,13], production of antibiotics[14], promotion of the plant growth[15] and induced or enhanced resistance within the host[16].

Singh et al.[17] showed that two PGPR strains (Pseudomonas fluorescens strain Pf4 and P. aeruginosa strain Pag) protected chickpea plants from Sclerotinia rolfsii infection when applied singly or in combination as seed treatment, induced the synthesis of specific phenolic acids. The phenolic acids being the

Corresponding Author: M. Cherif, Laboratoire de Phytopathologie, Institut National Agronomique de Tunisie, Cité Mahragène 1002 Tunis, Tunisie

35
natural constituents of all plants, they have been implicated in plant defense mechanisms. Beimen et al. noted that infection-induced responses in leaves of tomato to Clavibacter spp. include an increase in soluble phenolics, such as chlorogenic acid, rutin and tomatine.

The objective of this research was to determine the effect of pretreatment of chickpea with two Rhizobium isolates PchDMS and Pch43 on the control of the wilt disease and on the accumulation of phenolic compounds and defense related-enzymes such as peroxidases and polyphenoloxidases at the early stage of infection.

**MATERIALS AND METHODS**

**Fungal isolate:** Fusarium oxysporum f. sp. ciceris (Foc) race 0 was originally isolated from roots of infected chickpea grown in a naturally infested field at Oued Beja in North of Tunisia. Monoconidial fungal cultures of the pathogen were stored in sterile sand tubes at 4°C. Active cultures were obtained from small aliquots of a sand culture plated on Potato Dextrose Agar (PDA). Fungal cultures were incubated at 25°C for 8 days with a 12 h photoperiod of fluorescent and near ultraviolet light.

**Bacterial isolates:** Rhizobium spp. was isolated from the nodules of chickpea as described previously, bacterial cells were stored in yeast extract mannitol agar at 4°C. The two Rhizobium isolates PchDMS and Pch43 were selected after a nodulation test.

**Effect of Rhizobium isolates in dual culture:** In vitro antagonism tests were performed on PDA in 9 cm petri plates by applying a dual culture technique. Rhizobium isolates were streaked across the center of the plate. Two discs of 5 mm in diameter cut from the edge of a 7 day-old culture of Foc were placed at each side of the antagonist. The distance between the two microorganisms was 2.5 cm. Plates were incubated at 25°C for one week. Percent growth inhibition of Foc was calculated by the formula of Whipp.[25] (R1-R2)/R1×100, where, R1 is the farthest radial distance (measured in mm) grown by Foc after 7 days of incubation in the direction of antagonist (control value) and R2 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist.

**Bacterization and inoculation of seedlings:** The study was conducted to determine the efficacy of two Rhizobium isolates Pch43 and PchDMS to reduce the incidence of Fusarium wilt on two chickpea cultivars IL-C4R2 and INRAT87/1, respectively susceptible and moderately resistant to Foc race 0.

Seeds were surface disinfected in 2% NaOCl for 3 min, washed three times in sterile distilled water and germinated on autoclaved layers of paper towels in moist chamber at 25°C for 3 days. Germinated seeds, selected for the uniformity (length of radicle) were placed in a Rhizobium suspension (CFU=108) or in sterile distilled water (control) and incubated at 25°C for 24 h. Preinoculated and control seeds were sown in sterile soil (soil, sand and peat (1/1/1)) and seedlings were incubated in growth room adjusted to 25°C, 60-90% relative humidity and a 14 h photoperiod for 3 days. Rhizobium-inoculated seedlings were removed from the tray, washed free of soil under tap water and inoculated with Foc race 0 isolate by root dipping in a conidial suspension in sterile water (105 conidia/mL) for 24 h. Non challenged control seedlings were dipped in sterile water in the same conditions. The following treatments were included: (i) water/water; (ii) water/Foc; (iii) PchDMS/water; (iv) Pch43/water; (v) PchDMS/Foc and (vi) Pch43/Foc.

After challenge inoculation, seedlings were transplanted and incubated in growth room to give the same condition as above for three other days.

**Disease assessment and data analysis:** Disease incidence was assessed at the end of experiment (50 days after inoculation with Foc) by counting the number of wilted plants. Wilt incidence (%) = number of wilted plants/total number of plants ×100 Data were analyzed by ANOVA followed by the Duncan’s Multiple Range Test Using Sigma Stat. Statistical Software (SPSS, version 10).

**Extraction and analysis of peroxidases (Pox) and polyphenoloxidases (PO) activities:** Extraction of peroxidases was conducted with Phosphate buffer (0.1 M, pH 6.6). Pox activity was assayed spectrometrically at 470 nm using guaiacol as substrate. Twenty microliters of enzyme extract (250 mg FW/2 mL) was added to 2 mL of reaction mixture consisting of a solution of 0.1 M phosphate buffer (pH 6.6) and 25 mM guaiacol. Reactions were initiated with 20 µL of H2O2 (10%) and stopped after 3 min.

Experiments were performed with a minimum of three replicates per treatment and per time point.

Extraction of polyphenoloxidase was conducted with phosphate buffer (0.1M, pH 6.6). The enzyme activity was assayed spectrometrically at 420 nm using catechol as substrate. Two hundreds microliters of enzyme extract was added to 2 mL of reaction mixture consisting of a solution of 0.1 M phosphate buffer (pH 6.6) and 400 µL cathelic (0.2 M). Reactions were stopped after 3 min. Experiments were performed with a minimum of three replicates per treatment and per time point.
Extraction and analysis of phenolic compounds from roots: Frozen roots from different treatments were extracted three times with 80% eq. MeOH at 4°C under continuous stirring. The homogenate was centrifuged at 7000 *g* for three min and the supernatants were stored at -20°C until use. To estimate the concentration of total phenolics (milligram equivalent of catechin per milligram of Fresh Weight (FW)), Folin Ciocalteu reagent was used and the optical density was determined at 760 nm. Experiments were performed with a minimum of three replicates per treatments and per time points.

RESULTS

Fungal inhibition assays: We were interested in determining whether the two *Rhizobium* isolates PchDMS and Pch43 were directly inhibiting the growth of *Foc* in *vitro*. The results of *in vitro* dual culture revealed that the two bacterial isolates reduced the mycelial growth of *Foc* by forming an inhibition zone (Fig. 1). The inhibition growth percentage was up to 40%.

Disease assessment: *Rhizobium* treatment enhanced the resistance of chickpea to *Foc*. Indeed, inoculated control plants showed progressing wilt (leaf yellowing and stunting). Fifty days after inoculation with *Foc* 79.16% of the plants of the susceptible cultivar ILC482 and 54.16% of the moderately resistant cultivar INRAT87/1 had completely wilted. However, preinoculation of pregerminated seeds of chickpea plants with *Rhizobium*, reduced significantly the percentage of wilted plants in both cultivars. This percentage was 12.5 and 33.33% of plants treated, respectively by PchDMS and Pch43 of ILC482 Cv. and was 8.33 and 12.5% for INRAT87/1 Cv. (Table 1).

Non inoculated or inoculated seedlings only with *Rhizobium* showed no susceptible reaction and remained green for more than 50 days.

Peroxidases and polyphenoloxidases activities: The levels of peroxidases and polyphenoloxidases activities were analyzed in root tissues at different times after the challenge inoculation.

Peroxidases activities attained their maximal levels 24 h after the *Foc* inoculation and decreased thereafter (Fig. 2). The levels of the enzyme activity is in relation with the nature of inducer (PchDMS or Pch43) and the cultivar (ILC482 or INRAT87/1).

In susceptible ILC482 cultivar, a high level of peroxidases activity was observed in plants exposed to PchDMS/Foc compared to Pch43/Foc treatment. However, this level was much lower than those recorded in moderately resistant cultivar.

Polyphenoloxidase activities attained their maximal levels 72 h after the *Foc* inoculation. The level of the enzyme was much lower than those occurred in peroxidase activity (Fig. 3).

The level of Polyphenoloxidase obtained in resistant cultivar was a little higher than in susceptible cultivar and the plants treated by PchDMS/Foc possessed the high levels of Polyphenoloxidases.

Phenolic compounds: Higher amounts of soluble phenolics were recorded 72 h after the challenge with *Foc* in both ILC482 and INRAT87/1 cultivars (Fig 4).

The level of soluble phenolics in roots was much higher in *Rhizobium/Foc* treated plants than in inoculated control plants.

For the susceptible ILC482 cultivar, the content of root soluble phenolics, 72 h after challenging was 2.78 mg g\(^{-1}\) FW for PchDMS/Foc and 2.44 mg g\(^{-1}\) FW for Pch43/Foc treated plants. The same result was obtained in moderately resistant cultivar INRAT87/1. Indeed, the maximum level of soluble phenolics was attained in plants preinoculated by *Rhizobium* 72 h after challenging with *Foc*. The content was 3.23 mg g\(^{-1}\) FW for PchDMS/Foc and 2.96 mg g\(^{-1}\) FW for Pch43/Foc treated plants.

It seems that the inoculation of *Foc* to plants that were preinoculated with *Rhizobium* induced the increase of phenolic compounds to their highest level within 3 days after inoculation in both cultivars.

---

**Table 1:** Effect of *Rhizobium* isolate on wilt incidence (%) in both chickpea cultivars

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ILC482 Cv. (%)</th>
<th>INRAT87/1 Cv. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water/Water</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>PchDMS/Water</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>Pch43/Water</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>Water/Foc</td>
<td>76.2</td>
<td>54.2</td>
</tr>
<tr>
<td>PchDMS/Suc</td>
<td>12.5*</td>
<td>6.3*</td>
</tr>
<tr>
<td>Pch43/Suc</td>
<td>33.3*</td>
<td>12.5*</td>
</tr>
</tbody>
</table>

*The assays were repeated three times and each treatment was conducted with 20 plants. Mean values followed by "*" are significant (p<0.05) by Duncan's Multiple Range Test as compared to the inoculated control with *Foc***
DISCUSSION

The present study focused mainly on the ability of two *Rhizobium* isolates PchDMS and Pch43, to protect chickpea seedlings against *F. oxysporum* f. sp. *ciceris*. This protection seems to be the result of an antagonism between *Rhizobium* and *Foc* and through induction of host plant defense reactions.

The induction of host plant defense was demonstrated by the stimulating effects in term of peroxidases and polyphenoloxidases activities as well as in term of accumulation of phenolic compounds in challenged tissues. These enzymes and organic compounds might have a crucial role in the reduction of *Fusarium* wilt in chickpea as it was in other pathosystems\(^{(25-29)}\). In deed, the result showed a close correlation between accumulation of phenolics and enzymes owing to activities of PchDMS and Pch43 and protection of chickpea plants against *F. oxysporum* infection.

The reduction of fungal growth observed *in vitro*, by the two selected *Rhizobium* and formation of inhibition zone, were presumably due to the metabolites being released from bacteria into the culture medium. This metabolites could including antibiotics or cell-wall degrading enzymes. However there’s many investigators, that reported the implication of the antifungal secondary metabolites produced by *Rhizobium*, in the biocontrol of plant disease\(^{(22,24)}\).

In deed, some works explain the antagonistic properties of *Rhizobium leguminosarum* against *Fusarium oxysporum* f.sp. *lentis* to the excretion of antibiotics substances and the antimicrobial activity of *R. leguminosarum* is of protein nature and has fungicidal action on conidia of *F. oxysporum*\(^{(10)}\).

The analysis of peroxidases and polyphenoloxidases activities, in plant roots from both moderately resistant and susceptible cultivar, showed that *Rhizobium* were able to induce physiological changes in chickpea seedlings. In deed, the plants preinoculated with *Rhizobium* and exposed to Foc, displayed an elevated level of peroxidases and polyphenoloxidases activities compared to the inoculated control. Induced enzymatic levels were in general, higher for the most efficient
biocontrol (PchDMS) and for the moderately resistant cultivar (INRAT87/1).

This result corroborates findings from earlier studies conducted by Cushman et al.\textsuperscript{[59]} who reported the increase of peroxidase activity in chickpea roots preinoculated with non-host isolate of \textit{F. oxysporum} and challenged by a high virulent Foc.

Enzymes such as peroxidases and polyphenoloxidases are known for the oxidation of several compounds particularly phenolics increasing their toxicity\textsuperscript{[30-33]}. They are also known to be important in symptom expression, a close relationship between increased activity of peroxidases and polyphenoloxidases and appearance of symptoms in infected tissues was reported by several works\textsuperscript{[34-37]}

Peroxidase are usually associated with induced resistance response\textsuperscript{[36,39]} and they are also implicated in several plant defense mechanisms such as lignin synthesis, oxidative cross linking of different plant cell wall components or generation of oxygen reactive species\textsuperscript{[40].

Increase in total phenolics compounds, was observed in plant roots infected by Foc and preinoculated by \textit{Rhizobium}. However there's no great difference in levels recorded in both susceptible and moderately resistant cultivar. This result could be explained by the short period of analysis (4 days). In general, infection augmented the levels of phenolics and rapid increases occurred as lesions appeared\textsuperscript{[17]}. Many investigators reported the implication of phenolics in plant defense mechanisms\textsuperscript{[41-43]}. Earlier Mata\textsuperscript{[44]} had reported that inoculation of tomato plants with non pathogenic forms of \textit{Fusarium oxysporum} results in a sudden rise in phenolics concentration during the first 2-4 days, compared with inoculation with virulent race of \textit{F. oxysporum} f. sp. radicis-lycopersici.

In conclusion, this study shows the possible protection of chickpea culture by two \textit{Rhizobium} isolates. This protection is being in relation with the stimulating effects of plant defense implicating the phenolics metabolism.
Fig. 4. Time course of soluble phenolic compounds in chickpea roots of susceptible ILC482 (a and b) and moderately resistant INRAT87/1 (c and d) cultivars exposed to the different treatments [-O- water/water; -D- PchDMS/water; -∆- Pch43/water; -◆- water/Foc; - ■- PchDMS/Foc and - ▲- Pch43/Foc]

ACKNOWLEDGMENTS

Financial support for this study was provided by the Institut National De La recherche Agronomique De Tunisie (Laboratoire des Légumineuses à Graines) and by the action intégrée Tunisia-Maroc MT/24-02.

REFERENCES


