Phenolic compounds pattern in sweet clover (*Melilotus officinalis*) vs white clover (*M. alba*) revealed by 2D TLC (two-dimentional thin-layer chromatography) and its taxonomic significance

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Summary

Two closely related species: *Melilotus alba* and *M. officinalis* were compared in respect to chromatographically separated and visualized flavonoids. Several compounds were common for both species. Taking into account that phenolic compounds are genetically strictly controlled and not sensitive to environmental influences, the presence of characteristic compounds for each species confirmed their taxonomic values and differences.

Key words: *Melilotus alba, M. officinalis, TLC, phenolic compounds, genetic differences*

INTRODUCTION

Yellow sweet-clover (*Melilotus officinalis*) and white sweet clover (*M. alba*) originate from Eurasia but now they occur on all continents, including Australia, South America and the territory of both Americas, where they were introduced from Europe. They can be found throughout the lowlands and on lower mountainous stands. They do not have special requirements regarding climate and soilconditions, therefore, they occur on balks, roadsides, wastelands, ditches and railway embankments. They prefer sunny sites, and if they grow in shaded spots, they are less vital and the seeds production is limited[1].

Both species are cultivated: *M. alba* is valuable cattle feed. Successful researches on reducing the coumarin content in herbs were conducted[2-4]. *M. officinalis* is a medicinal plant since besides of coumarin compounds, it contains vitamin C, allantoin, tannins, mineral salts and flavonoids.

Thanks to specific effectiveness of the substances of the plant, herbal blends with *Herba Meliloti* have been used in the treatment of allergy [5], obesity [6], mucous membrane diseases [7], in the treatment of varicose veins and vein thrombosis, as well as to speed up healing wounds and ulcerations [8]. Both species are valuable melliferous plants.

Previously, various comparative studies had been carried out concerning their morphology, biochemistry of seeds, inheritance of the seeds and seedlings colour, flowering biology and the level of self-fertility [9].

In the last decade, there has been a Renaissance of interest in phenolic compounds [10] in relation with the search for substances that protect plants against harmful biotic and abiotic factors and play a preventive role in the development of cancer and heart disease. The research primarily aimed two groups of phenolic compounds: phenolic acids and flavonoids.

Phenolic acids take part in the biosynthesis of lignin. The orto-diphenol function is present in caffeic acid and its ester, chlorogenic acid, whose presence in the cell walls of plants is involved in their resistance to fungal pathogens. The inhibitory properties against fungal spores are also present in the case of the coumaric and feluric acids. Some phenolic acids play a significant role in allelopathy, especially caffeic and feluric ones, which are washed out from leaves during the rain and they may act as natural herbicides, in particular because some of them operate selectively [11]. Phenolic acids have been detected in all groups of plants: bryophytes [12-15] coniferous trees [16, 17] and other flowering plants [18].

Flavonoids give the colour to flower petals and fruits and they act as antioxidants [19-21]. These antioxidant properties of flavonoids determine their functions because they are counteractive to mutation and cancer effects. They also have an influence on delaying the aging processes. Flavonoids are the main component of anti-malaria preparations and arouse an interest as active compounds that act against AIDS and cancer [10].

Extensive functions of phenolic compounds make them an interesting subject of the research among biochemists, physiologists, environmentalists and geneticists. In the population genetics, the phenolic compounds may provide the data to determine the level of variability in plant populations. The usefulness of phenolic compounds in the research into plant populations is caused by the following characteristics:

– they are widespread;
– they are good markers on the species level (in contrast to basic metabolic products such as sugars, amino acids, citric cycle acids);
– their structural variability is genetically controlled and is independent from the environmental influences;
– they are relatively easy to detect, isolate and determine;
– their chemical structure is stable so they may be tested in dry tissues, e.g. in herbarium material [15, 17, 18].

MATERIAL AND METHODS

The seeds of white sweet clover (*M. alba* Med.) and yellow sweet clover (*M. officinalis* L.) were randomly collected in 12 natural populations with the use of transect method (fig. 1).

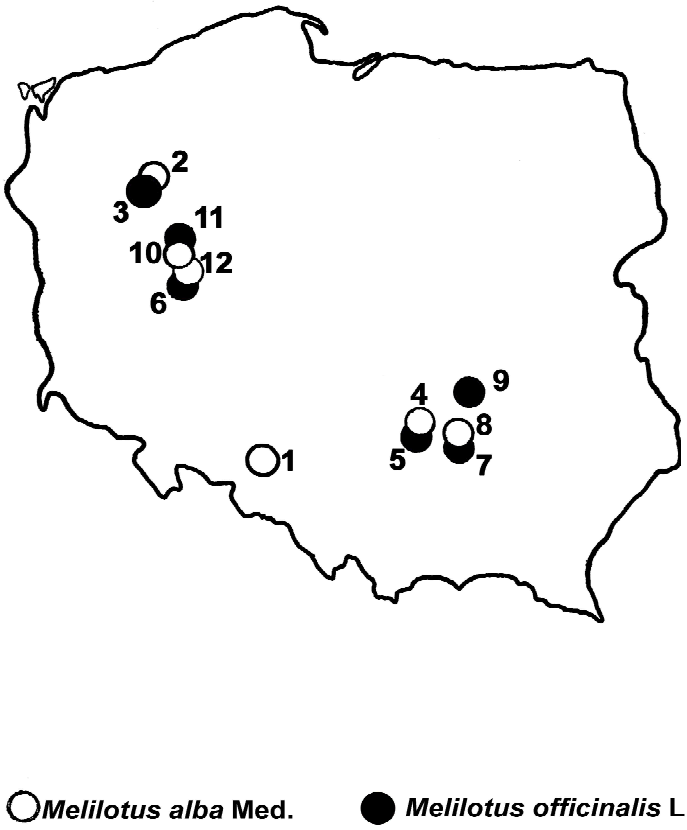

Figure 1. Geographical distribution of investigated populations
M. alba: 1 – Karłubiec, 2 – Tuczno, 4 – Tumlin, 8 – Łączna, 10 - Poznań Lutycka, 12 – Poznań Dębina,
M. officinalis: 3 – Tuczno, 5 – Tumlin, 6 – Poznań Dębina, 7 – Łączna, 9 – Skarżysko Kamienna, 11 – Poznań Lutycka.

Since the seeds of sweet clovers belong to so-called hard seeds, after being podded, they were subjected to scarification by making incisions with a razor blade, and next they were left to sprout on the Petri dishes [2]. The seedlings were transferred into the trays filled with soil and grown in identical greenhouse conditions. From two-month old seedlings the leaves were collected, dried and stored until performing the analyses. 200 mg of powdered plant tissue was flooded with 10 ml chloroform (in order to remove chlorophyll and essential oils) and then was

shaken in a shaker for 1 hour. The whole procedure was repeated three times. Dry, colourless powder was flooded with 20 ml 70% boiling methanol and shaken for 1 hour again. Then the extract was evaporated until dry in a vacuum (rotary evaporator Unipan 350). The residue was dissolved in 1 ml cooled methanol.

The chromatographic separation of phenolic compounds was carried out on glass plates 20x20 cm (Quickfit – England) coated with cellulose MN 300 layer, 0.2 mm thick. The extract (0.030 ml) was placed on one start up point in the corner of the plate (2 cm from the edges). After drying the spot in a stream of cool air, the plates were placed in airtight chambers (Quickfit) filled with a mobile phase. The mobile phase of the first development direction was 2% formic acid [22]. After drying, the plates were put in the chamber with the other mobile phase which developed the chromatogram in the second direction. The mobile phase II consisted of 65ml distilled water, 100 ml benzene and 210 ml propionic acid. In this way, the separation of phenolic spots, their colour observed in ammonia vapours, in UV light (254 nm), the position of spots (FR) and size were determined.

The results were elaborated using the coefficient S *phi* [23] applied to compare the prevalence of spots and to determine their taxonomic distances between the species.

$$S_{phi} = \frac{(AxD)x(BxC)}{A+B)(C+D)(A+C)(B+D)},$$

where
A – number of spots that are common to both species,
B – number of spots characteristic only for the first trial and absent in the second one,
C – number of spots occurring only in the second trial and absent in the first one,
D – number of spots not occurring in either of the trials.

After placing the values into the formula: $\sqrt{1-S_{phi}}$, the matrix of taxonomic distances was obtained. Each value of the matrix was divided by the smallest of them and that leaded us to create the so-called Matrix of transformed taxonomic distances. To illustrate the differences between populations, we also applied a Principal Component Analysis and an Unweighted Pair Group Method with Arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

In total, 66 unidentified phenolic compounds were detected in both species: 15 characteristic for *M. alba* (fig. 2) and 16 for *M. officinalis* (fig. 3), representing approximately 25% of all spots, the remaining spots were common to both species.

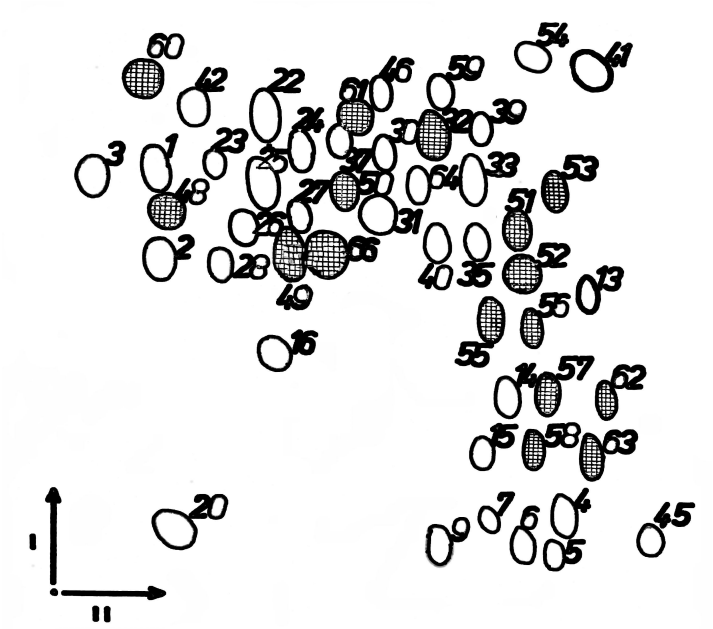

Figure 2. Chromatographic patterns of phenolic compounds in leaves of M. *officinalis*. Characteristic spots are shaded, the others are common to both species

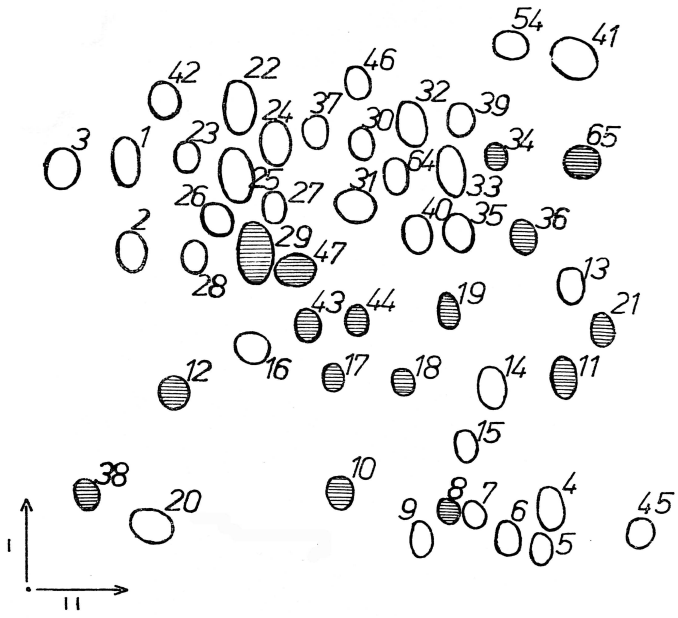

Figure 3. Two-dimensional chromatographic patterns of flavonoids in leaves of *M. alba*. The shaded spots are typical for the species

The spots differed in colour. The yellow ones probably correspond to flavonoids, and the blue colour usually indicates the phenolic acids. However, in these tests the discovered phenolic compounds were not identified.

The dendrite which was drawn basing on the distance coefficient S_{phi} (fig. 4) illustrates the differences between both species: the populations formed two groups divided by the greatest distance.

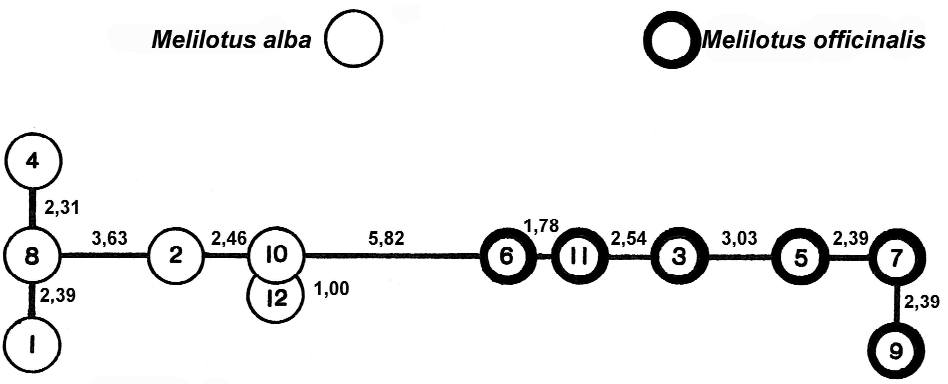

Figure 4. Dendrite constructed on the basis of Leuschner's genetic distances

The variability of two selected populations: *M. alba* (No. 1) from Karłubiec and *M. officinalis* (No. 5) from Tumlin was illustrated with the values of main components (fig. 5).

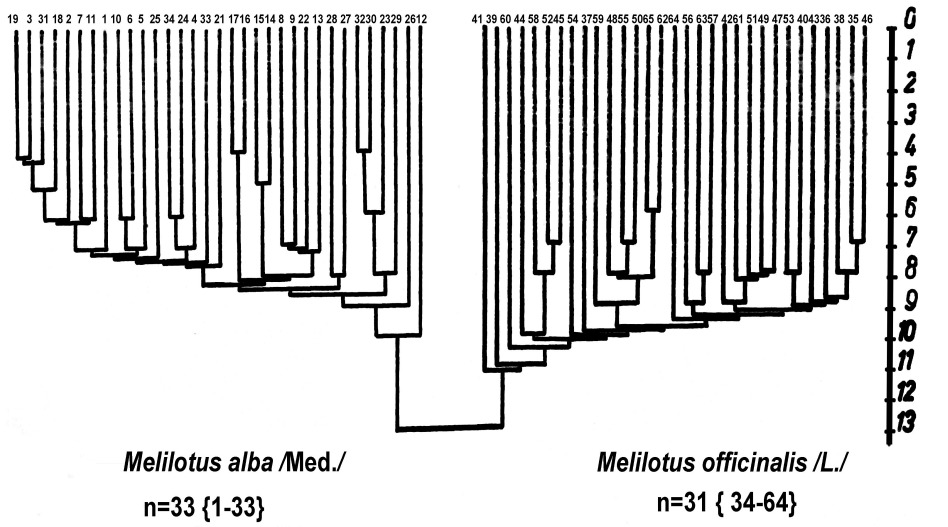

Figure 5. Dendrogram (UPGMA) constructed on the basis of genetic distances, according to Leuschner (1974)

The plants belonging to the population 1 are more grouped, which may indicate less differentiation within population, while the population 5 seems to be more diversified.

The comparison of the same populations with the Unweighted Pair Group Method with Arithmetic mean (UPGMA) confirms the differences between both species (fig. 6).

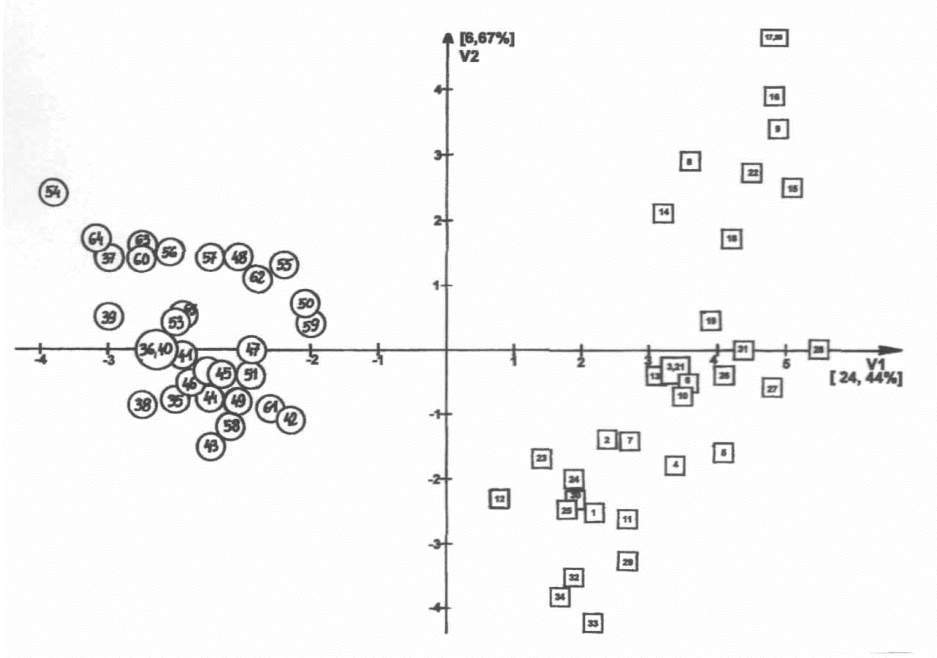

Figure 6. Individuals of *M. alba* and *M. officinalis* spread on the plane of the two Principal Components axes

The results of work must be regarded as preliminary due to the fact that each separate compound (chromatographic spot) may be eluted from the chromatographic plate and subjected to further, more precise analysis, for example using liquid chromatography (HPLC).

From a genetic standpoint, most phenolic compounds are determined by acting of polygenes and only few (e.g. anthocyanins) are governed by single genes [18] but frequently affected, directly or indirectly by multiple allelic series. The most important properties of phenolic compounds are as follows:

1) they give specificity of species, therefore newly discovered phenols are named after the plant in which the new compound was found, e.g. faseolin in *Phaseolus vulgaris*, pisatin in *Pisum sativum* [24].
2) they have regulatory properties:
   – in the process of plant growth through interactions with one or more classes of plant hormones;

- they have a direct nonspecific influence on many indirect biochemical transformations through the inhibition of ATP synthesis in mitochondria [25];
- they affect the transportation of auxins [26];
- the phenolic compounds occurring in chloroplasts protect them against the harmful effects of UV radiation [11];
- they are included in a lipid layer of pistil stigma and therefore are involved in the control of pollen germination characteristic for a particular species and enable the growth of a polen tube [27];
- they are a part of the defense mechanism of the plant;
- they play an ecological role as chemical signals in mycorrhiza and symbiosis phenomena, as well as they act as attractants or insect repellents;
- in ecosystems, a significant increase in concentrations of phenolic compounds in their producers may lead to a drastic decline of consumers [28].

Both *Melilotus* species differ clearly with regard to morphology. Thus, our chromatographic study did not serve as the confirmation of taxonomic differences, but it was done to show a genetically conditioned potential of each of them to produce various phenolic compounds. On the basis of phenolic compounds, the so-called "chemical varieties" in *Anthyllis vulneraria* were described [29], they were also helpful in indication of presumed parental forms, e.g. in the genus *Tilia* [30], or to assess the relatedness of ornamental plants [31]. It is possible that the phenolics typical for *M. officinalis* decide on its medicinal properties. The presence of the same compounds in both species confirms close relationship between them. It was proved earlier on the basis of numeric characteristics [32].

The dendrite drawn on the basis of similarity coefficients shows visible hiatus and divides the tested populations into two groups. These results correspond closely with earlier investigations on enzyme variability of the same *M. officinalis* populations [33]. Therefore, they additionally confirm the biochemical differences among the geographically isolated populations.

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SKŁAD ZWIĄZKÓW FENOLOWYCH W NOSTRZYKU LEKARSKIM (*MELILOTUS OFFICINALIS*) W PORÓWNANIU Z NOSTRZYKIEM BIAŁYM (*MELILOTUS ALBA*) OKREŚLONA METODĄ DWUKIERUNKOWEJ CHROMATOGRAFII CIENKOWARSTWOWEJ (2D TLC) I JEGO ZNACZENIE TAKSONOMICZNE

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S t r e s z c z e n i e

Dwa blisko spokrewnione gatunki: *Melilotus alba* i *M. officinalis* porównano pod względem obecności związków fenolowych. Część z nich okazała się wspólna dla obu gatunków. Biorąc pod uwagę, że związki fenolowe są ściśle kontrolowane genetycznie, a ich skład w roślinie nie podlega wpływom środowiska, obecność plam charakterystycznych dla każdego z gatunków potwierdza ich odrębność taksonomiczną.

Słowa kluczowe: *Melilotus officinalis, Melilotus alba*, *zmienność genetyczna*, *związki fenolowe*