www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(8): 629-633

www.thepharmajournal.com
Received: 19-06-2021
Accepted: 24-07-2021

RN Dhawale
Assistant Professor, VD College of Agril. Biotechnology, VNMKV, Parbhani, Maharashtra, India

MD Mahalle
PhD. Scholar, Department of Agril. Biotechnology, Assam Agril. University, Assam, India

KM Sharma
Assistant Professor, VD College of Agril. Biotechnology, VNMKV, Parbhani, Maharashtra, India

SD Karnewar
Assistant Professor, Agricultural Development Trust, College of Afril., Baramati, Maharashtra, India

Corresponding Author:
RN Dhawale
Assistant Professor, VD College of Agril. Biotechnology, VNMKV, Parbhani, Maharashtra, India

Assessment of genetic diversity in an elite set of restorer lines of *Helianthus annuus* L using PCR-based method

RN Dhawale, MD Mahalle, KM Sharma and SD Karnewar

Abstract
Genotyping of ten sunflower cultivars were done by 112 SSR primers generated differential banding patterns among different lines. All SSR primers were able to generate with an average 29.74 amplicons per primer. Out of total 3331 amplicons 21.08% were showed polymorphism among lines with 06.21 average numbers of polymorphic bands. Ha806-ar primer was the most informative primer as it generated the highest number of polymorphic amplicons while Ha357-ar stood least informative by generated less numbers of polymorphic amplicons. The lowest numbers of amplified fragments were generated by primer Ha360-ar. A perusal of dendrogram analyzed with a Euclidian distance of 0.26. The diversity in DNA level within 10 cultivars was indicating that sunflower cultivars did not show much variation at DNA level. Two groups (LSF-10, GMU-479 and LDM-72, LDM-02) were identified within which all the sunflower cultivars share 100% similarity for molecular (SSR) level, and also one group was also none of single line were identified with 100% dissimilarity.

Keywords: SSR, sunflower, GMU-479

Introduction
Worldwide, sunflower is fourth in importance as an oilseed after soybean, groundnut, rapeseed, and mustard and is grown on 23.4 million ha with a total annual production of 31.1 million tons and an average yield of 1.32 t.ha^{-1} (Bartholomew S.A. and Olubukola O.B., 2020). The cultivated sunflower (*Helianthus annuus* L.) is one of the three important edible oilseed crop grown in the world after soybean and groundnut. Sunflower is probably originated in Southern United States and Mexico from where it was introduced into Europe and later into former USSR. Sunflower (*Helianthus annuus* L.) belongs to family Asteraceae (formerly Compositae). Sunflower is essentially a cross-pollinated plant, besides showing varying degrees of self-incompatibility. The commercial sunflower has a narrow genetic base and as a consequence only a few introductions constitute the base material for the development of new cultivars or hybrids (Sujatha *et al*., 2006) [9].

The major reasons for the low productivity of the sunflower crop were, on the one hand, the highly fluctuating pedoclimatic conditions that were becoming more and more severe in the recent years and the different biotic stress factors, which are the bottleneck for increasing the productivity of sunflower, susceptibility to fungal diseases is a major limiting factor for increasing sunflower production in world. As all the currently grown varieties and hybrids are susceptible to Alternaria leaf spot, which is widespread in the world, it is necessary to identify durable resistance and this has to be a major thrust area in resistant breeding programmes (Sujatha M and Prabakaran A. J. 2001) [10].

As a first step in this direction, efforts are needed to identify the resistant sources. Only in some perennial tetraploid and hexaploid species particularly in *H. tuberosus* there are indications of existence of some resistance. Due to the limited natural variation, the genetic improvement of the cultivated sunflower for such agronomic traits has been broadly based on the ability to transfer the desired genes from wild relatives through conventional breeding methods, but unfortunately the utilization of many wild species has been limited by natural barriers to the reproductive process such as embryo abortion (Sujatha M. *et al*., 2001) [10].

Now-a-days, SSRs marker became a choice of molecular markers for assessment of population genetic analyses including DNA fingerprinting, genetic mapping, and molecular breeding in crop plants (Powell *et al*., 1996). In sunflower, several marker systems have been employed for assessment of genetic diversity in cultivated germplasm and wild sunflowers. Biochemical and molecular polymorphism were sufficient for distinguishing between closely or distantly related germplasm accessions in sunflower (Rieseberg and Seiler, 1990 [5].

The choice of a molecular marker technique depends on its reproducibility and simplicity. The best markers for phylogenic studies and crop conservation have low cost and labour requirements and high reliability, microsatellites or simple sequence repeats (SSRs), are short DNA sequences harboring motifs of 1-6bp that are tenderly repeated (Tautz and Renz, 1984) [12]. In this milieu, the present investigation aims at, analysis of genetic diversity using PCR based molecular markers (SSR).

Materials and Methods

Plant material

For the purpose of molecular comparative assessment of genetic diversity among three elite lines with other seven lines of sunflower (*Helianthus annuus* L.) through molecular marker, collected from Oilseed Research Station, Latur. Ten Cultivars of sunflower are DMLT-1Y, GMU 479, LSF-10, CMS-127 A, LDM O2, LDM72, R-274, DSF_{2A}, RHA-1-1, and 6D -1R. LSF-10, DMLT-1Y and GMU-479 (Figure 1). These lines are belonging to different maturity durations (Late, Early and Early respectively). Seeds of the above cultivars were grown in a greenhouse under natural light conditions with staggered planting dates to ensure continuous availability of explants.

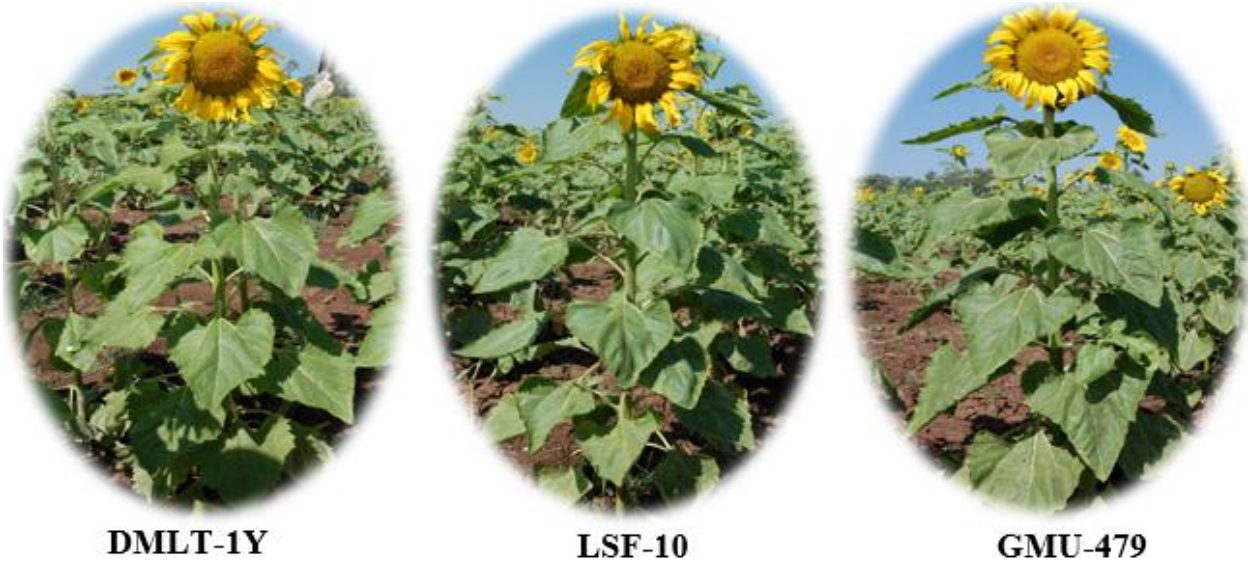

Fig 1: Elite cultivars of *Helianthus annuus* L.

Sample preparation

Each of 10 sunflower restorer lines was individually planted in pots in green house at Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, during *Kharif* season. These lines were germinated using sterile soil condition. After 15-20 days, fresh and primordial leaves were collected for bulk DNA extraction.

Chemicals and Solutions

The chemicals used in the investigation were of molecular biology grade or analytical grade of HiMedia Laboratories Pvt. Ltd, Mumbai, Qualigens Fine Chemicals, Mumbai, Merck Pvt. Ltd India. *Taq Polymerase*, Deoxyribonucleotides, Assay buffer, $MgCl_2$ and Agarose used for PCR was obtained from Bangalore Genei Pvt. Ltd. The primers used were obtained from Imperial Life Sciences (P) Limited, Gurgaon, Haryana, India. For all the molecular biology experiments, sterile double distilled water was used. PCR machine were used of eppendorf or biometric and electrophoresis unit of GeNei™ etc.

Genomic DNA isolation from mature leaves of sunflower

Genomic DNA was isolated from mature leaves of sunflower by CTAB method described by Saghai-Maroof *et al.* (1984) [7] with some modifications. Mature leaf samples of natural sunflower (600-700 mg) were harvested and kept frozen for at least 2 h. The frozen leaf samples were crushed and then ground to a fine powder. The powder (200-300 mg) was added in a volume of 1ml washing buffer was added, and the tube was kept on ice with mixing for 5 min. The tubes were centrifuged at 11,000 *g* for 10 min at 4°C. The supernatant was removed, and 700µl preheated high-salt CTAB buffer (60-65°C) was added with uniform mixing to avoid clumping at the bottom. The sample was incubated for 60 min at 65°C in a water bath. After the samples inversion 4-6 times during incubation, tubes were allowed to cool for 4-5 min, and then 500µl chloroform/iso-amyl alcohol (24:1) was added. The tubes were then mixed well by inversion for 1 min and allowed to stand for 5 min at room temperature. The tubes were centrifuged for 10 min at 11,000 *g*. The supernatant of about 750µl was transferred to a new tube. Steps 7-9 were repeated once. The aqueous phase was carefully transferred to a fresh tube, and 2 volumes of absolute alcohol were added. The tubes were mixed by gentle inversion to precipitate the DNA. DNA was pooled out with a yellow plastic tip and put into a new 1.5-ml tube with 1ml 70% ethanol to wash the DNA. Step 11 was repeated once, DNA was placed in a fresh tube, centrifuged for an additional 1 min at 5000 *g*, and the remaining liquid was removed as much as possible with a pipette. The tubes were allowed to drain inverted to dry the pellet for 10 min at room temperature. The DNA pellet was dissolved in 450µl TE I by incubating for 1 h at 37°C. An equal volume of phenol-chloroform-isoamyl alcohol (v/v, 25:24:1) was added to the tube. DNA precipitation was repeated once. DNA was re-suspended in 2.5 volumes of 100% absolute alcohol, with gentle mixing; there were some translucent floccules with some air bubbles present. DNA was centrifuged for 30 s at 5000 *g*. The supernatant was poured off, and the DNA pellet washed twice with 70% ethanol. The 70% ethanol was discarded, and 500µl absolute alcohol was

added, followed by mixing with gentle inversion for 1 min. The tubes were centrifuged for 1 min at 5000 *g*, and the absolute alcohol was discarded. The tubes were allowed to drain inverted, and the pellet was dried at 37°C for 15 min. A volume of 150µl TE II (RNase free) was added to dissolve the pellet and the DNA solution was stored at -20°C. DNA quality was assessed by electrophoresis and spectrophotometry. The extracted DNA was subjected to SSR analysis.

DNA quantification and estimation

The DNA samples were then quantified on 0.8% Agarose gel with known standards and showed a good quality of DNA. Quality of DNA was determined by horizontal submarine gel electrophoresis on 0.8% agarose gel. Purity of DNA was checked by taking the ratio of optical density (OD) using spectrophotometer, at 260 nm to that of 280 nm. The quantity, quality and integrity of isolated DNA were also checked by gel electrophoresis. 2 µl of DNA sample was isolated from each line and electrophoresis was done along with the λ Hind-III standard DNA in 0.8% gel concentration at 60 volts for 90 min. The DNA was stained by ethidium bromide and observed under UV-Trans-illuminator. The amount of fluorescence was proportional to the total mass of DNA. After quantification, the DNA was diluted by distilled water. The final concentration of DNA was 30ng/µl. DNA quantification as well as quality assessment was carried out spectrophotometrically using Bio photometer (Eppendorf-AG 22331, Germany) and checked by comparing DNA samples with known amount of DNA i.e. λ DNA. Quantities of all samples were found between 500-1000ng/µl. These samples were used as stock. Working samples were prepared by diluting it with nuclease free water. For SSR markers 20ng/µl of DNA was used. While amplified product by SSR markers were resolved in 3% agarose gel.

Results and Discussion

Electrophoretic analysis of PCR products for SSR Polymorphism on Agarose gel

The PCR products for SSR were resolved by electrophoresis on 3% (w/v) agarose gel (Bangalore Genei) stained with ethidium bromide (5µg/ml) and photographed under UV-light in gel documentation unit Alpha Imager® HP system. Molecular weight of bands was estimated using a wide range 1 Kb ladder (50-10000 bp) obtained from Bangalore Genei.

Estimation of the level of allelic polymorphism in an elite set of restorer lines using SSR marker

Genotyping of ten sunflower cultivars were done by one hundred twelve SSR primers generated differential banding patterns among different lines. All SSR primers were able to generate 3331 amplicons with an average 29.74 amplicons per primer (Table 1). Out of total 3331 amplicons 696 were detected polymorphism which showed 21.08% polymorphism among lines with 08.58 average numbers of polymorphic bands. The numbers of amplified fragments for primers ranged from (Ha9-ar) to (Ha357-ar) whereas the band size was ranged from 50bp to 750bp.

Ha806-ar primer was the most informative primer as it generated the highest number of polymorphic amplicons i.e. 18 polymorphic amplicons while forty four primers are stood least informative by generated less numbers of polymorphic amplicons. The lowest numbers of amplified fragments were generated by primer*s* (Table 1).

Genetic diversity analysis using SSR markers

Similarity coefficient

To assess the set of 10 cultivars of sunflower at molecular level by using SSR marker. The observations of SSR primers were scored for the presence (1) or absent (0) of bands on the DNA profiles and the data was then subjected to generate similarity matrix using the program similarity for qualitative data (SIMQUAL) dice coefficient (Sokal and Sneath, 1963).

Cluster analysis was carried out by the UPGMA method and dendrogram thus generated using SAHN subroutine of NTSYS-pc is summarized in (Table 2). A perusal of dendrogram (Figure 3 and 4) indicated that the similarity coefficient ranged from 0.60 to 0.86 among 10 sunflower restorer lines based on SSR markers. The diversity in DNA level was analyzed with a elucidian distance of 0.26 indicating that sunflower restorer lines did not show much variation at DNA level. The sunflower restorer lines were grouped into two major clusters A and B. The cluster A contains 02 lines while cluster B has 08 lines and shared 60% similarity. The cluster A consisted of 02 sunflower lines and shared 64% similarity.

The cluster B contains 08 lines while and shared 67% similarity. The cluster B consisted of two subgroups B_1 and B_2 sharing 67% similarity. Subgroups B_1 consisted of 05 lines (LDM-02, LDM-72, LSF-10, GMU479, and RHA 1-1) and shared 66% similarity. The subgroup B_1 was further subdivided into B_1 (1) and B_1 (2) and consisted of 03 and 02 lines, shared 76 % and 67% similarity respectively. Subgroups B_2 consisted of 03 lines (6D1R, DSF_2A, and DMLT-1Y) and shared 74% similarity. The subgroup B_2 was further subdivided into B_2 (1) and B_2 (2) and consisted of 01 and 02 lines, shared 74 % and 75% similarity respectively. Two groups was identified within which all the sunflower lines share 100% similarity for molecular (SSR) data (Table 3), and also one group was also none of single line were identified with 100% dissimilarity.

Table 2: Sunflower cultivars of cluster analysis based on DNA based SSR markers

Name of Cluster	Code of sunflower lines	Source
Cluster A (02 Sunflower lines)		O.R.S., Latur
Cluster B (08 Sunflower lines)		O.R.S., Latur / UAS, Bangalore
1.	**Cluster A**	
a.	R-274	
b.	CMS-127A	
2.	**Cluster B**	
a.	DMLT-1Y	O.R.S., Latur
b.	GMU 479	O.R.S., Latur
c.	LSF-10	O.R.S., Latur
d.	LDM O2	O.R.S., Latur
e.	LDM72	O.R.S., Latur
f.	DSF_{2A}	O.R.S., Latur
g.	RHA-1-1	U.A.S., Bangalore
h.	6D -1R	UAS, Bangalore
Cluster B		
Sub cluster B_1	(05 Sunflower lines)	O.R.S., Latur / UAS, Bangalore
Sub cluster $B_{1(1)}$	(01 Sunflower lines)	U.A.S., Bangalore
Sub cluster $B_{1(2)}$	(02 Sunflower lines)	O.R.S., Latur
Sub cluster B_2	(03 Sunflower lines)	O.R.S., Latur / UAS, Bangalore
Sub cluster $B_{2(1)}$	(01 Sunflower lines)	O.R.S., Latur
Sub cluster $B_{2(2)}$	(02 Sunflower lines)	O.R.S., Latur / UAS, Bangalore

Similarity matrix analysis for molecular (SSR) study: Genetic relationship between sunflower lines was determined on the basis of molecular (SSR) data in the form of '1'/'0' was subjected to NTSYS-pc software to calculate similarity among them (Table 4).

Table 3: R-lines of cluster analysis showing 100% similarity

Sr. No.	Group	Sunflower lines	Cluster
1	Group I	LSF-10, GMU-479	Sub cluster of $B_{1\ (1)}$
2	Group II	LDM-72, LDM-02	Subcluster$B_{1\ (2)}$

Table 4: Average similarity values of sunflower cultivars

Sr. No.	Sunflower lines	Average similarity value	Sr. No.	Sunflower Lines	Average similarity Value
1.	DMLT-1Y	0.67	6.	LDM72	0.69
2.	GMU 479	0.80	7.	R-274	0.59
3.	LSF-10	0.79	8.	DSF_{2A}	0.67
4.	CMS-127 A	0.59	9.	RHA-1-1	0.66
5.	LDM O2	0.69	10.	6D -1R	0.68
Total		6.83			
Average		0.68			

The value of similarity coefficient ranged from 0.59 to 0.80. Average similarity coefficient value per lines were calculated**.** The highest average similarity coefficient value (0.80) was calculated from GMU-479 and CMS-127A was represented lowest average similarity coefficient value (0.59). The average genetic similarity coefficient value was 0.68.

Molecular diversity analysis

Maintaining adequate supplies of essential food in the face of continued population presents a difficult challenge for Asia. Scientific advancements in molecular biology and its application for genetic advancement in staple food crops in the 1990s are now considered the most significant development in the entire history of plant breeding and crop improvement. Comprehensive integration of DNA marker technology with conventional breeding techniques if practiced will overcome the problems and to develop new inbred and hybrid sunflower cultivars with significantly improved yield potential and stability in a wide range of target environment. Specifically, fingerprinting and genetic diversity measures can be of use of breeders in following capacity.

1. To search for correlation of traits and markers in related individuals using a common database to store multiple type of data.
2. To narrow the search for new alleles in loci of interest.
3. To verify pedigree.
4. To choose parents for mapping, MAS and backcrossing schemes.
5. To monitor changes in allelic frequencies in population.
6. To study the evolutionary history of wild relatives,

These molecular markers are useful to fingerprint varieties, establish phylogenies, determining similarity among inbred and mapping entire genome molecular markers representing locus-specific DNA variation can be detected at morphological, gene protein level or directly at the DNA as with molecular markers. Presotto *et al.*, (2009) [4]. Reported that the populations of *H. annuus* L. naturalized in Argentina presented a high degree of phenotypic variability. Although it was smaller than that in populations from North America and lacks the extremes minimum values, it was enough to differentiate among populations into both groups.

These recombinant interspecific inbred lines are being utilized in the national sunflower network program for development of inbred lines and heterosis breeding. The pre-bred lines were characterized through cytological and molecular techniques using sunflower specific SSRs. Diploid perennial and hexaploid species have been utilized in the program aimed at introgression of resistance to *A. helianthus* through various ploidy manipulation procedures. The utility of the prebreeding material in the sunflower network program in India is presented, Sujatha M. (2006) [9].

PCR based SSR analysis for assessment of sunflower biodiversity: Fingerprinting is an absolute measure of genetic makeup of an individual or a line, and must be unique to that individual or line, in order to distinguish it from all others. Molecular genetic markers have been prepared to aid in plant genetic resource management in resource acquisition, maintenance assessment and utilization. Measurement of genetic diversity and the fingerprinting of lines or genotypes using molecular markers specially will be useful to germplasm bank curators and breeders.

The SSR analysis was found to be a valuable diagnostic DNA marker system to assess genetic diversity. The information about genetic similarity will be useful to avoid chance of elite line becoming genetically uniform and endangering long term productivity grains.

NTSYS-pc UPGMA clustering method with genetic similarity from DNA based SSR markers analysis

To assess the set of 10 cultivars of sunflower at molecular level by using SSR marker. The observations of SSR primers were scored for the presence (1) or absent (0) of bands on the DNA profiles. Cluster analysis was carried out by the UPGMA method and dendrogram thus generated using SAHN subroutine of NTSYS-pc.

A perusal of dendrogram figure 3 indicated that the similarity coefficient ranged from 0.60 to 0.86 among 10 sunflower restorer lines based on SSR markers. The diversity in DNA level was analyzed with an Euclidian distance of 0.26 indicating that sunflower restorer lines did not show much variation at DNA level.

The sunflower restorer lines were grouped into two major clusters A and B. The cluster A contains 02 lines while cluster B has 08 line***s*** and shared 60% similarity.

Two groups was identified within which all the sunflower lines share 100% similarity for molecular (SSR) data (Table 3), and also one group was also none of single line were identified with 100% dissimilarity.

Dissimilarity matrix analysis for molecular (SSR) coefficient study

Genetic relationship between sunflower lines was determined on the basis of molecular (SSR) data in the form of '1' / '0' was subjected to NTSYS-pc software to calculate similarity among them. The similarity coefficient values obtained were presented in Figure 3.

The value of similarity coefficient ranged from 0.59 to 0.80. Average similarity coefficient value per lines were calculated**.** The highest average similarity coefficient value (0.80) was calculated from GMU-479 and CMS-127A was represented lowest average similarity coefficient value (0.59). The average genetic similarity coefficient value was 0.68. Analysis of genetic diversity using molecular marker (SSR) in elite 10 sunflower cultivars using 12 SSR primers that detected polymorphism across all the genotypes.

1. Amplified products produced by SSR were resolved in 3% Agarose gel. Separation of fragments was visualized after staining with ethidium bromide under UV-transilluminator.
2. Bands were scored in the presence or absence (1 or 0) to produce molecular (SSR) marker data and were subjected to computer software NTYSIS-pc UPGMA for statistical analysis to calculate and analyze the diversity among the ten sunflower cultivars. Twelve SSR primers to generate 330 amplicons with an average 29.80 amplicons per primer. Out of total 330 amplicons 8.58% were detected polymorphic band per primer. Ha806-ar primer was detected to generate highest amount of polymorphic amplicons (47) while Ha357-ar were recorded lowest polymorphic amplicons. Dendrogram of similarity NTYSIS-pc method for SSR markers data was showed two groups of 100% similarity in which LDM-02, LDM-72, LSF-10 and GMU-479 sunflower cultivars collected from O.R.S., Latur. Polymorphic SSR markers revealed that a substantial variation existed in the sunflower cultivars studied. DNA fingerprinting using SSR proved to be powerful in characterizing the sunflower lines.

In the present context, investigation was taken up under conclusion as polymorphic SSR markers revealed that a substantial variation existed in the sunflower cultivars studied. DNA fingerprinting using SSR proved to be powerful in characterizing the sunflower lines. Present results indicated that out of 10 sunflower cultivars only few cultivars showed two groups of 100% similarity in which LDM-02, LDM-72, LSF-10 and GMU-479 sunflower cultivars collected from O.R.S., Latur. It will be fruitful for future hybrid development programme.

Acknowledgements

Author is grateful to the DBT, New Delhi, Government of India for financial support and Associate Dean and Principal, V.D.C.O.A.B., Latur for providing required facilities for this research work.

Authorship Contribution Statement

RN Dhawale conceived of the presented idea. MD Mahalle developed the theory and performed the computations. MD Mahalle and KM Sharma verified the analytical methods. SD Karnewar encouraged MD Mahalle to investigate [specifically data analysis aspect] and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

References

1. Arias DM and Reisberg LH. Genetic relationships among domesticated and wild sunflowers (Helianthus annuus, Asteraceae). Economic Bot. 1995;49:239-248.
2. Bartholomew Saanu Adeleke and Olubukola Oluranti Babalola. Oilseed crop sunflower (Helianthus annuus) as a source of food: Nutritional and health benefits, Food Science and Nutrition, Wiley, 2020, 4666-4684.
3. Powell W, Machray GC and Provan J. Polymorphism revealed by simple sequence repeats Trends Plant Sci., 1996;1:215-222.
4. Presotto. The Argentine wild Helianthus annuus L. genetic resources Helia. 2009;33(52):47-62.
5. Rieseberg LH and Seiler GJ. Molecular evidence and the origin and development of the domesticated sunflower (Helianthus annus, Asterceae), Econ Bot. 1990;44:79-91.
6. Reisberg LH, Choi HC and Chan R. Genomic map of a diploid hybrid species Heredity 1993;70:285-293.
7. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. Ribosomal DNA spacer-length polymorphism in barley: Mend Elian inheritance, chromosomal location and population dynamics. Proceedings of the National Academy of Sciences of the United States of America. 1984;81(24):8014-8019.
8. Sokal Robert R *and* Sneath Peter HA. Principles of Numerical Taxonomy, WH Freeman and Co., San Francisco and London, 1963, 16:359, Illus.
9. Sujatha M, Ranganatha AR, G Vishnuvardhan, Reddy A and Lakshmi P. Crop improvement. In Research achievements in sunflower. Edited by D.M. Hegde. Directorate of Oilseed Research, Hyderabad, India, 2006, 9-34.
10. Sujatha M and Prabakaran AJ. High frequency embryogenesis in immature zygotic embryos of sunflower, Plant Cell, Tissue and organ culture. 2001;65:23-29.
11. Sujatha M, Prabakaran AJ, Sangam L Dwivedi and S Chandra. Cytomorphological and molecular diversity in backcross-derived inbred lines of sunflower (Helianthus annuus L), Genome. 2008;51:282-293.
12. Tautz D and Renz M. Simple sequences are ubiquitous repetitive components of eukaryotic genomes Nucleic Acids Res. 1984;12:4127-4137.