Research Article

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Role of salicylic acid in induction of plant defense system in chickpea *Fusarium* wilt caused by *Fusarium oxysporum* f.sp *ciceris*

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The fungus *Fusariumoxysporum* f.sp. *ciceris* (FOC), the chickpea wilt pathogen, causes appreciable yield losses under favorable environmental conditions in Algeria. To evaluate the role of salicylic acid (SA) in induction of plant defense system in chickpea *Fusarium* wilt, chickpea seedlings were sprayed with a range of SA concentrations (0 (control), 0.05 and 0.5 mM). Obtained results revealed that the percentage of seedling plants was reduced at 23% in untreated seeds. By contrast, at the application of the different treatments, a maximum was about 50% for seedling and the ability to delay the apparition of necrosis lesions was observed. For the individual polyphenol separation assay we used high performance liquid chromatography (HPLC). Various peaks level in polyphenol for control and treated plants were noted. Application of SA on chickpea seedling resulted in increase of the growth percent and decrease of polyphenol, as well as an increase of flavonoid. As for their beneficial effects on disease control, the results revealed that SA may improve plant growth and disease control by maintaining the rate of polyphenols at a level without altering the growth of plants, and inhibit the effect of fungi invasion by increasing production of flavonoids implicated in resistance to antifungal compounds.

Key words: chickpea, seedling, polyphenol, flavonoid, HPLC assay.

Chickpea (*Cicerarietinum*L.) is the world's fourth most important legume crop after Soybean, common bean, and peas. In developing countries, chickpea is a rich complement to the cereal diet since it has a high nutritive value. Mainly grown for its edible seeds rich in proteins, this crop can be used for both seed and forage production (Yadav *et al.* 2011). In several countries (Algeria, Morocco, Syria, Turkey, Pakistan, India, etc.), production of chickpea did not increase because of low productivity and unstable output. Several causes (agronomic, abiotic and biotic) could be involved to explain this fact (Labdi 1995). In Algeria, a promising market Kabuli type chickpea, low yields and competition with other crops on limited land areas lead to a slow production's development (Pluvinage 1990). Chickpea *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *ciceris* (Padwick) Matuo& K. Sato (FOC) is a destructive disease agent, and an important pathogen in Algeria (Labdi 1990; Bouznad et al. 1996). Infected seeds, one of the main sources of inoculums, are responsible of introduction of these pathogens into disease-free areas. As a result, the pathogen has resulted in an important decrease of productivity and quality of chickpea productions **(**Ahmad et al. 2010; Ali et al. 2014). The sensing of biotic stress induces signaling cascades; these signals ultimately induce expression of specific sub-sets of defense genes that led to the

assembly of the overall defense reaction (Waseem et al. 2014). Salicylic acid has been shown to be a signaling molecule involved in diverse physiological processes, such as the control of flowering, seed germination, stomata functioning, gravity sensing and the activation of plant defense responses to fungal, bacterial and viral attacks (Raskin, 1992; Hayat and Ahmad, 2007). Impact on plant growth and development is also documented (Raskin, 1992, Idrees et al. 2011). Polyphenolic compounds are biosynthesized in plant as secondary metabolites, having an important role in sensorial and nutritional quality of fruits, vegetables and plants. Different studies showed that induced resistance, through accumulation of various phenolic compounds and phytoalexins may play a crucial role in control and resistance of chickpea to pathogenic attacks (Arfaoui et al. 2006; Cherif et al. 2007). The flavonoids are among the most efficient antioxidant molecules (Williams and Harbone, 1989; Chérif et al. 2007). Phenolic compounds seem to inhibit disease development through different mechanisms involving inhibition of extracellular fungal enzymes (cellulases, pectinases, laccase, xylanase) inhibition of fungal oxidative phosphorylation, nutrient deprivation (metal complexation, protein insolubilization), and antioxidant activity in plant tissues (Scarbel,1991).

In the context of search of novel alternative strategies to chemical control, the present study has been carried out to investigate the effect of low levels of exogenous salicylic acid on polyphenols and growth of chickpea under biotic stress.

MATERIALS AND METHODS

Salicylic acid treatments:

Salicylic acid (SA; 2-hydroxybenzoic acid) was initially dissolved in 1ml ethanol and concentrations of 0.05 and 0.50 mM (pH 6.0-6.5) were made up with distilled water (Khan et al. 2003). Chickpea seeds (cv ILC 3279) were imbibed for 8 hours with distilled water (control), or with SA solution.

Treated seeds were raised in earthen pots. At vegetative stage, the seedlings were transplanted after 30 days into clay pots. All plants were inoculated with the FOC and were sprayed with nutritive solution combined to salicylic acid doses (0- 0.05 and 0.5mM SA). Three replicates (05 pots, 02 plants/pot) were used for each observation and treatment.

Determination of total phenols and total flavonoids content in seedlings plants

Five g of fresh plant material were homogenized with 10 ml water/methanol (1:1) and the resulting pellet was extracted twice. The extracts were sonicated for 15 min on an ultrasonic bath (Branson model 3510, USA), centrifuged for 15 min at 4°C, 4000 xg, and the supernatants were concentrated in rotary evaporator at 37°C (Kim et al. 2006). The extract was diluted and used for the following analyses.

Total phenol content (TPC) was determined calorimetrically using the Folin-Ciocalteu method (Makkar, 1993) and *total flavonoid content* (TFC) was measured according to a colorimetric assay (Kim et al. 2006).

HPLC separation

Separation of phenolic compounds by HPLC was performed according to (Kajdžanoska et al. 2010). Chickpea extract (aqueous residue) was made up to 5 ml with 50% acetonotril in water, and it was filtered through 0.45 µm pore-size polyether-sulfone filter (Econofilter, 25/0.45 µm NL, Agilent 1100, series (UWD), Germany) before analysis. Separation of polyphenols from extracts was performed using narrow bore C18 Luna column (Phenomenex, 2 × 150 mm, 3µm) and monitored at 230 nm. The elution solution consisted of two solvents: formic acid (1%, *V*/*V*) in water à (45%) (A) and acetonitril (55%) (B). A linear gradient starting with 5% B, 5% B at 5 min, was installed to reach 80% B at 45 min, 100% B at 50 to 60 min. Elution was realized under following conditions: flow rate: 0.4 ml min^{-1}, injection volume: 20µl, column temperature: 40ºC. Spectral data from all peaks were accumulated in the range 230 nm.

Statistical analysis: Results are presented as mean ± Standard Error. Differences were compared using Student's test. Significant difference was statistically considered at the level of $p < 0.05$.

RESULTS AND DISCUSSION

Effect of salicylic acid on seedling percentage

Obtained results showed that the treatment of

seeds by different doses of the SA, has shown a significant effect on parameters of growth of the chickpea, infected with FOC, according to (Table 1), by Student's test ($p<0.05$). The percentage of the seedling plants was estimated in relation to the control. The number of plants is significantly higher at following the application of the SA doses (0.05 mM and 0.5 mM), respectively of (55%-44 %) in comparison with the control that do not exceed (25%). We have noticed a reduced rate of the necrosis plants, with the two doses of the SA (28.2% - 24.7%) compared with the wilted plants (43.1%). PLT 0 (Control) - PLT 0.05 SA (Plants treated by 0.05 mM SA) – PLT 0.5 SA (Plants treated by 0.5 mM SA).

The measurements made on the stems and the number of leaves had shown a different significant effect of the treatment depending on the doses of SA, among the two treatments, by Student's test ($p < 0.05$). The 0.05 mM SA recorded higher performance for Stalk length (26 cm) and leaves number (13.7). Weightings of the seedlings vegetation of each treatment were carried out at the harvesting; the maximum fresh weights of the vegetable parts were indicated for the plants watered with 0.05 mM SA (1.604), in comparison with 0.5mM the weights were less (1.03 g) (0.853 g). While in control it's did not exceed (0.853g) (Table 1). The 0.05 Mm SA showed higher vigilance and disease resistance in chickpea than 0.5mM SA ($P < 0.05$). PLT 0 (Control) - PLT 0.05 SA (Plants treated by 0.05 mM SA) – PLT 0.5 SA (Plants treated by 0.5 mM SA)

Our results showed the role of SA in the induction of the systemic resistance in plants following the attack by the phytopathogenic agents. Plants can be affected by *FOC* at any stage, there is no commercially viable way to eradicate this disease from the soil, and it can spread across fields, farms and regions. The infection of the plants with the *FOC* had created losses on the number of untreated plants in comparison with treated plants (Table 1). The FOC causes impair of growth and leaves of un-treated plants had developed chlorosis as demonstrated (Kumar et al. 2004; Muhammad et al. 2010). Kurmar et al. (2005) reported that chickpea seed yield under moisture stress significantly lowered biomass production, number of branches/m^2 and pods/m^2. The impact of SA on treated plants has been widely studied (Enyedi et al. 1992; Gharib and Hegazi, 2010). Susceptibility of the SA to limited moisture condition was shown through relatively germination rate and seedling parameters as compared to the untreated seeds.

Our results revealed that among different used concentrations, 0.05mM was more effective than 0.5 mM SA in chickpea, either on the growth parameters (lengths of stems, number of leaves) or on the weight of fresh matter (Table 1). Our results are in consistence with those previously published (Bezrukova et al. 2001**;** Fariduddin et al. 2003). Bezrukova *et al.(*2001**)** showed that concentration of SA as low as 0.05 mM increased levels of cell division within the apical meristem of seedling roots which caused an increase in plant growth. Fariduddin et al. (2003) reported that treatment of *Brassica juncea*'s leaves with 10^{-5} M SA resulted in increased biomass accumulation, but concentrations above that had inhibitory effect.

Effect of salicylic acid on total polyphenol content (TPC)

In the present work we tried to characterize the effects of SA on nutritional quality and to identify the role of some polyphenols interfering within the activation of plant's resistance against the *FOC*. Our results showed that higher induction of TPC was observed in un-treated than treated plants (Figure 1). Our findings are in line with those previously reported (Baker et al. 1997**;** Benhamou et al. 2000; Suprakash et al. 2012). Phenolic compounds exhibit promising effects regarding health and nutrition. Furthermore, they are involved in plant's defense against a large number of biotic and abiotic stresses (War et al. 2011; Usha and Jyothsna, 2010; Masood et al. 2015). Their accumulation was proved in infected sites of plants (Benhamou et al. 2000**;** Suprakash et al. 2012). Baker et al. *(*1997*)* have recently shown that secondary metabolites function in local acquired resistance and in the hypersensitive response. Different studies showed that induced resistance, through the accumulation of various phenolic compounds and phytoalexins may play a crucial role in the resistance of chickpea to pathogenic attacks, as *Fusarium*wilt (Cachinero et al. 2002; Arfaoui et al. 2006; Cherif et al. 2007). On the other hand we give evidence that SA application had diminished the polyphenol rate in plants.

Table 1: Effect of salicylic acid on seedling percentage, necrosis plants and growth parameters of Chickpea (cv ILC 3279), inoculated with *Fusarium oxysporum* f.sp. *ciceris* (1.6×10 ^{4} conidia/ml) in treated and untreated plants.

	Control	0.05mM SA	0.5mM SA
Seedling plants (%)	25	55	44
Necrosis plants / Seedling plants (%)	43.1	28.2	24.7
Leaves number	8.2 ± 2.6	13.7 ± 3.6	10.9 ± 3.9
Shoots length (cm)	18,7 ± 5.3	26 ± 5.7	24.2 ± 5.4
Fresh weight (g)	0.853 ± 0.1	1.604 ± 0.2	1.03 ± 0.11

We can suggest that SA treatment had a protective role in chickpea growth against FOC by maintaining the rate of polyphenols at a level without altering the growth levels and parameters. Also, a large accumulation of TPC content was induced in chickpea plants treated with 0.5 mM SA accompanied with low growth. On the other hand, 0.05mM SA gave the low TPC level and the high growth percent (Fig 1). Our findings are consistent with those demonstrated (War et al. 2011).

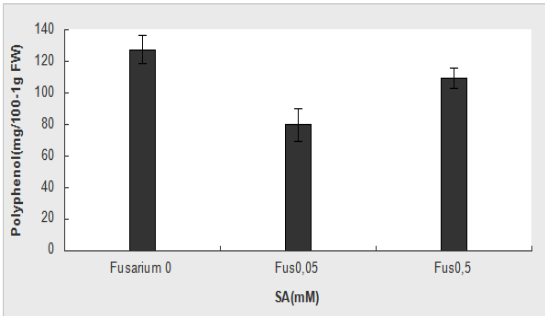

Figure 1: Effect of salicylic acid on total polyphenol content (TPC) of Chickpea (cvILC 3279) inoculated with *Fusarium oxysporium f.sp. ciceris* during seedling in treated and un-treated plants.

The TPC concentrations are significantly high in the plants untreated with SA. The TPC rate is much reduced with 0.05mM of SA, it reaches respectively (1.78–79.8 mg). While, these rates reach (1.7–109.4 mg) with 0.5mM as compared with the control (3.56 – 127.6 mg).

Effect of salicylic acid on total flavonoids content (TFC)

In contrast, an increase of flavonoids level was observed in treated plants when compared with the control (Fig. 2), which is in line with previous studies (Marfek, 2003; Amalesh et al. 2011; Javed et al. 2014). It's indicated that some flavonoids play the role of phytoalexin, which are very much synthesized by the leaves further to infection.

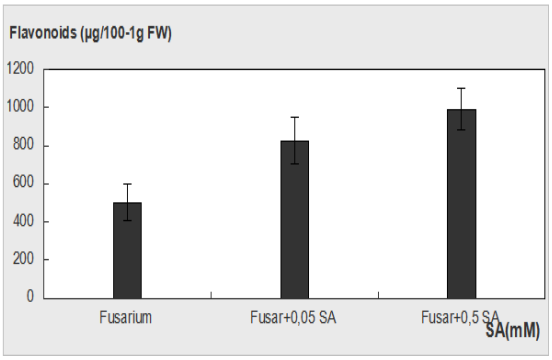

Figure 2: Effect of salicylic acid on total flavonoids content (TFC) of Chickpea (cvILC 3279) inoculated with *Fusarium oxysporum f.sp. ciceris*, during seedling, in treated and untreated plants.

A remarkable increase of (TFC) contents was noticed in chickpea seedling treated with SA. Moreover, in the plants, the (TFC) content reaches its maximum with 0.5mM (989.1 µg) than 0.05mM (824 µg) in comparison with the control (502.5 µg).

Their role in boosting plant resistance is well established for the leguminous family (Dixon et al. 1983; Dakora and Philips, 1996). Stevenson *et al*. (1997); Usha and Jyothsna. (2010) reported that higher concentrations of phytoalexin were detected in rice following SA application. Isoflavonoids are synthesized as part of the phenylpropanoid pathway (Dixon et al. 2002). Arfaoui *et al. (*2006) and Cherif (2007) demonstrated a significant effect of isoflavonoids on the decrease of pathogen's growth through inhibiting germination and

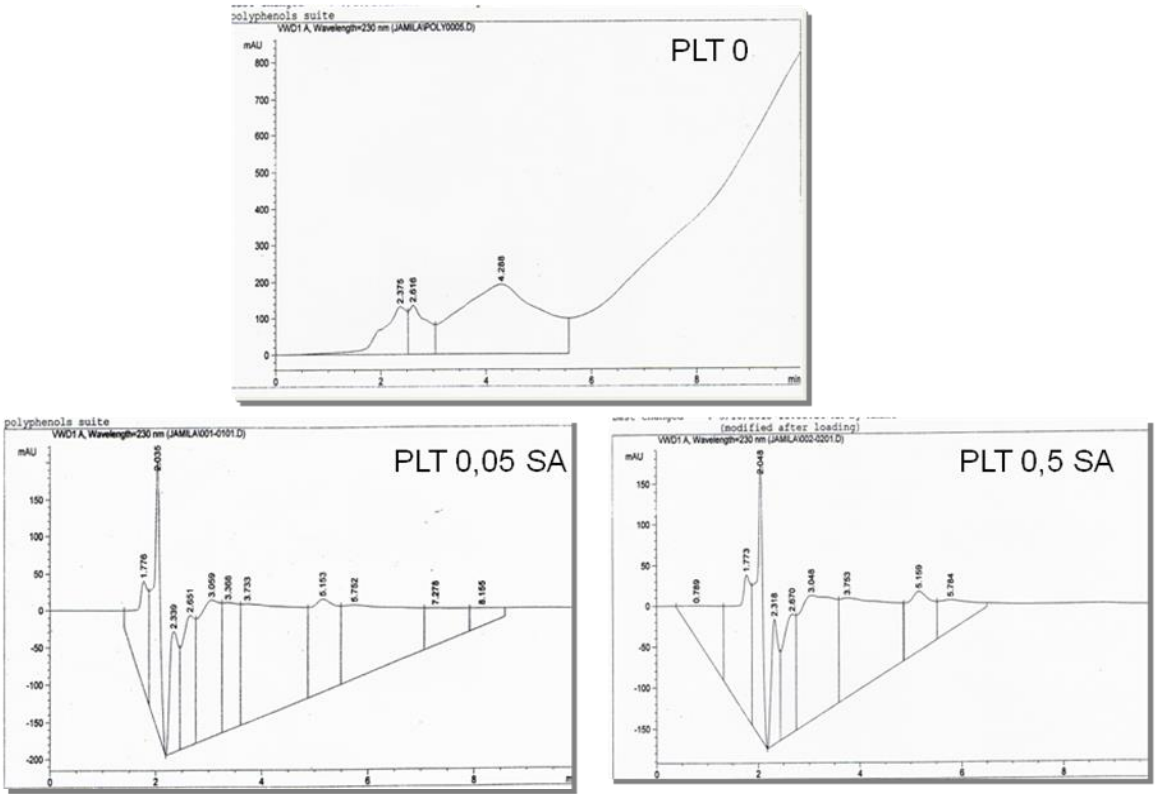

Figure 3: Phenolic substances by HPLC analysis at 280 nm obtained with methanol extracts of Chickpea plants (PLT)(cv ILC 3279) inoculated with *Fusarium oxysporium f.sp. ciceris*, during seedling, in un-treated and treated plants with salicylic acid.

hyphal growth and by causing changes in mycelium and disorganization of cells.

Effect of salicylic acid on phenolic substances by HPLC analysis

HPLC analysis of phenolic compounds in chickpea extracts revealed twelve peaks in plants treated with 0.05mM, nine with 0.5mM SA and only three peaks in controls (Fig 3). The separation of phenolic substances showed that many peaks values are observed in presence of SA, twelve with 0.05mM (PLT 0.05 SA) and nine with 0.5mM SA (PLT 0.5 SA), only three peaks in the control (PLT 0).These data suggest that exogenous SA treatment could be responsible of chickpea *Fusarium* wilt's protection and enhanced growth in chickpea. SA could play a biochemical role by maintaining rates of polyphenols at a level without altering the growth of plants. Moreover, it could inhibit the effect of fungi invasion by increasing production of flavonoids involved in resistance to antifungal compounds.

CONCLUSIONS

These data suggest that exogenous SA treatment could be responsible of chickpea *Fusarium* wilt's protection and enhanced growth in chickpea. SA could play a biochemical role by maintaining rates of polyphenols at a level without altering the growth of plants. Moreover, it could inhibit the effect of fungi invasion by increasing production of flavonoids involved in resistance to antifungal compounds.

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