Original Research

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in
Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/41/1/MRN-1116

Cytomorphological and molecular characterization of inter-specific hybrid between cultivated sunflower and *Helianthus argophyllus*

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Paper received: 25.02.2019 *Revised received*: 11.07.2019 *Accepted*: 03.10.2019

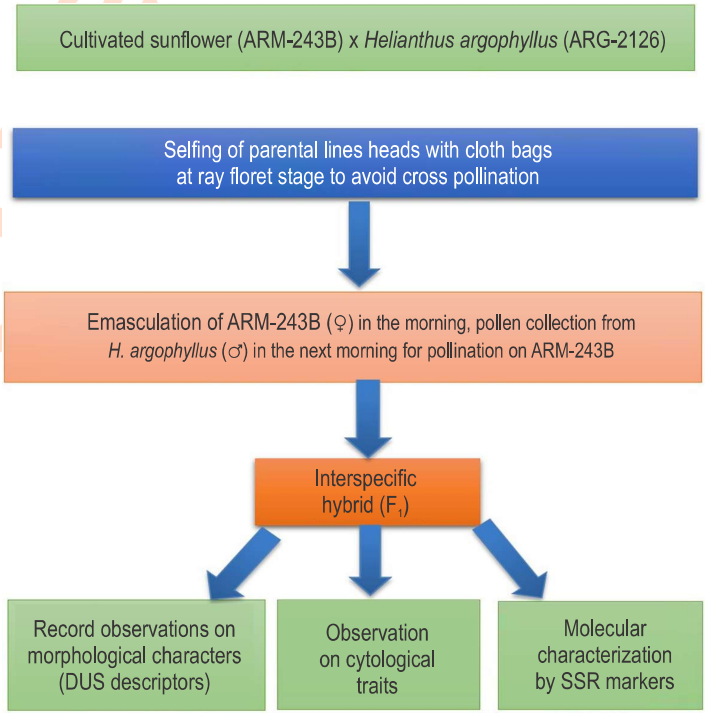
Abstract

Aim : To study useful variability generated in an inter-specific hybrid between cultivated sunflower inbred (ARM-243B) and silver leaf sunflower, *H. argophyllus* (ARG-2126) through cytomorphological traits and hybrid confirmation by molecular markers.

Methodology : The present study on hybridization was undertaken using cultivated sunflower inbred (ARM-243B) and silver leaf sunflower *H. argophyllus* (ARG-2126). Thirty one morphological characters were recorded of cultivated sunflower (ARM-243B), *H. argophyllus* (ARG-2126) and inter-specific hybrid. A total of 50 PMCs were analysed for cytological observations. 62 SSR primers were used for molecular characterisation and hybridity confirmation in inter-specific hybrid.

Results : Considerable variability was observed in inter-specific hybrid, with common traits including branched stems, anthocyanin coloration in petiole and disk florets, leaf petioles, and stems etc. Cytological abnormalities and reduction in pollen fertility indicated that generated inter-specific hybrid was true type. Out of sixty two SSR primers, nineteen showed parental polymorphism. ORS-1021 showed 10 bp allelic variation in inter-specific hybrid in a co-dominant manner and, hence, was used for hybrid confirmation.

Interpretation : The results of this investigation showed that inter-specific hybridization between cultivated sunflower and *H. argophyllus* and transfer of desirable traits into cultivated sunflower is possible.



Key words: Interspecific hybridization, Meiosis, Pollination, Sunflower

How to cite : Meena, H.P., M. Sujatha, H.D. Pushpa and J.J. Lal: Cytomorphological and molecular characterization of inter-specific hybrid between cultivated sunflower and *Helianthus argophyllu. J. Environ. Biol.*, **41**, 66-72 (2020).

Introduction

Changing climatic conditions essentially warrant broadening of genetic base to address the problems faced by sunflower cultivars. Because of narrow genetic base of this crop (Nooryazdan *et al.,* 2011), new approaches need to be adopted in sunflower breeding programme. In recent years, interest in inter-specific hybridization has been greater for transferring useful genes from wild species into cultivated lines to develop pre-breeding germplasm for sunflower improvement (Seiler *et al.,* 2017). Wild *Helianthus* spp. offer a significant amount of genetic diversity for further improvement of cultivated sunflower, including important traits such as disease (Fernandez-Martinez *et al.,* 2010) and insect pest resistance, cytoplasmic male sterility (Kantar *et al.,* 2015; Christov, 2013), fertility-restoration, agronomic and seed-oil characteristics, drought tolerance, protein content and fatty acid composition (Jan *et al.,* 2008; Warburton *et al.,* 2017). *Helianthus argophyllus* is known as 'silver leaf sunflower' since 1889 (Burpee seed catalogue).

Cultivated sunflower and *H. argophyllus* are closely related morphologically. The most conspicuous difference is found in the leaves, stems and bracts. Pubescence of *H. argophyllusis* covered with long silky hairs give plants a silvery grey colour whereas pubescence in *H. annuus* consists of short, rough hairs and the plants are green in colour. Most of the germplasm used in sunflower breeding programs have originated from limited genetic material, resulting in crop with an extremely narrow genetic base. Hence, sunflower breeders have explored and exploited variability found in wild *Helianthus* species. Wild *H. argophyllus* species possess useful variability for resistance to drought, diseases and parasitic Orobanche plant, which can be utilized for the improvement of cultivated sunflower (Jan *et al.,* 2008). The germplasm derived from *H. argophyllus* through several cycles of introgression have shown great value for sunflower breeding due to their resistance to drought with higher yield and stability (Griveau *et al.,* 1996). Therefore, it can be utilized as a source of favorable alleles for salt and drought tolerance (Rauf, 2008) and insect resistance (Sujatha and Lakshminarayana, 2007).

The first reported case of introgression of downy mildew resistance from *H. argophyllus* involved the resistance gene cluster containing *Pl8* (Bert *et al.,* 2001). The inbred line Arg 1575-2 carries *P. halstedii* resistance locus PlArg, introgressed from the wild species *H. argophyllus,* which confers resistance to all known races of fungus (Seiler, 1991). Soviet scientist Satsyperov first generated inter-specific hybrid between *H. annuus* and *H. argophyllus* to introgress rust resistance in cultivated sunflower. Kurnik and Walcz (1985) also reported brown stem canker resistance genes in *H. argophyllus.* Therefore, utilization of *H. argophyllus* in inter-specific hybridization with cultivated sunflower is a promising method for genetic enhancement of the crop for several agronomically desirable attributes. In light of the above scenario, the present investigation was carried out to study the cytomorphological variation and introgression of disease resistance genes or other major agronomic traits in cultivated sunflower.

Materials and Methods

Plant material: Cultivated sunflower seeds (ARM-243B; 2n=2x=34) were obtained from the Breeder Seed Production (BSP) Unit of ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad, India. Whereas seed material of wild diploid annual species *H. argophyllus* (2n=2x=34) [accession, ARG-2126 (PI-468651)] was obtained from the United States Department of Agriculture (USDA, USA) and established at Narkhoda Farm of ICAR-IIOR, Rajendranagar, Hyderabad.

Inter-specific hybridization: Inter-specific crosses were made between cultivated sunflower and *H. argophyllus*. Staggered sowing of female parent, twice at weekly interval, was done to synchronize flowering. The heads of cultivated parent (female) and wild *H. argophyllus* accession (male) 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 pollens from *H. argophyllus* in the following morning and re-bagged. The procedure was repeated till the opening of all the disc florets on the capitulum. Seeds collected from the crosses as well as from the parents were sown in the field at Narkhoda Farm, ICAR-Indian Institute of Oilseeds Research, Hyderabad during *kharif*-2015.

Morphological and phenological characterization: Morphological and phenological characterization of *H. argophyllus,* the cultivated sunflower inbred (ARM-243B) and the hybrid progenies was done. The observations were made on 3 plants grown in field conditions. The following traits were studied: hypocotyl pigmentation, days to 50% flowering, leaf size, leaf shape, leaf colour, leaf blistering, leaf serration, leaf hairiness, leaf base, leaf petiole pigmentation, stem pigmentation, stem hairiness at the top, ray floret number, ray floret shape, ray floret colour, disc floret colour, pollen colour, bract shape, bract anthocyanin colouration, position of later head 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,seed stripe colour and seed mottling.

Cytological analysis: For meiotic observations, flower buds at appropriate stage were collected from plant and fixed in freshly prepared Carnoy's fluid (ethanol : chloroform : acetic acid - 6 : 3 : 1), for a minimum period of 24 hrs at room temperature and subsequently stored in 70% alcohol at 10°C. For meiotic analysis, anthers were squashed in 1% acetocarmine (Georgieva-Todorova, 1990) and a total of 50 pollen mother cells (PMCs) obtained from parents and hybrids, were analyzed at diakinesis, metaphase I, anaphase I and telophase II stages. The results of meiosis are shown through chromosome configuration and regularity of meiosis. Observations were made on squash

preparations. Meiotic irregularities were expressed as percentage meiocytes with deviations in relation to the total number of PMCs studied.

Pollen fertility: Pollens were stained in 1:1 mixture of 1% acetocarmine and glycerol. Pollen viability was studied following the method of Alexander (1969). The method is based on differential staining of viable and non-viable pollen grains. Pollens from non dehiscent 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 grains were recorded as sterile.Viable and non-viable pollen grains were counted on three slides at 10 positions per slide. Pollen viability was expressed as percentage of viable pollen grains in comparison to the total number. The percentage of pollen fertility was worked out.

Molecular analysis: Total genomic DNA from fresh leaves of 4-week-old ARM-243B, ARG-2126 was extracted by Cetyl-Tri-methyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987) and quantification was done by Nanodrop spectrophotometer. DNA was diluted in 0.1 T.E buffer to a concentration of 50 ng-µ l^{-1} for PCR analysis. Using the information available in respect of marker distribution on genetic linkage map, SSR primers of ORS series (Knapp, 2004) were selected. PCR amplification was carried out in 15 µl of reaction mixture, containing 0.5 µM of each primer, 0.25 mM dNTPs, 0.6 U *Taq* DNA polymerase, 1X PCR buffer with 1.5 mM $MgCl_2$ (Bangalore Genei, India) and 50 ng of template DNA. Amplification was performed in a thermal cycler (Applied Biosystems Gene Amp 9700) using the following amplification conditions: 2 min. at 94 °C for 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 hr at 90 volts. The gels were stained with ethidium bromide (50 ng-m l^{-1}) and documented using the Syngene gel documentation system.

Results and Discussion

Peculiar differences observed in morphological characters among the parents and inter-specific hybrid plants are revealed in Table 1. The hypocotyl pigmentation differed in both the parents as it was absent in cultivated sunflower ARM-243B, and medium pigmentation was present in wild annual diploid *H. argophyllus.* The cross showed the character as that of wild parent (Table 1). There was considerable difference in days to 50 percent flowering. The cultivated species inbred ARM-243B flowered early (46 days) while *H. argophyllus* was late in flowering (78 days), the inter-specific hybrid was intermediate and flowered in 69 days. Nikolova and Christov (2004) reported similar results of 50 percent flowering in inter-specific cross between *H. annuus* L. line LHA-300 x *H. argophyllus* (E-091). Remarkable variations were detected in leaf parameters *i.e.,* size, colour, hairiness, pigmentation as unveiled in Table 1. The leaf size was medium in case of ARM-243B and *H. argophyllus* while the cross had medium sized leaves. The leaf colour of cultivated sunflower was medium green compared to *H. argophyllus* which was ashy green, however the cross exhibited dark green leaf colour which was more towards the female parent. Our results is in agreement with Encheva and Christov (2006) who reported dark green leaves and intermediacy for head diameter in inter-specific cross between *H. annuus*× *H. salicifolius.*

The leaf hairiness was less in cultivated sunflower ARM-243B, while dense pubescence was found in *H. argophyllus* however, the cross had low hairiness which was more towards female parent. Recently, similar results were reported by Vishnutej *et al.* (2016). Inter-specific hybrid was more similar towards wild species for stem hairiness, ray and disc floret colour, bract shape, position of lateral head to the central head, plant branching, seed base colour and seed stripe colour, while it was more towards female parent for few traits like leaf shape, ray floret shape and seed mottling. These characters indicate that the two inter-specific hybrids consist of genetic material from both cultivated and wild sunflower. It showed that most of the wild characters were dominant over cultivated sunflower characters. The inter-specific hybrid was similar to both the parents for leaf shape and head shape. More luxuriance was observed only for plant height compared to both the parents. Few seeds were set in F_1 plants due to self-incompatibility.

However, very few seed set was achieved under self-pollination and enough seed setting was obtained under open pollination followed by back crossing with pollens from cultivated sunflower. These results are in agreement with the study of Meena *et al.* (2017a) where F_1 hybrids failed to produce seeds from self-pollination, indicating a high degree of self-incompatibility. Three types of branching were observed: basal, axil, and branching both at the base and apical part of the stem. Similar results and observations were reported by Nikolova and Christov (2004). Branching in cultivated sunflower is controlled by recessive gene and in wild species by several dominant genes (Clement and Deihl, 1968). Two to three backcrosses can be required to eliminate the undesirable traits of wild sunflower. However, the backcrosses cannot be avoided as the presence of undesirable characters will not only hamper the quality but also reduce the commercial value of the crop.

Head shape in cultivated inbred and wild species was concave and same type of head shape was also observed in inter-specific hybrids. Nikolova and Christov (2004) observed convex head in hybrid combinations of *H. argophyllus* x L.1234, L.2607 x *H. argophyllus* and L.HA-300 x *H. argophyllus,* indicating similar results. New characters like leaf serration, ray floret shape and types of branching were also observed in inter-specific hybrids which may be due to more heterozygosity in both the parents (Meena *et al.,* 2017a). In both parental species, pairing between the homologous chromosomes were normal at diakinesis and metaphase I with 17 bivalents. Meiotic analysis of inter-specific

hybrid of *H. annuus* and *H. argophyllus* showed chromosomal abnormalities like univalents, bivalents, trivalents and quadrivalents in a total of 50 PMCs analysed (Fig. 1, 2 and 3). These results are in conformity with the findings of Meena *et al.* (2017b) and Narkhede *et al.* (1986) in which the chromosome association of 13II (bivalents) + 2IV (quadrivalents) was occasionally observed (Table 2). Quadrivalents observed in the present study were either in chain or ring form. This indicated that translocation might be the cause of quadrivalent formation and higher frequency of chain quadrivalents might be due to unequal size of segment exchanged between non-homologous chromosomes (Meena *et al.,* 2017b). The later stages of meiosis also showed abnormality with chromosome bridges and lagging chromosomes.

The presence of single quadrivalent at diakinesis/ metaphase I indicated that the genome of *H. argophyllus* differs from that of *H. annuus* by at least one reciprocal translocation. Meena *et al.* (2017a, b) reported that wild *H. argophyllus* genome was different from cultivated sunflower by at least one reciprocal translocation in *H. annuus* x *H. argophyllus* cross combination. In the present investigation, high frequency of ring bivalents was registered in inter-specific hybrids than other configurations like '8', 'V', loose chains, bracket, etc., in contradiction to the results of Yushkina *et al.* (2009) who observed high frequency of rod bivalents than ring bivalents in *H. annuus* x *H. argophyllus* hybrids. Higher chromosome associations such as trivalents and quadrivalents during meiosis were reported in inter-specific hybrids involving cultivated and wild diploid species of *Helianthus* (Prabakaran and Sujatha, 2004; Kesavaraman *et al.,* 2006). This led to the conclusion that the genomes of these species in the primary gene pool differed from each other by a limited number of reciprocal translocations. Exchange of unequal chromatin segments between the non-homologous chromosomes (translocation of chromosomes) may be a reason for the formation of quadrivalents. Presence of quadrivalents at diakinesis stage in inter-specific hybrids is well documented (Chandler *et al.,* 1986). Kulshreshta and Gupta (1979) also reported difference of one reciprocal translocation as the cause for the formation of single quadrivalent in *H. annuus* x *H. argophyllus* inter-specific hybrid. Frequency of pollen fertility varied among parents and inter-specific hybrid. The diploid parental species showed pollen stainability as high as 98.2% in *H.*

Table 1: Morphological characters of parents and their inter-specific hybrid (*H. annuus* × *H. argophyllus*)

Characters	*H. annuus* (ARM-243B)	*H. annuusx* ARG-2126 (F_1)	*H. argophyllus* (ARG-2126)
Hypocotyl pigmentation	Absent	Dark pigmentation	Medium pigmentation
Days to 50% flowering (days)	46	78	69
Leaf size	Medium	Broad	Medium
Leaf shape	Cordate	Cordate	Cordate
Leaf colour	Medium green	Dark green	Ashy colour
Leaf blistering	Very weak	Medium	Absent
Leaf serration	Medium	High	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 number	36	30	22
Ray floret shape	Elongated	Elongated	Rounded
Ray floret colour	Yellow	Orange	Orange
Disc floret colour	Yellow	Dark purple	Purple
Pollen colour	Yellow	Orange	Yellow
Bract shape	Rounded	Elongated	Elongated
Bract anthocyanin colouration	Absent	Present	Absent
Position of later head to the central head	-	Below	Below
Head shape	Concave	Concave	Concave
Head diameter (cm)	14.6	6.8	3.4
Plant height (cm)	144.3	328.6	278.0
Plant branching	Absent	Present	Present
Type of branching	Absent	Basal	Full
Seed shape	Elongated	Broad ovoid	Narrow ovoid
100-seed weight (g)	5.8	3.5	1.8
Seed base colour	Black	Brown	Brown
Seed stripe	Absent	On margin	Between margin
Seed stripe colour	Absent	Brown	Brown
Seed mottling	Absent	Absent	Present

Table 2: Frequency of PMCs showing different chromosome association at diakinesis

Species and crosses	PMCs scored	Number of cells with chromosomal association				Pollen fertility %
		17 II	15 II+1 IV	13 II+ 2 IV	15 II+1III+1I	
H. annuus	50	50	-	-	-	98.2
H. argophyllus	50	50	-	-	-	97.6
H. annuus x H. argophyllus	50	-	46	2	2	87.6

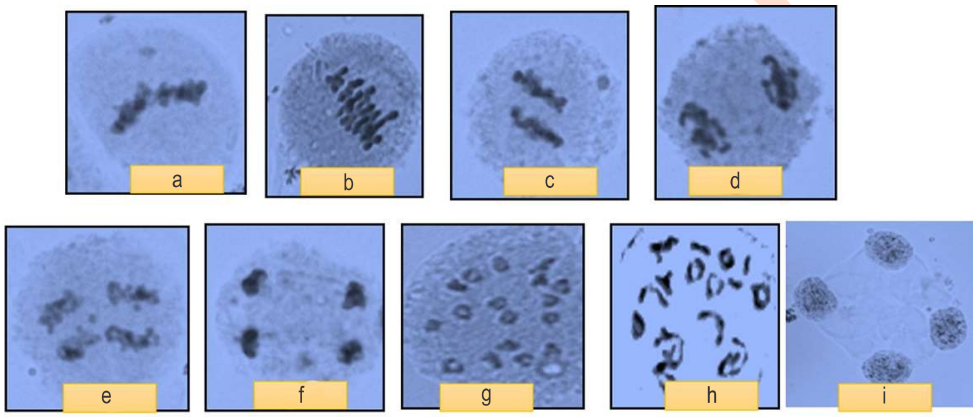


Fig. 1: Chromosomal behaviour during meiosis in inter-specific hybrid: (a, b) - Metaphase; (c). - Anaphase I; (d) - Telophase I; (e) - Anaphase II; (f) - Telophase II; (g) - 17 bivalents in ARM-243B; (h) - chromosomes of interspecific hybrid and (i) - Normal tetrad.

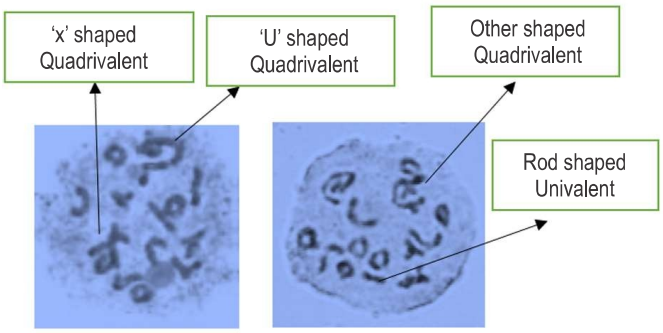


Fig. 2: Different chromosome configuration in interspecific hybrid.

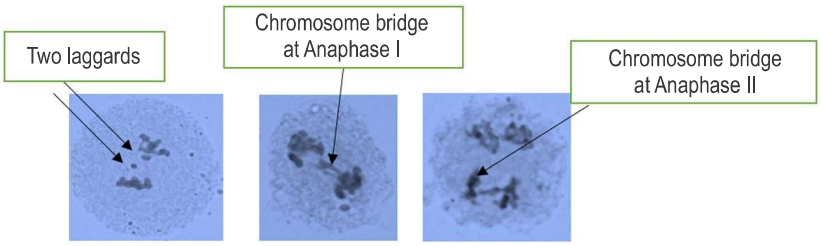


Fig. 3: Chromosomal alignment in Anaphase I, II stages of meiosis.

annuus and 97.6% in *H. argophyllus.* However, the inter-specific hybrid showed reduction in pollen stainability, recording only 87.6%. In the present study, chromosome bridges, laggards and presence of quadrivalents were observed possibly due to structural differences in the chromosome, leading to low pollen fertility in the inter-specific cross. Recently, Meena *et al.* (2017a,b) also detected reduced pollen viability in *H. annuus* x *H. argophyllus* inter-specific cross. Pollen viability in *H. eggertii* x *H. annuus* hybrid was 54.7%, which was very close to 42.8% as recorded by Christov (1988). These differences are probably due to the use of different populations (accessions) of same species. Reduced pollen-fertility in inter-specific hybrids has been reported by Manjula and Seetharam (2001).

Since inter-specific hybrid has genomes derived from both parental species, the presence of wild genome in hybrid causes meiotic irregularities that lead to reduced pollen fertility. Hybrid is usually identified on the basis of their morphological characteristics. However, identification based on morphological characteristics can be difficult (especially at early stages), ambiguous, time-consuming and dependent on environment (Lin *et al.,* 2010). In addition, the parents in some crosses might have high similar morphological characters or produce hybrid with morphological characters that cannot be visually distinguished from their respective female parents and, hence, progeny arising from self-pollination cannot be differentiated. In such cases, morphological characteristics alone are insufficient for the identification of hybrid and analysis at DNA level using molecular techniques (Verma *et al.,* 2017). In the present study, both the parental species ARM-243B and *H. argophyllus* were analyzed for parental polymorphism using sixty two SSR primers. Out of 62 sunflower SSR primers, 43 primers were monomorphic and 19 showed polymorphism between the parents, of which ORS-1021 showed 10 bp allelic variation and was used in the study.

Female parent ARM-243B showed amplification of 305 bp, whereas the male parent ARG-2126 yielded amplified product of 295 bp. Hence, the primer ORS-1021 was used to confirm hybridity and presence of amplicons specific to both the parents (Fig. 4) in F_1 plants in a co-dominant manner established hybridity of inter-specific hybrid (Fig. 4). Molecular markers are useful tools in identifying inter-species sunflower hybrids (Binsfeld *et al.,* 2001). Paniego *et al.* (2002) demonstrated the potential of SSR markers in identification of inbred lines of sunflower and assessment of their distinctness and genetic diversity. SSR markers are particularly useful for identifying inter-species hybrids between cultivated sunflower and diploid species from the genus *Helianthus* (Terzić *et al.,* 2006). Meena *et al.* (2017 a,b) also used SSR markers for confirming hybridity of inter-specific hybrids generated using cultivated *H. annuus* x *H. argophyllus.* The markers identified may be useful as reference markers in future breeding programme for identification of putative inter-specific hybrids.

In conclusion, *H. argophyllus* diploid annual species with leaf pubescence is reported to confer resistance to diseases like drought and insect pests like leaf hopper. Interestingly, in this study *H. argophyllus* accession (PI-468651) and the resultant inter-specific hybrids were found resistant to leafhopper as well as drought tolerant, which pose serious problem to sunflower, crop in India.

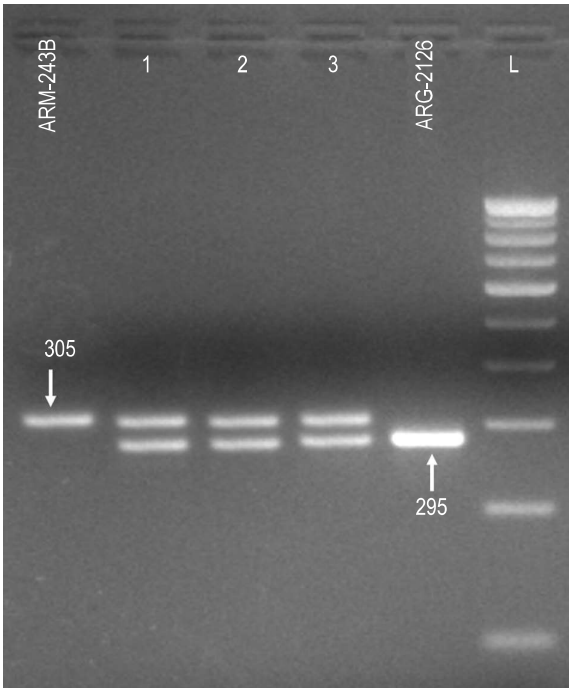


Fig. 4: Allelic variation of ARM-243B, ARG-2126 and three plants of inter-specific hybrid confirmed by using ORS-1021, L, 100 bp DNA ladder.

Acknowledgments

The authors are thankful to the Director, ICAR-Indian Institute of Oilseeds Research, Hyderabad (Telangana) India, for providing financial support and facilities to carry out this-research work. Dr. A.J. Prabakaran is also duly acknowledged for the support extended during cytological work.

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