



# Apomixis as a tool for development of high yielding clones and selections in *Jatropha curcas* L.

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**Abstract** *Jatropha curcas* L. has gained attention as an ideal feedstock for biofuel production. One of the major limitations for profitable cultivation of the crop is the lack of high yielding cultivars with predictable yields. This study describes the exploitation of the mixed mating system including apomixis prevailing in the crop for development of high yielding genotypes. Evaluation of 135 accessions (AC) originating from 15 countries showed apomictic mode of seed development in 33 accessions. Based on the apomictic behavior, crosses were effected between a non-toxic high yielding genotype of Mexican origin (AC-2) and an Indian toxic genotype with synchronous maturity (AC-86). The F<sub>1</sub> exhibited high heterosis and continued to produce seeds through both

apomixis and sexual reproduction. The F<sub>1</sub>A<sub>1</sub> progeny (n = 94) derived from the F<sub>1</sub> hybrid through apomixis were productive with mean seed yield of 335, 586 and 644 g/plant during the 1st, 2nd and 3rd years after planting. The F<sub>1</sub>A<sub>1</sub> progeny continued to reproduce through apomixis which ranged from 9.3 to 88.3%. A dendrogram based on genetic distances obtained from 1172 single nucleotide polymorphisms of 11 apomictic progenies along with the parents and the F<sub>1</sub> hybrid unequivocally established the maternal origin of the apomictic plants. Microscopic analysis of developing apomictic ovules showed adventitious embryos. This study demonstrates the possibility of development of high yielding cultivars in a short time frame and in a cost effective manner by combining different mating systems prevalent in the crop viz, hybridization of diverse parents, vegetative propagation of high yielding hybrids through cuttings, and perpetuation of the superior recombinants through apomixis.

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## Introduction

*Jatropha curcas* L. has attracted research and commercial interest because of the adaptable nature of the

plant to varied climate and soil conditions, the high oil content of its seeds, the suitability of its oil as a biofuel feedstock and the potential commercial value of the byproducts remaining after oil extraction from the seeds (Francis et al. 2005; Carels 2009; Achten et al. 2010). To fulfill its promise, *J. curcas* needs to be able to produce such quantities of seeds that makes its cultivation profitable to farmers under conditions that are disadvantageous (e.g. because of drought stress and nutrient poor soil) for conventional oilseed crops such as soybean, rapeseed, sunflower, etc. This is because the value of *J. curcas* cropping is seen in the plant's effectiveness in profitable cultivation of eroded lands. According to the "Global Land Outlook" of the UN Convention to Combat Desertification (UNCCD), a third of the planet's land is severely degraded and fertile soil is being lost at the rate of 24 bn tonnes a year ([https://knowledge.unccd.int/sites/default/files/2018-06/GLO%20English\\_Full\\_Report\\_rev1.pdf](https://knowledge.unccd.int/sites/default/files/2018-06/GLO%20English_Full_Report_rev1.pdf)).

According to the observation of the authors in India and several African countries, lands available for *J. curcas* cultivation as a biofuel crop are usually those that are not used for conventional cropping due to various reasons, and consequently, the low and unpredictable productivity of the crop. While *J. curcas* can thrive under such conditions, its seed yields need to be increased to make its cropping more broadly profitable. Thus, further improvement of the plant is required in order to realize its commercial potential effectively and to establish it as a profitable crop.

During the past two decades, there were many efforts that were aimed at genetic improvement of *J. curcas*. Realizing the need for development of high yielding cultivars, various strategies have been devised to widen the genetic base for exploitation in the breeding programs. Hybridization has been found to be one of the effective tools available for *J. curcas* improvement which requires a diverse parent population. Hence, during the first decade, the research focus was on assessment of the extent of genetic diversity available in the crop and intensive efforts of various research groups resulted in identification of exploitable genetic diversity in the Mexican and Central American accessions (Basha et al. 2009; Pecina-Quintero et al. 2011; Montes Osorio et al. 2014; Santos et al. 2016; Li et al. 2017). During the last decade, the major emphasis was towards development of high yielding cultivars and concerted efforts were made by few select groups for selection and

development of genotypes for high productivity and quality traits (Yi et al. 2014; Alfredo and Quintero 2017; Francis et al. 2018). For hybrid breeding, pistillate (only female) accessions are also available (Alfredo and Quintero 2017; Francis et al. 2018).

Despite the research over the last two decades, the challenge limiting the success of improving *J. curcas* is the low exploitable genetic variation (Yi et al. 2010; Basha and Sujatha 2007; Sun et al. 2008). One of the plausible reasons for the limited genetic variability is the existence of mixed mating system including the apomictic mode of reproduction (Alves et al. 2013; Bressan et al. 2013; Negussie et al. 2014; Bhattacharya et al. 2005). Based on flow cytometric analysis, Ambrosi et al. (2010) inferred the occurrence of non-gametophytic apomixis in *J. curcas*. Apomixis is an asexual mode of reproduction in which the plants bypass female meiosis and produce seeds without fertilization (Barcaccia and Albertini 2013). Apomixis is reported to occur in 10% of the 400 families of flowering plants and predominantly in gramineae, asteraceae and rosaceae (Asker 1979). Members of euphorbiaceae such as cassava (Nassar et al. 1998; Freitas and Nassar 2013), Hevea (Priyadarshan and Clément-Demange 2004) including *J. curcas* (Abdelgadir et al. 2009; Bressan et al. 2013; Nietzsche et al. 2014; Chang-Wei et al. 2007) are reported to reproduce asexually through apomixis. Apomixis usually coexists with sexuality (facultative apomicts) and has the advantages of rapid multiplication of genetically uniform individuals and also in fixing the heterosis through transgenerations. Apomixis assumes importance in plant breeding as it would help in fixing the heterosis, development of true-breeding hybrids precluding the need for cytoplasmic male sterility and its associated costs, rapid propagation of clonal genotypes, efficient exploitation of maternal effects, development of homozygous inbred lines, perpetuation of superior genotypes across generations (Hanna and Bashaw 1987; Koltunow and Grossniklaus, 2003; Mieulet et al., 2016; Hojsgaard and Hörandl 2019).

Hence, in the present investigation an attempt has been made to check for apomictic mode of reproduction in a representative set of germplasm comprising of 135 genotypes from 15 countries; develop hybrids between diverse parents and assess their hybrid vigour; evaluate the perpetuation of the heterotic potential of the F<sub>1</sub> hybrid through apomixis. Accordingly, crosses between a non-toxic Mexican genotype

with a toxic Indian accession were made and the  $F_1$  hybrid along with the progeny derived through apomixis were characterized for yield and related traits during the first 3 years of planting.

## Materials and methods

### Plant material and growth conditions

The plant materials subjected to apomixis included 135 accessions representing 15 source countries. These included toxic, non-toxic (edible), pistillate accessions and breeding lines. Each accession was represented by four plants (clones). Five-year old plants in the germplasm block were emasculated and bagged to check for apomictic development of seeds. For exploitation of heterosis, crosses were effected between a high yielding, non-toxic genotype from Mexico combining early maturity and semi-spreading habit (AC-2) with a toxic Indian accession with synchronous maturity (AC-86). The resultant  $F_1$  hybrid between these two diverse parents exhibited high heterosis. Both the parents and also the  $F_1$  hybrid were confirmed to produce seeds asexually through apomixis. The  $F_1$  hybrid was vigorous with profuse and continuous bearing and it was advanced to the next generation through selfing for production of  $F_2$  progeny and through apomixis for production of apomictic progeny ( $F_1A_1$ ) (Fig. 1). Further, for production of large quantities of seed and planting material besides evaluation of the performance of the  $F_1$  hybrid under varied conditions, the  $F_1$  hybrid was propagated vegetatively through stem cuttings. Seeds obtained through apomixis ( $F_1A_1$ ) were germinated in nursery bed and once the seedlings were at two-leaved stage, the saplings were planted in the field at a plant to plant and row to row spacing of 2 m  $\times$  2 m. The apomictic progeny (n = 94) derived from the  $F_1$  hybrid were analyzed for seed yield for three consecutive years. The  $F_1A_1$  plants were subjected to apomixis during the second year. Phorbol ester content of some promising and high yielding apomictic progeny (n = 33) was determined using HPLC according to the method described in Martinez-Herrera et al. (2006). The phorbol ester results are expressed as equivalent to a standard, phorbol-12-myristate 13-acetate.

The plant material was raised in an experimental farm located near Coimbatore, Tamil Nadu state, India (Latitude: N10 42.751; Longitude: E77 11.867). The soil is black sandy loam with a depth of 100–150 cm, pH 7.9, EC ( $dSm^{-1}$ ) 0.5, high in calcium carbonate, low in organic carbon (81 kg/acre), average phosphorous (5.5) and high potash (340). The rainfall during 2016, 2017 and 2018 as per the meteorological station in the region (IMD) was 589, 1120 and 1163 mm, respectively but the observations made at the farm site, showed below 400 mm actual rainfall in the years 2016 and 2017 and above 800 mm in 2018).

### Determining apomixis

*Jatropha curcas* is unisexual and monoecious producing male and female flowers on dichasial cymes. The male and female buds are easily distinguishable based on their shape, size and position on the inflorescence axils. The female buds are larger than the male buds, oval in shape and are located on the central axis of each of the paracladia while male buds are round and are borne on the lateral axils of the main inflorescence and the co-florescences. Inflorescences were selected in which the flowers are mature and ready for anthesis. Following emasculation, the inflorescences with only the female buds were covered with butter paper bags and tagged. The covered inflorescences were checked after 1 week for male buds, if any (Fig. 2a). The covers were removed after 2 weeks to allow proper growth and development of fruits and seeds. Observations were made on fruits per inflorescence, seeds per capsule, and seed weight (g) in the apomixed inflorescences. Apomixis (%) was calculated as follows

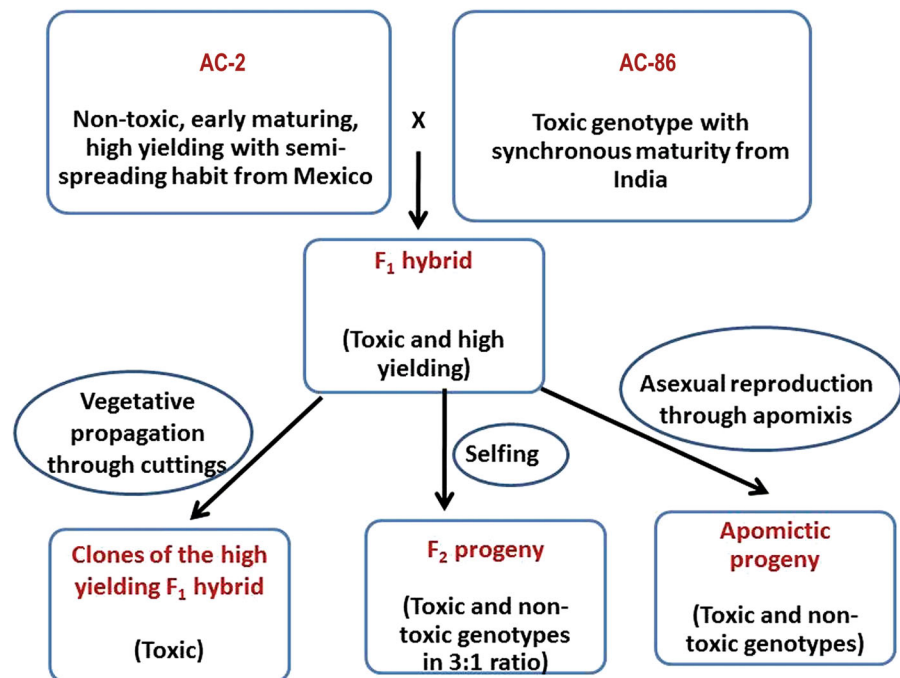
$$\text{Apomixis (\%)} = \frac{\text{Number of fruits formed} \times 100}{\text{Number of female flowers covered}}$$

The seed set (%) under apomixis was calculated as follows

$$\begin{aligned} \text{Seed set (\%)} \text{ under apomixis} \\ = \frac{\text{Number of seeds obtained} \times 100}{\text{Number of flowers covered} \times 2.8} \end{aligned}$$

The factor of 2.8 represents the average number of seeds obtained under the same set of conditions in open pollinated flowers. The seed yield under open pollination for the first 3 years after planting of the

**Fig. 1** Exploitation of different modes of propagation for development of superior clones and selections in *J. curcas*



apomixed offspring of the  $F_1$  hybrid ( $F_1A_1$ ) was also determined. The seed and kernel oil contents were determined through NMR and Soxhlet methods, respectively of seeds obtained through apomixis and open pollination.

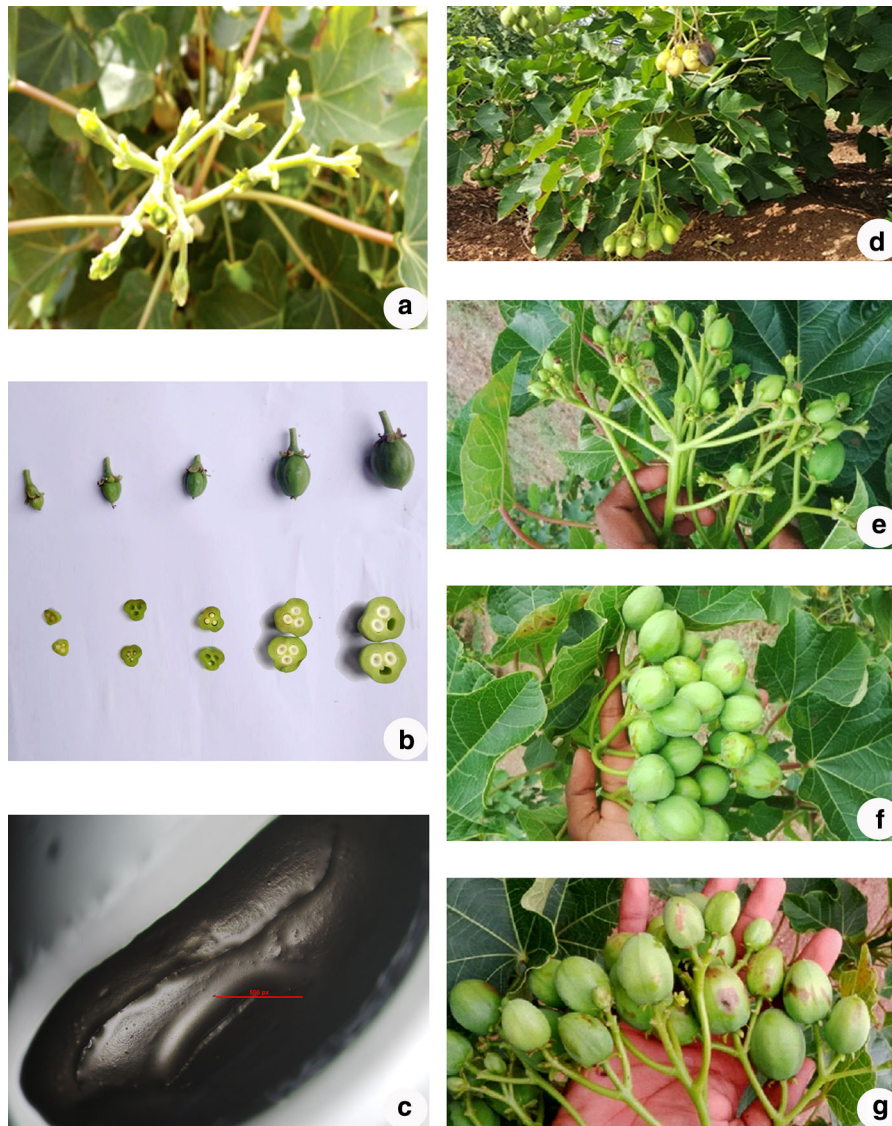
The ovule clearing technique was followed to observe the apomictic ovules. The fruits of *J. curcas* are tricarpellate and hence, gynoecia from each of the locules of developing apomixed fruits were dissected and fixed in formalin acetic acid alcohol (70% alcohol: formaldehyde: acetic acid in a ratio of 18:1:1) for 48 h. Dehydration was in alcohol series (70% alcohol; 85% alcohol; absolute alcohol-3 times) for 2 h duration in each solution. Clearing was done in methyl salicylate (MS) series (3 alcohol:1 MS; 1 alcohol:1MS; 3MS:1alcohol; absolute MS-thrice) with a duration of 2 h each and the processed samples in absolute MS were stored in the refrigerator. The cleared ovules were mounted in MS and observed under differential interference contrast microscope (Nikon Eclipse 80i).

#### Molecular characterization of apomictic progeny using SNPs

The  $F_2$  population of the same cross ( $AC-2 \times AC-86$ ) was subjected to single nucleotide polymorphism (SNP) analysis for identification of markers linked to

seed toxicity in *J. curcas* (Trebbi et al. 2019). The details with regard to DNA isolation, SNP discovery, genotyping assays, sequence analysis and appropriate software used are presented in Trebbi et al. (2019). As part of the research on identification of candidate SNPs linked to seed toxicity, whole genomes of the parents (AC-2 and AC-86) along with the toxic and non-toxic bulks were sequenced using NGS technology on Illumina massively parallel sequencing platform which resulted in identification of 6248 homozygous SNPs between the two parental lines of *J. curcas*. Since the  $F_1A_1$  progeny are derived from the  $F_1$  hybrid of the same cross, 1172 SNPs were used to calculate the genetic distances between the parents,  $F_1$  hybrid and 11 apomictic plants of which six were non-toxic and five were toxic. Genotypic data were used to carry out similarity analyses among these genotypes using NTSYSpc Version 2.1 (Exeter Software, Setauket, New York, USA) to confirm the apomictic origin of the  $F_1A_1$  progeny. Similarity matrixes for data were calculated using Jaccard (J) coefficient. To visualize the relationship between genotypes, the similarity matrix based on the J coefficient was used for the construction of dendrogram using SAHN (Sequential Agglomerative Hierarchical Nested) and UPGMA (Unweighted Pair-Group Method, Arithmetic average) procedure.





**Fig. 2** Seed and fruit development through apomixis in *J. curcas*. **a** Apomictic fruit development in the germplasm accession, **b** seed development in apomictic fruits showing development of 2 or 3 seeds, **c** apomictic ovule showing a

cotyledonary stage adventive embryo (35 $\times$ ), **d** apomictic fruits on the F<sub>1</sub> hybrid of AC-2  $\times$  AC-86, **e–g** increased fruit formation in F<sub>1</sub>A<sub>1</sub> progeny

## Results

### Growth and development of apomictic fruits

Formation of apomictic fruits varied with the flowering span and was pronounced during the first flowering peak from May to August. Apomixis was also influenced by season and weather conditions and during the 3 years of testing, apomictic fruit development was more during 2018–2019 under conditions of

high humidity and precipitation (slightly above 800 mm) rather than during the dry season and during the drought years 2015–2016 and 2016–2017. The number of seeds in apomictic fruits varied from 1 to 3 with an average of 2.45 seeds per fruit (Fig. 2b).

Apomictic mode of reproduction was found to be genotype dependent. Of the 135 accessions from 15 countries tested, 33 accessions displayed apomictic mode of reproduction at a frequency ranging from 1.1 (AC-16) to 37.5% (Chia) (Table 1). Regardless of the

**Table 1** *Jatropha curcas* L. accessions and breeding lines tested for apomixis responsiveness with their origin and toxicity status

Sl. nos.	Germplasm identity	Country of origin	Toxic (T)/non-toxic (NT)	No of flowers subjected to apomixis	Apomixis (%)
1	AC-1	Mexico	NT	110	–
2	<b>AC-2 (JP-2)*</b>	Mexico	NT	104	2.9
3	AC-3	Mexico	NT	79	–
4	AC-4	Mexico	NT	79	2.5
5	AC-5	Mexico	NT	84	–
6	AC-6	Mexico	NT	106	15.6
7	AC-7	Mexico	NT	24	–
8	AC-8	Mexico	NT	103	–
9	AC-9	Mexico	NT	110	–
10	AC-10	India	T	57	–
11	AC-11	India	T	76	–
12	AC-12	India	T	58	3.5
13	AC-13	India (origin uncertain)	T	64	–
14	AC-14	India	T	72	–
15	AC-15	India	T	20	5.0
16	AC-16	India (origin uncertain)	T	89	1.1
17	AC-17	Vietnam	T	19	–
18	AC-18	India	T	82	–
19	AC-19	India	T	34	2.9
20	AC-20	India	T	66	–
21	AC-21	Cape Verde	T	43	–
22	AC-22	India	T	29	–
23	AC-23	India	T	67	–
24	AC-24	Mexico	NT	39	–
25	AC-25	Uganda	T	56	–
26	AC-26	Mexico	NT	67	–
27	AC-27	Mexico	NT	63	–
28	AC-28	Mexico	NT	105	–
29	AC-29	Mexico	T	112	–
30	AC-30	Mexico	NT	127	4.1
31	AC-31	Mexico	NT	82	1.2
32	AC-32	Mexico	T	83	–
33	AC-33	Malaysia	T	73	–
34	AC-34	Madagascar	T	47	–
35	AC-35	Madagascar	T	85	–
36	AC-36	Madagascar	T	78	1.3
37	AC-37	Madagascar	T	62	–
38	AC-38	Madagascar	T	49	–
39	AC-39	Madagascar	T	41	2.4
40	AC-40	Madagascar	T	79	–
41	AC-41	Madagascar	T	58	–
42	AC-42	Madagascar	T	55	1.8
43	AC-43	Madagascar	T	42	–
44	AC-44	Madagascar	T	36	2.8

**Table 1** continued

Sl. nos.	Germplasm identity	Country of origin	Toxic (T)/non-toxic (NT)	No of flowers subjected to apomixis	Apomixis (%)
45	AC-45	Egypt	T	71	–
46	AC-46	India	T	66	–
47	AC-47	India	T	46	–
48	AC-48	Mexico	T	45	4.4
49	AC-50	Mexico	NT	87	–
50	AC-51	Mexico	NT	72	–
51	AC-52	Mexico	NT	81	–
52	AC-53	Mexico	NT	33	–
53	AC-54	Mexico	NT	82	–
54	AC-55	Mexico	NT	48	–
55	AC-56	El Salvador	T	72	–
56	AC-57	Mexico	T	61	–
57	AC-58	Philippines	T	43	4.7
58	AC-59	Philippines	T	77	4.0
59	AC-60	Burkina Faso	T	41	–
60	AC-61	India	T	69	–
61	AC-62	India	T	45	–
62	AC-63	India	T	35	2.9
63	AC-64	India	T	88	2.3
64	AC-65	China	T	91	–
65	AC-66	China	T	124	13.0
66	AC-67	China	T	84	–
67	AC-68	India	T	89	–
68	AC-69	Indonesia	T	98	5.1
69	AC-70	India	T	102	–
70	AC-71	Madagascar	T	160	–
71	AC-72	India	T	79	–
72	AC-73	Mexico	T	141	–
73	AC-74	India	T	41	–
74	AC-75	Mexico	NT	39	–
75	AC-77	Uganda	T	92	–
76	AC-78	India	T	56	–
77	AC-79	Mexico	NT	38	–
78	AC-80	Mexico	NT	71	–
79	AC-81	Mexico	NT	87	–
80	AC-82	Mexico	NT	51	–
81	AC-83	India	T	69	–
82	AC-84	India	T	54	–
83	AC-85	India	T	75	–
84	<b>AC-86 (JP-86)*</b>	India	T	82	3.7
85	AC-89	India	T	29	–
86	AC-90	India	T	76	–
87	AC-91	India	T	88	–
88	AC-92	India	T	72	–

**Table 1** continued

Sl. nos.	Germplasm identity	Country of origin	Toxic (T)/non-toxic (NT)	No of flowers subjected to apomixis	Apomixis (%)
89	AC-93	India	T	70	1.4
90	AC-94	India	T	51	5.1
91	AC-95	India	T	90	–
92	AC-96	India	T	63	–
93	AC-97	India	T	80	–
94	AC-98	India	T	50	–
95	AC-99	India	T	54	–
96	AC-101	India	T	66	2.8
97	AC-102	India	T	70	–
98	AC-103	India	T	83	–
99	AC-104	India	T	39	–
100	AC-105	India	T	69	–
101	AC-106	India	T	81	–
102	AC-107	India	T	72	–
103	AC-108	India	T	60	–
104	AC-109	India	T	80	–
105	AC-110	India	T	65	1.5
106	AC-111	India	T	78	–
107	AC-112	India	T	61	–
108	AC-113	India	T	31	–
109	AC-114	India	T	27	–
110	AC-115	India	T	30	–
111	AC-116	India	T	90	2.1
112	AC-117	India	T	93	–
113	AC-118	India	T	90	–
114	AC-119	India	T	68	–
115	AC-120	India	T	39	25.0
116	AC-121	India	T	48	–
117	AC-122	India	T	37	–
118	AC-123	India	T	41	–
119	AC-125	India	T	27	–
120	AC-131	Paraguay	T	38	–
121	AC-144	Bolivia	T	43	–
122	AC-1003	Indonesia	T	52	–
123	AC-1010	Indonesia	T	31	–
124	AC-1020	Mexico	NT	62	–
125	AC-1026	Mexico	NT	78	–
126	AC-156	Mexico	T	74	–
127	AC-157	Mexico	T	34	–
128	AC-159	Mexico	T	26	–
129	AC-158	El Salvador	T	43	–
130	AC-05-0265	Cape Verde (breeding line)	NT	87	34.5
131	AC-06-0018	Cape Verde (breeding line)	NT	30	23.3
132	AC-06-0020	Cape Verde (breeding line)	T	136	32.4



**Table 1** continued

Sl. nos.	Germplasm identity	Country of origin	Toxic (T)/non-toxic (NT)	No of flowers subjected to apomixis	Apomixis (%)
133	AC-MPec	Mexico (pistillate)	T	65	4.6
134	AC-Mtan	Mexico (pistillate)	T	69	11.6
135	AC-Chia	Mexico (pistillate)	T	32	37.5

Accessions marked in bold were used in the development of the  $F_1$  hybrid from which the  $F_1A_1$  offspring were obtained through apomixis and \* indicates the alternate identity of these accessions

– Indicates no seed set and hence, considered as non-apomictic

country of origin, the different categories of accessions viz, toxic, non-toxic, pistillate and breeding lines produced seeds through apomixis. The three breeding lines from Cape Verde had high frequency of apomixis (23.3–34.5%). It is interesting to observe apomixis in the three pistillate accessions as well and at a maximum frequency of 37.5% in the Chia pistillate accession.

Observations on ovules from developing apomictic fruits were made by following the ovule clearing technique. About 100 ovules were studied but it was difficult to understand the developmental ontogeny of apomictic embryos as the ovule size was not in agreement with the fruit size. In some ovules, clear development of adventive embryo has been observed (Fig. 2c).

#### Evaluation of apomictic progeny for yield and related traits

The clone AC-2 × AC-86 is an  $F_1$  hybrid which is obtained through selective hybridization between a non-toxic Mexican accession and an Indian toxic accession. The  $F_1$  hybrid was vigorous, fast growing, continuous bearing and produced seeds within the 1st year of planting. The cumulative yield was 10.2 kg/plant from 1st to 4th year of planting under good management and good rainfall. The  $F_1$  hybrid was propagated through stem cuttings for production of adequate quantity of hybrid seeds for varied purposes. The selfed seed was used to obtain the  $F_2$  population which was used for identification of molecular markers linked to seed toxicity. The  $F_1$  hybrid produced seeds through apomixis as well and the frequency of apomixis was 87.9% (Fig. 2d).

The apomictic progeny ( $F_1A_1$ ) also reproduced through apomictic mode and the frequency varied

from 9.3 to 88.3% (Fig. 2e–g; Table 2). There was no significant correlation ( $-0.22014$ ) between seed yield under open pollination (2nd year) and the apomixis frequency.

The average fruits per inflorescence in the  $F_1A_1$  progeny were 25–29 in most of the plants while few plants had 35–45 fruits per bunch. Comparison of Fig. 2a and e clearly shows the number of apomictic fruits in germplasm accessions and  $F_1A_1$  plants, respectively with higher fruit formation in the latter. The seed yield of the  $F_1A_1$  progeny ranged from 75.9 to 839.6 g/plant with an average of 335 g/plant during the 1st year. In the 2nd year, the seed yield varied between 93.4 and 1327.0 g/plant with a mean yield of 586 g/plant. The seed yield during the 3rd year of planting varied between 68.8 and 1508 g/plant with an average of 644 g/plant. The cumulative yields for the first 3 years of planting ranged from 304.4 to 3427.7 g/plant with a mean of 1557 g/plant. The single seed weights in open pollinated fruits of the apomictic plants varied from 0.45 to 0.75 g (mean = 0.59 g), 0.50–0.76 g (mean = 0.61 g) and 0.49–0.70 g (mean = 0.64 g) during the 1st, 2nd and 3rd years of planting, respectively (Table 2). The correlation between seed yield and seed weight was positive (0.308). The phorbol ester content determined for some of the high yielding accessions and few selected accessions showed the range in content from 0 (non-toxic) to 7.26 mg/g (highly toxic). Of the 33 plants analyzed for phorbol ester content, 26 plants were toxic while seven plants were non-toxic. It is interesting to note high yielding toxic and non-toxic plants with comparable seed yields such as AP-115 (T) and AP-138 (NT). The seed yield of the  $F_2$  population ( $n = 148$ ) derived from the same cross (AC-2 × AC-86) and raised under the same row to row and plant to plant spacing (2 m × 2 m) was low as compared to

**Table 2** Seed yield (under open pollination) for 3 years after planting (2016–2019), seed weight and seed set (%) under apomixis in the F<sub>1</sub>A<sub>1</sub> progeny obtained from the F<sub>1</sub> hybrid of the cross AC-2 × AC-86 along with the parents and the F<sub>1</sub> hybrid

Plant ID	1st year (2016–2017)		2nd year (2017–2018)		3rd year (2018–2019)		Cumulative seed yield for 3 years (g/plant)	Seed set (%) under apomixis	Phorbol ester (mg/kg full fat kernel powder)
	Seed yield per plant (g)	Single seed weight (g)	Seed yield per plant (g)	Single seed weight (g)	Seed yield per plant (g)*	Single seed weight (g)			
AP-169	93.8	0.52	291.3	0.66	NR	NR	385.1	35.4	–
AP-150	NR	NR	238.4	0.76	68.8	0.65	307.2	37.5	–
AP-159	75.9	0.58	148.0	0.64	80.5	0.66	304.4	36.1	–
AP-144	147.0	0.58	414.3	0.66	101.7	0.65	663.0	47.9	–
AP-163	351.3	0.52	448.8	0.55	104.3	0.64	904.4	63.1	–
AP-167	276.0	0.57	407.9	0.54	109.9	0.63	793.9	68.9	–
AP-168	318.3	0.55	384.7	0.59	129.8	0.53	832.8	10.2	–
AP-147	269.9	0.59	341.5	0.64	187.6	0.67	799.0	39.6	–
AP-175	230.6	0.58	306.5	0.66	203.7	0.61	740.8	50.0	–
AP-160	106.7	0.50	227.4	0.54	218.4	0.69	552.5	25.0	–
AP-176	380.1	0.62	782.7	0.64	237.2	0.69	1400.0	54.1	5.53 T
AP-162	436.6	0.66	568.0	0.67	266.6	0.69	1271.1	52.8	3.58 T
AP-86	108.7	0.63	206.8	0.62	267.7	0.64	583.3	26.7	–
AP-153	154.0	0.46	386.2	0.62	289.3	0.63	829.4	35.4	–
AP-134	215.0	0.51	372.0	0.55	294.9	0.58	881.9	33.3	–
AP-164	415.5	0.59	520.6	0.70	309.1	0.65	1245.2	22.2	–
AP-172	105.8	0.54	366.5	0.56	313.8	0.58	786.1	18.8	–
AP-141	164.2	0.62	93.4	0.57	316.5	0.66	574.0	41.7	–
AP-156	150.0	0.64	370.7	0.65	327.2	0.66	847.8	–	–
AP-140	459.3	0.57	437.9	0.55	333.6	0.49	1230.8	41.7	0.07 NT
AP-110	224.1	0.57	483.5	0.56	342.7	0.65	1050.3	36.1	–
AP-128	241.7	0.63	282.3	0.59	365.8	0.65	889.8	65.2	–
AP-126	326.0	0.53	544.6	0.59	367.9	0.63	1238.5	42.9	–
AP-177	261.5	0.58	471.8	0.57	370.3	0.62	1103.6	9.3	–
AP-173	302.7	0.54	454.7	0.55	372.6	0.59	1130.0	56.5	–
AP-131	249.1	0.51	513.3	0.63	397.2	0.66	1159.5	43.1	–
AP-165	437.7	0.59	587.4	0.64	403.7	0.63	1428.8	36.7	4.3 T
AP-154	448.2	0.71	568.1	0.66	422.9	0.73	1439.2	58.3	0 NT
AP-166	499.9	0.59	675.5	0.58	429.9	0.63	1605.3	44.4	3.14 T
AP-146	306.8	0.52	325.2	0.59	442.9	0.62	1074.8	48.3	–
AP-178	292.0	0.71	817.2	0.66	449.3	0.67	1558.5	48.6	5.8 T
AP-84	152.7	0.56	277.6	0.63	449.8	0.61	880.1	58.3	–
AP-148	527.2	0.63	676.3	0.63	456.6	0.62	1660.1	43.1	2.98 T
AP-152	355.2	0.65	699.3	0.66	469.7	0.67	1524.2	35.4	6.1 T
AP-106	217.7	0.62	541.3	0.60	475.3	0.68	1234.3	58.3	–
AP-174	533.4	0.57	698.6	0.56	489.3	0.58	1721.3	48.6	2.59 T
AP-121	379.8	0.58	295.0	0.55	509.2	0.58	1184.0	47.2	0.06 NT
AP-135	504.7	0.56	598.1	0.61	523.6	0.64	1626.4	45.5	3.01 T
AP-125	267.7	0.51	347.7	0.53	526.3	0.64	1141.7	20.8	–
AP-108	98.8	0.49	464.6	0.57	531.5	0.63	1094.9	37.5	–

**Table 2** continued

Plant ID	1st year (2016–2017)		2nd year (2017–2018)		3rd year (2018–2019)		Cumulative seed yield for 3 years (g/plant)	Seed set (%) under apomixis	Phorbol ester (mg/kg full fat kernel powder)
	Seed yield per plant (g)	Single seed weight (g)	Seed yield per plant (g)	Single seed weight (g)	Seed yield per plant (g)*	Single seed weight (g)			
AP-133	363.0	0.57	865.0	0.61	531.9	0.53	1760.0	33.3	–
AP-96	287.7	0.71	568.3	0.68	536.6	0.71	1392.7	40.0	–
AP-170	537.4	0.55	465.9	0.54	542.5	0.59	1545.8	88.3	7.26 T
AP-130	185.2	0.62	542.6	0.56	545.7	0.58	1273.4	47.9	–
AP-142	197.2	0.67	433.7	0.60	545.9	0.61	1176.8	45.8	–
AP-151	311.2	0.67	540.3	0.66	587.3	0.62	1438.8	41.7	–
AP-95	188.0	0.63	534.5	0.59	588.1	0.76	1310.6	–	–
AP-143	479.5	0.63	703.9	0.62	589.0	0.61	1772.4	31.3	5.53 T
AP-129	278.8	0.54	402.4	0.59	594.5	0.61	1275.8	64.3	–
AP-122	481.3	0.59	473.5	0.50	638.1	0.66	1592.9	53.7	–
AP-157	430.5	0.75	594.8	0.62	649.8	0.63	1675.1	55.6	0 NT
AP-103	392.5	0.53	652.0	0.62	657.8	0.65	1702.3	38.5	–
AP-149	525.7	0.66	756.4	0.65	658.7	0.61	1940.8	22.9	5.1 T
AP-112	467.1	0.65	1167.8	0.70	680.9	0.71	2315.8	50.0	7.1 T
AP-120	332.2	0.58	479.7	0.60	681.4	0.67	1493.3	42.9	–
AP-102	586.7	0.58	758.3	0.61	687.4	0.64	2032.4	44.4	5.51 T
AP-113	355.5	0.50	598.2	0.55	693.7	0.53	1647.4	51.2	–
AP-116	168.4	0.51	632.8	0.65	694.5	0.63	1495.7	27.0	–
AP-137	426.8	0.58	529.3	0.55	695.6	0.62	1651.7	19.4	–
AP-111	125.9	0.54	714.7	0.64	702.5	0.62	1543.0	22.9	–
AP-93	544.6	0.58	636.5	0.54	707.3	0.65	1888.4	14.6	–
AP-100	161.7	0.51	598.4	0.61	713.7	0.72	1473.8	26.7	–
AP-92	542.2	0.53	532.3	0.54	744.8	0.59	1819.3	16.7	0.19 NT
AP-145	223.4	0.62	391.0	0.61	755.7	0.67	1370.0	52.4	–
AP-155	675.1	0.62	787.5	0.72	761.7	0.71	2224.3	26.7	6.59 T
AP-161	465.5	0.65	778.0	0.66	765.2	0.74	2008.7	37.5	4.64 T
AP-132	266.6	0.58	545.6	0.56	774.9	0.61	1587.1	37.5	–
AP-124	301.5	0.63	520.8	0.57	801.3	0.66	1623.6	38.9	–
AP-139	647.3	0.62	976.6	0.61	809.4	0.62	2433.3	27.8	4.59 T
AP-114	374.3	0.61	787.6	0.59	809.8	0.67	1971.7	38.9	5.9 T
AP-123	463.3	0.59	835.4	0.63	853.9	0.67	2152.6	42.7	–
AP-94	465.0	0.49	599.2	0.59	861.4	0.63	1925.6	–	4.19 T
AP-107	125.6	0.48	490.7	0.59	866.6	0.66	1482.9	27.1	–
AP-109	250.8	0.63	861.8	0.66	867.6	0.67	1980.2	37.5	5.6 T
AP-104	335.2	0.58	611.4	0.55	892.1	0.67	1838.7	23.8	–
AP-117	294.3	0.52	416.7	0.50	930.8	0.54	1641.8	18.8	–
AP-101	341.1	0.59	489.6	0.61	933.1	0.75	1763.8	55.3	–
AP-127	224.0	0.55	352.0	0.56	933.8	0.65	1509.7	41.7	–
AP-88	191.5	0.63	662.0	0.66	1019.6	0.72	1873.0	–	–
AP-90	431.7	0.61	698.1	0.57	1021.3	0.62	2151.1	22.6	–
AP-119	263.3	0.58	976.2	0.61	1038.9	0.60	2278.4	25.0	–

**Table 2** continued

Plant ID	1st year (2016–2017)		2nd year (2017–2018)		3rd year (2018–2019)		Cumulative seed yield for 3 years (g/plant)	Seed set (%) under apomixis	Phorbol ester (mg/kg full fat kernel powder)
	Seed yield per plant (g)	Single seed weight (g)	Seed yield per plant (g)	Single seed weight (g)	Seed yield per plant (g)*	Single seed weight (g)			
AP-118	187.0	0.67	917.9	0.68	1076.2	0.71	2181.1	23.3	–
AP-79	416.3	0.57	531.1	0.54	1117.4	0.60	2064.7	19.4	–
AP-89	441.8	0.69	1014.7	0.63	1123.2	0.63	2579.6	–	3.6 T
AP-97	333.3	0.69	889.3	0.65	1204.7	0.74	2427.3	–	5.3 T
AP-91	496.4	0.61	784.7	0.67	1227.3	0.62	2508.4	–	–
AP-136	571.9	0.62	1278.3	0.62	1246.7	0.65	3096.9	22.9	4.35 T
AP-115	839.6	0.63	1327.0	0.64	1261.1	0.70	3427.7	–	3.49 T
AP-138	451.5	0.60	817.7	0.62	1328.1	0.66	2597.3	23.6	0 NT
AP-98	213.8	0.69	511.3	0.60	1343.0	0.62	2068.1	–	–
AP-105	314.2	0.67	787.6	0.60	1397.3	0.69	2499.1	16.7	–
AP-87	341.1	0.61	857.7	0.59	1486.8	0.65	2685.7	23.6	5.5 T
AP-83	364.3	0.66	883.7	0.62	1500.4	0.66	2748.4	23.6	–
AP-99	382.2	0.55	893.6	0.66	1508.4	0.65	2784.2	31.3	5.4 T
Range	75.9–839.6	0.45–0.75	93.4–1327	0.50–0.76	68.8–1508	0.49–0.76	304.4–3427.7	–	–
Mean	334.9	0.59	586.1	0.61	644	0.64	1557.2	–	–
Seed yield of the parents and the F <sub>1</sub> hybrid**									
AC-2	105.6	0.65	310.2	0.55	430.4	0.53	846.2	2.9	0.047 NT
AC-86	195.9	0.60	258.2	0.53	482.1	0.63	936.2	3.7	6.03 T
F <sub>1</sub> hybrid (AC-2 × AC-86)	581.7	0.64	460.9	0.59	752.1	0.64	1794.7	87.9	3.82 T

T Toxic, NT non-toxic, NR not recorded, – not determined

\*Data sorted based on seed yield per plant (g) during the 3rd year after planting

\*\*The first 3 years after planting in this case were 2014–2015, 2015–2016 and 2016–2017, the later 2 years were severe drought years at the experimental site

that of the apomictic progeny (F<sub>1</sub>A<sub>1</sub>). The seed yield (g/plant) of the F<sub>2</sub> population ranged from 3.0 to 165 (mean = 47.5); 3.8–426.4 (mean = 105), 34.3–707.6 (mean = 259.2) during the 1st, 2nd and 3rd years after planting, respectively (Figure S1). The seed yield of the F<sub>2</sub> population during the 4th year was comparable to the 3rd year yield of the F<sub>1</sub>A<sub>1</sub> progeny and ranged from 111.5 to 2103 g/plant (mean = 664.9). The seed yield of the clonally propagated plants of AC-2 × AC-86 F<sub>1</sub> hybrid (n = 70) during the 3rd year of establishment varied from 290 to 1905 g/plant (mean = 660 g/plant) with about 73% of the plants producing more than 500 g/plant. The seed weight ranged between 0.58 and 0.76 g (mean = 0.66 g).

The characters of seeds obtained through open pollination and through apomixis were compared and results are presented in Table 3. For this, samples were drawn from 1.0 kg bulks each for recording the observations. The seed weight of apomictic seed was high with a higher kernel to hull ratio. Despite a higher kernel content, the oil content determined on whole seed and also kernel basis was lower in the apomictic seed as compared to the open pollinated seed.

#### Confirmation of apomixis through SNP analysis

Genetic distances based on 1172 SNP mutations were used to draw the dendrogram (Fig. 3). The dendrogram clearly shows the clustering of the F<sub>1</sub>A<sub>1</sub> progeny

with the F<sub>1</sub> hybrid which in turn was grouped with the ovule parent (AC-2).

## Discussion

This study deals with the property of apomixis that is demonstrated by *J. curcas* and especially how this property can be used to increase the variability that would aid selection and hybridization. Apomixis is a boon for plant breeders and is preferred in hybrid crops for fixing heterozygosity and hybrid breeding; and in self-pollinated crops for production of cloned seed and maintenance of superior clones. *J. curcas* is a facultative apomict and predominantly reproduces sexually either through xenogamy, geitonogamy or occasionally through apomixis. In most of the crop plants, genes controlling apomixis were transferred from wild relatives to cultivated genotypes such as, cassava (Nassar et al. 1998, 2008), cereals like maize, pennisetum (Ozias-Akins et al. 2003; Savidan 2001) and forage grasses (Asker 1979). Introgression of apomixis from wild relatives is often hampered by interspecific crossability barriers and ploidy differences limiting the development of agronomically desirable genotypes. However, in case of *J. curcas*, apomixis occurs naturally and genes governing apomixis are identified in the cultivar germplasm that could be easily transferred to any desirable genetic background.

Sex in *J. curcas* is reported to be vulnerable to external factors and consequently, floral bud initiation, flower sex ratio, fruit maturation and seed set are influenced by the nutrient contents, physical and physiological factors and the micro-environment (Bhattacharya et al. 2005). Likewise, apomictic fruit development is influenced by the micro-environment under which the plants are grown. Apomictic seed development was pronounced during the first flowering flush as compared to the second flowering span.

Apomixis is reported by several research groups which indicates that the crop during the course of evolution has adapted itself for a mixed mating system (Abdelgadir et al. 2009; Bressan et al. 2013; Nietsche et al. 2014; Chang-Wei et al. 2007). The conditions favoring apomictic mode of development needs to be unravelled so as to enable the breeders to manipulate breeding program by switching the sexual and asexual modes of seed development. Light microscopic analysis has indicated development of somatic embryos in the apomictic fruits of *J. curcas*. Ambrosi et al. (2010) reported the occurrence of non-gametophytic apomixis in *J. curcas*. Adventitious embryos are seen to develop from the nucellus of abortive ovules during fruit development (Sowmyalatha et al. 1997). In cassava, both apospory and somatic embryony are reported to occur in the apomictic hybrids indicating plasticity among different types of apomixis (Freitas and Nassar 2013). *Hevea* is reported to be a facultative apomict, where both sexual and asexual embryogenesis can be active and adventive embryony is a prevalent phenomenon in Euphorbiaceae (Bhojwani and Bhatnagar 1992). As apomixis is found to be a common phenomenon in *J. curcas*, it should not be difficult to understand the mode of development of the apomictic embryos.

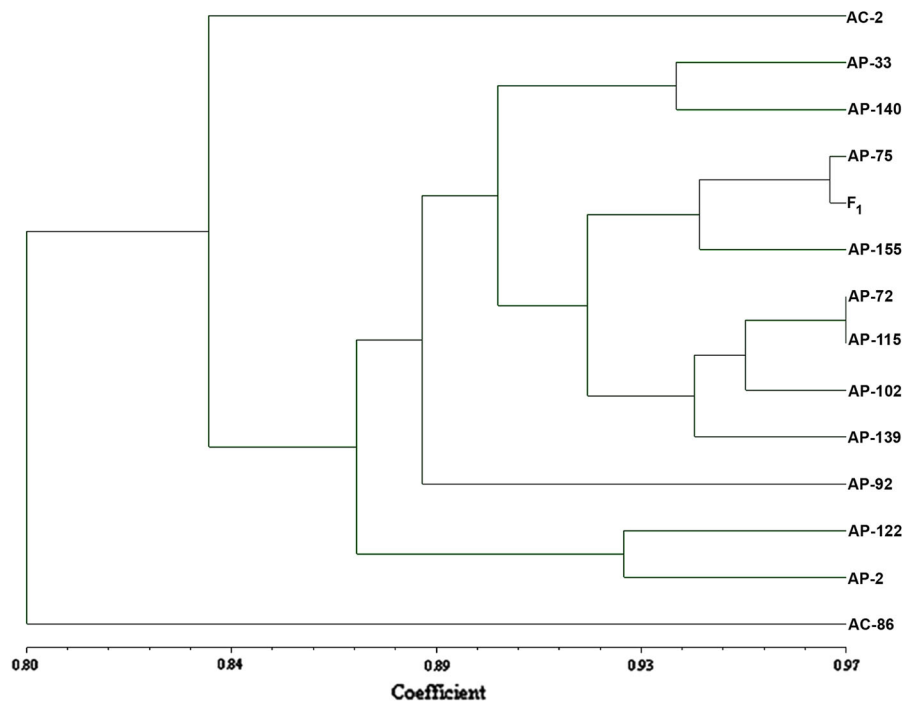
Fruit set under apomixis varied in different studies and was 4% (Abdelgadir et al. 2009), 5% (Juhasz et al. 2009), 10.1% (Nietsche et al. 2014), 12% (Chang-Wei et al. 2007), 13% (Bressan et al. 2013), 28.5% (Pranesh et al. 2010), 32.0% (Bhattacharya et al. 2005), 36.2% (Kaur et al. 2011) and 0.1 fruit per inflorescence (Rincón-Rabanales et al. 2016). Results in most of these studies are based on a single genotype. In the present investigation, frequency of apomixis based on fruit set varied from 1.1 to 37.5% in 33 accessions from a total of 135 accessions studied. Regardless of the geographical origin, apomixis was detected in the toxic, non-toxic, breeding lines and pistillate accessions. The frequency of apomictic fruit

**Table 3** Comparison of seed traits in seeds obtained through open pollination and apomixis

Seed	Seed weight	Hull (%)	Kernel (%)	Seed oil content (NMR)
Open pollinated seed	0.72 ± 0.05	36.3 ± 0.9	63.8 ± 0.9	35.9 ± 0.85
Apomixed seed	0.79 ± 0.06	34.7 ± 1.6	65.2 ± 1.6	34.4 ± 0.56

Differences are significant according to paired *T* Test, number of seeds in each sample = 20

**Fig. 3** Dendrogram based on genetic distances from 1172 SNPs. AC-2 and AC-86 are parents;  $F_1$  represents the AC-2  $\times$  AC-86 hybrid; AP-2, AP-33, AP-75, AP-92, AP-122, AP-140 are non-toxic apomictic plants; and AP-72, AP-102, AP-115, AP-139, AP-155 are toxic apomictic plants derived from the  $F_1$  hybrid



formation was high in the breeding lines and the pistillate accessions. It is interesting to note apomixis in pistillate plants which unarguably confirms asexual reproduction through apomixis in *J. curcas*. Likewise in cassava, apomixis was confirmed based on abundant fruit formation in plants producing sterile flowers (Freitas and Nassar 2013). Further, it is advantageous in breeding programmes aimed at hybrid development as the pistillate lines could be maintained through apomixis precluding the need of a maintainer line for maintenance of the pistillate trait. When both the parents with apomixis were hybridized, the  $F_1$  (87.9%) and also the  $F_1A_1$  progeny (9.3–88.3%) had higher frequencies of apomixis. This probably could be due to the additive action of the apomictic genes in the parents which needs further investigations.

Rincón-Rabanales et al. (2016) made observations with regard to open pollinated and apomictic fruits, and differences in fruit size were not observed but fresh fruit weight was high in apomictic fruits (13.8 g) as compared to open pollinated fruits (12.9 g) and conversely, seed fresh weight was lower in apomictic seeds (1.21 g) as compared to seeds obtained through open pollination (1.65 g). In our study, comparison of seed weight and oil content of seeds from apomictic mode of reproduction and open pollination were made

which indicated higher test weight, a higher kernel content and lower oil content in apomictic seeds as compared to those obtained through open pollination.

Perpetuation of the heterotic potential through apomixis is evident from the seed yield data of the  $F_1A_1$  progeny during the initial 3 years of establishment. The yields recorded for the  $F_1A_1$  progeny are apparently high when compared to most of the previous investigations (Rao et al. 2008; Biabani et al. 2012; Martin and Montes 2015; Francis et al. 2018). Keeping in view the small plant type of AC-2, a spacing of 2 m  $\times$  2 m between rows and plants was adopted in this study for the  $F_1A_1$  progeny, and the plant population was 2500 plants/ha. Accordingly, the seed yields was 189 kg to 2.1 tons with an average of 837 kg/ha during the first year; 234 kg to 3.32 tons with a mean of 1.5 tons/ha during the 2nd year; 172 kg to 3.7 tons with a mean of 1.6 tons/ha during the 3rd year and the cumulative yields for the 3 years ranging from 760 kg to 8.6 tons with an average of 3.9 tons/ha. Laviola et al. (2013) reported seed yield of 1093 kg/ha in the 4th year. Shabanimofrad et al. (2013) reported seed yields that ranged from 107 to 745.72 g/plant during the second year in Malaysian accessions evaluated under a high precipitation of 2429 mm while in the present study, apomictic



progeny produced seed yields ranging from 93.4 to 1327 g/plant during the second year where the rainfall during the year was only 16.5% (400 mm) of that in Malaysia. In the study of Biabani et al. (2012), the maximum seed yield during the 2nd year of planting was 399 g/plant. Evaluation of 57 diverse genotypes representing nine countries for seed yield also showed high yields only after the 4th year of establishment (Francis et al. 2018).

Breeding edible *J. curcas* assumes importance and this study indicates the possibility of making selections of elite toxic and non-toxic genotypes from the same population instead of breeding the edible and non-edible accessions in different breeding pipelines. For e.g. the apomictic progeny AP-115 (T) and AP-138 (NT) with comparable seed yields varied for toxicity. Combining heterosis and apomixis has resulted in selection of high yielding toxic and non-toxic accessions simultaneously. Evaluation of promising non-toxic accessions under similar conditions recorded yields of 253 and 329 g/plant during the 1st and 2nd years of planting (Francis et al. 2013) and a maximum of 1651 kg/plant during the 5th year after planting (Francis et al. 2018).

Apomixis is one of the reproductive strategies for production of offspring that are genetic replicas of the maternal parent. Progeny derived through apomixis display molecular patterns similar to that of the maternal parent. Bressan et al. (2013) investigated the mating system in *J. curcas* using five microsatellite loci and reported occurrence of apomixis at a frequency of 13% in a natural population. In the present study, SNPs based on whole genome sequencing of the parents were used to confirm the apomictic origin of the  $F_1A_1$  progeny. The  $F_2$  population derived from the same cross was used for identification of SNPs associated with seed toxicity, and has enabled simultaneous selection of high yielding toxic and non-toxic plants with homozygous SNPs associated with the respective traits (Trebbi et al. 2019). With the advent of high throughput NGS techniques, availability of draft genome sequence, genome wide association study (GWAS) tools and appropriate breeding populations, it would not be difficult to identify SNPs associated with the apomixis traits. Further, differential gene expression analysis of the developing sexual and apomictic gynoecia may shed more light on the candidate genes governing apomixis in *J. curcas*.

*Jatropha curcas* is reported to exhibit a mixed mating system (Bressan et al. 2013). In this study, attempts were made to exploit different modes of propagation for development of elite lines. Based on the information existing in literature that the accessions from Mexico are genetically diverse (Basha et al. 2009; Pecina-Quintero et al. 2011; Montes Osorio et al. 2014; Santos et al. 2016; Li et al. 2017), crosses were effected between a non-toxic Mexican accession and a toxic Indian accession. Both genotypes had different plant types and characteristics and the resultant hybrid exhibited high heterotic potential. The high yielding hybrid was propagated through stem cuttings for establishment of the hybrid plants orchard. The  $F_1$ s were selfed to obtain the  $F_2$  progeny which were used for studying the genetics of key traits including seed toxicity (Trebbi et al. 2019). Further, the  $F_1$  hybrid was propagated asexually through apomixis which has resulted in progeny with high yielding ability. This process has also facilitated selection of both toxic and non-toxic genotypes with high yield from the same progeny rather than breeding for the two types separately. In shrub breeding, development of elite cultivars is a time consuming process. However, exploiting different mating types naturally existing in *J. curcas* has led to the development of hybrids, appropriate breeding populations and superior selections simultaneously and within a limited time period. Taking advantage of the mating system and flowering spans (continuous, single, two flowering flushes), appropriate strategies could be devised to combine hybrid breeding, clone-based breeding program and apomixis to accelerate the breeding process towards development of high quality planting materials in a shortened period and in a cost effective manner in *J. curcas*.

In conclusion, *J. curcas* has great potential but the process of early domestication is delayed due to a genetic bottleneck. In recent past, extensive characterization of germplasm from in situ and ex situ collections and multi-locational evaluation of promising accessions provided valuable insights regarding the extent of exploitable genetic diversity in *J. curcas* that could be harnessed for adequate genetic gains. The present investigation successfully demonstrates the potential of sexual and asexual reproductive mechanisms inherent in this crop for development of elite cultivars with limited resources. Thus, exploiting apomixis in conjunction with other conventional

methods of hybrid breeding and vegetative propagation would accelerate breeding process and achieve genetic gains in a cost effective manner in facultative apomicts like *J. curcas*.

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

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