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Flowers Characteristics of Selected Species of Lime-Tree (*Tilia* spp.) in Terms of miRNA-Based Markers Activity, Mannose Expression and Biological Compounds Content

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Abstract: The significant healing effect of *Tilia platyphyllos* Scop. and *Tilia cordata* Mill. flowers are well known. However, the flowers of *Tilia tomentosa* Moench. are not suitable for harvest due to their toxic effects. To investigate the diverse background of this effect, we applied a functional miRNA-based marker, mannose expression analysis and determined the content of bioactive compounds. Out of nine tested markers, three (*miR160*, *miR167* and *miR408*) provided reproducible miRNA-based loci and two of them (*miR160* and *miR398*) enabled the acquisition of fingerprinting specific to flower and leaf samples of *T. platyphyllos* and *T. tomentosa*. The most pronounced profiling was specific for *miR408* marker, the function of which is connected to plant defense and adaptation mechanisms. We confirmed the suitability of microRNA-based markers for polymorphism determination of flowers of selected species of lime-tree. The highest values of antioxidant activity, flavonoids, total polyphenols and phenolic acids content have been reached in silver linden flowers. When comparing the transcription activity of mannose in flowers, more than 30 times higher levels of mannose transcripts for the silver linden flowers was observed.

Keywords: linden; flowers; microRNAs; mannose; antioxidants

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1. Introduction

The species of lime-tree (*Tilia* spp.) from the *Malvaceae* family are beautiful, stout trees with a dense, shady crown. They grow to a height of 30 m and can live up to 700 years. It is a symbol tree of the Slavs for its longevity, power and beauty. King Matej's lime tree, growing near Bojnice Castle in Slovakia, is one of the oldest trees in Europe [1,2]. In Europe, there are mainly found *Tilia platyphyllos* Scop. (large-leaved linden), *Tilia cordata* Mill. (small-leaved linden) and *Tilia tomentosa* Moench. (silver linden) [3]. The lime-tree is deeply rooted and rejuvenates from stumps. The flowers are yellowish, fragrant and honey-bearing. They are widely used in horticulture [2]. For proven healing effects, mainly flowers of large-leaved linden and small-leaved linden are collected. The pharmaceutical and environmental values make the lime-tree the "tree of the future" [1]. For silver linden (blooms in late June and early July), the collection of flowers for these purposes is not recommended, due to their toxicity. In terms of the presence of different pollinators, individuals of the honeybee dominate the large-leaved linden and the small-leaved linden.

However, the silver lime is dominated by various species of bumblebees, flies, or other, unspecified species of insects [4]. *Tilia cordata* Mill. (syn. *T. parvifolia* Ehrh.), a small-leaved linden, grows from the lowlands to the lower mountain ranges. The leaves are round heart-shaped, gray on the back and rusty hairy at the angles of the veins. Weakly ribbed drupes can be crushed between fingers. *Tilia platyphyllos* Scop. (syn. *T. grandiflora* Ehrh.), the large-leaved linden, has an overall appearance of the previous one. The occurrence is similar, but it extends to higher mountain ranges (up to a height of 1200 m above sea level). However, it buds and blooms more often. The leaves are larger and more asymmetrical, green on both sides, with whitish hairs at the veins of the veins. The drupes cannot be crushed between fingers. Both domestic species interbreed with each other. *Tilia tomentosa* Moench. (syn. *T. argentea* Desf.), the silver linden, has a conical crown with wide protruding branches. The shoots are hairy and the leaves on the back are grayish white felted with a shorter stalk. It blooms only in the second half of July and has a pleasant smell. It is very valuable and suitable for urban environments [1–3]. Due to its resistance to polluted air, drought resistance and frost resistance, it is one of today's most important trees in various major cities in Europe [5].

Several studies have been focused on genetic diversity and structure of lime-tree in urban and forest ecosystems assessed by RAPD [6,7], ISSR [8] and SSR markers [9–11], including plastid genome sequencing [12]. However, the miRNA-based markers have not yet been applied to this species. MicroRNAs molecules are an integral part of a gene regulation mechanism. As such, they are a part of comprehensive processes of plant development, plant organ formation, and they significantly participate in plant protection mechanisms against biotic and abiotic stress factors. For this reason, miRNA markers are commonly referred to as stress biomarkers [13–17]. Precursor molecules of miRNA (pre-miRNA) create specific stem-loop structures, which are characterized by genomic conservation of miRNA sequences. This unique feature of the stem-loop region provides an opportunity to develop a novel type of molecular markers [18]. The miRNA-based marker system is relatively polymorphic and reproducible and with putative functionality [19]. The high level of transferability across species demonstrates the usability of miRNA-based markers for genome mapping and phylogenetic studies [20]. Given the origin of markers, of which sequences correspond to the miRNA genomic sequences, they can be considered as functional markers at the DNA levels [19–21]. The microRNA-based genotyping technique has been applied in *Brassica species* [19], *Setaria italica* (L.) P. Beauvois and in related grass species [20], *Oryza sativa* (L.) [22,23], *Medicago truncatula* Gaertn. and related legume species [24], *Linum* species [25] and *Ginkgo biloba* (L.) [26].

A phenomenon linked to mass bee deaths has been reported for *Tilia tomentosa* due to the toxic components of nectar [27] but not for other linden species [28]. Different lime metabolites were identified as toxic, but mannose was reported to be toxic specifically for honeybees and bumblebees [29] and the toxicity effect has been further discussed in literature in opposite ways as the background molecule causing the bees death [30,31]. Mannoses disturb carbohydrate metabolism in bees [32] and beside the linden trees, this molecule was detected in nectar of other plants, such as *Tordylium apulum* (L.) or *Cistus salvifolius* (L.) [32].

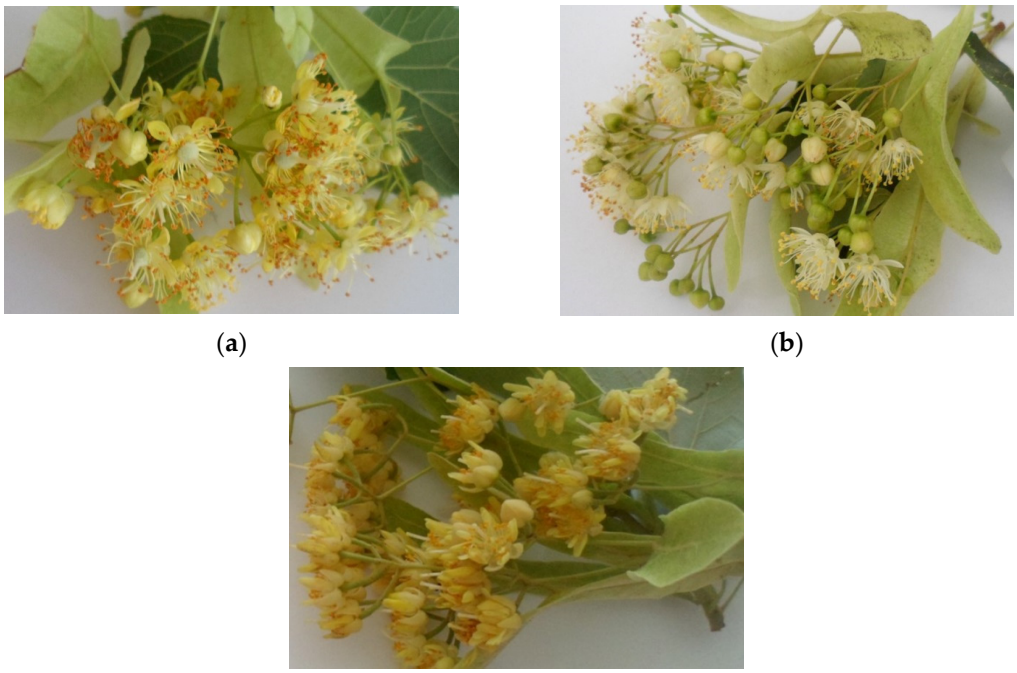
The purpose of our work was to point out possible differences connected to linden flowers properties using functional microRNAs markers, mannose expression and biological compounds content. We aimed to test the hypothesis that functional miRNA-based markers might be linked to some specific properties of silver linden flowers. To our knowledge, interdisciplinary, molecular-biochemical approaches to the study of linden flowers' properties, has not yet been applied.

2. Materials and Methods

2.1. Plant Materials and Sample Preparation

Plant material (whole inflorescences including bracts and leaves) for analysis was obtained from three species of lime tree: *Tilia platyphyllos* Scop. (large-leaved linden), *Tilia*

cordata Mill. (small-leaved linden) and *Tilia tomentosa* Moench. (silver linden) growing in an urban area at the time of intensive flowering (late May, June and early July 2019). Samples of flowers, bracts and leaves (Figure 1) were collected randomly from the tree and were obtained from two trees of each species. Separated parts (flowers, bracts and leaves) were stored at −20 °C till further analyses.



(**a**) (**b**) (**c**)

Figure 1. Flowers of (**a**) *Tilia platyphyllos* Scop. (large-leaved linden); (**b**) *Tilia cordata* Mill. (small-leaved linden); (**c**) *Tilia tomentosa* Moench. (silver linden).

2.2. Nucleic Acids Extraction

Subsequently, the pooled samples of flowers, bracts and leaves were prepared in liquid nitrogen and genomic DNA was isolated by NucleoSpin Plant II extraction kit (Macherey Nagel, GmbH & Co. KG, Düren, Germany). Isolated DNA was quantified by nanophotometer Implen P360 (Implen GmbH, Muenchen, Germany) and diluted to a concentration of 70 ng × μL^{-1}. Total RNA was extracted by Ribospin Seed/Fruit (GeneAll Biotechnology Co., Ltd., Seoul, Korea) from flowers and leaves and checked for its quantity and quality using the nanophotometer. Extracted RNAs were normalized to 30 ng × μL^{-1} and the transcription was performed by Tetro cDNA Kit (Bioline, Meridian Bioscience, TN, USA) following the instructions of the manufacturer and using oligo (dT)18 primers.

2.3. MiRNA-Marker Assay

The original protocol [19,20] was modified [33] and applied as follows: PCR was amplified in a PCR mix 20 µL containing 70 ng of genomic DNA, 10 pmol × dm^{-3} of each primer, 2 U of *DreamTaq* DNA polymerase (ThermoFisher Scientific Inc., Naarden, The Netherlands), 0.8 mmol × dm^{-3} dNTPs (Invitrogen, ThermoFisher Scientific Inc., Naarden, The Netherlands) and 1 × *DreamTaq* Buffer (KCl, $(NH_4)_2SO_4$, 20 mmol × dm^{-3} $MgCl_2$). The PCR amplification program used the 'touchdown' method as follows: initial denaturation at 94 °C for 5 min; 5 cycles of 30 s at 94 °C, 45 s at 64 °C (with a 1 °C decrease in annealing temperature per cycle), and 60 s at 72 °C; 30 cycles of 30 s at 94 °C, 45 s at 60 °C, and 60 s at 72 °C; and the final extension at 72 °C for 10 min. The samples were subsequently stored at 8 °C. PCR assays were carried out in duplicates. Amplificated products were separated on 3% agarose and Novex™ TBE-Urea gels, 15% (Invitrogen, ThermoFisher Scientific Inc., Naarden, The Netherlands), running in 1 × TBE Running

Buffer at constant power 180 V, 30 mA for 90 min. The gels were stained with PAGE GelRed™ Nucleic Acid Gel stain (Biotium, Inc., Fremont, QC, Canada) and visualized on G-Box (Syngene, Synoptics Ltd., Cambridge, UK) electrophoresis documentation system. For the recording of loci number and their position, as well as the identification of unique fragments, the gels were analyzed by GeneTools software (version4.3.10.0, Syngene, Synoptics Ltd., Cambridge, UK).

2.4. Design of miRNA Primers

The primers for miRNA-based markers were designed according to the mature or precursor sequences (pre-miRNA) (available in the miRBase database (http://www.mirbase.org release 22.1), accessed on 18 May 2020) [34] based on the methodology [19,20]. The database does not contain sequences of lime-tree species; within the family *Malvaceae*, it includes sequences of *Gossypium* species and *Theobroma cacao* L. We applied markers based on miRNAs sequences of the following species: *Gossypium*, *Malus*, *Linum*, *Hypericum* and *Glycine*. Our previous studies have confirmed species transferability of miRNA-based markers; therefore, we selected the following types of markers of above-mentioned species: *miR156*, *miR160*, *miR167*, *miR171*, *miR396*, *miR398*, *miR408* and *miR414*. All of them represent conserved miRNA families.

2.5. Primer Design for Mannose Expression Analysis and Real-Time PCR Analysis

Bioinformatic screening and BLAST based design was used for primers of mannose expression analysis, because the nucleic acid data specific for linden mannose gene does not exist. A total of five sequences for mannose pathway regulator are available in the NCBI database and these were BLASTed for finding a conserved part of them. The following nucleotides of the accession NM_105129.1 were found to be conserved and these were used for primer designation: nucleotides 909–941 and nucleotides 1001–1021. Actin was used in the analysis as a housekeeping gene. The reactions were performed in ElizymeMix with ROX 2X (Elizabeth Pharmacon, Ltd., Croydon, UK) and the following profile of real-time PCR was used: 95 °C 2 min (95 °C 5 s; 60 °C 25 s) 40× plus melt analysis. A delta delta Ct approach was used to compare the expression levels of mannose transcription activity among the individual combinations of linden species or tissues.

2.6. Analyses of Antioxidant Activity

2.6.1. Free Radical Scavenging Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to determine free radical scavenging activity in accordance with the method described by [35]. Briefly, the absorbance of samples (0.4 mL) and alcohol solution DPPH (3.4 mL) were measured at a wavelength of 515 nm using a spectrophotometer (6405 UV/Vis, Jenway, Stone, UK). The results of antioxidant activity were expressed as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent in mg/g.

2.6.2. ABTS Radical Cation Decolorization Assay

ABTS radical cation decolorization assay was determined by the method [36] with slight modifications. Trolox (100–100 mg/L; R^2 = 0.9991) was used as a standard, and the results were expressed in mg/g FM of Trolox equivalents.

2.6.3. Total Polyphenol Content

A standard method [37] was used to measure the total polyphenol content in analyzed samples. The results of absorbance at 700 nm measured spectrophotometrically (6405 UV/Vis, Jenway, Stone, UK) were then calculated and expressed as gallic acid equivalents expressed in mg/g.

2.6.4. Total Flavonoid Content

Total flavonoid content was measured spectrophotometrically (6405 UV/Vis, Jenway, Stone, UK) at 415 nm according to the method [38]. Briefly, to a 0.5 mL sample, ethanolic solution of aluminum chloride (10% *w/v*), potassium acetate (0.1 mL of 1 M) and distilled water (4.3 mL) were added. Then, the mixture was incubated in the dark for 30 min. Obtained results were expressed in mg/g quercetin equivalents.

3. Results

3.1. MicroRNA Analyses

In total, nine different types of microRNA-based markers belonging to eight different families of miRNAs were used in the diversity study- *miR156*, *miR160*, *miR167*, *miR171*, *miR396*, *miR398*, *miR408* and *miR414.* Positive amplification has been recorded only in three types of miRNA markers—*miR160*, *miR167* and *miR408*. In total 27 miRNA loci were amplified by markers based on *miR408*, where the most (48%) miRNA loci were detected in flower samples, 30% in leaf samples and 22% in bracts samples. The most significant amplification of this marker was observed in small-leaved linden flower sample (Figure 2). In the leaf samples, the amplification profile was similar in all three genotypes with the highest amplification effectivness in leaves of large-leaved linden. The most significant amplification by the marker *miR408* was recorded in bracts samples of silver linden (Figure 3).

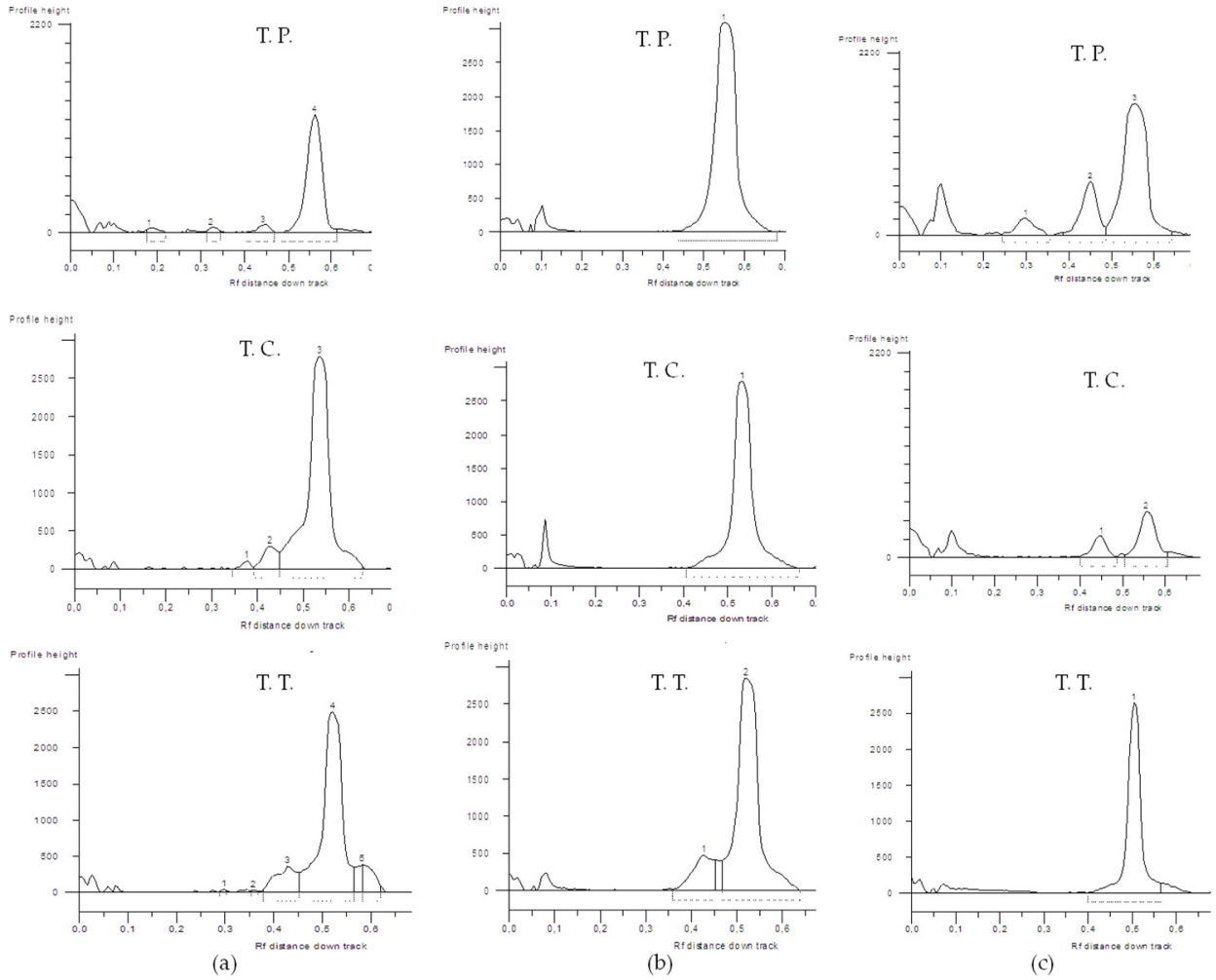


Figure 2. Amplified profiles of miRNA-based pattern of *Tilia platyphyllos* (T. P.), *Tilia cordata* (T. C.) and *Tilia tomentosa* (T. T.) in samples of flowers (**a**), leaves (**b**) and bracts (**c**) by *miR408* marker.

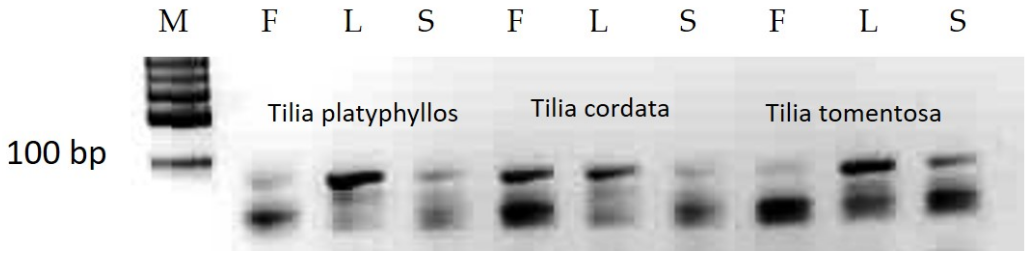


Figure 3. *Mir408*-based amplification in flowers (F), leaves (L) and bracts (S) of three linden genotypes. M—DNA Ladder.

In total, 26 miRNA loci were amplified by *miR160* marker, where leaf and bracts samples were amplified almost 39% and the flower sample was 23% of all amplified miRNA loci. The most significant amplification by the marker *miR160* was recorded in the flower sample of the small-leaved linden (*T. cordata*), leaf sample of large-leaved linden (*T. platyphyllos*) and bracts sample of silver linden (*T. tomentosa*) (Figure not shown).

The amplification product of *miR167*-based marker was observed mostly in flower samples of all three linden genotypes (*T. platyphyllos*, *T. cordata* and *T. tomentosa*). In the case of silver linden, fragments were also present in the leaf sample (Figure 4).

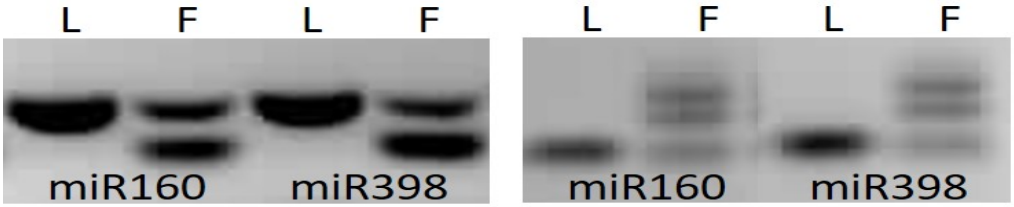


Figure 4. *Mir167*-based amplification in flowers (F), leaves (L) and bracts (S) of three linden genotypes. M—DNA Ladder.

The amplification product of *miR167*-based marker was observed mostly in flower samples of all three lime genotypes (*T. platyphyllos*, *T. cordata* and *T. tomentosa*). In the case of silver lime, fragments were present also in leaf sample (Figure 4).

One of the key features of microRNA-based markers is their species transferability, making it possible to derive them from various species and apply within the test species. This feature is dues to conserved nature of miRNAs sequences [19,20]. We decided to test the amplification affinity of markers *miR160* and *miR398* derived from apple (*M. domestica*) microRNA sequences. Interestingly, both markers have provided specific fingerprinting pattern distinguishing flowers´ samples from the leaves in large-leaved linden and silver linden (Figure 5).

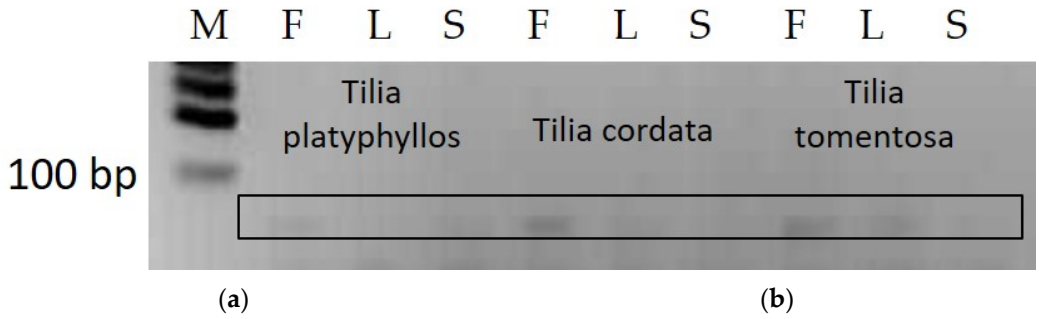


Figure 5. Fingerprinting of *miR160* and *miR398*-based pattern of flower and leaf samples of *T. platyphyllos* (**a**) and *T. tomentosa* (**b**).

3.2. Mannose Pathway Regulator Expression Analysis

Comparison of mannose transcription activity was analyzed for the following combinations: different linden species for flowers, different linden species for leaves and the comparison of expression change for flowers and leaves of the same linden species. After the optimization of the real-time PCR assay, the Tm of the mannose product was set to 72.2 °C.

When comparing the transcription activity of mannose in flowers of different linden species, the highest fold change was obtained for *Tilia tomentosa* and *Tilia cordata* (Figure 6) at the level of more than 30 times higher than the level of mannose transcripts for the flowers of silver linden. The expression of the mannose is nearly the same in the flowers of *Tilia platyphylos* and *Tilia cordata*.

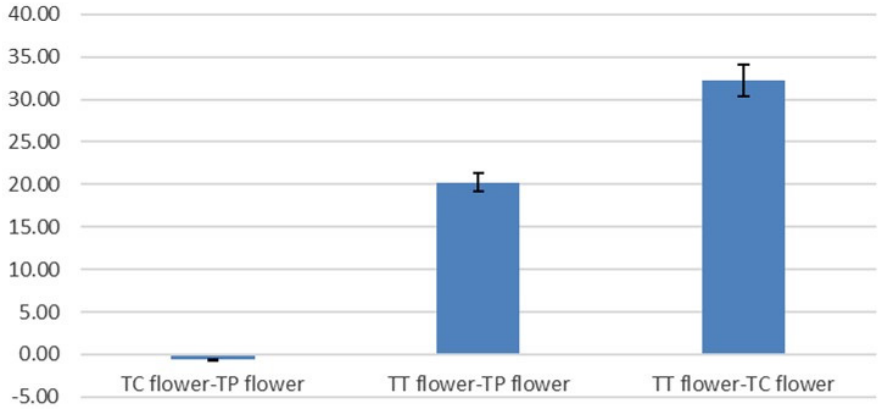


Figure 6. The expression change of mannose in the flowers of different *Tilia* species.

Similar results were obtained in the case of mannose expression comparisons in leaves of the analyzed linden species but the fold changes were much lower here as in the previous case with the highest change at the level of 2 (Figure 7).

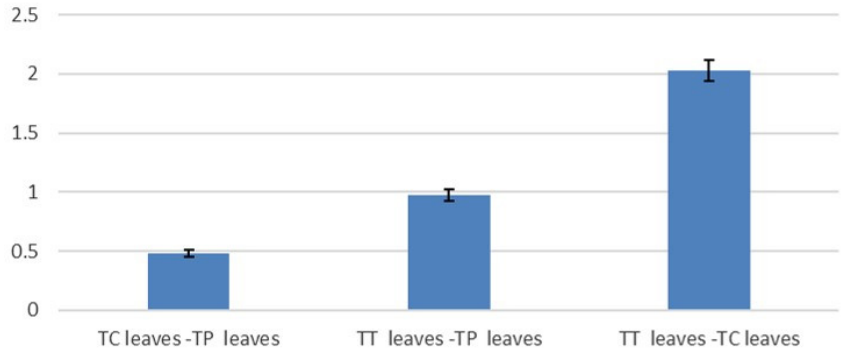


Figure 7. The expression change of mannose in the leaves of different *Tilia* species.

The lowest fold changes were found when the expression of mannose was compared between the flowers and leaves of the same species. Here, the changes were not higher than physiological differencies (Figure 8).

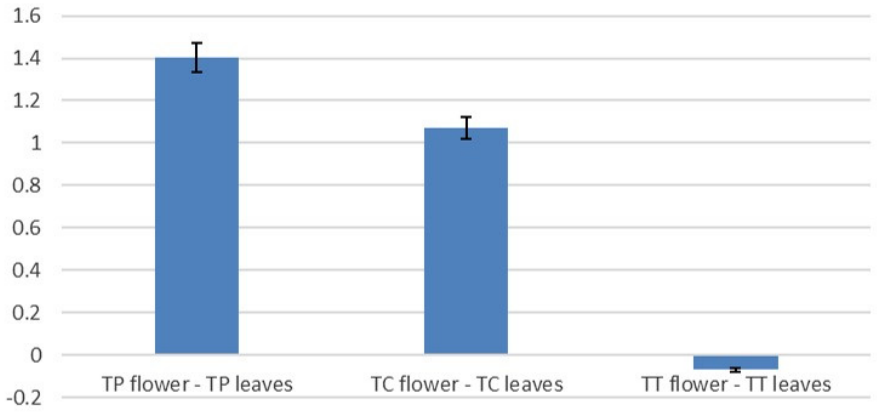


Figure 8. The expression change of mannose in leaves compared to the flowers of analyzed *Tilia* species.

3.3. Antioxidant Activity, Total Polyphenol and Total Flavonoid Content

Antioxidant activity, total polyphenol and total flavonoid content parameters were analyzed in flower samples of three lime genotypes because flowers are the main source of important medicinal properties. Flowers of silver lime showed the best antioxidant activity (Table 1). Almost similar values of antioxidant activity were detected in the flowers of large-leaved lime and small-leaved lime. The other analyzed parameters had a similar outcome. The results indicate that the highest content of total flavonoids, total polyphenols and phenolic acids is presented in flowers of silver lime (*T. tomentosa*). During the preparation of the extracts, the flowers of the silver lime had the most pronounced scent, and this scent penetrated the extract.

Table 1. Antioxidant activity, total polyphenol, total flavonoid content and total content of phenolic acids of flowers samples of *T. platyphyllos*, *T. cordata* and *T. tomentosa*.

Sample	DPPH (mg TEAC/g FM)	ABTS (mg TEAC/g FM)	TPC (mg GAE/g FM)	TFC (mg QE/g FM)	TPAC (mg CAE/g FM)
Tilia platyphyllos	0.593	6.952	4.274	1.904	1.538
Tilia cordata	0.561	6.716	3.968	2.296	1.576
Tilia tomentosa	0.657	9.620	5.759	3.419	1.909

TEAC—Trolox equivalent antioxidant capacity; ABTS—radical cation decolorization assay; FM—fresh matter; TPC—total polyphenol content; TFC—total flavonoid content; TPAC—total phenolic acids content; GAE—gallic acid equivalent; QE—quercetin equivalent; CAE—caffeic acid equivalent.

4. Discussion

Tilia is an ecologically important genus in the *Malvaceae* family. Lime flowers are an important source of nectar for pollinators, especially in urban areas. Their period of flowering from June to July provides sufficient time for food security of insects [1,2]. There is evidence that flowers of silver linden (*Tilia tomentosa* Moench) are toxic for insect' visitors [4] nor are they recommended to be collected for human consumption [39]. There is a lack of clinical research assessing the effects of silver linden flowers as well as safety and toxicity data. At the same time, no products containing silver linden flowers are available in the EU [39].

There are various hypotheses justifying the toxicity of silver linden flowers for bees either due to the presence of mannose or nicotine in the nectar or starvation due to insufficiency of the nectar to feed insect visitors [4,40]. Moreover, according to our observations, it is clear that the linden trees are rarely visited by bees, but mainly by other species of insects, such as bumble-bees. However, no trace of mannose or nicotine has been detected in nectar samples in any of the European lime species (*T. cordata*, *T. platyphyllos*. *T. tomentosa* and *T.* × *europaea*) [40]. Metabolic analyses of silver lime nectar have revealed that it does not contain mannose but does contain the pyridine alkaloid trigonelline [41]. The

authors suggest that behind the bee's mortality is a combination of environmental (low temperature), physiological/sociological (energy deficit of bees that collect nectar despite its shortage and are consequently unable to fly) and biochemical (alkaloids content) factors. A study of sedative and anxiolytic effects of silver lime nectar [42] identified constituents of a flavonoid nature with clear anxiolytic effect. Another study on the side effects of anxiolytic drugs has reported digestive problems [https://www.medicinenet.com/, accessed on 18 May 2020]; flavonoid concentration was one of the factors influencing anticonvulsant and antioxidant properties of *Tilia americana var. mexicana* flowers and leaves [43].

In all tested attributes (antioxidant activity, total flavonoids content, total polyphenols and phenolic acids content), our results indicate that the highest values were presented in the flowers of the silver linden (*T. tomentosa*). A number of bioactive compounds can be influenced by several parameters such as climatic conditions, soil, stage of maturity and genotype [44,45]. In our case, the trees from which the samples were obtained grow between residential blocks of an urban settlement (approximately 2 hectares), are the same maturity stage (40-year-old trees) and the soil and climatic conditions are similar. Therefore, from this point of view we can attribute the observed differences to different lime-tree genotypes. Flavonoid fraction is probably responsible for the antiradical properties of lime extracts. The content of biologically active compounds depends on their accumulation during the growing season [44,45]. Polyphenols are the main plant bioactive compounds, which act as natural antioxidants [46]. The medicinal potential of the small-leaved lime was tested based on the content of polyphenols in the dry matter of leaves [47]. The ethanolic extracts of *Tilia cordata* contained polyphenols (1.37 $\pm$ 0.0021 g/100 g DW) and indicate high antioxidant activity (IC_{50} = 0.3303 $\pm$ 0.0896 mg/mL calculated as DPPH scavenging activity). In comparison to our results, the levels of polyphenols and antioxidants were much higher but only in the fresh matter of flowers. A significant antioxidant activity of lime flowers (*T. platyphylos, T. cordata* and *T.* $\times$ *europaea*) was established in water (63.0 $\pm$ 3.8 mg/g) and ethanol (36.7 $\pm$ 1.8 mg/g) extracts [47]. In this study, the antioxidant activity was determined using DPPH radical and no statistically significant differences were observed in individual lime genotypes. The antioxidant activity of the water extract of *Tilia Argentea* Desf ex DC was concentration dependent, but the extract showed no antibacterial activity on the studied microorganisms [48]. Although, most of the studies are focused on antioxidant analyses of linden blossoms and flowers, *Tilia cordata* fruit extracts also contained 31 phenolic compounds [49].

The silver linden best tolerates polluted air and is characterized by drought and frost resistance, which makes it an integral part of cities [3,5]. Answers to the consequences of the death of pollinators may be found behind these properties. This observation might be supported by the study of local adaptation of 12 isolated populations of silver lime assessed by SSR markers [10]. Significant differences among the populations indicate ongoing adaptation process to local environmental conditions. The involvement of epigenetic mechanisms of regulation of the adaptation process to local environments with just the miRNA molecules representing one of the epigenetic mechanisms of gene expression regulation has been suggested [50,51].

One of the applied markers in our study, *miR408*, plays an important role in plant genome adaptation against abiotic stress [52]. This marker provided the most effective amplification in flower, leaf and bracts samples of *T. platyphyllos*, *T. cordada* and *T. tomentosa*. Activity of *miR408* is significantly affected by a variety of developmental and environmental conditions. Samples were collected from approximately 40-years-old trees, but from an urban area, which might participate in an abiotic stress response of tested lime trees. The *miR408* loci profile has reached the highest levels, mainly in leaf samples of large-leaved lime. One of the target sequences of *miR408* in the Malvaceae family are laccases, which are copper containing oxidases [53]. Laccases can oxidize a wide range of substrates, including environment pollutants [54], which could be one of the explanatory factors for environmental adaptability of lime trees.

Based on our results, the most significant amplification by the marker *miR160* was recorded in the flower sample of small-leaved linden (*T. cordata*), leaf sample of large-leaved lime (*T. platyphyllos*) and bracts sample of silver lime (*T. tomentosa*). This is in line with the function of *miR160* in development of flowers and floral buds [55]. Target sequences of *miR160* are an auxin response factor family of proteins (ARF), especificaly ARF 10, 16 and 17, which are included in flower organ development [56].

The presence of the *miR167* locus, especially in the flower samples of all tested lime genotypes, may be because *miR167* is a part of the regenerative organ development, embryogenesis and seed development mainly through controlling the auxin response factor transcriptional activities [57]. Lower intensity of *miR167* activity compared to other markers can be explained by tissue and spatial-specific miRNA activity in the given tissues [58,59].

In our previous experimental studies where miRNA-based markers were applied accross different species (ginkgo, milk thistle, lavender, flax, wheat and barley), the polymorphism level was much higher than in our current study. However, it should be noted that the level of polymorphism depends on the effectiveness of primers´ effectiveness as well as on the level of markers´ transferability. Some of the primers (*miR160*, *miR 396* and *miR398*) were designed based on *Gossypium* species or *Malus domestica* (BORKH) due to the lack of availability of the microRNAs sequences of *Tilia* spp. in the miRBase database. The markers *miR156*, *miR167*, *miR171*, *miR408* and *miR414* originated from miRNA sequences of other species (*Linum*, *Glycine* and *Hypericum*). A possitive reproducible amplification has been recorded in only three types of miRNA markers: *miR160* (originated from cotton and apple miRNA sequences), *miR167* and *miR408* (originated from flax miRNA sequences).

MiRNA-based markers usually demonstrate high polymorphism [19,20]. One of the reasons for the low polymorphism of some of the applied miRNA markers may be the difference in the length of noncoding region caused by insertions in the linden plastid genome compared to the cotton genome [12] based on which the primers were designed. Plastid genome of *Tilia* spp. is very similar to other sequenced plastid genomes of *Malvaceae* (*Gossypium* and *Theobroma* genera) and consists of 130 genes, of which 113 are unique [12]. Single nucleotide polymorphism in plastid genome of *Tilia* is defined by 41% of insertions and 59% of deletions. Most of these indels are associated with tandem repeats. Further research will be necessary to design and test a wider spectrum of miRNA-based markers.

Genomic analyses of *Tilia* spp. were so far related to population genetic structure. Microsatellite (SSR, simple sequence repeats) markers were able to discriminate the species *T. cordata* and *T. platyphyllos* and their hybrid (*Tilia* × *europaea*) as well as to evaluate the population genetic diversity [11]. The same type of markers reliably recorded a high level of genetic diversity of wild living populations of *T. cordata*; however, there was low differentiation among tested populations [9] with no evidence of geographic-related adaptation. The number of amplified alleles per locus varied from 5 to 32 by nine SSRs primers. A molecular phylogenetic study employing ISSR markers (inter simple sequence repeats) revealed that the groups of 20 populations of native silver lime have high genetic similarity despite different provenances. These populations were characterized by strong vitality and a large morphologic variability [8]. RAPD markers were used to determine genetic diversity of three *Tilia* species, including clones (*T. tomentosa, T. cordata* and *T. euchlora*). Nine of 12 RAPD primers produced suitable DNA fragments [7]. The authors suggest that the eco-geographic background is an important factor affecting the woody plant genome structure.

We applied miRNA-based markers as functional types of markers whose activity indicates a certain phenotype. Activity of miRNA-based markers *miR160*, *miR167* and *miR408* was in line with the role of these molecules in plant tissues. In addition, the *miR408*, whose proliferation was the most significant, plays an important role in the plant genome adaptation. We can state that our hypothesis to declare suitability of microRNA-based markers for determination of genetic polymorphism of selected species of lime-tree, has been confirmed. The mannose activity and level of bioactive compounds (antioxidant activity, total flavonoids content, total polyphenols and phenolic acids content) has reached

the highest levels in the flowers of silver linden (*T. tomentosa*). We can assume that these properties also contribute to the high adaptability of this genotype to urbanized environmental conditions and could have some connection with the properties of nectar of silver lime-tree. Continuing this research by applying more specific miRNA-based markers will broaden our understanding in this area and aid in any future applications of silver linden for urban areas or in the pharmaceutical industry.

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