Biological Journal of Microorganism
7th Year, Vol. 7, No. 28, Winter 2019
Received: April 16, 2017/ **Accepted:** February 21, 2018. **Page:** 53- 62

Effects of salicylic acid on the growth and pathogenicity of *Zymoseptoria tritici*

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Abstract

Introduction: *Zymoseptoria tritici*, is a species of filamentous fungus and causes the widespread wheat disease *Septoria tritici* blotch (STB). Salicylic acid has a key role in plant defence reactions and is also involved in the induction of systemic acquired resistance. However, the contribution of SA to the interaction of *Z. tritici* -wheat in STB is not entirely clear. In this study, it was shown that the *Z. tritici* mycelial growth and conidia germination were significantly inhibited the presence of increasing concentration of SA in both liquid and solid media. In addition, the effect of SA on pathogenicity of *Z. tritici* in wheat was investigated.

Materials and methods**:** In this study, the inhibitory effect of SA on *Z. tritici* at different concentrations (1 to 20 mM) *in vitro*, and also, the efficacy of its exogenous application in the suppression of STB in wheat under the greenhouse condition were investigated. *In vitro* evaluation was done on YMDA and YMDB to determine the effect of SA on the germination of conidia and growth of mycelium, respectively. Susceptible bread wheat cultivar was grown in pot and inoculated with fungus spores and SA in a three-leaf stage for green house experiments.

Results: The results showed that the germination of conidia was completely inhibited by 4 mM SA. Furthermore, in modified YMDA plates at over 0.8 mM, the colonies diameter was reduced significantly. The result of *in planta* assay indicated that the foliar application of 4 mM SA can significantly reduce the disease symptoms on the wheat leaves.

Discussion and conclusion: Regarding our data, it seems that SA shows more inhibitory effect in *in vitro* experiments than *in planta*. Moreover, according to the positive effects of SA on STB, the survey results can be considered as a potential approach in the management of this disease.

Key words: Salicylic Acid, Septoria tritici blotch, Wheat, *Zymoseptoria tritici*

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Introduction

Septoria tritici blotch (STB) is one of the most damaging fungal diseases of wheat which is caused by a fungus, *Zymoseptoria tritici* (Desm.), Quaedvlieg et al. (1) previously identified as *Mycosphaerella graminicola*. The common method in the management of this disease is mainly based on the use of fungicides (1). There are many reports about disease control failure in agricultural systems causing fungicide resistance (2-4). However, this method is not appropriate because of the high cost and appearance of the new virulence patterns. Moreover, the adverse environmental effects of using fungicides are notable and alternatives including biological agents are used more and more (5-8). In studies related to hemibiotrophic pathogenic pathosystem, *Z. tritici* is a model pathogen which according to the results of conducted surveys, ranked among the ten most important phytopathogenic fungi in the world (9).

Salicylic acid (SA) is one of the secondary metabolites which is produced in a broad range of both prokaryotic and eukaryotic organisms such as plants (10). This compound as a phenolic phytohormone has different roles in physiological processes including growth, development, photosynthesis, transpiration, ion uptake and transport (11). In plant immunity system, the most documented role of SA is mounting immune responses (12). As an endogenous signal molecule, SA is primarily involved in triggering immunity responses against biotrophic and hemibiotrophic pathogens, and also in the establishment of Systemic Acquired Resistance (SAR). Establishment of SAR by inducing the expression of pathogenesis-related (PR) proteins led to protection against a broad range of pathogenic organisms (13).

Although, the detailed mechanism of SA-mediated plant resistance is not known, the key role of SA in plant defence is generally accepted (14-16). Studies have shown that the exogenous usage of SA or its functional analogues, like benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) and 2,6-dichloroisonicotonic acid can induce SAR (17-19). Inversely, plants with *NahG* gene, which converts SA to catechol, are more sensitive to various pathogens (20). Primary knowledge on the role of SA in plant defence arises from the recognition of an *Arabidopsis thaliana* mutant (*sid2-2*) that is disabled in SA biosynthesis (21).

The detailed role of SA in the *Z. tritici*-wheat pathosystem is still not well determined. However, results of some studies have shown that the exogenous application of SA has produced resistance in wheat (22). Qi et al. (23) studied the effects of SA on head blight fungus, *Fusarium graminearum* in wheat. Their finding showed that SA has significant effects on the efficiency of germination and mycelium growth of the fungi, and also, based on their results, 400µM SA was sufficient to prevent diseases in susceptible and moderate cultivars.

Since the use of resistant varieties is one of the best approaches in the management of STB, the effect of SA and SA-mediated plant resistance in this pathogen was studied.

In the present study, for the first time, the direct effect of SA on *Z. tritici* conidia germination and mycelium growth was investigated. Finally, the positive effects of proper concentrations of SA were evaluated in wheat defence responses. The results of this study should be considered as promising new leads for the development of a new strategy of this pathogen management.

Materials and methods

Strain and growth conditions: *Z. tritici* virulent strain IPO323 was used for the experiments. IPO323 isolate was cultured on Yeast Malt Dextrose Agar (YMDA) and maintained at 18°C for five days. Conidia obtained from these plates were spread into 50 ml Falcon tubes containing 30 ml (YMDB). The inoculated YMDB were placed at 18°C at 135 rpm for five days. In order to prevent bacterial contamination, 50 mg/L kanamycin was added to all media. The experiment was conducted in both solid and liquid media.

The direct effect of SA on the germination of conidia and growth of mycelium was tested in liquid YMDB medium. To do this, spore concentration was adjusted to 200 spores per ml using a haemocytometer. Then, 500 µl of the prepared spore suspension was transferred into 50 ml YMDB media which amended with 0.3, 0.5, 0.8, 1, 2, 4, 5, 10 and 20 mM SA (Sigma). The inoculated YMDB media were maintained in shaker-incubator at 18°C at 135 rpm. At 0, 0.5, 2, 4, 8, 12 and 24 hours after the interaction, 500 µl was sampled from each flask and then inoculated on YMDA in three replications. Different concentrations of SA were provided from 1M stock solution in methanol. Plates with 1µl ml^{-1} of methanol were used as control treatments. Five days after inoculation, the images of colonies on the plates were captured by a digital camera. All inoculations and photographs were made by the same operator during a routine working day.

In the other method to study the direct effect of SA on the germination of conidia and growth of mycelium, fungi were inoculated on YMDA containing 0, 0.3, 0.5, 0.8, 1, 2, 4, 5, 10 and 20 mM of SA. All concentrations with three replicate were inoculated by 500 µl conidia suspension and placed at 18°C for five days.

The size of colonies was measured with the powerful Java-based image processing tool (ImageJ 1.48v). Each Colony's growth was determined by calculating the relative size of 50-70 colonies on each plate and then the average values were used for analysis (24-26). In the analysis, only colonies obviously grown from single spore were considered, and fused colonies which were developed from two or more spores were discarded.

Pathogenicity assay: The experiment was performed in a randomized complete block factorial design with three replications. Susceptible bread wheat (*Triticum aestivum*) Chamran cultivar was grown in 8-cm diameter pots. The IPO323 isolate was grown for 5-7 days on YMDB; spore concentration obtained from the media was adjusted to 10^7 spores/ml and supplemented with 0.15% Tween 20. Twenty-four hours before inoculation, seedlings were treated with 2 and 4 mM concentrations of SA. Ten-day-old wheat seedlings were inoculated using hand sprayer. After inoculation, plants were transferred into plastic bags to keep high humidity and maintained at 20-22°C under the dark conditions for 48 hours. Subsequently, they were brought out from the plastic bags and kept in the same greenhouse with high humidity (>75% RH). The first symptoms were visible between 14 and 16 days of post-inoculation. Finally, scoring was performed on first leaves at 21th day of post-inoculation by visual estimation of the percentage of leaf area with necrotic lesions bearing pycnidia (27).

Data analysis: To evaluate the data, analysis of variance (ANOVA) was performed with SPSS software version 20. Comparisons among means were conducted by Duncan at P= 0.01.

Results

In this study, the inhibitory effects of SA on *Z. tritici* at different concentrations in *in vitro* conditions, and also, the efficacy of its exogenous application in the control of STB in wheat under the greenhouse condition were investigated. In liquid media interactions based on the results of data analysis of variance, significant differences ($p \leq 0.01$) were observed among SA concentrations and time points. Also, the interaction between concentration and time point was also significant ($p \leq 0.01$) (data not shown). In our survey, low levels of SA had little inhibitory effect on conidia germination, but at high levels, a significant and proper impact was observed. At the first time points, conidia had more capacity of germination which, by increasing the duration of interaction, the reduction trend was observed in germination rate (Fig.1). On YMDB, by increasing the time period of interaction, conidia germination in lower concentration (up to 1mM) was affected negligibly. In 2 and 4 mM, the number of germinated conidia was decreased remarkably. However, the germination was observed at all time points. At 5mM, only a few numbers of zero time point spores were able to germinate and by increasing the time period of interaction, no increase in germination was observed. At 10 mM concentration only in time point of zero, one of the replications of plates contained three germinated spores. No colony growth and germination was observed in 20 mM concentration at all of the time points. Also, no significant changes were observed in colonies diameter on plates related to liquid medium interactions (Fig. 1).

In modified YMDA, based on the results, the germination ratio was significantly ($p \leq 0.01$) affected by different SA concentrations. The germination ratio of conidia was not significantly affected ($p \leq 0.01$) up to 0.8 mM (Fig. 2) and reduction in germination rate started from 1 mM concentration. However, germination was extremely prevented at 2 mM SA and halted by 4 mM. At 4 mM and higher concentrations, conidia were not able to germinate and in microscopic observation had wrinkled and shrunken appearance. Briefly, it was observed that there is a negative correlation between SA concentration and germination rate of conidia, i.e. as the concentration increases the germination rate decreases.

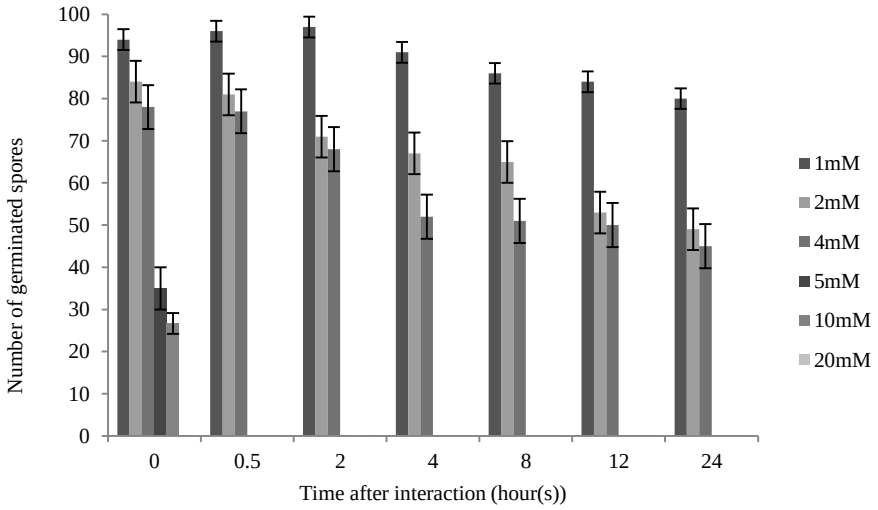

Fig. 1- The average values of germinated conidia on modified liquid YMDB media with 1, 2, 4, 5, 10 and 20 mM concentrations of SA at different time points.

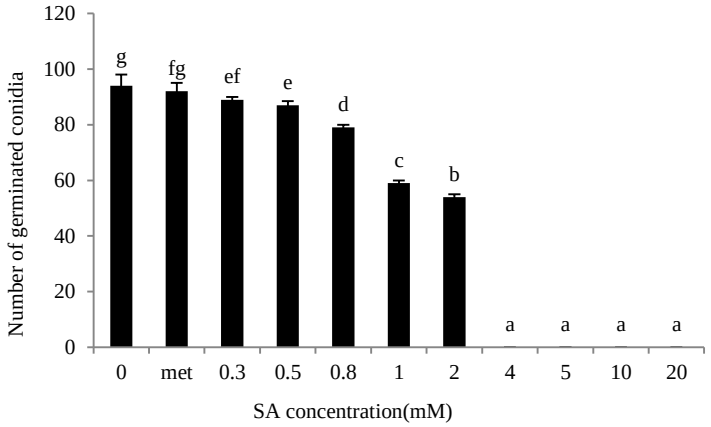

Fig. 2- The average values of germinated conidia on YMDA media with different concentrations of SA. met: Methanol in the same concentration was used as solvent. Values followed by the same letter(s) are not significantly different at $p \leq 0.01$.

Inhibitory effect of YMDA plates with different concentrations of SA on mycelium growth was not significant up to 0.5 mM. In spite of that the single colonies, diameter was reduced significantly at concentrations above 0.8 mM (especially at 1 and 2 mM). At 4 mM and higher concentrations, no colonies were grown (Fig. 3 and 4).

To further clarify the relationship between SA and *Z. tritici* growth and pathogenesis, we considered the foliar application of this component. As the results of the research confirmed the *in vitro* inhibitory effects of 2 and 4 mM of SA, for further investigation, their impact on wheat defence responses stimulation was evaluated in greenhouse examinations. Based on the results of analysis of variance, there were significant differences ($p \leq 0.01$) in pycnidium coverage percentage in interaction with different SA concentrations. The results showed that 2 and 4 mM SA were effective to reduce disease symptom in the susceptible cv. Chamran, but none of them completely prevents the symptoms development in infected wheat. With 2 mM SA, inoculated leaves were partially protected but the application of SA at 4 mM indicated lower values of diseases symptoms on wheat leaves (Fig. 5).

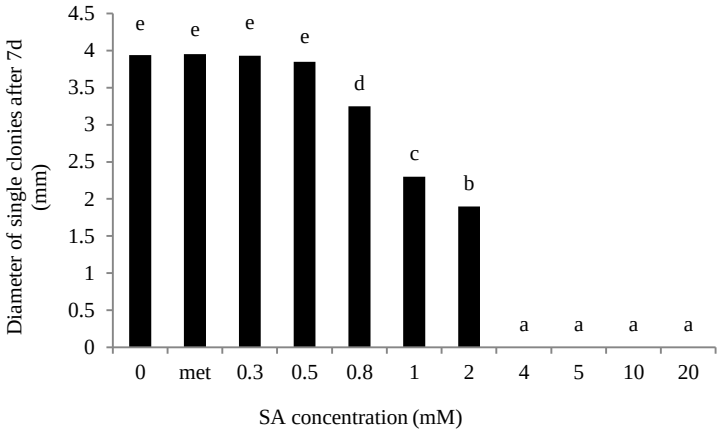

Fig. 3- The average values of grown colonies on YMDA media containing different SA concentrations. Values followed by the same letter(s) are not significantly different at $p \leq 0.01$.

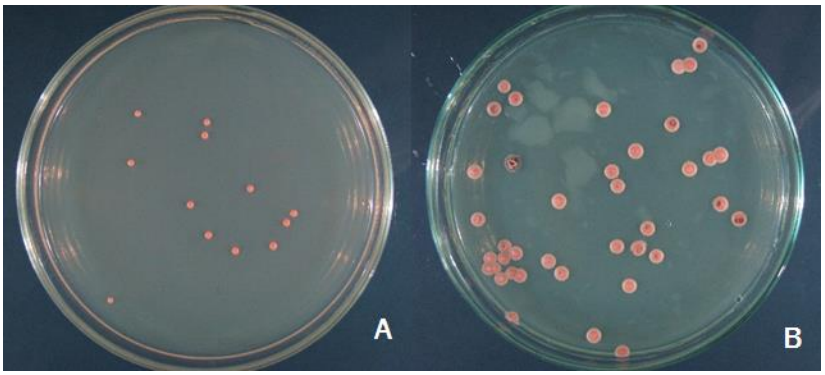

Fig. 4- The number and size of Z. tritici grown colonies in the presence of different concentrations of SA. A: 2 mM SA SA. B: 0.8 mM

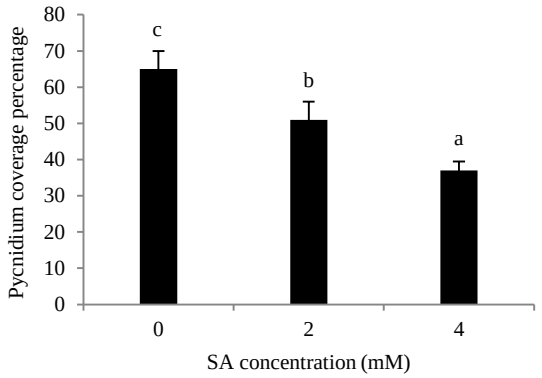

Fig. 5- Assessment of pycnidium coverage percentage at different concentrations of SA. Values followed by the same letter(s) are not significantly different at $p \leq 0.01$.

We did not observe any delay in symptom appearance in SA treated plants in comparison with control plants. Also, in comparing leaves with or without SA treatment after passing 21 days from inoculation, no significant visual difference was observed.

Discussion and conclusion

Wheat is the second most important source of nutrient after rice (28, 29). Among the pathogenic agents leading to the decline in wheat production, STB ranked as the 3rd after brown rust and powdery mildew (30, 31). Due to the high rate of evolution of this pathogen, the generation of resistant varieties is not efficient. Therefore, proper management strategies of the diseases have a critical role in the global wheat yield. Therefore, now the control of STB greatly relies on the use of fungicides (5, 32). Decrease in the use of chemicals is one of the principal goals in plant pathology. One of the promising strategies in this field is to promote the plant resistance to microbial pathogens by applying biotic or abiotic agents (33).

Plant infection by pathogens causes changes in the levels of phytohormones which lead to the suppression of the invasion and colonization of pathogen in the plant (34). The phytohormone SA is known to participate in the regulation of defence responses in plants (35-37). 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 (17, 38).

Recently, increasing attention to the application of non-chemical strategy in plant disease management has been started. White (39) initially demonstrated that exogenous SA application on tobacco can activate SAR. In limited fungi such as *F. graminearum*, direct effects of SA on the germination of spore and mycelium growth were studied (23). Most studies in this field have investigated the improving effect of SA on plant immunity system against pathogens (23, 40, 41). *In vitro*, SA was found to be the most effective inhibitor for the germination and growth of *Z. tritici*. At 4 mM of SA, it completely inhibited the germination and growth of *Z. tritici*.

In Shabana et al. (41) survey, SA was

tested against the linear growth of *Bipolaris oryzae* at different concentrations. They reported that at 9 mM SA, the linear growth of *B. oryzae* was inhibited completely. Qi et al. (23) showed that the *F. graminearum* mycelium growth and conidia germination were significantly inhibited, and eventually halted by an increase in the concentration of SA in both liquid and solid media. Cowan (42) explained the mechanisms responsible for the toxicity of the phenolics (salicylic acid, benzoic acid and hydroquinone) to microorganisms. He stated that it can occur via enzyme activity inhibition by the oxidized compounds, possibly on the basis of reaction with sulfohydryl groups or by more nonspecific interactions with the proteins. It is thought that the sites and number of hydroxyl groups on the phenol group plays a decisive role in their relative toxicity to pathogens (42). The toxicity increases by an increase in hydroxylation. Some studies have shown that phenols with high oxidation level have a more inhibitory effect on microorganisms (43). Farouk et al.(44) indicated that SA foliar application in the field can provide protection against cucumber downy mildew and also improve its growth and yield.

Anand et al. (40) showed the key role of the exogenous application of SA in reducing crown gall disease, *Agrobacterium*-mediated plant transformation. They showed that SA and SA-mediated plant defences can attenuate *Agrobacterium* infectivity on plants. In various postharvest diseases, exogenous application of SA on fruits at optimum concentration provided efficient management (45).

The exact concentration of SA required to inhibit *Z. tritici* growth differs between liquid and solid cultures of a given medium, however, inhibition was consistently observed in two phases. Inhibitory concentration in liquid culture is slightly higher than that of the solid medium. It seems that the duration of the interaction time with SA has an important role in this field. In the solid medium interaction, SA is always present in fungi substrate (7 days) while in broth media, this time is limited (Max. 24 hours). The permanent presence of SA in solid media causes an inhibitory effect in lower concentrations of SA when compared with liquid interaction.

The constant interaction of SA can also explain observed differences in colony diameter between two solid and liquid interactions. As it was shown, with an increase in the concentration of SA in the solid media, the diameter of colonies decreased, but no significant change was observed in pertinent interaction in liquid media.

In the present study, the interaction between concentration and time points was also significant. It was shown that in the liquid medium assay, with an increase in the duration of interaction, particularly in the high level of SA, the inhibitory effect on conidia germination increased.

Under greenhouse conditions, treatment with SA at 4 mM had significant effects on disease symptoms, as wheat plants showed lower level of diseases symptoms. Foliar application of 20 mM SA on *B.oryzae* showed the lowest values of diseases severity (DS) and diseases incidence (DI) on rice leaves (41). In other studies, 200 µM concentration of SA induced SAR in tomato against *Altenaria solani* (46). Furthermore, in another study, foliar application of 1000 µM SA induced systemic resistance to *A. cassiae* in sicklepod (47).

Like another foliar pathogen, *Z. tritici* reduces the rate of photosynthesis in the infected leaves. Wheat plants that were treated with SA showed a reduction in diseases symptom and an increase in the total photosynthetic pigments. This increase could be attributed to the anti-*Z. tritici* effect of SA.

In the present study, the effects of SA with different concentrations in both solid and liquid media and its impact on the rate of conidia germination and growth of mycelium on the wheat pathogen, *Z. tritici* were evaluated. The positive effects of some concentrations were recorded in this area. Furthermore, a significant decrease in the incidence of *Z. tritici* on wheat leaves was obtained in the treated plants. Based on the obtained results, applying SA can be recommended as an efficient, inexpensive and safe approach in the management of STB on wheat.

Acknowledgments

This work was supported by National Institute of Genetic Engineering and Biotechnology and Tarbiat Modares University. The authors would like to thank Mohammed Reza Atighi for his constant help in the greenhouse experiments.

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تاثیر اسید سالیسیلیک بر روی رشد و بیماریزایی قارچ *Zymoseptoria tritici*

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چکیده

مقدمه: *Zymoseptoria tritici* گونه‌ای از قارچ‌های رشته ای‌است و بیماری شایع سوختگی سپتوریایی برگ گندم (STB) را موجب می‌شود. اسید سالیسیلیک (SA) نقش کلیدی در واکنش‌های دفاعی گیاه داشته و مقاومت اکتسابی عمومی (SAR) را القا می‌کند. با این حال، سهم SA در برهمکنش *Z. tritici* – گندم در بیماری سوختگی سپتوریایی برگ کاملا روشن نیست. در این مطالعه مشخص شد در حضور غلظت‌های افزایشی SA از رشد هیف‌ها و جوانه‌زنی کونیدی‌ها *Z. tritici* هم در محیط مایع و هم در محیط جامد بطور معنی داری ممانعت می‌شود. علاوه بر این تاثیر SA بر روی بیماریزایی *Z. tritici* در گندم نیز بررسی شد.

مواد و روش‌ها: در این مطالعه اثر مهاری SA بر روی *Z. tritici* در غلظت‌های مختلف (از یک تا ۲۰ میلی مولار) در شرایط آزمایشگاهی، و همچنین اثر بخشی استعمال خارجی آن در کنترل STB گندم در شرایط گلخانه بررسی شد. ارزیابی‌های آزمایشگاهی برای تعیین تاثیر SA بر جوانه زنی کونیدی‌ها و رشد هیف‌ها به ترتیب در محیط های YMDA و YMDB انجام شد. جهت آزمایشات گلخانه‌ای واریته حساس گندم در گلدان کشت داده شده و در مرحله سه برگی با اسپور قارچ و SA تلقیح شد.

نتایج: طبق نتایج، جوانه‌زنی کونیدی‌ها توسط غلظت چهار میلی مولار SA به طور کامل مهار شد. بعلاوه در محیط‌های YMDA در غلظت‌های بالاتر از ۸/. میلی‌مولار، سایز کلنی‌ها بطور معنی‌داری کاهش یافت. نتایج آزمون *in planta* نیز مشخص کرد محلول پاشی غلظت چهار میلی مولار SA می‌تواند علائم بیماری در برگ‌های گندم را بطور قابل ملاحظه‌ای کاهش دهد.

بحث و نتیجه‌گیری: بر اساس داده‌های ما، بنظر می رسد تاثیرات مهارکنندگی SA در *in vitro* بیش از *in planta* است. علاوه بر این با توجه به اثرات مثبت SA بر STB، نتایج این پژوهش می‌تواند به عنوان یک روش بالقوه در مدیریت این بیماری محسوب شود.

واژه‌های کلیدی: اسید سالیسیلیک، سوختگی سپتوریایی، گندم، *Zymoseptoria tritici*

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* نویسنده مسؤول مکاتبات
تاریخ دریافت: ۱۳۹۶/۰۱/۲۷- تاریخ پذیرش: ۱۳۹۶/۱۲/۰۲