BREEDING FOR *ALTERNARIA* RESISTANCE IN SUNFLOWER: APPROACHES FOR INTROGRESSION FROM WILD SUNFLOWERS

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Summary:

Sunflower cultivation in India is seriously constrained by leaf spot disease caused by *Alternaria helianthi.* Successful breeding for resistance to *Alternaria* is limited by gene pool containing modest levels of resistance. Identification of resistant sources from wild perennial sunflowers in our laboratory viz., *H. simulans, H. mollis, H. divaricatus, H. maximilliani, H. occidentalis* (diploids), *H. decapetalus, H. pauciflorus* (tetraploids), *H. resinosus, H. tuberosus* (hexaploids) paved way for transfer of resistance through interspecific hybridization. However, successful transfer was limited by high degree of cross incompatibility and hybrid sterility owing to genetic distance, structural heterozygosity and ploidy differences. *In vitro* techniques and ploidy manipulations were attempted to overcome these problems. Among diploids, *H. mollis* and *H. divaricatus* were cross-compatible with sunflower, while interspecific hybrids involving *H. simulans* were reported for the first time. Cytological investigations revealed meiotic abnormalities resulting in high pollen sterility. Both *in vitro* and *in vivo* chromosome doubling using colchicine were employed to overcome the hybrid sterility. Interspecific hybrids between the tetraploids and cultivars were triploids showing complete sterility. Interestingly, the hexaploids were highly compatible with sunflower and the resultant tetraploid hybrids were fairly fertile. Keeping in view the expected chromosome abnormalities in the backcross progenies, a rapid, highly efficient and reproducible protocol for anther culture of these tetraploid hybrids was developed to reduce the ploidy. Cytological analysis of the anther plantlets resulted in the identification of dihaploids, tetraploids and aneuploids. Highly pollen fertile diploid anther culture derivatives were selected for backcross breeding to develop *Alternaria* resistant lines.

Introduction:

Recent years have witnessed a decline in the acreage under sunflower crop mostly in the traditional growing regions in India. Intensive cultivation of this crop in all the seasons enhanced the vulnerability of the high yielding cultivars to pests and diseases. Among the diseases, *Alternaria* leaf spot is economically important in India and results in seed losses from 27-80 % with 17-33 % loss in oil yield. Among the various approaches to manage these stresses, host plant resistance is the most reliable and economical to the end users. Owing to the narrow genetic base of the cultivated sunflower, concerted efforts are required to develop varieties and hybrids resistant to these biotic constraints by way of incorporation of additional genetic variability by integrating modern biotechnological tools with conventional breeding methods. Wild sunflowers possess considerable genetic variability in terms of resistance to biotic and abiotic factors, oil quality and continue to serve as sources of cytoplasm (Seiler, 1992). Wild sunflowers are distantly related to the cultivated sunflower and many severe problems need to be overcome for successful introgression of alien genes. Unique and innovative methods should be employed to manipulate chromosomes to obtain fertile interspecific derivatives with desirable attributes. Tissue culture techniques offer great promise in supplementing the breeding procedures by overcoming many of the difficulties encountered in wide crosses. Keeping in view the immediate concern of the breeders to incorporate resistance to *Alternaria* attempts were made to identify sources of resistance from wild sunflowers and to transfer the trait to the cultivated sunflower.

Materials and Methods:

A *Helianthus* collection was established with the seed materials obtained from USDA at the Directorate of Oilseeds Research, Hyderabad, India. Conservation and exploitation of the genetic variability in the wild sunflowers is a permanent challenge for the sunflower Breeders but the attempts made by several Researchers in India in the establishment and maintenance of the material being imported from time to time were unsuccessful. Nevertheless, careful understanding of the problems associated with the germination and establishment of the seedlings enabled the successful maintenance of 32 *Helianthus* species at the Directorate for the past five years. As most of the wild species are self-sterile and seed set is difficult, a rapid *in vitro* micropropagation protocol has been standardized and the wild species are being maintained in an *in vitro* garden (Sujatha and Prabakaran, 1997).

Screening against* Alternaria *leaf spot disease: A simple, rapid and reliable laboratory technique using detached leaves for screening germplasm against *Alternaria* leaf spot (Spore load: $1x10^6$ spores/ml; incubation at 25^o C & RH 80 %) has been developed to assist the field-based selection for resistant types. Thirty two wild *Helianthus* species were evaluated for their resistance to leaf spot disease incited by *Alternaria helianthi* both under laboratory and field screening (Sujatha *et al*., 1997).

Interspecific hybridization: Interspecific hybridization has become important as a means of introducing genetic variability of wild into the cultivated species. A wide gap exists between making initial hybrids and releasing cultivars with good agronomic performance and high yield. The genes from the closely related species from the primary gene pool can be easily transferred by following conventional breeding procedures, while the introgression of the traits of interest from species of the secondary gene pool requires several manipulations using recent techniques. Interspecific crosses between cultivars and species in the secondary gene pool are possible, but hybrids are partially sterile due to ploidy level differences, genomic incompatibility, cytoplasmic imbalances or other factors which cause sterility. Altering ploidy levels of either the parental species or their hybrid derivatives and/or applying embryo rescue techniques to recover hybrids are often necessary.

Anther Culture: Various basal media (Murashige and Skoog (MS), Nitsch, Gamborg, White), growth regulator concentrations and combinations (BA-NAA, 2,4-D-Kn, BA-IBA), temperatures (20^0, 25^0, 28^0, 30^0, 35^0) photoperiod (dark, 16:8 light/dark cycle) were tested. Best response was obtained when the anthers were incubated at 30 ^{o}C in dark on media with MS salts and organics, and supplemented with 0.5 or 1.0 mg/l each of benzyladenine (BA) and naphthaleneacetic acid (NAA). Regeneration was through direct embryogenesis and well formed embryos were visualized within 10-15 days. Frequency of embryogenesis was >90% and the number of embryos per regenerating anther varied between 1 to 10 with an average of 3.8. Transfer of embryogenic anthers to MS medium

with 1.0 mg/l each of BA and NAA and incubation under light at 28°C resulted in shoot differentiation. Multiplication and elongation of shoots was achieved on transfer to MS medium supplemented with 0.5 mg/l BA. Elongated shoots were rooted on basal MS medium or on medium with 1.0 mg/l NAA. Rooted shoots were acclimatized by covering with polybags and maintaining under high humidity and were subsequently grown to maturity.

Results and Discussion:

Intensive screening and evaluation of wild sunflowers at this Directorate has resulted in the identification of potential sources of resistance to *Alternaria* leaf blight for the first time (Sujatha *et al*., 1997). All the diploid annuals were found highly susceptible.

Table 1. Reaction of wild sunflowers to *Alternaria helianthi*

Species	Category	Disease index
H. mollis	Resistant	1.4 f
H. maximiliani	Resistant	4.1 f
H. divaricatus	Resistant	2.7 f
H. simulans	Resistant	4.8 f
H. occidentalis	Resistant	1.2 f
H. decapetalus	Resistant	1.3 f
H. pauciflorus	Resistant	5.4 f
H. resinosus	Resistant	5.1 f
H. tuberosus	Resistant	2.7 f
H. grossesseratus	Moderately resistant	14.2 ef
H. debilis	Moderately resistant	15.4 ef
H. strumosus	Moderately resistant	25.3 def
H. hirsutus	Moderately resistant	12.9 ef
H. praecox ssp	Susceptible	66.8 bcd
H. nuttalli	Susceptible	70.3 bc
H. annuus wild	Susceptible	92.9 abc
H. annuus cultivated	Susceptible	95.0 abc
H. niveus	Susceptible	95.2 abc
H. neglectus	Susceptible	95.6 abc
H. praecox ssp	Susceptible	96.0 abc
H. argophyllus	Susceptible	97.4 ab
H. deserticola	Susceptible	100 a
H. petiolaris	Susceptible	100 a

Percentage values were Arcsin angular transformed prior to analysis;
Means followed by same letters are not significantly different at p= 0.001

Maximum resistance was conferred by *H. mollis, H. maximiliani, H. divaricatus, H. occidentalis, H. decapetalus, H. pauciflorus, H. tuberosus, H. resinosus,* and *H. simulans*. Field evaluation under artificial epiphytotic conditions revealed a close agreement in the reaction of the wild sunflowers to *A. helianthi* under both laboratory and field conditions.

Transfer of desirable genes from identified sources

Species from primary gene pool: Interspecific hybrids were successfully produced between cultivated sunflower and other wild diploid annual sunflowers viz., *H. annuus* (wild), *H. argophyllus, H. praecox, H. petiolaris, H. debilis, H. niveus* and *H. neglectus* using conventional hybridization. The interspecific hybrids were studied for pollen fertility and meiotic chromosome behavior to understand the genomic relationships. The BC progenies showed dominance of wild traits in plant height, pigmentation, branching, number of heads, disc color etc. Families showing uniformity in height, good plant type, single stem and field resistance to pests and diseases were selected and evaluated in multilocations. Although all annual diploids were found susceptible to *Alternaria* leaf spot under very high disease pressure in artificial screening tests, yet it is interesting to note that these wild diploids, their interspecific hybrids and their derivatives were relatively tolerant to the disease in field evaluation at all the centers (Table 2) and have been included in the population improvement and inbred development.
Transferring genes from wild annual species into cultivated lines could be accomplished rather easily with conventional crossing and backcrossing. It has been demonstrated with the transfer of resistance genes for powdery mildew from *H. debilis* (Jan and Chandler, 1985), rust and downy mildew resistance genes from wild *H. annuus* (Quresh and Jan, 1993; Tan *et al.*, 1992) into cultivated sunflower.

Table 2. Introgression of resistance genes from the primary gene pool

Combination	Reaction of F_1s to *Alternaria helianthi*		Promising tolerant backcross populations tested at multilocations
	laboratory screening	field evaluation	
H. annuus x *H. annuus* (wild)	Susceptible	Tolerant	6
H. annuus x *H. debilis*	Moderately tolerant	Tolerant	4
H. annuus x *H. praecox*	Susceptible	Tolerant	-
H. annuus x *H. argophyllus*	Susceptible	Tolerant	13
H. annuus x *H. petiolaris*	Susceptible	Tolerant	2
H. annuus x *H. neglectus*	Susceptible	Tolerant	-
H. annuus x *H. niveus*	Susceptible	Tolerant	-
H. annuus x (*H. argophyllus* x *H. petiolaris*)	Susceptible	Tolerant	-
H. annuus x (*H. argophyllus* x *H.annuus* wild)	Susceptible	Tolerant	3
H. annuus x (*H. praecox* x *H. petiolaris)*	Susceptible	Tolerant	-

Species from the secondary gene pool: Reliable sources of resistance to *Alternaria* leaf blight were identified only from the perennial species viz., *H. mollis, H. simulans, H. maximiliani, H. divaricatus, H. occidentalis* (diploids), *H. pauciflorus, H. decapetalus* (tetraploids), *H. resinosus, H. tuberosus* (hexaploids). The crossability between sunflower and various perennial species being maintained at Directorate of Oilseeds Research and their reaction to *Alternaria* is presented in Table 3. The strong isolation mechanism between *H. annuus* and some of these perennial *Helianthus* species was circumvented by making repeated pollinations in either directions. The interspecific hybrids between *H. annuus* and a few other wild perennial species of economic importance were possible only through the use of embryo culture. The young embryos struggling to get nutrition due to the post fertilization causes could be rescued by culturing them in artificial medium under controlled conditions. The hybridity of the successful interspecific hybrids was confirmed through cytology and biochemical markers. The F_1 hybrid sterility was the major limitation in further advancement of the interspecific hybrids through backcrosses or self-pollination. This is because of the presence of dissimilar genomes in the hybrid or the differences in ploidy status of the species involved in the hybridization. Even when the chromosomes of diploid and polyploid species are homologous, some degree of sterility is encountered in triploid hybrids because of irregular chromosome segregation during meiosis. Jan (1988) used embryo-culturing technique to produce 26 interspecific hybrids of wild perennials x cultivated line P21 followed by chromosome doubling to improve the seed set on backcross and sib-pollination.

Table 3. Crossability between sunflower and perennial *Helianthus* species

Combinations	Crossability	Chro' no	Fertility	Reaction to *Alternaria*
H. annuus x H. nuttallii	X	-	-	-
H. annuus x H. simulans	H	34	Sterile	Resistant
H. annuus x H. occidentalis	H	34	-	Resistant
H. annuus x H. mollis	X	-	-	-
H. annuus x H. maximiliani	X	-	-	-
H. annuus x H. divaricatus	H	34	Fertile	Moderate
H. annuus x H. grosseserratus	X	-	-	-
H. annuus x H. atrorubens	H	34	-	-
H. annuus x H. hirsutus	H	51	Sterile	-
H. annuus x H. pauciflorus	X	-	-	-
H. annuus x H. decapetalus	H	51	Sterile	Moderate
H. annuus x H. strumosus	H	68	Sterile	Moderate
H. annuus x H. resinosus	H	68	Partial	Resistant
H. annuus x H. tuberosus	H	68	Partial	Resistant

H - Successful hybrids X - Cross failed

The results of the attempts made to transfer *Alternaria* leaf spot from wild perennial *Helianthus* species into cultivated sunflower overcoming the crossability barriers and hybrid sterility by

integrating various approaches are presented.

Sunflower x diploid perennial species

Among the five diploid species, interspecific crosses were successful with *H. simulans, H. divaricatus* and *H. occidentalis.* It is worth mentioning that the interspecific hybrid involving *H. simulans* (both directions) and cultivated sunflower is the first report of successful hybridization. However, exploitation of this cross combination was not possible owing to the hybrid sterility. Cytological investigation revealed irregular meiosis in the F_1 plants due to the presence of dissimilar genomes which led to the formation of univalents, bivalents and multivalents at metaphase I resulting in unequal separation of chromosomes in Anaphase I. The number of the sporads formed at the end of meiosis II was varying from 2-5 leading to polymorphic sterile pollen grains. The hybridity of this interspecific hybrid was also confirmed by isozyme analysis. The hybrid plants were screened under high spore load of *Alternaria* and were found to be highly resistant. There was no seed set either by backcrossing or open pollination indicating the presence of female sterility. A novel technique of chromosome doubling through *in vitro* colchiploidy has been standardized to overcome the sterility. Unlike the interspecific hybrid involving *H. simulans*, the hybrid between *H. divaricatus* and cultivated sunflower was highly fertile. As in the case of interspecific hybrids involving diploid annuals, dominance of wild characters was observed in F_1 and BC_1 generations. At BC_2 generations, plants resembling cultivated plant type were selected and intermated to avoid narrowing down of the variability for the polygenically controlled traits like resistance to *Alternaria*. The populations thus developed are being studied for further improvement. The interspecific hybrid between sunflower and *H. occidentalis* was successful only recently and the studies on genomic relationships are under progress.

Sunflower x tetraploid perennial species

Though the interspecific hybridization between the two tetraploid species with *Alternaria* resistance and sunflower was successful these combinations were not continued further because of the high degree of pollen sterility in the triploid F_1s. Chromosome doubling may improve the fertility but the utilization of the resulting amphiploids in further breeding is very much limited. Hence, attempts are being made to produce diploids from tetraploids using anther culture technique in order to improve the crossability and avoid hybrid sterility due to chromosome imbalance.

Sunflower x hexaploid perennial species

Cross-compatibility between sunflower and the two hexaploid species viz., *H. resinosus* and *H. tuberosus* was very high. The interspecific hybrids were tetraploids having three genomes from hexaploid species and one from cultivated sunflower. The pollen fertility of these hybrids was moderately low. Cytological study revealed the formation of quadrivalents, bivalents and univalents at metaphase I. Backcrosses were attempted on the tetraploid F_1s and the resultant BC_1F_1s were triploids and sterile. Hence, it was felt appropriate to bring down the ploidy of the F_1s to diploid level so that the problem of sterility can be overcome for facilitating backcrosses. A highly repeatable protocol for anther culture of the tetraploid interspecific hybrids was standardized.

Anther culture of interspecific hybrids: Anther culture of the F_1 hybrids of sunflower x *H. tuberosus* and sunflower x *H. resinosus* {2n=(1+3)x}was done in order to reduce the ploidy {2n=(1+1)x or (2)x}and improve the croassability with sunflower. A highly efficient protocol of plant regeneration was standardized for anthers of the interspecific hybrids (2n=68) between cultivated sunflower (2n=34) and *H. resinosus* (2n=102). Standardization of protocols for anther

culture of the hybrid between SF x *H. tuberosus* led to shoot regeneration at a frequency of 2.2 %. Further studies to enhance the frequency is being undertaken in this combination.

Pollen fertility of the anther plantlets varied between 0-58.33 %. Cytological studies were conducted to identify the chromosome number of the different anther culture plantlets which was found to vary from 34 to 68. Only the diploid plantlets (2n=34) with high pollen fertility were selected and screened against *Alternaria helianthi* under artificial conditions. The variability for the reaction to *Alternaria* was very high. It is expected that the diploid anther plantlets obtained in the present investigation would possess either one genome each from sunflower and the wild parent or both the genomes from the wild parent. The diploids carrying the genomes of both the parents are suitable for backcross breeding while those with only the wild genomes can serve as parent in the interspecific hybridization. The fertile diploid individuals with high degree of *Alternaria* resistance were selected and backcrosses were attempted with sunflower as recurrent parent. The progenies obtained in backcrosses are being evaluated and characterized using molecular and cytogenetical tools.

Several genes controlling resistance to many of the diseases of sunflower have been successfully identified from wild perennial *Helianthus* species and transferred to cultivated sunflower. Seiler (1993) released 12 interspecific germplasm lines derived from perennial accessions of *H. hirsutus, H. resinosus* and *H. tuberosus* with reasonably good fertility. Much of these introgressions were aimed at transferring resistance to diseases like rust, downy mildew, brown stem canker etc., mostly controlled by single gene. The resistance to *Alternaria* disease is a quantitative trait and has not received much attention as there were not much incidences of this disease in other major sunflower growing countries. However, this disease is a serious threat to sunflower in India occurring during *kharif* (rainy) season. It is also expected that many problems need to be understood to design appropriate breeding strategies to transfer this polygenic trait. It is interesting to note that many interspecific derivatives have been developed with acceptable levels of resistance to *Sclerotinia sclerotiorum* which is also a quantitative trait (Hammann *et al.*, 1994). Similarly, the present study clearly demonstrated the potential of wild *Helianthus* species and also the advantages of the integrated approaches in terms of conventional breeding methodologies and modern techniques for successful exploitation of the genetic diversity for *Alternaria* resistance available in the wild species for the genetic improvement of sunflower. The advanced breeding lines possessing the vast genetic variablity of the wild genomes has not only benefited the sunflower programs of the Directorate of Oilseeds Research but also are serving as base material for many of the sunflower network program in India.

References:

Hammann, T., H. Hohler and W. Friedt. (1994). Interspecific hybridization as a basis in sunflower breeding (*Helianthus annuus* L.) for disease resistance. Vortrage fur pflanzenzuchtung, 30: 151-157.

Jan, C.C. 1988. Chromosome doubling of wild x cultivated sunflower interspecific hybrids and its direct effect on backcross success. p. 287-292. In Proc. 12th Int. Sunflower Conf., Novi Sad. Yugoslavia. 25-29 July. Int. Sunflower Assoc., Paris, France.

Jan, C.C. and J.M. Chandler. 1985. Transfer of powdery mildew resistance from *Helianthus debilis* Nutt. to cultivated sunflower (*H. annuus* L.). Crop Sci., 25: 664-666.

Quresh, Z. and C.C. Jan. 1993. Allelic relationships among genes for resistance to sunflower rust. Crop Sci., 33: 235-238.

Seiler, G.J. 1992. Utilization of wild sunflower species for the improvement of cultivated sunflower. Field Crops Research, 30: 195-230.

Seiler, G.J. 1993. Registration of six interspecific germplasm lines derived from wild perennial sunflower. Crop Sci., 33: 1110-1111.

Sujatha M., A.J. Prabakaran and C. Chattopadhyay. 1997. Reaction of wild sunflowers and certain interspecific hybrids to *Alternaria helianthi*. Helia 20(27): 15-24.

Sujatha, M. and A.J. Prabakaran. 1997. Propagation and maintenance of wild sunflowers *in vitro*. Helia 20(27): 107-114.

Tan, A.S., C.C. Jan and T.J. Gulya. 1992. Inheritance of resistance to race 4 of sunflower downy mildew in wild sunflower accessions. Crop Sci., 32: 949-952.