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Endogenous Salicylic acid Estimation in Wheat leaves treated with Salicylic acid and infected with *Alternaria triticina*

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Abstract: This study was aime to measure the accumulation of endogenous salicylic acid, as indicator for systemic acquired re-sistance of wheat plants of "Utique" variety. The plant was sprayed with salicylic acid (SA) or water as control, at the five-leaf stage, later infected with leaf blight caused by *Alternaria triticina*. Leaf samples were removed after 10, 20, and 30 days of inoculation to test their endogenous content of salicylic acid as it is the primary internal signal indicating the emergence of systemic acquired resistance in plants, by the spectrophotometer measuring. Endogenous SA values were ascending dramatically from 10 days to 20 days and maximum with 30th day significantly, while control plants exhibit lower values in all day periods, insignif-icantly. SA treatments proved reduction in disease incidence after 10 days with 54% and after 20 days with 64%, while after 30 days the reduction recorded high percent-age of 80%. In comparison between the time intervals, disease severity was clearly reduction and reached to 83% after 10 days of inoculation then decreased to 72% for both 20th and maintained its stability on the 30th day of inoculation by 72%. This study was proved the reduction of wheat leaf blight incidence and disease severity as a result of treatment by using 1Millimolar (mM) of SA was leading to accumulation of significant levels of endogenous SA as indicator for internal induced resistance in plant.

تقدير حمض الساليسيلك الداخلي المنشأ في أوراق القمح المعاملة خارجيا بمنشط الساليسيلك والمصابة بـ *Alternaria triticina*

الكلمات المفتاحية :
حمض الساليسيلك الداخلي. لفحة الأوراق، *Alternaria triticina*، القمح

المستخلص : : تم رش نباتات القمح صنف "Utique" في مرحلة الخمسة ورقات بحمض الساليسيليك (1ميللي مول) أو الماء كشاهد، ثم أُجريت العدوى لاحقًا بمرض لفحة أوراق القمح المُتسبب عن *Alternaria triticina*. ازيلت عينات من الأوراق المُصابة بعد 10 و 20 و 30 يوماً من العدوى بلقاح الفطر لتقدير محتواها الداخلي من حمض الساليسيليك باعتباره الإشارة الداخلية الأولية التي تدل على نشوء المُقاومة الجهازية المُكتسبة في النباتات، وذلك عن طريق قياس امتصاص الأشعة فوق البنفسجية عند 540 نانومتر بواسطة المطياف الضوئي Spectrophotometer. سجلت النتائج تزايد قيم SA الداخلية المنشأ بشكل ملحوظ بعد 10 أيام إلى 20 يوم حتى بلغت الحد الأقصى في اليوم الـ30 من العدوى، بينما أظهرت نباتات الشاهد قيمًا أقل في جميع فترات القياس. أثبتت المعاملة بـ SA انخفاض في معدل ظهور المرض بعد 10 أيام بنسبة 54٪ وبعد 20 يومًا 64٪ ، في حين سجل الانخفاض نسبة عالية بلغت 80٪ بعد 30 يومًا من العدوى بلقاح الفطر، وعند قياس شدة المرض في نفس الفترات الزمنية اتضح أن شدة المرض انخفضت بنسبة 83٪ بعد 10 أيام من التلقيح وبنسبة 72٪ عند اليوم العشرين وحافظت على ثباتها 72% حتى اليوم الثلاثين من التلقيح أثبتت هذه الدراسة انخفاض معدل ظهور المرض وشدته نتيجة المعاملة بـ 1 ميللي مول من SA مما أدى إلى تراكم مستويات كبيرة من SA الداخلي وهو دليل استحثاث المقاومة في النبات.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops under Poaceae family grown throughout the world including Libya. It is one of the most important winter crops which are sensitive to temperature. In 2019 the world production of wheat

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was reached 765 million tons, making it the second most-produced cereal after maize (1.1 billion tons) (Faostat, 2019). In Libya, the average yield and total production of wheat have been estimated at 5510 tones on average area, 5312 hectares (Faostat, 2019). It is being a favorite food for millions of people which contains large amounts of carbohydrates and protein. There are several restrictions limiting the potential yield of wheat. Among them, foliar blight has recently emerged as a major concern throughout the world (Dubin & Ginkel, 1991).

The disease initially appears as small and irregularly scattered chlorotic lesions on the leaves in late December. As the disease advances, several spots are merge and cover the whole or part of the leaf showing a blighted appearance. Heavily infected fields show a burnt appearance (Kakraliya et al., 2017). *Alternaria triticina* is the causal agent of wheat leaf blight, and a small number of related cereals (Prasada & Prabhu, 1962). Infection occurs through seed-borne transmission, planting into infected soil, or from infested crop residues, where rain splash or leaves direct contact with the soil leads to infection. *A. triticina* is unable to infect young wheat seedlings under four weeks of age, with symptoms not seen until plants were reached seven weeks of age. Susceptibility was increased with plant age (Murray, 2009).

Following inoculation with *A. triticina*, phenol content increased in resistant leaves and decreased in susceptible ones. Free amino acids, especially those involved in aromatic metabolism, are raised markedly in resistant cultivars. In resistant cultivars, disease progress stopped after 5 days and changes in phenol and nitrogen content persisted for up to 10 days. Phenolic compounds accumulated more rapidly in resistant cultivars than in susceptible cultivars as a result of infection (Chalkley, 2012).

Systemic acquired resistance (SAR) is an inducible form of plant defense that confers broad-spectrum immunity to secondary infections beyond the initial infection site (Wang et al., 2018; Yasuda, 2007) summarized that in incompatible interactions between plants and pathogenic microorganisms, plants recognize the virulence gene products of individual pathogens using specific receptors, the R gene products. This interaction causes, at the infection site, a burst of reactive oxygen species (ROS), the rapid induction of a hypersensitive response (HR) involving controlled cell death, and the expression of pathogenesis-related (PR) genes. Following these events in the infected leaves, the uninoculated leaves exhibit an increased level of PR gene expression and usually develop long-lasting enhanced resistance to further attacks by pathogens, termed systemic acquired resistance SAR (Yasuda et al., 2008). Activation of SAR needs accumulation of the endogenous signaling molecule salicylic acid (SA). Exogenous application of SA is sufficient to trigger SAR and the concurrent induction of defense-associated genes (van den Burg & Takken, 2009). SAR can be induced by either pathogen infection or treatment with salicylic acid (SA) or its functional analogs 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH), which is associated with transcriptional activation of pathogenesis-related (PR) genes (Fu & Dong, 2013).

(Malamy et al., 1990) reported that the first evidence that SA is an endogenous signal molecule in plant defense came from studies with pathogen-challenged tobacco and cucumber plants. The detection of SA levels were increased in systemic leaves and in the phloem led many researchers to believe that SA might be a systemic signal for SAR (Ghanbari et al., 2015). Because *A. triticina*, is a seed-borne pathogen, and causes major damage to susceptible wheat varieties under wet or dry conditions. It is necessary to rely on stimulating systemic plant resistance through treatment with chemical inducers,

so this study was conducted to measure the accumulation of endogenous salicylic acid, as indicator for systemic acquired resistance.

MATERIALS AND METHODS

Wheat variety source: Seeds of wheat variety "Utique" F1 were obtained from Central Mutual Company of Seeds (Cosem), Manouba – Tunisia.

Experimental design: The experiment was conducted in complete randomized block design, by planting two basins with an area of 60 × 30 cm, at a rate of 300 wheat seeds in each basin, and irrigated by flooding method. The irrigation was continued once every five days.

Exogenous SA preparation and spray: Salicylic acid (SA) Sigma-Aldrich chemie-France was purchased from Sigma Co. branch, Cairo. The shoot system at the five-leaf stage plants in the first block was sprayed with Salicylic acid (1mM), while the other block was sprayed with sterile water.

Fungal isolate: *Alternaria triticina* isolate was obtained from fresh infection of wild wheat plants grown near the research station of the faculty of Agriculture farm. Isolation was carried out on fresh wheat leaves, showing early blight symptoms, carefully washed in running tap water, then surface sterilized with 10% sodium hypochlorite. The infected parts were cut into small pieces by using a sterilized scalpel at the zone of infection, then washed several times in sterile distilled water and dried between two sterilized filter papers, and transferred to the surface of Potato Dextrose Agar (PDA) medium in Petri dishes and incubated at 25 ˚C for 4-7 days to enhancing sporulation. Wheat plants of Utique variety (60 days old) which treated with SA or H_2O before, were inoculated with fungal spore suspension. A modified technique of (Fritz, 2005) was applied through this experiment. Fungal spores of 10 day-old cultures were harvested by gentle brushing to separate the spores from the mycelium. The spore suspension was quantified using a haemocytometer to 10^4 spores/ml. Plant leaves were inoculated by spraying the spore suspension until run-off. To ensure good spore germination, the plants were covered with transparent plastic bags for 24 hours to increase the relative humidity.

Plant extraction: Plant extraction was done according to the modified methodology of (Warrier et al., 2013). Leaf samples of wheat were cutting and grounded in 70% ethanol solvent to ensure the solubility of SA from tissues in the presence of interfering substances. Samples were mixed well in the solvent followed by centrifugation at 10,000g for 10 min. The supernatant was kept in ice for SA measurement.

Calibration curve of SA: The free phenolic hydroxyl group present in salicylic acid reacts with ferric chloride and forms a violet-colored mixture i.e., ferric salicylate which is proportional to the concentration of salicylic acid. The calibration curve of SA was constructed according to the method of (Venkataswamy, 2018). Ferric chloride reagent is prepared by adding 1 gm of $FeCl_3$ to 100 ml of 1% hydrochloric acid (HCL). Stock solution 1: Stock solution of salicylic acid (1mg/ml) is prepared by dissolving 100 mg of salicylic acid in a few ml of methanol and completed to 100 ml with distilled water in a volumetric flask. Stock solution 2: 10 ml of this stock solution 1 is diluted with 100 ml distilled water to get 100 µg/ml of salicylic acid solutions. Dilutions: The respective samples (1ml, 2ml, 3ml, 4ml, 5ml, and 6ml) each transferred in a test tube, reagent and distilled water were added to make a total volume of 10 ml to produce 10µg/ml, 20µg/ml, 30µg/ml, 40 µg/ml, 50µg/ml, and 60µg/ml.

Endogenous SA measurement by spectrophotometer: Measuring the absorbance of the prepared samples (violet-colored complex) was done by UV-Visible spectrophotometer

(Jenway-Model 6305) at a wavelength of 540 nm against blank sample (without salicylic acid). Using Microsoft Excel software, plotting a graph with the absorbance on Y-axis and concentration on X-axis, results in an equation formatted as follows: y= 0.0298x + 0.3466, where solving for x determines the SA concentration of the sample.

Disease incidence and severity: Disease incidence ($I = \Sigma x/N$) was the proportion of diseased plants, which consisted of the number of diseased plants (x) divided by the total number evaluated (N). Disease severity (S) was estimated by the equation $S = \Sigma (xi \times ni)/N$, in which xi represented disease grade (0 = free of infection, 1= trace - 25% leaf area spotted, 2= 26-50%, 3= 51-75%, 4= 76-100% leaf areas killed; ni, represented the number of diseased plants on the grades of disease scale and N was the total number of diseased plants evaluated (Cardoso et al., 1998)**.**

Statistical analysis: Data obtained were subjected to ANOVA and statistically analyzed (Gomez & Gomez, 1984) SA absorbance values were compared by Tukey HSD test, while disease incidence and severity were compared by Least Significant Difference (LSD) test at a confidence level of 95%. The package used for analysis was NCSS version 2021.

RESULTS

To detect the initiation of signaling pathway and plant response, a fast, sensitive method is required for determination of SA in the plant at different times. For the determination of endogenous salicylic acid, the property of its violet color production when bound to ferric chloride facilitates its detection by spectrophotometer.

In the present work, wheat plants infected with *Alternaria* leaf blight pre-treated with salicylic acid spray or water as control. Leaf samples were removed after 10, 20, and 30 days of *Alternaria* inoculation to test their endogenous content of salicylic acid by measuring the absorbance of UV light at 540 nm by spectrophotometer. Table (1) showed the increase of endogenous SA absorbance values by spectrophotometer over the days and more in the case of treatment with salicylic acid than distilled water (control). SA application compared to distilled water proved its success in recording high levels in endogenous SA after 10, 20, and 30 days of inoculation, (37.45, 45.31, 52.64) and were differs significantly. Therefore a spray of wheat leaves with H_2O (control) exhibit lower values (16.34, 16.62, 18.26) in all day's periods, respectively without significant differences between time intervals.

Table: (1). Colorimetric absorbance (540 nm) of endogenous SA of wheat leaves treated with SA or H_2O (control) after three time intervals (10, 20, and 30 days) of inoculation.

Treatments	Days after treatment		
	10	20	30
SA	37.45*a	45.31* b	52.64*c
H_2O (control)	16.34**d	16.62**d	18.26**d

According to Tukey HSD test (α=0.05): (*), (**) indicate significance in the same column, while rows with the same letters don't differ significantly.

As shown in Table 2, disease incidence values recorded at three periods; 10, 20, and 30 days of inoculation for wheat treated with exogenous salicylic acid indicated that *Alternaria* leaf blight disease was significantly decreased, it showed a low value of 4.19 after 10 days of inoculation, this value increased significantly after 20 days of inoculation (7.76), It has continued to increase significantly until the 30th day by 10.62. On the other hand, the use of water instead of salicylic acid showed high values of disease incidence with highest significant differences in all time periods during the experiment (9.05 after 10 days, 21.84 after 20 days and 54.15 after 30 days). Disease incidence reduction was 54% after 10 days, followed by 64%, after 20 days and 80% after 30 days.

Table: (2). Leaf blight disease incidence at three time intervals (10, 20 , and 30 days) of inoculation in treated

plants with exogenous SA compared to plants treated with H_2O (control).

Treatments	Disease Incidence			LSD (α=0.05)
	10	20	30	
SA	4.19[a]	7.76[c]	10.62[e]	2.81
H_2O (control)	9.05[b]	21.84[d]	54.15[f]	9.99
LSD (α=0.05)	1.5	7.37	10.27	

Rows and columns with the same letters don't differ significantly, according to LSD test (α=0.05).

Disease severity values listed in Table (3) indicated the decrease trend in disease severity for the plants were treated with a concentration of 1 Mm of salicylic acid compared to untreated plants with statistically significant differences. It is evident from Table (3) that the disease severity after 10 days recorded a low value of 2.43 and increased after 20 days of inoculation to 5.86 and then to 12.73 on day 30th of *Alternaria* inoculation with a significant difference between them. On the other hand, untreated plants (control) exhibited high values of disease severity in all periods, where it recorded 14.34, 21.13, and 46.04 in the day periods, respectively, with significant differences between them, except 10th and 20th day. In comparison between the time intervals, it is clear the severity reduced by 83% after 10 days of inoculation followed decrease to 72% for both the 20th and 30th days of inoculation compared to control.

Table: (3). Leaf blight disease severity at three time intervals (10, 20 , and 30 days) of inoculation, in treated plants with exogenous SA compared to plants treated with H_2O (control).

Treatments	Disease Severity			LSD (α=0.05)
	10	20	30	
SA	2.43[a]	5.86[b]	12.73[c]	3.06
H_2O (control)	14.34[d]	21.13[d]	46.04[e]	10.42
LSD (α=0.05)	5.17	9.3	8.12	

Rows or columns with the same letters don't differs significantly according to LSD test (α=0.05).

DISCUSSION

In plants, the positive correlation between endogenous levels of SA and resistance responses against biotrophic and hemibiotrophic pathogens is well established (Glazebrook, 2005). In this study, the applied of exogenous SA was suitable at concentration (1mM) as a plant activator to induce internal systemic resistance in wheat plants against leaf blight disease by enhancing significant levels of endogenous SA, which plays a key role in the emergence of systemic acquired resistance. (Vallad & Goodman, 2004) explained that exogenous application of SA on plants due to induction of endogenous SA accumulation causes SAR genes activation which leads to resistance against different kinds of pathogens. Exogenous application of SA or one of its active analogs is acceptable to induce plant defense against biotrophic and semibiotrophic pathogens (Koo et al., 2020; Lu, 2009; Vallad & Goodman, 2004) endorsed the use of SA at a concentration of 2 mM, stating that it was not only induces enhanced disease resistance but also has adverse effects on plant growth and productivity, which caused by imbalance between cost and benefit of limited energy that plant can use.

In this study a simple technique to detect endogenous SA in plant extraction by using the colorimetric method with a spectrophotometer was used, although there are other advanced techniques used in SA estimation such as HPLC, GC-MS; this was similar to (Warrier et al., 2013) who used the spectrophotometric method in comparison with the well-known sophisticated methods like HPLC, GC, MS; these extraction procedures are very cumbersome and time-consuming, the spectrophotometric method is simple, fast, reliable and accurate. Accumulation of endogenous SA level, throughout the present work, was associated with significant activation of systemic resistance against *A. triticina* in wheat plants grown under experimental conditions. SA was known to be an important signal molecule and its level may increase endogenously prior to the activation of SAR in each of the host-pathogen interactions (Malamy et al., 1990;

Métraux et al., 1990) Other authors concluded that endogenous levels of methyl salicylic acid (MeSA) increase in plants resisting pathogen infection (Tripathi et al., 2010; van den Burg & Takken, 2009). SA has been found to activate through a redox mechanism (Hadi & Balali, 2010). According to (Sticher et al., 1997), the SA signaling pathway can be triggered by exogenous SA, which improves disease resistance, because this pathway is related to systemic acquired resistance (SAR), which can emerge when endogenous SA accumulation and is triggered after plant pathogen infection.

In this experiment, after application of SA in wheat infected with *A. triticina* showed that an increase in the endogenous SA levels, the results agreement with (Gholamnezhad et al., 2016), who recorded increase in the endogenous SA levels which led to oxidation of phenolic compounds, that may limit the fungal growth. (Sahu & Sabat, 2011), after applying the SA in wheat plants, they found increased roots, superoxide dismutase (SOD) and malondialdehyde (MDA) activity, these enzymes protect the cell from oxidative stress

CONCLUSION

Our study proved the reduction wheat leaf blight incidence and severity as a result of treatment with 1mM of SA which leading to accumulation of significant levels of endogenous SA in which, it is the key role in initiation of systemic acquired resistance. It recommend to further investigations to determine the induced compounds responsible of resistance and other induced structures which, prevents the disease progression in the plant, and due to the lack of appropriate facilities, we were unable to make those estimates in our study.

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ETHICS

Authors should address any ethical issues that may arise after the publication of this manuscript.

Duality of interest: The authors declare that they have no duality of interest associated with this manuscript.

Author contributions: The first author did the practical experiments and wrote the results, while the second author did the chemical analyzes and calculations. Both authors contributed to the final version of the manuscript.

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