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ROLES OF INTERSPECIFIC HYBRIDIZATION AND CYTOGENETIC STUDIES IN SUNFLOWER BREEDING

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SUMMARY

The abundance and diversity of species within the genus *Helianthus* offer numerous and rewarding possibilities to sunflower breeders. All annual species and a large number of perennial species may be crossed to the cultivated sunflower by the conventional hybridization method. On the other side, the divergence and heterogeneity of the genus cause considerable difficulties, such as cross-incompatibility, embryo abortiveness, sterility and reduced fertility in interspecific hybrids. Because of that, methods of somatic hybridization, "*in vitro*" embryo culture, chromosome doubling, *etc*. are frequently used for interspecific crossing. Cytogenetic studies are used for determinations of chromosome number and structure and analyses of meiosis (microsporogenesis) and pollen viability, making it possible to establish phylogenetic relations between wild sunflower species and the cultivated sunflower and enabling the use of the former in sunflower breeding. Cytogenetic studies of the sunflower have evolved from cytology, through cytotaxonomy and classic cytogenetic to cytogenetic-molecular studies. Most intensive progress of cytogenetic studies has been associated with the use of interspecific hybridization in sunflower breeding.

Key words: ***Helianthus* sp., sunflower, interspecific hybridization, cytogenetic studies**

INTRODUCTION

Sunflower breeding has reached a plateau for a number of important agronomic traits. The major limiting factor for further improvements of the genetic potentials for seed yield and oil quality is the susceptibility of the sunflower to a large number of pathogens. Studies in the field of population genetics have shown that the genetic variability of the cultivated sunflower had been drastically narrowed. Molecular data on the origin and development of the cultivated sunflower

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are alarming, indicating that the possibility for further evolution of this economically important crop is limited (Rieseberg and Seiler, 1990). On the other hand, molecular studies have indicated the presence of large variability, *i.e.*, "primitive polymorphism", in both wild species and local populations of sunflower.

The term 'interspecific hybridization' implies the crossing between different species of the same genus. This method is frequently used in plant breeding, especially when variability of a cultivated form (primary gene pool) has been exhausted and it became necessary to search for desirable genes in its wild relatives (secondary and tertiary gene pools). This has been the case with the cultivated sunflower (*Helianthus annuus* var. *macrocarpus* (DC.) Ckll.) which has been crossed with wild sunflowers (*Helianthus* spp.). The classical hybridization method is typically used for that purpose, while "*in vitro*" embryo culture and somatic hybridization are less frequent. Interspecific hybridization is typically used for transferring resistance to disease agents, soil salinity and acidity, and drought as well as for finding new sources of *cms* and Rf genes and the development of new sunflower idiotypes.

The development of sunflower cytogenetics has progressed from cytology, via cytotaxonomy and classical cytogenetics to molecular -cytogenetic studies. The development and application of cytogenetic studies have been associated with the utilization of the germplasm of the genus *Helianthus* for improvement of the genome of the cultivated sunflower.

This paper reviews the systematics and taxonomy of the genus *Helianthus*, its genomic structure and the usefulness of wild *Helianthus* species as a source of desirable genes. Cytogenetic studies on sunflower are reviewed through the analyses of chromosome number (karyotype), meiosis (micro- and macrosporogenesis), pollen viability and cytoplasmic male sterility.

Systematics and taxonomy of the genus *Helianthus*

The sunflower belongs to the genus *Helianthus*, *Asteraceae* family. The genus is large and polymorphic. In the course of the 18^{th} and 19^{th} centuries, a number of authors had described more than 200 species from this genus. Sunflower systematics and taxonomy have been subject to continual changes and amendments. Heiser *et al.* (1969) described 66 species, 48 from North America and 18 from South America. The former group comprises 12 annual and 37 perennial species classified into 3 sections and 7 series. Robinson (1979) reclassified the latter group into a new genus that he named *Helianthopsis*. The North American group of the genus *Helianthus* as defined by Heiser *et al.* (1969) has been reconstructed following analyses of 42 morphological traits (Schilling and Heiser, 1981). Using the biosystematics and cluster methods, the authors classified the 49 species into 4 sections and 6 series. Section *Helianthus* covers 11 annual species including the cultivated sunflower. Section *Agrestis* includes one annual species. Section *Ciliares* includes two series, *Ciliares* and *Pumili*, each containing three perennial species from North America. Section *Corona solis*, *Microcephali*, *Atrorubens* and *Angustifolius*. Detailed descriptions of the species (plant habit, site, geographic distribution, period of flowering, ploidy level, *etc.*) were provided by Heiser *et al.* (1969) and Rogers *et al.* (1982).

Table 1: Infrageneric classification of *Helianthus* (after Schiling and Heiser, 1981)

Section	Series	Species
Helianthus		*H. annuus* L.
		H. Anomalus Blake[a]
		H. argophyllus T.&G.[a]
		H. Bolanderi A.Gray[a]
		H. debilis T.&G.[a]
		H. deserticola Heiser[a]
		H. neglectus Heiser[a]
		H. niveus (Benth.) Brandegee[a]
		H. paradoxus Heiser[a]
		H. petiolaris Nutt.[a]
		H. praecox Engelm.&A.Gray[a]
Agrestis		*H. agrestis* Pollard[a]
Ciliares	Ciliares	*H. arizonenzis* R.Jackson
		H. ciliaris DC.
		H. laciniatus A.Gray
Ciliares	Pumili	*H. cusickii* A.Gray
		H. gracilentus A.Gray
		H. pumilus Nutt.
Divaricati	Corona-solis	*H. californicus* DC.
		H. decapetalus L.
		H divaricatus L.
		H. eggertii Small
		H. giganteus L.
		H. grosseserratus Martens
		H. hirsutus Raf.
		H. maximiliani Schrader
		H. mollis Lam.
		H. nuttallii T.&G.
		H resinosus Small
		H. salicifolius Dietr.
		H. schweinitzii T.&G.
		H. strumosus L.
		H. tuberosus L.
Divaricati	Microcephali	*H. glaucophyllus* Smith
		H. laevigatus T.&G.
		H. microcephalus T.&G.
		H. porteri (A.Gray) Heiser[a]
		H. smithii Heiser
Divaricati	Atrorubentes	*H. atrorubens* L.
		H. occidentalis Riddell
		H. pauciflorus Nutt.
		H. silphioides Nutt.
Divaricati	Angustifolii	*H. angustifolius* L.
		H. carnosus Small
		H. floridanus A.Gray ex Chapman
		H. heterophyllus Nutt.
		H. longifolius Pursh
		H. radula (Pursh) T.&G.
		H. simulans E.E.Wats.

[a] Annual species; others perennial

Studies of Reiseberg *et al.* (2001), Reiseberg *et al.* (2002), Reiseberg *et al.* (2003a; 2003b) made special contributions to the knowledge of origin and speciation of *Helianthus* species.

Gentzbittel *et al.* (1992) compared their molecular classification of the genus *Helianthus* with the morphological taxonomy of Schilling and Heiser (1981). The former constructed molecular phylogenies for 44 *Helianthus* species on the basis of the distribution of their DNA fragments.

The extent of variability in the genus *Helianthus* has not been sufficiently studied. Heiser *et al.* (1969) indicated that subspecies, varieties and forms existed in some *Helianthus* species. The taxonomy of Schilling and Heiser (1981) retained subspecies only for some *Helianthus* species. This taxonomy is simpler to use but many researchers are baffled by the interspecies variability occurring in their collections. For example, a study of Miljanović *et al.* (2000) showed that there exists a large variability for some taxonomically stable traits in the perennial species *Helianthus giganteus* and *Helianthus maximiliani*, which could even justify the recognition of new infraspecific forms. However, questions may be raised here, first regarding the existence of natural hybrids and second growing the populations in a common environment vs. populations growing in their natural environment. Seiler (1992) concluded that changes in plant habit and distribution of species occur as consequences of natural adaptation and natural selection in the genus *Helianthus*.

The sunflower genome

Chromosome number in somatic cells of the cultivated sunflower (2n=34) was determined by Tahara (1915) and confirmed by Wagner (1932), Ševčenko (1936), and Kostoff (1939). Studying the chromosome number in different *Helianthus* species, Geisler (1931) found species with n=17, 34 and 51 chromosomes. This finding was later on corroborated by Heiser and Smith (1955) and Georgieva-Todorova (1976). While the basic chromosome number in the genus *Helianthus* is n=17, the genus is a polyploidy complex composed of diploid (2n=2x=34), tetraploid (2n=4x=68) and hexaploid (2n=6x=102) species.

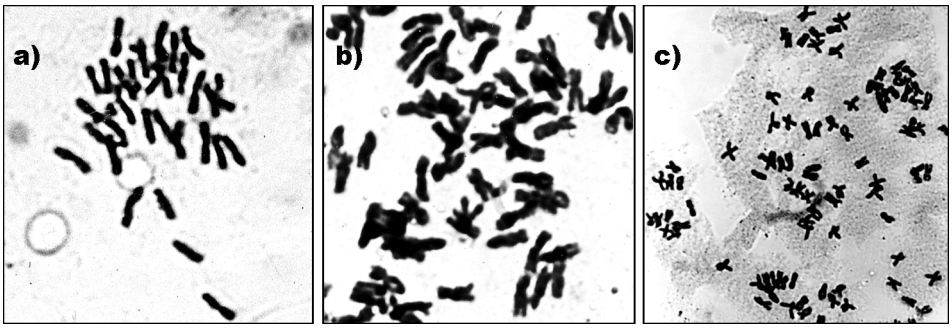

Figure 1: Polyploidy in the genus Helianthus: a) H. annuus, n=34, b) H. hirsutus, n=68 and c) H. rigidus, n=102

All 12 annual species are diploid; the 37 perennials include 25 diploid, 3 tetraploid, 6 hexaploid and 3 "mixoploid" species. *Helianthus ciliaris* and *Helianthus*

strumosus occur in the tetraploid and hexaploid forms, *Helianthus decapetalus* in diploid and tetraploid forms (Schilling and Heiser, 1981). Atlagić *et al.* (1992) found that the diploid species *Helianthus smithii* occurs also in the hexaploid form, while the species *Helianthus strumosus* occurs in diploid, tetraploid and hexaploid forms.

Most authors used to think that the basic chromosome number (n=17) comprised the sunflower genome. Hypotheses have been made on the origin of polyploidy, whether it was auto- or allopolyploidy. Some authors reported finding aneuploids following hybridization.

Although the annual diploids and the perennial diploids have the same chromosome number, they are either difficult to cross or cannot be crossed at all. Heiser and Smith (1964) concluded that these two groups of species had different genomes. Georgieva-Todorova (1976) arrived at a similar conclusion on the basis of an analysis of interspecific hybrids. The genome of the annual wild species evidently differs from that of the cultivated sunflower. Analyzing the meiosis in a group of annual diploids and their interspecific hybrids, Chandler *et al.* (1986) concluded that the basic chromosome number is not a single genome, *i.e.*, that the 17 chromosomes do not have the same origin. Thus they confirmed the finding of Kulshreshtha and Gupta (1979) who proposed that the basic number of chromosomes in the genus *Helianthus* had developed secondarily, by hybridization. Which are the original species that had hybridized in order to give rise to the diploid sunflower species? This question could not be answered because of the impossibility to mutually cross the species of the genus *Helianthus* (cross incompatibility) and the flaws in the cytogenetic methods (analysis of meiosis, C-bending and "*in situ*" hybridization). Great hopes have been invested in the method of molecular markers. Using the RAPD technique, Sossey-Alaoui *et al.* (1998) analyzed 40 *Helianthus* taxa, 36 identified and 4 non-classified. The analysis showed that there existed the following genomes:

1. C-genome, common for all species from the three analyzed sections,
2. H-genome, specific for section *Helianthus*,
3. P-genome, common for perennial species (sections *Atrorubens* and *Ciliares*), and
4. A-genome, specific for section *Atrorubens*.

The genomic constitution was therefore HC for section *Helianthus*, CPA for section *Atrorubens* and CP? for section *Ciliares*. The question remains if it is possible or not to find RAPD fragments which define the genome. It would also be important, when identifying the different genomes, to find an effective method of comparison of RAPDs against other molecular markers.

DNA content

Because of the different ploidy levels in the different *Helianthus* species, it was considered worthwhile to determine their total DNA contents. A study of DNA content in 22 *Helianthus* species and subspecies indicated that 2C DNA increased con-

tinuously from 6.4 pg in *Helianthus neglectus* to 12.02 pg in *Helianthus angustifolius* (Sims and Price, 1985). The highest DNA contents were found in *Helianthus divaricatus* and *Helianthus agrestis*, 16.90 pg and 25.91 pg, respectively. Such differences are difficult to explain without taking into account polyploidization. It has been noticed that DNA content was more similar among close species than among distant ones. This was an indication of small intraspecific variation. It was also an indication that DNA content depended on chromosome size. So, diploid perennials have a higher DNA content than diploid annuals or some diploid species have a higher DNA content than polyploid ones. It means that the origin of polyploid levels in the *Helianthus* species cannot be proved experimentally using DNA content.

Karyotype

Karyotype describes the haploid chromosome set of an organism, *i.e.*, the form and length of chromosomes, length index of chromosome arms, position of the centromere and secondary constrictions and the size and position of heterochromatic knots. The analysis of karyotype is most frequently performed on mitotic metaphase chromosomes, using the classical Feulgen technique.

Klimočkina (1940) performed a detailed karyological analysis of *Helianthus annuus*. Based on the position of the centromere, she divided chromosomes into four groups according to their morphology.

Numerous authors have conducted the analysis of karyotype in sunflower. The nomenclature used is based on the relations between chromosome arms. This classification distinguishes metacentric, submetacentric, subtelocentric and telocentric chromosomes in which the ratios longer vs. shorter arm are 1.0-1.7, 1.7-3.0, 3.0-7.0 and 7.0-?, respectively.

The karyotype of the species *Helianthus mollis* was analyzed by Georgieva-Todorova *et al.* (1974), *Helianthus annuus* and *Helianthus debilis* by Raicu *et al.* (1976), cultivated *Helianthus annuus* by Al-Allaf and Godward (1977), *Helianthus salicifolius* by Georgieva-Todorova and Lakova (1978), *Helianthus hirsutus* and *Helianthus decapetalus* by Georgieva-Todorova and Bohorova (1979), the hybrid *Helianthus annuus* × *Helianthus hirsutus* by Georgieva-Todorova and Bohorova (1980). Finally, Kulshreshtha and Gupta (1981) analyzed the karyotypes of 12 *Helianthus* species.

Raicu *et al.* (1976) found that the total length of the haploid chromosome set of the cultivated sunflower (the cultivar Record) was 73.82 μm. The lengths of the individual chromosomes varied from 3.76 to 5.15 μm. The karyotype consisted of 10 metacentrics, 3 submetacentrics and 4 subtelocentrics. Three chromosomes had secondary constrictions and a large variation of arm ratio, from 1.08 to 5.34. In *Helianthus debilis*, the length of the haploid chromosome set was 110.19 μm and the lengths of the individual chromosomes varied from 5.69 to 7.91 μm. Regarding their morphology, two of them were satellite chromosomes, 6 were meta-

centrics, 7 were submetacentrics and 2 were subtelocentrics (Georgieva-Todorova, 1976).

The karyotype of *Helianthus mollis* differed from those of *Helianthus annuus* and *Helianthus debilis* (Georgieva-Todorova *et al.*, 1974). The chromosomes were short, from 3.16 to 4.50 μm, and the karyotype formula was 2SAT + 11SM + 4ST. *Helianthus salicifolius* was similar to *Helianthus mollis* (Georgieva-Todorova and Lakova, 1978).

The closely related tetraploid species, *Helianthus decapetalus* and *Helianthus hirsutus*, had similar karyotypes, but the former had somewhat longer chromosomes. Both species had 4 SAT chromosomes.

Georgieva-Todorova and Bohorova (1980) presented the karyotype and ideogram of the F_1 interspecific hybrid *Helianthus annuus* (2n=34) × *Helianthus hirsutus* (2n=68). The somatic cells of the hybrid contained 51 chromosomes, the karyotype formula was 3 SAT+8M+11SM+4ST, and one chromosome was incomplete. The authors compared the karyotypes of the parent species with the karyotype of the F_1 hybrid. The total length of the chromosome set in the hybrid was 131.68 μm, while *Helianthus annuus* and *Helianthus hirsutus* had the lengths of 104.68 μm and 172.03 μm, respectively. It was difficult to identify chromosomes of the parent species on the basis of the karyotype of the F_1 hybrid.

Kulshreshtha and Gupta (1981) constructed the karyotypes of 12 *Helianthus* species. They identified only one SAT chromosome in each of the diploid *Helianthus tuberosus* species and three SAT chromosomes in the hexaploid *Helianthus tuberosus*.

Karyotypic characteristics of individual *Helianthus* species are useful for the study of interspecific relations as well as for the study of evolutionary changes.

The relatively small size and the large number of chromosomes in *Helianthus* species make it difficult to distinguish similar chromosomes on the basis of mitotic metaphase alone. Karyotype may be studied on the basis of meiotic pachytene chromosomes using C- or N- bending techniques. These techniques have allowed the identification of trisomics in many plant species (corn, tomato, rice, barley, and others).

Because of the limited possibilities to study karyotype exclusively by cytogenetic methods, a new direction of study has evolved, called molecular cytogenetic. Such studies allow us to understand the genomic organization of species with both large and small genomes. "*In situ*" hybridization, one of the methods employed in the cytogenetic-molecular studies, permits the identification of chromosomes and their arrangement which are indicators of the evolutionary history of the genome (Heslop - Harrison, 1995).

Chromosomal variability in *Helianthus annuus* var. *macrocarpus* was determined on the basis of heterochromatin distribution, number and position of NORs (Nuclear Organizer Regions) and the number and location of rDNA sequences using the method of Feulgen staining, C-bending, fluorochromium staining, silver staining and "*in situ*" hybridization (Cuellar *et al.*, 1996). Such complex studies using chromosome markers permit:

- determining whether a species is diploid, tetraploid or hexaploid, *i.e.*, is the

basic chromosome number n=17 maybe of polyploid origin (Chandler et al., 1986);
- determining whether the different *Helianthus* races are or are not chromosomally different, *i.e.*, whether they had been obtained from crosses between perennials and annuals or between diploids and polyploids (Kulshreshtha and Gupta, 1979), and
- gaining cytogenetic information from the analysis of meiotic configurations in interspecific hybrids. Such information is exceedingly important in interspecific hybridization.

Chromosome markers may be used for taxonomic purposes, for identification of chromosomes originating from different genomes, or for monitoring of introgression of an alien chromosome into the cultivated sunflower (Gustafson and Dille, 1992; Werner *et al.*, 1992). Sunflower linkage maps (Rieseberg *et al.*, 1993; Gentzbittel *et al.*, 1995) and polymorphism of ribosomal genes (Chonmane and Heizmann, 1988) permit the chromosomes with NOR_s to be labeled as a linkage group. Linkage groups may also be labeled on the basis of RFLP maps and "*in situ*" hybridization.

Studies of patterns of constitutive heterochromatin and fluorochrome response as well as the number and location of rDNA sequences of different species and cultivars from the genus *Helianthus* may provide complementary information of the evolutionary status of the genus. To be able to combine molecular and cytogenetic techniques in sunflower breeding, it is necessary to acquire an understanding of natural and artificial gene transfers (introgressions).

Meiosis - reduction division

Meiosis or reduction division is a process of micro- and macrosporogenesis taking place in plant stamens and ovaries, respectively. Microsporogenesis involves the development of pollen grains or male gametes in the anther. Macrosporogenesis involves the development of embryos or female gametes in the embryo sac.

Microsporogenesis

In *Helianthus* species, microsporogenesis is studied in immature anthers, most frequently by the acetocarmine method (Georgieva-Todorova, 1976).

Analyzing the meiosis in different *Helianthus* species, Atlagić (1989) made the following observations:

1. Leptonema and zygonema, early stages of prophase I, cannot be detected. Although pachynema occurs frequently in preparations, it is not suitable for analysis because sunflower chromosomes are thin and long. In diplonema, the chromosomes are short. Diakinesis is the most suitable stage within prophase I for determination of the numbers of bivalents, univalents, multivalents as well as chiasmata. Chromosome configurations may also be determined in metaphase I, when chromosomes are aligned along the equatorial plane. Anaphase I and telophase I occur frequently in preparations.

Meiosis II, metaphase II and anaphase II can seldom been be detected in the cultivated sunflower and almost never in the wild species, while telophase II is frequent in found in both. After telophase II, tetrads are most frequently formed, although diads, triads and pentads can be seen. Microspores are further divided mitotically, giving rise to pollen grains.

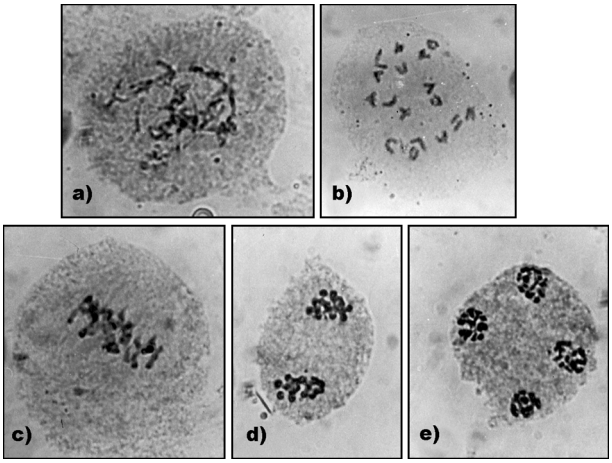

Figure 2: Normal meiosis: a) pachyten, b) diakinesis, c) metaphase I, d) anaphase I, c) telophase II

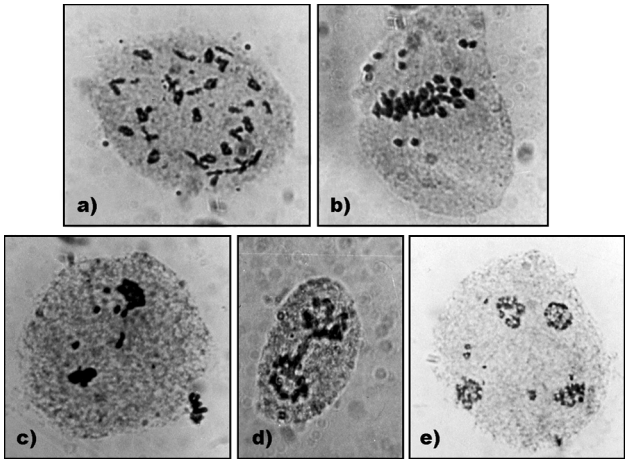

Figure 3: Irregular meiosis: a) diakinesis with univalents and multivalents, b) metaphase I with fast chromosomes, c) anaphase I with lagging chromosomes, d) anaphase I with chromosome bridge, e) telophase II with lagging chromosomes

2. Second, each sunflower head contains a large number of disk flowers which differ in age from the periphery to the center of the head. This is why a bud contains all phases of meiosis - microsporogenesis. Each disk flower has 5 anthers, which allows one to find all meiotic phases in a single preparation.

In sunflower, the meiotic division is asynchronous, *i.e.*, karyokines is not followed by cytokinesis.

Georgieva-Todorova (1976) made a detailed analysis of meiosis in the cultivated sunflower. At diakinesis, she observed 17 bivalents. The numbers of chiasmata per cell and per bivalent were 23.88 and 14, respectively. The number of ring bivalents ranged between 3 and 10. Meiotic abnormalities were observed even in normal fertile plants, but in less than 5% of meiocytes. These occurred as a result of spontaneous interruptions and changes.

The analysis of meiosis provides valuable data on the following:

- chromosome homology and translocations (configurations at diakinesis);
- changes in genetic material (number of chiasmata);
- unpaired chromosomes (univalents);
- non-included chromosomes (fast and lagging chromosomes);
- inversions (chromosome bridges and fragments).

The analyses of meiosis – microsporogenesis and pollen viability in interspecific hybrids (F_1, BC_1) are important for determination of phylogenetic relations among *Helianthus* species.

At maturity the pollen grains are yellow-orange, spherical, covered with spines (echinate), and have three apertures. Whelan (1978) screened sunflower pollen grains by electron microscopy. In polar view, the grains show three equidistant colpi in the wall. The equatorial view shows that each colpus extends almost from pole to pole, with an aperture near the middle. The germinating pollen tube emerges from one of these apertures. The diameter of the body of the pollen grain, without the spines, varies from 33 to 39 μ.

The abortive pollen grain is smaller and it has an increased number of spines. Gundaev (1971) reported that the diameter of sunflower pollen grains varied from 30 to 45 μ.

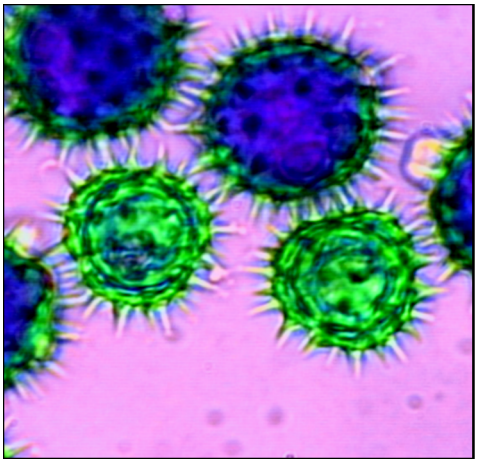

Figure 4: Fertile (red) and sterile (green) pollen grains

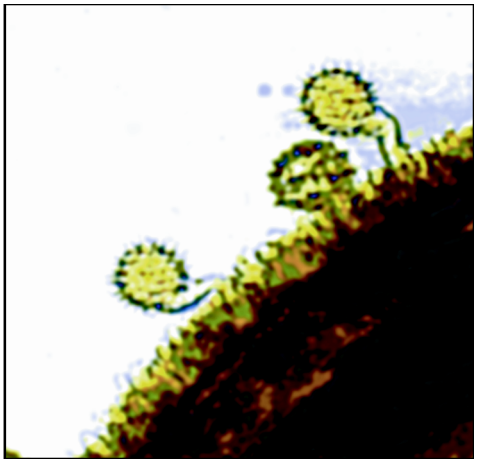

Figure 5: Pollen grains germination on stigma

Pollen viability is an important biological trait. It is typically assessed by staining methods (Georgieva-Todorova, 1976; Alexander, 1969; *etc.*). Pollen grain germination and pollen tube growth in sunflower are analyzed by fluorescent microscopy (Xanthopoulos, 1991).

Recent results of the QTL analysis have shown that it is possible to identify genetic factors that limit pollen viability in interspecific hybrids (backcross of the F_1 interspecific hybrid *Helianthus annuus* × *Helianthus argophyllus)* (Quillet *et al.*, 1996). Analyzing the meiosis in the interspecific hybrid *Helianthus annuus* × *Helianthus argophyllus*, Heiser (1951) found a single quadrivalent in a high percentage of PMCs. Chandler *et al.* (1986) found that the karyotypes *Helianthus argophyllus* and *Helianthus annuus* differed in 2 reciprocal translocations. Their F_1 hybrid was well developed, but both male and female fertilities were reduced. In a comparative analysis of meiosis, Quillet *et al.* (1996) detected more ring bivalents in the hybrid than in its parents. Abnormalities in meiotic behavior observed in BC_1 plants appeared to be in correlation with the reduced pollen viability. These results substantiate a hypothesis that chromosome arrangement affects to a large measure the viability of pollen in interspecific progenies. Genetic maps have shown that 3 regions of the genome covered about 80% of the variability of pollen viability in BC_1 progenies.

Macrosporogenesis

First data on the development and structure of the female gametophyte in the sunflower were reported three decades ago. Newcomb was the pioneer and he remained one of the only researchers to venture into the field macrosporogenesis. Using light and electron microscopy and the cultivar Peredovik, Newcomb (1973a) described development from the megaspore mother cell to the mature ovule. In another study, Newcomb (1973b) described embryo sac development after fertilization.

Female fertility in the sunflower has not received much attention. Although female fertility is definitively as important as male fertility, it is comparably more difficult to study. This appears to explain the present state of that field of study.

Male sterility

The sunflower is known to possess two types of male sterility, nuclear (*nms*) and cytoplasmic (*cms*). Generally, nuclear male sterility results from the action of individual recessive gene pairs. The number of genes determining this trait differs (ms1 to ms5, Vranceanu, 1970; ms6 to ms9, Jan, 1992). The cytology of *nms* lines has not been described in too much detail. Paun (1974) examined the *nms* lines AS-110 and AS-116, which contained the ms1 gene and which behaved similarly. He found that 2-4 univalents occur in diplonema-diakinesis as a consequence to asynapsis or desynapsis. The author noted unequal segregation, equatorial division and the elimination of univalents in subsequent phases. Chromosome clumping and agglutination were observed together with chromosome bridges and fragments. The

tetrad stage was mostly abnormal and it contained micronuclei. During flowering, 4-6% of the pollen grains were stained with acetocarmine, but only 75% of these had normal size and they lacked the spiny exine characteristic for *Helianthus* species. A question arises as to the viability of these 75% pollen grains. Numerous authors (Nakashima and Hosokawa, 1974; Pirev, 1968; Georgieva-Todorova, 1974) that have studied *nms* found that meiosis was normal until the tetrad stage, degenerations starting to occur at the stages of microspores or pollen grains.

Leclercq (1969) discovered the first source of *cms* in *Helianthus petiolaris* ssp. *petiolaris*. This discovery, together with the discovery of fertility restoration genes (Enns *et al.*, 1970; Kinman, 1970; Vranceanu and Stoenescu, 1971), made possible the development of hybrid sunflower.

Numerous *cms* sources have been discovered in programs of crossing between wild *Helianthus* species and the cultivated sunflower. Initially, the FAO list had registered 26 *cms* sources (Serieys, 1991). Jan (1997) reviewed 38 *cms* sources, which had been mentioned in publications released in the period 1972-1994. These sources typically belonged to the annual species *Helianthus annuus*, *Helianthus petiolaris* and *Helianthus argophyllus*. Serieys (2002) reported that the most recent FAO list included 70 *cms* sources. The list specified the origin, collection number of donor and author of the source. Of these 70 *cms* sources, 62 were derived from annual species in the section *Helianthus* (38 from *Helianthus annuus*, 24 from other annual species), and 8 were derived from perennial species in the section *Atrorubens*. Restorer genes have been found for most of these *cms* sources.

Cms in the sunflower is most frequently alloplasmatic. This term was coined by Pearson (1981) who uses it to describe male sterility resulting from interspecific and intergeneric crosses. He believed that alloplasmatic *cms* was a result of incompatibility between the nucleus and cytoplasm.

Considering the origin of *cms* and the expression of this trait after incorporation into different sunflower genotypes, cytogenetic studies in this field are both interesting and valuable for breeding.

Paun (1974) studied Leclercq's *cms*. He analyzed meiosis in 4 sterile lines and their fertile analogues. While meiosis was normal in the fertile lines, degeneration of sporogenous tissue occurred in the sterile lines. Degeneration occurred in pre-meiotic stages in two sterile lines and after tetrad stage in the other two lines. The author hypothesized that the degeneration was due to enzymatic reactions which inactivated the mechanisms for pollen development.

Studying microsporogenesis in sunflower male sterile lines, Rjabota (1969) registered the occurrence of chromosome bridges in anaphase I and degenerative changes in the post-meiotic cycle, *i.e.*, in uninuclear microspore, binuclear microspore and pollen grain stages.

Whelan and Dedio (1980) substituted *Helianthus annuus* nuclei into *Helianthus petiolaris* cytoplasm. Applying a series of backcrossing, they obtained progenies whose anthers were either empty or contained non-functional pollen. "*In vitro*"

testing of pollen fertility in 23 BC_5 plants showed that complete male sterility existed in 14 plants. Female fertility remained intact, as demonstrated by normal seed set. Meiosis was normal in the BC_5 plants. Meiotic abnormalities observed in the F_1 interspecific hybrids (univalents, chromosome bridges and fragments) were eliminated by backcrossing, resulting in the BC_5 generation being cytoplasmic male sterile.

Similar cytogenetic results were found when substituting *Helianthus annuus* into *Helianthus maximiliani* cytoplasm (Whelan and Dorell, 1980). Meiotic abnormalities (multivalents and chromosome bridges) were observed in early generations of crossing. The F_1 generation was observed to contain aneuploids (trisomic plants) which, together with the plants with modified anthers, were the most frequent *cms* sources.

A high percentage of abnormalities in the interspecific hybrid *Helianthus giganteus* × *Helianthus annuus* suggested that the parents differed in genomic structure, although they had the same number of chromosomes (Whelan, 1978). Based on the analysis of meiosis in this F_1 interspecific hybrid, Whelan concluded that *Helianthus giganteus* differed from *Helianthus annuus* in three translocations and one paracentric inversion. Whelan concluded that the consequence of these changes in chromosome structure was the occurrence of sterile plants. Backcrossing eliminated abnormalities, but sterility remained.

Whelan (1980) inferred that nuclear and cytoplasmatic factors are intermingled in early generations of interspecific crossing. Since meiotic abnormalities (nuclear factors) are eliminated by backcrossing, the male sterility remaining after BC_4 is inevitably cytoplasmic which produces normal fertile progeny when crossed with restorers.

Using light and electron microscopy, Horner (1977) compared microsporogenesis in a fertile line HA232 to its sterile analogue which contained *cms*-PET-1. He analyzed anthers, sporogenous tissue and chromosomes. He divided microsporogenesis into 11 stages, stages 1 to 4 ranging from the first sporogenous tissue to the initiation of tetrads, and stages 5 to 11 from late tetrad to mature pollen. Sterile and fertile analogues did not differ in microsporogenesis until stage 5. The elongation and degeneration of tapetal cells at the end of stage 5 caused degeneration of microspores in the tetrads, which ultimately resulted in sterility. Similar results were obtained by Vlčkova and Kovačik (1981) and Szabo *et al.* (1984).

Atlagić *et al.* (1996) studied the stability of 5 *cms* sources (PET-1, PET-2, MAX-1, GIG-1, ANN-6) during substitution into the inbred line HA89, and the stability of 5 *cms* sources (PET-1, PET-2, ANN-5, ANN-44, ANN-164) during substitution into the inbreeds L-1, L-98, L-74 and L-22. It was found that the anthers differed in development pattern from normal to rudimentary. Microsporogenesis developed normally until the tetrad stage in most of the cases. Some anthers contained deformed and sterile pollen grains. The authors concluded that the sources GIG-1 and PET-2 were unstable - their pollen viability was 10.42% and 1% to 63.43%,

respectively). Atlagić and Marinković (1998) conducted a cytogenetic study on potential *cms* sources (interspecific hybrids with 6 *Helianthus annuus* populations and one *Helianthus petiolaris* population). All plants in the BC_1F_1 generation were male sterile. Differences existed in the stage of anther development and related meiotic phases.

Numerous authors have observed differences between male sterile and male fertile cytoplasms in direct analyses of the mitochondrial and plastid genomes. Brown *et al.* (1986) found that the plasmid 1.45kb DNA was present in mitochondria of a male fertile line, but not in those of the sterile analogue. Similar results were obtained by Perez *et al.* (1986). They found differences between mitochondrial and plastid DNA not only in fertile and sterile analogues but also in different subspecies and populations of *Helianthus petiolaris*.

Relationships among restriction fragments in *cms* HA89 and HA89 have not been clearly defined. Crouzillat *et al.* (1987) found that a relationship existed between *cms* and *Helianthus* species with respect to the mitochondrial plasmid 1.45kb. Using three restriction enzymes and 12 probes, Crouzillat *et al.* (1991) found 13 different cytotypes. The relationship between *cms* cytoplasms and the wild species (their cytoplasm donors) observed in 1987 could not be observed in 1991, presumably because different collections of wild species were used. These wild species evidently differ in sterility type, as evidenced by the polymorphism in their mDNA and restriction fragment length.

The observed differences in *cms* types and their reactions to fertility restoration genes call for further studies at cytogenetic, biochemical and molecular levels.

Application of wild species in sunflower breeding

Wild sunflower species find their applications in field and laboratory work. Crossability is tested in the field. "*In vitro*" tissue culturing and cytogenetic analyses (meiosis and pollen viability) are conducted in the laboratory.

The annual wild species were studied in considerable detail by Chandler *et al.* (1986). They found that all annuals were crossable both mutually and with the cultivated sunflower. However, they frequently had to resort to embryo culture. The analyses of meiosis and pollen viability, which included all annuals and their F_1 interspecific hybrids, showed that the annuals differed in 0 to 6 translocations and 0 to 8 paracentric inversions. This was a further proof that the basic chromosome number (n=17) is not a single genome.

Meiotic abnormalities and reduced pollen viability in F_1 hybrids between wild annual species and the cultivated sunflower have been reported by Heiser (1947, 1961), Heiser *et al.* (1969), Georgieva-Todorova (1976, 1990), Whelan (1979) and Atlagić (1988, 1990).

One of the truly useful interspecific hybrid was made by Leclercq (1969) between *Helianthus petiolaris* and the cultivated sunflower. This was the first sta-

ble source of *cms* (PET1) which is still used exclusively for the development of commercial hybrids.

Study of wild annuals has lagged in recent years. Wild annuals are seldom used in inferspecific hybridization programs because they are as sensitive to major diseases as the cultivated sunflower.

Wild perennial sunflowers of various ploidy levels have been mutually crossed mostly for the purpose of cytotaxonomy (Heiser and Smith, 1955; Jackson, 1963; Heiser *et al.*, 1969; *etc.*). Wild perennials were also crossed with the cultivated sunflower (Christov, 1991), but these interspecific hybrids were seldom subject to cytogenetic analysis.

Diploid perennials are interesting for breeders as potential sources of resistance to diseases (*Helianthus giganteus*, *Helianthus maximiliani*), high oil content in seed (*Helianthus salicifolius*) or development of a new idiotype (*Helianthus mollis*). Crossability between diploid perennials and the cultivated sunflower is poor, as demonstrated in studies of Georgieva-Todorova (1976, 1990), Jan (1987), Atlagić (1994a), Atlagić *et al.* (1995), *etc.* Using the embryo culture method, Kräuter *et al.* (1991) succeeded in obtaining hybrids between *Helianthus mollis* and *Helianthus maximiliani* on one hand and the cultivated sunflower on the other.

Figure 6: H.salicifolius (2n=2x=34) and their F_1 hybrid with cultivated sunflower

Cytogenetic analyses of F_1 interspecific hybrids (Georgieva-Todorova, 1967, 1976, 1990; Whelan, 1978; Atlagić *et al.*, 1995) detected numerous meiotic abnormalities (uni- and quadrivalents in diakinesis, dislocated chromosomes in meta-,

ana- and telophases, and chromosome bridges in anaphase I). Also, these authors found complete sterility or reduced pollen viability in F_1 hybrids. These results indicated that the genomes of diploid perennials and diploid annuals differ (Heiser and Smith, 1964), which limits the use of the former in breeding programs.

The analyses of meiosis and pollen viability in the tetraploid species has raised the question of the origin of polyploidy in the sunflower. Georgieva-Todorova and Bohorova (1979) and Georgieva-Todorova (1990) reported that meiotic abnormalities and reduced pollen viability in tetraploid species *Helianthus hirsutus*, *Helianthus decapetalus*, *Helianthus strumosus* and *Helianthus scaberimus*, which indicated their allopolyploid nature. On the other hand, Atlagić (1991) reported that these species had regular meiosis, which suggests autopolyploidy. Hybridization between tetraploids and the cultivated sunflower was observed only in a few cases, by Heiser *et al.* (1962), Georgieva-Todorova *et al.* (1979), Pustovoit (1975), Christov (1991) and Atlagić (1994b). Georgieva-Todorova *et al.* (1979), Georgieva-Todorova (1984) and Atlagić (1994b) have successfully crossed the tetraploid species *Helianthus hirsutus*, *Helianthus decapetalus*, *Helianthus laevigatus* and *Helianthus strumosus* with the cultivated sunflower and conducted cytogenetic analyses of the hybrids.

Figure 7: H.hirsutus (2n=4x=68) and their F_1 hybrid with cultivated sunflower

The results of these analyses showed an exceedingly high percentage of meiotic abnormalities and a frequent occurrence of complete sterility. Obviously, it is difficult to transfer desirable genes from these species into the cultivated sunflower. In order to make these crosses, conventional hybridization methods have to be com-

bined with the embryo rescue method (Kräuter *et al.*, 1991) and chromosome doubling in the F_1 and BC_1 interspecific hybrids (Jan, 1988).

The species from the hexaploid group most frequently used in sunflower breeding are *Helianthus tuberosus* (a source of genes of resistance to *Phomopsis*, *Alternaria*, *Plasmopara*), *Helianthus pauciflorus* (*Helianthus rigidus*) (resistance to disease agents and high protein content in seed) and *Helianthus resinosus* (resistance to disease agents and high content of oleic acid in seed). These species have undergone extensive cytogenetic studies by a number of researchers. Kostoff (1934) was the first to conduct a detailed cytogenetic analysis of *Helianthus tuberosus* and he established two hypotheses on the genomic structure of the hexaploid species. First, *Helianthus tuberosus* is autohexaploid (AAAAAA); and second, *Helianthus tuberosus* is amphiploid (AABBCC) made from a cross of an autotetraploid and a diploid form. In 1939, the same author conducted a detailed study of a hybrid between *Helianthus tuberosus* and the cultivated sunflower, which confirmed his hypothesis on the different genomes in these two species.

Clevenger and Heiser (1963) claimed on the basis of cytogenetic analyses that *Helianthus tuberosus* is a natural hybrid but the results of Georgieva-Todorova (1990) and Atlagić *et al.* (1993) indicated that it is an original species.

Many researchers studied the meiosis and pollen viability in F_1 hybrids between *Helianthus tuberosus* and the cultivated sunflower (Kostoff, 1939; Heiser *et al.*, 1964; Cauderon, 1965; Heiser *et al.*, 1969; Pustovoit, 1969; Georgieva-Todorova, 1990; Atlagić *et al.*, 1993; Espinasse, 1995). Their results invariably showed that the complete sterility and reduced fertility in these interspecific hybrids were due to a large number of meiotic abnormalities occurring as a consequence of the differences in chromosome number and structure between the parent species.

Figure 8: H.rigidus (2n=6x=102) and their F_1, F_1BC_1 hybrids with cultivated sunflower

Helianthus pauciflorus (*Helianthus rigidus*) is another wild species extensively used in sunflower breeding programs. The species itself has not been extensively studied (Atlagić, 1996a), but F_1 interspecific hybrids between *Helianthus rigidus*

and the cultivated sunflower were studied by Whelan (1978), Georgieva-Todorova (1990) and Atlagić (1996a). All of these studies showed irregularities in chromosome pairing and diakinesis. Of all hexaploid species, Georgieva-Todorova (1990) found *Helianthus resinosus* to be most similar to the cultivated sunflower.

Within the scope of cytogenetic analyses of interspecific hybrids, the analyses of meiosis and pollen viability were studied by the largest number of researchers. In addition to crossability, sterility and reduced fertility in interspecific hybrids are most indicative of the applicability of these hybrids in sunflower breeding programs. Based on her long-term studies, Georgieva-Todorova (1984, 1990) concluded that pollen viability is invariably associated with meiosis as well as that it is genetically controlled. Conversely, Chandler *et al.* (1986) came to a conclusion that pollen viability is invariably affected by the number and type of meiotic abnormalities, but these effects do not necessarily have to be direct.

Cytogenetic studies have mostly been done on F_1 interspecific hybrids. However, analyses of BC_1F_1 hybrids showed even larger percentages of meiotic abnormalities, as well as the occurrence of aneuploids, plants with different chromosome numbers, reduced pollen viability, *etc.* (Whelan, 1979; Whelan and Dorrell, 1980; Atlagić, 1996b; Atlagić and Škorić, 1999).

Although Whelan (1979) and Whelan and Dorrell (1980) claimed that backcrossing eliminates meiotic abnormalities observed in F_1 interspecific hybrids, cytogenetic analyses of BC hybrids have shown that the elimination takes place in later generations of backcrossing.

Defining problems associated with the use of wild *Helianthus* species in sunflower breeding programs, Atlagić and Škorić (2000) pointed out that phylogenetic differences among species are as important if not more important than differences in ploidy level.

Recent studies of interspecies hybridization in sunflower have included various aspects of occurrence of partial hybrids in wide crosses between sunflower (*Helianthus annuus*) and perennial species (*Helianthus mollis* and *Helianthus orgyalis*) (Faure *et.al.*, 2002a; 2002b, 2002c).

Based on literature and the results of our own studies, it became clear that the method of interspecific hybridization, so extensively used in sunflower breeding programs, should not be used alone, without the aid of cytogenetic studies. On the other hand, the methods used in cytogenetics are conservative and they should be combined with novel methods of molecular biology.

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EL PAPEL DE LA HIBRIDIZACIÓN DE INTERSPECIES E INVESTIGACIONES CITOGENÉTICAS EN LA SELECCIÓN DE GIRASOL

RESUMEN

La multitud y la diversidad de las especies del género *Helianthus*, ofrece grandes posibilidades a los mejoradores de girasol. Por el método convencional de hibridización, es posible cruzar todas las especies anuales y gran número de las especies perennes con el girasol cultivado. Por otro lado, la divergencia y la heterogeneidad, presente en el género *Helianthus*, conlleva una serie de dificultades, antes de todo, la incompatibilidad "cross", la abortividad de embriones, la esterilidad y fertilidad de los híbridos interspecies disminuida. Por ello, a menudo en el cruzamiento interspecies se utilizan los métodos de hibridización somática, el cultivo de embriones "in vitro", duplicación de cromosomas etc. Para detectar las causas de tales acontecimientos, y hasta la superación de los mismos, se utilizan las investigaciones citogenéticas. La determinación de número y de la estructura de los cromosomas, el análisis de meiosis-microesporogénesis, vitalidad del polen, posibilita la determinación de los vínculos filogenéticos entre las especies silvestres y el girasol cultivado, y con eso, la posibilidad de su utilización en la mejora genética. El desarrollo de las investigaciones citogenéticas en girasol iba desde la citología a través de citotaxonomía y la citogenética clásica, hasta las investigaciones citogenéticas-moleculares. El especial desarrollo de las investigaciones citogenéticas, está vinculado con la aplicación de la hibridización de interspecies en la mejora genética de girasol.

RÔLES DE L'HYBRIDATION INTERSPÉCIFIQUE ET DES ÉTUDES CYTOGÉNÉTIQUES DANS LA CULTURE DU TOURNESOL

RÉSUMÉ

L'abondance et la diversité des espèces du genre *Helianthus* offrent de nombreuses possibilités dans la culture du tournesol. Toutes les espèces annuelles et un grand nombre d'espèces vivaces peuvent être croisées au tournesol de culture par la méthode d'hybridation conventionnelle. D'autre part, la divergence et l'hétérogénéité du genre *Helianthus* sont la cause de grandes difficultés comme l'incompatibilité de croisement, l'avortement d'embryon, la stérilité et la fertilité réduite dans les hybrides interspécifiques. C'est la raison pour laquelle des méthodes d'hybridation somatique, des cultures d'embryons "*in vitro*", de duplication de chromosomes, etc. sont souvent utilisées dans le croisement interspécifique. Les études de cytogénétique sont utilisées pour la détection et la résolution de ce type de phénomènes. La détermination du nombre et de la structure des chromosomes, l'analyse de la méiose (microsporogénèse) et de la viabilité du pollen permettent d'établir des liens phylogénétiques entre les espèces sauvages et les espèces cultivées de tour-

nesol et ainsi de les utiliser dans la culture. Le développement des études cytogénétiques sur le tournesol a évolué à partir de la cytologie en passant par la taxonomie cytologique et la cytogénétique classique jusqu'aux études de cytogénétique et moléculaire. Le développement particulier des études de génétiques cytologiques est lié à l'utilisation de l'hybridation interspécifique dans la culture du tournesol.