Some results of the study of alfalfa and meadow clover samples created by biotechnology methods

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Abstract. The conditions for creating alfalfa samples MN-2 (from the Selena variety) and P-67 (from the Lugovaya variety) with increased resistance to Fusarium pathogens using the method of gamete selection are described. The method is based on the selection of gametes in vivo under the influence of a selective factor (culture filtrate) introduced into the generative organs at the early stages of their development using vacuum infiltration. The method was developed in the biotechnology department of the V.N. V.R. Williams. The accessions are included in the breeding process and are currently the accessory varieties. In order to identify differences at the genetic level between the created varieties and the original forms, a comparative analysis was carried out using 2 types of molecular markers based on the PCR method. DNA polymorphism was detected using SRAP primers, which may indicate a change in the structure of genes associated with economic and biological traits. Research is ongoing. When growing under the conditions of a selection and greenhouse complex of acid-tolerant plants of meadow clover, created using cellular technologies in vitro, the infestation by powdery mildew of 120 acid-tolerant plants was studied. No signs of damage were found in 10% of plants. The largest number of affected plants (36.7%) had a score of 3. It was found that a high infestation with powdery mildew (scores 4 and 5) significantly reduces the number of inflorescences in the bush to 83.4 and 75.7%, respectively. Therefore, in further studies to create a population of acid-tolerant plants, the genotypes of meadow clover were used without signs and with a low score of powdery mildew damage.

Red clover and alfalfa play an important role in providing livestock feed. The most important role in realizing the potential of these perennial legume forage crops belongs to the variety. The selection of these crops is aimed at increasing resistance to unfavorable soil and climatic conditions of the environment and increasing the yield of forage mass and seeds. [1] The selection of alfalfa for high productivity, stable over the years, is associated with an indicator of resistance to the most common and harmful diseases. One of these diseases is fusarium (pathogens are fungi of the genus Fusarium Lk., More than 10 species). For the development of disease-resistant varieties, it is important to create a promising initial breeding material with increased resistance. One of the methods for creating such a material is the method of gamete selection [2]. In the biotechnology department of the V.N. VR Williams developed a method for increasing the resistance of alfalfa plants to Fusarium pathogens [3], based on
the selection of gametes in vivo. The generative organs of the plant are exposed to a solution of the culture filtrate (CF) of the pathogen using vacuum infiltration from the initial stages of development (from the beginning of the formation of male and female archesporia) to the formation of micro- and macrospores. In order to obtain seeds after development from the buds of mature flowers, artificial self-pollination is carried out. As a result of toxicity, the death of germ cells unstable to the selective factor, both microspores and macrospores, occurs. The fusion of selected gametes during fertilization leads to the formation of a sporophyte that is more resistant to the pathogen.

The resulting seeds are germinated on a medium containing the CF of the pathogen. Resistant seedlings are planted in soil and grown to the flowering phase. Then they are subjected to artificial self-pollination. Seeds from each plant are germinated separately on a medium with a culture filtrate of the pathogen. Plants with the highest percentage of surviving seedlings in their seed progeny are preserved as elite, and their seeds are sown on plots of breeding nurseries for field trials for resistance to the pathogen and assessment of the most important economically useful traits.

Varieties of alfalfa variable MN-2 (variety Selena) and P-67 (variety Lugovaya) were used in the work. In order to select gametes resistant to several species of fungi of the genus Fusarium, alfalfa buds of cultivar P-67 were treated with a mixture of CF of three fungal species: isolate No. 418 F. culmorum, No. 26 F. sambucinum, No. 533 F. oxysporum, and cultivar MH-2 EC of five species: (isolate No. 418 F. culmorum, No. 26 F. sambucinum, No. 533 F. oxysporum, No. 20 F.avenaceum, No. 702 F. gibbosum). The elements of the female generative sphere are very sensitive to the action of osmotic shock; therefore, CF was prepared in a solution of 10% sucrose: when processing sample P-67 at a dilution of 1: 1; sample MH-2 - 1: 2 (dilutions are selected experimentally) [4]. As a result of an assessment under the conditions of a breeding nursery of two forms, form No. 2 was selected from the MH-2 variety sample, which exceeded the initial one in resistance to Fusarium pathogens: the prevalence of internal root rot was 16.5% lower, the intensity of external root rot development was 12.5% lower, the intensity of development of internal root rot is 16.4% lower than that of the original form [5]. From the sample P-67, 2 forms were selected, in which the intensity of development of internal root rot is lower by 20-25%, external root rot by 15 and 20% compared to the original sample [4]. The created forms are included in the breeding process as an initial breeding material and are currently specimens.

In order to identify differences at the genetic level between the cultivars that underwent gamete selection and the original forms, a comparative analysis was carried out using 2 types of molecular markers based on the PCR method. The seeds of the studied forms MH-2, P-67 and varieties Selena and Lugovaya were germinated for 7 days in Petri dishes, and then DNA was isolated from a total sample, including 30 seedlings from each sample. DNA extraction was carried out by the method based on SDS lysis buffer [6]. For PCR analysis, 9 pairs of microsatellite (SSR) primers were used: RCS0017; RCS1640; RCS1307; RCS4100; TRSSR16; RCS4926; RCS033; RCS2183; RCS3711 [7.8]. Amplification products of varying intensity were generated from the test set of 5 primers; however, the DNA spectra of the studied samples were practically the same (Figure 1). The lack of information content of this marking technique in the analysis of alfalfa may be due to the origin of the primers originally developed for the microsatellite loci of the meadow clover genome. The material under study was further analyzed using 12 combinations of SRAP primers. SRAP (sequence related amplified polymorphism) is a marker system based on amplification of open reading frames or intron-exon regions of the genome of the organism under study. As a result of genotyping with primers F9-R8, F10-R8, F13-R7 [9], reproducible amplification fragments were obtained, revealing differences in the genome structure of the studied forms of alfalfa. Thus, the initial variety Selena and the sample MN-2 obtained on its basis differ in amplicons 350, 550 and
Figure 1. Electropherogram of cultivar amplification fragments and alfalfa cultivars with SSR primer TPSSR16
1 - grade Selena; 2 - specimen MN-2; 3 - cultivar Lugovaya 67; 4 - sample P-67; M - molecular weight marker (100 bp DNA Ladder, «Bio-Rad», USA)

1100 bp, 630 and 900 bp, 600 and 700 bp with combinations of SRAP primers F9-R8, F10-R8 and F13-R7, respectively. In cultivar P-67, an amplification fragment of 750 bp was found, which distinguishes it from cultivar Lugovaya 67, with a combination of primers F10-R8; at the same time, the 650 bp PCR product detected in cultivar Lugovaya 67 was absent in the DNA profile of cultivar P-67 with a combination of primers F13-R7 (Figure 2).

Figure 2. Electropherogram of amplification fragments of cultivars and alfalfa cultivars with SRAP primer combinations: F9-R8; F10-R8; F13-R7. 1 - grade Selena; 2 - specimen MN-2; 3 - cultivar Lugovaya 67; 4 - sample P-67; M - molecular weight marker (1 kb DNA Ladder, Eurogen, Russia)

The presence of DNA polymorphism in experimental alfalfa samples may indicate changes in the structure of genes associated with economic and biological traits, since SRAP markers are developed based on information about the nucleotide sequence in the coding regions of the genome. Research will continue with an expanded primer sample for the possibility of identifying DNA markers of genes associated with the resistance of the studied material to fusarium.

One of the important stages of obtaining the initial breeding material by in vitro biotechnology methods is the study of the created regenerant plants under the conditions of the breeding and greenhouse complex. In the experiments carried out, it was found that regenerated plants obtained under controlled conditions of climatic chambers are especially sensitive to greenhouse conditions: high or low temperatures, not always sufficient illumination, air humidity. All this often contributes to the development of pathogenic microflora, in particular powdery mildew. In this regard, the influence of the degree of infestation with powdery mildew on the assessment of acid-tolerant plants of meadow clover, created in vitro by our method, was studied [10]. Morpho-biological characteristics of plants are
important indicators of acid tolerance: height, number of shoots and inflorescences. The counts were carried out in the phase of maturation of the heads before mowing during the period of the greatest development of powdery mildew. Depending on the genotype, the degree of pathogenic damage was different. The assessment of plant infestation was carried out according to the guidelines for the selection of perennial grasses (1985) [11]. It was found that 10% of meadow clover plants had no signs of powdery mildew damage (Table 1).

Table 1. Evaluation of the degree of damage by powdery mildew (Erysiphe trifolii) to meadow clover plants.

| % of the affected surface | Score | Number of plants | % |
|---------------------------|-------|------------------|---|
| to 10                     | 0-1   | 12               | 10,0 |
| from 10 to 25             | 2     | 16               | 13,3 |
| 26-50                     | 3     | 44               | 36,7 |
| 51-75                     | 4     | 18               | 15,0 |
| Over 75                   | 5     | 30               | 25,0 |

In other plants, as they developed from the stage of rosette, stemming, budding, flowering to maturation of the heads, a white cobweb, and then a powdery bloom, consisting of mycelium and conidial sporulation, developed on the leaves and stems. With weak development (point 2), small and medium-sized chlorotic spots were formed on the leaves of the lower layer of 13.3% of plants. In 36.7% of plants (point 3), by the beginning of the maturation of the heads, 25-50% of the leaf surface of the lower and middle tiers had spots of medium and large size, the mycelium on which was compacted, and the sporulation was abundant. In 40% of plants (scores 4 and 5), from 50 to 75% or more, the surface of leaves and internodes of the lower, middle and upper tier was covered with a powdery bloom, the resulting spots merged and the leaves became chlorotic, brown and died. In a comparative study of acid-tolerant meadow clover plants with different scores of damage

Table 2. Comparative study of acid-tolerant meadow clover plants with varying degrees of powdery mildew damage (Erysiphe trifolii).

| Defeat score | Number of plants, pcs. | Высота | Quantity | % | pcs. | % |
|--------------|------------------------|--------|----------|---|------|---|
|              |                        | cm     | stems    | % | inflorescences | pcs. | % |
| 0-1          | 12                     | 122,6  | 100      | 11,2 | 100 | 141,1 | 100 |
| 2            | 16                     | 122,1  | 99,6     | 11,0 | 98,2 | 138,6 | 98,2 |
| 3            | 44                     | 122,0  | 99,5     | 10,8 | 96,4 | 132,6 | 94,0 |
| 4            | 18                     | 121,0  | 98,7     | 10,5 | 93,8 | 117,7 | 83,4 |
| 5            | 30                     | 110,4  | 90,0     | 10,4 | 92,9 | 106,8 | 75,7 |

powdery mildew and plants without signs of damage, it was found that plants with points of damage of 1.2 and 3 in height, the number of stems and inflorescences do not differ significantly from plants with a point of 0 (table 2). Whereas plants with points 4 and 5 formed 16.6% and 24.3% fewer inflorescences, which ultimately leads to a decrease in seed productivity. In addition, meadow clover plants with a pathogenic damage score of 5 were 10.0% lower than plants without signs of damage and had, on average, 7.1% fewer stems.

Thus, a high pathogen infection significantly affects the decrease in the number of formed inflorescences, as well as the height of plants and the number of stems, which leads to both a decrease in seed productivity and the productivity of green mass of acid-tolerant original breeding material of meadow clover. Therefore, in further studies, to create a population of acid-tolerant plants, the genotypes of meadow clover without signs and with a low score of powdery mildew were used.
References

[1] Kosolapov VM, Shamsutdinov ZSh, Ivshin GI et al. 2015 Main species and varieties forage crops Moscow (Nauka) 544 p

[2] Mielyan LG and Balashova NN 1994 Method of pollen breeding of plants for resistance to phytopathogens (for example, tomatoes) Agricultural biology №1

[3] Solozhentsev PD, Solozhentseva LF and Agafodorova MN 2006 Method of increasing resistance of alfalfa plants to fusarium Patent No. 2278508 RF Byul. No. 18

[4] Solozhentsev PD, Solozhentseva LF and Agafodorova MN 2007 Biotechnological techniques creation of forms of fodder crops with increased resistance to root rot Fodder production: ways and solutions Moscow p 352-357

[5] Agafodorova MN, Solozhentsev PD, Shamustakimova AO, Klimenko IA and Solozhentseva LF 2014 Biotechnology methods in alfalfa breeding Current breeding trends and the use of alfalfa in fodder production Moscow p 91-96

[6] Klimenko IA, Kozlov NN, Kostenko SI, Shamustakimova AO and Mavlyutov YM 2020 Identification and certification of forage grasses varieties (meadow clover, alfalfa volatile, sowing and hop-like) based on DNA markers Methodical recommendation Moscow 34 p

[7] Sato S, Isobe S, Asamizu E, Ohmido et al. 2005 Comprehensive structural analysis of the genome of red clover (Trifolium pratense L) DNA Research 12 p 301-364

[8] Kölliker R, Enkerli J and Widmer F 2006 Characterization of novel microsatellite loci for red clover (Trifolium pratense L.) from enriched genomic libraries Mol Ecol Notes 6 50-53

[9] Li G and Quiros C F 2001 Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica Theoretical and Applied Genetics 103 p 455-461

[10] Solodkaya LA, Agafodorova MN and Lapotyshkina LI 2020 In vitro selection method acid-tolerant forms of meadow clover (Trifolium pratense L.) Patent No. 2711781 RF Bulletin No. 3

[11] Smurygin MA, Novoselova AS, Konstantinova AM et al 1985 Guidelines on the selection of perennial grasses Moscow 188 p.