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Full Length Research Paper

Priming against Asiatic citrus canker and monitoring of *PR* genes expression during resistance induction

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Plant activators provide an appealing management option for Asiatic citrus canker. The ability of β-aminobutyric acid (BABA) and green tea extract (GTE) to induce resistance in lime (*Citrus aurantifolia*) plants against *Xanthomonas citri* subsp. *Citri* (*X*cc) was investigated. Green house experiments showed that BABA (250 ppm) and GTE {5 % (w/v)} treatment protects lime plants against *X*cc. Lime plants treated with BABA, green tea extract (*Camellia sinensis*), copper oxychloride and distilled water were inoulated with *X*cc. Lesion expansion of inoculated leaves were evaluated 5,10 and 20 days after treatment. The results showed that BABA and GTE had inhibitory effects on disease development. Base on previous experiments, none of the applied agents for plant treatment had direct antimicrobial activity on *Xcc*, except copper oxychloride. This indicated that the inhibitory effects of BABA and GTE was as a result of strengthening the defense capacities of the plant. To support this claim, two pathogenesis-related (*PR*) genes from lime, β-1,3-glucanase and chitinase, used for defense signaling pathway was studied. Analysis of *PR* genes expression showed increased mRNA levels of β-1,3-glucanase and chitinase, after treatment. Reduction in lesion size and lack of antimicrobial activity indicates that BABA and GTE might have an important role in turning on defense signaling pathway against *Xcc* infection.

Key words: Pathogenesis-related genes, *Xanthomonas citri* subsp. *Citri*, plant activator, disease resistance.

INTRODUCTION

Asiatic citrus canker (ACC) is the most important disease of citrus caused by xanthomonads. The bacterial agent, recently reclassified as *Xanthomonas citri* subsp. *Citri* (Schaad et al., 2006) (synonym = *X. axonopodis* pv. *citri* pathotype A) is widely distributed in southern Iran citrus growing areas (Alizadeh and Rahimian, 1989; Khodakaramian and Swings, 2002). ACC is most severe on grapefruit (*Citrus paradisi*), limes (*Citrus aurantifolia* and *Citrus limettioides*) and, trifoliate orange (*Poncirus trifoliata*) (Gottwald and Graham, 2000). Typical symptom of ACC is a crateriform lesion surrounded by a water-soaked margin on leaves, fruits and stems (Gottwald and Graham, 2000). There are no highly effective canker disease suppression strategy for the most susceptible cultivars (Leite and Mohan, 1990). Copper reduces bacterial populations on leaf surface, but multiple applications are needed to achieve adequate control on susceptible citrus host (Graham et al., 2004). Disadvantages of long term use of copper bactericides include induced copper resistance in xanthomonad populations (Marco and Stall, 1983; Rinaldi and leite, 2000) and accumulation of copper in soils with potential phytotoxic

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Abbreviations: ACC, Asiatic citrus canker; **SAR,** systemic acquired resistance; **PR,** pathogenesis-related; **PR3,** chitinase; **PR2,** glucanase; **BABA,** β-aminobutyric acid; **BTH,** benzothidiazoles; **ASM,** acibenzolar-S-methyl; **RT-PCR,** reverse transcriptase-polymerase chain reaction; **NAS,** nutrient-agar-sucrose; **EF1,** elongation factor 1-alpha; **GABA,** γ-aminobutyric acid.

and adverse environmental effects.

Plant-pathogen interaction during infection induces signal cascades which activate a cellular response to minimize lesions (Dixon et al., 1994). When a plant detects pathogen attack, it triggers several categories of defense mechanisms which are involved in resistance to disease. Rapid signaling events are generally followed by a broad spectrum of metabolic modifications such as reactive oxygen species, systemic acquired resistance (SAR) and induced systemic resistance (Hammond-Kosack and Jones, 1996). The SAR group includes several pathogenesis-related (PR) proteins such as chitinase (PR3) and glucanase (PR2) which are either antifungal or antibacterial (Ryals et al., 1996; Sticher et al., 1997). In recent years, the importance of various synthetic compounds (such as β-aminobutyric acid (BABA), benzothidiazoles (BTH), acibenzolar-S-methyl (ASM) etc) as disease control agents without displaying a direct antibiotic effect themselves has been emphasized (Jakab et al., 2001). In addition, other numerous inorganic and organic substances, as well as plant extracts made of neem (*Azadirachta indica*), ginger (*Zingiber officinale* Roscoe), curcuma rhizomes (Curcuma longa L.) (Vechet et al., 2009), mistletoe (*Viscum album*) and ivy (*Hedera helix*) (Zeller, 2006) compounds have been described which are capable of controlling plant disease without displaying a direct antibiotic effect themselves.

In this research, we studied priming of 2 years juvenile lime plants against *Xcc* using compounds with disease suppressive activity such as BABA and green tea extract (GTE) and monitoring of plant *PR* genes expression during suppression of disease development.

MATERIALS AND METHODS

Plant materials and treatments

The 2 years juvenile plants raised from lime (*C. aurantifolia*) seeds were used as plant materials. The greenhouse used for these experiments, was maintained at 27 to 31°C (day) and 23 to 26°C (night) with 14 h of natural light and approximately 60% relative humidity.

Plants were treated by foliar spray application of BABA 250 ppm, GTE (*Camellia sinensis*) 5% (w/v), copper oxychloride 0.6%, and distilled water, on the entire plant. All chemicals were purchased from Sigma-Aldrich Chemical Co. except for green tea, which was obtained from the local market.

Inoculum preparation

Bacterial inoculum was prepared with *Xcc* strain, J1 originally isolated from a lime leaf showing canker symptoms and confirmed by polymerase chain reaction (PCR) using specific primer, XACF [CGTCGCAATACGATTGGAAC] and XACR [CGGAGGCATTGTCGA AGGAA] (Park et al*.,* 2006). *Xcc* cultured on nutrient-agar-sucrose (NAS) plate and grown at 28°C for 24 h. Colonies were transferred to sterile tap water using a sterile bacterial loop. The density of the suspension was adjusted to O.D.600 = 0.3, which corresponded to 10^6 cells ml^{-1}.

Immature leaves (75% expanded) were inoculated using a tuberculin syringe (1.0 cm^3) with no needle as previously described (Graham et al*.*, 2004). In brief, *Xcc* inoculum was infiltrated by pressing the needleless syringe tip against the abaxial leaf surface to produce a zone of water-soaked tissue 2 mm beyond the diameter of the syringe opening. At least ten leaves were inoculated per plant. The inoculated plants were covered with plastic bags for 1 day to maintain high humidity conducive for bacterial growth in the leaves.

Total RNA extraction from lime leaves

Approximately 1 to 2 g of leaves were ground with a mortar and pestle in the presence of liquid nitrogen. The powder was transferred to a tube containing ice cold extraction buffer (100 mM Tris-HCl pH 8.5/10 mM EDTA pH 8/100 mM LiCl/1% SDS) and mixed. Tris-equilibrated phenol was added, mixed before chloroform/ isoamylalcohol was added and mixed. After centrifugation, the upper aqueous phase was extracted two times with chloroform/ isoamylalcohol (24:1). Addition of 10 M $LiCl_2$ to the supernatant precipitated the RNA over night. After centrifugation, the RNA pellet was resuspended in RNase-free H_2O and ethanol was precipitated. The final RNA pellet was resuspended in RNase-free H_2O. RNA quantity and purity was analyzed by measuring the ratios of absorption at 260/280 and 260/230 nm, and RNA integrity was evaluated from the 28S and 18S rRNA bands by ethidium bromide staining after agarose gel electrophoresis.

Preparation of cDNA

1 µg total RNA was DNase treated and used to make cDNA. First-strand cDNA was prepared using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions.

Real-time quantitative reverse transcriptase (RT)-PCR

To analyze lime *PR* genes expression during priming against *Xcc,* specific primers were used, these include: F (5'-AATGTTGCTAGC ATTGTGACTCC-3') and R (5'-GCAGCATTCAGAAACGCATCT-3') for chitinase (CHI1) AF090336, F (5'-TTCCGACGGATCGTTAA GTTACC-3') and R (5'-CCACCCACTCTCTGATATCACG-3') for β-1,3-glucanase (GNSL) AJ000081, F (5'-GGTCAGACTCGTGAG CATGC-3') and R (5'-CATCGTACCTAGCCTTTGAGTACTTG-3') for elongation factor 1-alpha (EF1) AY498567. The assays were carried out within the IQ5 System thermocycler including the associated software program from Bio-Rad (USA). All reactions were set up in 96-well reaction plates (Bio-Rad, USA). Each 25-µl reaction was performed in the IQ SYBR Green super mix Kit (Bio-Rad, USA), containing the SYBR Green I dye as a fluorophor. A primer concentration of 500/500 nM (forward/reverse) was chosen for all genes based on optimization of each primer set using standard curves. Negative control reactions replacing the cDNA template contained sterile water. Each reaction was repeated twice. PCR cycling parameters were 95°C for 5 min (denaturation), followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. After the 40 cycles, melting curves were gotten by heating to 95°C for 1 min and

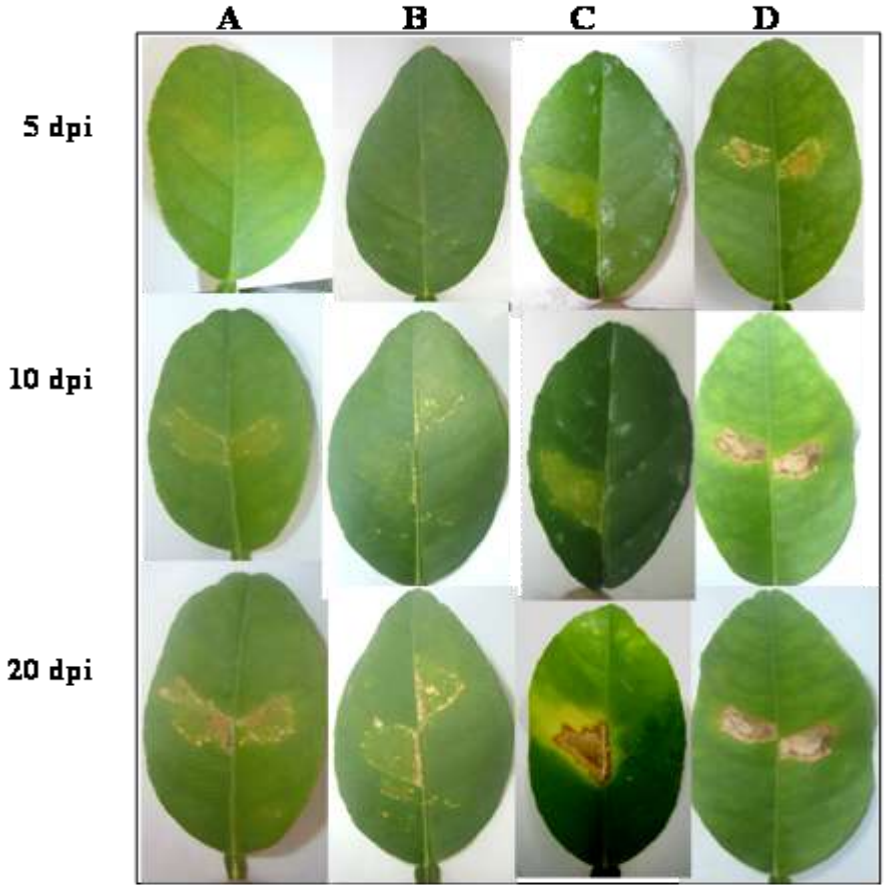

Figure 1. Treated leaves with BABA and GTE showed smaller necrotic lesions than control and other treatments. Leaves were infected by injection with 1×10^7 cfu ml^{-1} *Xanthomonas citri* subsp. *Citri*. Leaves were photographed at the indicated time with post chemical application . A, BABA; B, GTE; C, copper oxychloride; D, control.

cooling to 60°C for 30 s, followed by ramping up the temperature to 95°C with 0.5°C per second where the temperature was maintained for 30 s. The fluorescence data were continuously collected during the ramping from 60 to 95°C. Identical thermal cycling was used for all targets. The transcript level was calculated by standard curve method and normalized against citrus elongation factor 1-alpha (*EF*1) gene as an internal control. Results from several works indicated *EF*1 as one of the best usable housekeeping genes to normalize gene expression levels since it appeared that it was not influenced during biotic stress (Nicot et al., 2005), various phases of development and in different environmental conditions (Jain et al., 2006). For each gene, the lowest sample value at the non-inoculated time was defined as the 1X expression level, and results were expressed as the fold increase of mRNA over this sample.

Statistical analysis

The experiments were arranged based on completely randomized design (CRD) with ten biological replicates. Ten leaves from each replicate were evaluated for necrotic lesion diameter and their mean were used as each replication value. The means were compared for statistical significance using Duncan's multiple-range test (P = 0.05).

RESULTS AND DISCUSSION

Symptom development assay indicated that primed lime plants with of BABA 250 ppm and green tea extract (*C. sinensis*) 5% (w/v) had least *Xcc* symptom development at 20 days after inoculation. No reduction in development symptoms as compared to the water control was observed following treatment with copper oxychloride (Figure 1). Result of the previous *in vitro* experiment showed that applied agents had no direct effect on bacterial survival. The idea was that this inhibition of symptom development after priming with GTE and BABA may have resulted from induction of *Xcc* defense signaling pathway genes of lime plant. We cloned, by RT-PCR a 117bp fragment of a β-1,3-glucanase (HM593870)

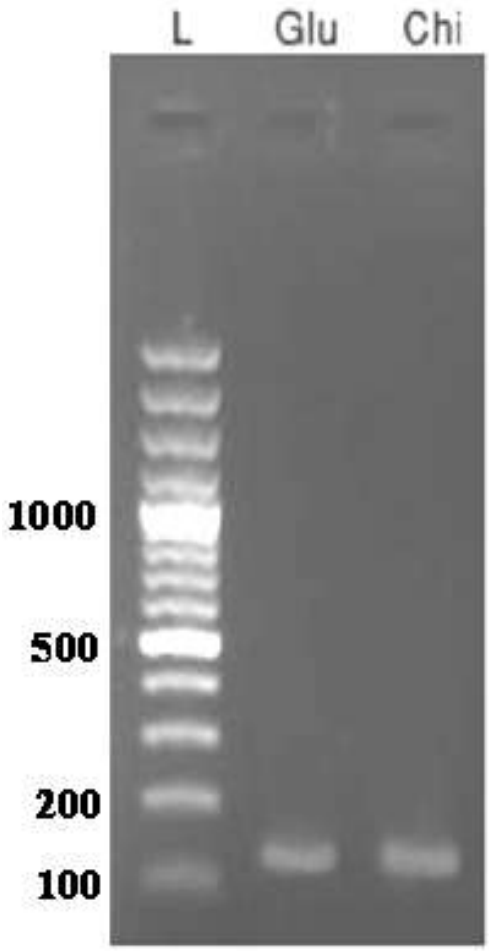

Figure 2. RT-PCR bands of β-1,3-glucanase and chitinase genes of lime. Numbers on the left of photo show marker band sizes. L; ladder 3 kb, Glu; β-1,3-glucanase, Chi; chitinase

and a 111 bp fragment of a chitinase (HM593869) gene as two important pathogen related genes from lime (Figure 2). We investigated the expression pattern of these two genes at three time points: 24 h before priming, 24 h after priming with BABA and GTE and 120 h after infection with *Xcc* (when primary symptom was expressed) by quantitative real time PCR. Gene expression at 24 h before priming was considered as calibrator (gene expression rate = 1). Results showed that 24 h after priming with BABA, both genes had higher transcripts. *Chitinase* gene and *glucanase* gene transcripts increased to 20 and 11 fold, respectively (Figure 3). Similarly, after priming with GTE, increase in *chitinase* and *glucanase* genes expression was 7.4 and 2.5 fold, respectively (Figure 3). However, expression of both mentioned genes in control plants had no change (Figure 3). However, expression of these two *PR* genes had no significant change at 5 days after inoculation for both priming agents.

Our results indicated that among all applied agents, BABA and GTE significantly reduced symptom development in lime leaves. Also, *PR* genes expression analysis showed that *glucanase* and *chitinase* genes expressed more after priming with these two mentioned agents. These results are in agreement with previous studies which reported that BABA induced PR-1, chitinase and β-1,3-glucanase protein accumulation in pepper, tomato and tobacco (Cohen, 1994; Cohen et al., 1994; Hwang et al*.,* 1997). Also, previous research showed that increased *PR* genes (like chitinase) expression lead to increased resistance to *Xanthomonase campestris* pv. *Vesicatoria* (Hong and Hwang, 2005). Brisset showed that increase in *chitinase* and *glucanase* gene expression was in relation with high resistance to apple tree to fire blight disease. Based on their result, resistance to this pathogen came from systhemic acquired resistance (Brisset et al., 2000). The mode of action of BABA in inducing resistance against *Xcc* is not yet fully understood. There are reports showing the induction of *PR* genes after BABA treatment (Cohen, 1994). Green tea is full of components with anticancer, antiviral and anti pathogen characters like polyphenols (catechins), fragrance components, amino acids (theanine), saponins and γ-aminobutyric acid (GABA). In plants, GABA accumulation was observed in response to biotic and abiotic stresses (Shelp et al., 1999; Roberts, 2007). Data on the resistance-inducing activity of plant extracts are rather rare. Inducers from plant origin have been reported by Paul and Sharma (2002), who found that a water extract made from the leaves of neem provided control against leaf stripe disease on barley (*Drechslera graminea*) was as effective as the fungicide Bavistin (carbendazin). The infiltration method immediately exposes a large number of plant cells to bacteria, thus it likely facilitates a better picture of the early events following recognition of *Xcc* by citrus cells.

But understanding of the signaling pathways of BABA and green tea in resistance of lime to *Xcc* need more investigation. Application of BABA and GTE in citrus possesses promising results in the control of the disease and could serve as an alternative to traditional chemical compounds which are harmful to the environment.

In green agriculture, eco-friendly agents are safe and pro environment. Since most synthetic bacteriocides do harm the ecosystem to some extent, their usage should be minimized and safer strategies as biological compounds application should be more publicized in future. We believe that characterization of signaling pathway of induced resistance by GTE and BABA can help to have promising environmentally safer measures in plant pathogens management.

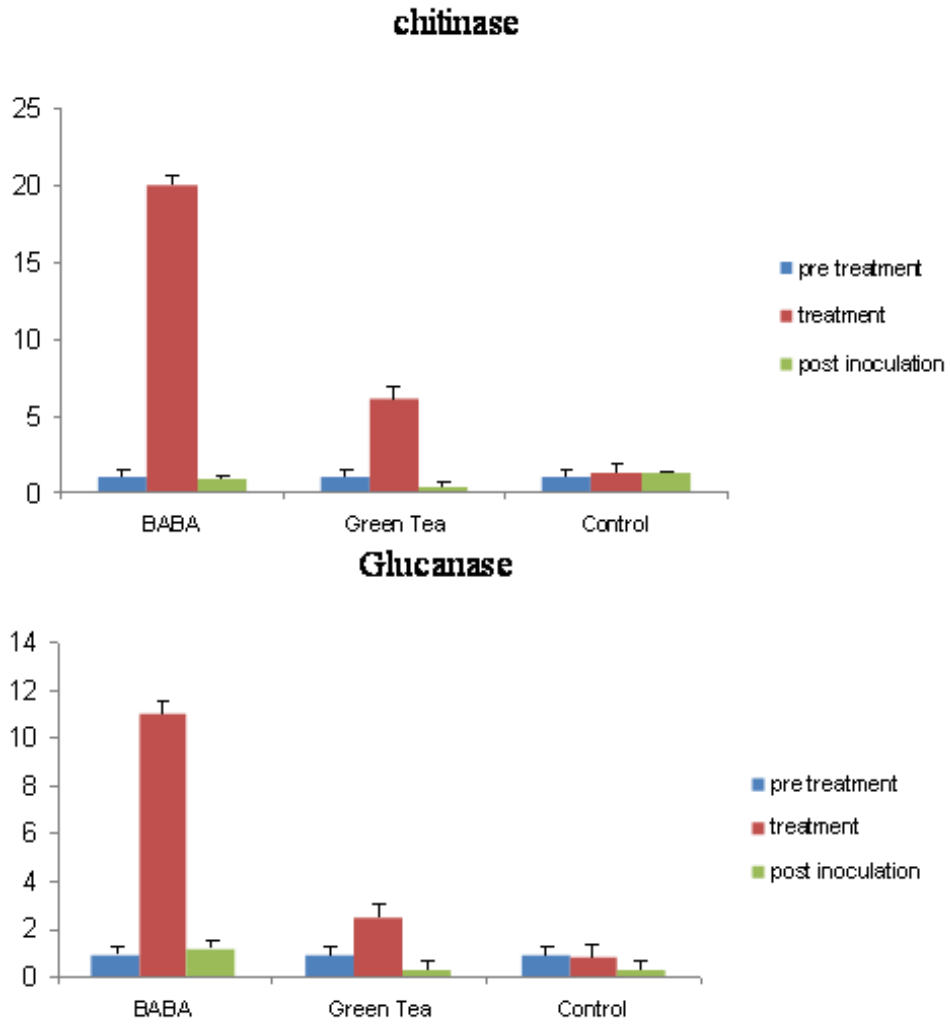

Figure 3. Relative expression of the chitinase and β-1,3-glucanase genes in lime leaves after a single application of treatments. Leaves were inoculated by injection with 1×10^7 cfu ml^{-1} *Xcc* after treatment. Relative gene expression was calculated using the standard curve method. Values represent the mean of three biological replicates, bars represent standard error. Treatments are: BABA and GTE and *Xcc* untreated inoculated (control). Gene expression was assessed three times: pre treatment, treatment and post inoculation.

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