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BIOTECHNOLOGIES AND MOLECULAR METHODS IN VEGETABLE CROP BREEDING (to 95th Anniversary of VNIISSOK)

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Abstract

The resource consumption and costs for an increased agricultural production can be lowered only due to effective breeding for which biotechnologies and molecular genetics provide powerful tools. Among them, tissue cultures, clonal micropropagation, androgenesis, gynogenesis, and genetic transformation are widely used to obtain diversified forms and homozygous constant lines, and to speed up breeding process (J.M. Dunwell, 2010). Androgenesis and gynogenesis involve individual gametoclonal variability, rare recessive alleles, and unique genetic recombinations into breeding (T. Winkelmann et al., 2006). An interspecific hybridization is a way to develop initial breeding material. Due to it, novel traits (for example, resistance to biotic and abiotic stresses) can be transferred from wild species to cultivated crops, and the range of genetic variability expands (R. Hajjar et al., 2007). An interspecific hybridization incompatibility can be overcome by biotechnological methods, too. Molecular markers are helpful for detecting DNA changes and desired genes' introgression from one genotype to another, for germplasm fingerprinting, gene mapping, etc. Marker-assisted selection is powerful facility to maintain germplasm collections, to plan crosses, to predict useful gene combination, and to protect varieties and hybrids authenticity. In the review we summarized the results on biotechnology, molecular genetics and their practical use in All-Russian Research Institute of Vegetable Breeding and Seed Production (Moscow Province). In particular, onion (Izumrudnii, Sigma, Zolotye Kupola, Tseparius), salad (Izumrudnii, Tvorets, Aleks, Korall, Malakhit), and physalis (Lakomka, Desertnii) varieties were recently obtained by distant hybridization. Carrot and wild aubergine are involved into interspecial crossings, and pepper plants as sources of resistance to viral infection are selected. Microclonal protocol has been developed for unlimited in vitro propagation of male sterile cabbage. Clonal micropropagation and in vitro cultivation were used to provide embryo viability under interspecial hybridization. A novel protocol developed for in vitro cell cultivation made it possible to create double haploids in carrot and pepper. Due to optimized conditions, double haploids in rape, Chinese cabbage, broccoli and white cabbage were selected. Double haploids of carrots, onion, cucumber, pumpkin, sugar beet, etc., were derived from in vitro cultivated seed-bud without pollination. In this, the ISSR, IRAP, AFLP и SSR markers are involved to assess genome variability and genotyping in vegetable crops.

Keywords: interspecific hybridization, clonal micropropagation, androgenesis, gynogenesis, molecular markers.

The Gribovskaya Vegetable Breeding Research Station founded in 1920 was transformed into the All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops (VNIISSOK) in 1971. Hear, within 95 years, consistent formation of vegetable breeding on a scientific basis was in process in close cooperation with the development of life sciences in the world. At the beginning of the last century, N.I. Vavilov noted in his writings that the selection as a science is a synthesis of the data of many disciplines and linked to genetics, systematics, embryology, cytology, ecology, biochemistry, physiology, and technology [1]. At the Gribovskaya station, in 10 years after its foundation, new theoretical laboratories were established: the Laboratory of Physiology and Bio-

chemistry in 1931, and the studies of plant resistance to disease started at the same time, Laboratory of Cytology in 1933, Laboratory of Plant Protection in 1943, Laboratory of Genetics and Cytology, on the basis of the Laboratory of Cytology, in 1972.

The most important method of enriching the gene pool of plants is distant hybridization, through which valuable traits are transmitted from wild species to cultured ones. This direction is given much attention in world breeding practice. Over the past 20 years, about 100 beneficial traits have been transferred from 60 wild species to various crop varieties [2]. As a result of the research on distant hybridization started at the Gribovskaya vegetable experimental breeding station in 1930, the first forms of onion interspecific hybrids of *Allium cepa, A. fistulosum, A. vavilovii, A. oschaninii, A. schoenoprasum,* and *A. nutans* were created [3-6]. The varieties of Emerald, Sigma, Golden Domes, Tseparius, highly resistant to peronosporosis and stably high yield were obtained on their basis [7-9]. Interspecific hybridization of the *Lactuca* genus made it possible to isolate the original forms with new morphological characteristics and resistance to *Alternaria*, on the basis of which the lettuce varieties of Emerald, Creator, Alex, Coral, and Malachite were created [10]. The Gourmand and Dessert varieties characterized by resistance to abiotic stressors, by high content of sugar, vitamin C, pectin, and the lack of bitterness have been obtained in VNIISSOK from the interspecific hybridization of tomatillo *Physalis longifolium* (non-alkaloidal small-fruited form) and *Ph. angulata* (low-alkaloidal large-fruited form) [11-12]. In the studies on involving wild aubergine species in breeding for the transfer of resistance to abiotic stressors, interspecific hybrids of *Solanum melongena* L., *S. integrifolium* L. and *S. aethiopicum* L. have been obtained in vitro using the method of embryo culture; the offspring has been assessed by morphological and economically valuable traits, the promising samples have been selected [13, 14].

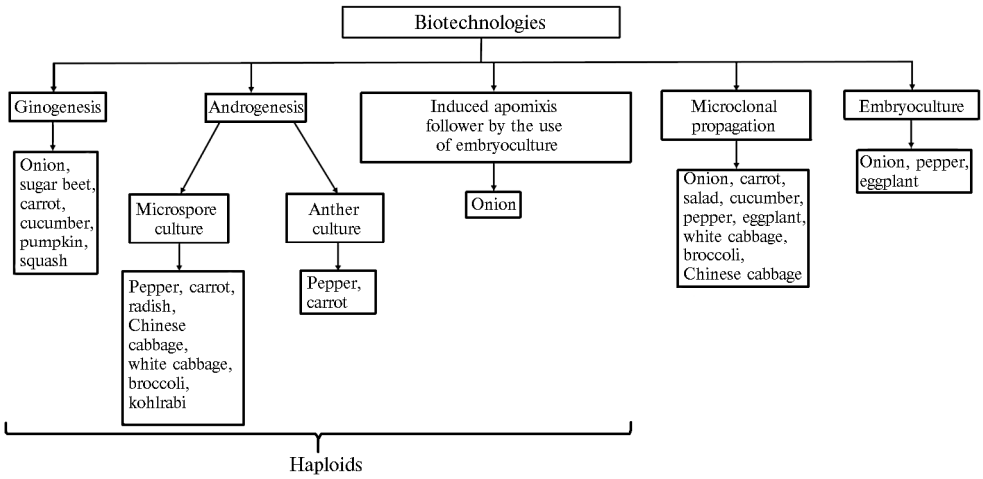
A method of producing pepper source material resistant to viral diseases has been developed using the interspecific hybridization. The *Capsicum annuum* L., *C. frutescens* L. and *C. chinense* L. species have been involved in hybridization. In this, both the classical techniques of breeding and evaluation of the resulting material, and biotechnological techniques (e.g., in vitro culture of isolated embryos to overcome the incompatibility) and modern molecular genetic approaches (i.e., molecular control for R-resistance genes in wild species, and interspecific hybrids using the RGA-marking) were applied. As the result, the pepper, lines resistant to the tomato spotted wilt virus (TSWV) were obtained: L-(Health × *C. frutescens*); L-(Health × *C. chinense*); L-[Chimes × (*C. annuum* × *C. frutescens*)]; L-(*C. annuum* × *C. chinense*); Л-[*C. annuum* × *C. frutescens*) × Health] [15] (the research was supported by the GK № 1282/13 grant from the Ministry of Agriculture ofthe Russian Federation). Studies on the interspecific hybridization of carrots are being performed in VNIISSOK within the past 20 years. Among the hybrid offspring of the combinations of crosses *Daucus carota* × *D. hispidifolius*, *D. carota* × *D. gi-ngidium*, *D. carota* × *D. carota* ssp. *libanotifolia*, etc. the forms with a new combination of high resistance to *Alternaria*, intense orange root-crops and other features of cultural carrots have been selected [10].

The development and evaluation of interspecific hybrids is not impossible without cytogenetic studies, which are conducted in VNIISSOK with the involvement of high-performance methods of hybrid form diagnostic (genomic in situ hybridization, GISH; fluorescence in situ hybridization — FISH) [16, 17].

One of the most important purposes of today's selection is the fast achievement of constancy of breeding material, which is especially important when creating heterosis hybrids that require homozygous lines with high combin-

ing ability. Usually, these lines are obtained with prolonged inbreeding (5-10 generations), but modern biotechnological approaches can almost twice reduce this process. Among the methods of cell technology, the most demanded ones are clonal micropropagation, androgenesis, gynogenesis, and genetic transformation that are widely used in agricultural programs to obtain diversified forms, create constant lines with the specified characteristics, and to speed up the breeding process [18].

The methods of androgenesis and gynogenesis can realize the individual gametoclonal variability in individual plants, detect rare recessive alleles, and create unique forms. Clonal micropropagation subject to high rates of reproduction and preservation of genetic stability can be included in the selection process and occupies an important place in the world crop practice [19]. In the breeding of vegetables it is especially important to maintain the male-sterile or self-incompatibility plants.



Biotechnological methods used in the creation of agricultural varieties in the All-Russian Research Institute of Breeding and Seed Production of Vegetable Crops (Moscow Region)

The in vitro tissue and cell culture techniques (Fig.) have been actively developing in VNIISSOK since the end of the 1980s, the start for the research was given by obtaining the virus-free seed garlic in the meristem culture [20]. The use of improved seed garlic increases the yield (70 % maximum growth) and is widely used throughout the world [21]. Virus-free seed stachys and yakon were also obtained through meristem culture with heat treatment and clonal micropropagation using antibiotics [22, 23].

In the Institute the range of vegetable crops under biotechnological research increased over time. The methods of cell technology were improved. Thus, cauliflower clonal micropropagation using perifloral meristem has been optimized [24]. In world practice, the first study on clonal micropropagation of cabbage crops started at the beginning of the 1970s, however, the achievement of a high rate reproduction in vitro failed. Effective protocol for clonal micropropagation of Chinese cabbage, broccoli and rape were developed in the last decade only [25-27]. Microclonal protocol has been developed for unlimited in vitro propagation of male sterile cabbage in the Laboratory of Biotechnology of VNIISSOK [28] with support by GK № 566/13 grant from the Ministry of Agriculture of the Russian Federation. Clonal micropropagation may become the basis for many cell technologies. Thus, in aubergine and pepper, our clonal micropropagation technology [29, 30] was used for embryo cultivation to provide embryo viability under interspecies hybridization [31].

Producing doubled haploids (DH-technology) makes it possible to

quickly create homozygous lines and speed up the breeding process. By 2010, about 300 varieties have been produced in the world using the DH-method [32]. However, this approach was less effective in vegetable crops, so now they have become the objects for the experiments on the induction of haploid embryogenesis [18, 33, 34]. First doubled haploids in carrot and cabbage in anther culture were obtained in the Laboratory of Biotechnology of VNIISSOK in the early 1990s [35, 36]. The cytological study of embryogenesis in the anther culture of carrot has identified the regularities of embryoid formation of microspores, and the change in ploidy has been shown to start in the early stages of primary embryoid development [37]. Later, cultivation of carrot anthers was optimized, doubled haploid variety samples of different origin were produced (NIIOH 336, Vitamin, Moscow Winter A-515, Losinoostrovskaya 13, Leander, Shantane 2461, Nape, Rondo, F_1 hybrids Karatan and Calisto, etc. The obtained regenerated plants possess gametoclonal variation which has been confirmed by morphological and molecular genetic analysis [38].

Currently, the methods for producing doubled haploids in microspore culture for a variety of vegetables are being improved in the Laboratory of Biotechnology. A domestic technique for creating doubled haploid lines of pepper via anther (microspores) culture was developed [39], and doubled haploids of different varieties and interspecies hybrids were obtained based on it [40]. This technique is not inferior to the best foreign methods developed for hot pepper [41, 42]. This research was supported by a grant from the Ministry of Education and Science of the Russian Federation GK № 16.M04.11.0004 and continued with funding from the Ministry of Education of the Russian Federation, a grant GK № 14.M04.12.0013. The first hybrids of sweet pepper (Natalie, Hussars) have been produced with doubled haploid lines.

The methods for obtaining doubled haploids in the microspore culture of vegetable cabbage crops are actively developing abroad [43-48]. The basic protocol for the rape microspore culture was optimized, and doubled haploid lines of Chinese cabbage [49], broccoli line BR 1-1 and white cabbage involved in the creation of heterotic hybrids were produced in the Laboratory of Biotechnology of VNIISSOK.

Despite the numerous attempts to obtain doubled haploids in the carrot anther culture [50-54], the progress in the development of microspore culture technique for these important vegetable crops was achieved just in the recent years [55, 56]. A technique for producing DH-plants in the carrot microspore culture is under development in our Institute, with regenerated plants have been grown.

To produce haploid plants, female gametophytes are also used in breeding. In vitro cultivation of non-pollinated ovaries and ovules makes it possible to produces haploids when anther or microspore cultures do not provide good results. Sometimes this is the only way to isolate the DH-lines (for example, in plants with cytoplasme male sterility) [57]. The developed methods mostly are cultivating non-pollinated ovules in onion [58-60], cucumber [61, 62], red beet [63], pumpkin [64], and zucchini [65]. These studies have been conducted in VNIISSOK over the past 20 years. There are technologies for producing doubled haploids in the culture of non-pollinated ovules for carrot, onion, cucumber, zucchini, squash, and red beet [37, 66-69]. The effectiveness of the doubled haploid cucumber plant technique developed in the Laboratory is a few times superior to the foreign analogues [68], and the study is supported by grant of Russian Foundation for Basic Research 08-04-13513-ofi_ts.

In the Laboratory of Biotechnology, genetic transformation of plants is also performed. In collaboration with the scientists from the branch of the Insti-

tute of Bioorganic Chemistry of the Russian Academy of Sciences (Pushchino), transgenic carrots with the genes of *GUS*, defensin *Rs* and thaumatin II have been obtained, and the thaumatin II gene expression has been identified in the leaves and root-crops, the families of transgenic plants that are resistant to the *Fusarium avenaceum* pathogen have been selected [70].

Over the past decade, physical and functional organization of genomes of many crops has been studies due to the significant advances in molecular genetics. The molecular marking technologies make it possible to monitor the transfer of commercially valuable genes from one organism to another, and allow to accelerate and optimize the breeding process [71]. The systems of molecular marking of vegetable crops began to be developed in VNIISSOK in the 1990s together with the worldwide development of DNA technology. While at the initial stage the RAPD-markers (random amplified polymorphic DNA) only were used in tomato, aubergine, and onions [72-75] for the identification of intra- and interspecies polymorphisms, currently ISSR- (inter-simple sequence repeat), IRAP- (inter-retrotransposon amplified polymorphisms), AFLP- (amplified fragment length polymorphism) and SSR-labeling (simple sequence repeat) are used in VNIISSOK to study the variability of genomes, to genotype varieties (lines), and determine the purity of hybrid offspring in tomato, cabbage, onion, parsley, aubergine, peppers and other vegetables [76-81]. To maintain and improve the collection of varieties, copyright, authenticity verification of high-grade material and seed certification, molecular-genetic passports for pepper varieties have been composed on the basis of multilocus marking [82]. The possibility of using the data of molecular genetic analysis (AFLP and SSR) in the selection of pairs for hybrids with high heterosis effect has been shown, and promising heterotic hybrid combination of sweet pepper with a complex of major economically important traits have been selected [83].

In VNIISSOK, the development and application of DNA markers for vegetable crops are mainly of applied nature and are aimed at identifying genetic donors of economically important traits, one of which is the cytoplasmic male sterility (CMS) widely used to create hybrids on the sterile basis. Using molecular SCAR-markers (sequence characterized amplified region), *coxII* and *atp6* mitochondrial genes responsible for the CMS have been identified in samples of sweet pepper bred in VNIISSOK and in the *Capsicum frutescens* and *C. shinense* interspecies hybrids obtained using the embryo culture [84].

Screening of onion samples from the VNIISSOK collection using PCR markers for the mitochondrial genes of *orfA501* and *cob* made it possible to identify the samples with sterile and fertile cytoplasm, as well as to determine the types of sterile cytoplasm (S- or T-plasma-type) that do not differ phenotypically [85]. The selected samples of onion with a sterile S-plasma type are recommended for obtaining hybrids on the basis of CMS, as the system of male sterility in onions is easier to inheritance and stable in various environmental conditions [86]. Using multiplex PCR, the type of sterility in cabbage, broccoli, Beijing, radish and daikon have been identified, and the primer system allowing selecting the plants with the cytoplasm type of *Ogura, Ogu-NWSUAF, nap, pol, cam,* and *rad* has been matched. A new subtype of sterile cytoplasm of *Ogura* has been found in cabbage based on the *orf138* nucleotide sequencing [87].

Indirect selection based on molecular marking makes it possible to detect the desired alleles and haplotypes in the early stages of plant development, which significantly reduces and simplifies the selection process [88]. The *pun1* gene markers responsible for the hotness of pepper became an example of the practical application of this approach (*C. annuum* L.) [89]. The screening of the segregating population produced as a result of sweet and hot peppers crossing made with the help of this marker made it possible to select the genotypes with the desired com-

bination of alleles of *Pun1/Pun1* at the stage of seedling [90].

Thus, the results of theoretical research on biotechnology and molecular genetics along with traditional methods are used to accelerate the breeding objectives, create qualitatively new varieties and heterosis hybrids of vegetable crops combining high productivity and quality with complex resistance to the most dangerous diseases, pests, and abiotic stressors.

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