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Assessing the Influence of the Initial Forms of Melilot on the Quality of Fodder Mass in the Conditions of Northern Kazakhstan

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Abstract

As a result of the hybridization in the conditions of freely-limited cross-pollination on isolated plots, 122 new hybrid forms of the valuable high-yield and high-protein crop of melilot have been obtained. Preliminary assessment of the breeding stock for yield, quality and nutritional value of the forage mass, and the content of coumarin has been performed. The best low-coumarin genotypes that combine several valuable characteristics have been selected and used.

Keywords: *coumarin, hybridization, melilot, protein, selection.*

Introduction

The most common leguminous herbs in Kazakhstan include alfalfa, yellow melilot, and the Volga white sainfoin. However, despite the fact that melilot is a valuable high-yield high-protein crop, which can be used for green mass (both in the pasture and stall keeping), silage raw materials, hay, vitamin-and-grass flour, its chemical composition includes harmful secondary metabolites.

In agriculture and livestock breeding, the toxicity of secondary metabolites, and the corresponding changes of their concentration may have serious consequences for livestock breeding, health, and negative economic consequences. Poisonous plants that have toxic sapogenins and alkaloids, which affect livestock in their composition, draw attention to the problem [1].

The negative feature of melilot chemical composition used as forage plant is the presence of glucoside named coumarin, which is strongly fragrant, bitter and toxic. Vegetable toxins, being products of plants' secondary metabolism, are for the most part not toxic to the plant organisms themselves and for other plants, and, on the contrary, perform a specific protective function. The physiological role of coumarins has not yet been fully determined. It is known that they are involved in the regulation of plant growth, being antagonists of auxins, absorb ultraviolet rays, thus protecting young plants from excessive solar radiation, and protect plants from viral diseases. Coumarins accumulate mainly in the fruit, seeds, flowers, roots, and in particular perform the role of plant hormones that inhibit growth in the periods of seasonal dormancy [2].

At the same time, they are poisonous for most animals. In case of poisoning with melilot, cattle has overall weakness, unsteadiness of gait, tremor, dilated pupils, reduced coagulation and formation of thrombincgen in the blood, bruises appear all across the body; poisoned animals may have cerebral haemorrhage. It should be noted that coumarin can accumulate in the meat of animals; therefore, the standard coumarin level has been determined, which is 0.02% in food products [3]. With that, the norm for humans has been established, which is 0.1 mg/kg of body weight [4]. In this respect, the actual task of melilot breeding is creating varieties for various areas of economic use, which should be characterized by high forage mass productivity and the best quality, and have sustainable seed yield and low coumarin content. The first works in this area were started in the 50s of the XXth century in Canada and in the United States, then in Hungary, Poland and Germany. Several coumarin-free varieties have been obtained there: Kumino, Polara, Denta, Norgold, Gülzev, Acumar and others [5].

During genetic and breeding studies with the melilot crop, American scientists paid special attention to the characteristic of being coumarin-free, and were the first to use inter-generic hybridization in genus *Mellilotus* Mill. The Kumino variety is based on the hybrid line obtained at the Wisconsin University (USA) by crossing white melilot with a low-coumarin form of dentated melilot. Sown genetic hybrids gave quickly dying white sprouts (albinos), which they managed to bring to the flowering stage by inoculations onto plants of yellow melilot. In the second stage of hybridization, the inoculated hybrids were pollinated with the pollen of white clover, which resulted in obtaining several viable seeds. Later, at the forage crops' breeding laboratory of the University of Saskatchewan (Canada), 5 saturating crosses of the hybrids with white melilot were made. After the repeated selection, almost coumarin-free line was obtained. Later, based on this line, as a result of the selection work at the Melfort Agricultural Experiment Station (Saskatchewan province), new varieties of white melilot (Polara [6] and Denta (https://www.southwestfarmpress.com/NEW-LOW-COUMARIN-SWEET-CLOVER-ONLY-FEW-YEARS-AWAY) [7]), and yellow melilot (Norgold) with low coumarin content [8] were obtained.

Other researchers like Stevenson T. M. [9], K W. Smith [10] and Goplen B. P [11] indicate the possibility of using inter-generic hybridization of white and yellow melilot with coumarin-free dentated melilot for creating low-coumarin plants.

The research was aimed at selecting and assessing the source material for creating productive hybrids of melilot with low coumarin content, and ensuring high quality of forage.

Materials And Methods

The experimental work was performed at the base of stationary field experiments laid at the Research and Production Center of Grain Farming n.a. A. I. Barayev in Shortandy district, Akmola region, in the subzone of arid herbaceous mat-grass steppes.

Plants and seeds of varieties and collection samples of wild populations of various species of melilot were used as the objects of the research. The total of 132 samples of melilot was studied. In laying nurseries in 2016, original seeds of ARIPB (The All-Russian Institute of Plant Breeding n.a. N. I. Vavilov) reproduction were used. The collection included a number of low-coumarin varieties of melilot. A series of analyses were performed at the laboratory for determining the quality of fodder mass and the anti-nutritive substances. The crude protein content was determined according to GOST 13496.4-93 «Fodder, mixed fodder and animal feed raw stuff. Methods of nitrogen and crude protein determination with instrument UDK 142 (by the Kjeldahl method)». The fat content was determined according to GOST 13496.15-97 «Fodder, mixed fodder and animal feed raw stuff.

Methods of determining the content of crude fat (ISO 6492:1999. Animal feed raw stuff, determination of fat content)». GOST 26226-95 «Fodder, mixed fodder and animal feed raw stuff. Methods of crude ash determination (ISO 5984:2002 Animal feed raw stuff. Determination of crude ash content)». Crude fiber was determined by the method of Kürschner and Ganek.

The method of determining coumarin is based on gravimetric determination of the total coumarin compounds. To remove organic acids, the chloroform extract was treated with sulfuric acid. After that, coumarin was extracted with chloroform, followed by stripping, drying, and weighing of the residue.

Fodder units and exchange energy were calculated in accordance with the regression equations and the guidelines for assessing quality and nutritional value of fodder. The amount of exchange energy (EE) in MJ/kg of dry matter was determined by the following formula:

$$EE = 13.1 \times (1.0 - CF \times 1.05),$$

where CF was the content of crude fiber, kg/kg of hay dry matter; and 13.1; 1.0; 1.05 were the constant coefficients. The amount of fodder units was determined by the following formula:

$$\text{Fodder units} = EE^2 \times 0.0081,$$

where EE was the exchange energy, MJ/kg of dry matter; and 0.00

81 was the constant coefficient.

RESULTS AND DISCUSSION

In order to engage various species of melilot in crossbreeding of high-yield breeding material, the best forms from the local collection and collections of ARIPB (All-Russian Institute of Plant Breeding n.a. N. I. Vavilov) reproduction were used, the samples were assessed by the yield, quality and nutritional value of the fodder mass, and the content of coumarin.

Table 1 shows the yield rate of green and dry mass of the most promising forms in terms of these characteristics. The maximum formation of green mass was characteristic of yellow melilot - 155.7 to 185.5 kg/ha, with the yield of dry mass between 40.6 and 49.2 hw/ha, the Volga melilot provided the yields in the range between 148.0 and 181.7 hw/ha, and the variation of the productivity index in terms of dry weight was between 43.6 and 46.9 hw/ha.

For involving the highest quality forms in crossing, the quality of fodder mass was studied, and 39 high-protein nutritious ecologically distant forms of melilot from the USA, Hungary, Canada, Ukraine, Belarus, Russia and Kazakhstan were identified (Table 2).

The lowest levels of coumarin were detected in the samples from the ARIPB collection: yellow melilot K-36677 - 0.18%, K-36673 - 0.28%, and K-36675 - 0.33%. Sample K-36673 differed by the highest nutritional and energy value, crude protein content of 19.56%, crude fiber content of 14.26%, exchange energy of 11.14 MJ, and fodder units of 1.005 kg/kg.

In addition, seeds of melilot genotypes were screened for coumarin content. A seed of melilot was placed on a glass plate into a drop of 0.5% alkali solution, and irradiated with UV light for 10-15 minutes.

The seeds with light green fluorescence were rejected; only the seeds that did not have fluorescence in a drop of alkali in accordance with Figure 1 were left for sowing. After treatment, the seeds were rinsed with water and dried.

As a result of screening, 3.1% of seeds of white melilot, 5.1% of seeds of yellow melilot, and 5.2% of seeds of the Volga melilot were rejected, Table 3.

To confirm the low content of coumarin, NMR analysis was performed on spectrometer JNN-ECA Jeol 400 in the engineering laboratory at the KSU n.a. Sh. Ualikhanov (Kokshetau). Extracts of total coumarin of 2 melilot samples were handed over. Extracts of coumarins were analyzed in the deuterated chloroform medium ($CDCl_3$). As a result of NMR spectrometry, spectra of 2 types of melilot extracts ^1H and ^{13}C were obtained in accordance with Figure 2.

According to the literature, it is known that signals of coumarin protons and derivatives should manifest themselves in the area of weak field (6-8 ppm), due to the peculiarities of the matter structure and mutual influence of atoms. Molecules of coumarins are dominated by aromatic fragments, which manifest themselves in the above mentioned region.

In the proton spectrum (^1N) of the studied compounds, very few protons are observed. Their integral intensity is only 1.40 N, which indicates low content or absence of coumarin derivatives in the sample.

In the carbon spectrum (^{13}C), signals of coumarin and its derivatives are observed in about 120-140 ppm. Low intensity of carbon nuclei signals also indicates low content of coumarin in the tested samples.

Table 1. The yield rate of the best samples of melilot involved in crossing as parental forms, on average for 2016-2017

Origin	Yield rate, the total for two hay mowings, hw/ha	
	green mass	dry weight
Yellow melilot (Melilotus officinalis)		
Early ripening Omsk variety	163.0	44.6
K-2440	163.4	45.5
K 2745	165.9	43.4
KD 1699	176.7	49.2
KD 1847	177.7	47.4
KD 1596	161.9	43.7
KD 1819	155.7	40.6
KD 1841	171.6	45.8
KD 1768	165.5	42.2
White melilot (Melilotus albus)		
KD 1889	131.8	33.6
KD 1894	138.6	34.2
KD 1892	144.6	38.4
Volga melilot (Melilotus wolgicus)		
KD 1793	157.1	43.6
IK 2603	161.8	43.7
Variety Akbas	166.0	44.2
KD 1840	148.0	45.2
KD 1690	181.7	47.4
Variety Bars	176.7	46.2
KD 1807	172.5	46.9

Table 2. Characteristics of high-quality genotypes of various species of melilot selected to be used as parental forms in crossing, the harvest of 2016-2017

Crop	Content of coumarin, %	Mass fraction in dry matter, %						
		crude protein	crude fiber	crude ash	crude fat	Nitrogen-free extractive substances	EE MJ	EC, kg/kg
Yellow melilot (Melilotus officinalis)								
Early ripening Omsk variety	1.06	20.73	13.18	10.21	2.18	53.7	11.29	1.030
IK 2440	0.75	16.64	18.68	10.72	2.16	51.80	10.53	0.898
IK 2745	0.94	17.22	18.30	10.16	2.07	52.25	10.58	0.907
KD 1699	1.06	18.54	15.52	9.74	2.11	54.09	10.97	0.970
KD 1847	1.13	16.20	16.58	9.16	2.16	55.90	10.82	0.950
KD 1596	1.05	17.37	18.50	10.74	2.22	51.17	10.56	0.900
KD 1819	1.01	20.00	18.66	9.62	2.15	49.57	10.53	0.899
KD 1841	0.92	19.56	16.96	9.95	2.12	51.41	10.77	0.939
KD 1768	0.82	18.54	18.40	9.66	2.02	51.38	10.57	0.905
White melilot (Melilotus albus)								
K-2793	0.83	13.43	20.58	7.43	2.00	56.56	10.27	0.854
IK 2797	0.90	14.60	17.96	7.77	1.99	57.68	10.63	0.915
IK 2623	0.89	18.25	20.34	9.96	1.91	49.54	10.30	0.860
KD 1892	0.76	19.56	23.94	8.41	1.90	46.19	9.81	0.779
KD 1891	0.92	17.22	21.88	9.84	1.92	49.14	10.09	0.825
KD 1894	0.62	16.49	23.08	8.85	2.03	49.55	9.93	0.798
KD 1889	0.58	19.41	23.26	10.12	2.23	44.98	9.90	0.794
Volga melilot (Melilotus wolgicus)								
KD 1793	0.79	18.68	18.20	8.60	2.03	52.49	10.60	0.910
IK 2603	0.94	20.80	18.50	9.11	2.15	49.44	10.56	0.902
KD 1828	1.13	16.93	16.74	9.78	2.14	54.41	10.80	0.940
Variety Akbas	0.86	19.41	20.52	8.89	2.15	49.03	10.28	0.856
KD 1687	1.00	19.56	19.62	9.11	2.04	49.67	10.40	0.876
KD 1840	0.97	20.14	18.94	9.36	2.11	49.45	10.49	0.892
KD 1690	0.86	21.17	17.50	10.14	2.29	48.90	10.69	0.926
IK 2775	0.82	18.98	15.64	9.85	2.13	53.40	10.95	0.971
Variety Bars	0.92	20.44	19.00	9.23	2.06	49.27	10.49	0.891
KD 1807	0.91	18.83	19.38	9.15	2.00	50.64	10.43	0.882
ARIPB collection								
Yellow melilot (Melilotus officinalis)								
K-36673	0.28	19.56	14.26	9.83	2.12	54.23	11.14	1.005
K - 36674	0.98	19.56	14.64	9.47	2.19	54.14	11.09	0.996
K - 36675	0.33	18.98	15.06	9.75	2.18	54.03	11.03	0.985
K - 36676	0.70	17.81	15.72	9.25	2.18	55.04	10.94	0.969
K - 36677	0.18	18.39	16.42	8.36	2.15	54.68	10.84	0.952
K - 9036679	0.51	19.56	11.96	9.58	2.14	56.76	11.45	1.063
K - 36680	0.74	18.54	13.38	9.50	2.18	56.40	11.26	1.027
K - 38925	0.83	17.66	15.14	10.28	2.04	54.88	11.02	0.983
K - 30500	0.66	19.85	12.96	10.96	2.28	55.95	11.32	1.039
K-30502	0.70	19.56	11.86	11.15	2.20	55.23	11.47	1.065
White melilot (Melilotus albus)								
K-30495	0.46	17.81	14.60	11.09	2.05	57.80	11.09	0.997
K - 24894	0.54	19.56	16.82	7.73	2.16	53.73	10.78	0.941
K-24997	0.55	15.33	16.58	8.27	2.26	57.56	10.83	0.950

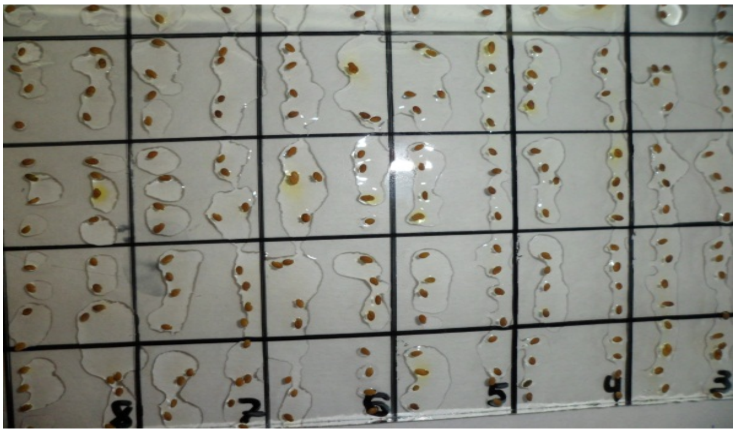

Figure 1. Fluorescence of melilot seeds containing coumarin

Table 3. Coumarin content in the samples of melilot seeds, data for 2015-2016

Crop	Number of seeds before treatment with ultraviolet light, pcs	Rejected seeds	
		pcs	%
Melilotus officinalis	8,042	414	5.1
Melilotus albus	7,061	213	3.1
Melilotus wolgicus	3,735	194	5.2

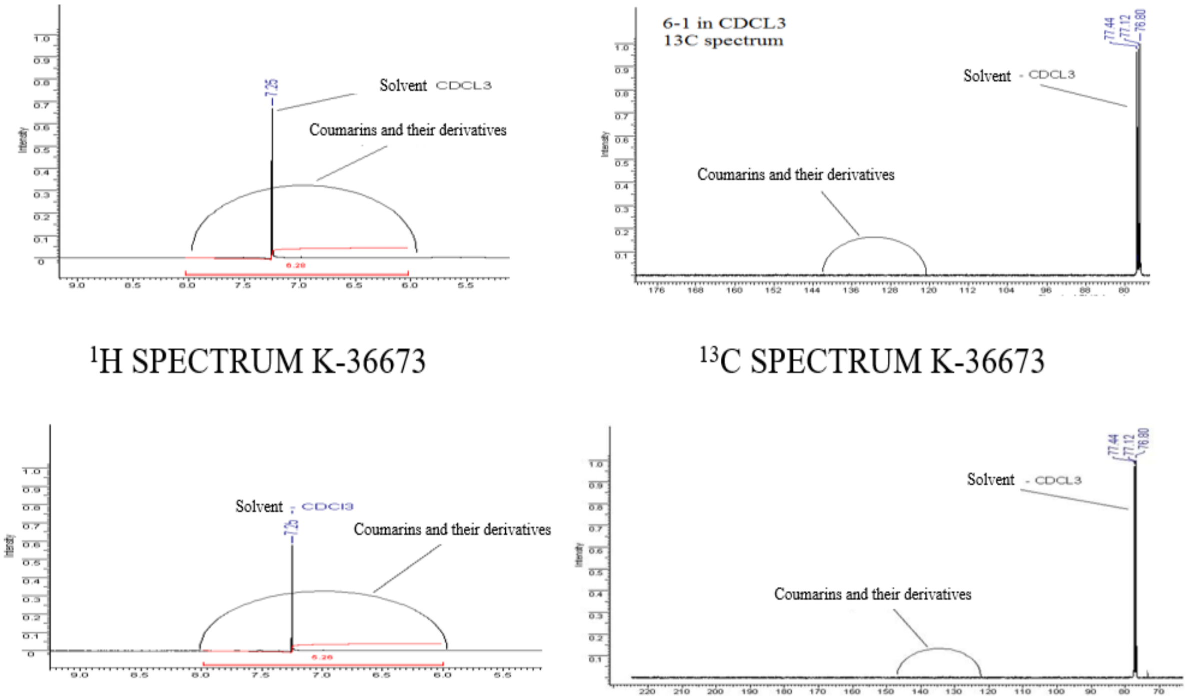

Figure 2. NMR spectra of melilot samples

CONCLUSIONS

With the aim of creating high-quality melilot breeding material with low content coumarin for crossing, hybridization nurseries were laid in 2015-2016 in the conditions of spatial isolation in the field: Volga melilot (13 crossing combinations), white melilot (14 crossing combinations), and yellow melilot (21 crossing combinations). Forage mass of the parent forms of melilot in terms of the yield, nutrient and energy values were preliminarily assessed, and coumarin content was determined. Low content of coumarin in two of the most valuable samples - K-36673 and K-36675 - was proven by NMR spectroscopy on the JNN-ECA Jeol 400 device.

Over three years, during the hybridization in the conditions of freely-limited cross-pollination, 122 hybrid forms of melilot were obtained for further studies.

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