

ELICITATION OF ANTHOCYANIN PRODUCTION IN ROOTS OF *KALANCHOE BLOSSFELDIANA* BY METHYL JASMONATE

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Received December 4, 2014; revision accepted March 18, 2015

The influence of methyl jasmonate on anthocyanin accumulation in roots of *Kalanchoe blossfeldiana* plants was studied. Methyl jasmonate (JA-Me), at a concentration of 5.0 to 40.0 mg.l⁻¹, substantially increased anthocyanin accumulation in roots of intact plants, when it was applied as a solution under natural light conditions. The production of anthocyanin depended on the concentration of methyl jasmonate and the age of the plant. The stimulatory effect was higher in older plants of *K. blossfeldiana* than in younger ones. When leaves were removed methyl jasmonate slightly stimulated anthocyanin accumulation compared with intact plants. The obtained results indicate that leaves are necessary for the anthocyanin accumulation in the roots. In isolated roots methyl jasmonate did not affect the accumulation of anthocyanins in light conditions. Seven anthocyanins were documented in the roots of control plants and 8 anthocyanins in the roots of JA-Me treated ones. JA-Me increased the level of anthocyanins in roots of old *K. blossfeldiana* plants 6.8, 6.0 and 3.6-folds, after 4, 8 and 14-days of treatment, respectively.

Key words: Anthocyanin, *Kalanchoe blossfeldiana*, methyl jasmonate (JA-Me), root, light

INTRODUCTION

Root flavonoids, including anthocyanin, play a significant role in regulating root growth and function, influencing different aspects of nitrogen cycle and protecting the plants against diseases (Rao, 1990). Production of anthocyanin also takes place in hairy roots genetically transformed with *Agrobacterium rhizogenes* strains such as *Daucus carota* (Kim et al., 1992) and *Lobelia chinensis* (Tada et al., 1996).

Anthocyanins are accumulated in plants subjected to stresses and various elicitors. The factors affecting the level of anthocyanins include: culture filtrates and cell extracts of fungal and bacterial origin, UV light, fluridone, jasmonates, ethylene, β -glucan, chitosan and inorganic ions (Zhang et al., 2002).

Jasmonic acid (JA) and its methyl ester (JA-Me) modulate many physiological processes in plants (Creelman and Mullet, 1997). These compounds can diffuse to the distal parts of the plant via the vapour phase or by intercellular migration, possibly

through the phloem. JA and JA-Me may participate in signal transmission over long distances in plant, as well as they may affect other plants (Cheong and Choi, 2003). Therefore, jasmonates are involved in many processes of the plant life cycle and their adaptation to environmental stress conditions. Jasmonates are important signal transducers in plant secondary metabolism. Treatment with JA-Me of a variety of cell suspension cultures led to elicitation of a wide range of secondary metabolites, such as flavonoids, guaianolides, anthraquinones and alkaloids (Gundlach et al., 1992).

Enhanced anthocyanin accumulation in response to jasmonates has been observed in several plant species, such as soybean seedlings (*Glycine max* Merr.) (Franceschi and Grimes, 1991), *Arabidopsis thaliana* (L.) Heynh. seedlings (Feys et al., 1994), in stems and leaves of the tulip (*Tulipa gesneriana* L.) (Saniewski et al., 1998), in a suspension culture of *Vitis vinifera* cells (Zhang et al., 2002; Curtin et al., 2003) and in cell cultures of *Vaccinium pahalae* (Fang et al., 1999). JA-Me

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added to growth medium induced anthocyanin accumulation and influenced on the composition in the sweet potato cell suspension cultures (Plata et al., 2003). Anthocyanin synthesis was also induced by wounding of *Petunia* corollas and this effect was enhanced by JA-Me (Tamari et al., 1995).

Kalanchoe blossfeldiana is a short day plant. Leaves of the plant become red during the flowering stage, especially at the apical end and on the underside (Neyland et al., 1963).

Saniewski et al. (2003) showed that JA-Me applied in lanolin paste on the middle part of the stem of *K. blossfeldiana* greatly stimulated accumulation of anthocyanins in the main and lateral stems. It can suggest that JA-Me is transported in *K. blossfeldiana* shoots in acropetal as well as basipetal directions. When leaves were removed from the plant, almost no anthocyanin formation was observed in the stem treated with JA-Me.

The results from previous studies on shoots (Saniewski et al., 2003) were the basis to conduct further research on accumulation of anthocyanin in the roots of *Kalanchoe blossfeldiana*.

MATERIALS AND METHODS

PLANT MATERIAL

The study was conducted on *Kalanchoe blossfeldiana* Poelln. with purpurea flowers belonging to the family Crassulaceae. Plants were grown in a mixture of soil, peat moss and sand (2:1:1) in a greenhouse under natural light conditions. In the roots of *K. blossfeldiana* cultivated in soil hardly any accumulation of anthocyanin was observed.

The cuttings used for experiments had the same number of leaves (12). The shoots from old plants (cultivated for 12–14 months) and young plants (cultivated for 3–4 months) were cut off and rooted in distilled water. Young *K. blossfeldiana* plants had green leaves and shoots while old plants had red leaves especially at the apical end and on the underside. For the experiments both vegetative and generative cuttings were used. The roots of intact plants or after excision of all leaves were soaked in methyl jasmonate solutions (5, 10, 20 and 40 mg·l⁻¹). The control plants were stored in distilled water only. The isolated roots were treated with middle concentration of JA-Me 20 mg·l⁻¹. The plants were subjected to experiments at day/night (22±3°C/18±3°C) and in dark conditions.

ANTHOCYANIN MEASUREMENT

Plant samples were taken for anthocyanins analysis after 4, 8 and 14 days of JA-Me treatment. Total anthocyanin content was determined using Mancinelli

et al. (1988) method with small modification. The pigments were extracted with 5 ml of acidified (1% HCl, w/v) methanol for 24 h at 4°C in darkness with occasional shaking. The extract was centrifuged for 10 min at 6000 g. Anthocyanin content was determined at 530 nm using an ε-Helios spectrophotometer. The results are expressed as cyanidin-3-glucoside equivalent using 29,600 as a molecular extinction coefficient.

For each treatment 5–7 plants were used. The results are the mean of five independent replicates.

They were statistically elaborated, using variance analysis. Duncan's multiple range t-test was used for assessment of differences between the means, adopting the significance level of P=0.05.

For separation, identification and determination of the content of individual anthocyanins, the roots were lyophilized and extracted with Dionex ASE 200 automatic system. Solvent used for the extraction was 0.1% HCl in 60% MeOH (Ju and Howard, 2003). The extract was purified and analyzed by mass spectrometry (LCQ-MS, Thermo Finnigan). Mass analyses were performed in the positive ionization mode, spray voltage was 3.9 kV, capillary voltage 35 V, tube lens offset 55 V, capillary temperature 180°C, nitrogen sheath 75, and auxiliary gas flows were 10. One of the compounds present in the extract was identified as 3,5 di-O-glucocyanidin chloride on the basis of its molecular ion, fragmentation pattern and literature data (Barnes and Schug, 2011). The content of this compound and seven other anthocyanins present in the extract were analyzed by analytical HPLC system (Waters S 600), a method described by Kammerer et al. (2003). The 3,5 di-O-glucocyanidin chloride from Extrasynthase (Lyon, France) was used as the standard.

RESULTS

It has been shown that treatment with JA-Me used at all concentrations from 5 to 40 mg·l⁻¹ stimulated anthocyanin accumulation in the roots of the old *K. blossfeldiana* plants growing in a greenhouse. The accumulation of anthocyanins depended on the light conditions in the greenhouse at the temperature 22±3°C/18±3°C (day/night). Temperature higher than 25°C caused lower accumulation of anthocyanin in the roots of *K. blossfeldiana* (only morphological observation).

The first anthocyanin measurement was performed after 4 days of treatment with JA-Me. There were no significant differences in the level of anthocyanin accumulation in the roots treated with JA-Me at concentrations ranging from 5 to 20 mg·l⁻¹ (Fig. 2). After 8 days of treatment, the content of anthocyanins in the roots increased. Its level in the

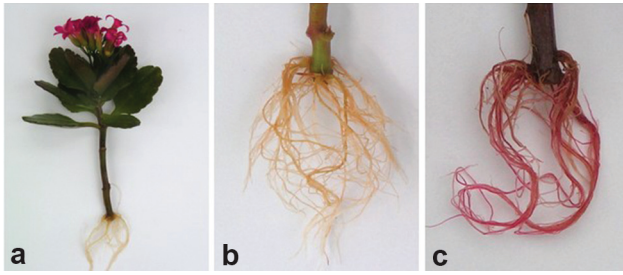


Fig. 1. *Kalanchoe blossfeldiana* plant (a); roots of the control plant (b); roots of the plant treated with JA-Me 40 mg·l⁻¹ for 14-days (c).

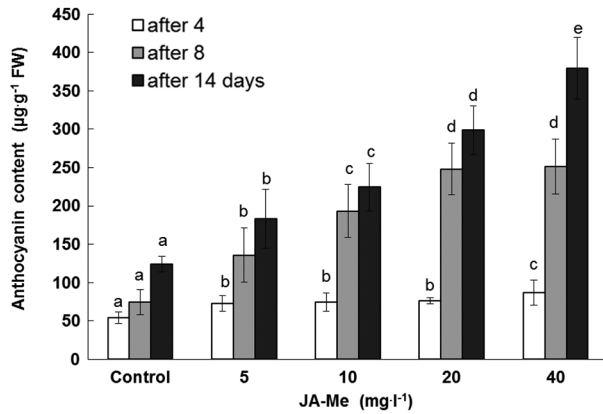


Fig. 2. Effect of treatment time and concentration of methyl jasmonate (5, 10, 20, 40 mg·l⁻¹) on anthocyanin accumulation in roots of old *K. blossfeldiana*, under natural light conditions. The results were elaborated statistically after 4, 8 and 14 days of treatment, separately. Means followed by the same letter are not significantly different at P=0.05 according to Duncan's test. Error bars indicate ±SD (n=10–12).

control plants was 74.69 µg·g⁻¹ of fresh weight (FW), whereas plants treated with 40 mg·l⁻¹ of JA-Me reached 251.40 µg·g⁻¹ FW. Anthocyanin accumulation in the roots after 14 days depended on the concentration of JA-Me. The highest level of anthocyanins (379.31 µg·g⁻¹ FW) was found in the roots treated with the highest concentration of JA-Me (40 mg·l⁻¹) (Fig. 1 and 2).

In the young plants (cultivated for 3–4 months) treated with JA-Me for 14-days, the accumulation of anthocyanin in the roots was lower than in the roots of old *K. blossfeldiana* plants (cultivated for 12–14 months). The level of anthocyanins in the roots of the plants treated with JA-Me 5 mg·l⁻¹ amounted to 145.8 µg·g⁻¹ FW and in the plants treated with JA-Me at a concentration of 40 mg·l⁻¹ was 138.3 µg·g⁻¹ FW (Fig. 3).

Figure 4 presents the results of anthocyanin content in the roots of old *K. blossfeldiana* plants

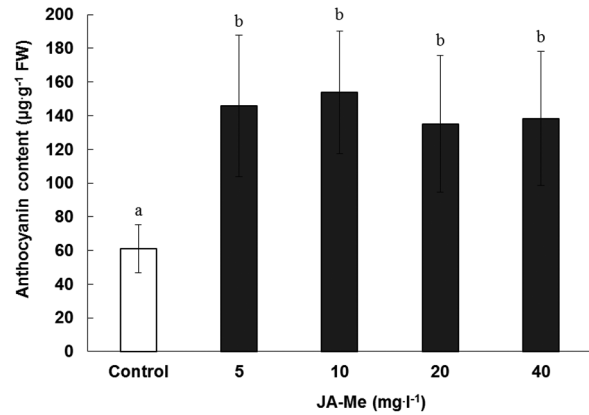


Fig. 3. Effect of methyl jasmonate (5, 10, 20, 40 mg·l⁻¹) on anthocyanin accumulation in the roots of young *K. blossfeldiana* plants, under natural light conditions; measured 14 days after treatment with methyl jasmonate. Means followed by the same letter are not significantly different at P=0.05 according to Duncan's test. Error bars indicate ±SD (n=10–12).

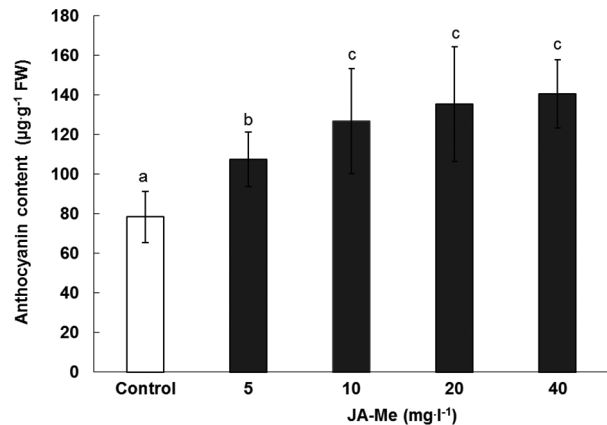


Fig. 4. Effect of leaves excision and treatment with methyl jasmonate (5, 10, 20, 40 mg·l⁻¹) on anthocyanin accumulation in roots of old *K. blossfeldiana* plants, under natural light conditions; measured 14 days after treatment with methyl jasmonate. Means followed by the same letter are not significantly different at p=0.05 according to Duncan's test. Error bars indicate ±SD (n=10–12).

after excision of all leaves. All the used JA-Me doses (5 to 40 mg·l⁻¹) slightly stimulated anthocyanin formation in roots under natural light conditions. In this case anthocyanin content did not depend on the concentration of JA-Me. This indicates that leaves are necessary for anthocyanin accumulation in the roots of *K. blossfeldiana* treated with JA-Me.

It was found that JA-Me had no effect on accumulation of anthocyanins in the isolated roots under natural light conditions. The content of anthocyanins in the roots of the intact plants treated with JA-Me was 311.14 µg·g⁻¹ FW, while in the isolated

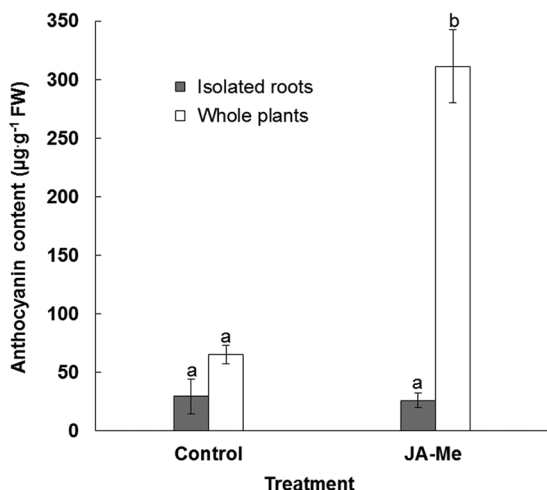


Fig. 5. Effect of methyl jasmonate (20 mg·l⁻¹) on anthocyanin content in intact and isolated roots of old *K. blossfeldiana* plants. The results were elaborated statistically for intact and isolated roots, separately; measured 14 days after treatment with methyl jasmonate. Means followed by the same letter are not significantly different at P=0.05 according to Duncan's test. Error bars indicate ±SD (n=10–12).

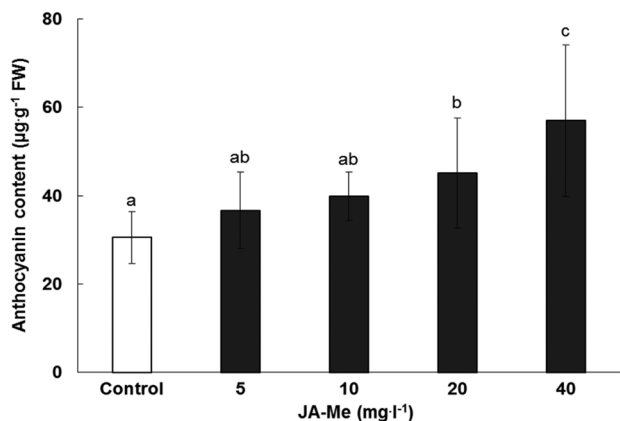


Fig. 6. Effect of methyl jasmonate (5, 10, 20, 40 mg·l⁻¹) on anthocyanin accumulation in roots of intact old *K. blossfeldiana* plants in the dark conditions; measured 14 days after treatment with methyl jasmonate. Means followed by the same letter are not significantly different at P=0.05 according to Duncan's test. Error bars indicate ±SD (n=10–12).

roots it was ~ 10-folds lower, and reached the level of only 25.96 µg·g⁻¹ FW (Fig. 5).

Figures 6 and 7 summarize the results of the experiment on JA-Me treatment of the old *K. blossfeldiana* plants in darkness. In plants possessing leaves, which were subject to the experiment, accumulation of anthocyanins in roots almost did not occur. (Fig. 6) compared with the roots of plants

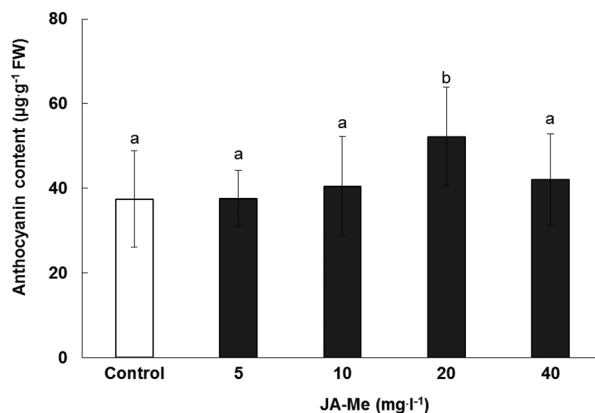


Fig. 7. Effect of methyl jasmonate (5, 10, 20, 40 mg·l⁻¹) on anthocyanin accumulation in roots of old *K. blossfeldiana*, plants without leaves in the dark conditions; measured 14 days after treatment with methyl jasmonate. Means followed by the same letter are not significantly different at P=0.05 according to Duncan's test. Error bars indicate ±SD (n=10–12).

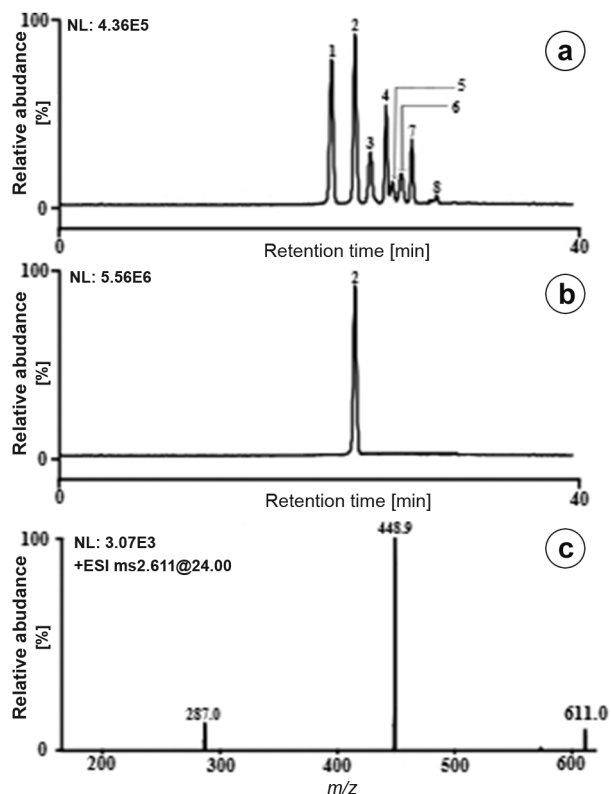


Fig. 8. Exemplary chromatograms obtained for extract of roots of *K. blossfeldiana* (a), standard of 3,5 di-O-glucocyanidin chloride (b), and mass spectrum of standard (c).

under natural light conditions (Fig. 2). Thus, in roots of *K. blossfeldiana*, light plays an important role in formation of anthocyanins and increases

TABLE 1. Effect of methyl jasmonate (20 mg·l⁻¹) and light irradiation on anthocyanins content in the roots of *K. blossfeldiana* after 4, 8 and 14 days, based on the 3,5-di-O-glucocyanidin chloride - compound No 2.

Treatment	Anthocyanins content [µg/100 mg lyophilized weight]								The total content of anthocyanins
	Compound								
	1	2	3	4	5	6	7	8	
Control (water) – light, after 4 days	4.18	1.95	1.40	1.14	0.00	0.93	0.75	0.00	10.34
JA-Me – light, after 4 days	31.33	14.43	9.27	6.24	1.75	5.15	2.09	0.00	70.26
Control (water) – light, after 8 days	7.33	2.77	2.25	1.51	0.00	1.48	0.68	0.00	16.01
JA-Me – light, after 8 days	34.71	17.17	16.12	7.59	3.99	14.25	2.10	0.87	96.10
Control (water) – light, after 14 days	13.09	4.49	4.50	2.24	1.00	3.78	0.78	0.00	30.04
JA-Me – light, after 14 days	24.03	21.12	11.12	21.27	3.60	10.45	15.36	3.36	110.30
Control (water) – dark, after 14 days	2.49	1.36	0.95	0.80	0.00	0.85	0.00	0.00	6.45
JA-Me – dark, after 14 days	6.49	2.82	1.91	1.29	0.00	1.12	0.73	0.00	14.36

production of these compounds. Also, in the plants in the dark after excision of all leaves, roots did not accumulate anthocyanins (Fig. 7).

Mass spectrometry analysis (LCQ-MS, Thermo Finnigan) of *K. blossfeldiana* roots was performed, and on the basis of interpretation of the fragmentation pattern, it was found that the compound 2 was 3,5 di-O-glucocyanidin chloride (Fig. 8). In order to confirm the structure, chromatographic analysis of the purchased standard was performed. The retention times – 23.23 min and mass-to-charge ratio (m/z) – [M]⁺ = 611.0 m/z; [M-Glc]⁺ = 448.9 m/z; [M-2 Glc]⁺ = 287.0 m/z of the compared peaks were identical.

The presence and content of individual anthocyanins in the roots of *Kalanchoe* plants treated in the light with middle concentration of JA-Me (20 mg·l⁻¹) were analysed after 4, 8, and 14 days of the experiment duration. Seven anthocyanins were found in the roots of control plants and 8 anthocyanins in the roots of JA-Me treated plants. In the roots of JA-Me increased the anthocyanin level by 6.8, 6.0 and 3.6 times, after 4, 8 and 14-days of treatment, respectively (Tab. 1).

DISCUSSION

Anthocyanins are accumulated in some plants during their lifetime, but in others they occur in a specific developmental period or in response to environmental stress (Chalker-Scott, 1999). Accumulation of anthocyanin in the leaves of *Kalanchoe blossfeldiana* is a photoperiodic response of the plant (Neyland et al., 1963).

Our study showed that accumulation of anthocyanin in the roots of *K. blossfeldiana* is greatly

stimulated by JA-Me applied as a solution to the roots at natural light irradiation in a greenhouse, when cuttings with red leaves were used for rooting.

Also Franceschi and Grimes (1991) reported that 5-day treatment with JA-Me induced five to seven-fold increase in anthocyanin accumulation in light-grown soybean seedlings, but inhibited anthocyanin biosynthesis in etiolated seedlings. Light can induce a photooxidative stress, and JA-Me stimulates the stress response by a signalling mechanism. For instance, jasmonic acid and light increased accumulation of anthocyanins in *Vitis vinifera* cell culture, and when combined caused a synergistic effect (Curtin et al., 2003). Enhanced anthocyanin accumulation in response to jasmonates has also been observed in plant cell cultures of *Vaccinium pahalae* (Fang et al., 1999). According to Takos et al. (2006), light stimulates signal transduction and expression of genes involved in anthocyanin biosynthesis.

Roots of some plants exposed to light can accumulate anthocyanins (Rao, 1990). Accumulation of anthocyanin in roots exposed to daylight has been observed in *Salix* and *Zea* (Tselas et al., 1979), *Fragaria × ananasa* (Nemec, 1973), *Impatiens* spp. (Thakur and Nozzolillo, 1978) and in *Metrosideros excelsa* (Solangaarachchi and Gould, 2001).

Shimizu et al. (2010) reported that light irradiation was essential for anthocyanin accumulation induced by JA-Me treatment in *Gynura bicolor* cultured roots. Anthocyanin content in roots of the plants treated with JA-Me in darkness decreased remarkably compared with the plant treated in light conditions.

The effect of light on anthocyanin biosynthesis is generally attributed to the requirement for a photomorphogenic signal, mediated by photoreceptors

such as phytochromes (Ubi, 2004). Under natural conditions many plants are exposed to a very large range of light intensities. Anthocyanin pigments accumulate especially in young plant seedlings in response to light. This accumulation is preceded by increased transcription of genes encoding enzymes in the anthocyanin-biosynthetic pathway (Noh and Spalding, 1998).

Biosynthesis of anthocyanin in leaves of *K. blossfeldiana* is a photoperiodic response of the plant and occurs under short day conditions (Neyland et al., 1963). In comparison to old plants the level of anthocyanin in the roots treated with JA-Me in young plants was much lower. It seems that leaves of older plants produce metabolite(s) which is/are transported to the roots and in interaction with JA-Me induce anthocyanin accumulation.

Our results indicate that leaves are necessary for anthocyanin accumulation in the roots of old *K. blossfeldiana* plants treated with JA-Me under light conditions. Removing leaves led to disappearance of JA-Me elicitation properties of anthocyanin accumulation. It is the first evidence that JA-Me induced anthocyanin in the roots of intact plants of *K. blossfeldiana* in light conditions, and that shoots (leaves) are necessary for anthocyanin accumulation. The obtained results confirm the data presented by Shimizu et al. (2010) that JA-Me induced anthocyanin accumulation in roots of intact cultured plants of *Gynura bicolor*. However, the above-ground part of the plant is not needed for production of anthocyanin in hairy root culture. Anthocyanin production was noted in light-cultured hairy roots of *Ipomoea batatas*. The level of anthocyanin production in these roots depended on the composition of the growth medium (Nishiyama and Yamakawa, 2004).

Roots of intact old plants grown in darkness and treated with JA-Me or plants after excision of leaves accumulated lower content of anthocyanins than the roots of plants exposed to light. It is known that light is an important factor in anthocyanin biosynthesis for many plants. The stimulatory effect of light on accumulation of anthocyanins was observed in plant leaves of: *Ipomoea batatas* L. (Islam et al., 2005), *Capsicum annuum* (Lightbourn et al., 2007), *Arabidopsis thaliana* (Page et al., 2012) and fruits (Merzlyak et al., 2002; Zhou and Singh, 2004). Only a few plants have been found to produce anthocyanin in darkness. These include callus cultures of *Ajuga reptans* flowers (Callebaut et al., 1997), callus cultures of *Aralia cordata* Thunb. (Sakamoto et al., 1993), *Vitis* sp. cells in suspension culture (Yamakawa et al., 1983). In *Aralia cordata* the highest anthocyanin yield was obtained when medium contained 1.0 mg·l⁻¹ 2,4-D and 0.1 mg·l⁻¹ kinetin (Sakamoto et al., 1993). Some *Solanum tuberosum* L. cultivars can also produce

anthocyanin in darkness but light enhanced the level of these pigments (Lewis et al., 1998). Illumination of the leaves initiated anthocyanin in foil-covered minitubers at a slower rate compared to that for light-exposed minitubers. Lewis et al. (1998) suggest that a 'trigger' compound was produced in the leaves as a result of exposure to light, and it was transported to the tubers.

It is well known that in long day leaves of *K. blossfeldiana* contain high levels of soluble phenolic compounds, in opposite to those growing in short day conditions (Balsa et al., 1979). Leaves of *K. blossfeldiana* plants become red and accumulate anthocyanins under short day conditions (Neyland et al., 1963).

The composition of various metabolites in green and red coloured leaves of *K. blossfeldiana* is different and it is possible that some of them are translocated to the roots and interact with exogenously applied JA-Me causing anthocyanin accumulation. Sucrose can be one of the factors affecting accumulation of anthocyanin in *K. blossfeldiana*. Shimizu et al. (2010) showed in their studies on *Gynura bicolor* that sucrose is the rate-limiting factor in interaction with JA-Me for anthocyanin biosynthesis in roots.

In leaves of flowering plants, the major pigment is chrysanthemins (cyanidin-3-monoglucoside) with minor amounts of cyanin (cyanidin-3,5-diglucoside). The major anthocyanin of the flower petals is cyanin with minor amounts of chrysanthemins and probably pelargonin (pelargonidin-3,5-diglucoside) (Neyland et al., 1963). We showed that one of the anthocyanins in the roots of *K. blossfeldiana* was cyanidin-3,5-di-*O*-glucoside. Seven anthocyanins were documented in the roots of the control plants and 8 anthocyanins in the roots of JA-Me treated plants.

Tissues of *Kalanchoe blossfeldiana* cultivars contain 3,5-*O*-β-D-diglucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Nielsen et al., 2005). In the cultures of *Gynura bicolor* treated with JA-Me, the composition of anthocyanins in roots was the same as in the leaves of intact plants (Shimizu et al., 2010). In the roots of *Metrosideros excelsa* exposed to light cyanidin and delphinidin were found (Solangaarachchi and Gould, 2001). Cyanidin was also identified in the roots of *Impatiens* (Thakur and Nozzolillo, 1978) and the presence of cyanidin and pelargonidin was recorded in the roots of *Raphanus sativus* (Harborne, 1963; Ishikura and Hayashi, 1962). Cyanidin is the most common among plants.

Adaptive advantages of anthocyanins in non-reproductive tissues are definitely less clear. They may allow the plant to develop resistance to a number of environmental stresses. It is well known that there is a rapid increase in the endogenous level of jasmonate in plants or their organs under stress

conditions such as mechanical wounding, pathogen infection and insect attack (Saniewski, 1997). JA-Me as a signal molecule participates in induction of gene transcription leading to L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), the enzymes involved in synthesis of flavonoids (Richard et al., 2000; Dao et al., 2011).

This raises a question about the mechanism of inducing action of methyl jasmonate on anthocyanin accumulation in the roots of intact *K. blossfeldiana* plants under natural light conditions, and the place of biosynthesis of anthocyanins. It is possible that biosynthesis of anthocyanin takes place in roots or anthocyanins (or their precursors) are transported from the stem and leaves to the roots. Further studies will be undertaken on pathways of anthocyanin biosynthesis induced by JA-Me in roots of *K. blossfeldiana*.

AUTHORS' CONTRIBUTIONS

JG-K carried out the experiments, analysis and interpretation of data and wrote the manuscript; MS contributed to the conception and design, and wrote the manuscript; JM carried out an experiment on the identification and determination of anthocyanins and interpretation of data; AS, WO interpretation of results concerning the identification of anthocyanins and drafting or critical revision of the paper. The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

The research was supported by the Ministry of Science and Higher Education of Poland as part of the statutory activities (7.2.1) of the Department of General Biology, Research Institute of Horticulture in Skierniewice.

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