The analysis of the suitability of using waste from the production of planting material for apple trees to obtain natural anthocyanin dyes

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Abstract. The analysis of the content of pigments in the stems of clonal apple rootstocks bred by Michurinsky State Agrarian University showed a high content of anthocyanins (more than 200 mg/100 g for rootstock 54-118). When determining the qualitative composition, it was revealed that anthocyanins in the stems of the rootstocks are represented by three groups, while there are differences in the shape of the rootstocks. As a source of pigment production, it is necessary to select rootstocks with a high endogenous synthesis of anthocyanins (i.e. red-leaved forms 54-118, 57-491, 98-7-77, etc.), during the cultivation of which, in addition to the main product—detachable rooted cuttings—one can additionally receive from 3 to 10 kg of anthocyanin dyes from 1 hectare of mother plant.

1. Introduction

The presence of anthocyanin pigments is observed in all parts of plants: generative, vegetative, fruits and seeds. If their content is not constant, then they can be synthesized at certain stages of ontogenesis or organogenesis, but more often when exposed to stress factors. The scientific and practical interest in these substances contributes to the fact that the knowledge of the biochemical composition, biosynthesis, its regulation, as well as the fields of application of pigments, to date, is quite extensive and is constantly being replenished [1–3].

In the scientific community, interest in anthocyanins has especially increased in the context of the use of pigments as express test systems, which make it possible to quickly obtain and analyze information on the degree of stressfulness of technogenic impact on plant biogeocenoses. More often, methods are used that use indicators of the optical properties of plant tissues that have been exposed to stress, and therefore their changes are associated with the accumulation of anthocyanins. There are data on the use of such methods for environmental monitoring of plant communities and remote sensing of plants under stress [4–6].

A special object is plants with a high endogenous level of anthocyanin pigments, which may be an indicator of a stable physiological state of both cells and the plant as a whole [5, 7]. In this regard, the clonal rootstocks of the apple tree obtained at the Michurinsky State Agrarian University are of
particular interest for research, which is associated with the uniqueness of their origin. In the process of breeding, scientists used various species and varieties of apple trees as parental forms. Some of them, in particular the Nedzwiecki apple tree (*Malus niedzwetzkyana* Diesk., or *M. pumila var. niedzwetzkyana* Hemsl.), have an intense anthocyanin color of tissues, and therefore this trait was inherited by many hybrid forms, zoned and promising rootstocks (Paradyzka Budagovsky, 62-396, 54-118, etc.) [8–11].

Researchers have repeatedly noted the high environmental stability, plasticity, immune properties of clonal apple rootstocks obtained at the Michurinsky State Agrarian University: high frost and winter hardness of the aboveground part and root system, heat resistance, salt tolerance, resistance to bacterial burns, root rot (with soil fatigue of the garden area) and a number of other diseases [6, 11–13].

In addition, the study of plants with a high endogenous level of pigments, the search for new sources of physiologically active compounds is relevant for the food and pharmaceutical industries in connection with the prospect of the practical use of plant objects to obtain biologically active plant products on their basis with a high pharmacological and medical effect.

Considering the above, the purpose of our research was to analyze the quantitative content and qualitative composition of anthocyanins in annual shoots of two groups of clonal apple rootstocks— "green-leaved" and “red-leaved”—in connection with their possible use as raw materials for the production of food dyes.

2. Materials and methods

The biological objects of research were the genotypes of clonal rootstocks of apple trees obtained as a result of many years of breeding work at the Michurinsky State Agrarian University. The rootstocks were planted according to the scheme 150x30 cm in the mother plant of competitive study located on the territory of the structural subdivision of Michurinsk State Agrarian University, Research and Educational Center (REC) named after V.I. Budagovsky. Laboratory studies were carried out at Michurinsk State Agrarian University and TSTU. The pigment content was evaluated by two methods.

M.A. Solovieva [4] proposed a method in which the extraction was carried out from 0.2 g of crushed shoot bark with 0.1 N hydrochloric acid. The color intensity was determined on an FEK-56 photocolorimeter at 490 and 360 nm (light filters No. 5 and No. 2) in a cuvette with a thickness of 10 mm.

The obtained result of measuring the optical density in arbitrary units (E = mg/cm·g) was calculated by the formula: $x = E \cdot \frac{U}{p \cdot e}$, where:

- $x$ is the optical density of the analyte in the calculation of the bark extracted from one gram in one milliliter of solution with a layer thickness of 1 cm;
- $E$ is optical density at 490 nm;
- $U$ is the volume of the extract, ml;
- $e$ is the thickness of the solution cuvette, cm;
- $p$ is weight in grams.

The quantitative determination of the sum of anthocyanins in the whole shoot was carried out by the method of triple extraction with 0.1 M HCl solution. For the obtained extracts, spectra were recorded on a PE-5400 UV spectrophotometer at the wavelength of 515 nm in a 10-mm-thick cuvette.

The anthocyanin content was calculated using the formula based on the Bouguer-Lambert-Beer law:

$$ C = \frac{D}{\varepsilon \times l} \times n \times \frac{M}{m} \times V \times 100 $$

(dimentional quantity is g/100 g),

where $D$ is the optical density of the solution; $\varepsilon$ is the extinction coefficient (26900 l/(mol·cm)); $l$ is the cuvette thickness, cm; $n$ is dilution; $M$ is the molar mass of anthocyanin (cyanidin-3-glucoside); $m$ is the mass of the sample; $V$ is the volume of the flask.

The qualitative determination of anthocyanins was carried out by thin-layer chromatography. Solvent system was as follows: “n-butanol - glacial acetic acid – water” in a ratio of 4:1:2. To identify the substances on the plate, the coefficient of relative chromatographic mobility $R_f$ was calculated using the following formula:
$Rf = \frac{a}{b},$

where $a$ is the distance from the center of the spot to the starting line, $b$ is the distance from the solvent front to the starting line [14].

3. Results and Discussion

Considering the uniqueness of the forms of apple tree clonal rootstocks obtained as a result of breeding at the Michurinsky State Agrarian University, the idea of practical use of their ability to endogenously synthesize anthocyanins in all parts of plants was expressed in the last century, when the forms created by V.I. Budagovsky in the fifties-sixties of the twentieth century were tested and implemented. Professor, head of the department of fruit growing V.A. Potapov [15], suggested that the same plantings, for example, rootstock-raw orchards, can be used both for their main purpose—to obtain cuttings for propagation, and additionally as a raw-material base for obtaining organic anthocyanin dyes. In addition, the possibility of using painted wood rootstocks as finishing materials was expressed (Fig. 1). The colored fruits of the rootstocks were supposed to be used in processing, which was implemented in the works of the followers of scientists [13].

![Figure 1. Saturated anthocyanin staining of the xylem of the shoot of the red-leaved clonal rootstock of the apple tree 98-7-77](image)

Studying the issues of winter hardiness of apple clonal rootstocks, we used the content of anthocyanins in the bark of clonal rootstocks determined by the method of M.A. Solovieva as an indirect indicator [4]. This indicator was evaluated in dynamics over a number of years [7, 11]. Its value is influenced by many factors: genotype, sampling time, temperature, etc. The data presented (Table 1) show that the amount of pigment varies within a fairly wide range. However, there is a single tendency towards an increase in the pigment content in the period from November to December in all forms. At this time, the rootstocks are in a state of deep dormancy and form the greatest resistance to the stress factors of winter.

Most clonal rootstocks that do not have the phenotypic manifestation of anthocyanin coloration (green-leaved) generally contain significantly less amount of this pigment than red-leaved ones. However, among green-leaved rootstocks, there are some forms (for example, 2-9-49) that synthesize pigment at a level or even higher than some red-leaved forms (62-396).

The technological operation of separating the rooted cuttings in the mother plants of our natural and climatic zone usually begins in the first decade of October and lasts for a month or more, depending on the cultivated area and the availability of labor.

Table 2 shows data on the content of pigment in the bark of zoned and widespread rootstocks 54-118 and 62-396, as well as forms that differ most contrastingly in the presence of pigment in the vegetative parts of the plant. The analysis was carried out in the 3rd decade of October. The greatest
amount of pigment is found in the bark of forms 54-118, 98-7-77, 2-12-10. Green-leaved forms accumulate a small amount of pigment during this period. It has been repeatedly noted in the scientific literature that pigment synthesis increases with decreasing temperature [1, 6, 11].

Table 1. Anthocyanin content in the bark of annual shoots of rootstock forms, mg/cm·g (average for 2015-2020)

| Rootstock forms | Months         |         |         |
|-----------------|----------------|---------|---------|
|                 | September      | October | November |
| Red-leaved      | 54-118         | 95.0    | 120.0   |
|                 | 57-491         | 92.0    | 95.0    |
|                 | 62-396         | 45.0    | 42.5    |
| Malys Budagovskiy | 51.0  | 68.0    | 68.5    |
| 98-7-77         | 89.5           | 88.0    | 110.0   |
| 2-12-10         | 120.0          | 124.0   | 134.0   |

| Rootstock forms | Months         |         |         |
|-----------------|----------------|---------|---------|
|                 | January        | February | March   |
| Red-leaved      | 157.0          | 160.0   | 135.0   |
|                 | 100.5          | 100.5   | 98.5    |
|                 | 68.0           | 67.5    | 67.0    |
| Malys Budagovskiy | 68.5 | 68.0   | 67.5   |
| 98-7-77         | 108.0          | 90.5    | 90.0    |
| 2-12-10         | 134.0          | 128.0   | 134.0   |

| Rootstock forms | Months         |         |         |
|-----------------|----------------|---------|---------|
|                 | September      | October | November |
| Green-leaved    | 23.0           | 34.0    | 23.0    |
|                 | 22.0           | 22.0    | 24.0    |
|                 | 34.0           | 42.0    | 34.0    |
|                 | 33.0           | 33.0    | 18.0    |

Table 2. Anthocyanin content in the bark of annual shoots of rootstock forms, October (mg/cm·g)

| Rootstock forms | Years of observation |         |         |         |         |         |         |
|-----------------|-----------------------|---------|---------|---------|---------|---------|---------|
|                 |                       | 2008    | 2009    | 2010    | 2011    | 2012    | 2013    | 2014    |
| Red-leaved      | 54-118                | 85.0    | 120.0   | 100.5   | 130.0   | 95.0    | 120.5   | 100.5   |
|                 | 62-396                | 40.5    | 48.5    | 45.0    | 48.0    | 48.0    | 57.0    | 50.5    |
|                 | 98-7-77               | 88.0    | 95.0    | 104.0   | 100.0   | 112.0   | 120.0   | 95.0    |
|                 | 2-12-10               | 110.5   | 125.0   | 115.0   | 130.0   | 120.0   | 100.0   | 110.0   |

| Rootstock forms | Years of observation |         |         |         |         |         |         |
|-----------------|                       |         |         |         |         |         |         |
|                 |                       |         |         |         |         |         |         |
| Green-leaved    | 14.5                  | 16.5    | 8.5     | 21.0    | 5.0     | 12.0    | 10.0    |
|                 | 18.0                  | 19.0    | 12.5    | 24.0    | 16.5    | 17.0    | 19.0    |

In order to more accurately estimate the amount of pigment that can be obtained from the waste of planting material, in 2020 the content of anthocyanins was determined in the whole stem and the calculation was made according to the formula based on the Bouguer-Lambert-Beer law. The use of this technique made it possible to preliminarily determine the potential yield of anthocyanins from the stem part of non-standard layering of clonal apple rootstocks in the mother plants (Figs. 2-4).

The results of determining the quantitative content of pigment in the whole stem and calculated using the formula based on the Bouguer-Lambert-Beer law differ from the average long-term data obtained using the method of M.A. Solovieva. The data obtained are influenced by the fact that the rootstocks accumulate pigment not only in the bark, but also in other tissues (xylem of stems and roots, parenchyma of leaf blades, etc.), which were not taken into account when applying the method of M.A. Solovieva [4]. Comparison of the quantitative content of pigment in the tissues of the stem using these techniques showed that if in red-leaved forms its concentration increases, then in green-leaved forms it decreases. It can be assumed that in green-leaved rootstocks, much less anthocyanins are synthesized in the xylem and parenchyma of the pith than in the bark.

In connection with the revealed features of such rootstocks, using the methodology and calculation by the formula based on the Bouguer-Lambert-Beer law, we obtained results that allow quantitatively
determining the potential output of anthocyanins from 1 hectare of the mother plant (Figs. 3, 4).

Many green-leaved forms (83-1-15, 2-9-49) form no smaller number of non-standard layering (taking into account their total mass per unit area) than red-leaved ones, such as 57-491, 2-12-10, etc. (Fig. 3), but it must be borne in mind that the low level of endogenous synthesis of pigment in the stem part will not allow them to obtain a significant amount of the substance. However, among the green-leaved rootstocks, there are some forms that are superior to the red-leaved ones in terms of the studied indicator. Thus, in the green-leaved form 2-9-49, the pigment yield can potentially be 1534.8 g/ha, while when using the red-leaved stock 62-396 it is only 887.7 g/ha (Fig. 4). In this case, the biological characteristics of each particular rootstock play a role—the thickness of the shoots and their ability to rooting—which allows obtaining the maximum number of standard layering, which we did not consider as a source of pigment production.

**Figure 2.** The content of anthocyanins in the shoots of red-leaved and green-leaved clonal rootstocks of an apple tree in December

**Figure 3.** Specific mass output of non-standard layering of clonal rootstocks of apple trees from the mother plant
The highest pigment yield can be obtained using clonal rootstocks capable of high endogenous synthesis of anthocyanins, i.e. from the group of red-leaved ones, among which the stock 54-118 stands out—the most common in our country and capable of their formation up to 11106.25 g/ha in rejected plant material from mother bushes. Forms that do not have a phenotypic manifestation of the presence of anthocyanins in vegetative parts (i.e. green-leaved ones) will not allow obtaining a high yield of pigment, since its content both in the bark of the shoots and in the entire stem is much lower than in red-leaved ones.

Figure 4. Potential yield of anthocyanins from the stem part of non-standard layering of clonal apple rootstocks in the mother plant

Analysis of the qualitative composition of anthocyanins in the main organs of annual shoots of mother bushes—leaves and stems—showed that anthocyanins in the studied rootstocks are represented by three main groups (Table 3).

Table 3. Qualitative composition of anthocyanins in the main organs of shoots of zoned and promising red-leaved clonal apple rootstocks (September 2020)

| No. | Specimen, analyzed organ | Cyanidine-3,5-diglucoside | Malvidin-3,5-diglucoside | Peonidine-3,5-diglucoside |
|-----|--------------------------|--------------------------|--------------------------|--------------------------|
| 1   | 62-396 leaf              | +                        | +                        | +                        |
|     | 62-396 stem              | +                        | +                        | +                        |
| 2   | 54-118 leaf              | +                        | +                        | +                        |
|     | 54-118 stem              | +                        | +                        | +                        |
| 3   | 98-7-77 leaf             | +                        | +                        | +                        |
|     | 98-7-77 stem             | +                        | +                        | +                        |
| 4   | Мalysh Budagovskiy leaf  | +                        | +                        | +                        |
|     | Мalysh Budagovskiy stem  | +                        | +                        | +                        |
| 5   | 2-12-10 leaf             | +                        | +                        | +                        |
|     | 2-12-10 stem             | +                        | +                        | +                        |

These compounds are purple and purple-blue in color. Mainly two groups of anthocyanins are
synthesized in the rootstock stems: malvidin-3,5-diglucoside and peonidin-3,5-diglucoside. However, the leaves of rootstocks 62-396, 54-118 and 98-7-77 also contain cyanidin-3,5-diglucoside, which can be metabolized into peonidin-3,5-diglucoside.

Despite the content of anthocyanins in the parenchyma of leaf blades of a number of genotypes of clonal rootstocks, their isolation is technologically difficult and ineffective due to the simultaneous presence of a complex of other leaf pigments—chlorophylls a and b, carotenoids. One should also take into account the mandatory defoliation before the direct separation of layering on the mother plant, which may complicate the use of leaves in the process of obtaining anthocyanins.

Thus, the stem part of non-standard shoots of red-leaved clonal apple rootstocks is the most convenient raw material for the isolation of anthocyanins. In significant quantities, this plant material is formed on the mother plants during the separation of cuttings in the fall, when all non-standard cuttings (thin, unrooted or thickened and highly branched) are rejected, and after the standard cuttings are tied into bundles, their tops are trimmed and also fragments of shoots remain. Another possible way to use unnecessary plant material is bud trimming of the grafted tree in the spring in the second field of the nursery; in this case, many cut shoots of clonal rootstocks are also formed. The practical use of these plant residues for the isolation of anthocyanins simplifies their utilization and significantly increases the technological level of nursery.

4. Conclusion

As a result of the production of apple planting material, a large amount of non-standard plant products is formed, which must be disposed of annually after the separation of the clonal rootstocks of the apple tree in the mother plant and bud trimming of the grafted trees. In our zone, with standard technology, plant waste from nurseries is most often incinerated or processed into compost and mulch [16]. Both quantitatively and qualitatively, wastes from the production of apple planting material—rejected non-standard shoots of red-leaved clonal rootstocks—can serve as an alternative and very affordable source of natural, environmentally friendly food dyes for the production of which valuable food raw materials are often used. It is better to select rootstocks with a high endogenous synthesis of anthocyanins (i.e. red-leaved) as their source. So, when cultivating red-leaved rootstocks (54-118, 57-491, 98-7-77, etc.) from 1 hectare of mother plant, in addition to the separated rooted cuttings, which are the main product, it is possible to annually obtain from 3 to 10 kg of anthocyanin dyes.

Acknowledgments

The research was carried out within the framework of the State task of the Ministry of Agriculture of the Russian Federation “Breeding of winter-hardy low-growing clonal rootstocks using biotechnology methods” for 2021 (state registration No. AAAA-A21-12011190007-9) in the facilities of the Center for Collective Use “Selection of agricultural crops and technologies for the production, storage and processing of functional and therapeutic-prophylactic food products” of the Michurinsky State Agrarian University.

References

[1] Zaprometov M N 1993 Phenolic compounds (Moscow: Nauka) 271 p
[2] Andersen O M, Markham K R (Eds) 2006 The anthocyanins, in: Flavonoids: chemistry, biochemistry and applications (Boca Raton, FL: CRC Press) pp 452-471
[3] Tarova Z N, Churikova N L, Dubrovsky M L, Kruzhkov A V, Savelyeva N N 2020 Agrobiological evaluation of new apple clonal rootstocks of the Michurinsk State Agrarian University selection using different breeding methods BIO Web of Conferences 23 01002
[4] Solovieva M A 1982 Methods for determining winter hardiness of fruit crops: Methodical manual (Leningrad: Gidrometeoizdat) 36 p
[5] Maslennikov P V, Chupakhina G N 2001 Biosynthesis of anthocyanins in plants with a deficiency of some macronutrients Actual problems of agriculture: Collection of scientific papers (Kaliningrad) Part 2, pp 187-195
[6] Dubrovsky M L, Kruzhkov A V, Churikova N L, Papikhin R V, Ussova G S 2020 Patterns of development of advanced clonal apple rootstocks of the Michurinsk State Agrarian University selection in the mother plantation BIO Web of Conferences 23 01004

[7] Papikhin R, Muratova S, Dubrovsky M, Grigoryeva E 2020 Development of methods for introducing hybrid progeny of Malus sieboldii Rehd. at in vitro conditions E3S Web of Conferences 210 06024

[8] Drenova N V, Shamshin I N, Dubrovsky M L 2019 Marking of QTL-resistance to bacterial blight in apple varieties and hybrids Fruit and berry growing in Russia 59 219-226

[9] Dubrovsky M L, Papikhin R V 2019 Analysis of the karyotype of the Russian apple tree clonal rootstocks bred at the Michurinsk State Agrarian University Amazonia Investiga 8(21) 688-698

[10] Shlyavas A, Trifonova A, Shamshin I, Boris K, Kudryavtsev A 2019 Genetic diversity of apple landraces from VIR collection based on SSR markers XV EUCARPIA Fruit Breeding and Genetics Symposium p 23

[11] Tarova Z N, Bobrovich L V, Krivolapov I P, Astapov A Yu, Korotkov A A, Grechushkina K S 2020 Analysis of taxation assessment results and development of a method for applying digital technologies in the assessment of garden agroecosystems stability Journal of Physics: Conference Series 1679 22101

[12] Savel'ev N I, Lyzhin A S, Kudryavtsev A M, Boris K V, Shamshin I N 2015 Use of molecular markers for identification of genotypes of columnar apple trees Russian Agricultural Science 41(5) 323-325

[13] Solomatin N M, Tarova Z N, Astakhova L V, Kolotso V A, Bocharova T E 2015 Nutritional qualities of both red pulp apples and their products Ecology, Environment and Conservation 21 AS41-AS44

[14] Kirchner J 1981 Thin-layer chromatography, vol. 2 (Moscow: Mir) 522 p

[15] Papikhin R V, Dubrovsky M L 2018 Cytological features of male gametophyte formation from distant hybrids pyrus X Malus and ribes X Grossularia Journal of Pharmaceutical Sciences and Research 10(10) 2524-2527

[16] Grigoreva L V 2018 Biological growth peculiarities of the cuttings of various rootstocks in a horizontal nursery International Journal of Pharmaceutical Research 10(4) 632-640