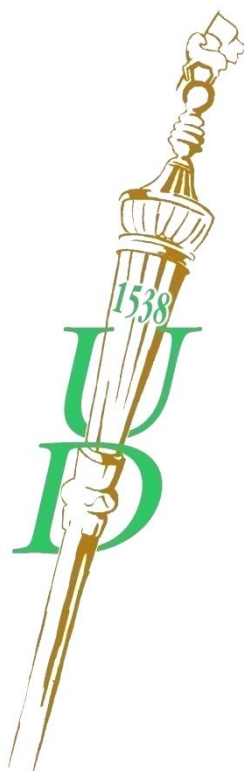


THESIS OF DOCTORAL (PH.D) DISSERTATION

HORTICULTURAL USES OF SPECIAL BOTANICAL VARIETIES (*FORMS OF LUSUS*) OF WOODLAND SAGE (*SALVIA NEMOROSA* L.)

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1. INTRODUCTION AND AIMS OF STUDY

1.1. The importance of theme

Today, I clearly see that *Dr. Zoltán Kováts's* (1924-2010), the former famous external expert of our department, the honorary professor of the University of Debrecen, investigatory thoughts on future was proved, as he said: the "*Species of Salvia genus are able to act interdisciplinary (botany, genetics, breeding, biotechnology) leading to new discoveries, which cannot be planned in advance.*"(KOVÁTS, 2009).

An expedition launched in 2009 to search and collect the *lusus* forms of woodlands sage discovered in Zoltán Kováts's childhood. During my PhD research, divided into eleven research sub-areas, using this genetic material to find the answer that *Salvia nemorosa* what kind of future value has in several areas of application not discussed before, from the horticulture through bio-industry to the aspect of forage industry.

1.2. Aims

- Searching for natural woodland sage populations located in Hungary.
- Creation of an *ex situ* gene bank at a new venue using new, „non-destructive” cloning method.
- Characterization, botanical description of the valuable varieties, as well as study of their hereditary characteristics, reproductive biology and flowering possibilities.
- Setting and evaluation of seed biological experiments.
- Creation of an *in vitro* gene bank by grafting sterile shoot tips of the valuable varieties and maintaining them in sterile medium.
- Biochemical examination of *Salvia nemorosa* using green parts of the plant, inflorescence and crop.
- DNA analysis of valuable *Salvia nemorosa* varieties.
- Recording and evaluation of the weed coverage rate of woodland sage (*Salvia nemorosa* L.).
- Determination of biomass production of woodland sage (*Salvia nemorosa* L.).

2. MATERIAL AND METHOD

2.1. Location of the experiment, geographical conditions

2.1.1. The original habitat of *Salvia nemorosa* varieties

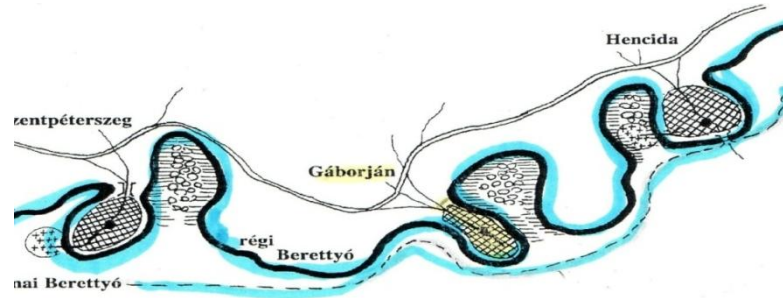


Figure 1. Sites of *Salvia nemorosa* color varieties (Gáborján, 2009).
(KOVÁTS, 2010)

2.1.2. Location of the field experiment, conditions

Place of the field experiment located in University of Debrecen, Centre of Agricultural Sciences, Plants of Future Biomass Demonstration Garden. The soil is very heterogeneous, because it was previously filled.

2.1.3. Location of the laboratory experiment, facilities

The laboratory experiments were carried out in Ottó Orsós Plant Biotechnology Laboratories located in Life Science Centre opened in 2005. In 2013, the experiments were continued under a new name and location (University of Debrecen, Faculty of Agricultural and Food Sciences and Environmental Management, Department of Agricultural Botany, Plant Physiology and Biotechnology).

2.2. Plant material of the experiment

The place of the field experiment located at University of Debrecen, Centre of Agricultural Sciences, Plants of Future Biomass Demonstration Garden. The mother plants originated from the flood zone of Berettyó river. Applying this mother plants and clones of the varieties, developing by splitting, were further propagated under field conditions (VÁRADI, 2013).

2.3. Introduction of the field experimental parcel

Because of the additional water supplementing irrigation (flooding) of the area in summer time, we transferred the plants in "cassette". Five varieties were placed into one cassette, all together 25 plants of different colors were the plant material of the experiment. The plants were spaced 50 x50 cm distance from each other.

2.4. Methods of propagation and recording

2.4.1. Establishment of field population

In 2010, by the guidance of Zoltán Kováts, the members of our department organized an expedition for mapping some natural woodland sage population located in Hungary. The exploratory work continued in the next year at the end of the flowering phenological phase (June). After cultivating the broke cuttings in plots, the mother plants and clones were growing under field conditions (University of Debrecen, Centre of Agricultural Sciences, Plants of Future Biomass Demonstration Garden, Debrecen). In 2013, 12 varieties were picked from the varieties having high shape and color aesthetic value by positive selection and they were individually placed in separate circles in order to prevent seedmixing.

2.4.2. Botanical measurements in the new population

We measured height, habit, length of inflorescence axis, number of inflorescence axis, leaf color of each variety (VÁRADI, 2013). The individual stamps were continuously recorded in the period of flowering and counted the amount of the flowers per plant.

2.4.3. Broke cuttings as a new method of vegetative propagation of woodland sage (*Salvia nemorosa* L.) species

We examined the root developing percentage of broke cuttings of the wild population, the different cultivars (*S. n.* 'Violett Königin', *S. n.* 'Blaukönigin', *S. n.* 'Rosakönigin', *S. n.* 'Rosenwein') and varieties found valuable during the experiment.

2.4.4. Reblooming examination of woodland sage varieties

The phytotechnical intervention occurred approx. 10 cm under the inflorescence axis two weeks after the main flowering and the second flowering. During the biological testing the plant reblooming, as well as the date and number of re-blooms was recorded.

2.5. Biological examination of seeds

The difference between the thousand seed weight of versions are an important aspect, therefore the data was evaluated by statistical analysis.

2.5.1. Examination of germination percentage

2.5.1.1. Germinationtest in laboratory

Experiment venue: University of Debrecen, Centre for Agricultural and Applies Economic Sciences, Ottó Orsós Laboratory - Department of Plant Biotechnology. The seeds collected from field planting of *Salvia nemorosa* versions were placed on moist filter paper. In another experiment, the seeds from the field varieties were treated with different temperature waterbath and different duration of cooling.

2.5.1.2. Germinationtest in greenhouse

The venue of the experiment the greenhouse of the University of Debrecen, Centre of Agricultural Sciences, Plants of Future Biomass Demonstration Garden was. The materials of the experiment were the seeds collected from the field population in the middle of June, which were germinated in moist filter paper under natural lighting (VÁRADI, 2013).

2.6. Applied *in vitro* methods

We planned to propagate and maintain (*in vitro*) the *Salvia nemorosa* varieties based on the Italian colleagues. The propagation of the 12 varieties was conducted by

sowing seed in a sterile way (MS medium -MURASHIGE AND SKOOG, 1962) and by grafting the shoot apex.

2.7. Biochemical experimental methods

2.7.1. Determination of total monomeric anthocyanin content (TMAC) of woodland sage inflorescences by pH differential method

The measurement was performed using samples stored at -20°C, under two different pH (pH 1.0 and pH 4.5) and wave lengths (530 and 700 nm) (LEE *et al.*, 2005). The samples were petals characterized by the four basic colors (white, pink, blue, purple) petals (2 g/sample).

2.7.2. Phytochemical examination of *Salvia nemorosa* L.

The determination of the composition and ratio of *Salvia nemorosa* L. essential oils was performed with the method applied by BÖSZÖRMÉNYI (2010). Tree color (white, pink and blue) varieties of *Salvia nemorosa*'s dried leaf was the material of the samples.

2.7.3. Determination of photosynthetic pigment content by spectrophotometry

The determination of the chlorophyll and carotene content was carried out by the method published by PORRA *et al.* (1989), with some modifications. We used the most developed leaves in every case because of the comparability.

2.7.4. Determination of LPC (leaf protein concentrate) and protein fiber content of *Salvia nemorosa* L. by the Bradford method

To the measurement we selected three different color (white, pink, purple) woodland sage varieties and we used the fresh green mass of these plants without roots after flowering. The pressing occurred by GreenStar 3000 twin screw press machine. The most widely used protein determination assay, the Bradford method was applied (I1).

2.7.5. Determination of whey protein content in woodland sage by the Kjeldahl method

During the measurement we used the fresh weight of varieties which selected in case of the Bradford method without roots after flowering. The pressing occurred by Green Star 3000 twin screw press machine. Amaranths and lucernewhey were used as comparison material.

2.7.6. Determination of dry matter content of woodland sage

The comparative analysis of the dry matter contents of inflorescence axis removed from the commercially available four cultivars of woodland sage (*Salvia nemorosa* L. 'Rosakönigin', *S. n.* 'Rosenwein', *S. n.* 'Blaukönigin', *S. n.* 'Violett Königin') and the selected 12 varieties were performed by Alpha REF 113 Brix 0~32 % ATC Portable Refractometer.

2.7.7. Nutritional indicators of *Salvia nemorosa* L. seed

In 2014, the nutritional substances and their values of the seeds originated from the wild population were revealed by the staff of Agricultural Instrumentation Centre, University of Debrecen. The seeds collected after the main flowering and pulverized by coffee grinder. The nutritional composition of the seed was measured by GC (gas chromatography), one-way split method. The GC-FID examinations were made with Agilent 6890N device connected to Agilent flame ionization detector.

2.8. Molecular genetics equipments and methods

2.8.1. Comparison of ploidy level in woodland sage varieties by flow cytometry

After collecting the shoot apices originated from the field population the sample was used on 1 cm² leaf surface. Measurement device: Becton Dickinson FACScan flow cytometer (LISZTES-SZABÓ, 2015). The calculations were conducted according to the database of the Kew Royal Botanic Gardens (I2).

2.9. The role of woodland sage in ecological farming

2.9.1. Weed coverage rate of woodland sage

During the spring of 2014, we carried out the weed mapping with absolute sampling method. Within this, we chose the method based on total counting. The quadrature method was the subunit of the method (SZABÓ, 2014).

2.9.2. *Salvia nemorosa* as bioherbicide

The radishes, onions, pepper and lettuce are plant species belonging to different families; therefore we chose them as a purpose of the analysis. Another direction was to monitorize the germination inhibitory effect of the seed of woodland sage. In this experiment, we evaluated the germination of the stinging cocklebur and the thorn apple.

2.10. Applications such as biomass plant

We examined the possibility of utilization in case of the flowered green mass, as biomass plant. This made possible comparing the habit of the isolated varieties. We cut the plant samples approx. 10 cm above the soil surface and put them into bags separately by varieties, then measured and compared the obtained data.

2.11. Evaluation and analysis of experimental data

The applied statistical method: ROPstat 2.0, the date of the last significant revision: June of 2011 (VARGHA, 2007).

3. RESULTS

3.1. Botanical evaluation of valuable *Salvia nemorosa* varieties (2012-2014)

3.1.1. Evaluation of woodland sage varieties in aspect of horticultural usability

According to the literature, it has extremely broad morphological variability. **Table 1/a. and b.** illustrates the genetic variability of different varieties which was unknown for botanists and gardeners so far.

Table 1/a. Evaluation of different woodland sage varieties in 2011

	SN1	SN2	SN3	SN4	SN5	SN6	SN7	SN8	SN9	SN10	SN11	SN12	SN13	SN14	SN15	SN16
inflorescence axis length	medium	medium	medium	long	medium	short							medium	short	short	medium
inflorescence axis colour	green	purple spots	purple	green	green	green							green	green	green	green
rachised		X		high										X		
inflorescence compact					X	X								X	X	
leaf colour	green	green	green	light green	green	green							light green	light green	light green	green
upper lip colour	blue	light purple	purple	light pink	light blue	purple							white	white	white	light purple
back of upper lip	X		big lip												X	
white spots on upper lip						X										
over lip colour	blue	light purple	purple	light pink	light blue	purple							white	white	white	light purple
back of lower lip	X		big lip										X			
white spots on lower lip					X	X										
epil colour from above	purple	purple	purple	pink	purple	purple							dark green	grayish	light green	green
epil colour from below	purple	purple	purple	pink	purple	purple							green	light green	light green	green
nect colour from above	purple	purple stripe	purple	light purple	light green	purple stripe							light green	grayish	grayish	purple
nect colour from below	purple	purple stripe	purple	pink	green	purple stripe							green	green	green	purple

Table 1/b. Evaluation of different woodland sage varieties in 2011

	SNC17	SNC18	SNC19	SNC20	SNC21	SNC22	SNC23	SNC24	SNC25	SNC26	SNC27	SNC28	SNC29	SNC30	SNC31
inflorescence axis length	short	medium	long	short	medium	short	medium	short	short	short	medium	medium	very short	short	short
inflorescence axis colour	green	grayish	green	green	green	green	green	green	green	green	purple stripe	green	purple stripe	green	green
branched axis								X							
inflorescence compact	X			loosely		X		X		X			X	X	X
rad colour	green	green	green	green	green	green	green	green	green	green	green	green	green	green	green
upper lip colour	white	light pink	pink	pink	light purple	blue	dark blue	light blue	blue	light purple	dark blue	light blue	light purple	light blue	light blue
ack of upper lip					small	X									
white spots on upper lip							X		X		X	X			
lower lip colour	white	light pink	pink	pink	light purple	dark blue	dark blue	light blue	blue	light purple	dark blue	light blue	purple	light blue	light blue
ack of lower lip	X		X							X					
white spots on lower lip					X		X		X		X	X		X	
equal colour from above	green	light purple	purple	light purple	light purple	purple	light purple	purple	light purple	light purple	light purple	purple	purple	purple	purple
equal colour from below	green	purple	purple	purple	purple	purple	light purple	purple	purple	purple	purple	purple	purple	purple	purple
racist colour from above	grayish	light purple	purple	purple	purple	purple	light purple	purple	purple	purple stripe	purple	purple	purple stripe	purple edge	purple
racist colour from below	green	purple	purple	purple	purple	purple	purple	purple	grayish	purple	purple	purple	purple stripe	purple edge	purple

3.1.2. Comparison parameters of woodland sage varieties

Figure 2. shows versions according to the comparison of their height and width.

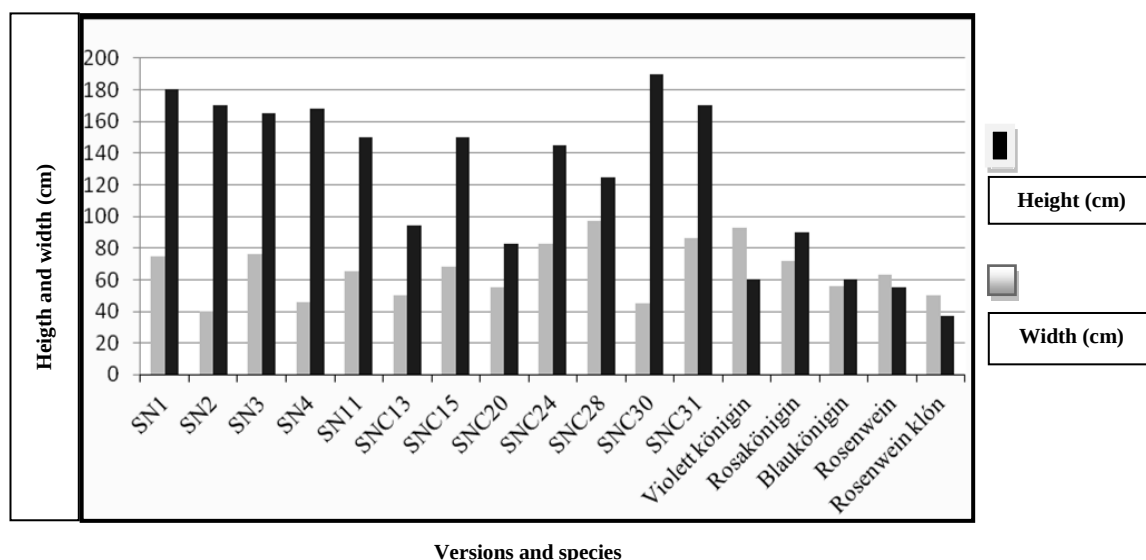


Figure 2. Height and width parameters of *Salvia nemorosa* cultivars and clones in 2014 (cm)

12 valuable woodland sage varieties according to habit:

Erect habit: SN3; SNC13; SNC20

Half-prostrate habit: SN1; SN11; SNC15; SNC24; SNC28; SNC31

Prostrate habit: SN2; SN4; SNC30

In 2014, the shoot developing tendency was measured, which represents the number of stems located on one plant. Variety SNC30 was significant (**Figure 3.**) the stem developing value of clone SNC20 and clone 'Rosenwein' was equal.

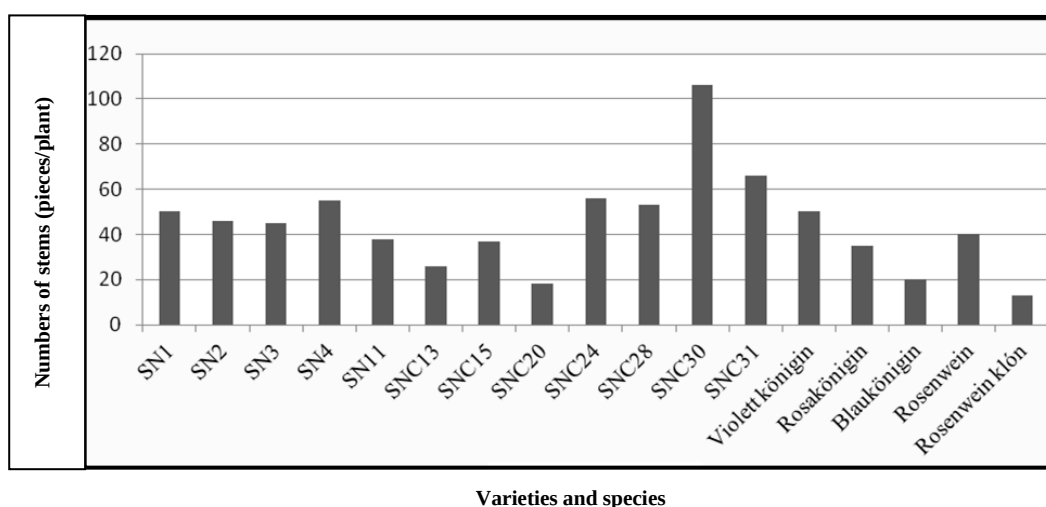


Figure 3. Stem development tendency of woodland sage varieties and cultivars in 2014 (pieces/plant)

Figure 4. shows the differences between the main inflorescence axis length of the varieties and cultivars. The maximum value was measured in case of the 'Violett Königin' and SNC24 variety. Among the ecotypes the smallest main inflorescence axis length was recorded in case of SN4 and SNC31 varieties, among the cultivars the smallest values were measured in the case of 'Blaukönigin' and 'Rosenwein' cultivar.

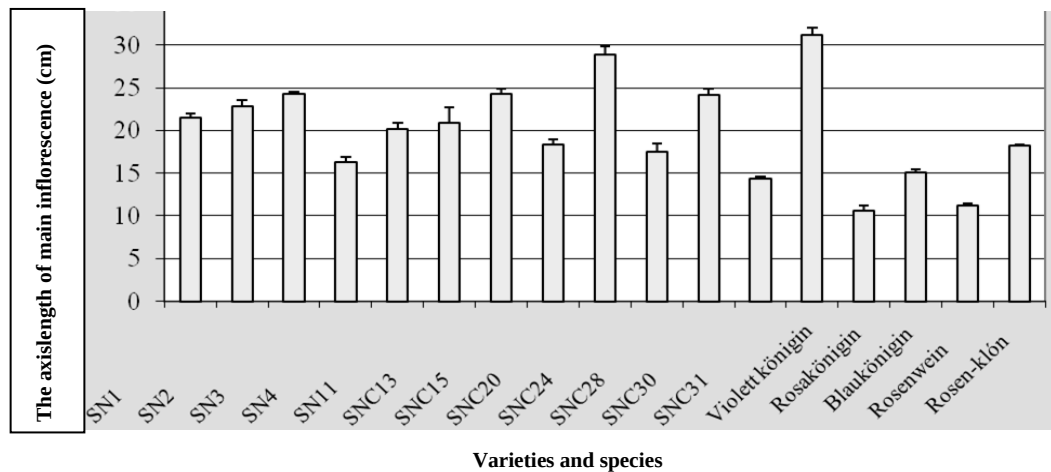


Figure 4. The axis length of main inflorescence in *Salvia nemorosa* cultivars and varieties in 2014 (cm)

Based on the comparison of main inflorescence weight (**Figure 5.**) the SN2 and SN3 varieties showed the highest weight and the main inflorescence of SNC20 was the smallest. The inflorescence weight of clone 'Rosenwein' is appreciable between the cultivars in comparison with the 'Rosenwein' cultivar.

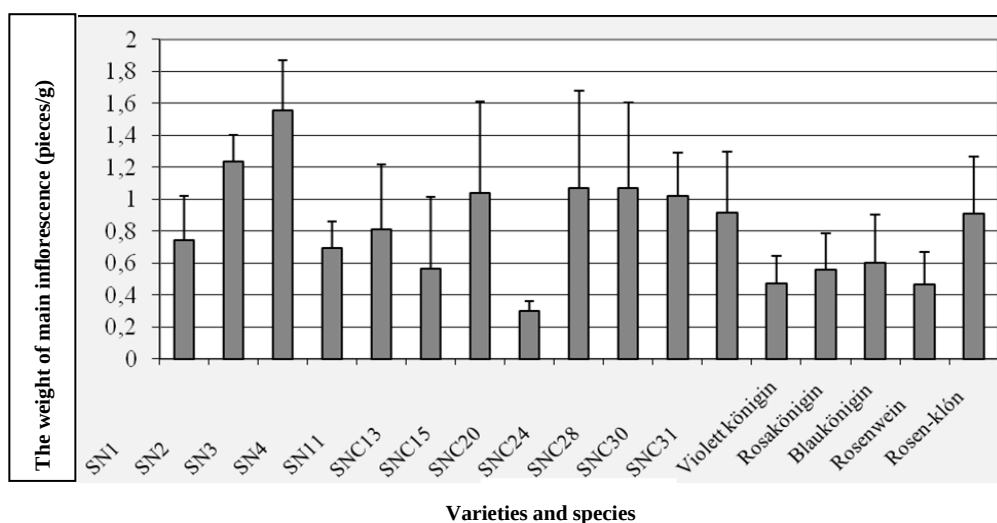


Figure 5. The weight of main inflorescence in *Salvia nemorosa* cultivars and varieties in 2014 (pieces/g)

Based on the number of petals located in inflorescence axis (**Figure 6.**) the value of SN2 and SN3, as well as the SNC31 varieties were the highest, the SNC13 and SNC20 varieties had less petals. Among the cultivars a conspicuous contrast was observed as the 'Rosenwein' has little petal comparing to its own clone.

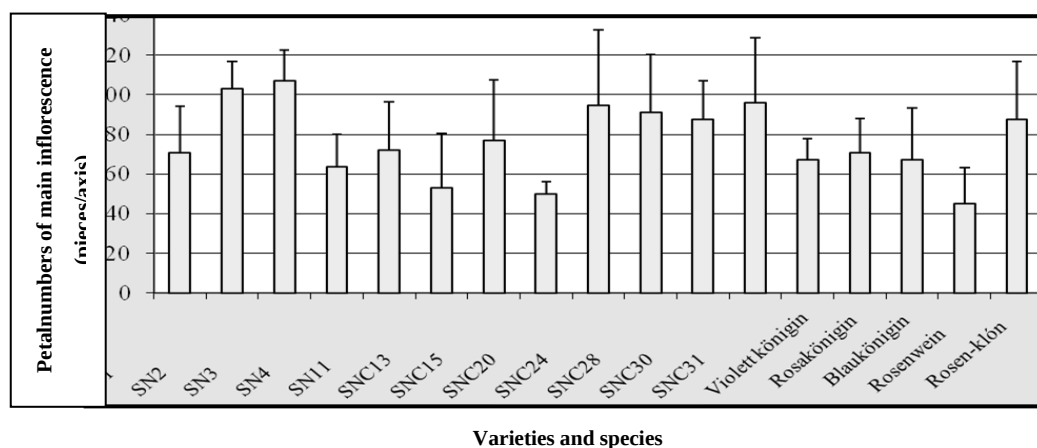


Figure 6. Petalnumbers of main inflorescence of woodland sage in 2014 (pieces/axis)

3.2. Broke cuttings as a new method of vegetative propagation of woodland sage (*Salvia nemorosa* L.) species

In case of the wild population the root developing ratio was 95%, while this value was approx. 30-50% in case of the cultivars. The cuttings of the selected varieties showed a strong deviation between 10-70%. The root developing ratio of clone SN3 and SN4 cuttings was the best, the least of all the SN6 and SNC15.

3.3. Reblooming examination of woodland sage varieties

The inflorescence stems appeared approx. 3 weeks later between basal leaves of the plant in due to combined effect of pruning and applying nutrient solution after flowering (late June) by the purpose of lengthening the flowering period. The next flowering started at in the middle of August and lasted for approx 3 weeks. In 2014 due to the mild wintermonths it was observed the flowering weight was less. The largest masses of flowers were recorded in case of the SNC14, SN1 and SNC23 plants. In the next two years we continued the comparison, but the measurements were only made in case of the 12 varieties selected by positive selection.

3.4. Examination of germination percentage

3.4.1. Germination test in laboratory

According to the germination percentage the seeds of SNC14 germinated the best, the SN2, SNC27 and SNC20 varieties also reached the 40% value. In the following year, the value of SN4, SNC24 and SNC20 was the highest, in case of the selected 12 varieties.

During the different thermal treatments, the maximum 35% germination percentage was achieved by storing in refrigerator (+4°C) for 1 hour, cooling for 10 minutes and using 60 °C waterbath. The lowest germination was observed using one month cooling.

3.4.2. Germination test in greenhouse

In 2011 the experiment set up in 3 replicates showed that the germination percentage of seeds of wild population was 41.11%. In the following year the germination percentage increased to 61.6%.

3.5. Applied *in vitro* methods

After sowing the seeds in a sterile way the germination began on the 4th and 5th days. In case of the varieties the white SNC13 and SNC15, as well as the purple SNC20 seeds did not germinate. 10 weeks after the steriles owing the grafting were carried out (passages).

3.6. Biochemical experimental methods

3.6.1. Determination of total monomeric anthocyanin content (TMAC) of woodland sage inflorescences by pH differential method

According to the statistical analysis the greatest deviation was observed in case of the SNC31 varieties. The highest anthocyanin content was detected in the SN1 variety having dark purple petals.

3.6.2. Phytochemical examination of *Salvia nemorosa* L.

Regarding to the three different color varieties, the highest amount of essential oil was recovered from the white woodland sage, while the blue SNC31 variety contained the most β -caryophyllene. The pink SN4 version has more carvacrol and borneol components than the other two varieties.

3.6.3. Determination of photosynthetic total pigment content by spectrophotometry

From the examined 12 varieties, the SNC 20 had the highest total photosynthetic pigment content.

3.6.4. Determination of LPC (leaf protein concentrate) and protein fibers content of *Salvia nemorosa* L. by the Bradford method

In case of the pink variety (SN3) the determination could not be performed due to the small amount of whey protein content. Comparing the white (SNC15) and purple (SNC28) versions the purple showed the higher value of whey protein content. SNC15 had the highest fiber content among the three color varieties.

3.6.5. Determination of whey protein content in woodland sage by the Kjeldahl method

Table2. shows the percentage of protein content measured in the whey of *Amaranthus sp.*, alfalfa and woodland sage. According to the results the alfalfa's protein content was the highest and the woodland sage had the lowest value among the three species.

Table 2. Comparison of protein contents in different plant species

Sample	<i>Amaranthus sp.</i> whey	Lucerna whey	<i>Salvia nemorosa</i> whey
Protein % (m/m)	1,11	1,26	1,03

The following measured features of woodland sage's and *Amaranthus sp.*'s whey showed that drymatter, vitamin C, iron, zinc and magnesium content of *Salvia*

memorosa are lightly higher. Calcium, potassium, manganese and nickel content of *Salvia memorosa* are far exceeded the values measured in case of *Amaranthus sp.*

3.6.6. Determination of dry matter content of woodland sage

SNC15, SNC30 varieties had highdry matter contents. Also, significant amount of dry matter was measured in case of SN3, SN4, SNC28, SNC31 and 'Rosakönigin', 'Violett Königin' cultivars.

3.6.7. Nutritional indicators of *Salvia memorosa* L. seed

The seed of woodland sage in the highest amount linoleic acid was measured, but it contains significant amount of in the linoleic acid and oleic acidas well (**Figure 7.**). Beside these, palmitic and stearic acid are also constitutes of its nutritional components.

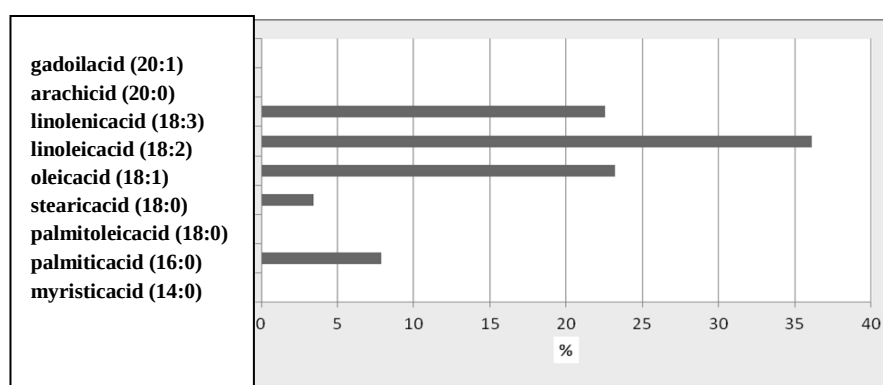


Figure 7. Nutritional ingredients of woodland sage seeds (%)

3.7. Molecular genetics equipments and methods

3.7.1. Comparison of ploidy level in woodland sage varieties by flowcytometry

Based on the nucleus DNA content of daisy we estimated the DNA content of the individuals ranged from 0.87 - 1.37 pg. The mean value of this data corresponds to 1.09 pg value found in the Kew's database. There was no detect able difference in ploidy level between the varieties.

3.8. The role of woodland sage in ecological farming

3.8.1. Weed coverage rate of woodland sage

In the sampling area hoary cress (*Lepidium-draba*) occurred in the largest amount, followed by spiny cocklebur (*Xanthium spinosum*).

3.8.2. *Salvia nemorosa* as bioherbicide

In the set up control germination test the radish and lettuce seeds germinated over 70%. The fresh woodland sage leaves decreased this germination percentage by more than 20%. In case of weed seeds, the amount of the germinated seed of spiny cocklebur was 30% less than the control when in the presence of *Salvia nemorosa* seeds. The control value of thorn apple was 30%, but in the presence of woodland sage seeds none of the thorn apple seeds germinated.

3.9. Applications such as biomassplant

The versions having large amount of greenmass can be used as biomassplant. The SN1, SN2, SN3, SN4, SNC24 and SNC30 varieties belong to this type.

4. NEW SCIENTIFIC RESULTS

- I evaluated the collected woodland sage populations based on morphological parameters. I selected the valuable types having ornamental horticulture value (leaf color, upper and lower lip color, habit, etc.).
- I was the first, who applied the broken cutting method, which is a successfully usable method in case of woodland sage.
- With the aim of lengthening of the flowering period I was carried out phytotechnical intervention combined with fertigation. With this combination I proved that three flowering is possibility instead of two.
- I determined the nutritional parameters of the tested varieties. During the phytochemical examination of *Salvia nemorosa* variations I proved that the white color woodland sage contained the highest quantity of essential oil. I was the first, who examined the protein- and dry matter content, as well the bioactive components of the woodland sage seed. Based on the results the species may be suitable for perspective animal foraging.
- Examining the bioherbicide effect of the *Salvia nemorosa* I proved the germination inhibitory effect of the species.

5. PRACTICAL UTILIZATION POSSIBILITIES OF THE RESULTS

- The collected valuable varieties can be planted with each other or together with other drought-tolerant annuals on public spaces.
- The new propagation method enables homogeneous, strong seedlings regardless of the season.
- As a result of cutting back after flowering and fertigation even three flowering can be achieved in one year.
- The costs can be reduced significantly with the utilization of woodland sage in comparison with annuals planted twice in a year.
- Based on the results of the biochemical tests the *Salvia nemorosa* may be suitable for use as medicinal and fodder plant.
- The results of the germination tests and weed survey of different plant species proved the germination inhibitory effect of *Salvia nemorosa*. In the future, it can be a promising research line as bioherbicide.

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