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Characterization of endosperm development in Guggul genotypes based on flow cytometry

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ABSTRACT

A comprehensive investigation on guggul endosperm development pattern was done by studying fruit set development under bagged condition and checked their ploidy level through flow cytometry. The study was carried out in 59 accessions maintained in the field gene bank of DMAPR. The flowers were bagged to avoid pollen grain and studied the fruit development pattern. It was found that, 48 accessions showed fruit set under bagged condition whereas 11 accessions failed to set the fruits. In these 11 accessions, the flowers were allowed for pollination and developing fruits (30-40 Days After Anthesis [DAA]) were collected and flow cytometric study was carried out to confirm triple fusion of endosperm development if there any. From the study it was observed that these showed 2x: 4x ploidy level, 2x:4x: 8x along with 2x: 3x which confirmed that, this apomictic species retains a low frequency of sexual reproduction. Hence it can be concluded that, both sexual and apomictic pathways are still active in the species which can be used as fixation of heterosis towards crop breeding improvement programme.

Figurs : 02 References : 21 Tables : 03

KEY WORDS : Apomixis, Autonomous endosperm, *Commiphora wightii*, Flow cytometry.

Introduction

Commiphora wightii (Arnott) Bhandari (Guggul) is a small tree, native to Africa and inhabiting drier parts of Western India, mainly Rajasthan and Gujarat. 'Sushruta Samahita' the classical ancient treatise on Ayurvedic medicine describes the use of Guggul for a wide number of diseases like rheumatism, obesity and arteriosclerosis. It acts as hypolipidemic, hypocholestrolemic, astringent, antiseptic, anticancer agents also which reduces the risk of heart disease and stroke[18]. It is included in red data list in the category of critically endangered in IUCN 2015[10].

Apomixis is reported in guggul and the species is non pseudogamous (not involving male participation) with nucellar polyembryony and autonomous endosperm development[7]. Interestingly a worker[6] reported the obligate sexual genotype of *C. wightii* for the first time from Gujarat populations. The discovery of such obligate sexual genotype has made our interest to screen out germplasm collected from various natural habitats for the study of endosperm development. It was reported that different types of apomixis can co-exist within an individual plant[19]. Most apomicts are facultative, *i.e.,* both apomictic and sexual reproductions occur at different levels and even in a same plant. There are species, such as *Poa pratensis*, in which its reproductive behavior ranges from nearly obligate apomixis to complete sexuality[1]. So the present work was carried out to screen out different plant types based endosperm development pattern. The study will help to study the mode of reproduction of the species.

Materials and Methods

The study was conducted in 59 accessions collected from Gujarat and Rajasthan and maintained in the field gene bank of DMAPR. The buds (n= 50 to 200 buds per plant) of the plants were bagged to avoid pollen

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TABLE-1 : Study of fruit set under bagged condition (restricting pollen availability) in Guggul

S. No.	Accession No.	No. of flower buds bagged	Fruit set success with no. of fruits formed under bagging condition
1.	DMAPR CW 2	192	√ (three)
2.	DMAPR CW 3	103	√ (five)
3.	DMAPR CW 5	69	√ (five)
4.	DMAPR CW 6	58	√ (two)
5.	DMAPR CW 8	178	√ (three)
6.	DMAPR CW 9	52	×
7.	DMAPR CW 10	50	√ (nine)
8.	DMAPR CW 11	262	√ (three)
9.	DMAPR CW 13	130	√ (eight)
10.	DMAPR CW 16	175	√ (three)
11.	DMAPR CW 20	50	√ (one)
12.	DMAPR CW 22	176	×
13.	DMAPR CW 24	50	√ (four)
14.	DMAPR CW 26	107	√ (six)
15.	DMAPR CW 28	195	√ (one)
16.	DMAPR CW 29	59	√ (two)
17.	DMAPR CW 30	86	×
18.	DMAPR CW 31	173	√ (ten)
19.	DMAPR CW 33 F12	86	×
20.	DMAPR CW 35	90	√ (one)
21.	DMAPR CW 37	85	No fruit
22.	DMAPR CW 39	236	√ (one)
23.	DMAPR CW 41	140	×
24.	DMAPR CW 42	50	√ (three)
25.	DMAPR CW 43	163	√ (two)
26.	DMAPR CW 44	50	√ (three)
27.	DMAPR CW 45	185	√ (one)
28.	DMAPR CW 46	130	√ (eight)
29.	DMAPR CW 47	68	√ (one)
30.	DMAPR CW 48	190	×
31.	DMAPR CW 49	140	√ (one)
32.	DMAPR CW 50	213	√ (eleven)
33.	DMAPR CW 51	145	√ (one)
34.	DMAPR CW 33 F16	135	√ (two)
35.	DMAPR CW 52	131	√ (one)
36.	DMAPR CW 53	84	√ (twelve)

S. No.	Accession No.	No. of flower buds bagged	Fruit set success with no. of fruits formed under bagging condition
37.	DMAPR CW 54	158	×
38.	DMAPR CW 55	75	√ (seven)
39.	DMAPR CW 56	98	√ (one)
40.	DMAPR CW 57	75	√ (ten)
41.	DMAPR CW 58	114	√ (eight)
42.	DMAPR CW 59	70	√ (seven)
43.	DMAPR CW 60	228	√ (eleven)
44.	DMAPR CW 61	50	×
45.	DMAPR CW 62	50	√ (ten)
46.	DMAPR CW 63	50	√ (one)
47.	DMAPR CW 64	50	×
48.	DMAPR CW 65	50	√ (one)
49.	DMAPR CW 66	56	√ (one)
50.	DMAPR CW 67	147	√ (three)
51.	DMAPR CW 68	78	√ (eight)
52.	DMAPR CW 69	50	√ (one)
53.	DMAPR CW 70	50	√ (nine)
54.	DMAPR CW 71	84	√ (eight)
55.	DMAPR CW 72	90	√ (five)
56.	DMAPR CW 73	50	√ (three)
57.	DMAPR CW 74	52	√ (one)
58.	DMAPR CW 75	61	√ (two)
59.	DMAPR CW 76	100	×

grains. Absence of fruit set indicates the absence of sexual reproduction or non-autonomous apomixis. The accessions which failed to set fruits under bagged conditions were used for flow cytometry study after allowing natural pollination to confirm sexual seed development. For flow cytometry, ovules were collected from open pollinated flowers (5 developing fruits/plant) and checked for the ploidy level of the developing endosperm.

Flow cytomtery was efficiently utilized by various workers for sex determination, study of interspecific hybrids, aneuploid detection, ploidy detection[5,15,16]. Fluorescence Assorted Cell Sorting (FACS) studies were performed according to the procedure described[5]. Developing ovules of about 30-40 days old were used for the analysis. Although the embryo maintains the parental ploidy, in amphimixis, triple fusion gives rise to a 2x: 3x endosperm, whereas in apomictic reproduction it was expected to produce 2x: 4x or its multiples due to endore duplication. The analysis gives an indirect assessment of the events of endosperm formation on the basis of their nuclear DNA contents.

Developing ovules/ seeds of about 30 days old (n = 5) from the open pollination were collected from the accessions which failed to develop fruits under bagged condition. Developing ovules from each fruit was dissected out and tested individually. According to the manufacturer's instructions, Nuclei samples were prepared using the kit (Partec high resolution Kit type P, Partec GmbH, Münster, Germany). Matured fruit wall was used as standard. With the help of a sharp razor blade, samples were chopped in nuclei extraction buffer (Partec Cystain UV Precise P) and the suspension nuclei were filtered through a CellTrics disposable filter (mesh size 30 ìm) directly in to the sample

Fig. 1 : (a) Fruiting twig of guggul, (b) and (c) close up view of developing ovules (30-40 Days after anthesis) Scale bar (b,c) = 1000 µm

tube followed by adding DAPI staining buffer (Partec Cystain UV Precise P). The stained sample nuclei were analysed with UV excitation with the flow cytometer (Partec PA II system, equipped with HBO lamp). Endosperm ploidy from the developing ovules of the bagged flowers of 22 selected accessions were also tested by flow cytometry as a control. These 11 accessions were used to study the type of endosperm development under open pollination condition. Developing fruits (Fig.1a to c) were collected from these accessions and the ovules were used for flow cytometric study to confirm sexual/apomictic seed development.

Results and Discussion

It was found that 48 accessions showed fruit set under bagged condition whereas 11 accessions failed to set the fruits (Table-1). It means that 81.35% accessions had autonomous endosperm development which is an indication of apomictic embryo development.

Since the species bear seeds of “exalbuminous” or “cotyledonous” type, the endosperm is absorbed during embryo development; developing ovules of about 30-40 days old were used for the analysis. Developing ovules were collected from open pollinated pistils (n= 5 developing fruits/plant) of the studied accessions. Although the embryo maintains the parental ploidy, in amphimixis, triple fusion gives rise to a 3x endosperm, whereas in apomictic reproduction it was expected to produce 2x: 4x or 2x:4x: 8x and higher ploidy levels. The analysis gives the indirect assessment of the events of endosperm formation on the basis of their nuclear DNA contents.

FACS of the somatic nuclei extracted from fruit wall gave a single major peak and it was adjusted at channel value 200 (Fig.2a). Under the same instrument settings, developing ovules were tested. The FACS results of developing ovules showed cells were at either 2x or 2x: 4x or peaks of higher ploidy levels or 2x: 3x also. The 2x: 4x levels its higher levels of ploidy from the developing ovules of open pollinated flowers indicated the autonomous development of ovule in the species.

From the study it was observed that the ovules collected from of the 48 accessions of controlled conditions (bagged) showed peaks corresponding to 2x or 2x:4x ploidy or peaks of higher ploidy levels (2x:4x:8x) (Fig.2 b, c, e & Table-2). Thus, the result revealed

TABLE-2 : Ploidy analysis of ovules of the selected 22 accessions by Flow cytometry

Accession No.	Ovule No.	Ploidy level	Accession No.	Ovule No.	Ploidy level	Accession No.	Ovule No.	Ploidy level	Accession No.	Ovule No.	Ploidy level
DMAPR CW 2	1	2x, 4x	DMAPR CW 43	1	2x,4x	DMAPR CW 58	1	2x,4x	DMAPR CW 71	1	2x,4x,8x
	2	2x, 4x		2	2x,4x		2	2x,4x		2	2x
	3	2x, 4x		3	2x,4x		3	2x		3	2x,7x
	4	2x,4x, 8x		4	2x,4x		4	2x,4x		4	2x
	5	2x, 4x, 8x		5	2x,4x		5	2x		5	2x
DMAPR CW 5	1	2x, 4x	DMAPR CW 44	1	2x,4x	DMAPR CW 59	1	2x,4x	DMAPR CW 72	1	2x,4x
	2	2x, 4x		2	2x,4x		2	2x		2	2x,4x
	3	2x		3	2x,4x,8x		3	2x,4x		3	2x,4x
	4	2x, 4x		4	2x,4x,8x		4	2x,4x		4	2x
	5	2x		5	2x,4x		5	2x		5	2x
DMAPR CW 10	1	2x	DMAPR CW 46	1	2x,4x	DMAPR CW60	1	2x,4x	DMAPR CW 73	1	2x,4x
	2	2x		2	2x,4x		2	2x,4x		2	2x, 4x
	3	2x,4x		3	2x		3	2x,4x		3	2x, 4x
	4	2x		4	2x,4x		4	2x,4x		4	2x, 4x
	5	2x		5	2x,4x		5	2x		5	2x,4x,8x

Accession No.	Ovule No.	Ploidy level	Accession No.	Ovule No.	Ploidy level	Accession No.	Ovule No.	Ploidy level	Accession No.	Ovule No.	Ploidy level
DMAPR CW 24	1	2x, 4x	DMAPR CW 53	1	2x,4x	DMAPR CW 62	1	2x	DMAPR CW 75	1	2x
	2	2x, 4x		2	2x, 4x		2	2x		2	2x,4x
	3	2x, 4x		3	2x,4x,8x		3	2x		3	2x,4x
	4	2x, 4x		4	2x, 4x		4	2x, 4x		4	2x,4x
	5	2x, 4x, 8x		5	2x, 4x		5	2x		5	2x,4x
DMAPR CW 26	1	2x	DMAPR CW 55	1	2x,4x	DMAPR CW 67	1	2x			
	2	2x,4x		2	2x,4x		2	2x			
	3	2x		3	2x,4x,8x		3	2x			
	4	2x		4	2x,4x		4	2x, 4x			
	5	2x		5	2x,4x		5	2x			
DMAPR CW 31	1	2x	DMAPR CW 57	1	2x,4x	DMAPR CW 70	1	2x, 4x			
	2	2x		2	2x		2	2x, 4x			
	3	2x		3	2x,4x,8x		3	2x, 4x			
	4	2x,4x		4	2x		4	2x, 4x			
	5	2x,4x		5	2x		5	2x			

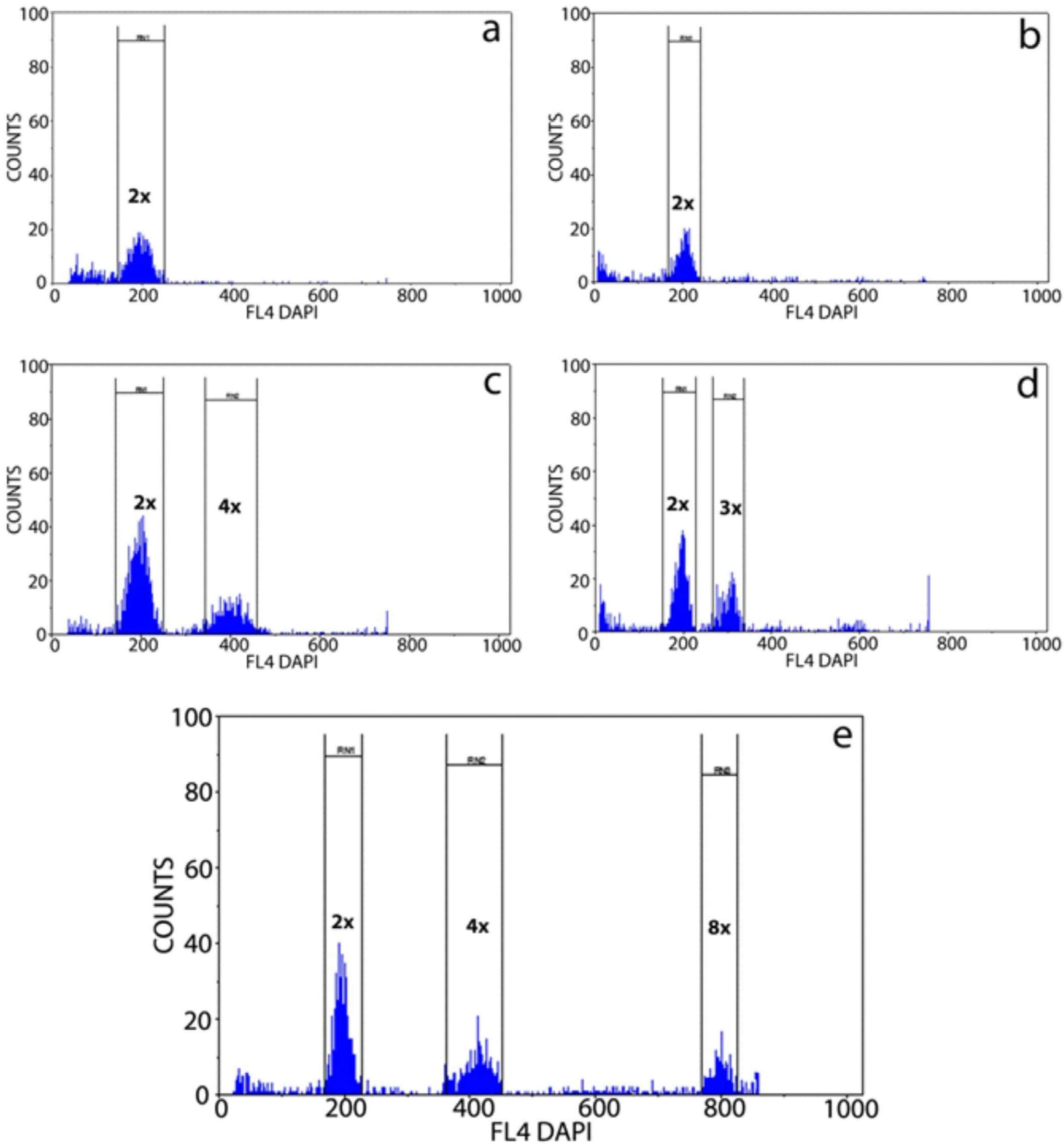

Fig. 2 :

(a) FACS histogram of nuclei from fruit wall used for standardization. Peak value was adjusted at channel 200.

(b) FACS histogram of studied accessions showing autonomous endosperm development at 2x ploidy status

(c) FACS histogram showing autonomous endosperm development showing 2x: 4x ploidy status

(d) FACS histogram showing triple fusion (2x:3x) endosperm development

(e) FACS histogram showing 2x: 4x: 8x and higher ploidy status

TABLE-3: Ploidy analysis of ovules of the selected 11 accessions by Flow cytometry

S.No.	Accession No.	Ovule No.	Ploidy Level
1	DMAPR CW 9	1	2x,3x
		2	2x
		3	2x,3x
		4	2x,3x
		5	2x,4x
2	DMAPR CW 22	1	2x, 4x
		2	2x, 4x
		3	2x, 3x
		4	2x, 3x
		5	2x
3	DMAPR CW 30	1	2x
		2	2x,3x
		3	2x
		4	2x
		5	2x
4	DMAPR CW 37	1	2x,3x
		2	2x,4x,8x
		3	2x,3x
		4	2x,4x
		5	2x,4x,8x
5	DMAPR CW 41	1	2x,3x
		2	2x,4x,8x
		3	2x,4x

		4	2x,4x
		5	2x,4x
6	DMAPR CW 48	1	2x,4x
		2	2x,4x
		3	2x,4x
		4	2x,5x
		5	2x,3x
7	DMAPR CW 54	1	2x,4x
		2	2x
		3	2x
		4	2x,3x
		5	2x,3x
8	DMAPR CW 61	1	2x,4x
		2	2x,3x
		3	2x, 4x
		4	2x,4x
		5	2x, 4x
9	DMAPR CW 64	1	2x,3x
		2	2x,4x,8x
		3	2x,4x,8x
		4	2x,4x
		5	2x,4x
10	DMAPR CW 76	1	2x,4x
		2	2x,4x

		3	2x,4x
		4	2x,4x
		5	2x,3x
11	DMAPR CW 33 F12	1	2x,4x
		2	2x,3x
		3	2x,4x,8x
		4	2x,3x
		5	2x,4x

autonomous endosperm development which indicated the absence of triple fusion. Whereas 11 accessions (out of 59 accessions) such as DMAPR CW 9, DMAPR CW 22, DMAPR CW 30, DMAPR CW 37, DMAPR CW 41, DMAPR CW 48, DMAPR CW 54, DMAPR CW 61, DMAPR CW 64, DMAPR CW 76 and DMAPR CW 33 F12, showed the peaks at 2x: 3x level (Fig. 2 d) along with autonomous endosperm products (2x or 2x:4x ploidy or 2x:4x:8x) which reflected the presence of triple fusion of endosperm nucleus also. However, the frequency of triple fusion (2x: 3x ploidy level) were very low (Table 3) among these accessions.

Result of FACS analysis of the developing ovules from the studied accessions confirmed the autonomous endosperm development in 48 accessions whereas, in 11 accessions, there was endosperm developed through autonomous pathway as well as by triple fusion. The study revealed that those accessions having low % of 2x: 3x ploidy level confirm the hypothesis that most apomictic species retain a low frequency of sexual reproduction due to stimulation of environment condition[11,12]. This hint of sexual reproduction in apomictic plants is also critically important for the evolution of environmental adaptation. The cytometric analysis thus provided evidence of endosperm formation through triple fusion or endoduplication in guggul.

In most of the cases, adventitious embryony occurs in the presence of normal sexual reproduction. Usually, pollination is followed by the double fertilization of a reduced sexual embryo sac. The embryo and endosperm initiate to develop and the stimulus of the embryo development results in the growth of further adventive embryos in the nucellus and has been named “induced adventitious embryony”[17].

Although apomixis is scattered throughout the plant kingdom, few important agricultural crops possess this trait. Adventitious embryony is common in *Citrus*, orchids, mangoes and mangosteen[14]. In facultative apomicts there is a coexistence of sexual and apomictic reproduction in an individual ovary and often within a single ovule. Apomixis and sexuality are not mutually exclusive events, because in facultative apomicts the processes already coexist[12].

Given that both sexual and apomictic processes can coexist within a given ovule, experiments could be conducted that should provide an indication about the degree of relatedness between the genes being expressed during sexual and apomictic reproduction[12].

It was reported that *E. binata* is an autonomous apomixis species and involves fertilization independent of endosperm development[13]. Flow cytometry analysis was performed to identify the sexual reproduction in the population of *E. binata* and the study revealed the presence of certain rate of sexual reproduction in this population of *E. binata*[21]. Presence of sexuality along with apomixis was reported in the germplasm of *Brachiaria decumbens* stapf mentioned[20]. Our obtained results *i.e.,* Apomixis and sexuality have been demonstrated in Buffel grass and other species of *Paspalum, Bothriochloa* and *Dichanthium*, *Coprosma robusta* and *Coprosma waima*[2-4,8,9].

Conclusions

Hence in the present work, a wide range of guggul germplasm accessions were screened based on endosperm development. The study proved that the sexual and asexual processes can occur in the same plant of guggul as the ûow cytometric analysis provided evidence of endosperm formation through triple fusion or endoduplication. The study will help in molecular and genetic study of apomixis and to introduce the desired trait/s to the crop varieties for fixation of heterosis.

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