Evaluation of Salicylic Acid effect on antioxidant activity in *Cicer arietinum L.* inoculated with *Fusarium redolens*.

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Abstract:

Effect of pathogenesis stress on plants depends on different developmental stage and it significantly affects yield and other physiological traits. So it is possible to use different agro-techniques to increase total yield and maintain the components value of the crops. In this frame, seeds of chickpea cultivars (***Cicer arietinum L*** "ILC 3279") were treated with a range of SA concentrations (0,05 and 0,5 mM) and subjects to *Fusarium redolens*. Also, Seedlings plants were inoculated by this pathogen and sprayed with SA concentration for eight weeks. Obtained results showed that chickpea seed priming with SA resulted in high germination percentage comparing to no treated sample, with an increase of plant growth number against a decrease in necrosis plants rates. As well as an increase in antioxidant capacity, according to the studied stage (analyses seeds and plants) were exhibited by treatment. SA at 0,05 mM reached higher antioxidant activity and proteins accumulation, although, higher induction of CAT and SOD activities besides a decrease in APX activity were observed. In conclusion, we can suggest that the application of salicylic acid at 0,05mM may be safe to this plant and could be utilized for the induction of plant defense, and also could urge on antioxidant capacity of chickpea in the course of germination and growth.

Keywords: Salicylic acid; *Cicer aretinium*; *Fusarium redolens*; Antioxidant activity; catalase enzyme; peroxidase activity; superoxide dismutases.

Introduction:

Plant defense against environnemental stresses is mediated through various signaling pathways that lead to the production of many defensive proteins and non-protein compounds (Ghasemzadeh and *al*, 2013). Plant phytohormones such as abscisic acid, jasmonic acid, ethylene and salicylic acid (SA) are important components of different signaling pathways involved in plant defense (Shraiy and *al*, 2009, Ghasemzadeh and *al*, 2013). However, SA and its derivatives are present in various soils types, they are absorbed by plant roots and affect the uptake of other compounds (Singh et al., 2004). Moreover, endogenous SA is a key signal, involved in the activation of plant defense responses to fungal, bacterial and viral attacks (Krasavina, 2007).

Its involvement in hypersensitive response (HR) and systemic acquired resistance (SAR), the main components of SA signaling pathway are revealed, and relation of this pathway to generate reactive oxygen species (ROS) has been studied (Alvares, 2000; Singh et al., 2010). Recently, SA pretreatment was shown to directly or indirectly activate cellular antioxidant enzymes during stress (Horvath et al. 2002; Kang et al. 2003). Salicylic Acid stimulates expression of the production genes of PR proteins (Ward and al,1991, Metraux,2002), it is a transducer and a messenger. It also adjusts cell death combined with hypersensitive response by tactivation of the peroxydation of lipids and generation of free stems, as well as activation or inhibition of the antioxidant enzymes, for several plants (Conrath ,1995, Yousuf,2008, Peleg and Blumwald, 2011).

Chickpea (*Cicer arietinum* L.) is an important legume crop in semi-arid tropics of the world; they represent in poor countries an important food source because of the great wealth of seeds and leaves in vegetal proteins (Harborne, 1999, Cavaillès, 2009). However, it is under heavy stresses due to various biotic and abiotic factors that results in yield losses, more than 120,000 tons are imported each year to Algeria (Pluvinage, 1990). As a result of this progressive deterioration and extent and severity of the disease per hectare production of chickpea grains has decreased and his quality has significantly diminished (Muhammad et *al*, 2010).

In order to attract the attention towards early impact of exogenous application of SA on chickpea inoculated with pathogen on germination, seedling and to examine its effect on antioxidant activity among total protein and antioxidant enzymes (CAT, APX, SOD) activities in plants under normal (control) and pathogens conditions of growing.

Materials and methods:

Seeds SA treatments:

Salicylic acid (SA; 2-hydroxybenzoic acid) was initially dissolved in 1 ml 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). Seeds were treated for 8 hours with distilled water (control), or with the solutions of SA (0,05 and 0,5 mM).

Seed germination:

Control and treated seeds were inoculated with *Fusarium. redolens.sp* and distributed to germinate in Petri dishes on two filter-paper discs, each dish contains 50 seeds and sprayed with 20 ml of sterilized water, and incubated at 25 °C. Germination is spotted by the output of radical from the integuments as described by (Gilles,2009, Jaouadi,2010). Germinated grains are counted, every 24 hours. The test ends when after two successive counts no germination is registered. After 96 h, germinated grain of each dish is taken for biochemical analyses.

Seedling growth:

The second lot of control and treated seeds was raised in earthen pots. At vegetative state, seedlings were transplanted into clay pots (35 cm diameter) containing sterilized sandy loamy soil (1:2 w/w). All plants were inoculated with the *Fusarium redolens* and were also irrigated with nutritive solution combined to salicylic acid doses (control, 0,05 and 0,5mM SA).Three replicates (5 pots and each pot contained 2 plants) were used for each observation under each treatment. In the end of the experiment the percentage of healthy and wilted plants in each row was counted. Vegetative parts were weighted and used for biochemical analyses. *Fusarium redolens.* was procured from the laboratory of agronomy (phytopathology).

Biochemical analysis

1-Preparation of the methanol extracts

For the extraction, about 5.0 g of fresh plant material were homogenized with 10 ml water/methanol (1:1) under magnetic stirring at 4°C for 20 min. After centrifugation of the mixture (15 min at 4°C, 4000 xg), the resulting pellet was extracted twice following the same protocol. The supernatants were collected, pooled and centrifuged. The obtained extract was concentrated by rotary evaporation at 30°C (Kim *et a*l., 2006). The residue was dissolved in deionized water and used for antioxidant activity analyse.

Antioxidant activity was measured by DPPH determination (Awika et *al*. 2003). To 2,9 ml de DPPH à 0,004% (P/V) in methanol- H_2O (8 : 2), added to 100 µl of the plant methanolic extract was added. The mixture was shaken and allowed to stand at 20 °C in dark for 30 minutes. After the decrease in absorbance, the resulting solution was monitored at 517 nm.

The DPPH radical scavenging activity of phenolic compounds was expressed as mg /100 g of dry matter and as mg /100 ml of VCEAC in 30 minutes. The control solution was consisted by 100 µl of methanol and 2.90 ml of DPPH solution. The radical solution was prepared daily. The percentage inhibition of the DPPH radical (IP50) by the samples was calculated using the formula: IP50 = $[(A_c - A_x)/A_c] \times 100\%$. A lower absorbance indicates a higher free radical scavenging activity

2-Preparation of the protein extracts:

For the extraction, about 200 mg of fresh plant material were homogenized with nitrogen liquid in tampon Tris-HCl (pH 8,0) and polyvinylpyrrolidone (PVP) 10 % (p/p), at 4°C. After centrifugation of the mixture, the supernatant was used as the determination of **the protéines soluble, catalase, ascorbate peroxydase and Superoxide dismutases.**

- Soluble protein content was determined by the method of Bradford (1976)

- Enzymes essay (Geoffroy et *al*, 2004**): Catalase activity (CAT)** was measured using the method of Aebi (1984). It's basis on reduction of substract (H2O2) by the catalase, absorbance was read immediately in spectrophotometer (Shimadzu) at 240 nm. About 100 µl of H_2O_2 (10 mM) was added to 50 µl of protein extract during 2 min. A**scorbate peroxydase (APX)** activity was measured by the reduction of ascorbate in the presence of H2O2, the enzymatic reaction was measured after 3 min at 290 nm. About 50 mM tampon phosphate (pH 7,5) and 0,5 mM H2O2 were put in test tube with 100 µl of protein extract, than 50µl ascorbate (250mM) was added. The nitroblue tetrazolium (NBT) method was used for determination of **Superoxide dismutases (SOD)** activity. One unit of SOD activity was defined as the amount of enzyme required to produce 50% inhibition of NBT reduction at 560 nm.

Statistical analysis

Results are presented as mean ± standard error; statistical analyses of experimental result were subjected to analysis of variance. Significant difference was statistically considered at the level of $P < 0.05$.

Results and Discussion:

Salicylic acid (SA) is an endogenous growth regulator of phenolic nature, which participates in the regulation of different physiological processes in plants (Raskin,1992, Hayat,2007, Idrees,2011, Siamak and *al*,2014). Recently this substance has drawn attention of researchers because of its ability to induce system acquired resistance (SAR) in plants to different pathogens, which is manifested as appearance of pathogenesis related proteins (PR), while SA is considered to serve as a signal in the induction of expression of these genes (Ward et al., 1991; Metraux, 2002; Kachro and *al*,2005).

Effect of SA on germination and seedling:

According to the Figure 01, seeds of chickpea were germinated since 24 h, either for all lots. The percent of germinated seeds were higher in control until (80%). But the presence of the *Fusarium.redolens* has a depressive effect on the kinetics and the percent of germination in control seeds, during the 72 h, respectively of (21,6 to 23,5%), but this rate has fallen in 96 h by the root of the no treated seeds at (0%). On the other hand, the percent of germination seeds pre-treated with the two concentrations of SA were both linear for the duration of the experiment, it has reached a very high rate, respectively (55% and 40,5% in 96h) with 0.05 and 0.5 mM AS, except for the presence of 1 mM of the SA, no germination was noticed.

The (0,05mM SA) dose had a positively significant effect than (0,5mM SA)($P < 0.05$), in the course of the germination.

Tab 1: Effect of salicylic acid on germination of chickpea (ILC 3279), inoculated with *Fusarium redolens* (1,6.10 ^{4} conidies/ml).

SA(mM)	Contro	0	0,05	0,5	1
% de germination	89,4±13,5	23,5±2,5	55,03±3,46	40,5±4,2	0,5±0

The percentage of developed plants was estimated in relation to control ones. The number of developed plants is significantly higher following the application of SA doses (0,05 mM and 0,5 mM), respectively of (55% - 44 %) in comparison with the control plants that do not exceed (25%). In parallel, we have noticed a very reduced rate of necrosis in treated plants, (28,2% - 14,7%) compared with witness plants (43,1%) (Fig 2).

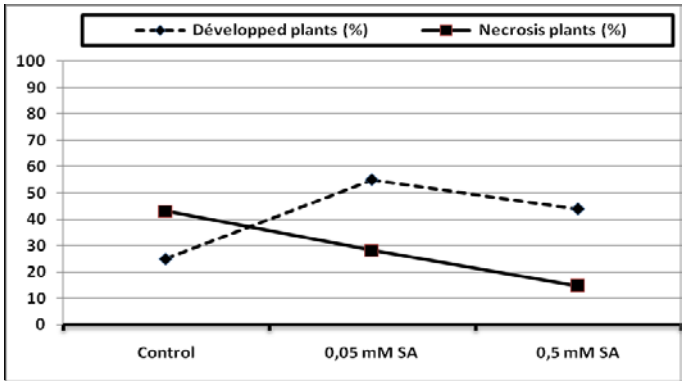

Fig 2: Effect of salicylic acid on seedling parameters of chickpea (ILC 3279), inoculated with *Fusarium redolens* (1,6.10 ^{4} conidies/ml)

Moreover, we can observe the impact of SA on the yield of treated plants in relation with non treated plants and control. Indeed, the infection of plants with the *Fusarium redolens* had created losses on the number of non treated plants in comparison with treated plants.

However, the leaves to no treated plants had developed chlorosises. Plants infecting causes lacks at rising, impair of growth, necrosis of plants and a bad quality of seeds (Halila and al,1996; Pande and *al*,2007, Muhammad and *al*,2010). On the contrary, a less harmful effect was noticed in plants treated with SA, either on the yield or on the growth parameters, Boroumandjazi et al (2011) had reported that SA increased length and dry weight of root and shoot, leaf area, specific leaf area, specific leaf weight and leaf weight raito under stress condition. SA enhanced level of developed plants and diminishes level of necrosis and positively affected the storage fresh weight (Fig 2), Similar results were proved (Enyedi and *al*,1991; Shakirova and *al*,2003; ,2007). These authors have shown that SA stimulates cell division of roots, as well as the increase of foliar mass and weight of plants, by stimulating mitotic system of apical meristem. Gharib (2006) and Khan et *al*.(2003) reported that application of SA enhanced photosynthesis rate so that leaf area has been increased.

This positive effect of SA may be related to increasing of CO2 assimilation and photosynthetic rate and increasing of mineral uptake in stressed plant under SA application (Szepesi et al., 2008). The 0,05 Mm SA showed higher disease resistance in chickpea than 0,5mM SA with ($P < 0.05$). These alterations would injure plant general metabolism and reduce overall growth and photosynthetic attributes, these results were observed in a study by (Uzunova and Popova,2000 and Ghasemzadeh and *al*,2013).

Effect of the Salicylic Acid on the biochemical parameters:

In order to characterize effect of SA on nutritional quality and identify some interfering molecules in the activation of resistance in seeds and plants, against *Fusarium redolens*, the following results were observed.

Proteins Contents:

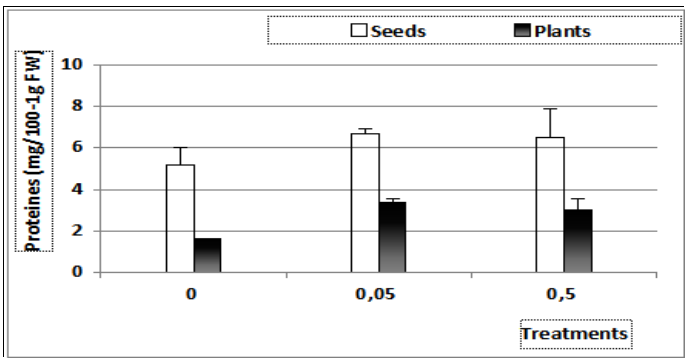

Fig 3: Effect of salicylic acid on protein content (*mg/g*$^{-1}$*PF*) in seeds and plants of Chickpea (ILC 3279*)* inoculated with *Fusarium redolens* (*1,6.10*^{4} *conidies/ml*)

In general, a great accumulation of proteins was noticed in seeds in relation to plants. Following application of SA, these contents have reached in seeds and plants for all treatments respectively (6,68 – 3,36 mg) with 0,05 mM SA and (6,54m -3,04mg) with 0,5mM SA, except for both controls this rate has declined to (5,2 mg -1,66 mg), seeds and plants treated with SA exhibited higher protein content than controls. These results were shown by (Gary and Murray ,2007). Many authors had noted that SA plays role of a signal molecule in the induction of defense proteins (Pr) in contact with parasites (Ward,1991, Klessig and Mamaly J.1994, Hoyos and Zhang,2000). Indeed, proteins play an important role in plant defense in the form of various defense enzymes and other non enzymatic protein (War and *al*, 2011, Chen and *al*,2009).

The Antioxidant Activity:

Recorded to figure 3, the calculated values of antioxidant activity for treated plants as well as control ones were shown. First we noted that this activity is significantly higher in plants than seeds. In second part, this activity had decreased in no-treated seeds and plants (16,35 - 55,75). In contrast, application of SA enhanced antioxidant activity, with elevated raise with 0,05 mM SA (11,56-29,11) than 0,5 mM SA (11,97-36,8).

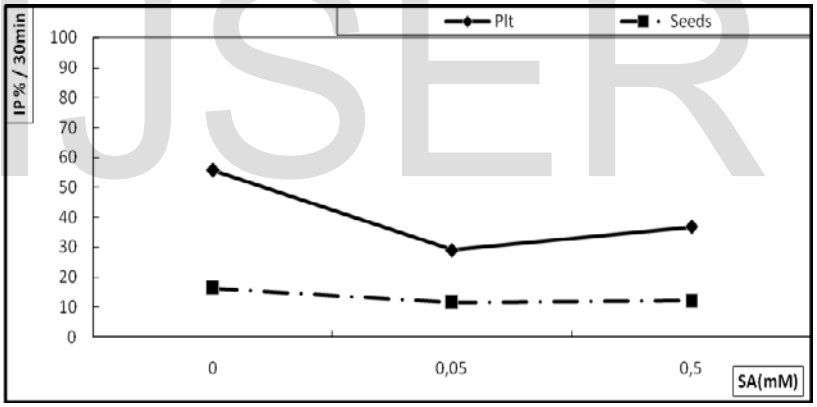

Fig 3: Effect of salicylic acid on antioxidant capacity of Chickpea (ILC 3279*)* inoculated with with *Fusarium redolens* (*1,6.10* ^{4} *conidies/ml*), during germination and seedling

Exogenous application of SA increased antioxidant activities; the greatest responses were obtained in plants sprayed with 0,05 mM than 0,5 mM SA. Sukand and Kulkarni (2006) had reported that oxidization of biochemical compounds are involved in weakening of resistance of plants towards pathogenesis. The effects of salicylic acid (SA) and fungi on antioxidative protection in chickpea were recently studied (Hayat and *al*,2009, Sangmin and *al* (2010). They proposed that SA affects the plant growth under stress through nutrient uptake, water relations, stomatal regulation and photosynthesis. Smirnoff (2005) and Shraiy and al (2009) had reported that antioxidant ability depends on the specie and the stage of development of plants. While, Chen (1997) and Fodor (1997), had mentioned that SA degrades antioxidant ability in plants.

Influence of salicylic acid on the activity of the antioxidant enzymes in chickpea:

Recently, SA pretreatment was shown to directly or indirectly activate cellular antioxidant enzymes during stress (Horvath et al. 2002; Kang et al. 2003).

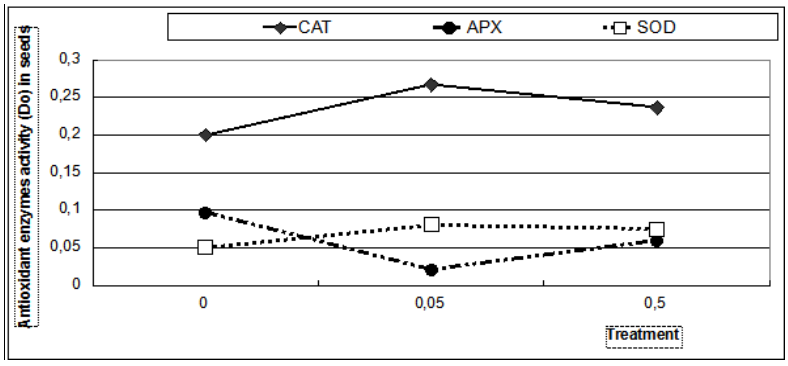

Fig 4: Effect of salicylic acid on Catalase (CAT), Peroxidase (APX) and (superoxide dismutases) SOD activity of chickpea seeds (ILC 3279*)* inoculated with *Fusarium redolens* (1, 6.10 conidies/ml) on germination

CAT and SOD activity increased in seeds respectively (0,267 and 0,081) with 0,05mM SA and (0,23 and 0,075) with 0,5mM SA in comparision with control seeds (0,20 and 0,051). Indeed APX activity decreased with SA application, especially 0,05 mM(0,021) compared to 0,5mM SA (0,06) and controls (0,097).

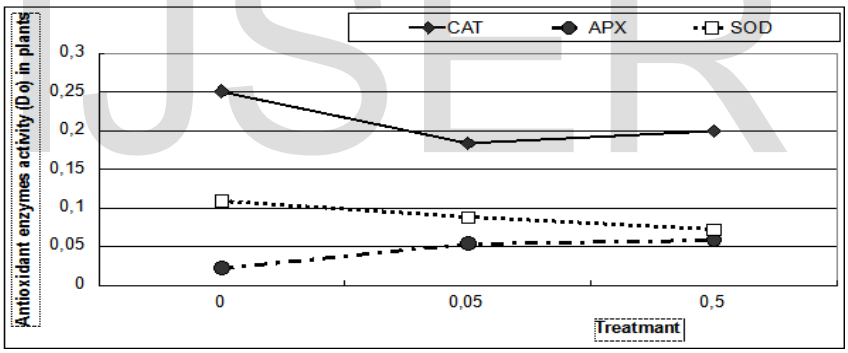

Fig 5: Effect of salicylic acid on Catalase (CAT), Peroxidase (APX) and Superoxide dismutases (SOD) activity of Chickpea plants (ILC 3279*)* inoculated with *Fusarium redolens* (*1,6.10* ^{4} *conidies/ml*), during seedling

Enzymes CAT and SOD activity increase in no-treated plant (0,25 and 0,109) in comparison to SA application. Also these enzymes activity decline to (0,18 and 0,088) with 0,05 mM SA and to (0,2 and 0,072) with 0,5 mM SA. APX activity raised in treated plants (0,054) with 0,05 mM SA and (0,059) with 0,5 mM SA. But don't exceed the rate of CAT and SOD.

In our study, however, neither of these processes was favored by the application of SA, activity of CAT, APX and SOD was different depending on treatments doses and of development state. We can note with SA application, activity of CAT was significantly higher in seeds and plants, although lower activity was observed in APX. However, Application of 0,05 mM SA showed significantly superior activity to 0,5mM SA and no treated ones. Also, we can observe that SA

increase CAT and SOD activity in seeds but decrease these enzymes activity in plants. In contrast SA application increased APX activity in plants but decreased it in seeds, in accordance with other studies (Hayat and *a*l, 2005, Yousuf, 2008).

In the present study, enhanced activities of SOD and CAT due to SA application might have been one of the factors contributing to improved germination and growth in plants under stress. Similar to our results, Ghasemzadeh and *al* (2013) suggest that increased SOD activity was accompanied by increases in CAT activities because of the high demands of H_2O_2 quenching, Superoxide dismutase (SOD), a key enzyme in cellular defence, catalyses the dimutation of superoxide radicals to H_2O_2 and O_2 (Foyer and Noctor, 2000). Although, Durner and Klessig (1995) showed that SA also inhibits APX, the other key enzyme for scavenging H2O2, its supports the hypothesis that SA-induced defense responses are mediated, in part, through elevated H_2O_2 levels or coupled perturbations of the cellular redox state.

SA regulates the activities of various antioxydant enzymes which are the major components of induced plant defense against biotic and abiotic stresses (Paulraj and al,2011, Zhao and *al* ,2009, Idrees and *al*,2011). SA induced effects on the rate of generation of O_2^- and H_2O_2 (Chen et al., 1993; Minibayeva et al., 2001).

Antioxidant system plays an important role in the process of neutralization of the effects of oxidative stress, plants possess a well-defined enzymatic antioxidant defense system to protect themselves against these deleterious effects by scavenging ROS (Neill et al., 2002). The existence of positive relationship between antioxidant activity and antioxidant enzyme activity in chickpea was shown. Salicylic acid was found to enhance the activities of antioxidant enzymes such as catalase, peroxidase and superoxide dismutase. Similar studies were shown by (Yousuf, 2008, Ghasemzadeh and *al* 2013).

Salicylic Acid is a transducer and a messenger, it also adjusts cell death combined with hypersensitive response by the activation of peroxydation of lipids and generation of free stems, as well as the activation or the inhibition of antioxidant enzymes, for several plants (Conrath ,1995, Yusuf, and *al*,2008, Peleg and Blumwald, 2011).This increased antioxidant enzyme activity might be due to SA's regulatory role at transcriptional and/or translational levels (Szepesi, 2008; Yousuf, 2008). Increases in SOD activity and in turn decrease in APX activity due to exogenous SA treatment have been reported previously and strongly support our observed results (Hayat and al,2005, Ghasemzadeh and al,2013). These results may had a relationship with the different effect of 0,05 mM and 0,5 mM SA on the different ability of germination, plants seedling as well as a decline in necrosis apparition degree. The rise of the antioxidant ability seems to have an important link with growth stage of chickpea as well as the applied doses of SA. At lower SA concentration, germination, growth, protein, antioxidant activity and activities of antioxidant enzymes such SOD and CAT were

significantly elevated, in accordance with other studies (Hayat and al,2005). However, higher SA concentration may have caused permanent changes to cell membrane-level organization (Uzunova and Popova, 2000).

These alterations would injure plant general metabolism and thereby reduce overall growth and photosynthetic attributes. Analysis of variance and mean comparison of this experiment shows that using salicylic acid could increase plant tolerance to fungal stress during germination and seedling stage.

In conclusion, overall the results one of the more significant findings to emerge from this study is that exogenous SA application may have a role in pathogenesis tolerance of chickpea. We can support the suggest that low SA concentrations (0,05 mM) induce raise of germinated seeds and number of developed plants, by improving antioxidant system activity which was accompanied by increases in CAT and SOD activities, although reducing oxidative stress damages, it therefore appears that SA can generally be used as a growth regulator to enhance germination and plant growth.

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