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Original article

Spectrophotometric Quantification of Endogenous Salicylic Acid in Priming Wheat Tissue with Puccinia *Triticina F. Sp. Tritici*

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ABSTRACT

Aims. *To prove the importance of application exogenous SA that leading to accumulation of endogenous SA as an indicator to the initiation of SAR, by measuring the levels of endogenous SA in priming wheat plant.* ***Methods***. *The experiment was conducted in complete randomized block design, by planting two basins, at a rate of 300 wheat seeds in each basin.* ***Place and Duration of Study.*** *This study was conducted at research station farm of the agriculture faculty in Soluqe area, between December 2020 and April 2021. Fungal spores and extraction: spores of Puccinia triticina f. sp. tritici were harvested and inoculated in Wheat plants sprayed with Salicylic acid or distilled water. Wheat leaves were extracted in ethanol solvent. Samples were swirled well in the solvent followed by centrifugation at 10,000 g for 10 min. Absorbance was measured using UV-Visible spectrophotometer at wavelength of 540nm against blank sample (without salicylic acid) to determines the SA concentration, disease incidence and severity were measured too.* ***Results.*** *Endogenous SA values were ascending dramatically from 10 days to 20 days with significant differences, while control plants exhibit lower values in all day's periods, insignificantly. SA treatments proved reduction in disease incidence after 10 days with 50% and after 20 days 45.4%, while after 30 days the reduction recorded high percentage of 63.6%. In comparison between the time intervals, it is clear the severity reduced by 70.9% after 10 days of inoculation and by 71.9% after 20 days, but the reduction was the highest by 80.8% after 30 days of inoculation.* ***Conclusion.*** *We proved the reduction of wheat leaf rust incidence and severity as a result of treatment with 1mM of SA, leading to accumulation of significant levels of endogenous SA; the key role in initiation of systemic acquired resistance.*

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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. It was reported in 2019 that the world production of wheat was 765 million tons, making it the second most-produced cereal after maize (1.1 billion tons) [1]. In Libya, average yield and total production of wheat has been estimated 5510 tones on average area, 5312 hectares in 2019 [1]. It is being a staple food of millions of people which contains large amount carbohydrates and protein.

Wheat leaf rust, caused by *Puccinia triticina f. sp. tritici*, is one of the most devastating diseases of wheat (*Triticum aestivum*). Leaf rust is favored by warm temperatures up to 35 °C [2] and can develop quickly, causing severe yield losses when conditions for its development are favorable. Wheat leaf rust (*Puccinia triticina f. sp. tritici*) had largely been controlled from the late 1970s due to the widespread use of host-plant resistance and the elimination of the alternate host Berberis spp. in North America and Europe [3].

Systemic acquired resistance (SAR) is an inducible form of plant defense that confers broad-spectrum immunity to secondary infections beyond the initial infection site [4-5] Summarized that in incompatible interactions between plants and pathogenic microorganisms, plants recognize a virulence gene product 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 regulated cell death, and the expression of pathogenesis-related (PR) genes. Subsequent to 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 [6].

Activation of SAR requires 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 [7]. 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 [8-9] 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 increased SA levels in systemic leaves and in the phloem led many researchers to believe that SA might be a systemic signal for SAR [10]. Due to spread of wheat leaf rust in wide Libyan areas which threatens wheat cultivation in the favorable conditions for this disease, this study came to prove the importance of application exogenous SA that leading to accumulation of endogenous SA as an indicator to the initiation of SAR, by measuring the levels of endogenous SA in priming wheat plant.

MATERIAL AND METHODS

Wheat variety source

Seeds of wheat variety "Utique" F1 was 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 the 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. 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 H2O.

Fungal isolate

Puccinia triticina f. sp. tritici isolate was obtained from fresh infection of wild wheat plants grown near the research station of the faculty of Agriculture farm. Fungal spores were harvested by gentle brushing the uredosori of infected wheat leaves to separate the uredospores and then rinsed with a sterile H2O. The resulting spore suspension was quantified using a haemocytometer to 104 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 cover for 24 hours to increase the relative humidity.

Plant extraction

Plant extraction was done according to the modified methodology of [11]. Leaf samples of wheat for both treatments; SA and H2O (30 leaves in each) were washed under tap water, then dried and grounded in ethanol solvent to ensure the solubility of SA from tissues in the presence of interfering substances. Samples were swirled well in the solvent followed by centrifugation at 10,000g for 10 min. The supernatant was stored on 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 complex i.e., ferric salicylate which is proportional to the concentration of salicylic acid. Calibration curve of SA was constructed according the method of [12]. Ferric chloride reagent is prepared by adding 1 gm of FeCl3 to 100 ml of 1% HCL. Stock solution 1: Stock solution of salicylic acid (1mg/ml) is prepared by dissolving 100 mg of salicylic acid in few ml of methanol and made up 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 salicylic acid solutions. Dilutions: the respective samples (1ml, 2ml, 3ml, 4ml, 5ml, and 6ml) was taken in each test tube, the reagent and distilled water was added to make 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 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.

2.8. Disease incidence and severity: Disease incidence (I = Σ 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 = Σ(xini)/n, in which x 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; [13], ni represented the number of diseased plants on the ith grade of the disease scale and n was the total number of diseased plants evaluated.

Statistical analysis

Data obtained were subjected to ANOVA and statistically analyzed [14] 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

SA is one of the target chemicals in the pathway to signal transfer in SAR induction. In the following step Methyl Salicylate, an airborne signal chemical, further induces disease resistance in other parts of the plant and even transfers resistance to other plants. 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 is taken advantage of, this facilitates its detection by spectrophotometer.

Wheat plants of Utique variety infected with stem rust caused by Puccinia triticina f. sp. tritici were sprayed with salicylic acid or water as control, leaf samples were removed after 10, 20, and 30 days of disease inoculation to test their endogenous content of salicylic acid by measuring the absorbance at 540 nm by spectrophotometer. The data recorded in (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 in the case of distilled water. The application of SA compared to distilled water proved its capability to record high values in endogenous SA by periods 10, 20, and 30 days after inoculation and differs significantly. Endogenous SA values were ascending dramatically from 10 days to 20 days with significant differences, but insignificantly with 30 days where the endogenous SA value stabilizes greatly, while a spray of wheat leaves with H2O (control) exhibit lower values in all day's periods with insignificant differences between time intervals (Fig.1).

Table 1. Colorimetric absorbance of endogenous SA of 30 wheat leaves treated with SA or H2O (control) after three-time intervals (10, 20, and 30 days) of disease inoculation.

Treatments	Endogenous SA absorbance		
	Days after treatment		
	10	20	30
SA	22.55± 2.24^{*a}	28.14± 1.99$^{* b}$	29.70± 2.03^{*b}
H_2O (control)	10.79± 2.55^{**a}	11.27± 2.28^{**a}	12.84± 1.86^{**a}

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

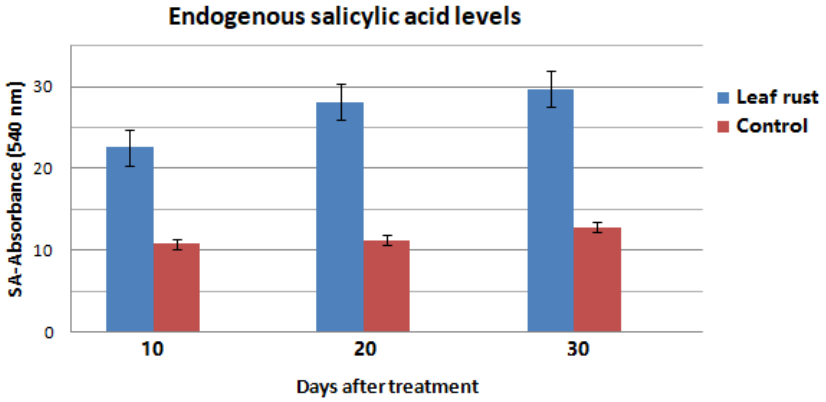

Fig. 1. Shows wheat plants content of endogenous SA levels which, ascending from 10 days after leaf rust inoculation in exogenous SA treatment to 20 and 30 days, while plants treated with H_2O has close values.

As shown in (Table 2), disease incidence values recorded at three periods; 10, 20, and 30 days after disease inoculation in case of exogenous salicylic acid treatment indicated that stem rust disease was significantly low, as it showed a low value of 4.44 after 10 days of inoculation, this value increased slightly after 20 days of inoculation, as it recorded 13.33, which remained constant until the 30th day of inoculation and without significant differences between them. On the other hand, the use of water instead of salicylic acid showed high values of disease incidence with clear significant differences in all time periods during the experiment. There was a large discrepancy in the values of disease incidence: 8.88 after 10 days, 24.44 after 20 days, and 36.66 after 30 days (Fig. 2). Disease incidence reduction after 10 days was 50%, and after 20 days 45.4%, while after 30 days the severity reduction recorded the high percentage of 63.6%.

Table 2. Leaf rust disease incidence at three-time intervals (10, 20, and 30 days) of inoculation in treated plants with exogenous SA compared to plants treated with H2O (control).

Treatments	Disease Incidence			LSD (α=0.05)
	10	20	30	
SA	4.44[a]	13.33[c]	13.33[c]	7.73
H_2O (control)	8.88[b]	24.44[d]	36.66[e]	11.26
LSD (α=0.05)	2.92	10.53	12.49	

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

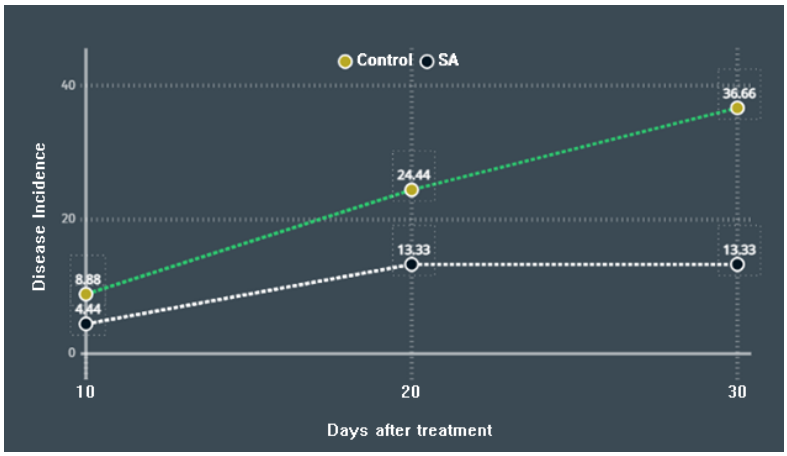

Fig. 2. Leaf rust caused by Puccinia graminis tritici incidence during time intervals (10, 20, and 30 days) of inoculation in wheat plants treated with exogenous SA or H2O (control).

Results of disease severity values listed in (Table 3) indicated that the disease severity decreased in plants treated with a concentration of 1 mM of salicylic acid compared to untreated plants with statistically significant differences. It is clear from the Table 3 and Fig. (3) that the disease severity after 10 days of inoculation recorded a low value of 3.093 and increased after 20 days of inoculation to 6.930 and then to 8.435 on day 30th with a significant difference between them. On the other hand, untreated plants (control) exhibited high values of disease severity in all periods (Fig. 3), where it recorded 10.630, 24.714, and 44.102 in the day periods, respectively, with significant differences between them. In

comparison between the time intervals, it is clear the severity reduced by 70.9% after 10 days of inoculation and by 71.9% after 20 days, but the reduction was the highest by 80.8% after 30 days of treatment.

Table 3. Leaf rust 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	3.093[a]	6.930[c]	8.435[e]	1.08
H_2O (control)	10.630[b]	24.714[d]	44.102[f]	5.47
LSD (α=0.05)	3.74	2.83	5.08	

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

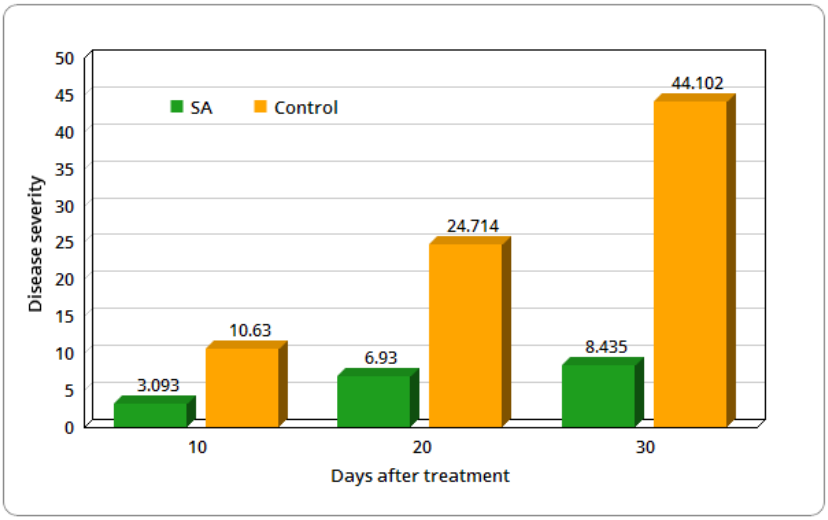

Fig. 3. Leaf rust caused by Puccinia triticina f. sp. tritici severity during time intervals (10, 20, and 30 days) after disease inoculation in wheat plants treated with exogenous SA or H_2O (control).

DISCUSSION

In plants, the positive correlations between endogenous levels of SA and resistance responses against biotrophic and hemi biotrophic pathogens are well established [15]. In this study, we applied a suitable concentration (1mM) of exogenous SA as a plant activator to induce internal systemic resistance in wheat plant against leaf rust disease by enhancing significant levels of endogenous SA, which plays a pivotal role in the emergence of systemic acquired resistance. [16] Summarized similar findings; dose of SA (>2 mM) not only induces the enhanced disease resistance, but also has adverse effects on plant growth and productivity, which caused by undesirable balancing between cost and benefit of limited energy that plant can use.

In the present study we tried unusual technique to detect endogenous SA in plant extraction by using colorimetric method with spectrophotometer, although there are other advanced techniques used in SA estimation; this was in harmony with [11] who found that spectrophotometric methods in comparison with the well-known sophisticated methods like HPLC, GC, MS where 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 *Puccinia triticina f. sp. tritici* in wheat plants grown under experiment 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 [9, 17]. Other authors concluded that endogenous levels of methyl salicylic acid (MeSA) increase in plants resisting pathogen infection [7, 18]. SA has been found to activate through a redox mechanism [19]. According to [20], the SA signaling pathway can be triggered by exogenous SA, which increases disease resistance, because this pathway is related to systemic acquired

resistance (SAR), which can occur when endogenous SA accumulates and is activated after plant pathogen infection. Our findings show that the accumulation of endogenous SA content in treated wheat plants after leaf rust infection is approximately 50% higher than in non-treated plants.

In our experiment, after application of SA in wheat infected with *P. triticina* we showed increase in the endogenous SA levels, and less values of disease incidence and severity, which could be explained as a process of oxidation of phenolic compounds, which may limit the fungal growth, this was in agreement with [21, 22], after applying the SA in wheat plants, they found increased roots, increased SOD and MDA activity, these enzymes protect the cell from oxidative stress and play an active role in metabolism and creating physical barriers at biochemical and cellular levels on host plants [23]. [24] Reported that applying SA to plants improved the initiation of pathogenesis-related gene expression, as well as the synthesis of defensive compounds involved in local and systemic acquired resistance. This was proved by the results of disease incidence and measuring the disease severity, which was found to be low in plants treated with SA compared to untreated plants. Many researchers have proven the success of salicylic acid in reducing the disease incidence and severity such as: [25; 26; 27]. It was also found that the increase in the content of endogenous SA at intervals of 10, 20 and 30 days led to a slight increase in the rate of disease incidence and severity, and there was no significant increase after 20 days of treatment, which indicates the success of the treatment in stopping the progression of the disease, where systemic acquired resistance is certain to arise in the plant.

CONCLUSION

Our study proved the reduction of wheat leaf rust 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 recommends to further investigations to determine the induced compounds responsible of resistance and other induced structures which, prevents disease progression in the plant.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

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