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Possible structure-activity profile of salicylate derivatives: their relationship on induction of systemic acquired resistance

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In nature plants can be induced systemically to become more resistant to diseases through some biotic or abiotic inducers. One of the commonly used chemicals inducers is salicylic acid (SA), which appears to mimic the systemic effects of localized infection. The potential of some chemical inducers of systemic acquired resistance (SAR) to reduce *Alternaria* leaf spot disease on tomato was assessed in glasshouse trials. Possible effects of using SA and some of its 5-position derivatives including, 5-chlorosalicylic acid, 5-methoxy salicylic acid, 5-aminosalicylic acid, 5-fluorosalicylic acid, 5-methylsalicylic acid and 5-nitrosalicylic acid in host resistance induction have been investigated. Foliar applications of salicylate derivatives with concentrations of 400 µM have been tested for disease resistant induction. Among the selected mono-substituted salicylate derivatives that have been tested, applications of SA and 5-methoxysalicylic acid have induced disease resistance significantly as well as increasing the SA accumulation in plant tissues, compared with not treated control plants. Treatments such as 5-chlorosalicylic acid and 5-nitrosalicylic acid had a deleterious effect on SA accumulation in plant tissues as well as in disease resistance induction. The analysis results obtained by high performance thin layer chromatography (HPTLC) indicated a high correlation between the accumulation of endogenous level of free SA content in treated plants and the reduction of Alternariosis symptoms on tomato plant. The results indicated that, among the salicylate derivatives, the biochemical activators containing electron donating groups are more suitable for inducing disease resistance in tomato crop.

Key words: Salicylic acid; *Alternaria*; Tomato; HPTLC.

Introduction

One of the major problems concerning the production of food crops is the difficulty of controlling plant diseases to maintain the high quality and yield which the producer and consumer expect. Resistance to disease can be induced systematically in a number of plant species by biological and chemical means

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(Ryals *et al*., 1994). One of the potential management approaches is the use of SAR to activate host defense mechanisms, which would not involve the application of toxic compounds to plants (Durrant and Dong, 2004).

Chemical activation of disease resistance in plants represents an additional option for growers to protect their crops from losses due to plant diseases. One of the commonly used chemical inducers is salicylic acid (SA), which appears to mimic the systemic effect of localized infection (Kessmann *et al*., 1994; Vallad and Goodman, 2004; Gaffney, 1993), and appears to be a central signaling component in SAR (Klessig and Malamy, 1994). Exogenous application of SA and some other chemicals including: polyacrylic acid, acetyl salicylic acid, 2,6-dichloroisonicotinic acid, methyl salicylate, jasmonic acid and jasmonic methyl ester, benzothiadiazole derivatives, DL-β-aminobutyric acid and oxalic acid, can induce accumulation of pathogenensis-related proteins (PRp) and lead to reduced incidence of several diseases on many crops (Gozzo, 2003; Vallad and Goodman, 2004).

Based on the structure and reactivity theory, the activity of substrates would be affected by their molecular structures. Recently Almeras *et al*. (2003) found that, reactive electrophile species such as small α, β-unsaturated carbonyl compounds (eg. acrolein and methyl vinylketone) are highly active and proved to be potent stimulators of expression of the pathogenesis-related gene (PR4). Also the structure relationship of 47 mono-substituted and multi-substituted salicylate derivatives regarding their effects on disease resistance to tobacco mosaic virus and pathogenesis-related protein (PR1) accumulation were evaluated (Silverman *et al*., 2005). Among the selected derivatives, fluorinated or chlorinated in the 3- and /or 5- position induced more PR1 protein than SA. In contrast, substitutions in positions ortho (3- position) or para (5- position) to the hydroxyl group with electron-withdrawing groups other than chlorine or fluorine decreased induction, and electro-donating groups in these positions also had a deleterious effect on PR1 induction (Silverman *et al*., 2005).

More recently in South Africa, Basson and Durbery (2007) have found that 3, 5- dichlorosalicylic acid, as a priming agent of plant defense, and 2, 6-dicholoroisonicotinic acid elicited an effective and rapid responses in Arabidopsis plant. However, to date, cytological and biochemical studies to address the mechanisms involved in the resistance have not been conducted in the tomato-*Alternaria alternata* pathosystem.

Alternaria leaf spot or early blight is a common foliar disease of tomato (*Lycopersicon esculentum* Mill.) and potato (*Solanum tuberosum* L.) occurring in most regions of the world. *A. alternata* is one of the causal agents of Alternariosis disease in tomato and potato is responsible for significant

economic losses sustained by tomato or potato producers each year (Esmailzadeh *et al*., 2008).

Since endogenous SA is an important signal molecule that plays a critical role in plant defense against pathogen invasion (Malamy *et al*., 1990; Vallad and Goodman, 2004), the *in vivo* disease resistance effects of SA and its derivatives were investigated on *Alternaria* leaf spot on tomato as a model crop (Arie *et al*., 2007), for plant-pathogen interactions.

In continuation of our studies on SAR mechanisms (Soleimani and Esmailzadeh, 2007; Esmailzadeh *et al*., 2008), and in order to define a possible structure-activity relationship, regarding the development of new synthetic resistance activators, we decided to choose a suitable range of salicylate derivatives containing electron donating groups (EDGs) and electron withdrawing groups (EWGs).

Materials and methods

Chemicals

SA and all other salicylate derivatives were obtained from Merck, KGaA, Germany. These salicylate derivatives were selected from compound with both electron-withdrawing groups and electro-donating groups and with different substituted ions as described in Table 1. SA and other salicylates were dissolved in water to final concentration of 400 µM (Esmailzadeh *et al*., 2008).

Table 1. List of applied chemicals and their manufacturers.

Applied chemicals	Chemical formula	Manufacturer
1) Salicylic acid	$C_7H_6O_3$	Merck KGaA, Darmstadt, Germany
2) 5- Chloro-2-hydroxybenzoic acid(5- Chlorosalicylic acid)	$C_7H_5ClO_3$	Merck-Schuchardt, Germany
3) 5- Amino-2-hydroxybenzoic acid(5- Aminosalicylic acid)	$C_7H_7NO_3$	Merck KGaA, Darmstadt, Germany
4) 2- Hydroxy-5-methoxybenzoic acid(5- Methoxysalicylic acid)	$C_8H_8O_4$	Merck KGaA, Darmstadt, Germany
5) 2- Hydroxy-3,5-dinitrobenzoic acid(3,5- Dinitrosalicylic acid)	$C_7H_4N_2O_7$	Merck KGaA, Darmstadt, Germany
6) 5- Methylsalicylic acid	$C_8H_8O_3$	

Plant Materials and Inoculation

Seeds of tomato cv. Ergon were grown in pots of 25cm diameter and kept in an environmentally controlled glasshouse (Temp. 25-28°c) till desired stage.

After 3 weeks the four-leaf stage plants were selected and used in the experiments.

Inoculum suspensions containing conidia of *A. alternata* f.sp. *lycopersici*, were made from fresh culture on potato carrot agar (PCA). The inoculum density was adjusted with sterile distilled water to 1×10^5 conidia/ml using haemocytometer. Ten days prior to conidial application, four- leaf tomato plants were sprayed by a concentration of 400 μM of SA solution and salicylate derivatives. Tween 20 was added (0.01%) as a surfactant to SA and salicylate derivatives. Control plants were sprayed with Tween20/distilled water mixture and kept at the same conditions as the corresponding treatment.

Forty-eight hours before inoculation they were keeping in growth chamber with relative humidity near 90% and remained in a fairly dark condition at 20-22 ^{0}C. Plants were evaluated daily for apparent symptoms of disease. The extend of disease symptoms was determined by measuring the percentage of infected area of necrotic lesion (visual assessment), and counting the lesions number on four newly emerged compound leaves 14 days after inoculation, with the aid of low magnification of microscope. Treatments consisted of four pots each containing two plants, and were arranged in a randomized complete design and replicated four times. This experiment was conducted three times, and representative data from one of the trials are shown in the results.

Determination of Free-SA

A densitometric method, using high performance thin layer chromatography (HPTLC) a technique previously reported for the rapid and accurate identification and quantitative determination of acetylsalicylic acid, ascorbic acid, and salicylic acid by Krzek and Starek (1999) was performed in this study.

Inoculated and non-inoculated leaf tissues samples from different treatments were harvested at 14 days after SA application, and were examined and assessed in terms of their free salicylic acid content. Tissue samples (0.5 g fresh weight) were ground in 20 ml methanol and sonicated for 20 min. The extracts were filtered and the solvent was evaporated to dryness *in vacuo*. Then the extracts were resuspended in 5 ml of 3- fluoroacetic acid. The free SA phase was extracted by ethylacetate: cyclohexan: isopropanol (50:50:1, v/v), two times, each with 10 ml of solvent mixture. Finally the organic phase was collected and re-evaporated to dryness, and resuspended in 1 ml of acetone.

HPTLC system include TLC Scanner 3 and Linomat 5 sprayer samples winCAST 1.2.2 software (CAMAG, Switzerland, Muttenz) was used for determination of free SA in leaf samples. TLC was performed on silica gel

plate (Merck, $60F_{254}$, 20×30cm). The solvent system which has been used was: normal hexane: dimethyl ether: formic acid (0.4: 1: 1).

Sample spraying was performed with Linomat 5 sprayer samples and under liquid nitrogen atmosphere, and a micro liter Hamilton syringe was used for sampling. Ten ppm of SA was applied as internal standard and maximum absorption was obtained at 305 nm wavelength of spectra region (Krzek and Starek, 1999).

Statistical Analysis

Data were subjected to analysis of variance, and means were separated by Duncan's and least significant difference (LSD) test at P= 0.05. The relationship between free SA accumulation and reduction in early blight disease indexes was determined by regression analysis using Plot IT software (Scientific Programming Enterprises, Haslett, MI).

Results

The results from glasshouse and laboratory study indicated that, challenge inoculation of SA and 5-methoxysalicylic acid treated tomato plants using conidia of *A. alternata* f.sp. *lycopersici* resulted in fewer lesions per leaf (data not shown) as well as in reduced blighted leaf area as compared with control plants not receiving salicylate treatment (Fig.1). The foliar application of 400 µM SA and some other salicylate derivatives significantly increased the endogenous SA content of leaves (Fig. 2). It has been demonstrated previously that the concentration of SA is proportional to the number of lesions in a TMV inoculated leaf (Yalpani *et al*., 1991). However, in this study there was no significant difference between treatments concerning early blight lesion numbers (Table. 2).

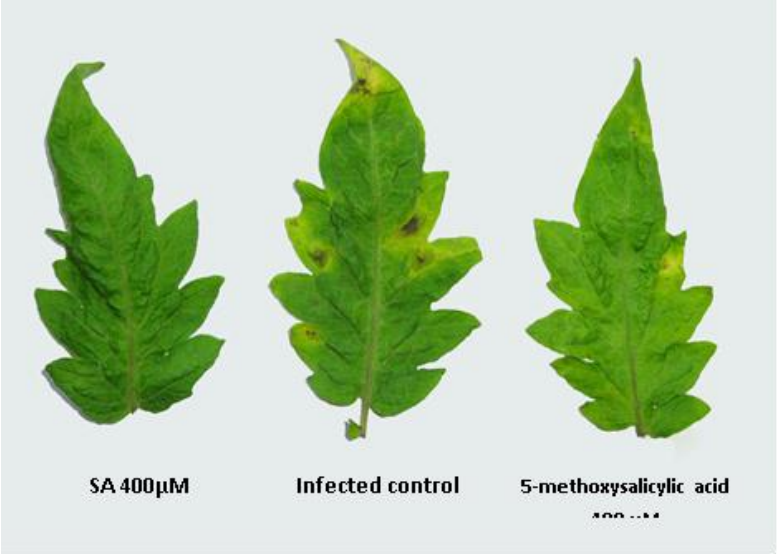

Fig. 1. Protection of tomato leaves against *Alternaria alternate* f.sp. *lycopersici* by a foliar spray with 400 µM salicylic acid (left), distilled water (middle) and 400 µM of 5-methoxysalicylic acid (right) fourteen days after inoculation with *Alternaria* conidial suspension (1×10^5 conidia ml^{-1}).

Table 2. Effects of salicylate derivatives treatments (400 µM) on the free-SA content (µg/g Fresh Weight), blighted area (mm^2) and lesion number in tomato leaves, inoculated by *Alternaria* conidial suspension (1×10^5 conidia/ml) comparing with untreated-inoculated and healthy control

Treatment	Mean of lesion area (mm^2)	Mean of lesion number	¥Mean of free SA(µg SA/g Fresh Weight)
5-chlorosalicylic acid	11.68 ± 3.8	20 ± 7.7	8.33 ± 1.7
Infected control	10.31± 3	17.75 ± 7.5	8.7 ± 2.8
5-aminosalicylic acid	8.68 ± 3	10.75 ± 3	9.16 ± 3.2
5-methylsalicylic acid	7.12 ± 2.7	15.75 ± 8	8.4 ± 2.5
3,5-dinitrosalicylic acid	5.81 ± 2	14.25 ± 5.9	8.46 ± 1.8
5-methoxysalicylic acid	3.21 * ± 1.5	7.75 ± 3.3	12.2* ± 0.7
Salicylic acid	2.25 * ± 1.1	10.25 ± 5	10.36* ± 3.8
Healthy control	0 *	0*	1.5 ± 2.2

¥SA concentrations are expressed as µg g^{-1} fw. Values are means ± s.e.m. of three independent experiments.

Data points represent averages with SE of measurements on more than nine samples out of three independent experiments.

*Denotes a significant ($p \leq 0.05$) difference

To determine the relationship between SA levels in inoculated leaves and SAR induction, we treated and inoculated compound leaves of tomato plants and then measured free-SA contents and SAR induction in newly emerged

leaves. The range of doses was chosen based on the results of our previously reported experiment (Esmailzadeh *et al*., 2008).

In this study we have performed a densitometric method, using high performance thin layer chromatography (HPTLC) for the first time as a rapid and accurate identification and quantitative determination of salicylic acid, in leaf tissues. The results obtained in present study indicated that exogenous application of SA and some derivatives including; 5-methoxysalicylic acid and 5-fluorosalicylic acid have reduced disease index significantly comparing with untreated infected control plants (Table. 2). That means application of these salicylates on tomato plants can activate SAR which is effective against early blight disease. However, application of salicylate derivatives such as 5-choloro and 5- nitrosalicylic acid was ineffective to induce disease resistance (Table. 2).

Discussion

In recent years, there has been an increasing interest in the non-chemical control methods in plant disease management. In the present study, we undertook experiments with exogenous application of SA and salicylate derivatives in order to explore their potential effects to activate systemic acquired resistance and suppression of early blight disease of tomato plants. As a quantitative means of assessing induced resistance, we measured the blighted area on treated and non-treated tomato plants. The results of present study indicated that, challenge inoculation of SA and 5-methoxy salicylic acid treated tomato plants using conidia of *A. alternata* f.sp. *lycopersici* resulted in fewer lesions per leaf (data not shown) as well as reduction in blighted leaf area as compared with control plants not receiving salicylate treatment (Fig. 1). We found that, foliar application of 400 µM SA and some other salicylate derivatives significantly increased the endogenous free-SA content of tomato leaves (Fig. 2). So it has been demonstrated that the ability of a compound to accumulate SA correlates with its potential to induce resistance of tomato crop against Alternariosis disease. This is in accordance with the results obtained by Spletzer and Enyedi (1999), showing that the application of SA to the root system significantly increased the endogenous SA content of leaves, resulted in 77% reduction in blighted leaf area (caused by *Alternaria solani*) as compared with control plants not receiving SA. However, this is the first report showing that application of 5-methoxysalicylic acid on plant is effective on inducing the systemic acquired resistance.

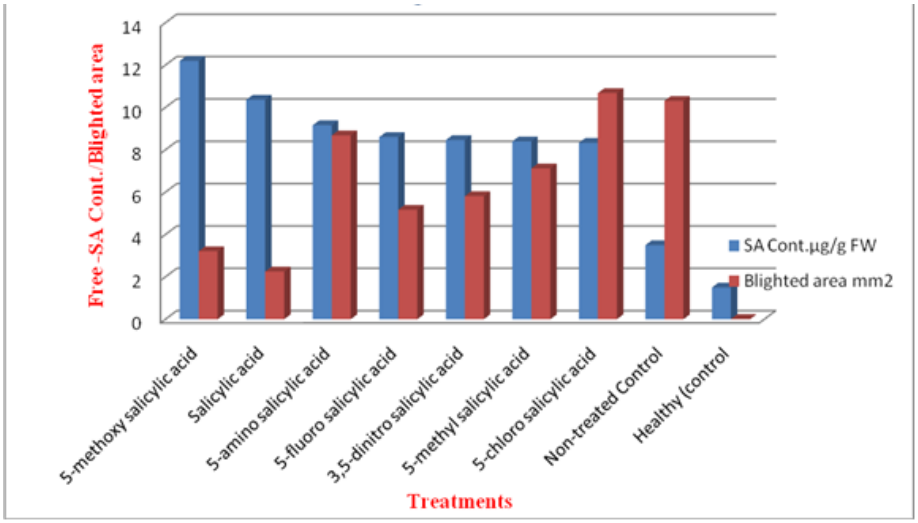

Fig. 2. Effects of salicylate derivatives treatments (400 µM) on the free-SA content (µg/g Fresh Weight) and blighted area (mm^2) in tomato leaves, inoculated by *Alternaria* conidial suspension (1×10^5 conidia/ml) comparing with untreated and healthy control.

Determination of the free-SA contents in leaf tissues obtained by using high performance thin layer chromatography (HPTLC) indicated significant differences between inoculated and not inoculated treated plants (Table. 2). In non-treated and inoculated plant the free-SA contents (3.5 µg/g FW), is roughly twice more than in untreated healthy (control) plants (1.5 µg/g FW), the more free-SA accumulation in inoculated plants due to pathogen-induced necrosis. This is in agreement with previous publications suggesting that SA is synthesized at the site of pathogen-induced necrosis (Metraux *et al*., 1990; Rasmussen *et al*., 1991: Yalpani *et al.*, 1991).

As it has been indicated in Fig. 3 the 5-substituted SA derivatives (5-SSAD) have a positive correlation with accumulation of free-SA in tomato leaves and disease index. The results obtained in present study indicated that exogenous application of SA and some derivatives including 5-methoxysalicylic acid and 5-fluorosalicylic acid which can release an electron via resonance and decreased the acidity of salicylic acid have reduced disease index significantly comparing with infected control (Fig. 2). That means application of these salicylates on tomato plants can activate SAR which is effective against early blight disease. However, application of salicylate derivatives such as 5-choloro- and 5- nitrosalicylic acid was ineffective to induce disease resistance (Table. 2). This is consistent with the results obtained by Almeras *et al.* (2003) as they found that reactive electrophile species such as small alpha, beta-unsaturated carbonyl compounds are highly active and proved to be potent stimulators of expression of the pathogenesis-related gene (PR4),

thus resulting in resistance induction in *Arabidopsis* plant. Similar results have been reported in recent years, as Silverman *et al*. (2005) found that, substitutions in positions ortho (3-position) or para (5-position) to the hydroxyl group with electron-withdrawing groups other than chlorine or fluorine decrease resistance induction, and electro-donating groups in these positions also have a deleterious effect on PR1 induction in tobacco.

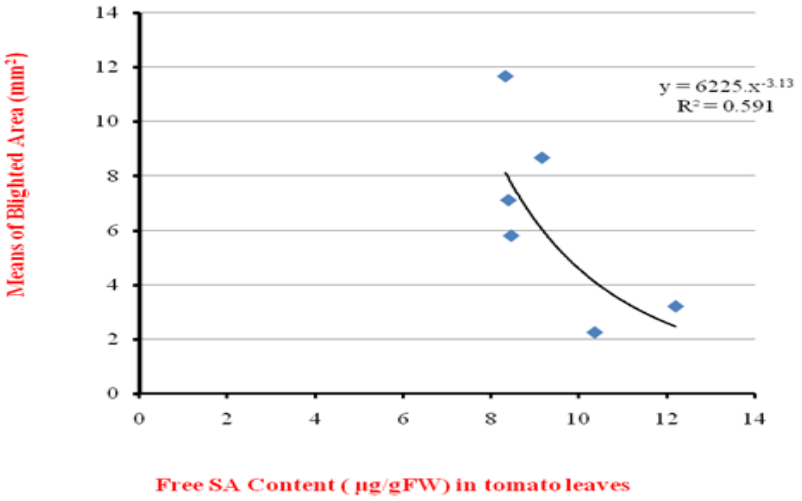

Fig. 3. Correlation between the level of free SA in tomato leaf tissues (µg/g FW) and means of blighted area (mm^2) in treated tomato leaves.

Involvement of pathogenesis-related (PR) proteins has been implicated in *Alternaria* resistance in tomato through the observation that it correlates with high and rapid accumulation of PR-proteins. However, it is speculated that those PR-proteins, many of which possess hydrolytic activity, are not necessarily directly involved in arresting the pathogen but rather release elicitors from the pathogen cell wall, thus triggering a hypersensitive response (Lawrence *et al*., 2000). The HR is considered to be a major element of plant disease resistance as it disrupts the pathogen from food supply and confines it to the initial infection site.

From the data presented in Table 2 it can be concluded that a threshold concentration of endogenous SA is required for host protection. Indeed, when SA concentration is below µ8 g/g FW the protection is low whereas the protection is significant when SA levels raise 10-12 µg/g FW. Furthermore, this relationship was very reproducible, as we obtained similar results in three separate experiments. Moreover, the results may provide insights that can be used both to engineer plants resistant against a wide spectrum of pathogens and

to develop compounds capable of inducing systemic resistance. Although, according to the described data we can conclude that chemical activators containing electron-donating groups are more suitable for inducing disease resistance in tomato crop. However, several factors are likely to regulate the activity of a particular molecule and further studies will be needed to better understanding the structure/activity rules. So, a future challenge will be needed to identify the relative contribution of more potential molecules involved, and introduce a new set of biochemical SAR inducer with a unique insight to systemic acquired resistance.

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