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ORIGINAL ARTICLE

Ploidy analysis of *Helianthus* species by flow cytometry and its use in hybridity confirmation

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Abstract Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops cultivated worldwide. It is one of the few crops that has been improved for several characteristics through interspecific hybridization. In the present investigation, the ploidy of 43 *Helianthus* species belonging to diploid, tetraploid and hexaploid status along with 2 accessions of cultivated sunflower was determined. As wide variation exists in the *Helianthus* species for vegetative characteristics including pigmentation, processing of the leaf samples was tried with incorporation of 1 % polyvinylpyrrolidone while chopping the leaf which gave sharp peaks with low coefficient of variation. Ploidy as determined by flow cytometry was in agreement with the reported status of the *Helianthus* species used in the study. The mean 1C values for diploids, tetraploids and hexaploids were 3.67, 6.73 and 10.07 pg, respectively. The technique was validated on an interspecific hybrid of cultivated sunflower (2n = 2x) with *H. hirsutus* (2n = 4x) which confirmed the triploid status of the hybrid.

Keywords Flow cytometry · Genetic resources · *Helianthus* · Ploidy · Sunflower

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops cultivated globally for oil, confectionary and ornamental purposes. Interspecific hybridization played a vital role in introgression of the desirable traits from wild sunflowers to cultivated sunflower including cytosterility with appropriate maintainer and restorers, thus, transforming an ornamental plant to an oilseed crop with high oil productivity potential in the cultivated varieties and hybrids. Despite differences in ploidy levels (diploid, tetraploid, hexaploid), growth habit (annual, perennial), interspecific hybrids were successfully produced between cultivated sunflower and several wild species [13]. Most of these hybridization experiments resulted in complete or partial hybrids with varying chromosome number and structural rearrangements (*H. mollis, H. occidentalis, H. orgyalis* and *H. maximiliani*) [2, 10, 19, 20]. Natural hybridization between cultivated and wild forms resulting in intermediate morphologies is also reported [3, 25]. Some of the *Helianthus* species viz, *H. decapetalus* and *H. strumosus* have two forms of ploidy. Often difficulty is encountered in counting the accurate chromosome number in *Helianthus* with 34–102 chromosomes and with the lengths varying from 37.1 to 191.0 μm [13]. Hence, it is essential to use alternate methods for determining the ploidy of the parents and the resultant hybrids and for success of the introgressive breeding programmes. Aryavand et al. [1] successfully used the stomatal frequency and size to differentiate ploidy forms in *Aegilops neglecta*. Preliminary studies were done in our laboratory to correlate the stomata per unit area (mm^2) with ploidy status but these two variables were significantly negatively correlated (−0.33814). One of the simpler alternatives is determination of ploidy using flow cytometry, which to some extent has substituted the conventional chromosome counting and densitometry as the analysis besides being fast, precise and convenient can help in analysis of large number of samples within a

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short time [5]. However, reliability of the results depends on the tissue being processed and the stains used as there is a possibility of nonspecific binding of fluorochrome, auto fluorescence from various pigments and interference of secondary metabolites with DNA stains leading to erroneous interpretations [7].

In wild sunflowers, in addition to differences in chromosome number and growth habit, large differences exist with regard to the leaf characteristics particularly with regard to the texture, hairiness, colour, pubescence and such characters are known to interfere in the fluorescence. A wide range of variation in DNA content (3–8.2 pg) was observed among plants of the same sunflower variety by using laser flow cytometry of propidium iodide (PI) stained nuclei [18, 22]. Price et al. [21] reported the presence of unidentified endogenous compounds in sunflower leaves that interfere with propidium iodide (PI) interaction resulting in reduced fluorescence from sunflower nuclei. Gohde et al. [12] proposed the use of DAPI fluorochrome (4,6-dimino-2-phenylindole) for flow cytometry, and since then its utilization in flow cytometry has been widely used because of its specificity and high fluorescence which is very well suited for FCM in whole cells, nuclei and chromosomes.

The coefficient of variation (CV) value was considered as an elementary criterion that reflects the quality of the applied methodology [11, 17]. Reports using DAPI instead of PI resulted in lower CV values [6, 9, 26]. Flow cytometric analysis using DAPI was done in anther culture plants of the interspecific hybrids (triploids) derived from the cross between a hexaploid species *H. tuberosus* with the diploid cultivated sunflower to determine the ploidy status of the hybrids and anther culture plantlets [24] and intermated F_1 populations of *H. annuus* × *H. tuberosus* population [15]. The CV values of the study were in a range from 1.2 to 10 %. A CV value of less than 5 % was the acceptance criterion in plants [11]. However, the use of DAPI for estimation of ploidy might be affected by negative effect of cytosol. Some species contain cytosolic compounds (polyphenols), which interfere with DNA staining and might distort FCM measurements [7]. In sunflower, high content of polyphenols, polysaccharides and secondary metabolites resulted in low quality of nucleic acid extraction [16] which resulted in histograms of high background noise.

The present study has been undertaken with the objective of optimization of the sample preparation procedure, determine the ploidy levels of wild *Helianthus* species using flow cytometry and validation of ploidy in an interspecific hybrid between species of two different ploidies.

Materials and methods

Plant material

Seeds of wild *Helianthus* species obtained from USDA, USA, and the Institute of Field and Vegetable Crops, Novi Sad, Serbia, were established in the *Helianthus* species garden at the Indian Institute of Oilseeds Research, Hyderabad, India. These accessions varied in ploidy and growth habit (Table 1). Forty-three accessions of *Helianthus* accessions along with two accessions of cultivated sunflower (RHA 6D-1, PS 2023) were analyzed for ploidy by flow cytometry. These included one accession each of *H. deserticola*, *H. exilis*, *H. hirsutus*, *H. laevigatus*, *H. multiflorous*, *H. occidentalis*, *H. resinosus, H. salicifolius;* two accessions each of wild *H. annuus*, *H. divaricatus*, *H. microcephalus, H. mollis;* three accessions each of *H. decapetalus*, *H. giganteus*, *H. grosseserratus*, *H. tuberosus;* four accessions each of *H. nuttalli*, *H. praecox*, *H. strumosus* and five accessions of *H. maximiliani*.

Ploidy determination

Preparation of samples for ploidy determination in a PA-I flow cytometry (Partec, Monster, Germany) was done as per the manufacturer's instructions. Approximately 0.5 cm^2 of freshly harvested leaf tissue material was chopped with a sharp razor blade in a Petri dish in 400 µl of nuclei extraction buffer (Partec cystain UV precise P). As the CV was high, modifications such as use of 1 % PVP (polyvinylpyrrolidone) or 15 mM β-mercaptoethanol in nuclei extraction buffer and grinding in liquid nitrogen were done. As leaves of some species (*H. tuberosus, H. resinosus*) had tough texture, leaf samples were processed using the tissue processor to release nuclei into the lysis solution according to Cousin et al. [4]. Also, the histograms of fresh and frozen tissues (10, 20, 30 days after freezing) were studied. The nuclei suspension was filtered through a cell trics disposable filter (50 µm) into the sample tube. Nuclei were stained for 1 min with 1.6 ml of DAPI staining buffer (Partec Cystain UV precise P). Flow cytometry analysis was done with UV excitation (by mercury arc lamp) of the ploidy instrument with fluorescence excitation below $\lambda = 420$ nm and blue emission for DAPI at wavelength between $\lambda = 435$ and $\lambda = 500$ nm. The sample processing was done on ice and maintained in cold conditions till analysis. The gain of the instrument was calibrated with trout erythrocytes to give a CV of less than 1.0 %. Analysis of samples processed on a particular day was completed on the same day. The start and end samples and every 10th sample included cultivated sunflower RHA 6D-1 ($2n = 2x = 34$) as control. In each sample, 5000–10,000 nuclei were counted and a minimum of three measurements were made for each

Table 1 Ploidy of *Helianthus* accessions as determined by flow cytometry (FCM)

Species	IIOR Identity	Growth habit	Chromosome number (n)	FC-determined ploidy	1C value (pg)
H. annuus	RHA 6D-1	A	17	2x	3.4
	PS 2023	A	17	2x	3.5
H. decapetalus	DEC-01	P	17, 34	2x	3.8
	DEC-1887	P	17, 34	2x	3.0
	DEC-1992	P	17, 34	2x	3.8
H. deserticola	DES-873	A	17	2x	4.9
H. divaricatus	DIV-830	P	17	2x	3.6
	DIV-1885	P	17	2x	4.1
H. exilis	EXIL-891	A	17	2x	4.4
H. giganteus	GIG-184	P	17	2x	4.5
	GIG-1616	P	17	2x	3.3
	GIG-2014	P	17	2x	2.6
H. grosseserratus	GRA-10	P	17	2x	3.1
	GRA-977	P	17	2x	4.4
	GRA-2043	P	17	2x	4.4
H. hirsutus	HIR-1536	P	34	4x	6.4
H. laevigatus	LAE-5709	P	34	4x	7.4
H. maximiliani	MAX-11	P	17	2x	3.6
	MAX-30	P	17	2x	4.2
	MAX-1631	P	17	2x	4.1
	MAX-2010	P	17	2x	4.9
	MAX-33001	P	17	2x	2.5
H. microcephalus	MIC-1585	P	17	2x	4.1
	MIC-1887	P	17	2x	2.9
H. mollis	MOL-1530	P	17	2x	4.3
	MOL-1629	P	17	2x	4.6
Helianthus x multiflorus	MUL-RU	P	17	2x	4.0
H. nuttalli	NUT-05	P	17	2x	3.3
	NUT-1517	P	17	2x	3.2
	NUT-1962	P	17	2x	3.3
	NUT-SA	P	17	2x	4.7
H. occidentalis	OCC-52	P	17	2x	3.3
H. praecox	PRA-H-849	A	17	2x	3.0
	PRA-H-853	A	17	2x	2.9
	PRA-H-855	A	17	2x	2.3
	PRA-R-849	A	17	2x	2.8
H. resinosus	RES-09	P	51	6x	8.3
H. salicifolius	SAL-241	P	17	2x	3.8
H. strumosus	STR-15	P	34, 51	6x	11.0
	STR-216	P	34, 51	6x	10.2
	STR-1532	P	34, 51	4x	7.5
	STR-1934	P	34, 51	4x	5.6
H. tuberosus	TUB-03	P	51	6x	9.7
	TUB-15	P	51	6x	11.2
	TUB-2729	P	51	6x	10.0

Growth habit: *A* annual, *P* perennial

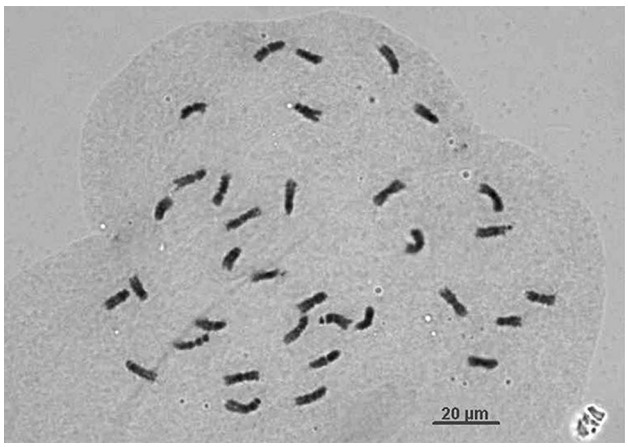


Fig. 1 Chromosome counting of RHA 6D-1(*H. annuus*, 2n = 34)

sample. The chromosome number of the diploid cultivated sunflower RHA 6D-1 was checked by direct counting on root-tip cells (with the kind help of Dr. Zhao Liu, USDA-ARS, Fargo, ND) (Fig. 1), which served as reference for the 1C measurements and based on previous reports for sunflower [13, 23], the 1C for the reference was taken as 3.4 pg.

Confirmation of hybridity of the interspecific hybrid of *H. annuus* (2n = 2x) and *H. hirsutus* (2n = 4x) by ploidy analysis and molecular analysis

To validate the ploidy results, a interspecific hybrid (2n = 3x) between *H. annuus* (2n = 2x) and *H. hirsutus* (2n = 4x) was characterized using flow cytometry and microsatellite markers. Total genomic DNA of the parents (*H. annuus* and *H. hirsutus*) was extracted by following the standard CTAB method with minor modifications [8]. The PCR amplification reaction (15 µl) consisted of 25 ng of DNA, 1X PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, and 1.5 mM $MgCl_2$), 7.5 mM of each of the four dNTPs, 3.75 pM each of forward and reverse primers of the SSR markers ORS 610 and ORS 1114 and 0.6 U of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplifications were performed in a GeneAmp 9700 Thermal Cycler (Perkin Elmer Applied Biosystems) with an initial denaturation at 94 °C for 2 min followed by 35 cycles at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min with a final extension at 72 °C for 5 min. The SSRs used were selected based on the parental polymorphism from 56 primers. The PCR products were separated on 3.0 % agarose gel in 1x TAE buffer.

Results

Optimization

Release of phenolics and oxidation was observed in some of the samples. Hence, representative material of 2 genotypes was chopped in extraction buffer with and without 1 % PVP. The addition of 1 % PVP to the chopping procedure produces isolated nuclei free of cytoplasm and sunflower inhibitors and makes the DNA accessible to DAPI dye. The addition of PVP used in lysis buffer had a measurable effect on reducing the inhibition and showed distinct peaks (Fig. 2). The peaks measured with a coefficient of variation (CV) less than 5 %, which was the acceptance criterion in ploidy determination (Table 2). Inclusion of β-mercaptoethanol in the nuclei isolation buffer failed to show significant improvement in peak quality or the CV. There were no peaks in samples ground in liquid nitrogen while fluorescence was negligible in frozen samples and samples processed with tissue homogenizer.

Ploidy analysis

All the 43 *Helianthus* accessions subjected to flow cytometric analysis with the optimized protocol gave histograms with single peak. Most of the accessions gave narrow peaks with a CV ranging from 4 to 10. FCM histograms obtained were with good resolution (Fig. 3). Based on 1C DNA content, the 45 sunflower accessions analyzed were separated into three different ploidy groups (Table 1). Thirty-five of the accessions were diploid with 1C value varying between 2.3 and 4.9 with a mean of 3.67 pg; four were tetraploid with 1C content ranging from 5.6 to 7.5 with a mean of 6.73 pg; six were hexaploid with the 1C level varying from 8.3 to 11.2 with a mean of 10.07 pg. Accessions belonging to species like *H. decapetalus* and *H. strumosus* were reported with different ploidy levels. Ploidy analysis differentiated the 4X and 6X accessions of *H. strumosus* while all the *H. decapetalus* accessions used in the study were diploid.

Hybrid confirmation using SSR primers and Flow cytometry

The hybridity of the interspecific hybrid (*H. annuus* × *H. hirsutus*) was confirmed with SSR primer which produced bands of both the parents and the polymorphism observed with ORS 1114 is presented in Fig. 4. Further confirmation of hybridity was done by flow cytometric estimation of ploidy of both the parents and hybrid. The mean value of the peak was 91 for *H. annuus* (diploid), 182 for *H. hirsutus* and 131 for the F_1 hybrid indicating the triploid nature of the hybrid.

Discussion

Chromosome counting and identification in wild *Helianthus* species and their derivatives is laborious due to high number (2n = 2x, 4x, 6x = 34, 68, 102), small size, poor spread resulting in inconsistencies. Interspecific

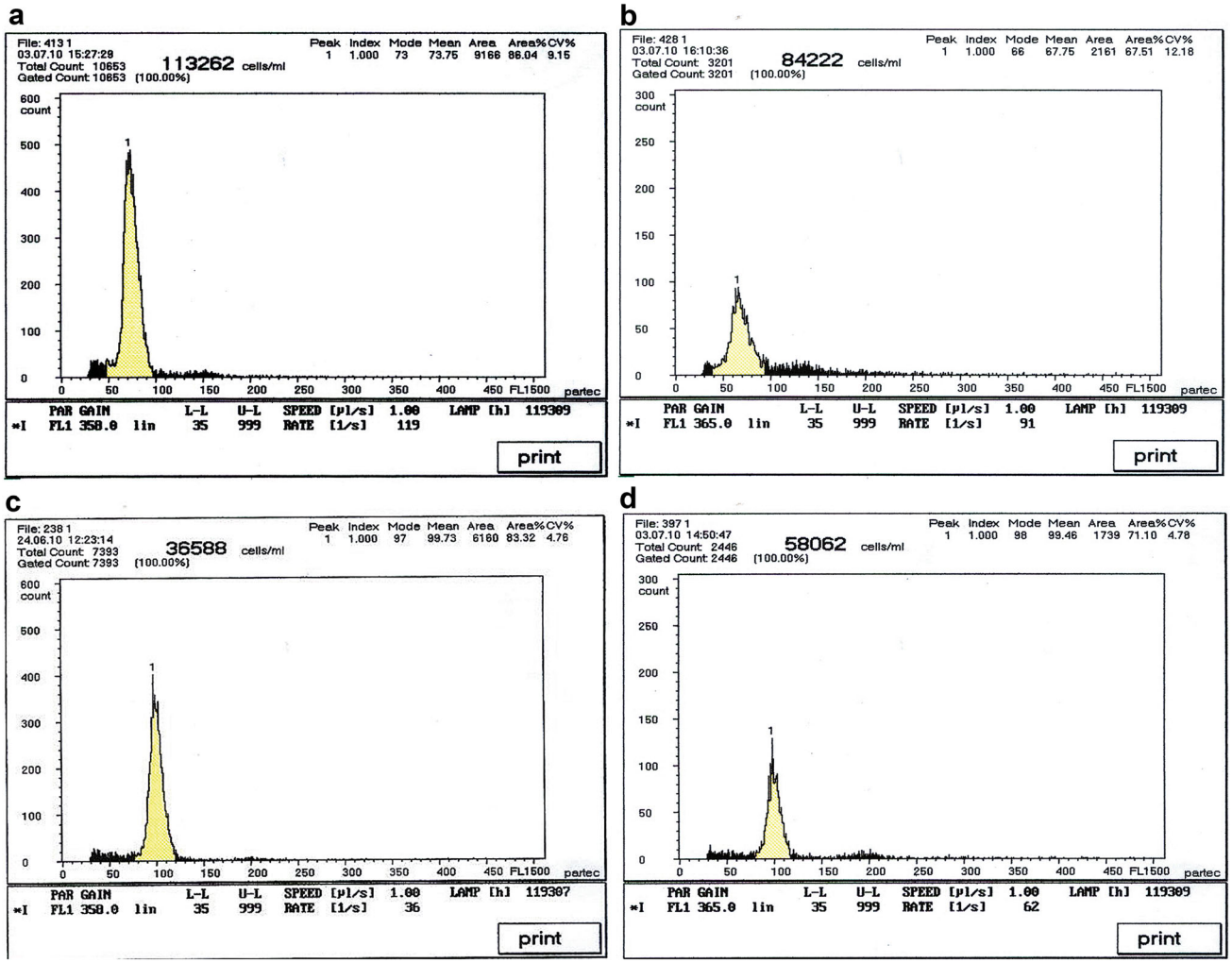


Fig. 2 Representative histograms obtained with different sample preparation methods in sunflower. **a** and **b**. histograms of PS 2023 and RHA 6D-1, respectively with chopping without polyvinylpyrrolidone, **c** and **d**. chopping with inclusion of 1 % polyvinylpyrrolidone

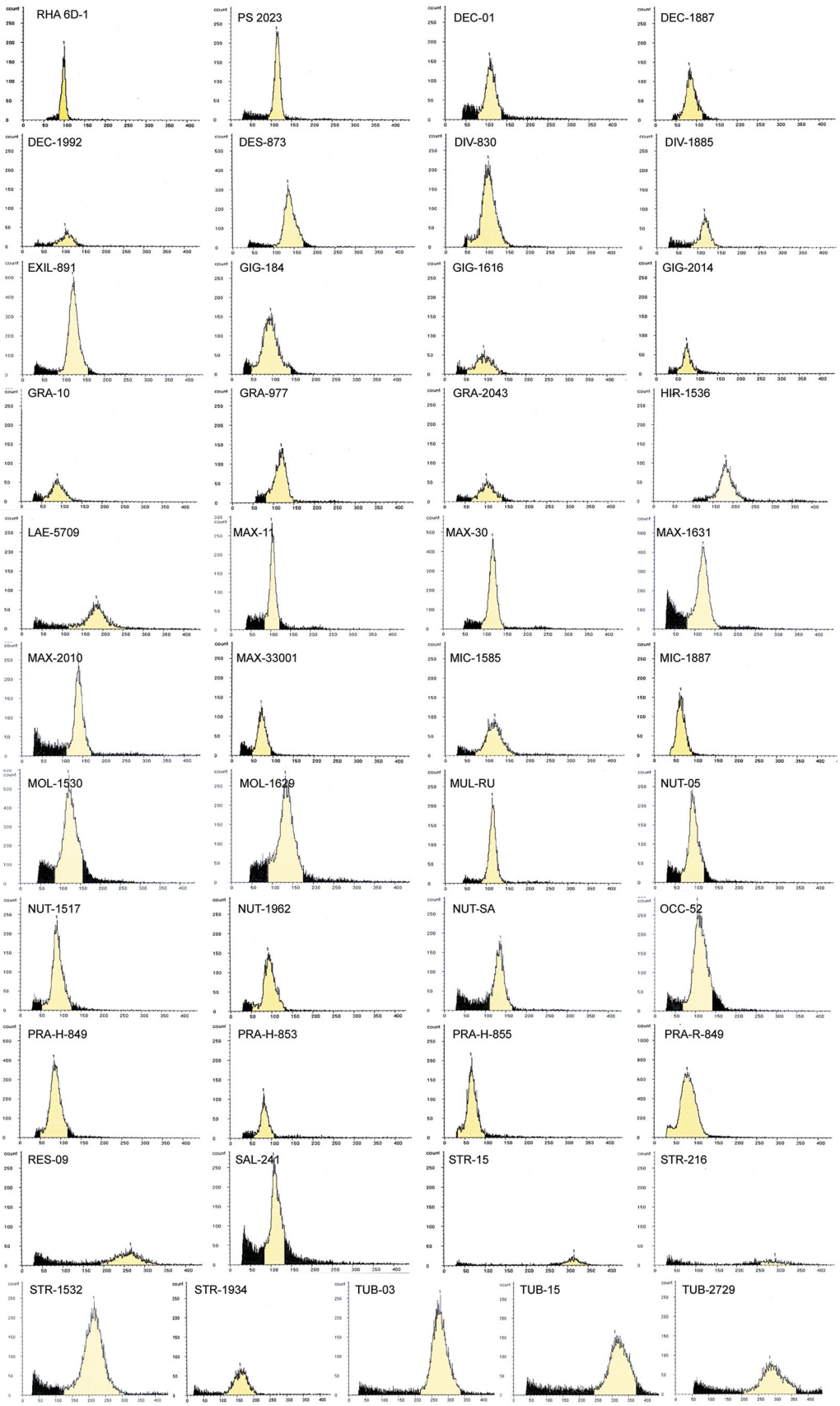
Table 2 Effect of incorporation of PVP in preparing nuclear suspension of sunflower for flow cytometry

Method of processing leaf sample	Plant material	Histogram	CV (%)
Chopping of leaf	PS 2023	Yes	9.15
	RHA 6D-1	Yes	12.16
Chopping of leaf with 1 % PVP	PS 2023	Yes	4.17
	RHA 6D-1	Yes	4.78
Processing of frozen leaf	PS 2023	No	–
	RHA 6D-1	No	–
Grinding of leaves in liquid nitrogen	PS 2023	No	–
	RHA 6D-1	No	–
Grinding of leaves in tissue homogenizer	PS 2023	Yes but very broad peaks	15.27
	RHA 6D-1	Yes but very broad peaks	18.28

CV (%) coefficient of variation

hybridization requires accurate identification of not only the parental material but also the resultant hybrids and the subsequent derivatives through backcrossing.

It is reported that sunflower leaves have unidentified compounds which lead to low fluorescence with propidium iodide (PI) staining of nuclei [21]. In this present


RHA 6D-1
PS 2023
DEC-01
DEC-1887
DEC-1992
DES-873
DIV-830
DIV-1885
EXIL-891
GIG-184
GIG-1616
GIG-2014
GRA-10
GRA-977
GRA-2043
HIR-1536
LAE-5709
MAX-11
MAX-30
MAX-1631
MAX-2010
MAX-33001
MIC-1585
MIC-1887
MOL-1530
MOL-1629
MUL-RU
NUT-05
NUT-1517
NUT-1962
NUT-SA
OCC-52
PRA-H-849
PRA-H-853
PRA-H-855
PRA-R-849
RES-09
SAL-241
STR-15
STR-216
STR-1532
STR-1934
TUB-03
TUB-15
TUB-2729

◀**Fig. 3** Histograms of relative fluorescence (relative nuclear DNA content) obtained from fresh leaves of *Helianthus* species

investigation, flow cytometric analysis has been carried out in Partec Ploidy analyzer in which the kits are based on the DAPI fluorochrome (4,6-dimino-2-phenylindole). Flow cytometric analysis with the optimized protocol gave histograms with single peak. Most of the species gave narrow peaks with a CV of less than 10. The addition of 1 % PVP for extraction of nuclei might have reduced the oxidation of polyphenolics and resulted in good yield and purity of nucleic acids [16]. The procedure yielded FCM histograms of the nuclear DNA content with good resolution and allowed detection of small increase of DNA content variability. Failure to get histograms or low fluorescence in samples ground in liquid nitrogen, tissue homogenizer and frozen samples could be due to the damage to the nuclei or poor release of intact nuclei.

In the present study, the 1C values reported for sunflower are in the range of values reported for cultivated sunflower. The 1C values were between 3.0 and 3.98 in different sunflower inbreds [18] and 3.1–3.19 in a natural populations of sunflower collected from different sites [22].

Well spread metaphase plates are possible in cultivated sunflower (Fig. 1) but accurate determination of the chromosome number is difficult in sunflower and the interspecific hybrids because of the existence of different ploidy levels (2n = 34, 68, 102) [14]. Chromosome counting is laborious, time consuming and is often a challenge to get good spread of metaphase chromosomes. Flow cytometry allows ploidy level determination with speed and precision. In the present study, ploidy levels of 45 sunflower accessions of cultivated and wild sunflowers were determined accurately. The species with existence of two ploidy levels (DEC-01, DEC-1887, DEC-1992, STR-15, STR-216, STR-1532 and STR-1934) were easily distinguished through ploidy analysis. Most of the ploidy levels represented in the present study corroborates the findings from chromosome counts [13].

Studies of interspecies hybridization in sunflower have included wide crosses between annual sunflower and perennial species. The F_1 hybrids are confirmed either by cytological examination or using molecular markers [2, 14, 20]. The present investigation had successfully proved the hybridity and ploidy status of F_1 hybrid between *H. annuus* (2n = 2x) and *H. hirsutus* (2n = 4x). This method of ploidy determination by flow cytometry is rapid and accurate and can be utilized in conjunction with molecular markers for characterization of interspecific hybrids and their derivatives. FCM has become the method of choice because of it is convenience, rapidity, reliability and ploidy can be determined at initial stage of growth of the interspecific hybrids.

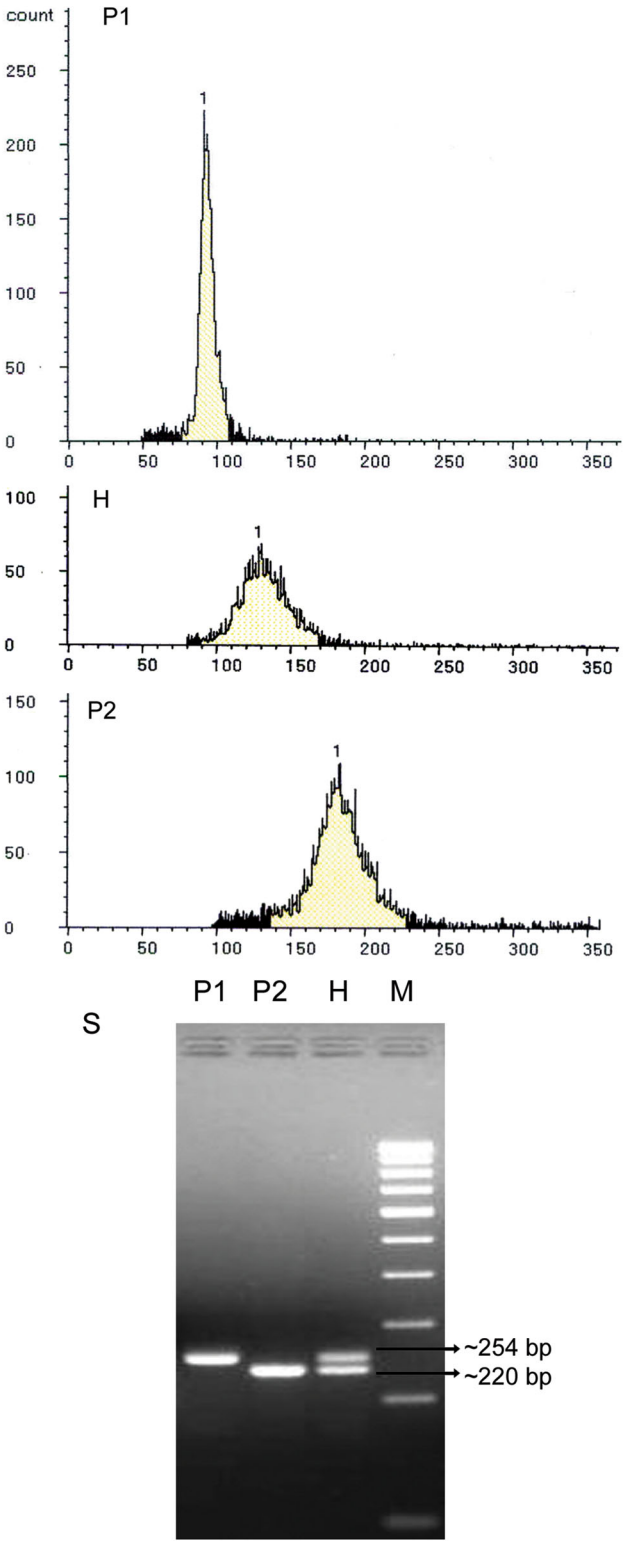


Fig. 4 Hybridity confirmation of an interspecific hybrid developed from two *Helianthus* species with different ploidy levels. P1. Histogram of diploid *H. annuus* (2n = 2x), P2. Histogram of the tetraploid *H. hirsutus* (2n = 4x), H. Histogram of the triploid F_1 (2n = 3x), S. Hybridity confirmation of F_1 with SSR marker, ORS 1114, P1 (*H. annuus*, 2n = 34) and P2 (*H. hirsutus*, 2n = 68) are the parents, H is the hybrid between *H. annuus* and *H. hirsutus*, M represents 100 bp marker

Conclusions

In the present investigation, the ploidy analysis procedure for sunflower has been optimized and used for determination of ploidy of different *Helianthus* species. Further, the utility of the technique was checked through confirmation of interspecific hybrid between *H. annuus* × *H. hirsutus* with different chromosome numbers using sunflower specific SSR primers and flow cytometry. Thus, the technique of flow cytometry with minor modifications could be employed to confirm the ploidy status of wild *Helianthus* species and also the interspecific hybrids between *Helianthus* species from different ploidy levels reliably and can be successfully used in introgressive breeding programmes in sunflower.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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