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Improved crossing technique and identification of true F_1 hybrids of *Ziziphus mauritiana* Lam. by molecular markers

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ABSTRACT

Breeding in *Ziziphus mauritiana* Lam. through hybridization is limited by its small sized flowers, cross-incompatibility, low fruit-set and poor retention. In the present study, emasculation of flowers 2 h before anthesis and pollination by placement of dehisced flowers on stigma in inverted position resulted in increased fruit-set. Crossing of *Z. mauritiana* cultivars Gola and Thar Sevika (early maturity and fruit quality) with cultivars BS-1 (powdery mildew resistant) and Tikadi (fruit fly and frost tolerant) showed that Thar Sevika is cross compatible with BS-1 and Tikadi whereas Gola is cross compatible with BS-1 only. Among the crosses fruit-set range was 2.31–10.92%. Based on the presence of male parent-specific DNA fragment produced by RAPD and ISSR markers 13 out of 14 F_1 progeny seedlings were found to be true hybrids. This is the first report in *Z. mauritiana* on the identification of true hybrids among F_1 progenies using molecular markers.

1. Introduction

Indian jujube (*Ziziphus mauritiana* Lam.) commonly known as '*ber*' is one of the most ancient and common fruits indigenous to India, belonging to the family *Rhamnaceae*. Though it is an important fruit of the arid and semi arid parts of India, it is considered an under-utilized fruit crop. *Ber* is drought-tolerant and grows in the wild as well as in cultivated forms throughout the warmer regions of India, Pakistan, Bangladesh, Sri Lanka, Central to South Africa and northern parts of Australia (Pareek, 2001). It is a multipurpose tree although use of fruits is a major focus of interest. *Ber* fruits are richer than apple or citrus in protein, minerals and vitamins A and C (Jawanda and Bal, 1978). The fruits are eaten raw, dried, used for making pickles or made into confectionery and fruit juice (Awasthi and More, 2009).

Evolution and distribution of *Z. mauritiana* is believed to be in the Indo-Malaysia region (Pareek, 2001). This species has a wide range of morphologies from shrub to small or medium sized trees which might be erect, semi-erect or semi-spreading. The tree is semi-deciduous shedding its leaves during hot summer. Usually the shrub or tree is spinuous with short, strait or hooked spines. Leaves are alternate, oblong-ovate, obtuse or acute, nearly asymmetric, dense with dark green depressed longitudinal veins. Lower surface of leaf is whitish due to persistent dense hairs but may be buff colored while the upper surface is glossy. Flowers are very small (2–4 mm) in cymes, predominantly green or yellow or white, pedicellate (5–6 mm long pedicle). The pollen is sticky, thick and heavy and pollination is done by honey bees (*Apis* spp.), yellow wasp (*Polister hebraeus*) and house fly (*Musca domestica*) (Morton, 1987). Fruit is a drupe, fleshy, yellow or red brown. The ripe fruit is sweet and sour in taste. Single stone per fruit, hard, rough, central stone contains 1–2 elliptic brown kernels (Pareek, 2001; Jagwan and Singh, 2009; Vashishtha, 2001).

High genetic variation exists in *Z. mauritiana* germplasm because of cross pollination, self-incompatible and polyploid nature. These genetic variations provide ample opportunities for selection of genotypes for desirable traits (Vashishtha and Pareek, 1989; Vashishtha, 2001). Most of the commercially growing cultivars of *Z. mauritiana* were identified by selection from the natural variants for their fruit quality and yield; and their true to type being maintained by budding on the rootstock (Azam-Ali et al., 2006). These cultivars are susceptible predominantly to powdery mildew disease and fruit fly infestation and to some extent, frost. Powdery mildew (*Oidium erysiphoides* f. sp. *ziziphi*) disease results in defoliation and fruit drop (Pardeep and Jambhale, 2001) and causes yield loss up to 60% (Jamadar et al., 2009). Fruit damage caused by fruit fly has been recorded up to 100% (Sharma et al., 1998). Low temperature (frost) at the time of fruit development causes fruit drop and deformities which result in reduction of yield and quality of

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Table 1a
Genotypes of *Z. mauritiana* used in this study and their important characters.[a]

S. No.	Characters	Gola	Thar Sevika	BS-1	Tikadi
1.	Cultivar class	Early maturing	Early maturing	Late maturing	Mid season maturing
2.	Total soluble solids (TSS)°	14.7	17.1	14.4	15.0
3.	Time of Anthesis	12 Noon	8.30 AM	8.30 AM	1.00PM
4.	Leaf shape	Ovate	Oblong	Oblong	Cordate
5.	Fruit shape	Oval/Apple shape	Ovate	Oblong	Pyriform
6.	Resistance to powdery mildew disease	Susceptible	Susceptible	Resistant	Moderate resistant
7.	Resistance to fruit fly	Susceptible	Susceptible	Moderate resistant	Resistant
8.	Tolerance to frost	Susceptible	Susceptible	Susceptible	Resistant

[a] Pareek (2001), Vashishtha (2001), Azam-Ali et al. (2006), Sharma et al. (1998), Sharma and Panwar (2002), and Anonymous (2005).

fruits (Azam-Ali et al., 2006). Damage to foliage and fruits of *ber* due to frost was noticed in the arid regions of Rajasthan (Anonymous, 2004, 2008). The yield loss due to frost was recorded up to 40%. Therefore, to develop varieties resistant to powdery mildew and fruit fly infestation and tolerant to frost a breeding programme was initiated at the Central Institute for Arid Horticulture (CIAH), Bikaner during 2008. The large germplasm collection of *Z. mauritiana* maintained at the field gene bank of CIAH (Awasthi and More, 2009) were screened under natural conditions at the CIAH farm and other different powdery mildew and fruit fly hot spot/endemic locations of India through All India Coordinated Research Project (AICRP) on Arid Fruits for several years. The germplasm line, Tikadi, was found highly resistant to fruit fly damage (1–10% infestation) (Anonymous, 2005; Sharma et al., 1998) while BS-1 was highly resistant to powdery mildew (Anonymous, 2005; Sharma and Panwar, 2002). In addition, Tikadi has shown tolerance to frost/low temperature (Anonymous, 2008). These genotypes can be used as donor parents with commercial cultivars like Gola and Thar Sevika to impart resistance to powdery mildew disease, fruit fly and frost damage through hybridization, which otherwise have useful characters (Table 1a). The main bottlenecks in ber breeding through hybridization are lack of improved crossing techniques to handle small size flowers for emasculation, self- and cross-incompatibility, prevalence of polyploid genotypes, long juvenile phase and lack of techniques to identify true hybrids among F_1 progenies at an early stage (Azam-Ali et al., 2006; Pareek et al., 2007). Identification of hybrid nature of F_1 progenies by morphological markers is difficult as they are limited and easily influenced by the environment. However, these can be identified using molecular markers which are abundant and not affected by environment and developmental stages of plant. The molecular markers such as Random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), and simple sequence repeat (SSR) have been used to identify hybrids, compatibility groups, examination of genetic relationship and diversity, and cultivar identification in various perennial plants (Weekley et al., 2002; Golein et al., 2011; Bendokas et al., 2007; Bhat et al., 2010; Wunsch and Hormaza, 2002; Ross-Davis et al., 2008). Therefore, improving crossing techniques, cross compatibility between cultivars having useful traits in *ber* and identification of true hybrids among F_1 progenies are essential to accelerate the breeding programme. Here, we report the cross compatibility of selected genotypes of *Z. mauritiana* and the identification of F_1 progenies using molecular markers.

Table 1b
Details of ISSR primers.

Primer name[a]	UBC[b] code	Sequence (5′–3′)
P1	808	AGAGAGAGAGAGAGAGAGC
P2	809	AGAGAGAGAGAGAGAGG
P3	814	CTCTCTCTCTCTCTCTA
P4	825	ACACACACACACACACT
P5	829	TGTGTGTGTGTGTGTGC
P6	240	GAGAGAGAGAGAGAGACTT
P7	841	GAGAGAGAGAGAGAGACTC
P8	848	CACACACACACACACAAGG
P9	850	GTGTGTGTGTGTGTGTCTC
P10	854	TCTCTCTCTCTCTCTCAGG
P11	855	ACACACACACACACACCTT
P12	856	ACACACACACACACACCTA
P13	876	GATAGATAGACAGACA
P14	880	GGAGAGGAGAGGAGA
P15	889	AGTCGTAGTACACACACACACAC
P16	890	ACGACTACGGTGTGTGTTTGTGT
P17	894	TGGTAGCTCTTGTCAGGCAC
P18	900	ACTTCCCCACAGGTTAACACA

[a] Primer name used in this study.
[b] University of British Columbia.

2. Materials and methods

2.1. Standardization of crossing techniques

Standardization of crossing technique was done during the flowering season (mid August–November) of *Z. mauritiana* in the year 2008. *Z. mauritiana* cv BS-1 (Bawal Selection-1) was used as female parent while cv. Gola was the male parent. Emasculation was done by two methods namely (i) removal of anthers, sepals and petals 1 day before anthesis and (ii) removal of anthers and petals 2 h before anthesis. In each emasculation method, three different pollination techniques were employed. They were (i) placement of anthers on stigma, (ii) rubbing of dehisced flowers on stigma, and (iii) placement of dehisced flowers on stigma in an inverted position (Table 2). For emasculation, branches of the tree were selected randomly during peak period of flowering. Approximately a 20 cm long terminal end of the branch possessing three to four cymes was selected. From the selected cymes immature flower buds, opened and open-pollinated flowers were removed. One to two flower buds at the appropriate stage were emasculated in each cyme. After emasculation, the remaining cymes on the branch and terminal part of the growing shoot tip were also removed. The emasculated flowers were covered with butter paper bags to prevent pollinating insects and were tagged. For pollination, flowers about to dehisce were collected from male parent (cv. Gola) in butter paper bags and kept in sunlight for 30 min. Pollination was done as per the three methods described above. Pollinated flowers were covered immediately by fresh butter paper bags and stapled. The covers were retained for 3 days as the duration of stigma receptivity is 24–48 h after anthesis. After 3 days, the cover was removed and observations on flower retention and successful fruit-set were taken on the basis of color change of the swollen ovary from cream to green. The immature ovaries/set fruits were allowed to grow in the open until pea size stage. At pea stage of fruit, the branches bearing them were again covered with perforated butter paper bags to prevent damage to fruits by birds and infestation by fruit fly. Fully developed fruits were carefully plucked and stones were extracted. The F_1 seeds were obtained by carefully breaking the stones.