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Test for L-glutamate inhibition of growth of *Alternaria alternata* by inducing resistance in tomato fruit

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ABSTRACT

Although numerous studies have reported the involvement of glutamate in plant abiotic stress, relatively little is known about the role of glutamate in plant defence against pathogens. To gain further knowledge, we investigated the effect of glutamate on *Alternaria alternata* in tomato fruit. A multidisciplinary approach was pursued, combining exogenous glutamate applications, enzymatic activity measurements and real-time quantitative PCR analysis. The results showed glutamate significantly reduced the disease incidence in tomato caused by *A. alternata,* by inducing resistance (Duncan's test, $p < 0.05$). A large variety of defence-related enzymes and genes involved in the glutamine synthetase/glutamate synthase cycle, energy-generated metabolism, such as the γ-aminobutyric acid shunt, glycolysis and the tricarboxylic acid cycle, and the salicylic acid signalling pathway were activated by glutamate. The activation of these pathways as mentioned above might play a potential role in the resistance mechanisms underpinning glutamate-induced plant immunity.

1. Introduction

Diseases caused by fungal pathogens result in significant economic losses of fruit in the field and during the postharvest phase (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010). Tomato (*Solanum lycopersicum* L.) is economically important, worldwide, with a recent increase in demand. However, due to the high moisture content and rich nutrition, tomato is highly susceptible to fungal pathogens. *Alternaria alternata* has been described as one of the most economically important necrotrophic pathogens in tomato fruit (Nair, Kolet, Thulasiram, & Bhargava, 2015). Currently, the application of synthetic fungicides is the simplest and most effective approach for disease management. However, the growing public concern about pollution of the environment and health hazards for consumers have led to the development of alternative approaches. Recently, increasing natural resistance in plants, using physical, biological and chemical elicitors, has emerged as a viable alternative strategy for disease control, to reduce the use of chemical fungicides. A large number of substances, such as γ-aminobutyric acid (GABA), arginine and salicylic acid (SA), have been used as elicitors to control postharvest diseases, with positive results (Cao, Glazebrook, Clarke, Volko, & Dong, 1997; Yu et al., 2014; Zheng et al., 2011).

Plant resistance to disease is a result of highly coordinated effects on the various layers on the pathogen (Prusky et al., 2009). In the past various studies have been carried out to decipher plant hormones, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), as the key regulators in the signalling networks that are involved in induced defence responses (van Loon, Rep, & Pieterse, 2006). SA is essential for the activation of systemic acquired resistance (SAR), which is a form of long-term immunity to a broad-spectrum of pathogens including viruses, bacteria and fungi (Rahman, Oirdi, Gonzalez-Lamothe, & Bouarab, 2012).The SA-mediated signalling pathway often parallels the expression of pathogenesis-related (PR) proteins thought to contribute to resistance (Chuanfu & Zhonglin, 2011). It is also well documented that some plant defence mechanisms are associated with the activation of induced systemic resistance (ISR), which is dependent on JA and ET.

There is evidence showing a picture regarding the function of primary metabolism in plant defence. During plant-pathogen interactions, primary metabolism provides not only energy to support extensive defence responses, but also a source of signalling molecules to stimulate plant resistance (Bolton, 2009; Rojas, Senthil-Kumar, Tzin, & Mysore, 2014). It was observed that after challenging with pathogens, transcripts were up-regulated from

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energy production, such as glycolysis, the tricarboxylic acid (TCA) cycle and biosynthesis of some amino acids (glutamate, arginine, serine, lysine, methionine and glycine) (Less, Angelovici, Tzin, & Galili, 2012). Recent research has proposed a new perspective to explain the part played by plant glutamate metabolism in the resistance response (Seifi, Van Bockhaven, Angenon, & Höfte, 2013).

L-Glutamate is a multi-functional amino acid which occupies a central position in amino acid metabolism and nitrogen assimilation (Forde & Lea, 2007). In addition to its structural role in proteins, glutamate functions as a precursor for some defence compounds, such as GABA, arginine and proline (Galili, Tang, Zhu, & Gakiere, 2001). It was suggested that glutamate conferred tolerance to plants against abiotic stresses. Exogenous glutamate was shown to alleviate inhibition of cucumber seed germination under salt stress (Chang et al., 2010). In dehydrated leaves, glutamate might be converted into proline and GABA which were important for the acquisition of desiccation tolerance (Martinelli et al., 2007). However, understanding of the role of glutamate in plant defence against pathogens is still poor. Glutamate, a highly abundant *N*-rich amino acids, could be served as the N source of both pathogen and plant. The competition for N nutrition between the pathogen and its host is complicated and dynamic. The contradiction of the dual function of N supply has been studied in various plant–pathogen interactions (Hoffland, Jeger, & van Beusichem, 2000). Seifi, Curvers, De Vleesschauwer, Delaere, Aziz, & Hofte, et al., 2013; Seifi, Van Bockhaven, et al., 2013 found glutamate metabolism resulted in resistance or susceptibility, largely depending on the lifestyle of the pathogen.

With the concept of safe food for human health, the interest in finding safe and effective elicitors of plant resistance has greatly increased. Glutamate is one of the most important amino acid and extensively consumed in the medical and food fields. It could be an interesting tool in managing fungal rots because it is safe, cheap, readily available, and suitable for postharvest practices. Accordingly, this study aimed to gain further knowledge on the effect of exogenous glutamate as a potential commercially valuable elicitor for resistance to *A. alternata* in tomato fruit, and to present the potential defence mechanisms involved.

2. Materials and methods

2.1. Fruit material

Tomato (*Solanum lycopersicum* L. cultivar 'Qianxi'), without synthetic fungicide application, were hand-harvested at the red stage without damage or infections from a local commercial orchard in Hangzhou (Zhejiang Province, China). They were selected for uniform size, colour and maturity in the experiment. To remove dust and any pathogens landing on the fruit surface, freshly harvested fruit were immediately surface sterilized by immersion in of 0.1% sodium hypochlorite solution (active chlorine $\geq$56.8 g L^{-1}) for 2 min, rinsed thoroughly with tap water, and dried at room temperature.

2.2. Fungal strains

Alternaria alternata was obtained and cultured as described by Feng and Zheng (2007). Petri dishes containing potato dextrose agar (PDA) were inoculated with *A. alternata* and incubated at 25 °C for 7–10 days. Conidia were rubbed from the agar surface with a sterile glass rod and transferred to sterile distilled water with 0.01% (w/v) Tween 80. The spore quantity was counted using a haemocytometer and adjusted to the desired concentration.

2.3. l-Glutamate treatment and pathogen inoculation

L-Glutamate (purity $\geq$98.5%) was purchased from Sangon Biotech (Shanghai, China). To avoid being influenced by pH, glutamate solution was adjusted to about 7 by sodium hydroxide.

2.3.1. l-Glutamate at different concentrations

Tomatoes were immersed in a glass container with glutamate solutions at the concentration of 0 (control), 10, 100 and 1000 μg ml^{-1}, respectively, for 10 min and then air-dried for approximately 60 min. Afterwards, treated tomatoes were sealed in polyethylene-lined plastic boxes and kept in the thermostatic cultivation room at 25 °C with 90–95% relative humidity. After 24 h, the fruit were punctured (5 mm diameter and approximately 2 mm deep) gently with a sterile borer in the equatorial region to form a hole and inoculated with 20 μl of *A. alternata* conidial suspension adjusted to 10^4 conidia ml^{-1}, then stored in the thermostatic cultivation room to allow pathogen development. All treatments were conducted with 3 replicates and 30 tomatoes per replicate.

2.3.2. Different time interval between l-Glutamate treatment and pathogen inoculation

Tomatoes were immersed in a glass container with glutamate solutions at the concentration of 0 (control) and 100 μg ml^{-1}, respectively, for 10 min. The fruit were then air-dried and incubated in the thermostatic cultivation room at 25 °C with 90–95% relative humidity. Tomatoes were punctured as described above. At various time intervals (0, 12, 24 and 36 h) after treatment, 20 μl of 10^4 conidia ml^{-1} suspension of *A. alternata* was applied to each wound. 30 tomatoes were chosen randomly as replicates, and each treatment included 3 replicates.

2.3.3. Disease assessment

When white mycelium develop in the wound area on each fruit, it could be counted as infected. The number of infected fruit were examined daily during inoculation. Disease incidence was expressed as percentage of infected fruit.

2.4. Enzymatic activity measurement

The samples were taken following 100 μg ml^{-1} glutamate or sterile distilled water treatment at various time intervals (0, 12, 24, 36 and 48 h) and immediately frozen in liquid nitrogen.

Soluble proteins were extracted in a buffer containing 50 mmol l^{-1} Tris-Cl (pH 7.5), 0.1% (v/v) 2-mercaptoethanol, 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 1 mmol l^{-1} $MgCl_2 \cdot 6H_2O$. The crushed tissue was suspended in the pre-cooled extraction buffer at a ratio of 1:3 (w/v) and centrifuged at 10,000*g* and 4 °C for 20 min. Protein content in extracts was measured by the Bradford method in enzymatic assays (Bradford, 1976). Glutamine synthetase (GS), malate dehydrogenase (MDH), succinate dehydrogenase (SDH) activities were analyzed using colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), following the manufacturer's specifications. β-1,3-glucanase (GLU) activity was estimated by the method of Zheng et al. (2011). The enzymes activities were expressed as units per mg protein.

2.5. Real-time quantitative PCR (qPCR) analysis

Total RNA was isolated from frozen tissue using TRIzol reagent (Takara, Japan). First-strand cDNA was synthesized from 1 μg of total RNA with a PrimeScriptR RT reagent Kit with gDNA Eraser (Takara, Japan) according to the manufacturer's instructions. Quantificative PCR amplifications were performed with SYBR® Premix