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Analytical Methods

Anti-inflammatory and antioxidative activity of anthocyanins from purple basil leaves induced by selected abiotic elicitors

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ABSTRACT

This paper investigates changes in the anti-inflammatory and antioxidative activity of anthocyanins from purple basil (*Ocimum basilicum* L.) leaves induced by arachidonic acid (AA), jasmonic acid (JA) and β-aminobutyric acid (BABA). The anthocyanins content was significantly increased by all elicitors used in this study; however, no increase was observed in the antioxidant activity of the analyzed extracts. Additionally, a significant decrease by about 50% in the ability to chelate Fe(II) was noted. Further, an increase in the potential anti-inflammatory activity of basil anthocyanins was observed after treatment with each the abiotic elicitor. The IC_{50} value for lipoxygenase inhibition was almost twice as low after elicitation as that of the control. Also, cyclooxygenase inhibition by anthocyanins was stimulated by abiotic elicitors, except for JA-sample. Additionally, HPLC-analysis indicated that elicitation with AA, JA and BABA caused increases in content most of all anthocyanin compounds.

1. Introduction

Consumption of vegetables, fruit and other food of plant origin, especially fresh spices, has been growing in recent years because these products are relatively rich in vitamins, minerals and other bioactive compounds.

Basil (*Ocimum basilicum* L.) is one of the most popular and readily available culinary herbs originating from India, Africa and southern Asia (Makri & Kintzios, 2007). These days, basil is cultivated worldwide: in particular because it is a rich source of natural compounds, such as monoterpenes, sesquiterpenes, phenylpropanoids, anthocyanins and phenolic acids (Hussain, Anwar, Sherazi, & Przybylski, 2008).

Purple basils are very valuable herbs both for their ornamental value and a good source of anthocyanins (Phippen & Simon, 1998). Anthocyanins belong to flavonoids which represent a large class of plant secondary metabolites. This group of flavonoids are water-soluble pigments which occur in most species in the plant kingdom. Anthocyanins are accumulated in cell vacuoles, especially in flowers, fruits and vegetables, but they are also found in leaves, stems, seeds and other tissues (Goto & Kondo, 1991). Anthocyanins functions within plants include UV protection, attraction of animals for pollination and seed dispersal, defense against pathogens and pests, and protecting DNA and the photosynthetic apparatus from high radiation fluxes. Additionally, they can serve both as antioxidants and in the acquisition of tolerance to many different kinds of environmental stressors, such as cold or frost, heavy metal contamination, desiccation and wounding (Hatier & Gould, 2009).

Increasing interest in anthocyanins from fruits, vegetables and herbs has been focused recently on their biological activities, such as antioxidant, anti-inflammation, cardioprotective, antitumor, antidiabetic and eye function properties (Ghosh & Konishi, 2007; Pallavi, Elakkiya, Tennety, & Devi, 2012). Antioxidant and anti-inflammatory activities of anthocyanin are manifested via its ability to neutralize free radicals as well as inhibition of lipoxygenase and cyclooxygenase, enzymes metabolizing arachidonic acid (Bowen-Forbes, Zhang, & Nair, 2010).

Since modern horticultural production concentrates on increasing crop yield and improvements in the quality and the safety of plant and herbal food, it is recommended to cultivate herbs using ecological methods. Thus, cultivation without the use of pesticides has become increasingly common. For this reason, there is growing interest in studies using alternative methods of crop protection such as wounding or elicitor treatment. Apart from this, significant interest in increasing the amount of functional phytochemicals, such as phenolic compounds and terpenoids is observed (Kim, Chen, Wang, & Rajapakse, 2006; Złotek, Świeca, & Jakubczyk, 2014).

It is well known that the level of phenolics in plants is generally affected by genetic and environmental factors such as the cultivar, illumination and temperature (Świeca, Gawlik-Dziki, Kowalczyk, &

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Złotek, 2012). In recent years, scientists have been looking for new alternatives to conventional methods for plant protection, and simultaneously for improvements in health properties and bioactive compound content. One of these methods is elicitation, that is induction of natural plant resistance mechanisms using biotic or abiotic factors. Elicitation can be an important strategy towards obtaining improved production of plant secondary metabolites *in vivo*. In cell and organ cultures, biotic and abiotic elicitors have effectively stimulated production of wide range of plant secondary metabolites (Hussain et al., 2012). Phenolic compounds, especially anthocyanins, are known from their antioxidant, anti-inflammatory and anticancer properties (Bowen-Forbes et al., 2010). Anthocyanins responsible for the various attractive colours in plants are also becoming important alternatives to many synthetic colourants due to increased public concerns over the safety of artificial food colours.

It has been reported that various biotic and abiotic elicitors significantly increased the production of secondary metabolites such as anthocyanins in plants (Horbowicz, Mioduszewska, Koczkodan, & Saniewski, 2009; Saw, Riedel, Kütük, Ravichandran, & Smetanska, 2010).

Elicitors are biotic and abiotic substances that induce resistance in plants. Commonly known abiotic elicitors include jasmonic acid (JA) and arachidonic acid (AA). Jasmonic acid and arachidonic acid represent plant hormones which can induce biosynthesis of compounds directly associated with the defense system to protect plant from stresses and pathogens, but also secondary metabolites such as phenolic compounds and terpenoids (Kim et al., 2006; Savchenko et al., 2010; Złotek et al., 2014).

However, the effect of abiotic elicitors on the induction of anthocyanins in basil has not been actively investigated.

In this study, changes in production and biological properties of anthocyanins in purple basil (*O. basilicum* L.) as a result of elicitation with jasmonic acid, arachidonic acid and β-aminobutyric acid, were investigated. For consumers, dieticians and product quality, it is essential that the value of the health benefits of the entire pool of anthocyanins is assessed rather than individual compounds.

2. Materials and methods

2.1. Plant materials and growth conditions

Purple basil seeds (*O. basilicum* L. cv. Dark Opal) were purchased from W. Legutko Breeding and Seed Company. Basil seeds were sown into sowing boxes filled with universal soil for sowing seeds. Seven-day-old seedlings were transplanted to 600 mL pots containing universal garden soil (four plants per pot and four pots for each variant of treatment). Plants were grown in a growth chamber (SANYO MLR-350H) at 25/18 °C, photoperiod 16/8 h day/night, with PPFD (photosynthetic photon flux density) at a plant level of 500–700 μmol m^{-2} s^{-1} and a relative humidity of 75%. The seedlings were fertilized twice (before transplantation and one week after transplantation) at the following levels (in mg L^{-1}): N – 50, P – 50, K – 100, Mg – 60. Twenty-one-day-old plants were sprayed with a water solution of 10^{-6} M jasmonic acid (JA) (Sigma) and 10^{-6} M arachidonic acid (AA) (Sigma) and 10^{-2} M β-aminobutyric acid (BABA) (Sigma), respectively (AA, JA and BABA had previously been dissolved in a very small amount of ethanol). The control plants (C) were sprayed with a very small amount of ethanol in deionized water. The concentrations of elicitors were selected based on literature data (Oikawa, Ishihara, & Iwamura, 2002; Pajot, Le Corre, & Silué, 2001; Rozhnova, Gerashchenkov, & Babosha, 2003) and previous screening experiments (data not published), to avoid negative effects on the health of the plants. Fifteen days after elicitation the herb was collected and frozen.

2.2. Extraction procedure

Frozen leaf tissue (2 g in triplicate) was ground in a mortar and pestle with 15 mL of acidified methanol (0.1% HCl) and the anthocyanins were extracted for 1 h at 4 °C, then centrifuged at 9000×*g* for 30 min. This procedure was repeated three times and the supernatants were combined and adjusted to 50 mL of final volume with acidified methanol – this was a crude extract of anthocyanins. The extraction procedure was followed by removing of the methanol using a vacuum at a temperature of 40 °C in a rotary evaporator (RVO 200A, Ingos), then making up remaining aqueous extract to 5 mL with acidified deionized distilled water. Anthocyanin separation was carried out according to the method described by Rodriguez-Saona and Wrolstand (2001). The water extracts were loaded on a C-18 cartridge (Supelco, PA) activated with acidified methanol followed by 0.1% HCl (v/v) in deionized water. Anthocyanins were adsorbed onto the column while carbohydrates, acids, and other water-soluble compounds were washed out by flushing with 0.1% HCl. Anthocyanins were recovered with methanol containing 0.1% HCl (v/v). The methanol fractions were evaporated using a rotary evaporator at 40 °C and the pigments were redissolved in acidified methanol HPLC grade (0.1% HCl) and used in further analyses. Cartridges were washed with ethyl acetate to remove phenolic compounds other than anthocyanins.

2.3. Anthocyanin determination

The total anthocyanin content in *O. basilicum* was determined using the pH differential method (Giusti & Worlstad, 2001). Two dilutions of the same sample were prepared in 0.025 M potassium chloride solution and in 0.4 M sodium acetate solution adjusted to pH 1.0 and 4.5 with HCl, respectively. The absorbance of each dilution was measured at 520 and 700 nm against a distilled water blank using a Lambda 40 UV–Vis spectrophotometer. Absorbance (A) was calculated as follows:

$$A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$$

The anthocyanin concentration in the original sample was calculated using the following formula:

$$\text{Anthocyanin content } (mg/L) = (A \times MW \times DF \times 1000)/(\varepsilon x1)$$

where MW is the molecular weight of cyanidin-3-O-glucoside (449.2 g mol^{-1}), DF is the dilution factor, and ε is the molar extinction coefficient of cyanidin-3-glucoside (ε = 26 900 L cm^{-1} mol^{-1}).

Total anthocyanin content was calculated in the sample as mg per g of fresh weight (FW).

2.3.1. Anthocyanin analysis

Purified extracts were used for the quantitative analysis of anthocyanins via HPLC. Samples were analyzed with a Varian ProStar HPLC System separation module equipped with a Nucleosil 100-5 C18 reverse-phase column (250 × 4.6 mm, particle size 5 μm, pore size 100 Å) and a ProStar DAD detector. The column thermostat was set at 25 °C. An elution gradient with 4.5% formic acid in water as solvent A and 2% formic acid (v/v) in acetonitrile as solvent B was used with the following profile: 0–30 min: 100–73% A, 30–35 min: 73–87% A, 35–55 min: 87–0% A with a flow rate 1 mL min^{-1}. At the end of the gradient, the column was washed with acetonitrile and equilibrated to the initial condition for 10 min. Injection volume of the sample was 10 μL and detection was carried out at the wavelength 520 nm. Quantitative determinations were carried out with an external standard calculation, using calibration curves of the standards, at each of the following concentrations: 1.25, 2.5, 5, 10 and 20 μg mL^{-1} dissolved in 0.1% HCl in 100% methanol. Anthocyanins were separated and analyzed