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ORIGINAL PAPER

Effect of natural light on the development of adventitious roots in stem cuttings of *Salix babylonica* 'Tortuosa': Histological and metabolic evaluation*

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Abstract

During the vegetative propagation of willow *Salix babylonica*, stem cuttings develop adventitious roots that accumulate high levels of anthocyanins in the presence of light. This metabolic response has been known earlier, but there are no data in the available literature regarding the profile of these pigments. The aim of this study has been to determine the composition and content of anthocyanins and other accompanying phenolic compounds and salicinoids in adventitious roots of *S. babylonica*. Identification and analysis of anthocyanins were carried out using micro-HPLC-MS/MS-TOF, while HPLC-MS/MS was applied to analyze phenolic acids, flavonoids and salicinoids. In addition to these analyses, histological observations were made using a microscope. On microscopic cross-sections, a red color was found only locally, namely in some sub-epidermal cells of the exodermis and parenchyma cells of the cortex. In *S. babylonica* roots, presence of eleven glycosides of cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin has been demonstrated. Quantitatively, the main anthocyanin in roots developed in darkness was delphinidin glycoside, while the main anthocyanin in light-exposed roots was cyanidin glucoside. The increase in the anthocyanin content in roots exposed to light for three weeks was several hundred times greater than in those kept in the dark, and their total level was more than a hundred times higher than that of flavonoids, phenolic acids and/or salicinoids. This likely means that anthocyanins are major participants of the protection system in response to stress caused by exposure of *S. babylonica* roots to light.

Keywords: weeping willow, stem cuttings, roots, light, anthocyanins, salicinoids

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INTRODUCTION

The willow and poplar species, members of the Salicaceae family, are known for their rapid growth and are cultivated for their wood used in construction, furniture and other wooden products and as ornamental plants in gardens and landscapes (Christenhusz, Byng 2016). Tissues of willow species contain many anti-inflammatory and antibacterial compounds (Hijazi et al. 2022). Therefore, since ancient times, willow bark has been used in the production of various pharmaceutical preparations (Shara, Stohs 2015, Brereton et al. 2017, Petruk 2019, Förster et al. 2021, Curtasu, Nørskov 2024). Cultivars of *Salix babylonica* 'Tortuosa' belong to so called weeping willows, and are widely distributed all over the world (Tawfeek et al. 2019). In addition to *S. babylonica*, the name *Salix matsudana* Koidz is also used (Singh et al. 2017, Petruk 2019). Willow species have an excellent ability to reproduce vegetatively from cuttings (Chmelar 1974). A mature willow forms the primordia of adventitious roots in the peripheral tissue of the stems. These primary roots remain dormant when the stem is intact, but usually develop rapidly into roots when pieces of the stem are removed from the tree and placed in water or soil (Lux et al. 2004).

Phenylalanine ammonium lyase (PAL) is a crucial enzyme in the biosynthesis of phenolic compounds in plants. Its catalyzes the elimination reaction of ammonia from phenylalanine, yielding cinnamic acid, and it mediates carbon transfer from primary metabolism (carbohydrates) to secondary metabolism, resulting in the biosynthesis of hundreds of phenolic compounds (Barros, Dixon 2020). The most numerous group of phenolic compounds are flavonoids, which include flavones, flavonols, flavanones, anthocyanins, chalcones, isoflavones and aurones (Koes et al. 2005, Marin-Recinos, Pucker 2024). Phenolic acids are the next major group of secondary metabolites produced by plants (Mattila et al. 2006). Phenolic acids can be divided into two subgroups: hydroxycinnamic acids, e.g. *p*-coumaric, ferulic, caffeic and synapic acids and hydroxybenzoic acids, e.g. *p*-hydroxybenzoic, vanillic, protocatechuic and syringic acids (Mattila et al. 2006). Anthocyanins are plant pigments that are biosynthesized in the last stages of the flavonoid biosynthesis pathway (Grotewold 2006, Ferrer et al. 2008, Deng and Lu 2017). Molecular aspects of their biosynthesis have been studied in detail and reviewed many times (Saslowsky et al. 2005, Grotewold 2006, Agati et al. 2012, Saito et al. 2013, Marin-Recinos and Pucker 2024).

Previous investigations of the chemical composition of tissues of the genus Salix have focused mainly on leaves, young shoots or bark. During the growing season, the content of salicin and flavonoids, luteolin-7-O-glucoside, myricetin, apigenin-3′-oxyethyl-7-O-glucoside, rutin, quercetin, luteolin, kemferol and apigenin in the leaves of Salix matsudana was examined (Li et al. 2013). It was shown that salicin, myricetin and rutin were present in the highest concentrations (Li et al. 2013). In young shoots of nine willow

species, the following phenolic acids were found: *iso*-vanillic, caffeic, vanillic, protocatechuic, *p*-coumaric, ferulic, chlorogenic, cinnamic and sinapic acids, and their content depended on the analyzed species (Budny et al. 2021). Generally, in these willow tissues, the highest concentrations of iso-vanillic, caffeic, protocatechuic and *p*-coumaric acids were noted. Furthermore, nine flavonoids were found in the shoots of the studied willow species: quercetin, kaempferol, iso-rhamnetin, naringenin, luteolin, apigenin and catechin. Flavonoid content varied widely, from trace amounts (naringenin) to much higher concentrations (catechin). Among salicinoids, salicin, saligenin and prunin were at the highest content (Budny et al. 2021).

A detailed review published by Tawfeek et al. (2021) summarized data on important pharmacological components in the genus Salix. According to this review, 94 flavonoids, 76 phenolic glycosides, 28 phenolic acids and 13 other phenols were found in the tissues of various willow species. The largest number of different classes of phenolic compounds was detected in the leaves, and the lowest in the roots.

Since the main source of compounds in the Salix genus is its bark, most of the research focuses on this tissue (Brereton et al. 2017, Curtasu, Nørskov 2024). Therefore, the analysis of the anthocyanin content of this species was mainly concerned with their content in the bark. Since the 1970s, it has been known that cyanidin 3-glucoside is the predominant anthocyanin in the bark of Salix spp. and delphinidin 3-glucoside was present in smaller amounts (Bridle et al. 1973). Recently, a total of 43 flavonoids have been identified in the green, purple, and red willow barks of Salix species, and among them there are seven anthocyanins: cyanidin rutinoside, pelargonin chloride, cyanidin glucoside, pelargonidin rutinoside, petunidin rutinoside, pelargonidin and malvidin (Zhou et al. 2022).

Light plays an important role in the biosynthesis and accumulation of flavonoids, both in its intensity and spectral range (Karimi et al. 2013, Tusevski et al. 2013, Dębski et al. 2017, Do et al. 2023). Increasing light intensity, an elevated level of total phenolics in leaves, stems and roots of three varieties of *Labisia pumila* (Karimi et al. 2013) and enhanced flavonol and anthocyanin contents, but reduced flavone levels in cotyledons of buckwheat seedlings (Dębski et al. 2017) were obtained. Also, in light-exposed root cultures of the hairy roots of Hypericum perforatum, there was an increased content of flavan-3-ols (Tusevski et al. 2013). Similarly, in Agastache rugosa, the contents of most phenolic compounds were significantly higher in transgenic hairy root cultures grown under light conditions (Do et al. 2023).

Anthocyanins are widely distributed in plants giving orange, pink, red, purple or blue colors to plant organs (He, Giusti 2010). They are responsible not only for the color of plant organs, but are also involved in protection against many biotic and abiotic stresses (Chalker-Scott 1999, Gould 2004, Marin-Recinos, Pucker 2024). Plants produce and accumulate anthocyanins

mainly in flowers, fruits and leaves, or as a result of various stresses (Hatier, Gould 2008, Zhang et al. 2014). They are generally accumulated in peripheral tissues exposed to light, although there are exceptions, such as the accumulation in abaxial leaf tissues and in shade-tolerant plants (Steyn et al. 2002).

Besides their commonly known presence in floral and fruit tissues, anthocyanins can also be found in roots, shoots and leaves. Relatively little research has been done on the nature and function of anthocyanins in roots although root reddening is common among dicotyledonous plants (Harborne, Grayer 1988). Anthocyanins have also been reported in the root cap of *Impatiens glandulifera* seedlings (Mumford 1990), maize roots (Tselas et al. 1979) and in roots subjected to osmotic or toxic stress (Kaliamoorthy, Rao 1994, Wetzel et al. 1995).

There is one report from the 1950s about the color of willow roots and, according to its author, in plants that generally do not produce anthocyanins, roots can accumulate considerable amounts of them (Alston 1959). The root coloring under the influence of light also occurred in other plant species (Alston 1958, Pilet, Takahashi 1979, Rengel, Kordan 1988 Hughes et al. 1999, Hemm et al. 2004,).

Thus, the anthocyanin accumulation in the roots under the effect of light seems to be quite common. However, the role of anthocyanins and other phenolic compounds accumulated in roots is not fully known. Perhaps the antioxidant properties of these compounds are a reason of their accumulation in roots. Therefore, the aim of this study was to investigate the effect of natural light on the accumulation of various phenolic compounds, especially anthocyanins and salicinoids, in the developing adventitious roots of *Salix babylonica*. The histological analyses were also carried out and the composition of anthocyanins, flavonoids, phenolic acids and salicinoids was determined by micro-HPLC-MS/MS-TOF, and HPLC-MS/MS.

MATERIALS AND METHODS

Plant material and growth condition

The source of stem cuttings was a 15-year-old *Salix babylonica* 'Tortuosa' tree. Segments of branches, three to four years old, with a length of 0.20 to 0.25 m, were used for experiments. Experiments on the rooting of *S. babylonica* stem cuttings were carried out from February to April, in a greenhouse at the National Institute of Horticultural Research in Skierniewice, Poland, under natural sunlight intensity, 60 to 100 µmol m^{-2} s^{-1}. Willow stem cuttings were kept in 0.25 dm^3 glass beakers containing distilled water, and placed in a greenhouse where the temperature was between 292 and 296 K. The reference samples (control) had stem segments whose lower

parts, before immersion in water, were wrapped in black foil opaque to light. Other samples of stem cuttings were kept under the same conditions, but with full access to light. Fifteen stem cuttings were used in each experiment, and the experiment was repeated three times. During the experiments, morphological observations were carried on the appearance of adventitious roots and the growth of new shoots. After three weeks of the experiments, adventitious roots were cut from the plantlets and pre-frozen at 245 ± 1 K in a laboratory freezer for 24 h. The frozen samples were then placed in Petri dishes and freeze-dried for 48 h, during which the temperature of the condenser was 218 K, and the final pressure was 600 pA. This process was carried out in a LABCONCO laboratory freeze-dryer (Kansas City, USA). The freeze-dried material was stored in a refrigerator at 277 K until chemical analysis.

Microscopic observations

The following samples of *S. babylonica* stem cuttings were taken for microscopic observation: (1) stem cuttings rooted in water for fifteen days in the dark (control), (2) stem cuttings rooted in water for fifteen days in natural light. The following tissues were evaluated in each sample: root surface, root surface after epidermal isolation, and root sections. The observations were made free-hand and without staining, using a VHX-7000N digital microscope (Keyence, Japan).

Determination of anthocyanins, phenolic compounds and salicinoids

Details of the analyses of anthocyanins, phenolic acids, flavonoids, and the determination of salicinoids have recently been published by Wiczkowski et al. (2024). Identification of these compounds was based on a comparison of their retention time and MS/MS fragmentation spectrum (m/z values) with data of standards analysis, the published data, or/and on the interpretation of the fragmentation spectrum obtained. Quantification of compounds was based on external standards. All analyses were carried out in three replicates and the results of the analyses were statistically elaborated using analysis of variance followed by the Duncan's multiple range test at $p<0.05$ (Statistica 13.1, StatSoft Inc., Tulsa, USA).

RESULTS AND DISCUSSION

Willow species have a very good ability to reproduce by vegetative propagation through stem cuttings (Chmelar 1974). A mature willow forms primary adventitious roots in the peripheral tissue of the stem. These primary roots remain dormant when the stem is intact, but they usually quickly develop adventitious roots when stem cuttings are placed in water or soil

(Lux et al. 2004). In our study, after only 5 days of immersion in water, *Salix babylonica* seedlings developed adventitious roots (Figure 1). This physiological response occurred in both seedlings growing in the dark and those exposed to light. Developed adventitious roots kept in the darkness were etiolated (colorless), while roots developed under light conditions were intensively red (Figure 1). Growth rates of adventitious roots and new shoots in the darkness and in those exposed to light were similar (Figure 1).

Tselas et al. (1979) declared that the first information on the coloration and presence of anthocyanins in willow and maize roots exposed to daylight was reported as early as the 19th century. The formation of beet-red color in willow roots after exposure to light was also mentioned by Alston (1959). However, these were general reports, without determination of the type of pigments and their content.

Fig. 1. Red pigment accumulation in light exposed stem cuttings roots of *Salix babylonica*. (A, B, C) Basal part of stem cuttings kept in water for 2 weeks (A, B) and 3 weeks (C) in darkness, upper part of stem was exposed to natural light; etiolated adventitious roots were formed. (D, E, F) Entire stem cuttings, including basal part of stem kept in water for 2 weeks (D, E) and 3 weeks (F), were exposed to natural light; adventitious red roots were formed. Bars represent the length of 20 mm

Microscopic observation of adventitious roots in stem cuttings of *S. babylonica*

Adventitious roots of *S. babylonica* kept in darkness were colorless (Figure 2). In contrast, roots developed from stem cuttings exposed to light

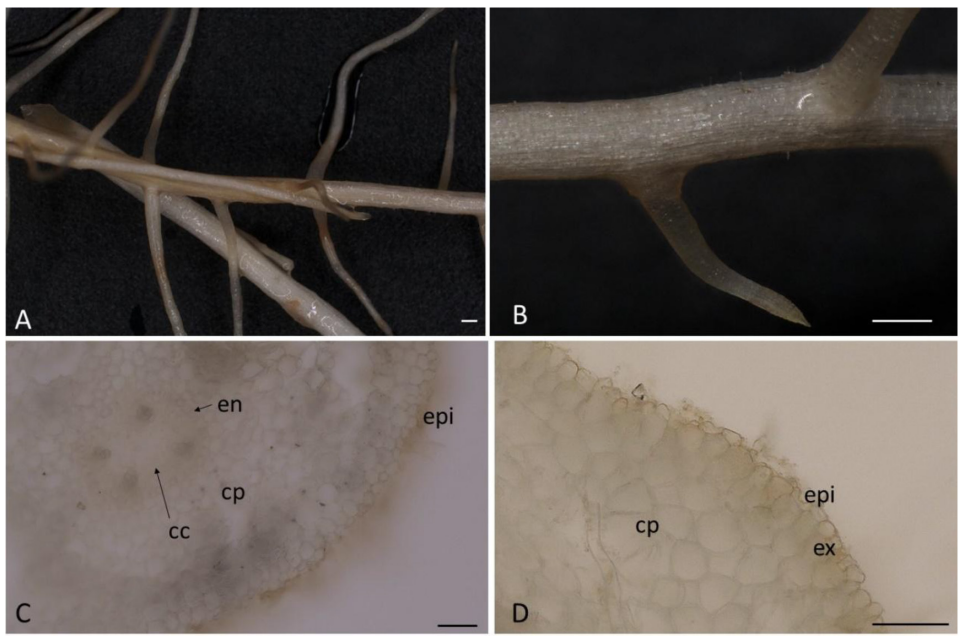

Fig. 2. Morphology and histology of adventitious roots developed from *Salix babylonica* stem cuttings kept in the dark. (A) Two-week-old roots. (B) Detached etiolated roots. (C) Presentation of a cross-section of the root at 2.5 cm from the apex. (D) Higher magnification of root cortex and epidermis (A-B) Bars represent 500 µm; (C-D) Bars represent 60 µm. Abbreviations: epi, epidermis; cc, central root cylinder; ex, exodermis; en, endodermis; cp, cortical parenchyma

produced a red color indicating the presence of anthocyanins (Figure 3). The pigmentation did not occur uniformly, but formed pinkish-red spots with a kind of checkerboard pattern (Figure 3 B-D). It was also noted that the newly formed lateral roots initially were colorless (Figure 3 B and C, marked by a white arrow).

On microscopic cross-section, a red color was found only locally – in some sub-epidermal cells of the exodermis and parenchyma cells of the cortex, while in the epidermis and central cylinder the color was absent (Figure 3E and F). The role of light in the biosynthesis and accumulation of anthocyanins in plant tissues has been the subject of extensive research. However, it mainly concerned leaf tissues (Rengel, Kordan 1988). Furthermore, it was shown that sunlight caused anthocyanin accumulation in the adventitious roots of *Metrosideros excels* (Solangaarachchi, Gould 2001), in etiolated maize seedling (Singh et al. 1999) and in *Impatients* species (Thakur, Nozzolillo

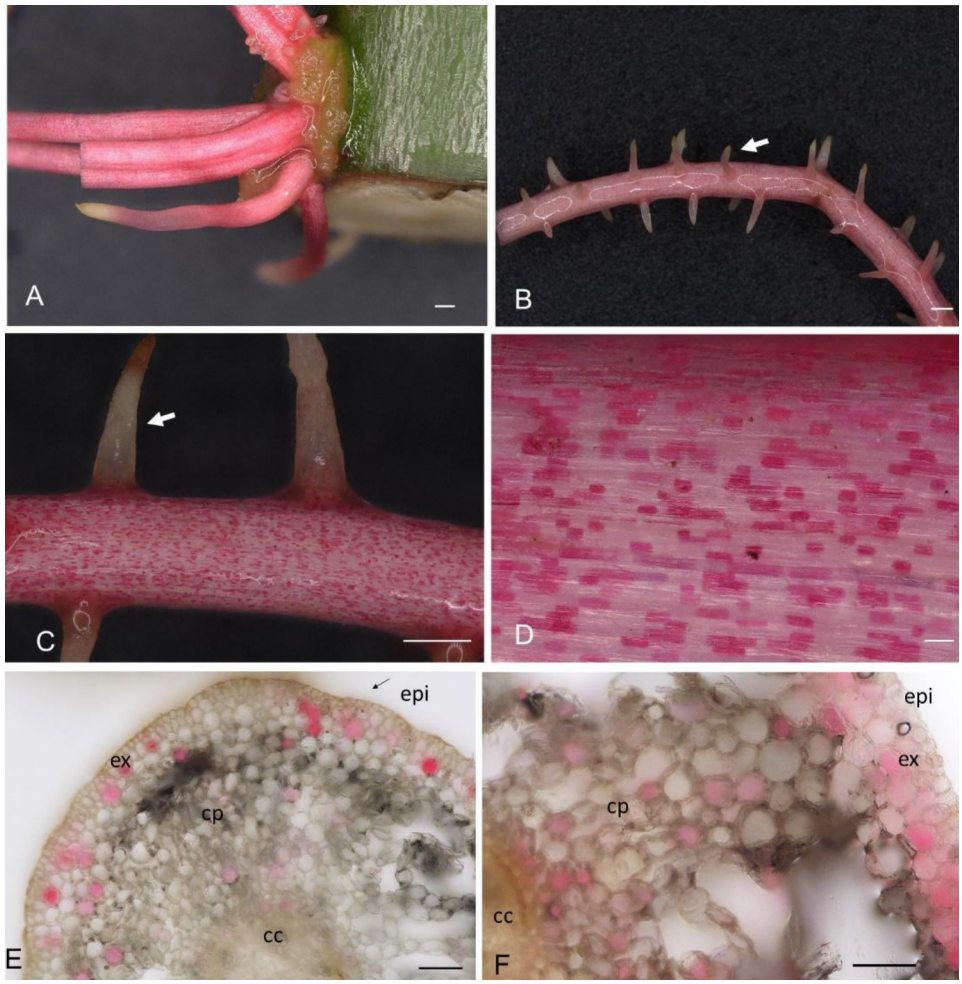

Fig. 3. Morphology and histology of adventitious roots *Salix babylonica* exposed to light. (A) Newly formed adventitious roots in stem cuttings (B, C) Lateral roots are initial colorless (marked by a white arrow). (D) Root surface shows a local occurrence of anthocyanins. (E, F) Cross section of root showing accumulation of anthocyanins in exodermis and cells of cortex parenchyma. (A-D) Bars represent 500 µm; (E, F) Bars represent 60 µm. Abbreviations: epi, epidermis; cc, root central cylinder; ex, exodermis; cp, cortex parenchyma

1978). Nevertheless, it has not been conclusively established what role anthocyanin pigments play in roots (Solangaarachchi, Gould 2001). In roots and shoots of *Zea mays* L., anthocyanin accumulation was high in the presence of blue light, lower in red light and much lower in far red light (Pilet, Takahashi 1979).

Identification and composition of anthocyanins in adventitious roots of *S. babylonica*

The micro-HPLC-MS/MS-TOF method was used to identify the parent structures of the anthocyanins and their aglycones, and during this study 11 anthocyanins were identified (Table 1). These identifications were based

Table 1

Retention times (RT), mass spectral data, and UV/Vis details of anthocyanins present in the adventitious roots of *Salix babylonica*

No.	Anthocyanin	RT (s)	$[M^+H]^+$	Fragment MS^2	UV/Vis max
1	delphinidin glucoside	195	465	303	277; 526
2	cyanidin glucoside	202	449	287	280; 517
3	cyanidin galactoside	203	449	287	280; 528
4	cyanidin rutinoside	204	595	287	280; 518
5	petunidin glucoside	205	479	317	278; 523
6	pelargonidin glucoside	206	433	271	274; 503
7	peonidin glucoside	207	463	301	280; 518
8	peonidin rhamnoside-glucoside	218	609	463, 301	280; 522
9	delphinidin acetylglucoside	221	507	303	276; 528
10	malvidin glucoside	226	493	331	278; 530
11	delphinidin rutinoside	233	611	303	279; 528

on the mass spectra of the molecular ions with the added hydrogen cation [M + H]+ and the molecular fragment after the loss of the glycosidic moiety. The m/z ions 271, 287, 301, 303, 317 and 331 correspond sequentially to the following anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Peak 2 and 3 was identified as cyanidin-glucoside and cyanidin-galactoside, respectively. Both possessed a molecular ion $[M]^+$ with an m/z (mass-to-charge ratio) value of 449 mass unit (mu) and a fragment ion with a m/z value of 287 mu. A mass reduction of 162 mu corresponds to a loss of one molecule of dehydrohexose (glucose or galactose).

Peak 4 was identified as cyanidin rutinoside because its molecular ion $[M]^+$ had an m/z value of 595 mu and a fragment ion [M]+ with an m/z value of 287 mu. The decline of 308 mass unit corresponds to the loss of one molecule of rutinose. Similarly, peak nr 11 was identified as delphinidin rutinoside due to decline of 308 mu. Peaks 1, 5, 6, 7 and 10 are glucosides of delphinidin, petunidin, pelargonidin, peonidin and malvidin, respectively. In all the compounds, a mass reduction of 162 mu corresponding to a loss of one molecule of glucose appeared. There was also delphinidin acetylglucoside (peak 9) identified, in which mass reduction 204 ha appeared. Besides, there was found peonidin rhamnoside-glucoside (peak 8), which possesed molecular ion 609, and two ions 463 and 301, which corresponded to a loss of 162 mu (glucose) and 146 (rhamnose).

The contents of all eleven anthocyanins contributing to the intense red color of adventitious roots growing on *S. babylonica* stem cuttings were many times higher in the tissues of roots growing in the presence of light than those growing in the dark (Figure 4, Table 2). Total anthocyanin contents reached 37.3 μg g^{-1} DW in roots growing in the dark (Experiment 1) and 2107 μg g^{-1} DW and 2713 μg g^{-1} DW in roots growing in the light, respective-

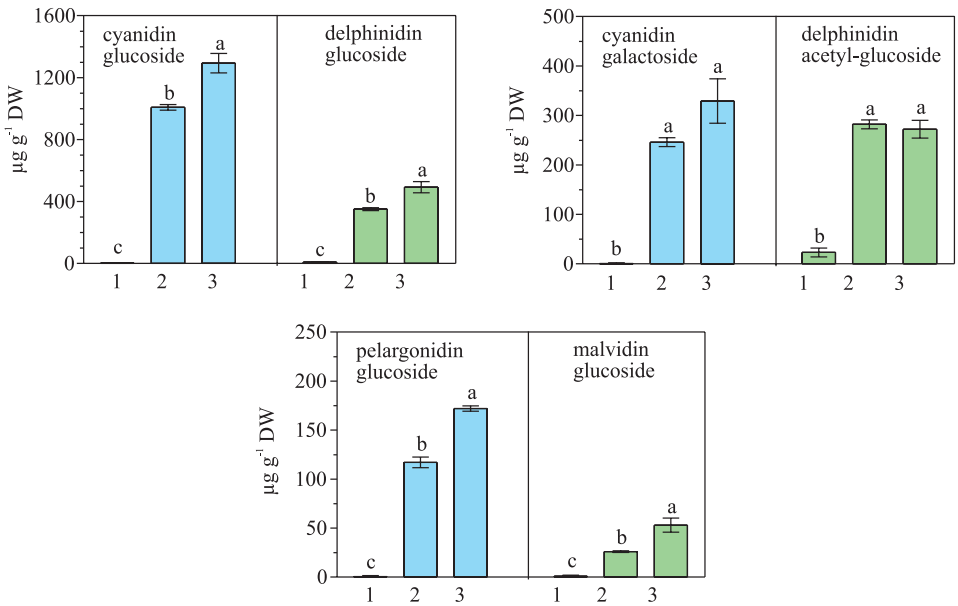

Fig. 4. Content (means ± standard deviation; DW – dry weight) of major anthocyanins in the adventitious roots of *Salix babylonica* grown in the darkness and under natural light conditions: 1 – Experiment 1, roots grown in darkness within the March 1-21, 2 – Experiment 2, roots grown in light within the March 1-21, 2023, 3 – Experiment 3, roots grown in light within the March 10-31, 2023. Bars marked with the same letter do not differ at the significance level of $p<0.05$ according to the Duncan's test

Table 2

Content (μg g^{-1} dry weight, means ± standard deviation) of minor anthocyanins in the adventitious roots of *Salix babylonica* grown in the darkness and under natural light conditions

Anthocyanin	Experiment 1	Experiment 2	Experiment 3
Cyanidin rutinoside	0.03 ± 0.01^b	5.42 ± 0.25^a	5.12 ± 0.09^a
Delphinidin rutinoside	Tr	Tr	3.83 ± 0.97
Petunidin glucoside	Tr	11.15 ± 0.15^a	12.11 ± 0.34^a
Peonidin glucoside	1.08 ± 0.04^b	39.79 ± 0.95^a	42.84 ± 0.78^a
Peonidin rhamnoside-glucoside	0.57 ± 0.01^c	20.86 ± 0.33^b	38.01 ± 0.90^a

Experiment 1 – roots grown in darkness within the March 1-21, Experiment 2 – roots grown in light within the March 1-21, 2023, 3 – Experiment 2 – roots grown in light within the March 10-31, 2023, tr – traces, below 0.01 μg g^{-1} dry weight. Means in the rows marked with the same letter do not differ at the significance level of $p<0.05$ according to the Duncan's test

ly (Experiment 2 and 3). Moreover, the contents of most anthocyanins were higher in adventitious roots from Experiment 3 than from Experiment 2. Our experiments have shown that the intensity and duration of exposure to natural light has a remarkably strong effect on anthocyanin levels in adventitious roots of willow *Salix babylonica*. It was also interesting that the red color of the roots formed during exposure to natural light had no effect on the shoot growth of *S. babylonica* plantlets. Higher anthocyanin contents occurred in roots from the experiment conducted in the second half of March (Experiment 3) than those conducted earlier (Experiment 2).

Quantitatively, the major anthocyanins in the adventitious roots of *S. babylonica* were glucosides of cyanidin, delphinidin, pelargonidin and malvidin, as well as cyanidin galactoside and delphinidin acetyl-glucoside (Figure 4). Among the mentioned anthocyanins, cyanidin glucoside was present in the highest content, with 48% share in the content of all anthocyanins in roots exposed to light. The content of this anthocyanin was about 500 times higher in roots exposed to light (Experiments 2 and 3) than in those growing in the dark (Experiment 1).

Since no data are available on the anthocyanin content of willow roots, our results can only be compared with the anthocyanin composition of other tissues in the genus Salix. The profile of anthocyanins in Salix bark showed differences between species (Lux et al. 2004). *S. daphnoides* and *S. alba* contained only cyanidin 3-glucoside, while both cyanidin and delphinidin 3-glucosides occurred in *S. phylicifolia*, *S. nigricans*, *S. calodendron* and *S. viminalis*. On the other hand, *S. triandra* and *S. amygdalina* contained 3-glucosides of delphinidin, cyanidin and petunidin (Lux et al. 2004). Earlier, it was found that the quantitatively dominant anthocyanin in the bark of the genus Salix was cyanidin-3-glucoside, while delphinidin-3-glucoside was present in smaller amounts (Bridle et al. 1973). Similarly, in our study cyanidin glucoside was quantitatively the major anthocyanin in the roots of *S. babylonica*. Recently, the following anthocyanins have been shown to be present in the green, purple and red bark of willows: cyanidin rutinoside, cyanidin glucoside, pelargonidin rutinoside, petunidin rutinoside, pelargonidin chloride, pelargonidin and malvidin (Zhou et al. 2022). Therefore, it can be generally indicated that in both the bark and roots of willow, the main anthocyanin is cyanidin glucoside, with delphinidin glucoside present in lesser content.

Composition of phenolic acids, flavonoids and salicinoids in adventitious roots of *S. babilonica*

The adventitious roots of *S. babylonica* contained derivatives of two flavonols, i.e. quercetin and kaempferol, one flavone, apigenin, and one flavanol, epicatechin (Table 3). However, their content in roots exposed to light was much lower than that of anthocyanins (Figure 5, Table 2). Among them, quercetin glycosides and the free form of epicatechin were quantitatively dominant. Roots exposed to light accumulated many times more glycosides

Table 3

Contents (μg g^{-1} dry weight ± standard deviation) of free forms (F), esters (E), glycosides (G), and total contents of all forms of flavonoids in the adventitious roots of *Salix babylonica* grown in the in darkness or in natural light conditions

Flavonoid	Experiment 1	Experiment 2	Experiment 3
Quercetin, total	0.95 ± 0.08^b	6.53 ± 0.10^a	7.01 ± 0.10^a
F/E/G	0.16/0.55/0.24	0.62/1.33/4.58	0.62/2.23/4.16
Apigenin, total	tr	0.10 ± 0.01^a	0.19 ± 0.01^a
F/E/G	tr/tr/tr	0.01/tr/0.09	0.03/tr/0.16
Kaempferol, total	0.07 ± 0.02^b	0.41 ± 0.06^a	0.66 ± 0.07^a
F/E/G	0.01/0.02/0.04	0.04/0.06/0.31	0.05/0.16/0.45
Epicatechin, total	3.30 ± 0.15^b	3.18 ± 0.06^b	4.36 ± 0.07^a
F/E/G	3.30/tr/tr	3.18/tr/tr	4.26/tr/tr

Experiment 1 – roots grown in darkness within the March 1-21, Experiment 2 – roots grown in light within the March 1-21, 2023, Experiment 3 – roots grown in light within the March 10-31, 2023. The means in the rows marked with the same letter do not differ at the significance level of $p<0.05$ according to the Duncan's test. F/E/G – indicates polyphenols in free (F), ester (E) or glycosidic (G) form. tr – traces, below 0.01 μg g^{-1} dry weight

of quercetin, kaempferol and apigenin than those growing in darkness. However, the presence of light had little effect on the epicatechin content. In the roots of growing in the presence of light, the total content of flavonoids, i.e. free forms, glycosides and esters, was more than twice as high as in roots growing in the dark (Figure 5). Among the total flavonoid forms

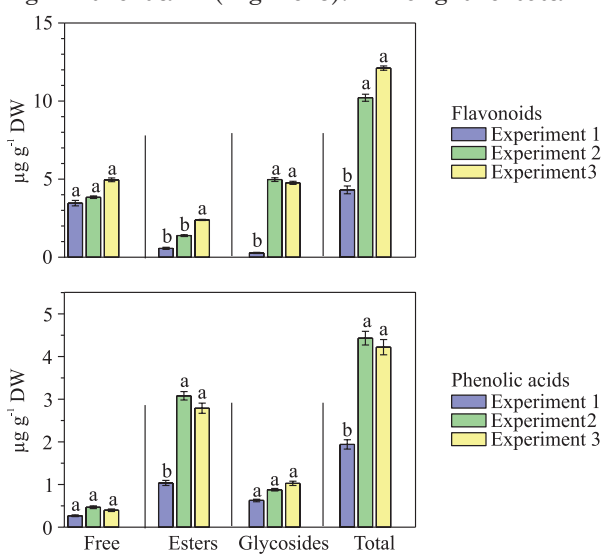

Fig. 5. Total content (means ± standard deviation; DW – dry weight) of free forms, esters, glycosides and total flavonoids and phenolic acids in the adventitious roots of *Salix babylonica* grown in the darkness and under natural light conditions: Experiment 1 – roots grown in darkness within the March 1-21, Experiment 2 – roots grown in light within the March 1-21, 2023, Experiment 3 – roots grown in light within the March 10-31, 2023. Bars marked with the same letter do not differ at the significance level of $p<0.05$ according to the Duncan's test

evaluated, exposure of roots to light resulted in a large increase in glycosides, a smaller increase in esters, and there was no significant effect of growth conditions on free flavonoid contents.

Five phenolic acids have been found in the adventitious root tissues of *S. babylonica*: ferulic, *p*-coumaric, 3-*hydrox*y-benzoic, protocatechuic and coffee acids. Quantitatively, the major phenolic acids in the roots were *3-hydroxy*-benzoic and protocatechuic (Table 4). *3-hydroxy*-Benzoic has been

Table 4

Contents (μg g^{-1} dry weight ± standard deviation) of free forms (F), esters (E), glycosides (G), and total contents of all forms of phenolic acids in the adventitious roots of *Salix babylonica* grown in the in darkness or in natural light conditions

Phenolic acid	Experiment 1	Experiment 2	Experiment 3
Ferulic, total	0.02 ± 0.01^a	0.04 ± 0.01^a	0.03 ± 0.01^a
F/E/G	0.01/0.01/tr	0.01/0.03/tr	0.01/0.02/tr
p-Coumaric, total	0.19 ± 0.01^b	0.78 ± 0.05^a	0.56 ± 0.04^a
F/E/G	0.04/0.15/tr	0.11/0.65/0.02	0.06/0.49/0.02
3-hydroxy-Benzoic, total	1.20 ± 0.05^b	1.63 ± 0.04^a	1.55 ± 0.03^a
F/E/G	0.16/0.58/0.46	0.14/0.76/0.73	0.11/0.64/0.79
Protocatechuic, total	0.35 ± 0.03^b	1.29 ± 0.06^a	1.42 ± 0.07^a
F/E/G	tr/0.18/0.17	tr/1.16/0.13	tr/1.20/0.22
Caffeic, total	0.18 ± 0.01^b	0.69 ± 0.03^a	0.66 ± 0.06^a
F/E/G	0.06/0.12/tr	0.21/0.48/tr	0.22/0.44/tr

Experiment 1 – roots grown in darkness within the March 1-21, Experiment 2 – roots grown in light within the March 1-21, 2023, Experiment 3 – roots grown in light within the March 10-31, 2023. The means in the rows marked with the same letter do not differ at the significance level of $p<0.05$ according to the Duncan's test. F/E/G – indicates polyphenols in free (F), ester (E) or glycosidic (G) form. tr – traces, below 0.01 μg g^{-1} dry weight

found mainly in glycosylated or esterified form. Protocatechuic acid was in the form of esters or glycosides and not in free form, while caffeic acid was in free and esterified form.

The contents of *p*-coumaric, protocatechuic and caffeic acids were 3 or 4 times higher in roots growing in the light compared to those growing in the dark, while in the case of m-BA, the presence of light caused a relatively small increase in its content.

The following compounds specific to the genus Salix (salicinoids) were found in the adventitious roots of *S. babylonica*: prunin, taxifolin, helicin, tremuloidin, salicortin and salicin (Table 5). The contents of taxifolin, helicin and tremuloidin were significantly lower in roots growing in the light compared to those growing in the dark. In the case of prunin, its content was lower in roots exposed to light, but the differences were not significant. In turn, the presence of light increased the content of salicortin and salicin in the roots of *S. babylonica*.

Table 5

Content (μg g^{-1} dry weight ± standard deviation) of salicinoids in the adventitious roots of *Salix babylonica* grown in the darkness and under natural light conditions

Analysed compound	Experiment 1	Experiment 2	Experiment 3
Prunin	1.02 ± 0.22^{a}	0.67 ± 0.06^{a}	0.85 ± 0.03^{a}
Taxifolin	3.32 ± 0.03^{a}	1.92 ± 0.07^{c}	2.63 ± 0.11^{b}
Helicin	0.08 ± 0.01^{a}	0.07 ± 0.01^{a}	0.06 ± 0.01^{a}
Tremuloidin	1.77 ± 0.02^{a}	0.89 ± 0.01^{b}	0.81 ± 0.02^{b}
Salicortin	2.24 ± 0.09^{b}	2.28 ± 0.41^{b}	7.95 ± 0.10^{a}
Salicin	1.52 ± 0.16^{c}	3.91 ± 0.13^{a}	2.29 ± 0.09^{b}

Experiment 1 – roots grown in darkness within the March 1-21, Experiment 2 – roots grown in light within the March 1-21, 2023, 3 – Experiment 3 – roots grown in light within the March 10-31, 2023. Means in the rows marked with the same letter do not differ at the significance level of $p<0.05$ according to the Duncan's test

The results of chemical analyses of *S. babylonica* roots obtained in our study show a huge quantitative advantage of anthocyanins over other phenolic compounds. This could mean that in the phenylpropanoid pathway *p*-coumaroyl CoA is almost entirely used for the biosynthesis of anthocyanins. Only to a small extent, is it a precursor of phenolic acids. This could also mean that anthocyanins are major components in the protective system of root tissues against the stress caused by an inadequate light-method of propagation of *Salix babylonica*. Earlier, it was found that increased light intensity contributed in the hypocotyl of buckwheat seedlings to a decrease in the content of flavones, such as orientin, *iso*-orientin, vitexin and *iso*-vitexin, while there was a significant increase in the content of cyanidin glycosides (Dębski et al. 2017). Flavonoids appear to be important in willow bark, where their content can account for up to 20% of bark dry weight (Förster et al. 2021). The low content of the glycosides quercetin, apigenin, kaempferol, and epicatechin in the roots of *S. babylonica* means that they play a much smaller protective role compared to anthocyanins.

The photoinduced process of anthocyanin accumulation is accompanied by slight changes in the content of compounds typical of the Salix genus, i.e. salicinoids. Thus, the influence of natural light does not seem to be important for a possible increase in their levels in the roots, which is important for the pharmaceutical industry.

Some unknown endogenous factor may be necessary for the anthocyanin level to accumulate in adventitious roots exposed to light. Such a phytohormone could be methyl jasmonate (JA-Me), which can be transported from the upper green part of plantlets to the roots. This hypothesis is supported by the observed phenomenon that exposure to light of cut etiolated Salix roots did not result in anthocyanin accumulation. Previously, it was shown that JA-Me markedly increased the anthocyanin content in the roots of intact *Kalanchoe blossfeldiana* plants exposed to natural light (Góraj-Koniarska

et al. 2015). After leaf removal, JA-Me only slightly increased the anthocyanin level compared to intact plants. Moreover, the stimulating effect on anthocyanin accumulation was higher in roots taken from older than from younger plants. These results indicate that leaves are essential for the anthocyanin content in *K. blossfeldiana* roots exposed to light. In roots excised from *K. blossfeldiana* plants, JA-Me did not affect anthocyanin accumulation in the presence of light.

Similarly, Shimizu et al. (2010) reported that light was essential for the anthocyanin content induced by JA-Me in cultured roots *of Gynura bicolor*. The authors also found that the anthocyanin level decreased remarkably in roots of the plants treated with JA-Me in darkness compared with the plants treated and grown in light conditions. It was also shown that maize roots exposed to light accumulated anthocyanins, but application of abscisic acid (ABA) inhibited the root growth and caused a significant decrease in the levels of these pigments (Pilet, Takahashi 1979). Thus, it seems that the accumulation of anthocyanins in the roots of *Salix babylonica* exposed to light is a complex phenomenon and requires further detailed research.

CONCLUSIONS

The results obtained in the experiments show in detail that the developing adventitious roots of *Salix babylonica* 'Tortuosa'responded rapidly to exposure to daylight, resulting in the formation of an intense red color due to the accumulation of various anthocyanins. This may indicate that anthocyanins are major contributors to the protection system in response to stress induced by exposure of roots to light. Quantitatively, the main anthocyanins in the light-exposed roots were cyanidin and delphinidin glucosides, as well as cyanidin galactoside and delphinidin acetyl-glucoside. This accumulation has been observed morphologically and confirmed by histological studies and chemical analyses. When the adventitious roots were kept in the dark, the anthocyanin levels in these roots were very low, and then quantitatively the main anthocyanin was delphinidin glucoside. Beside high anthocyanin contents, 3-hydroxybenzoic and protocatechuic acids and epicatechin were present in relatively high concentrations in the roots of *S. babylonica* exposed to light. Moreover, salicinoids such as salicortin, salicin and taxifolin occurred in the highest contents in the tissues of these roots.

It is likely that unknown compounds produced in the upper green part of stem cuttings may be transported to the roots, where they may play an important role in anthocyanin formation and accumulation, but light is an essential factor in this process. The focus of our further research will be on identifying compounds transported into the adventitious roots of *S. babylonica* exposed to light. Another putative reason could be light-induced changes in the activity of certain enzymes in the phenylpropanoid

metabolic pathway that resulted in a tremendous increase in the anthocyanin content and minor changes in the content of other flavonoids and phenolic acids. These suggestions require further enzymatic and/or genetic studies.

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Author contributions

A.M.-C. – conceptualization, investigation, visualization, writing – original draft, funding acquisition, W.W. – methodology, formal analysis, D.S.-N. – investigation, W.K. – investigation, J.G.-K. – methodology, J.M. – formal analysis, visualization, M.S. – conceptualization, methodology, writing – original draft, supervision, project administration, M.H. – writing – original draft, writing – review and editing, visualization. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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