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Short communication

Determination of polyphenol levels variation in *Capsicum annuum* L. cv. Chelsea (yellow bell pepper) infected by anthracnose (*Colletotrichum gloeosporioides*) using liquid chromatography–tandem mass spectrometry

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ABSTRACT

Healthy fruits of *Capsicum annuum* L. cv. Chelsea (yellow bell pepper) and one infected by *Colletotrichum gloeosporioides* were analysed for polyphenols via high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS). Among seven polyphenols characterized, four components in the *C. annuum* fruits were identified for the first time. To investigate the characteristics of the polyphenols as defence materials, the content change of the fruit polyphenols inoculated with *C. gloeosporioides* was monitored by HPLC. It was observed for the first time that *de novo* induced *N*-caffeoyl putrescine (**1**) and caffeoyl *O*-hexoside (**2**) appeared to act as a phytoalexin in the defence mechanism of the *C. annuum* fruits against *C. gloeosporioides*, and constitutively formed feruloyl *O*-glucoside (**3**), kaempferol *O*-pentosyldihexoside (**4**) and dihydroxyflavone *O*-hexoside (**7**) as a phytoanticipin in the diseased *C. annuum* fruits.

1. Introduction

Anthracnose, one of the most destructive pepper diseases, is caused by several *Colletotrichum* species, including *Colletotrichum gloeosporioides, Colletotrichum acutatum, Colletotrichum* capsici, and *Colletotrichum coccodes*. It causes extensive pre- and post-harvest damage to the pepper family (*Capsicum* spp.) in the rainy and warm seasons. Under circumstances favourable to the disease development, up to 100% of the fruit's marketable yield can be lost. Even tiny anthracnose lesions on the pepper fruits drastically reduce the market value (Harp et al., 2008).

Pepper is an important crop that is heavily consumed worldwide as a season-free vegetable and has a total annual production value in South Korea of about US $1.4 billion. About 10% of Korea's total annual pepper production is lost due to anthracnose (Kim, Park, Choi, Lee, & Kim, 2008). Several strategies have been proposed for controlling the pathogen development, including cultural control methods, such as planting pathogen-free seeds, crop rotation with non-host crop, elimination of alternate host, chemical and biological fungicide application and the use of intrinsically resistant cultivars (Than, Prihastuti, Phoulivong, Taylor, & Hyde, 2008). Among the available options, the breeding of intrinsically resistant cultivars remains the goal of pepper breeders. The use of *Colletotrichum*-resistant varieties can effectively reduce the total cost of disease control (Agrios, 2005). Some pepper varieties are reportedly resistant to anthracnose and, especially, inoculation with *Colletotrichum* pathogen resulted in only limited lesions for certain varieties (Than et al., 2008; Yoon, 2003). However, information available on the resistance against the disease is scattered (Babu et al., 2011) and sufficient overall resistance has not yet been achieved in the pepper varieties (Park, 2007). Although little is known about the biochemical and physiological resistance mechanisms of peppers against anthracnose, this resistance may be ascribed, at least in part, to the defence compounds produced by pepper itself. Plants have a potency to utilise a series of defence materials against pathogen attack during infection (Chaves & Gianfagna, 2007). Therefore, an investigation of the defence materials by

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which the plants respond to pathogen attack promises to facilitate the selection of genotypes with higher disease resistance and the establishment of alternative disease control strategies utilising the materials. A number of *de novo* induced and constitutive polyphenols act as plant defence materials against the diseases (Koskimäki et al., 2009; Treutter, 2006).

In this work, the polyphenols of the healthy *Capsicum annuum* L. cv. Chelsea (yellow bell pepper) and of one infected by anthracnose are characterised using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) and the defence materials and molecular markers against the diseases were investigated. Metabolomic profiling of the polyphenol defence materials will be the starting point to investigate their physiological role in the defence mechanism of the *C. annuum* plant, and breeding a variety with stronger natural defence power against the pathogen attack.

2. Experiments

2.1. Materials and chemicals

C. annuum L. cv. Chelsea fruits were obtained from Gyeongsangnam-do Agricultural Research and Extension Services Technology Education Center (ATEC) in November 2010. The fruits were authenticated as homozygous in genetic background by Chae-Shin Lim of ATEC. Caffeic acid (±98%), ferulic acid (99%), 6,2′-dihydroxyflavone (98%), luteolin (≥98%), quercetin 3-*O*-rhamnoside (98%) and kaempferol (≥97%) used as the external standards were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All solvents and pure water were purchased from Duksan Pure Chemical Co. Ltd. (Ansan, Republic of Korea). *C. gloeosporioides* isolated from *C. annuum* (red pepper) (KACC No. 40690) was obtained from the Korean Agricultural Culture Collection.

2.2. Pathogen inoculation

The fresh *C. annuum* fruits were sterilized with 1% *aq*-NaOCl solution by dipping for 1 min, washed with distilled water three times and air-dried in the laboratory. *C. gloeosporioides* was subcultured on potato dextrose agar (PDA, Difco, MD, USA) for two weeks at 28 ± 2 °C. Spore concentrations were adjusted to 1.5×10^6 spores/ml with sterile distilled water. The conidial suspension was then dropped on the fruit surface (10 μl per a site) wounded by a syringe needle. After inoculation, the fruits were placed in a beaker (500 ml) overlaid with five layers of paper towel (Whatman Kimtowel L25) to maintain high humidity and were incubated at 25 ± 2 °C. Samples were collected at 1-day intervals for up to 8 days after wound inoculation. Liquid nitrogen was immediately poured over the collected fruits which were then ground with a pestle in a mortar.

2.3. Extraction

The ground fruits (2 g) were added to 70% methanol (20 ml). The mixture was homogenised using a Polytron blender (Brinkman Instruments, Westbury, NY, USA) for 5 min, treated in a sonicator (100 WATTS, 42 KHZ, Bransonic® 3510R-DTH, Danbury, USA) for 10 min at room temperature and filtered through filter paper (Whatman No. 1, Whatman International Ltd. Maidstone, UK) under reduced pressure. The filtrate was centrifuged at 4000*g* using a model SCT4B centrifuge (HITACHI, Ibaraki, Japan) for 15 min. The supernatant was filtered through a PTFE syringe filter (Titan, 0.45 μm, SMI-Lab Hut Co. Ltd. Maisemore, UK). The filtrate was stored at −20 °C until analysis.

2.4. HPLC–MS/MS

HPLC–MS/MS experiments were performed according to the method reported by Kim et al. (2011) without solvent system. The binary solvent system consisted of 1% aqueous acetic acid (A) and methanol (B). The gradient conditions of the mobile phase were from 2% to 45% of B over 55 min, followed by isocratic elution for 15 min.

2.5. Quantification

All of the polyphenols were quantified using chromatograms extracted at 360 nm. Since none of the polyphenols in *C. annuum* except quercetin 3-*O*-rhamnoside (**6**) were commercially available, the individual components were quantified using the calibration curves of external standards containing the same aglycone. Thus, *N*-caffeoyl putrescine (**1**), caffeoyl *O*-hexoside (**2**) and feruloyl *O*-glucoside (**3**) were quantified as their corresponding acid, kaempferol *O*-pentosyldihexoside (**4**) as kaempferol, luteolin 7-*O*-(2-apiosyl)glucoside (**5**) as luteolin, and dihydroxyflavone *O*-hexoside (**7**) as 6,2′-dihydroxyflavone. Plant phenols for which standards are not available were routinely quantified using the standard curve of a related compound (McGhie, Hunt, & Barnett, 2005). The calibration curves were constructed by using seven different concentrations (0.1, 1.0, 10, 50, 100, 500, and 1000 mg/l) of each standard and by plotting the concentration of the standard against the peak area.

2.6. Statistical analysis

The repeated measurements ($n = 3$) were subjected to analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($p < 0.001$), using SAS version 9.1.3.

3. Results and discussion

3.1. Separation and characterisation

The polyphenols were isolated from the *C. annuum* L. cv. Chelsea fruits by extraction with 70% aqueous methanol. The isolated polyphenols were characterised by HPLC over a C_{18} column, MS/MS in negative ion mode, UV data and a comparison with reported data. Seven polyphenols (Table 1) were detected in healthy and/or diseased fruits 6 days after inoculation; in the Fig. 1 chromatogram they are observed between 10 and 60 min. The structures and HPLC–MS/MS data of the seven polyphenols are shown in Fig. 2 and Table 1, respectively. Polyphenols **3**, **5** and **6** were recently characterised for other *C. annuum* varieties (Marín, Ferreres, Tomás-Barberán, & Gil, 2004), while the other four polyphenols, **1**, **2**, **4** and **7** were characterised for the first time by their mass fragmentation patterns, along with the literature data. Polyphenol **1** was characterised as *N*-caffeoyl putrescine using positive ion mode experiment. Its MS/MS produced $[M + H]^+$ at m/z 251, which fragmented into m/z 234 (*N*-(4-butenyl)caffeamide, $[M + H]^+$-NH_3), 192 (*N*-methylenecaffeamide, $[M + H]^+$-C_3H_9N), 163 (caffeoyl, $[M + H]^+$-putrescine), and 135 ($[M + H]^+$-putrescine-CO) (Camacho-Cristóbal, Lunar, Lafont, Baumert, & González-Fontes, 2004). Polyphenol **2** yielded $[M-H]^-$ at m/z 341 as a base peak, which fragmented to give m/z 179 ($[M-H]^-$-hexosyl), 161 ($[M-H]^-$-hexose), and 135 ($[M-H]^-$-hexosyl-CO) (Gouveia & Castilho, 2010). Polyphenol **2** was identified as caffeoyl *O*-hexoside. Polyphenol **4** was identified as kaempferol *O*-pentosyldihexoside. Its MS/MS produced $[M-H]^-$ at m/z 741, which fragmented into m/z 609 ($[M-H]^-$-pentosyl), 447 ($[M-H]^-$-pentosyl-hexosyl), and 285