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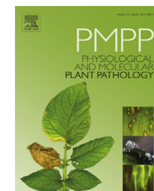

# Study of catechin, epicatechin and their enantiomers during the progression of witches' broom disease in Mexican lime (*Citrus aurantifolia*)

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ABSTRACT

Witches'broom diseases of Mexican lime (WBDL) caused by '*Candidatus Phytoplasma aurantifolia*, is a big threat to lime production in Southern Iran. In this investigation, concentration of catechin, epicatechin and their enantiomers were monitored during WBDL progression for 6 months. In addition, the activities of peroxidase (POD), phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) as well as the content of malondialdehyde (MDA) were assayed. The results showed that after 30 days of inoculation, levels of catechin and epicatechin were considerably increased up to 390% and 290% respectively, but both of them significantly decreased after 150 days. These changes in the levels of catechin and epicatechin are in well agreement with the activities of PAL, CHS and POD as well as the level of MDA. Does WBDL influence on biosynthesis of enantiomers in lime? The analysis of catechin enantiomeric ratio of infected leaves demonstrated that after 30 days of inoculation, the ratio of (+)-catechin changed from 61:39 to 79:21 and then remained almost constant. Finally, the infection of lime by WBDL, not only induced the production of resistance enzymes (PAL and CHS) as well as catechin and epicatechin but also enhanced the accumulation of (+)-enantiomer.

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## 1. Introduction

The devastating effect of *Candidatus Phytoplasma aurantifolia,* the causal agent of witches' broom disease of lime (WBDL), resulted in economic loss of some Mexican lime producing countries such as United Arab Emirates, Oman and Iran [9,27]. Phytoplasma-infected plants could be diagnosed by some methods such as polymerase chain reaction (PCR), polyclonal antibodies as well as symptom expression [4,5]. One of the major areas of research in plant disease is directed toward the identification of metabolite biomarkers that can accurately predict the state of plants [7], reported that the natural compounds such as hesperidin, naringenin, and quercetin present in the leaves could be used as a biomarker to identify diseases in citrus trees. In other study, biomarkers such as gallic and azelaic acids could be tested for the monitoring of infection of grapes at the early infection stage in the vineyard [3,38], indicated that the enantiomeric ratios of linalool and its oxides could be biomarker for the determination of orange honey authenticity. Hetch and co-workers [13] showed benzo[a]pyrene tetraol enantiomers are the best biomarker to show high risk of lung cancer for smokers [33], introduced a method for the determination of biomarker candidates of chiral amins and carboxyls in Alzheimer's disease brain homogenates.

Catechin and epicatechin are epimers belonged to the class of flavan 3-ols (phenolic metabolites) which not only find in many plants, as well as Mexican lime, but also induce in different infected plant tissues as a defense reaction [20,34,36,10,21], reported that the husk catechin content of infected green walnut fruits by *Xanthomonas arboricola* pv. *Juglandis* increased up to 23 fold more than

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it is in healthy plants. Infection of pear leaves by *Gymnosporangium sabinae* increased the production of flavan-3-ols [35].

In addition, catechin and epicatechin have two chiral centers, so four stereoisomeres exist for them, but (−)-epicatechin and (+)-catechin are the most optical isomers found in the nature [24]. Some stresses such as disease, temperature and pH could change the ratio of enantiomers for catechin [18]. In previous investigation, we demonstrated that the infection of wheat by *Puccinia triticina* caused to increase catechin and epicatechin contents and also changed enantiomeric ratio of catechin in the leaves [10]. Now, the main question is what happens to both catechin and epicatechin and their chirality in phytoplasma-infected lime trees. To find a metabolite biomarker as an alternative method of diagnosing the infected plants as well as PCR technique, we should investigate the metabolites through the development of the disease in order to identify the metabolite and also control the disease in further investigations.

However, when plants are subjected to some stresses, reactive oxygen species (ROS) would be produced. ROS can disrupt normal metabolism through peroxidation of lipids [2]. The rate of lipid peroxidation in terms of malondialdehyde (MDA) can be used as an indicator to evaluate the tolerance of plants to oxidative stresses [16]. Furthermore, there are numerous reports of different plants synthesizing a variety of pathogenesis-related (PR) proteins in response to abiotic and biotic stresses [1]. One of the PR proteins includes peroxidases (PODs), which are the most important components in the scavenging system of ROS. Stressed plants also increase the production of Phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) which are involved in the production of phytoalexins. PAL and CHS activities are considered as excellent markers of plant disease resistance against pathogens [6,29,41].

The aim of this study is to monitor the concentration of catechin, epicatechin and their enantiomers in leaves of infected Mexican lime by *Ca. Phytoplasma aurantifolia*, and also some physiological responses such as POD, PAL and CHS activities as well as MDA-content during WBDL progression.

## 2. Materials and methods

### 2.1. Plant material and inoculation

Twenty healthy 1-year-old Mexican lime trees grown in the greenhouse were arranged on the greenhouse bench. Specimens from Mexican lime trees infected with witches' broom disease were grafted to half of them randomly and were covered for 30 days with plastic bags to increase their humidity. All trees were kept under natural light conditions at a temperature of 25–28 °C. Diagnoses of WBDL in trees were based on polymerase chain reaction (PCR) using P1/WB3 primers [42].

The experiment was conducted using a randomized complete block design (RCBD). Randomly, leaves of five infected trees by *Ca. Phytoplasma aurantifolia*, as a treatment and five healthy trees without *Ca. Phytoplasma aurantifolia* inoculation, as a control were sampled every 1 month over a period of 6 months and used for further analysis.

### 2.2. Chemical materials

The standard chemicals were purchased from Merck (Darmstadt, Germany). HPLC grade solvents were also obtained from Merck. MilliQ-water was prepared by a MilliQ-System (Mil-lipore, Saint-Quentin-en-Yvelines, France).

### 2.3. Instrumentation

The instruments used in this study were as follows: a LC-10AD VP liquid chromatography pump (Shimadzu Co. Japan), a SPD-10A UV–VIS detector, a computer-controlled system with GC_CHROM software and 20 μL sample loop. The analytical HPLC columns were ODS-120A Varian column (250 mm × 4.6 mm, 5 μm) and Eurecel 01 column (120 mm × 8 mm, 5 μm) packed with cellulose (tris 3, 5-dimethylphenylcarbamate (Knauer, Germany).

Thermo Fisher LCQ ion trap mass spectrometer (Bremen, Germany) with the ability of scanning the range of 10–2000 *m/z* was used for the analysis of the samples. Also, software of X-calibur was utilized. Negative mode of ESI-MS under capillary voltage of −2.0 KV and skimmer cone voltage of +20 V was applied to determine the molecular weights of the sample components.

### 2.4. Extraction of catechin and epicatechin from leaves

The leaves of healthy and infected lime were dried using liquid nitrogen and then powdered. Total phenols of each plant (0.4 g) were extracted by ethanol, under continuous stirring at 300 × g, during 2 h at room temperature. The extracts were filtrated and diluted with water (1:1 v/v). Less polar compounds were discarded using chloroform. Afterwards, catechin and epicatechin were extracted by ethyl acetate and then injected to HPLC [26].

### 2.5. Separation and identification of catechin and epicatechin

To separate and identify catechin and epicatechin in lime, the reversed phase HPLC method was evaluated using C18 column with different length. The optimum condition was obtained by an ODS-120A Varian column (250 mm × 4.6 mm i.d. 5 μm) which was connected to the HPLC system (Shimadzu, Kyoto, Japan), 20 μl loop for each sample, methanol and water (20:80v/v) elution at a flow rate of 1 ml/min and the UV–Vis detector at 280 nm. The resolution of catechin and epicatechin was evaluated by the analysis of spiked crude extract with the standard solution of catechin and epicatechin using the optimum chromatographic separation conditions.

### 2.6. Determination of enantiomeric ratio of catechin and epicatechin

Catechin and epicatechin were collected in the effluent from a C18 column. Then, the solvent was evaporated and the extracted sample was dissolved in ethanol. The column Eurecel 01 column (120 mm × 8 mm, 5 μm) was used for these analysis and n-hexane:ethanol:acetic acid (64.5:35:0.5 v/v/v) was applied as the mobile phase at a flow rate: 1 ml/min and temperature 40 °C [15].

### 2.7. Preparation of enzyme solution

One gram of each plant was homogenized in 5 ml of phosphate buffer (0.1 M; pH 7.2) at 4 °C and was centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was diluted 5-fold and subsequently used as the enzyme source [12,14].

### 2.8. Assay of phenylalanine ammonia-lyase (PAL)

PAL activity was determined by Ref. [12] method as follow: 0.1 ml of enzyme solution was incubated with 2 ml of 3 mM L-phenylalanine solution and 0.9 ml of water at pH 8.5. Then, absorbance was measured at 270 nm. This reaction was as a test sample. Blank was prepared with the same composition. The only difference between them was using 0.1 ml 150 mM Tris–HCl buffer,

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