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Brief Note

Stimulation of jasmonic acid production in *Zea Mays* L. infected by the maize rough dwarf virus - Río Cuarto. Reversion of symptoms by salicylic acid

A. Vigliocco*, B. Bonamico*, S. Alemano*, O. Miersch** and G. Abdala*

* Facultad de Ciencias Exactas, U.N.R.C, 5800-Río Cuarto, Argentina.
** Leibniz-Institut für Pflanzenbiochemie, Weinberg 3, 06120-Halle, Germany.

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ABSTRACT: In the present paper we study the possible biological relevance of endogenous jasmonic acid (JA) and exogenous salicylic acid (SA) in a plant-microbial system maize-virus. The virus disease "Mal de Río Cuarto" is caused by the maize rough dwarf virus - Río Cuarto. The characteristic symptoms are the appearance of galls or "enations" in leaves, shortening of the stem internodes, poor radical system and general stunting. Changes in JA and protein pattern in maize control and infected plants of a virus-tolerant cultivar were investigated. Healthy and infected-leaf discs were collected for JA measurement at different post-infection times (20, 40, 60 and 68 days). JA was also measured in roots on day 60 after infection. For SDS-PAGE protein analysis, leaf discs were also harvested on day 60 after infection. Infected leaves showed higher levels of JA than healthy leaves, and the rise in endogenous JA coincided with the enation formation. The soluble protein amount did not show differences between infected and healthy leaves; moreover, no difference in the expression of soluble protein was revealed by SDS-PAGE. Our results show that the octadecanoid pathway was stimulated in leaves and roots of the tolerant maize cultivar when infected by this virus. This finding, together with fewer plants with the disease symptoms, suggest that higher foliar and roots JA content may be related to disease tolerance. SA exogenous treatment caused the reversion of the dwarfism symptom.

Abbreviations: AcOH, acetic acid; GC-MS, gas chromatography-mass spectrometry; JA, jasmonic acid; JIP, jasmonate induced protein; MeOH, methanol; MeJA, methy-jasmonate; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Introduction

Jasmonic acid (JA) is considered a member of the signal transduction pathway in the defense mechanism of plants. The biosynthesis of JA from α-linolenic acid occurs through octadecanoid pathway (Vick and Zimmerman, 1984) and is activated by pathogen attack (Farmer and Ryan, 1992), wounding (Conconi *et al.* 1996), and osmotic stress (Kramell *et al.*, 1995, 2000).

JA has been found to be involved in the induction of several genes encoding proteins related to stress plant defense, such as PR1 (unknown enzymatic activity antifungal), PR2 (β-1,3-glucanase), PR3 (chitinase), PR5 (traumatin-like) and PR9 (peroxidase) (Schweizer *et al.*, 1997). In cell culture of numerous plant species, fungus elicitors produced JA and phytoalexins accumulation (Blechert *et al.*, 1995). Moreover, pathogen and MeJA induced the expression of defensin gene *PDF1.2*

Address correspondence to: Dra. Guillermina Abdala. Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto. (5800) Río Cuarto, Córdoba, ARGENTINA.
Tel: (+54- 358) 467 6532. E-mail: gabdala@exa.unrc.edu.ar
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and the thionin gene *Thi2.1* in *Arabidopsis* (Penmickx *et al*., 1996; Epple *et al*., 1997). On the contrary, rice inoculated with the blast fungus did not increase the levels of (-)JA during the time required for PR gene expression (Schweizer *et al*., 1997).

Different studies established that JA might be involved with replication and/or viral concentration. Petrovic and Ravnikar (1995) and Petrovic *et al*. (1995) reported interactions between potato virus M and Y^{NTN} and jasmonate treatments of systemically infected *in vitro* culture plantlets of this specie. The addition of 0.1 µM JA to the growth medium reduced the virus content. On the other hand, it has been suggested that viral infections could determine changes in the jasmonate-endogenous levels (Petrovic and Ravnikar, 1995; Clarke *et al*., 1998, 2000).

Another compound related to the plant defense is salicylic acid (SA). It has been postulated that there exists some type of croos-talk between both signals (JA and SA) in the establishment of defense status (Mur *et al*., 1997; Reymond and Farmer, 1998). On the other hand, SA is essential for the systemic acquired resistance (SAR) of dicotyledoneous plants against a broad range of pathogens (Yang *et al*., 1997).

The "Mal de Río Cuarto" is a maize disease caused by the maize rough dwarf virus - Río Cuarto (MRDV-RC) which causes serious inhibition of maize growth and important yield losses. The characteristic symptoms of the infected plants are the appearance of galls or "enations" in leaves, shortening of the stem internodes, poor radical system and general stunting (Nome *et al*., 1981). Enation formation appears in the abaxial epidermis of leaves and are constituted by a cell hyperplasia of leaf vascular bundles (Vigliocco *et al*., 1993). Thus, we investigated the endogenous levels of JA in healthy and infected leaves and roots of maize plants cv. Morgan, tolerant to the Mal de Río Cuarto virus, during 68 days after infection. In addition, analysis of the protein pattern was performed in leaves. Then, we observed the expression of the disease symptoms after exogenous SA application in order to see if this compound was able to revert those symptoms.

Materials and methods

Plant material

Maize seeds of a tolerant cultivar (cv. Morgan) were sown in pots containing sterile soil. At the coleoptile emergence, they were infected by putting the seedlings for 72 h in contact with the vector insect *Delphacodes kuschelli* collected in field. Ten days after infection, plants were transplanted to field under isolated condition. Control plants were grown under the same conditions, without a previous exposure to vector insects.

To test the virus occurrence in plants without visible symptoms of the disease a segment of root of each plant was subjected to serological assays (DAS ELISA) performed according to Clark and Adams (1977).

Leaf discs from twenty healthy and infected maize plants were collected for JA measurement 20, 40, 60 and 68 days after infection. JA determination was also performed in roots on day 60 after infection. Protein analysis was performed in leaf discs harvested also on day 60 after infection.

JA evaluation

JA quantification was done in duplicate. 1 g FW of tissue was homogenized with MeOH and 200 ng D_6-JA. The MeOH fraction was purified by gel chromatography on DEAE-Sephadex A25, eluted first with 3 ml MeOH and 3 ml 0.1 N AcOH in MeOH, then with 5 ml 1 N AcOH in MeOH. JA eluted in this fraction, was dried and applied onto a C_{18}-minicolumn which in turn was eluted with MeOH in 0.2% AcOH and water. JA fractions were pooled, evaporated and analysed by C_{18}-HPLC (Eurospher 100, 5 mm column) with an isocratic mixture of MeOH:water in 0.1% H_3PO_4 (60:40 v/v); solvent flow rate was 1 $ml.min^{-1}$. Fractions corresponding to D_6-JA Rt (9.2 min) were derivated with diazomethane and subjected to GC-MS for JA identification and quantification. The GC-MS system was

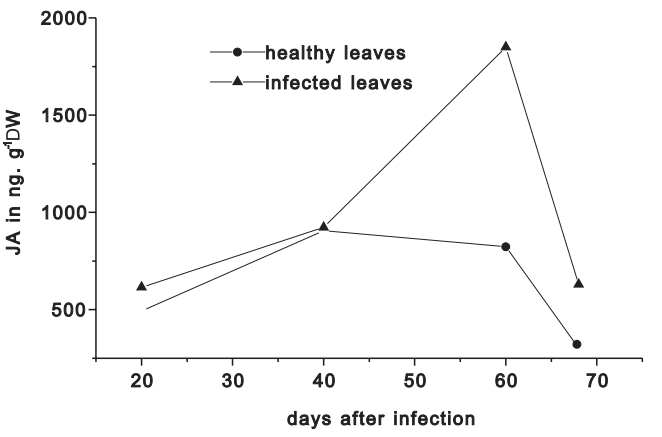

FIGURE 1. Endogenous JA level in leaves of tolerant maize plants infected by MRDV-RC. Measurements done at 20, 40, 60 and 68 days after infection.

equipped with a Hewlett Packard 9000/300-9133 computer set. A Hewlett Packard quadrupole mass spectrometer (model 5970B) combined with an HP 5890 gas chromatograph was used. GC was performed on a 25 m x 0.2 mm inner diameter cross-linked methyl silicon-fused silica column, film thickness was 0.11 mm, helium was used as carrier gas (2.5 $ml.min^{-1}$) and splitless injection. Settings were: injection at 275°C; direct inlet interface at 230°C; ion resource at 250°C. The temperature program was as follows: from 60°C (1 min) to 180°C (30°C min^{-1}); from 180°C (1 min) to 200°C (10°C min^{-1}); from 200°C (1 min) to 27°C (5°C min^{-1}). The electron impact energy was 70 eV.

Protein analysis

Leaf tissue (500 mg) were homogenized with 1 M NaCl 0.1 M phosphate buffer pH 7 and saoked for 2 h at 25°C. The extract was centrifuged at 1,000 g for 30 min. Supernatant containing the soluble proteins in the leaf extracts were measured in triplicate by the method of Bradford (1976). SDS-PAGE was performed by method Laemmli (1970). Sample of 6 μg were separated in 12% acrylamide gel by electrophoresis. Protein bands were resolved with silver staining according to Nesterenko *et al*. (1994).

Exogenous application of SA

To analyse the expression of the disease symptoms after SA exogenous application, twenty infected maize plants of approximately 10-12 cm of height were watered three times with 1 mM SA every 5 days. Infected plants without SA treatment and healthy plants (without virus presence) were considered as controls. Plants were observed periodically for the appearance and evolution of symptoms. Finally, the height of ten plants from each treatment was recorded at the end of the experiment.

Statistic analysis

Statistic analysis of protein measurements was performed with three samples constituted by healthy and infected leaf discs taken at random. Data were analyzed using an ANOVA test. Data of plant height were subjected to Multiple-Sample Comparisons Analysis and Multiple Range Test a posteriori. Likewise, a Box-and-Whisker Plot graphical option was also performed. The software used was Statgraphics Plus, version 3, Manugistics 1997.

Results

The virus-infected plants showed the symptoms characteristic of the disease: stunted growth, shortening of internodes (dwarfism), appearance of swollen vein or "enation" on the abaxial epidermis of leaves and underdevelopment of root system (Nome *et al*., 1981; Vigliocco *et al*., 1993).

A remarkable increase in JA level was found in infected leaves 60 days after infection, whereas in the healthy ones no variation in JA was observed between the 40 and 60 days. The highest endogenous JA level was detected at the stage of enation initiation, when the veins began to swell; at this moment, the increase of JA was two fold in the infected leaves with respect to control (Fig. 1). However, on day 68 JA decreased near the value obtained on day 20 after infection.

In the same way, in roots of infected plants an important raise of endogenous JA was found, on day 60 after infection. The infected roots showed a JA increase around 47% in respect to the control ones (data not shown).

Control Infected + SA Infected

FIGURE 2. Reversion of the dwarfism symptom caused by SA. Infected plants were watered three times with 1 mM SA every 5 days. Infected plants without SA treatment and healthy plants (without virus presence) were considered as controls.

The soluble protein quantification did not show statistically significant differences ($P < 0.05$) between infected and healthy leaves. Moreover, SDS-PAGE did not reveal differences in the expression of protein obtained from infected and heatlhy leaves (data not shown).

The tolerant infected plants treated with SA did not show the dwarfism symptom of the disease. On the other hand, the infected plants without SA-treatment showed a pronounced shortening of the internodes length (Fig. 2). Statistic analysis showed that means of plant height were significantly different among treatments, at 95% of confidence level. The Box-and-Whisker Plot graphical shows important features about the data (Fig. 3).

Discussion

An important role for JA in the interaction plant-pathogen has been postulated. However, the proposed function of JA as a signal in these interactions has been based mainly on model systems such as treating detached leaves with elicitors or cell suspension cultures (Mueller *et al*., 1993; Blechert *et al*., 1995), although few cases have reported true model plant-pathogen interactions (Cohen *et al*., 1993; Schweizer *et al*., 1993; Kogel *et al*., 1995). Thus, in the present paper we have made an approach studying the possible biological relevance of JA and SA in a plant-microbial system such as the resulting from the interaction of maize and the Mal de Río Cuarto virus.

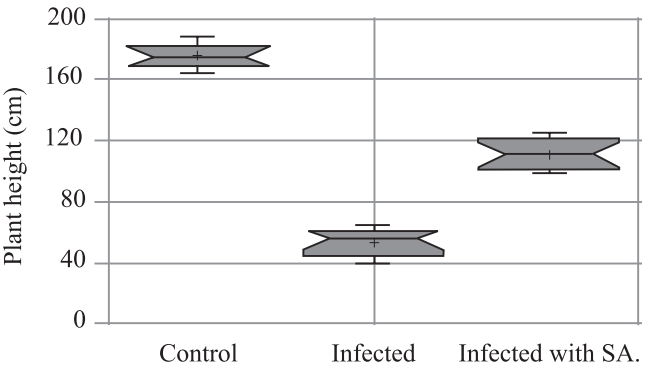

FIGURE 3. Statistic analysis of plant height treated with SA. Box-and-Whisker Plot divides data for each treatment into four equal areas of frecuency. The box encloses the 50% of the data and the median is drawn as a horizontal line. The other 50% is observed as a vertical line known as whisker, which is extended upward and downward the box.

Our results showed an increased content of JA in leaves and roots of the tolerant maize cultivar infected by the virus MRDV-RC. One plausible explanation for the high JA-level registered in this cultivar might be due to: i) a stimulation of the octadecanoid pathway, or ii) an inhibition of JA-degradation. Likewise, the increased level of JA in tolerant maize plants infected leaves compared to the healthy ones could be considered responsible of the lower incidence of the disease. Possibly, JA might be involved in the inhibition of the virus replication as was shown by Clarke *et al*. (1998) in *Phaseolus vulgaris* infected with white clover mosaic potexvirus. These authors informed that SA and JA did not prevent systemic spread of the virus, but virus titre was reduced by these compounds in the systemic leaves.

It has been demonstrated that a mutant plant of *Arabidopsis*, *fad3-2 fad7-2 fad8,* that does not accumulate jasmonate is extremely susceptible to root rot caused by the fungus *Pythium mastophorum*. However, application of exogenous MeJA reduces the incidence of disease and protects mutant plants from the infection (Vijayan *et al*., 1998). In potato and tomato plants, exogenously treatments with JA and MeJA induced SAR against *Phytophthora infestans* (Cohen *et al*., 1993).

When we analyzed the protein pattern in maize leaves, the techniques used did not reveal correlation between high levels of JA and quantitative and qualitative changes of particular bands of proteins. So far, there is no evidence available on the minimum quantities of these compounds that can activate defense genes or their products. In contrast, several examples of JA-inducible genes being activated without increase in endogenous JA level are known. For example, no enhancement of JA level was detected in rice seedlings attacked with the blast fungus *Magnaporthe grisea* during the time required for PR gene expression (Schweizer *et al*., 1997). Hence, different signalling pathways may exist for exogenously given and endogenous raised jasmonates.

Likewise, differences in timing and localization of the pathway signal transduction components from the initial release of systemin to the production of proteinase was determined in tomato leaves after wounding. In this way, the increases in JA and in the defense genes transcription occur at different time and localization (Ryan, 2000).

A similar temporal framework was observed by Keton *et al*. (1999) in tobacco plants (cultivar SR1) infected with *Pseudomona syringae* pv. *phaseolicola*, which induced a rapid hypersensitive response (HR). The accumulation of JA in the HR lesion preceded protein, fresh weight loss and cell death and followed the

initial increases in SA. These facts could explain why in the interaction maize-MRDV-RC virus the increased JA level did not show correlation with changes in the leaf discs protein pattern.

Both compounds, SA and JA, have been implicated in plant pathogen responses. A set of pathogen-induced genes have been shown to be expressed differentially upon SA and JA treatment (Penninckx *et al*., 1996; Manners *et al*., 1998). This could be related to the fact that SA exogenous application to infected plants determined the reversion of the dwarfism symptom.

In summary, the tolerant maize plants responded to the virus infection increasing their content of JA in leaves and roots, and exogenous SA treatment to infected plants reverted the symptoms caused by this virus.

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