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Analytical Methods

Determination of flavonoid level variation in onion (*Allium cepa* L.) infected by *Fusarium oxysporum* using liquid chromatography–tandem mass spectrometry

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ABSTRACT

In order to evaluate the flavonoid level variation in an onion (*Allium cepa* L.) infected by *Fusarium oxysporum*, the bulbs of a healthy onion and of an infected one were analysed for flavonoids via high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS). Among eleven flavonoids characterised, isorhamnetin 4′-*O*-galactoside (**8**) was identified in an onion for the first time. When the healthy bulb was inoculated with the fungus, the two quercetin derivatives (**4** and **7**) and the two isorhamnetin derivatives (**5** and **9**) underwent concentration changes typical for the defense materials against pathogens. The yellow granules that were accumulated on the abaxial epidermal cell layers after 8 days of inoculation were confirmed as quercetin (**10**) and isorhamnetin (**11**). It was deduced that they were produced from flavonoids **4**, **5**, **7** and **9** by hydrolysis enzyme of the fungus.

1. Introduction

Onions (*Allium cepa* L.) are the oldest vegetable in continuous cultivation, dating back to at least 5000 BC. They can be cultivated from the tropics to sub-polar regions (http://en.wikipedia.org/wiki/Onion). As with other crops, onions are also susceptible to the attack of numerous fungal pathogens that reduce yield and quality. Fusarium basal rot (FBR) arising from the invasion of the fungus *Fusarium oxysporum* through skin wounds is one of the most destructive soil-borne disease in onions worldwide (Bayraktar, Türkkan, & Dolar, 2010). Once there is a wound in the onion tissue, the fungi can enter and begin to spread up into the leaf system. Onions may become infected at any point in the field or during storage (Bayraktar et al., 2010; Cramer, 2000). The disease begins with yellowing from the leaf tips, which is followed by progressive wilting, curling and brown discolouration on infected plants and the symptoms move downward. In the advanced stage of infection, the pathogen causes pitting and decaying of the basal plate of bulbs, watery rot and the development of white, fluffy mycelium (Sumner, 1995). Several cultural strategies including crop rotation, pre-plant soil fumigation, avoiding wounds, storing bulbs in cool, biological control using fungal and bacterial antagonists and growing varieties resistant against FBR have been proposed for controlling the pathogen development. Among the available options, the use of FBR-resistant varieties can effectively reduce losses to FBR. A number of intermediate and long-day onion varieties are resistant against FBR infection (Cramer, 2000). However, little research has examined the chemical and physiological resistance mechanisms of onions against FBR. This resistance may be attributed, at least in part, to the role of the defense materials produced by the onion itself. Plants have a potency to activate a series of defense mechanisms to withstand the pathogen attack during infection (Chaves & Gianfagna, 2007). Therefore, understanding the defense materials and their functions by which the plant responds to pathogen attack may elucidate molecular markers for selecting genotypes with higher disease resistance and reveal an alternative disease control strategy. Flavonoids with a basic skeleton of phenylbenzo-γ-pyrone are a

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member of the family of secondary metabolites ubiquitously found in the plants. Plant flavonoids have attracted great interest as antioxidants providing beneficial effects for human health. According to epidemiological reports, they have diverse biological activities such as reducing the risk of various types of chronic disease (Erlund, 2004; Le Marchand, 2002), and exerting antioxidant, anti-aging and antibacterial effects (Xu et al., 2007). They also play an important role in plants themselves as physiologically-active components, including stress-protecting agent, attractants, feeding deterrents, signaling materials between plants and soil microorganisms and defense materials against biotic and abiotic stresses (Harborne & Williams, 2000; Treutter, 2006; Vierheilig & Piché, 2002). Since several flavonoids are toxic toward disease germs but not mammals, research interest has focused on their possible application in subduing plant pathogens in an environmentally friendly manner (Benavente-Garcia, Castillo, Marin, Ortuño, & Del Rio, 1997).

This work aimed at characterising the flavonoids of onion (Hanaro cultivar) infected by FBR using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) and at elucidating the defense materials and molecular markers against the disease. The flavonoid defense materials were profiled as the first step in understanding their physiological role in the defense mechanism of onions and breeding onion varieties with stronger natural defenses against the FBR pathogen attack.

2. Materials and methods

2.1. Materials and chemicals

Onion (*A. cepa* L.) bulbs were harvested from Gyeongsangnam-do Agricultural Technology Education Center (GATEC), Republic of Korea, in November 2010. The bulbs were authenticated as homozygous in genetic background by Dr. Chae-Shin Lim of GATEC. The bulbs were lyophilised (PVTED50A, Ilsin Bio Base Co. Ltd., Yangju, Republic of Korea), and stored at −70 °C until needed. Quercetin 3,4′-*O*,*O*′-diglucoside, quercetin 4′-*O*-glucoside and isorhamnetin were purchased from Extrasynthese (Genay, France), and quercetin, quercetin 3-*O*-glucoside and kaempferol from Sigma–Aldrich Co. (St. Louis, MO, USA). The substances were used as standards for calibration after recrystallisation in ethanol. The purity of all standards was confirmed by HPLC to be higher than 99%. All solvents and pure water were purchased from Duksan Pure Chemical Co. Ltd. (Ansan, Republic of Korea). *F. oxysporum* (KACC No. 6076) was obtained from the Korean Agricultural Culture Collection.

2.2. Pathogen inoculation

The fresh onion bulbs were sterilised with 70% ethanol by dipping for 10 s, washed with distilled water three times and air-dried on a clean bench. *F. oxysporum* was sub-cultured on potato dextrose agar (Difco, MD, USA) for 1 week at 27 ± 2 °C. The bulbs were placed in rectangular plastic boxes that contained five layers of paper towel (Whatman Kimtowel L25) on the bottom to maintain the humidity. The bulbs were wounded with a needle and inoculated with an agar disc containing mycelium of *F. oxysporum*. The inoculated bulbs were incubated at 25 °C. The agar was removed from the diseased samples. The samples were collected at one-day intervals for up to 9 days after inoculation. After liquid nitrogen was poured onto the samples, they were ground with a pestle in a mortar, lyophilised and stored at −70 °C.

2.3. Extraction and purification of flavonoids

The lyophilised onion bulb (3 g) was poured into 70% methanol (50 mL), homogenised using a Polytron blender (Brinkman Instruments, Westbury, NY, USA) for 2 min, and treated with a sonicator (100 W, 42 kHz, Bransonics 3510RDTH, Danbury, USA) for 10 min at room temperature. After repeated extraction ($n = 3$), the samples were combined and centrifuged for 10 min at 3200*g*. The supernatant was transferred into a 200 mL flask. The solvent was removed under reduced pressure with a rotary evaporator (Eyela NVC-2100, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The residue was dissolved in methanol/CH_2Cl_2 (1:5, v/v, 2 mL) and loaded onto a homemade silica gel column (1 × 5 cm, 70–230 mesh, Merck 7734). The column was washed with hexane (5 mL) and eluted with ethyl acetate (20 mL). The solvent was removed under reduced pressure. The residue was reconstituted in methanol (1 mL), filtered through 0.45 μm cellulose membrane, transferred into glass vials and stored at −20 °C until analysis.

2.4. HPLC–MS/MS

HPLC–MS/MS experiments were performed according to the literature method (Kim et al., 2011) except for the use of a solvent system consisting of 0.1% aqueous acetic acid (A) and methanol (B). The gradient conditions of the mobile phase were from 20% to 30% of B over 10 min, increased to 70% B over 15 min, decreased to 20% B over 5 min, and then isocratic elution for 10 min.

2.5. Statistical analysis

All determinations were performed in quintuplicate and the mean ± standard deviation values were calculated. The data were subjected to analysis of variance for repeated measurement (SAS version 9.1.3) and significant differences were between means ($p < 0.001$).

3. Results and discussion

3.1. Separation and characterisation

The flavonoid mixture was isolated from the onion bulbs by extraction with 70% aqueous methanol. Extraction with aqueous alcoholic solvent followed by isolation by reverse phase chromatography over C_{18} column has been extensively used for purification of plant flavonoids (Dou, Lee, Tzen, & Lee, 2008). In this work, the aqueous methanol extract was chromatographed over silica gel using a mixture of methanol and CH_2Cl_2 as an eluent. Silica gel is a cheaper absorbent than C_{18}. It has been demonstrated that flavonoid mixture can be effectively isolated from the plant extract by chromatography over silica gel (Lee et al., 2011). The isolated flavonoids were characterised by reverse phase HPLC (RP-HPLC), MS/MS in negative ion mode and compared with reported data. Since flavonoid compounds are moderately polar, they are usually analysed primarily on RP-HPLC coupled to ESI-MS (Choi et al., 2010). After testing several RP-columns for their suitability, a Zorbax SB-C_{18} column was chosen because it provides good peak separation and symmetry. The binary solvent system of 0.1% aqueous acetic acid and methanol was used as the mobile phase because it provided good separation between the chromatographic peaks. A mass spectrometer with electrospray interface was operated over the scan range m/z 100–1000 in negative ion mode.

The eleven flavonoids (**1**–**11**) are labeled between 10 to 60 min in the chromatogram of the healthy and diseased onion bulbs recorded at 360 nm at 8 days after inoculation (Fig. 1). The structures and HPLC–MS/MS data of the eleven flavonoids are shown in Table 1. Ten flavonoids (**1**–**7**, **9**–**11**) have previously been characterised for other onion varieties (Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2005; Grzelak, Milala, Król, Adamicki, & Badełek, 2009; Lee &