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Interspecific hybrid between cultivated sunflower (*Helianthus annuus* L.) and silver leaf sunflower *H. argophyllus* T. & G.: Cytomorphological and molecular characterization

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Abstract

Successful interspecific hybrids were obtained through hybridization between cultivated sunflower (*Helianthus annuus* L.; 2n=2x=34) variety ARM-243B and a wild species (*H. argophyllus*; 2n=2x=34) accession no. PI-468649 for transferring desirable traits like downy mildew resistance, oil content and hopper resistance from wild species into cultivated background. Morphological, cytological and simple sequence repeats (SSR)-based molecular analyses were carried out to confirm the hybrid nature of the F_1 plants. The hybrids exhibited morphological features intermediate to both the parents for few attributes and showed greater similarity to wild *Helianthus* species for traits like leaf and stem hairiness, flower colour, stem size, branching, disc floret pigmentation, plant height, seed size and seed shape, etc. A reduction (89.9%) in pollen fertility was recorded in F_1 plants. Meiotic analysis revealed the presence of univalents, bivalents, trivalents and quadrivalents in all the pollen mother cells (PMCs) analysed. Multivalents were observed in few PMCs, indicating segmental homology between chromosomes. Higher level of chromosome configurations like quadrivalents were observed in 42 out of 50 PMCs. Frequently observed chromosome configurations in diakinensis were 15 II + 1 IV and 13 II + 2 IV. The results suggested that the species *H. argophyllus* and *H. annuus* differ by 1-2 translocations. SSR primers ORS-05, ORS-896 and ORS-908 were found to reveal highly polymorphic bands in the parents.

Key words: Chromosomal configurations, *H. argophyllus*, interspecific hybridization, meiotic study, pollen viability, SSR.

Introduction

Plant breeding is a perpetual task to develop high yielding varieties which are well adapted to the target environment. Cultivated sunflower has an extremely narrow genetic base which has left the crop potentially vulnerable to diseases (Seiler 1992). A series of biotic and abiotic stresses continue to limit the productivity of cultivated sunflower. There are 53 wild species in the genus *Helianthus*, including 14 annual and 39 perennial (Seiler and Jan 2014). Wild *Helianthus* annual species are diploid, with chromosome number (2n=2x=34) similar to cultivated sunflower, whereas wild *Helianthus* perennial species include 29 diploids (2n=2x=34), 4 tetraploids (2n=2x=68) and 6 hexaploids (2n=2x=102). Wild species of *Helianthus* genus are an important reservoir of useful genes and can be exploited both to broaden the existing narrow genetic base and to enrich the existing varieties with desired agronomically important traits. Interspecific hybridization is an additional technique to create new sources of genetic variability for the improvement of sunflower (Christov 2013). Apart from biotic stresses resistance, genes for tolerance to soil salinity, acidity, drought and diversification of CMS and fertility restorer genes for the development of new sunflower idiotypes are the need of the hour (Atlagic 2004).

Silver sunflower *H. argophyllus* T. & G. is closely related to wild *H. annuus* L. (Heiser et al. 1969). The two species display common morphological characteristics such as general plant architecture and large leaves in contrast to other annual species with small leaves. Several genetic pools have been derived from interspecific hybrids between *H. annuus* and *H. argophyllus* in order to introgress useful traits from wild species into the cultivated background (Miller et al. 1992; Seiler 1992). *H. argophyllus* is a source of several desirable traits such as salt tolerance,

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resistance to downy mildew and some races of rust, tolerance to several insect pests including the sunflower beetle and the sunflower midge, altered fatty-acid composition, *Rf*-genes, etc. (Thomson et al. 1981). It is also a source of a dominant gene for all known races of downy mildew (Seiler 1991; Jan and Gulya 2006; Wieckhorst et al. 2010). A new dominant downy mildew resistance gene (Pl_{18}) was transferred from wild *H. argophyllus* (PI-494573) into cultivated sunflower (Qi et al. 2016) which resulted in development of the downy mildew resistant germplasm HA-DM1. It has been reported that silver leaf sunflower constitutes a potential source of genes for drought tolerance (Jamaux et al. 1997; El Midaoui et al. 2003). Apart from biotic and abiotic stresses, Ziebell et al. (2013) reported that silver sunflower is a potential source of biofuel and improved lignocellulosic biofuels traits, namely, increased biomass, decreased lignin, and increased glucan. Heiser et al. (1969) stated that this species is sometimes cultivated as an ornamental plant for the striking effect of the foliage. In the light of the above scenario, the present investigation was carried out with the objective of studying the morphological variation and chromosomal behaviour of interspecific crosses.

Materials and methods

Plant material

Seeds of cultivated sunflower, ARM-243B were obtained from the Breeder Seed Production Unit of the ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad, Telangana, India and an accession, PI-468649 of wild diploid annual *H. argophyllus* (2n=2x=34) were established from seed material obtained in 1980 from United States Department of Agriculture, Texas, USA.

Interspecific hybridization

Field grown plants of the cultivated sunflower var. ARM-243B and accession no. PI-468649 crossed to produce interspecific hybrids. Staggered sowings of female parent, twice at weekly interval, was done to synchronize the flowering. The capitula of ARM-243B was used as female and the wild *H. argophyllus* accession I468649 as male and were covered with cloth bags and white butter papers, respectively at the ray floret stage to avoid cross pollination. Unopened flower buds of cultivated sunflower were emasculated in the morning, covered with cloth bags and pollinated with freshly collected pollen from five plants of one accession of wild *H. argophyllus* in the following morning and rebagged and the procedure repeated till the opening of all the disc florets on the capitulum. The seeds collected from the crosses and parents were sown in field at Narkhoda Farm, IIOR, Hyderabad during *kharif* 2014.

Morphological and phenological characterization

For morphological and phenological characterization of the wild *H. argophyllus*, the cultivated sunflower inbred line and the hybrid progenies, observations were recorded on at least 5 plants grown in the field. The traits *viz*., hypocotyl pigmentation, days to 50% flowering, days to maturity, leaf size, leaf shape, leaf colour, leaf blistering, leaf serration, leaf hairiness, leaf base, leaf petiole pigmentation, number of leaves/plant, stem pigmentation, stem hairiness at the top, ray floret number, ray floret shape, ray floret colour, ray floret length (cm), ray floret width (cm), disc floret colour, pollen colour, bract shape, bract anthocyanin colouration, position of axillary heads to the central head, head shape, head diameter (cm), plant height (cm), plant branching, type of branching, seed length (mm), seed shape, 100-seed weight (g), seed base colour, seed stripe (present/absent), seed stripe colour and seed mottling were studied.

Cytological analysis

For meiotic analysis, flower buds of appropriate stage were collected and fixed in freshly prepared Carnoy's fluid (ethanol : chloroform : acetic acid in a ratio of 6 : 3 : 1), for a minimum of 24 hours at room temperature and subsequently stored in 70% alcohol at 10^{o}C. Anthers were squashed in 1% acetocarmine and a total of 50 pollen mother cells (PMCs) each obtained from parents and hybrids were analyzed at diakinesis, metaphase I, anaphase I and telophase II stages.

Pollen fertility

For study of pollen fertility, pollen grains were stained in a 1:1 mixture of 1% acetocarmine and glycerol. Pollen viability was studied by the method of Alexander (1969). The method is based on differential staining of viable and nonviable pollen grains. Pollen grains from nondehiscent anthers were taken from 3 to 5 plants per population or hybrid combination and suspended in a drop of stain. The fully stained and round pollen grains were scored as fertile, while shrivelled and unstained pollen grains were recorded as sterile. Viable and nonviable pollen grains were counted on three slides, at 10 positions per slide. Pollen viability is shown as a percentage of viable pollen grains. The

percentage of pollen fertility was worked out by using the following formula.

$$\text{Pollen fertility (\%)} = \frac{\text{No. of fertile pollen}}{\text{No. of fertile pollen + sterile pollen}} \times 100$$

DNA extraction and SSR analysis

To confirm the hybridity of interspecific cross, young leaves were harvested from ARM-243B, PI-468649 and their F_1s individually grown in controlled condition at ICAR-IIOR Research farm. DNA was extracted from fresh leaves of 4 week old plants by cetyl-tri-methyl ammonium bromide (CTAB) method (Doyle and Doyle 1987) and quantification was done by Nanodrop spectrophotometer. DNA was diluted in $T_{10}E_1$ buffer to a concentration of 50 ng/µl for PCR analysis. Using information on marker distribution on genetic linkage map, SSR primers of the ORS series (Knapp 2004) were selected. The PCR amplification was carried out in 15 µl of reaction containing 0.5 µM of each primer, 0.25 mM dNTPs, 0.6 U *Taq* DNA polymerase (Genei, Bangalore), 1X PCR buffer with 1.5 mM $MgCl_2$ (Bangalore Genei, India), and 50 ng of template DNA. Amplification was performed in thermal cycler (Applied Biosystems GeneAmp 9700) using the following amplification conditions: 2 min at 94°C for the initial denaturation, followed by 35 cycles reaction profile involving 45 sec of denaturation at 94°C, 45 sec of annealing at 58°C, and 1 min of extension at 72°C with a final extension at 72°C for 5 min. The PCR amplified products were run on 3% agarose gel along with 100 bp DNA ladder (Bangalore Genei, India) in 1X TAE for 1 hour at 90 volts. The gels were stained with ethidium bromide (50 ng/ml) and documented using the Syngene gel documentation system.

Results and discussion

Morphological characterization of parents and F_1 hybrid

The data on comparative morphology of the F_1 hybrid and parents are presented in Table 1. The hypocotyl pigmentation was absent in cultivated sunflower, while pink pigmentation was present in *H. argophyllus* and the F_1 plants. Meena et al. (2017) reported similar results in interspecific cross between *H. annuus* L. x *H. argophyllus.* The cultivated species inbred ARM-243B flowered early (48.5 days) while the *H. argophyllus* was late in flowering (79.4 days), the F_1 was intermediate and flowered in 74.6 days. Nikolova and Christov (2004) reported similar results of 50 per cent flowering in interspecific cross between *H. annuus* L. line LHA-300 x *H. argophyllus* (E-091). Encheva and Christov (2006) showed that the hybrid progenies of the interspecific cross *H. annuus* (hybrid Albena) × *H. salicifolius* were two to three days earlier in flowering than that of the female parent. The hybrid was taller than both the parents and exhibited small head diameter, higher stem girth, and higher leaf length and width than the cultivated species. The F_1 plants were profusely branched, had medium head diameter, number of ray florets, achene size, test weight and were more similar to their wild parent for most of the other morphological and inflorescence attributes. The leaf colour of cultivated sunflower was medium green compared to *H. argophyllus* which was a shy green but the cross exhibited leaf colour which was more towards the female parent. Encheva and Christov (2006) reported dark green leaves in the hybrid plants similar to the female parent in F_1 between *H. annuus* (hybrid Albena) × *H. salicifolius* and the head size was medium as compared to the parents. Nikolova and Christov (2004) and Vassilevska-Ivanova and Tcekova (2005) also reported intermediate head diameter in interspecific hybrids between cultivated sunflower and *H. argophyllus*. Atlagic and Skoric (1999) reported large leaf size in F_1 hybrids derived from cross between *H. annuus* x *H. laevigatus*. While, Encheva and Christov (2006) reported intermediacy in cross between *H. annuus* (hybrid Albena) × *H. salicifolius*. The occurrence of intermediate phenotypes of the progeny is explained by inheritance pattern based on polygenic control with additive effects and reported in several plant genera including *Helianthus* (Kuligowska et al. 2016).

Three types of branching were observed: basal, axil, and branching both at the base and the apical part of the stem. Branches in some plants were situated above the central head of the plant. In general, branching in cultivated sunflower particularly the restorer lines is recessive and consequently, the hybrids are predominantly monoheaded (Sandu and Marinescu 1998). However, branching in wild species is controlled by several dominant genes resulting in interspecific hybrids with branching (Clement and Deihl 1968). Similar results and observations of interspecific hybrids with varied types of branching were reported earlier (Saciperov 1961; Christov 1988). The vegetation period of the hybrids varied between 96 and 117 days and was intermediate than that of the wild species. Manjula and Seetharam (2001) and Nikolova and Christov (2004) also reported intermediate maturation

Table 1. A comparison of morphological characters of parents and F_1 hybrid (*H. annuus* × *H. argophyllus*)

Characters	*H. annuus* (ARM-243B)	*H. annuus* x PI-468649 (F_1)	*H. argophyllus* (PI-468649)
Hypocotyl pigmentation	Absent	Dark pigmentation	Medium pigmentation
Leaf size	Medium	Broad	Medium
Leaf shape	Cordate	Cordate	Triangular
Leaf colour	Medium green	Medium green	Ashy green
Leaf blistering	Very weak	Medium	Absent
Leaf serration	Medium	Medium	Fine
Leaf hairiness	Very low	Low	Medium
Leaf base	Cordate	Cordate	Triangular
Leaf petiole pigmentation	Absent	Present	Absent
Stem pigmentation	Absent	Very weak	Weak
Stem hairiness at the top	Medium	Strong	Strong
Ray floret shape	Elongated	Elongated	Rounded
Ray floret colour	Yellow	Orange	Orange
Disc floret colour	Yellow	Dark purple	Purple
Pollen colour	Yellow	Yellow	Yellow
Bract shape	Rounded	Elongated	Elongated
Bract anthocyanin colouration	Absent	Present	Absent
Position of lateral head to the central head	-	Below	Below
Head shape	Concave	Concave	Concave
Plant branching	Absent	Present	Present
Type of branching	-	Basal & top	Full
Seed shape	Elongated	Broad ovoid	Narrow ovoid
Seed base colour	Black	Brown	Brown
Seed stripe	Absent	On margin	Between margin
Seed stripe colour	-	Brown	Brown
Seed mottling	Absent	Absent	Present

period in hybrids between *H. annuus* and *H. argophyllus*. Maturation of the main head began 5-10 days earlier than the cultivated sunflower. The highest quantity of seeds was obtained after open pollination, followed by backcrossing with pollen from cultivated sunflower. These results are in agreement with the studies of Hristova-Cherbadzi (2004) and Jan and Feng (2004) where the F_1 hybrids failed to produce seed from self-pollination, indicating a high degree of self-incompatibility.

Concave head shape was observed in the F_1 interspecific hybrids. In contrary, Nikolova and Christov (2004) observed convex head in *H. argophyllus* (E-007) x L.1234, and L.2607 x *H. argophyllus* (E-091) and LHA-300 x *H. argophyllus* (E-091).

Cytology of parents and their F_1 hybrids

Meiotic studies were carried out on parents and the F_1 to know the behavior of chromosomes at diakinesis of metaphase I and in anaphase I and II. Both the parents showed normal pairing between the homologous chromosomes at diakinesis and metaphase I with the formation of 17 bivalents in different forms during diakinesis. There were no laggards and chromosome bridges during anaphase and no micronuclei during tetrad formation (Fig. 1a-j). indicated that meiotic division proceeds normally and resulting in normal pollen and seed fertility. Similar results were recorded by Binsfeld et al. (2001) and Kesavaraman et al. (2006). Sujatha et al. (2008) reported that diakinesis is not suitable stage for

Table 2. Morpho physiological characteristics of F_1 hybrid and parents

Characteristic	*H. annuus* (ARM-243B)	*H. annuus* (ARM-243B) x *phyllus* (PI-468649)	*H. argophyllus* (PI-468649)
Morphological characteristics			
Days to 50% flowering (days)	48.5	74.6	79.4
Plant height (cm)	144.3	318.6	267.0
Leaf number	28.4	60.2	62.1
Leaf length (cm)	18-22	24-36	22-26
Leaf width (cm)	14-19	20-27	13-14
Ray floret number	36.2	30.3	22.5
Ray floret length (cm)	5.1	3.2	3.1
Ray floret width (cm)	1.5	1.6	0.6
Head diameter (cm)	14.6	6.3	4.2
Seed length (mm)	1.6	0.8	0.5
100-seed weight (g)	5.8	2.3	1.7
Physiological development			
Period of vegetation (days)	94.1	127.5	140.1

studying various chromosome associations (univalents, bivalents and multivalents) and different configurations like rod, ring, chain,'8'and other unique shapes. In the present study, 34 chromosomes were observed in the meiotic stages of interspecific cross as also reported previously by Narkhede et al. (1986).

At diakinesis stage, several bivalent shapes were observed (Table 3) *viz.,* ring, rod and 'U' bivalents occurred more frequently than other configurations, like '8', 'V', loose chains and bracket. Georgieva-Todorova (1984) considered autosyndesis of chromosomes as the cause of ring bivalents and heteromorphic nature (formed by conjugation between homeologous chromosomes) of chromosomes as the cause of more frequent rod bivalents (Cedeno et al. 1985). In the present investigation, high frequency of rod shaped bivalents was registered similar to the results of Narkheda et al. (1986) who observed high frequency of rod bivalents than ring bivalents in *H. annuus* x *H. argophyllus* crosses. They attributed loose pairing of bivalents was the cause for high frequency of rod shaped bivalents and open ring shaped bivalents or 'U' shaped bivalents. In this study, the occurrence of univalents ranged from zero to three, exceptionally a few PMCs showed as high as four univalents during diakinesis (Fig. 2). Dolgova et al. (2007) reported zero to four univalents in cross, *H. annuus* x *H. argophyllus*.

Out of 50 PMCs (Table 4) studied, it was observed that four chromosome bridges were noticed in which only single bridges was observed rather than double bridges (Fig. 3). The maximum number of chromosome bridges and fragments observed which may be likely due to the number of paracentric inversions differentiating parents species (Chandler et al. 1986; Narkhede et al. 1986; Georgieva-Todorova 1984). Manjula et al. (1999) reported the appearance of chromosome bridges, fragments and laggards during Anaphase I in the interspecific hybrids of sunflower with wild *Helianthus* species. The results of the present study indicated that the parental species used may differ for at least zero to four translocations with same number of laggards.

Pollen fertility

The pollen fertility of ARM-243B and PI-468649 observed was 97.6 and 96.4%, respectively while in interspecific crosses was 89.9% indicating good cross compatibility between *H. annuus* L. and wild annual diploid *H. arogophyllus*. An interspecific hybrid comprises genome from both the parents and presence of wild genome in the hybrid is enough to cause meiotic irregularities that lead to reduced pollen fertility in the interspecific hybrids. Earlier we had recorded reduction in pollen stainability (87.6%) in the F_1 interspecific hybrids between cultivated sunflower and *H. argophyllus*. Reduced pollen viability in different F_1 hybrids has also been reported by others (Georgieva-Todorova 1984; Espinasse et al. 1995). These differences are probably due to the use of different populations (accessions) of the same species. Manjula and Seetharam (2000) observed lower pollen fertility in the interspecific hybrids and explained that pairing abnormalities like quadrivalents in earlier stages and abnormal disjunction in later stages of meiosis resulted in low pollen fertility.

Confirming hybridity using SSR markers

Identification of hybrids based on morphological intermediacy can be difficult (especially at early stages), ambiguous, time consuming, and dependent on environment (Lin et al. 2010) and therefore analysis at the DNA level using molecular techniques is required. In the present study, both the parents were analyzed for parental polymorphism using 94 SSR primers of which 14 showed polymorphism between

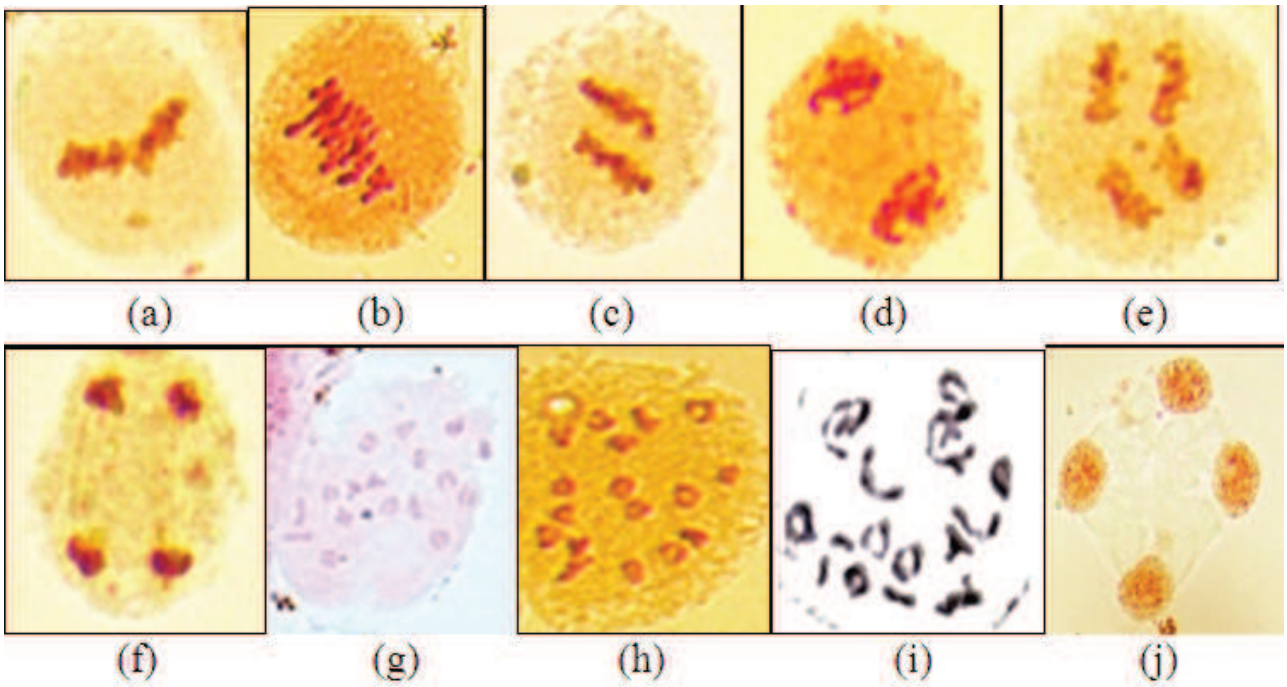

Fig. 1. Chromosomal behaviour during meiosis in the interspecific hybrids - (a & b) = Metaphase, (c). Anaphase I, (d) = Telophase I, (e) = Anaphase II, (f) = Telophase II, (g) = 17 bivalents in ARM-243B; (h & i) = chromosomes of interspecific hybrids and (j) = Normal tetrad

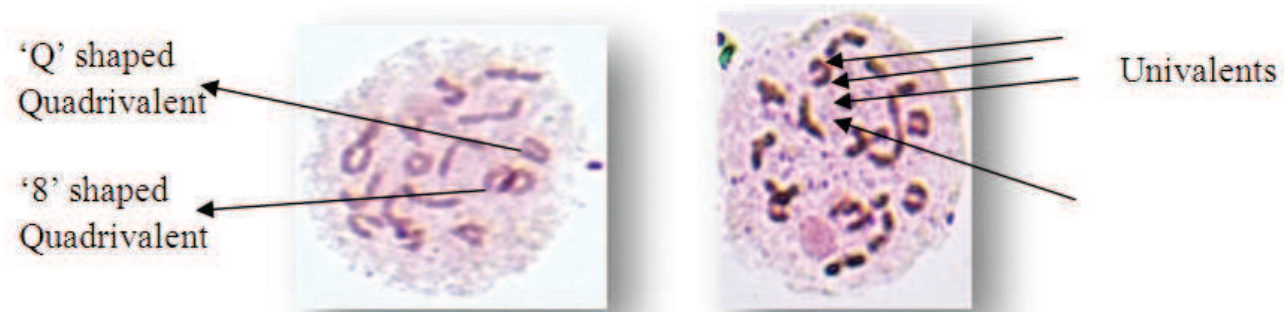

Fig. 2. Different chromosome configurations in interspecific hybrids

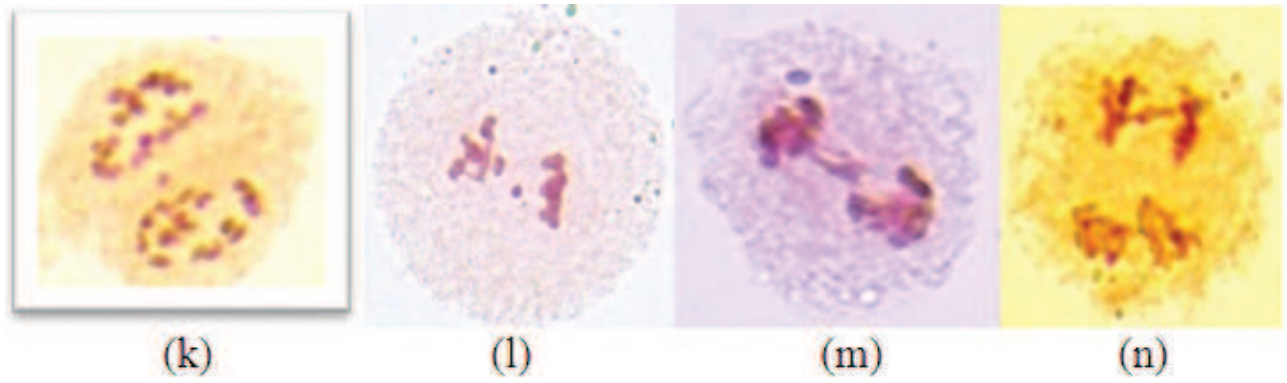

Fig. 3. Chromosomal alignment in Anaphase I, II stages of meiosis in cross; *H. annuus* x *H. argophyllus*; k) = Single laggard; l) = Two laggards; m) = Bridge formation at Anaphase 1; n) = chromosomal bridge at Anaphase II

Table 3. Frequency of various forms of chromosomes observed in *H. annuus* L. (ARM-243B), *H. argophyllus* (PI-468649) and interspecific cross (*H. annuus* × *H. argophyllus*)

	Univalents	Bivalents										Trivalents	Quadrivalents					
		U	V	Ring	Rod	8	χ	Chain	δ	γ	( )	Other		Ø	8	χ	δ	other
ARM-243B	-	0-3	0-8	2-7	0-3	0-2	0-2	0-1	0-1	0-2	0-2	0-1	-	-	-	-	-	-
H. argophyllus (PI-468649)	-	1-8	0-12	1-6	0-6	0-1	0-2	0-1	0-1	0-1	0-1	0-2	-	-	-	-	-	-
Interspecific cross (F_1)	0-6	0-8	0-5	0-6	1-12	0-3	0-1	0-1	0-1	0-1	0-2	0-2	0-3	0-2	0-2	0-1	0-2	0-2

Table 4. Observations on meiotic abnormalities during Anaphase I, Anaphase II and Telophase II of parents and interspecific hybrids (*H. annuus* L. x *H. argophyllus* T. & G.)

	No. of PMCs observed	No. of PMCs showing chromosome bridges					No. of PMCs showing laggards in Anaphase I		No. of cells with precocious chromosome in Anaphase I	No. of cells with micronuclei in Telophase II
		Anaphase I			Anaphase II					
		0	1	2	0	1	1	2		
H. annuus L. (ARM-243B)	50	50	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
H. argophyllus (PI-468649)	50	50	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Interspecific cross (ARM-243B x PI-468649)	50	46	4	0	47	3	4	3	Nil	Nil

Fig. 4. Allelic variation of ARM-243B, ARG-1317 (PI-468649) and hybrid confirmation by using ORS-05, ORS-896 and ORS-906, L-100bp DNA ladder, P_1- ARM-243B and P_2 ARG-1317 (PI-468649)

the parents. Of the polymorphic primers, ORS-05, ORS-896, and ORS-906 showed 50, 30 and 230 bp allelic variations, respectively and were used in the study. The marker ORS-05 amplified alleles of size of 350 bp/300bp ORS-896 amplified alleles of size 210 bp/180 bp while ORS-906 amplified alleles of 480 bp/250 bp in ARM-243B and PI-468649 (Fig. 4). Hence, these three primers ORS-05, ORS-896 and ORS-906 with amplicons specific to both the parents in the F_1 plants in a co-dominant manner, established the hybridity of the F_1s (Fig. 4).

Molecular markers are useful tools in the identification of interspecies sunflower hybrids (Rieseberg et al. 1995; Binsfeld et al. 2001). SSR markers are particularly useful for reliable identification of interspecies hybrids between cultivated sunflower and diploid species from the genus *Helianthus* (Terziæ et al. 2006; Hristova-Cherbadzi 2009).

In conclusion, this is the first successful report at production of

interspecific hybrids of cultivated sunflower with *H. argophyllus* (PI-468649). This diploid annual species with leaf pubescence is reported to confer resistance to drought and downy mildew. Interestingly, in our study the *H. argophyllus* accession (PI-468649) and the resultant interspecific hybrids were found to be resistant to leafhopper which is a serious problem on sunflower in India. Further work on the confirmed interspecific hybrids with regard to generation advancement and selection of promising pre-breeding lines with agronomically exploitable traits is in progress.

Authors' contribution

Conceptualization of research (HPM, MS); Designing of the experiments (HPM); Contribution of experimental materials (HPM, MS); Execution of field/lab experiments and data collection (HPM, MS, PKS); Analysis of data and interpretation (HPM, MS); Preparation of manuscript (HPM, MS).

Declaration

The authors declare no conflict of interest.

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