

Effect of amino acid application on induced resistance against citrus canker disease in lime plants

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Abstract: Citrus bacterial canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), is a destructive disease. So far, the chemicals used to control this pathogen are either ineffective or harmful to the environment. To improve control of this disease, lime (*Citrus aurantifolia*) were treated with L-arginine, L-methionine, L-ornithine, and distilled water. Plants were inoculated with *Xcc*, 48 hours post treatment. Lesion diameters of inoculated leaves were evaluated four weeks after inoculation with a bacterial suspension. Changes in β -1,3-glucanase transcript levels and activity of antioxidant enzymes, catalase, peroxidase, and phenylalanine ammonia-lyase were investigated at 48 hours post treatment and 24, 48, and 72 hours post inoculation. Based on the results of phenotypic, antioxidant enzyme activity and a molecular study of the stressed plants, it was found that those plants treated with the amino acid methionine significantly increased the plant induced resistance as well as decreased the severity of disease by reducing necrotic lesion size.

Key words: amino acids, bacterial citrus canker disease, induced resistance

Introduction

Citrus bacterial canker is one of the most feared of citrus diseases. It affects all the important citrus crops and causes extensive damage to citrus. Severity of infection varies with different species and varieties, and prevailing climatic conditions. The disease is caused by *Xanthomonas citri* subsp. *citri* (*Xcc*, syn. *X. citri* pv. *citri*, Gabriel *et al.* 1989), and is characterised by the occurrence of conspicuous raised necrotic lesions that develop on the leaves, twigs, and fruits. Severe infections can cause a range of symptoms from defoliation, blemished fruit, premature fruit drop, and twig dieback, to general tree decline (Francis *et al.* 2009; Dewdney *et al.* 2012). Non-marketable quality due to lesions and premature fruit drop are the most economically important damages. There are no highly effective canker disease suppression strategies for the most susceptible cultivars of citrus. Copper reduces bacterial populations on leaf surfaces, but the long-term uses of copper bactericides induce copper resistance in xanthomonad populations. Also, accumulation of copper in citrus soils has potential phytotoxic and adverse environmental effects. However, other contact bactericides are not as effective as copper because they lack sufficient residual activity to protect leaf and fruit surfaces for extended periods (Graham *et al.* 2000). The development of more effective alternative compounds

to control citrus canker is crucial. Systemic acquired resistance (SAR) is a mechanism of induced defense that may confer long-lasting protection against a broad spectrum of microorganisms (van Loon *et al.* 2006). Induced resistance requires the signal molecule salicylic acid and is associated with the accumulation of pathogenicity-related (PR) proteins, and induction of defense enzymes. The latter two are thought to contribute to resistance. For example, soil application of salicylic acid (SA) and the biocontrol agent, *Trichoderma harzianum* (TH), increased the activities of both peroxidase and polyphenol oxidase enzymes in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* (Ojha and Chandra Chatterjee 2012). Foliar application of acibenzolar-S-methyl (ASM), neonicotinoid imidacloprid (IMID) and 2,6-dichloroisonicotinic acid (INA) induces resistance against a broad range of disease-causing organisms including fungi, bacteria, viruses, and nematodes, and induces resistance in citrus plants against canker disease (Jakab *et al.* 2001; Dekkers *et al.* 2004; Graham and Leite 2007). β -Aminobutyric acid (BABA) is a non-protein amino acid which induces resistance against a broad range of disease-causing organisms including fungi, bacteria, viruses, and nematodes (Jakabet *et al.* 2001; Francis *et al.* 2009). β -Aminobutyric acid was shown to induce resistance in tomato plants against fungal pathogens like *Phytophthora infestans* (Cohen 1994),

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F. oxysporum f. sp. *lycopersici* (Li *et al.* 1996), *Botrytis cinerea* (Cohen 2002), and the nematode *Meloidogyne javanica* (Oka *et al.* 1999). Pretreatment with BABA can also be effective against bacterial diseases by inducing systemic resistance against *Xanthomonas vesicatoria* in tomato (Cohen 2002) and *X. citri* pv. *citri* (Graham and Leite 2007; Francis *et al.* 2009). Resistance to diseases in plants was induced by BABA, either through the activation of a signaling pathway that depends on SA or through the activation of a novel signaling cascade not dependent on SA but on jasmonic acid or ethylene (Zimmerli *et al.* 2000). Also, enhanced disease resistance induced by the amino acids L-methionine and riboflavin against powdery mildew infection has been demonstrated on melon, cantaloupe, squash, pea, and strawberry (Tzeng *et al.* 1996; Sarosh *et al.* 2005). However, the research on the effect of amino acids on the induction of resistance in plants is very limited. So, we tested the effect of the amino acids: L-arginine, L-methionine, and L-ornithine on induced resistance against citrus canker disease in lime plants.

Materials and Methods

Plant material and treatments

For the greenhouse experiments, two-year-old lime juveniles were prepared and they were maintained at 25–30°C and 60% relative humidity, in a greenhouse. Lime plants were foliar spray-treated with different concentrations viz., 0, 5, 10, 25, and 50 mM of amino acids L-arginine, L-methionine, L-ornithine (Merck Millipore, Germany). Distilled water was also used in the preparation of the plants (Sarosh *et al.* 2005).

Bacterial culture and inoculation

Bacterial inoculum was prepared with *Xcc* strain 88 obtained from the National Institute of Genetic Engineering and Biotechnology (NIGEB), and was stored at –80°C. For the preparation of the bacterial suspension, the bacterial strain was cultured in Nutrient Agar (NA) (Merck Millipore, Germany) and grown at 28°C for 24 h. Then, a single colony was transferred to Yeast Extract Peptone (YP) medium and grown at 28°C for 24 h to log phase. The final bacterial suspension was pelleted at 10,000 g for 20 min and re-suspended in distilled water to reach to an OD₆₀₀ of 0.4, equivalent to a 7×10^8 colony forming unit (CFU/ml) (Graham and Leite 2007).

Immature leaves were inoculated using a needleless tuberculin syringe to produce a zone of water-soaked tissue, 2 mm beyond the diameter of the syringe opening. The infiltrated area of the leaf was approximately 6 mm diameter and contained 5 µl of bacterial suspension. Two injection infiltrations were performed on each side of the mid-vein and eight leaves were inoculated per plant.

Disease severity

For an estimation of disease severity, the diameter of the necrotic lesion was evaluated four weeks after treatment. The optimum concentration of L-methionine, which does

not affect toxicity and reduced the necrotic lesion diameter significantly (25 mM), was analysed and compared with L-arginine and L-ornithine at the same concentration.

Reverse transcription PCR (RT-PCR)

Treated and inoculated leaves were collected 48 hours post treatment (hpt) and 24, 48, and 72 hours post inoculation (hpi) to monitoring the expression of the *PR-2* gene and frozen in liquid nitrogen. The leaves were then stored at –80°C until processed.

Total RNA was extracted by using the RNX-Plus kit (Cinnagen Co.). One µg total RNA was DNase treated and used to make cDNA by OligodT primer and M-MuLV Reverse Transcriptase kit (Vivantis Co.), according to the manufacturer's instructions.

The expression of β -1,3-glucanase was monitored by the relative transcription levels (mRNA) of *PR-2* gene using primers designed for the β -1,3-glucanase gene from *Citrus sinensis* (NCBI data base accession AJ000081, *PR-2*) (<http://www.ncbi.nlm.nih.gov>). Analysis was done by comparing the PCR band signals. Each PCR reaction contained 0.8 µl of each primer, 1 µl of cDNA template, 0.75 µl of MgCl₂, 0.5 µl of dNTPs 10 mM, 0.3 µl *Taq* DNA polymerase, and 2.5 µl of 10× PCR Master Mix (Cinnagen Co.); a total volume of 25 µl. The cycles were programme: the initial denaturing cycle at 94°C for 2 min, followed by 40 cycles of denaturing at 94°C for 15 sec, annealing at 59°C for 40 sec and elongation at 72°C for 20 sec, followed by a final cycle at 72°C for 2 min. Expression was analysed before and after inoculation, and after treatment.

Enzyme extraction

Extraction of the enzymes from the frozen leaf was carried out using the method originated by Gapinska *et al.* (2008). Samples were powdered with liquid nitrogen. A sample that was 0.5 g was homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7) containing 1% (w/v) polyvinyl-pyrrolidone (PVPP) and 1 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatants were used as crude enzyme extracts to assay the enzyme activities and protein contents. The soluble protein content was assayed according to the Bradford method (1976) using bovine serum albumin as a standard protein.

Catalase activity assay

Catalatic activity was measured spectrophotometrically according to Dhindsa *et al.* (1991). The reaction mixture consisted of 50 mM phosphate buffer (pH 7), 15 mM of H₂O₂, and 100 µl diluted enzyme extract in a total volume of 2 ml. The decomposition of H₂O₂ was followed by a decline in absorbance at 415 nm (Dhindsa *et al.* 1981).

Peroxidase activity assay

The peroxidase activity was determined spectrophotometrically according to the method described by Reuveni *et al.* (1995). The assay mixture contained 700 µl of 50 mM potassium phosphate buffer (pH 7), 100 µl of 4% guaiacol, 100 µl

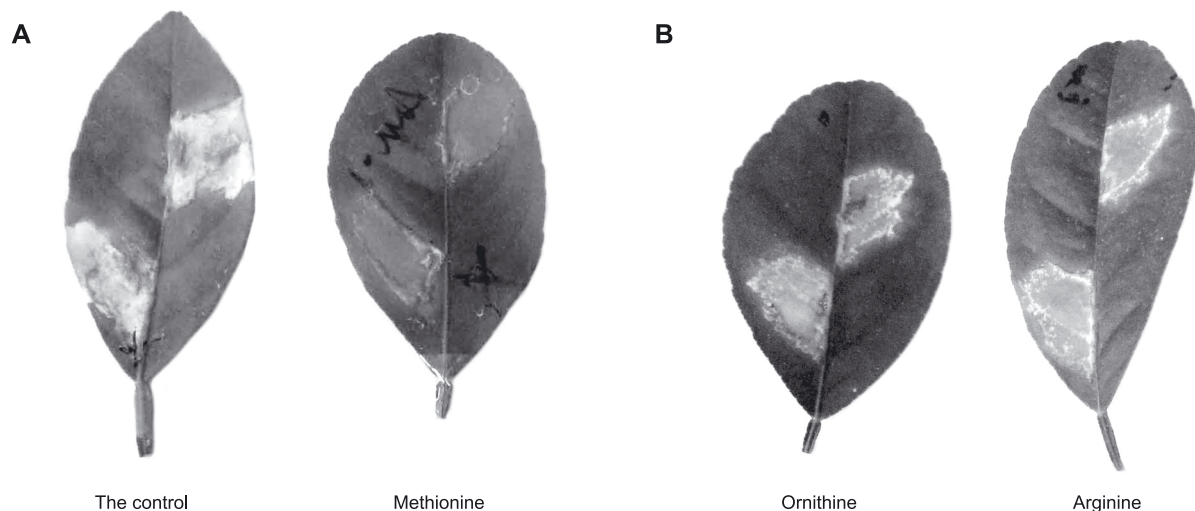


Fig. 1. Effect of foliar applied amino acids L-methionine (A) and L-arginine and L-ornithine (B) compared to water control on disease severity of citrus canker on lime leaves, four weeks after inoculation. Spray treatment with L-methionine, reduced lesion size by about 78.5% as compared to the water control. No significant reduction in lesion size was observed following treatment with L-arginine or L-ornithine

diluted enzyme extract, and 100 μ l of 1% H_2O_2 . Changes in the absorbance at 470 nm were measured for 3 min. Peroxidase activity was expressed as $\Delta A_{470}/\text{min}/\text{mg}$ protein.

Phenylalanine ammonia-lyase activity assay

Enzyme activity was determined based on the production of trans-cinnamic acid following the method of Yao and Tian (2005). A reaction mixture of 100 μ l of enzyme extract, 1 ml of 50 mM potassium phosphate buffer (pH 7), and 500 μ l of 1 mM L-phenylalanine (Merck Millipore, Germany) was incubated for 1 h at 37°C. The reaction was terminated by keeping the samples on ice and the absorbance of the supernatant was measured at 290 nm. The amount of trans-cinnamic acid produced, was determined based on its standard curve (Merck Millipore, Germany). The specific activity of phenylalanine ammonia-lyase was defined as the amount of enzyme extract that produces an increase in the trans-cinnamic acid per mg of total protein per hour.

Statistical analyses

All treatments were arranged in a randomised complete design. All statistical analyses were done using the Statistic Analysis System program (SAS/IML, version 9.1) and Duncan's multiple range tests. Mean differences were considered to be significant at $p < 0.05$.

Results

Disease severity

Disease severity of treated plants inoculated with a *X. citri* subsp. *citri* suspension, was evaluated by calculating the necrotic lesion diameter, four weeks after inoculation. The results showed that a spray treatment with L-methionine, reduced lesion size by about 78.5% as compared to the water control. Thus while water-treated leaves showed lesion sizes of about 1.4 cm, methionin-treated plants had lesions of only about 0.3 cm. No significant

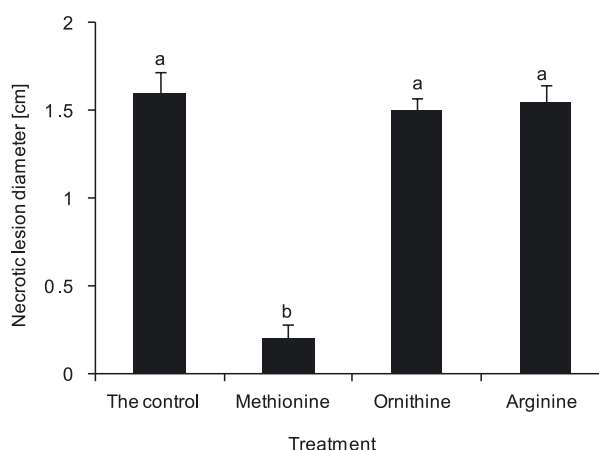


Fig. 2. The diameter of necrotic lesions in plants treated with: distilled water (the control), methionine, ornithine, and arginine four weeks after inoculation with bacterial suspension. Different letters showed significant difference based on Duncan's test in $p < 0.05$

reduction in lesion size was observed following treatment with L-arginine or L-ornithine (Fig. 1–2).

RT-PCR

Semi-quantitative reverse transcription PCR was used to compare the relative transcript amounts of the *PR-2* gene, β -1,3-glucanase, 48 hpt, and 24, 48, and 72 hpi in plants treated with L-methionine, L-arginine, L-ornithine, and in the control plants.

The results of RT-PCR showed that relative expression of *PR-2* genes increased in treated plants with amino acid methionine as compared with the control plants. The increased expression was observed 48 and 72 hpi. It should be noted that the maximum increase in the sampling time was at 72 hpi. The amount of gene expression in the control plants was fixed at all times, which indicates this gene is expressed uniformly at all times (Fig. 3).

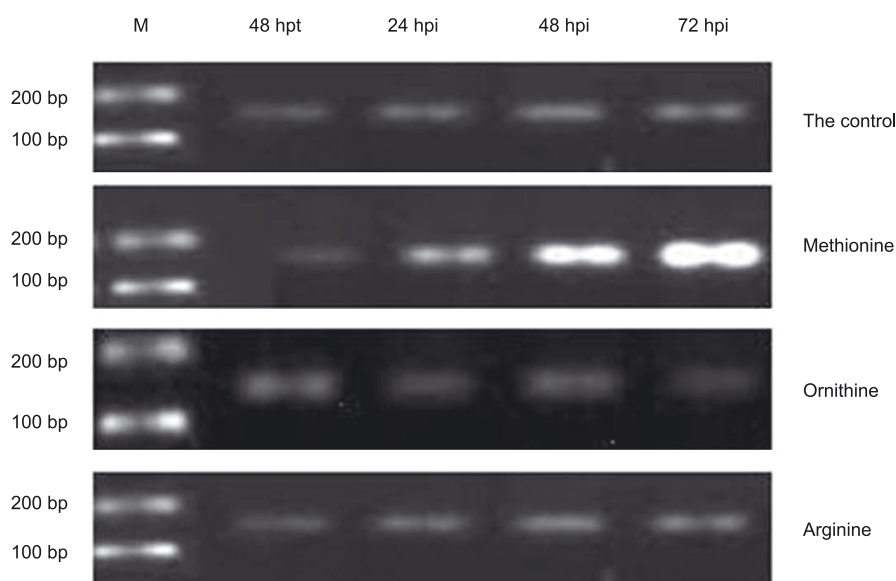


Fig. 3. Relative transcript amounts of the *PR-2* gene, β -1,3-glucanase, 48 hours post treatment (hpt) and 24, 48 and 72 hours post inoculation (hpi), in plants treated with L-methionine, L-arginine, L-ornithine and control plants. The increased expression of *PR-2* genes was observed 48 and 72 hpi in treated plants with L-methionine

Catalase activity assay

The results showed that catalase activity in plants pre-treated with methionine, was significantly reduced 48 hpi. It seems that, in these conditions, the antioxidant activity of plant catalase was active (Fig. 4). The results of the analysis indicate that changes in the enzyme activity, in plants treated with L-methionine, L-arginine, and L-ornithine compared to the control, was not significant.

Peroxidase activity assay

The results of the analysis of peroxidase activity showed that pretreatment of plants with amino acid methionine increased the activity of this enzyme at 48 and 72 hpi. In contrast, L-arginine or L-ornithine treatments were not effective in increasing enzyme activity (Fig. 5).

Phenylalanine ammonia-lyase activity assay

Phenylalanine ammonia-lyase activity significantly increased at 48 and 72 hpi in plants treated with methionine compared to the control ($p < 0.05$). However there was not a significant difference between phenylalanine ammonia-lyase activity in plants treated with L-arginine or L-ornithine, compared to control (Fig. 6).

Discussion

Xcc is the causal agent of Asiatic citrus canker disease which causes economical damage to many citrus species and Rotaceous plants (Dewdney *et al.* 2012). No highly effective strategy has been presented to control the disease. One of the management strategies against pathogen attacks, is an activation of the plant's defense system

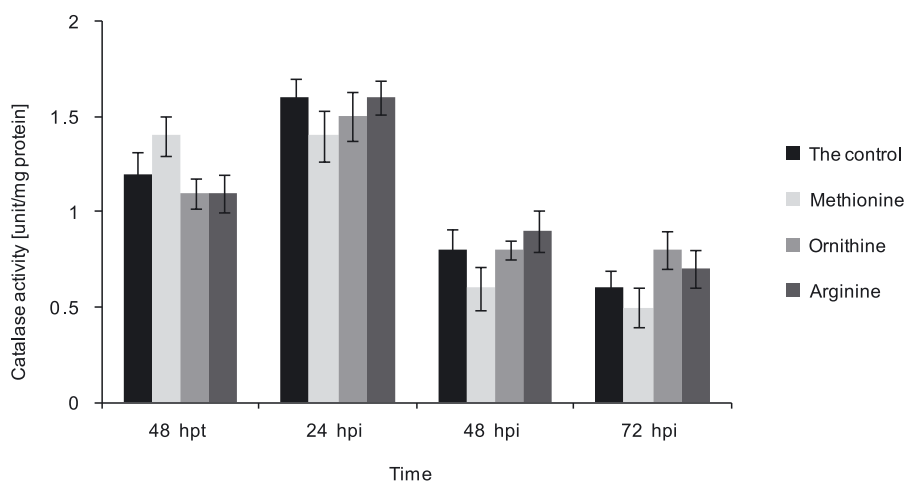


Fig. 4. Catalase enzyme activity in plants treated with: distilled water (the control), methionine, ornithine, and arginine, at 48 hours post treatment (hpt) and 24, 48, and 72 hours post inoculation (hpi)

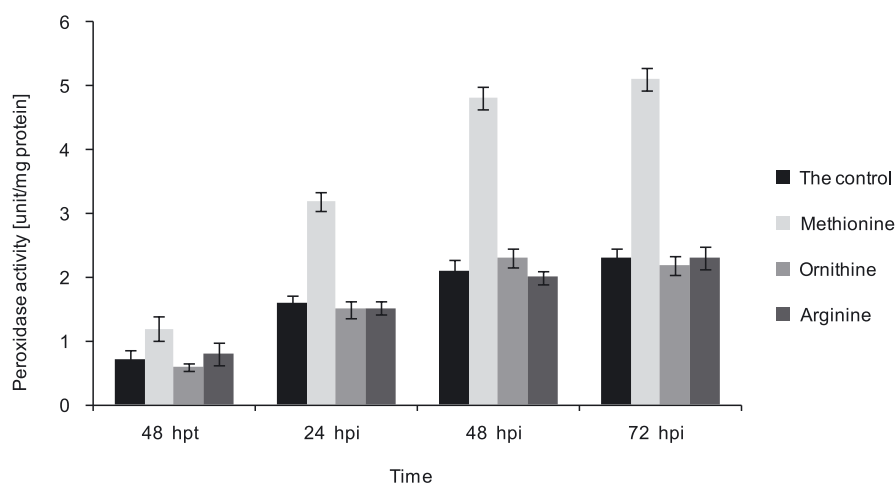


Fig. 5. Peroxidase enzyme activity in plants treated with: distilled water (the control), methionine, ornithine, and arginine, at 48 hours post treatment (hpt) and 24, 48, and 72 hours post inoculation (hpi)

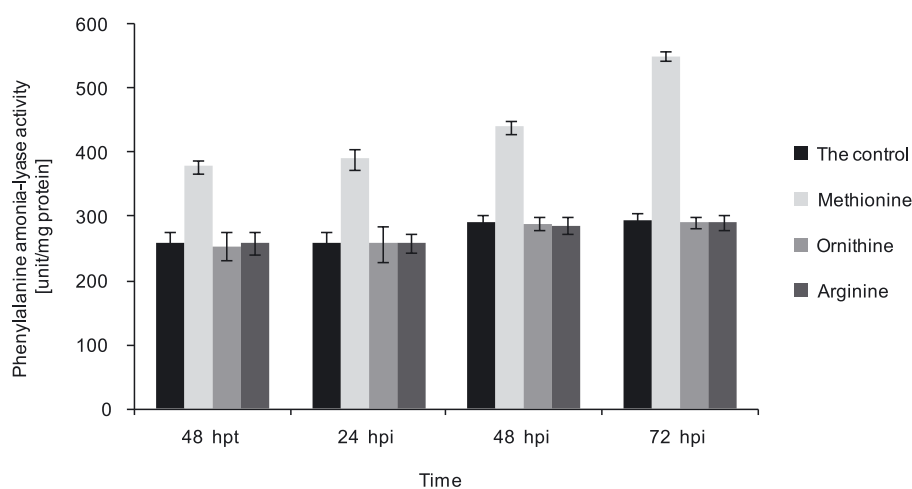


Fig. 6. Phenylalanine ammonia-lyase enzyme activity in plants treated with: distilled water (the control), methionine, ornithine, and arginine, at 48 hours post treatment (hpt) and 24, 48, and 72 hours post inoculation (hpi)

characterised by the accumulation of salicylic acid and the expression of *PR* genes (Hammerschmidt *et al.* 2001). Our study aimed at identifying agents that limit pathogen damage to the plant. For this purpose, we tested the effect of the amino acids L-arginine, L-ornithine, and L-methionine on induced resistance in lime plants against citrus canker disease. Our results indicate that among the tested treatments, L-methionine significantly reduced canker lesions in lime leaves. Analysis of the mRNA levels of *PR-2* gene by semi-quantitative RT-PCR, showed that *PR-2* gene (β -1,3-glucanase) enhanced expression in response to *Xcc* infection. The activity of β -1,3-glucanase was significantly induced 48 and 72 h after inoculation, but especially 72 h after inoculation. This result is supported by previous studies that reported β -1,3-glucanase accumulation in plants correlated with increased levels of induced resistance elicited by various elicitors (Graham and Leite 2007; Francis *et al.* 2009; Graham *et al.* 2011). Furthermore, an increase in peroxidase and phenylalanine ammonia-lyase enzyme activity was observed in plants treated with methionine compared to ornithine or arginine and the control treated plants.

Peroxidase controls the availability of H_2O_2 in the cell wall, which is a prerequisite for the cross-linking of phenolic groups in response to various external stresses, such as wounds, pathogen interactions and environmental constraints, through the formation of a physical barrier of lignin or suberin. High concentrations of phenolic compounds around wounds or pathogen-infected areas can restrict or weaken pathogen growth (Reimers and Leach 1991). Ballester *et al.* (2010) found that soluble peroxidase contributes to the beneficial effect of pathogen infection treatment by reducing disease incidence. Thus, the increase in peroxidase is one of the markers of induced resistance.

Phenylalanine ammonia-lyase is the first enzyme in the metabolic pathways of phenylpropanoids, which is responsible for the biosynthesis of p-coumaric acid derivatives, phytoalexin, and lignins that contribute to plant defense systems. Phenylalanine ammonia-lyase also participates in the biosynthesis of the defense hormone salicylic acid, which is required for both local and systemic acquired resistance in plants (Dixon and Paiva 1995). Induction of phenylalanine ammonia-lyase activities in-

creased significantly in plants treated with L-methionine. This result is in agreement with previous findings that phenylalanine ammonia-lyase is involved in increasing resistance and significantly increases in response to the stimulation of different resistance elicitors in citrus fruit (Droby *et al.* 2009; Ballester *et al.* 2010). The present study assessed the effect of the L-methionine on disease development. Enhanced disease resistance induced by L-methionine and riboflavin against powdery mildew infection has been demonstrated for melon, cantaloupe, squash, pea, and strawberry (Tzeng *et al.* 1996; Sarosh *et al.* 2005). The results from this study indicated that pre-treatment of lime plants with L-methionine induced the levels of disease resistance against *Xcc*, and significantly reduced the severity of disease.

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