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RESEARCH ARTICLE

Development of novel interspecific hybrid between cultivated and wild species of okra [*Abelmoschus esculentus* (L.)] Moench through embryo rescue

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Abstract

A reproducible embryo rescue technique has been developed for producing YVMV resistant okra in which embryos developed through hand pollination between a cultivated okra species *Abelmoschus esculentus* (Cv. Pusa Sawani) and wild species, *A. moschatus* subsps *tuberosus* (IC 470750) were excised at a very specific stage and germinated on agar solidified medium containing a basic salt mixture and carbohydrate. The choice of carbohydrate and the growth conditions employed were demonstrated to markedly affect the germination percentage and subsequent plantlet development. This technique has been proved successful for the production of F_1 hybrid when compared to conventional methods of hand pollination followed by post-zygotic development on plant itself, which generally leads to embryo abortion in case of interspecific hybridization between two sexually cross-incompatible species of okra. The trueness of interspecific hybrid plant obtained through embryo rescue technique from a cross between cultivated and wild species (tertiary to primary gene pool) of okra was confirmed by morphological and molecular characterizations. The two parents and F_1 hybrid developed were characterized morphologically using DUS parameters set for okra. The optimization of this technique will overcome pre and post-fertilization barriers coming across in the interspecific hybridization process and will pave the way further for gene introgression through distant hybridization in okra.

Keywords: Okra, Embryo rescue, Interspecific hybridization, YVMV, *A. moschatus* subsps. *tuberosus*

Introduction

Okra [*Abelmoschus esculentus* (L.) Moench] is one of the most popular vegetable crops cultivated throughout world for its characteristic tender, fleshy fruits. It belongs to the Malvaceae family and is an allopolyploid plant with considerable chromosome numbers and ploidy levels variations. The chromosome number of cultivated okra has a vast range of variation. The lowest chromosome number reported is 2n=56 for *A. angulosus* (Ford 1938), whereas the highest is 2n= 200 for *A. manihot* var. *caillei* (Siemonsma 1982a and 1982b; Singh and Bhatnagar 1975). Even within *A. esculentus*, chromosome numbers 2*n* = 72, 108, 120, 132 and 144 are observed in a regular series of polyploids with x = 12 (Datta and Naug 1968). The geographical origin of okra is found to be Africa (National Horticulture Board 2018). It is an important vegetable crop in the tropical and subtropical regions of the world (Wammanda et al. 2010). In the world, India is the largest producer of okra (6.3 mt) with a contribution of 61.9%, followed by Nigeria (2.04 mt) and Sudan (0.28 mt) [FAOSTAT 2013]. India occupies the highest area under cultivation (0.53 mha) followed by Nigeria (1.10 mha) and Cote d'Ivoire (0.05 mha). But in terms of productivity, India ranks 12th (12 t/ha) which is almost half as compared to Bahamas (24 t/ha) and Senegal (23 t/ha), which rank first and second, respectively in terms of productivity. Major okra-producing states in India are Andhra Pradesh, West Bengal, Bihar, Gujarat, Odisha, Jharkhand, Maharashtra, Madhya Pradesh, Chhattisgarh, Assam and Uttar Pradesh.

Gujarat has a production and productivity of 859.47 '000 mt and 11.65 mt/ha, respectively with 73.79 thousand hectares area under cultivation, which is the highest

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amongst all the states of India in the year 2018-19 (National Horticulture Board 2018). There is a necessity to improve the yield per unit area to achieve increased production from limited land. In okra, yield levels have been improved substantially through intensive and concerted breeding efforts, and further yield advances seem to be more difficult, necessitating the application of newer breeding approaches.

A number of diseases caused by viruses, fungi, bacteria, phytoplasma, nematodes and insect pests attack this crop (Prakasha et al. 2010). Yield loss of okra due to crop pests has been estimated up to 20 to 30%, and may increase up to 80 to 90% in case of a severe infestation (Ali et al. 2005). Okra Yellow Vein Mosaic Virus (OYVMV) transmitted by white fly (*Bemisia tabaci* Gen.) is the most serious disease of okra (Fajinmi and Fajinmi 2010). As 100% infection in the field is very usual and yield loss ranges between 50 and 94% depending on the stage of crop growth at which infection occurs (Sastry and Singh 1974). If plants are infected within 20 days after germination, their growth is retarded, a few leaves and fruits are formed and loss may reach up to 94% (Sastry and Singh 1974). The extent of damage declines with delay in infection of the pathogens. Plants infected 50 and 65 days after germination suffer a loss of 84 and 49%, respectively (Ali et al. 2005; Sastry and Singh 1974).

The resistant varieties developed in okra by various research organizations through interspecific hybridization are very meager and have started showing signs of susceptibility. Hence, it is imperative to find diverse source of resistance to YVMV and evolve resistant varieties by suitable gene introgression programme. Interspecific hybridization for YVMV resistance followed by selection in the segregating generations is an effective method for obtaining desirable recombinants (Aanchal et al. 2021). Several wild species of okra showed high degree of YVMV resistance. Among the okra wild taxa, *A. tetraphyllus*, *A. ficulneus*, *A. crinitus*, *A. angulosus*, *A. pungens* and *A. tuberculatus* are truly wild. *A. moschatus* and *A. manihot* are semi wild, and show a greater diversity than the cultivated forms (Bates 1968). It is important to note here that all the wild species of okra are not equally crossable with the cultivated one (*Abelmoschus esculentus* L.) and though a few are crossable, but there is problem of non-viability of seeds, sterility of the hybrid or hybrid breakdown etc (Aanchal et al. 2021). Therefore, in order to introgress resistance genes from wild species into cultivated, a study has to be planned to do the intervention of *in vitro* techniques like embryo rescue and somatic hybridization (Ahsan et al. 2014; Puja and Sanjeev 2020; Rajamony et al. 2006).

Abortion of embryos at one or the other stage of development is a characteristic feature of distant hybridization. Laibach demonstrated successful embryo culture to obtain an interspecific cross between *Linum perenne* x *L. austriacum* for the first time (1925, 1929). Since then several refinements have been made in embryo culture/embryo rescue techniques and considered a popular approach for raising hybrids from several incompatible crosses by several researchers in different crops. Currently, embryo rescue holds great promise not only for effecting wide crosses, but also for obtaining plants from inherently weak embryos, obtaining haploid plants, and shortening the breeding cycle (Sharma et al. 1996).

Molecular markers provide improved tools for diversity assessment, mainly varietal identification and validation of hybrid purity. There are a few reports regarding molecular markers used in okra, and these have been limited to the use of random amplified polymorphic DNA (RAPD) markers (Aladele et al. 2008) and sequence related amplified polymorphism (SRAP) for cultivars and germplasm characterization (Gulsen 2007). Simple sequence repeats (SSRs) developed for *Medicago truncatula* were used in okra cultivars (Sawadogo et al. 2009). Recently, 402 SSRs markers (ESM1) were developed successfully through next generation sequencing (NGS) (Ravishankar et al. 2018). Studies on genetic diversity in *Abelmoschus* species, viz., *A. esculentus, A. tuberculentus, A. moschatus, A. moschatus* subsp. *tuberosus* and *A. manihot* have been earlier carried out earlier using SSR marker (Kumar et al. 2016). SSRs produce much more polymorphism than most other DNA markers, co-dominant and numerous. Therefore, SSR has become an ideal molecular marker in plant variety/hybrid purity identification. A number of other similar studies have been conducted in different crops including maize (Senior et al. 1998), rice (Yashitola et al. 2002; Garland et al. 1999), wheat (Phogat et al. 2023) and vegetables such as tomato hybrids (Bredemeijer et al. 2002; Liu et al. 2007). With the present availability of fewer SSR markers in okra, a huge importance has been realized to develop SSR markers for okra, especially to examine the purity of hybrids and distinguish the hybrids from parental lines. Keeping the above in view, the present study was conducted to develop embryo rescue technique for overcoming cross-incompatible barriers across the distant species in okra and validation of interspecific hybrids of okra was also done through morphological and molecular markers.

Materials and methods

Screening of okra accessions for YVMV

Two genotypes, Pusa Sawani (*A. esculentus*) and IC 470750 (*A. moschatus* subsp. *tuberosus*) were grown at Distant Hybridization Farm, Department of Agricultural Biotechnology (AAU), Anand Agricultural University, Anand to carry out the hybridization programme. The okra accessions taken for the research were maintained at Distant Hybridization Farm, Department of Agricultural Biotechnology, AAU, Anand. All the experiments were conducted during the years 2014-2018 under natural light and temperature conditions. All the required plant

protection measures were followed from time to time to maintain the healthy and optimum plant growth condition to be utilized for crossing and further rescuing embryo. To screen the 87 lines of okra, experiment was designed as RBD with three replications. In one row 10 plants were grown. Spacing between plants was 60 cm and between rows was 90 cm. The selection of the parents in okra in this experiment was made on the basis of screening and evaluation of 87 diverse lines of okra against YVMV for consecutive three years at distant hybridization farm. The 87 accessions comprised of six wild species namely, *A. angulosus* var. *grandiflorus*, *A. manihot, A. manihot* var. *tetraphyllus*, *A. ficulneus, A. tetraphyllus, A. tuberculantus, A. moschatus* and A. *moschatus* subsp. *tuberosus*. The wild species, *A. moschatus* subsp. *tuberosus* was found highly resistant against YVMV under screening for three years, where the Pusa Sawani taken in the programme of hybridization was susceptible but a well-adopted cultivated variety of okra.

Hybridization and the development of embryo rescue technique

Crosses were made by hand pollination between cultivated (Pusa Sawani) and wild species (*A. moschatus* subsp. *tuberosus*) of okra. The emasculation was carried out in the evening between 4 and 5 pm. With the onset of anthesis in the morning, the pollen was removed from a flower of the male parent using a camel hair paint brush and placed on the stigma of the flower of the female parent emasculated a day before. Pollen was only placed onto the surface of stigmas considered receptive, when they are yellow in color and possess secretions to which the pollens adhere. Following reciprocal crossing, the emasculated/pollinated flowers were enclosed in a greaseproof paper bags in order to prevent rogue fertilization/pollen contamination by airborne or insect vectored pollen. The greaseproof paper bags were removed when ovary-to-fruit formation was seen to have been initiated, which was normally 3 to 4 days of post-crossing. Okra pods of 3-4 days post-crossing were harvested from the plants because embryo rescue could not be carried out immediately. Collected pods were stored in sealed glass jars in the dark at 40°C for 2-3 days.

For surface sterilization, the okra fruits were thoroughly washed with running tap water and scratched with bristle brush (soft tooth brush was used as it is very handy and cost-effective). Fruits were soaked in Tween-20 (2–3 drops/L) for 10 minutes and gently stirred intermittently with detergent (1 tablespoon/L). Again the fruits were soaked for 10 minutes in 5% (v/v) NaOCl solution. Afterwards fruits were gently washed 3 to 5 times with sterile D/W without any damage to fruit. The okra fruits were then dipped in ethyl alcohol (70-80%) for 10 seconds and flame sterilized rapidly such that heat would not affect the ovule. All the process was done under laminar hood except first step of this surface sterilization.

Effect of culture medium composition on germination of excised okra embryo

In order to determine the optimum media composition for the germination of okra embryos, excised embryos were placed on the surface of 40ml aliquots of agar solidified (0.6% Sigma; pH 5.7) medium in 175 mL capacity screw-capped glass jars (5 embryos/jar). Media contained 3% sucrose as carbohydrates and growth regulators BAP and GA_3. The carbohydrates were chosen because they had been employed in the embryo rescue of other species (Ulas et al. 2015) and likewise, both the growth regulators had been used in other embryo rescue protocols (Rajamony et al. 2006; Barzali et al. 2011). They had also been shown to have an effect on breaking the dormancy of intact okra seed (Rajamony et al. 2006).

A total of 5012 embryos obtained from cross, Pusa Sawani x IC 470750 (*A. moschatus* subsp. *tuberosus*) were cultured on medium, which were initially maintained in the dark (for 14 days at 22°C), followed by their transfer to a 16 hours photoperiod provided by 35 µEm-$2s^{-1}$ daylight fluorescent illumination at the same temperature. After 20-35 days, the number of germinated embryos was recorded and the germination percentage was calculated. Likewise, different media were optimized for different stages of development such as, Shooting, rooting, sub-culturing of shoots, multiplication of shoots, maturation of shoots, rooting and maturation of plantlets (Tables 1-7). However, the best formulation which showed highest result was highlighted. Thus, all the media compositions were originally developed by us by standardizing on different cultural explants.

Excision of embryos

Using a razor blade and forceps, ovules were isolated from the fruit. After flame sterilization, the fruit was kept on sterilized paper to absorb extra ethyl alcohol and for drying. With the help of forceps, the stalk of fruit was held slantly (Fig. 1a) and with another hand, scalpel having razor blade was held. The ridges of the fruit were cut gently without any damage to the ovule. The base and apex part of the fruit was cut and removed (Fig. 1b). Using forceps, the outer surface of the fruit from end to end was pressed gently for loosening the surface and easily removal of inner part containing ovule (Fig. 1c-d). After separation of fruit wall, the ovules attached on ovary wall were taken gradually with forceps for culturing by holding another end by forceps (Fig. 1e). Excised ovules were then immediately placed on the standardized culture medium. Twenty embryos (5 embryos/jar; 4 replicates/ treatment) were then subjected to optimized cultural media. All steps were carried out under Laminar hood. The data for days after pollination to ovule culture, total number of ovules in culture, days to ovule germination after pollination, number of germinated ovules and percentage of ovule germination were recorded.

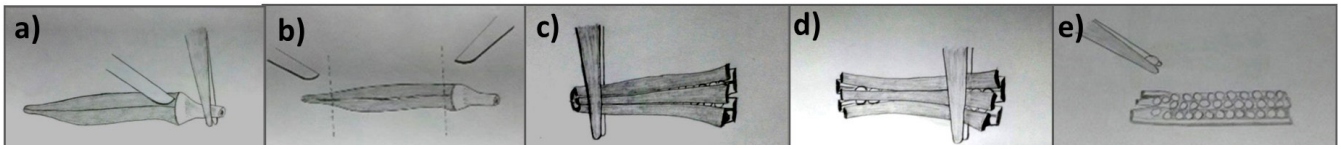


Fig. 1. Stepwise method involved in isolation of ovules from fruit of okra

Fig. 2. Harvested hybrid fruit pods of okra at different time intervals. a) Hybrid fruit pods harvested after 3 days of pollination in cross Pusa Sawani x IC 470750 (*A. moschatus* subsps. *tuberosus*) suitable for ovule culture. b) Dried mature hybrid fruit pod contained non-viable mature seeds of Pusa Sawani x IC 470750 (*A. moschatus* subsps. *tuberosus*)

cultured every after two weeks upto four times. The data for shoot induction percentage, number of shoots/explant, shoot multiplication rate and shoot length were calculated on the basis of response to the media (Table 1).

Observation on in-vitro root regeneration from shoots

Once roots appeared, the data for root induction (%) and roots length (cm) were recorded on the basis of response to the media (Table 1). Initial roots were transferred to semi-solid media formed from either agar or phytagel, then transferred to the liquid rooting media as described in Table 2. Glass

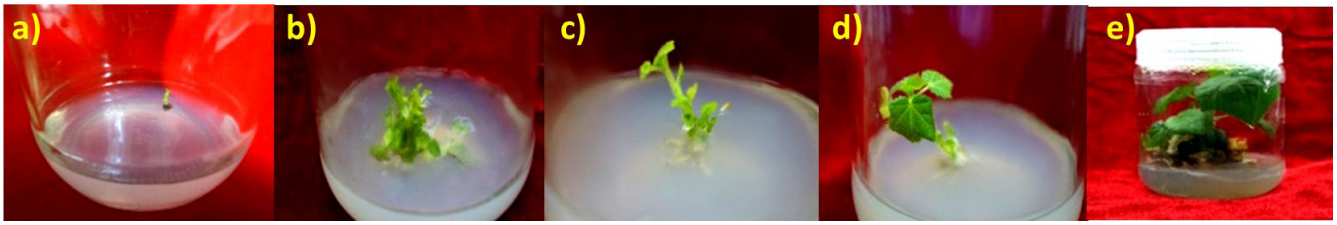


Fig. 3. Embryo rescue techniqueutilizedin cross [Pusa Sawani x IC 470750 (*A. moschatus* subsps. *tuberosus*)]. a) Ovule cultured on suitable media and initiation of direct shoot regeneration. b) Shoot establishment from ovule explant. c) Root initiation of shoot. d) Plantlet stabilization and adaptation to media. e) Shoot maturation in media

Fig. 4. Multiplication, maturation and pre-primary acclimatization of hybrid plantlets [(Pusa Sawani xIC 470750 (*A. moschatus* subsps. *tuberosus*)] a) Multiplication of plantlets. b) Roots maturation in liquid media. c)-d) Various techniques utilized for better adaptation of plantlets in pre-primary conditions

Table 1. The efficiency of the shoots and roots establishment in hybrid okra

Cross Combinations	Shoot Induction (%)	No. of Shoots/ Explant	Shoot Multiplication Rate	Shoot Length (cm)	Root Induction (%)	Root Length (cm)
Pusa Sawani (*A. esculentus*) x IC 470750 (*A. moschatus* subsps. *tuberosus*)	0.02	1.0 ± 0	30.0 ± 0	12.0	100	7.0

Observation on in vitro shoot regeneration from ovule culture

When shoots were regenerated from ovule upto a length of 1-2 cm, they were transferred to new media, examined and photographed (Fig. 3). Any blackening or yellowing of the leaves or shoots was discarded. Shoots were timely sub-

bottle/jar was preferred for liquid media and for support to the plantlets; inverted sterilized plastic cups were kept. Mature plantlets were kept under controlled environment for stabilization. The humidity, light and temperature regime of cultured bottles was checked timely (Fig. 4).

Transfer and acclimation of embryo rescue derived seedlings to greenhouse conditions

When plantlets, produced from the excised cultured embryos, reached a height of approx. 15-20 cm, they were considered suitable for transfer to greenhouse conditions for a number of reasons (Fig. 5). Specifically: i) they had reached a stage equivalent to that at which plantlets in a conventional breeding programme would be transferred from seed trays to pots; ii) they had reached a size at which they could easily be handled and iii) further growth under *in-vitro* conditions would be limited by the size of the culture vessel. Plantlets were removed from the culture vessels and any agar-medium adhering to the roots was washed away with distilled water. Plantlets were planted in 7.5 cm diameter plastic pots, containing equal volumes of cocopeat and perlite. The plantlets were initially watered with a 0.2 gL^{-1} solution of Ridomil: Bavistin (1:1) fungicide and later watered when required to ensure that the soilless culture remained moist. Prior to their transfer to the greenhouse, each pot was covered with a 20 x 13 cm polythene bag to maximize relative humidity and to prevent desiccation. Fourteen days after transfer to the greenhouse, polythene bag was punctured everywhere entire surface, followed by complete removal of the bag after 7 days to facilitate a gradual acclimatization to conditions of lower humidity and temperature (Fig. 5).

Bags were removed by 35 days after transfer to the

Fig. 5. Primary hardening of embryo rescued hybrid [Pusa Sawani x IC 470750 (*A. moschatus* subsps. *tuberosus*)] under greenhousea) Eight plantlets obtained in first cycle of multiplication ready to be shifted in primary green house; b) Plantlets acclimatization in primary fan pad green house

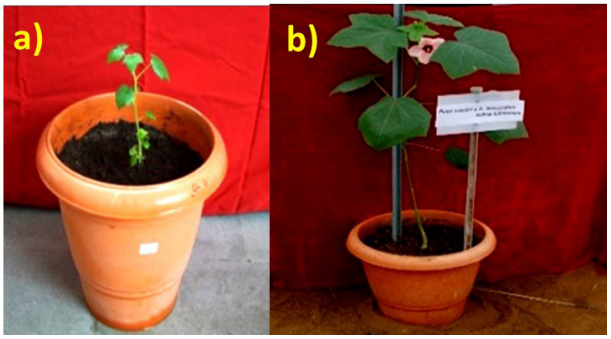


Fig. 6. Secondary hardening of embryo rescued hybrid [Pusa Sawani xIC 470750 (*A. moschatus* subsps. *tuberosus*)] in Polyhouse. a)-b) Secondary hardening of two plantlets resulted in initiation of pink color flower

Fig. 7. Embryo rescued hybrid plants transferred to the field in *kharif*-2015a) Initial stage (Flowering) of hybrid plant transferred to field b) Fruiting stage of hybrid plant c) Hybrid plants reached upto 12 ft height.

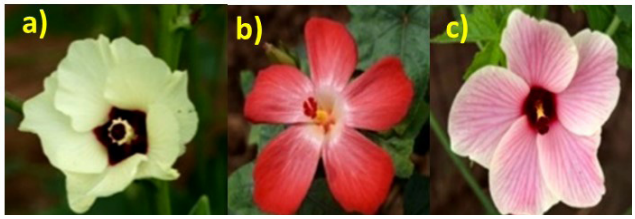


Fig. 8. Comparision of three types of flowers a) cultivated $Parent_1$-Pusa Sawani, b) wild $Parent_2$- *A. moschatus* subsps. *tuberosus*, c) F_1 hybrid-Pusa Sawani x IC 470750 (*A. moschatus* subsps. *tuberosus*)

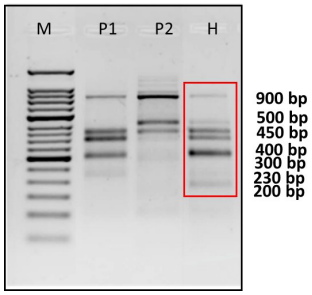


Fig. 9. SSR profile of two parents and interspecific hybrid in okra generated through embryo rescue technique by SSR primer OKRA1: M-Marker (50- 15, 00 bp), P1- Parental line 1 (Pusa Sawani), P2- Parental line 2 (*A. moschatus* subsps. *tuberosus*), H- Interspecific hybrid [Pusa Sawani x IC 470750 (*A. moschatus* subsps. *tuberosus*)] There were seven bands present in the hybrid of molecular wt. size 900 bp, 500bp, 450 bp, 400 bp, 300 bp, 230 bp and 200 bp. Out of seven bands, five bands from cultivated species (Pusa Sawani) viz., 900bp, 450 bp, 400 bp, 300 bp and 230 bp and six bands from wild species (*A. moschatus* subsps. *tuberosus*) viz., 900 bp, 500bp, 450 bp, 400 bp, 300 bp and 200 bp were present

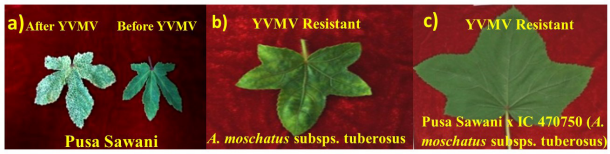


Fig. 10. Morphological evidence of YVMV resistance in F1 hybrid plant Pusa Sawani xIC 470750 (*A. moschatus* subsps. *tuberosus*); a) Leaf of cultivated species Pusa Sawani infected with YVMV disease, having yellow vein all over the leaf as compared to the normal leaf. b) Leaf of wild species *A. moschatus* subsps. *tuberosus* showing inbuilt resistant to YVMV disease. c) Leaf of F1 hybrid Pusa Sawani xIC 470750 (*A. moschatus* subsps. *tuberosus*) acquired resistance from wild parent through crossing and embryo rescue

Table 2. Media composition used to investigate the requirements for the rooting of shoots

S.No.	Basal salt/ vitamin mixture	Sucrose (%)	Growth regulator (mg/L)			Coconut Water (ml/L)	Agar (Ag)/ Phytagel (Ph) (%)	pH
			BAP	*GA*	*NAA*			
1.	Initial stage							
	MS	3.0	0.3	-	1.0	-	0.8 (Ag)	5.7
2.	Intermediate stage							
	½MS	2.0	2.0	-	0.5	150	0.75 (Ag)	5.7
3.	Mature stage							
	MS	2.0	1.0	0.5	0.2	-	1.0 (Ph)	5.7

Table 3. Establishment of the plantlets in the green house and field

Cross combinations	Number of plantlets	Number of acclimated plants transferred to green house	Number of transferred plants to field
Pusa Sawani (*A. esculentus*) x IC 470750 (*A. moschatus* subsps. *tuberosus*)	30	25	3

greenhouse. Plants were maintained under natural daylight at 25 ± 2°C for 8-10 weeks in secondary greenhouse (Fig. 6). Six weeks after transfer to the greenhouse, the number of seedlings survived was recorded. The embryo rescue derived plants reached to a fully mature flowering stage were transferred to the field (Fig. 7, Table 3). The differences in flowers of cultivated, wild and hybrid species were compared (Fig. 8).

Validation of putative inter-specific hybrids

DNA extraction from putative hybrid tissues

The DNA was extracted from samples of parent tissues and putative interspecific hybrid tissues as described by Edwards et al. (1991), slightly modified by us. Fresh tissues (0.25 g fresh wt.) were macerated (15 minutes) at room temperature in sterile 1.5 mL eppendorf tubes containing metal balls using Tissue Lyser. Following maceration, 400 µL of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1% β-mercaptoethanol) were added to each sample and vortexed for 5 seconds. The samples were left at room temperature for at least 30 minutes (usually until all the sample extractions had been completed). The extracts were centrifuged (13000 rpm; 1 min) and 300 µL of the resulting supernatants were transferred to clean, sterile eppendorf tubes. An equal volume (300 µL) of isopropanol was added and the samples left at room temperature for a further 2 min. The samples were centrifuged (13 000 rpm, 5 min) and the supernatants was removed by evaporation in the sterile air flow of a laminar air flow cabinet. The resulting pellets were each dissolved in l00 µL of TE buffer (10 mM Tris HCl; 1 mM EDTA; pH 8.0). A 10 µL of 3M Na acetate was added and the samples were well mixed. This was followed by addition of 250 µL of ice-cold 100% absolute ethanol and the samples were again thoroughly mixed. The samples were stored at -20°C (10 minutes) prior to centrifugation (13000 rpm, 5 min). Following centrifugation, the supernatants were removed (by pouring away) and the resultant pellets well washed in ice-cold 70% (v/v) absolute ethanol. The samples were again centrifuged (13000 rpm, 2 minutes) and the pellets dried by placing the open eppendorf tubes inside a laminar air flow cabinet. The remaining DNA pellets were each dissolved in 50 µL of TE buffer and stored at -20°C until further used for

Table 4. Observations on number of flowers pollinated, fruit set, number of crossed fruits retained and average number of ovules per fruit among different cross combinations

Cross combinations	No. of flowers pollinated	Fruit set (%)	Crossed fruits retained (%)	Average number of Ovules per fruit
Pusa Sawani (*A. esculentus*) x IC 470750 (*A. moschatus* subsp. *tuberosus*)	1000	86.7	85.1	15.78

Table 5. The efficiency of the ovule culture and ovule germination in hybrid okra

Cross combinations	Total Ovules cultured (40 DAP)	Ovule germination		
		DAP of Ovule Germination	Number of Germinated Ovules	Ovules Germination (%)
Pusa Sawani (*A. esculentus*) x IC 470750 (*A. moschatus* subsps. *tuberosus*)	5012	35	52	1.04

DAP = Days after pollination

Table 6. Media composition used to investigate the requirements for germination of ovule, shoot multiplication and maturation

S. No.	Basal salt/vitamin mixture	Sucrose (%)	Growth regulator (mg/L)		Coconut Water (ml/L)	Agar (%)	pH
			BAP	*GA*			
1.	Germination of hybrid ovules						
	MS	3.0	0.5	5	100	0.7	5.7
2.	Shooting of hybrid ovule						
	MS	3.0	0.3	3	70	0.7	5.7
3.	Subculture of shooting						
	MS	3.0	1.0	3	-	0.7	5.7
4.	Shoot multiplication						
	MS	3.0	2.0	5	-	0.7	5.7
5.	Shoot maturation						
	MS	3.0	0.1	-	-	0.7	5.7

Table 7. Media composition used to investigate the requirements for the maturation of plantlet

S. No.	Basal salt/ vitamin mixture	Sucrose (%)	Agar (%)	pH
1.	Initial stage			
	MS	3.0	0.7	5.7
2.	Mature stage			
	½MS	2.0	0.7	5.7

polymerase chain reaction (PCR) amplification.

Amplification of extracted DNAand PCR analysis

Out of 20 SSR primers screened (obtained from Operon Technologies Inc, Alameda, California, USA), one primer was able to validate the interspecific hybrids. The SSR primer sequence is Okra1_F ATGGAGTGATTTTTGTGGAG and Okra1_R GACCCGAACTCACGTTACTA. The amplification reaction was carried out in 25 µL volumes containing 10X Taq buffer followed by $MgCl_2$ (2.5 µL), dNTPs (0.5 µL), Taq DNA polymerase (0.5 µL), Millipore sterilized water (17.5 µL) and template DNA (2.5 µL) in sequence and finally the primer (0.5 µL). The reagents were mixed thoroughly by a short spin using microfuge. The tubes were then placed on the Thermal Cycler for cyclic amplification. PCR was performed using an Eppendorf vapor Protect Thermal Cycler. The total reaction was carried out for 45 cycles. PCR products were separated by electrophoresis through 2.5% agarose gels having 5 to 6 µL ethidium bromide. The gel was run on SUB6 Mini Submarine gel electrophoresis Unit system with an electrophoresis power Supply and visualized under UV illumination of gel documentation system (Alfa Innotech Corporation, USA).

Results and discussion

The present work provided novel method of *in-vitro* regeneration of entire plant upto the field from a crossed ovule between two sexually incompatible *Abelmoschus* species, *A. esculentus* and *A. moschatus* subsp. *tuberosus*. Although other wild species have been used earlier by previous authors (Rajamony et al. 2006) which were crossable and resistance for YVMV resulted in successful hybrids but has been broken down later.

The emasculation of flowers in female plant was done in the evening from 4 to 6 pm prior to a day before pollination appropriately and the pollination was done the next day between 9 and 11 am through male parent *A. moschatus* subsp. *tuberosus* (IC 470750), as afterwards the flowers get withered. It was also observed that the actual rate of fertilization may be higher because some embryos died before being rescued, the number of rescued embryos could be used to estimate the minimum rate of fertilization. About 1000 crosses were made for the cross combination between the selected parents but the fruit setting was observed as 86.7% in the cross (Fig. 2, Table 4), whereas the average number of ovules per fruit was found to be 15.78 per cent (Table 5).

The fruit pods were surface sterilized by dipping in methanol/ethanol followed by heat sterilization, and 90% of microbial contamination was removed. Ovules inside fruit pods were innate sterilized, so direct placement of ovules in culture media was performed. Inter-specific hybrid ovules obtained in the cross responded very variedly to the culture medium MS 2 for embryos cultured after 20-35 days. The cytokinin BAP played an important role in shoot regeneration (Table 6). Embryos rescued from interspecific crosses on media MS 3 having 0.5 mg/L 2-4, D promoted an enhanced growth of the shoots, when compared to the results obtained in response to other MS media for shoot regeneration. Timely subculturing to MS media (Table 6) resulted in profound shooting. This was followed by multiplication for three cycles in media composition mentioned in detail (Table 6). The growth regulator BAP has been found to be implicated in breaking of dormancy in

seeds of *R. arvensis*. This may be because BAP is thought to affect the balance between endogenous growth promoters and inhibitors that normally prevent germination (Jackson 1963). The presence of one or more growth regulators was required for the efficient germination of excised embryos in the genera *Manihot, Lupinus* and *Gossypium* (Biggs et al. 1986; Kasten and Kunert 1991; Liu et al. 1992).

Shoot maturation was done in media MS 1 (Table 6). Different root stages were obtained when sequentially kept on different MS media (Table 2). These root stages were described as initial, intermediate and mature stage. Growth regulators such as NAA responded very well and quickly with BA and GA combinations.

Maturation of plantlets is very critical step, as 90% of plant loss was seen in this stage only. Thus, correct combinations for hybrid cross were designed as initial stage MS 3 and mature stage ½ MS 5 media (Table 7). Successful production of seedlings through embryo culture largely depends on both the developmental stage of the embryo and the composition of the culture medium (Stelkens and Seehausen 2009). The promotion of precocious embryo germination promoted by growth regulators has also been reported in other species like *Chrysanthemum*, legumes, oilseeds and cashew nut (Anderson et al. 1990; Bajaj 1990; Das et al. 1999).

Morphological evidence of YVMV resistance in F_1 hybrid plant [Pusa Sawani x IC 470750 (*A. moschatus* subsp. *tuberosus*) were found (Fig. 10) in all plant leaves: a) Leaf of cultivated species Pusa Sawani infected with YVMV disease, having yellow vein all over the leaf as compared to the normal leaf; b) Leaf of wild species *A. moschatus* subsp. *tuberosus* showing inbuilt resistance to YVMV disease and c) Leaf of F_1 hybrid acquired resistance from wild parent through crossing and embryo rescue.

Figure 11 depicts the anatomy of cultivated, wild and hybrid species of stem part against YVMV disease: a) Stem transverse section of cultivated species Pusa Sawani showed intact anatomy having complete epidermis, collenchyma, pith, vascular bundle, xylem, phloem and cortex; b and c) Stem transverse section of cultivated species Pusa Sawani infected with YVMV showed defective anatomy having damaged pith (hollow from inside), shrunken bundle sheath, least xylem and phloem, decreased layer of cortex and broken epidermis; d) Transverse section of stem of wild species *A. moschatus* subsp. *tuberosus* showed intact anatomy having inbuilt resistance towards YVMV and. e) Transverse section of stem of F_1 hybrid acquired resistance from wild parent through crossing and embryo rescue showed intact anatomy after infection with YVMV disease.

Tthe parents, Pusa Sawani and IC 470750 and the inter-specific hybrid developed between them through embryo rescue were subjected to characterization for 37 morphological characters (Supplementary Table S1). The morphological analysis was performed according to DUS

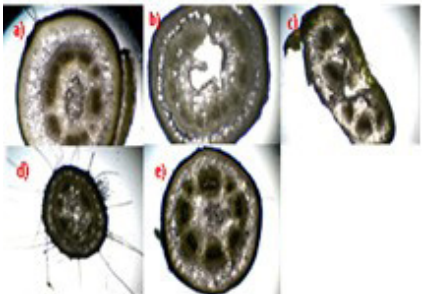


Fig. 11. Anatomy of cultivated, wild and hybrid species of stem part against YVMV disease. a) Stem transverse section of cultivated species Pusa Sawani showed intact anatomy having complete epidermis, collenchyma, pith, vascular bundle, Xylem, Phloem and cortex. b) & c) Stem transverse section of cultivated species Pusa Sawani infectedwith YVMV showed defective anatomy having damaged pith (hollow from inside), shrunken bundle sheath, least xylem and phloem, decreased layer of cortex and broken epidermis. d) Transverse section of stem of wild species *A. moschatus* subsps. *tuberosus* showed intact anatomy having inbuilt resistance towards YVMV. e) Transverse section of stem of F_1 hybrid Pusa Sawani x IC 470750 (*A. moschatus* subsps. *tuberosus*) acquired resistance from wild parent through crossing and embryo rescue showed intact anatomy after infection with YVMV disease

characterization and additional information related to morphological features. Identification of traits was carried out starting from fully grown plant to the end of the harvest. Morphological characters were distinguishable in all the three genotypes, the parents and their interspecific hybrid. These traits were identified based on comparative vegetative and reproductive morphological features (Fig. 12). The vegetative characters are depicted in detail (Fig. 12) included leaf (color, shape, size, type, rim, venation, margins, serration and hairs); petiole (color, shape, size, type and hairs); stem (color, shade, diameter, nodes, internodes and stipules); plant (branching patterns and height). On the other hand, reproductive characters (Fig. 12) included flower bud (shape, color, epicalyx, receptacle, pedicel, anther arrangement, anther color and stigma receptors); flower (color, shape, arrangement, shades, size and petal basal color); ovary (shape, size, arrangement and apex shape); fruit (shape, size, basal part constriction, locules, diameter, pubescence, apex shape and surface); ovule (shape, size, color, quantity and density). All the above detailed descriptions confirmed the validity of interspecific hybrid plant and exhibited traits of both the parents and also superiority over both the parents (heterobeltosis) for many traits including YVMV resistance and height of the plant.

This superiority is known as hybrid vigour. Plant breeder always tries to find hybrid vigour of their interest because of beneficial effects on the yield and quality of cultivated plants. Many workers found that the superior yield of F_1 hybrids of polyploid okra is the result of small increases in several components of yield, for example, a better stand of plants per plot (Udengwu 2009), increased height of plant (Makdoomi et al. 2018), more number of flowers per plant (More et al. 2015), better pod length and girth (Khanokar

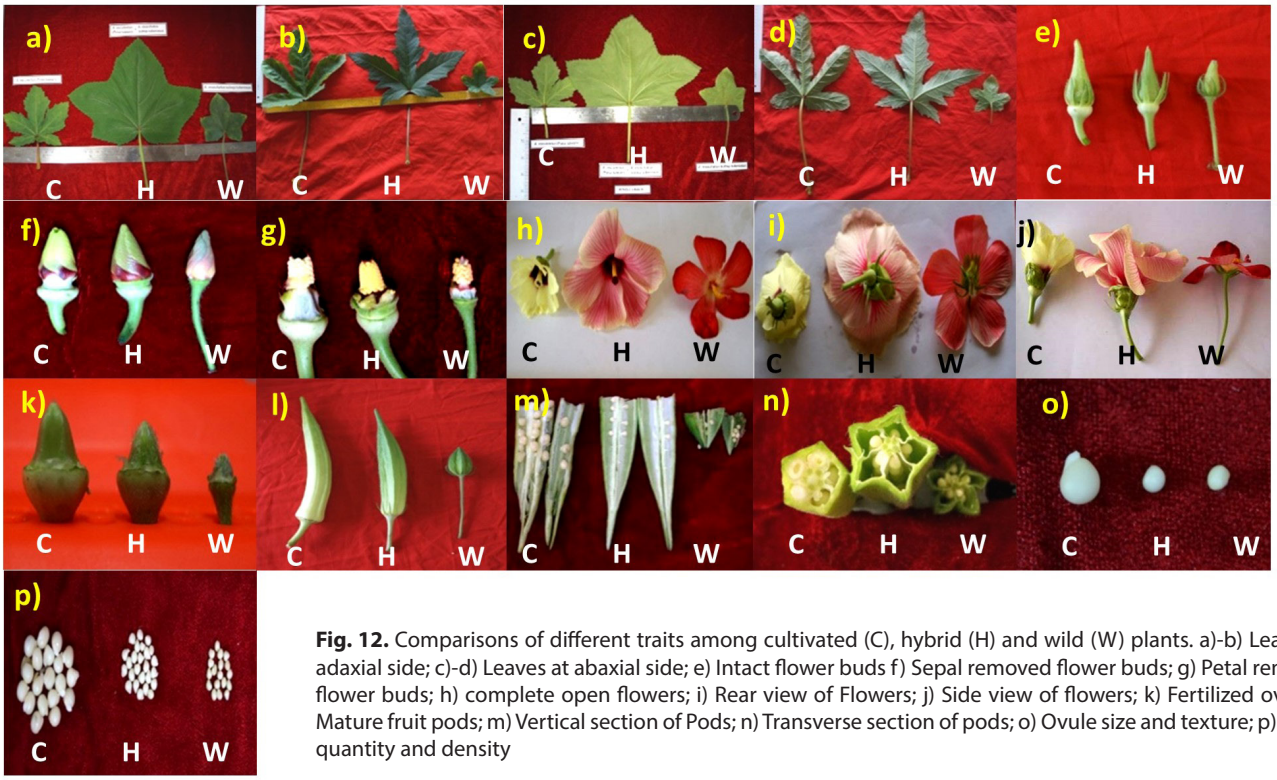


Fig. 12. Comparisons of different traits among cultivated (C), hybrid (H) and wild (W) plants. a)-b) Leaves at adaxial side; c)-d) Leaves at abaxial side; e) Intact flower buds f) Sepal removed flower buds; g) Petal removed flower buds; h) complete open flowers; i) Rear view of Flowers; j) Side view of flowers; k) Fertilized ovary; l) Mature fruit pods; m) Vertical section of Pods; n) Transverse section of pods; o) Ovule size and texture; p) Ovule quantity and density

and Kathira 2010; Makdoomi et al. 2018), increased pod yield per plant (Patel and Patel 2016; Makdoomi et al. 2018), and improved set of seeds per pod and per plant (Makdoomi et al. 2018). Enhanced vigour may enable the plant to overcome environmental hazards and thereby increased yield (Fig. 7 c). Many authors have assumed that transgressive characters (characters showing values beyond the range of the parental species) expression is the result of a complementary action of genes (Rieseberg et al. 2003; Bell and Travis 2005; Stelkens and Seehausen 2009). Transgressive character expression in hybrids results from loci interactions between alleles having opposing effects on phenotypes within each parental species but have reinforcing effects (i.e., complementation) in hybrids (Bell and Travis 2005) (Fig. 12)

Maximum expressions of heterosis are shown in F_1 hybrids and it does not produce seeds. Hence, those crops which are raised from seed, the crosses must be repeated to keep up a continuous supply of planting material. This is an expensive process and a serious obstacle to the commercial utilization of hybrid vigour, particularly with agricultural crops. Therefore, present protocol for regeneration of hybrid plant from embryo rescue technique has been developed to introgress the YVMV resistance into the cultivated okra variety as well as to explore the change for developing male sterile line in okra as distant hybridization often leads to male sterility.

Morphological characters were thoroughly employed during the last century as the main marker for hybrid recognition; nowadays it is known that pattern of inheritance is complex and usually unpredictable. PCR based marker system overcomes most of the limitations prevailing in morphological markers. DNA fingerprinting techniques such as Microsatellite markers or Simple Sequence Repeats (SSR) have many advantages (Kalia et al. 2011). SSR consists of sequences of repetitions, comprising short motifs generally between 2 and 6 base-pairs long. The polymorphism within a specific locus is due to the variation in length of the microsatellite sequence, which depends on the number of repetitions of the basic motif. SSR primer OKRA 1 showed good amplification as well as polymorphism.

A reproducible embryo rescue technique has been developed successfully for producing YVMV resistant okra. The choice of an agar solidified medium containing a basic salt mixture and carbohydrate and the growth conditions employed were demonstrated to markedly affect the germination percentage and plantlet development. The result obtained has confirmed the hybrid purity of cross Pusa Sawani (*A. esculentus*) x IC 470750 (*A. moschatus* subsp. *tuberosus*) (Fig. 9). The optimization of this technique will overcome pre and post fertilization barriers coming across in the interspecific hybridization process and will pave a way further for gene introgression through distant hybridization in okra.

Supplementary material

Supplementary Table S1 providing morphological descriptions of okra species online www.isgpb.org

Authors' contributions

Conceptualization of research (AP); Designing of the experiments (AP); Contribution of experimental materials (AP, MZ); Execution of field/lab experiments and data collection (AP, MZ); Analysis of data and interpretation (AP, MZ); Preparation of the manuscript (AP, MZ).

References

Aanchal C., Deepa S. and Sanjeev K. 2021. Potential, challenges and strategies involved in gene introgression from wild relatives of vegetable crops: A review. Agril. Reviews, **42**(4): 390-397.

Ahsan M. Z., Majidano M. S., Channa A. R., Panhwar F. H., Soomro A. W., Khaskheli F. I. and Rashid K. 2014. Regeneration of Cotton (*Gossypium hirsutum* L.) through asexual methods, A Review. Am. Eurasian J. Agric. Environ. Sci., **14**(12): 1478-1486.

Aladele S. E., Ariyo O. J. and de La Pena R. 2008. Genetic relationships among West African okra (*Abelmoschus caillei*) and Asian genotypes (*Abelmoschus esculentus*) using RAPD. Afr. J. Biotechnol., **7:** 1426–1431.

Ali S., Khan M., Habib A., Rasheed S. and Iftikhar Y. 2005b. Management of Yellow Vein Mosaic Disease of Okra through pesticide/bio-pesticide and suitable cultivars. Int. J. Agric. Biol., **7**(1): 145-147.

Anderson N.O., Ascher P.D., Widmer R.E. and Luby J.J. 1990. Rapid generation cycling of chrysanthemum using laboratory seed development and embryo rescue techniques. J. Am. Soc. Hortic. Sci., **115:** 329-336.

Bajaj Y.P.S. 1990. Wide hybridization in legumes and oilseed crops through embryo, ovule, and ovary culture. In: BAJAJ, Y.P.S. (Ed.). Legumes and oilseed crops I. Berlin: Springer-Verlag, Biotechnol. Agricult. forestry, **10**: 3-37.

Barzali P., Mofidabadi A. J., Zangi M. R. and Bozorgi R. 2011. The using of gibberellic acid hormone on cotton mature embryo resulted by crossing between wild and commercial species on artificial medium. Afr. J. Biotechnol., **10**(46): 9322-9327.

Bates D. M. 1968. Notes on the cultivated Malvaceae (*Abelmoschus).* Baileya, **16:** 99–112.

Bell M. A. and Travis M. P. 2005. Hybridization, transgressive segregation, genetic covariation, and adaptive radiation. Trends Ecol Evol., **20:** 358–361.

Biggs B.L., Smith M.K. and Scott K.L. 1986. The use of embryo culture for the recovery of plants from cassava (*Manihot esculenta* Crantz) seeds. Plant Cell Tissue Organ Cult., **6:** 229-234.

Bredemeijer M., Cooke J., Ganal W., Peeters R., Isaac P., Noordijk Y., Rendell S., Jackson J., Roder S., Wendehake K., Dijcks M., Amelaine M., Wickaert V., Bertrand L. and Vosman B. 2002. Construction and testing of a microsatellite containing more than 500 tomato varieties. Theor. Appl. Genet., **105:** 1019-1026.

Das S., Jha T. B. and Jha S. 1999. Factors affecting in vitro development of embryonic axes of cashew nut. Sci. Hortic., **82:** 135-144.

Datta P. C. and Naug A. 1968. A few strains of *Abelmoschus esculentus* (L) Moench. Their karyological in relation to phylogeny and organ development. Beitr, Biol, Pflanzen, **45:** 113-126.

Fajinmi A. A. and Fajinmi O. B. 2010. Incidence of okra mosaic virus at different growth stages of okra plants (*Abelmoschus esculentus* (L.) Moench) under tropical condition. J. Gen. Mol. Virol., **2:** 28-31.

FAOSTAT, (http://www.fao.org), (2013).

Ford C.E. 1938. A contribution to a cytogenetical survey of the *Malvaceae*. Genetica, **20:** 431-452.

Garland S. H. L., Lewin L., Abedinia M., Henry R. and Blakeney A. 1999. The use of microsatellite polymorphisms for the identification of Australian breeding lines of rice (*Oryza sativa* L.). Euphytica, **108:** 53–63.

Gulsen O., Karagul S. and Abak K. 2007. Diversity and relationships among Turkish germplasm by SRAP and Phenotypic marker polymorphism. Biologia, Bratislava, **62**(1): 41-45.

Jackson, G.A.D. 1963. Hormonal control of fruit development, seed dormancy and germination with particular reference to *Rosa.* Society of Chemical Industry, Monograph No. 31.

Kalia R. K., Rai M. K., Kalia S., Singh R. and Dhawan A. K. 2011. Microsatellite markers: an overview of the recent progress in plants. Euphytica, **177:** 309–334.

Kasten W. and Kunert R. 1991. A culture method for isolated immature embryos of different *Lupinus* species. Biol. Zent., **110:** 290-300.

Khanokar S. M. and Kathira K. B. 2010. Heterobeltosis, inbreeding depression and heritability studies in okra. Electron. J. Plant Breed., **1**(4): 731-741.

Kumar S., Parekh M. J., Fougat R. S., Patel S. K., Patel C. B., Kumar M. and Patel B. R. 2016. Assessment of genetic diversity among okra genotypes using SSR markers. J.Plant Biochem. Biot., DOI 10.1007/s13562-016-0378-2.

Laibach F. 1925. Das Taubwerden von Bastardsamen und die kuntlichea ufzuchtfrut absterbender Bastardem-bryonen. Z. Botan., **17:** 417-459.

Laibach F. 1929. Ectogenesis in plants. J. Hered., **20:** 201-208.

Liu C., Shun J. and Liu J. 1992. *In vitro* interspecific fertilization, embryo development and formulation of hybrid seedlings between *Gossypium hirsutum* and G. *arboretum.* Euphytica, **60:** 79-88.

Liu Li-Wang., Y Wang., Yi-Qin Gong., Tong-Min, Zhao., G. Liu., Xiao-Yan, and LiandFa-Min, Yu. 2007. Assesment of genetic purity of tomato (*Lycopersicum esculentum* L.) hybrid using molekuler markers. J. Hortic. Sci. Biotech., **115:** 7–12.

Makdoomi M. I., Wani K. P., Dar Z. A., Hussain K., Nabi A., Mushtaq F. and Mufti S. 2018. Heterosis studies in okra (*Abelmoschus esculentus* L. Moench). Int. J. Curr. Microbiol. Appl. Sci., **7**(2): 2319-7706.

More J. S., Chaudhari K. N., Bhanderi D. R., Saravaiya S. N. and Chawla S. L. 2015. Heterosis study in okra (*Abelmoschus esculentus* L. Moench). Trends in Biosciences, **8**(12): 3252-3255.

National Horticulture Board. 2018. Indian Horticulture Database, Ministry of Agriculture, Government of India.

Pabendon M. B., Mejaya M. J. and Subandi D. M. 2005. Sidikjari empatvarietasjagung hibri dabesertatet uanyaber dasarkanm arkami krosatelit. Zuriat., **16**(2): 192 – 200.

Patel B. G. and Patel A. I. 2016. Heterosis studies in okra (*Abelmoschus esculentus* (L.) Moench). Ann. Agr. Environ. Sci., **1**(1): 15-20.

Phogat S., Poudel A., Bhurta R., Kalwan G., Madhavan J., Padaria J.C., Singh P.K., Vinutha T. and Mandal P.K. 2023. An improved in-vitro regeneration protocol using scutellum of mature and

immature embryos of wheat. Indian J. Genet. Plant Breed., **83**(2): 195-204. https://doi.org/10.31742/ISGPB.83.2.5

Prakasha T. L., Patil M. S. and Benagi V. I. 2010. Survey for bhendi yellow vein mosaic disease in parts of Karnataka. Karnataka J. Agr. Sci., **23:** 658-659.

Puja R. and Sanjeev K. 2020. Development of interspecific hybrids (*Abelmoschus esculentus x A. tetraphyllus*) in okra using Embryo Rescue approach. Biosci. Biotech. Res. Asia., **17**(3): 517-523.

Rajamony L., Chandran M. and Rajmohan K. 2006. *In Vitro* embryo rescue of interspecific crosses for transferring virus resistance in okra (*Abelmoschus esculentus* L. Moench). Acta Hortic., **725**.

Ravishankar, K. V., Muthaiah, G., Mottayan, P. and Gundale, S. K. 2018. Identification of novel microsatellite markers in okra (*Abelmoschus esculentus* (L.) Moench) through next-generation sequencing and their utilization in analysis of genetic relatedness studies and cross-species transferability. J. Genet., **97**: 39-47.

Rieseberg L. H., Widmer A., Arntz A. M. and Burke J. M. 2003. The genetic architecture necessary for transgressive segregation is common in both natural and domesticated populations. Philos T Roy Soc B., **358**: 1141–1147.

Sastry K.S.M. and Singh S. J. 1974. Effect of yellow vein mosaic virus infection on growth and yield of okra crop. *I*ndian Phyto pathol., **27**: 294–7.

Senior M. L., Murphy J. P., Goodman M. M. and Stabungr C. W. 1998. Utility of SSR for determining genetic similarities and relationships in maize using an agarose gel system. Crop Sci., **38**: 1088–1098.

Sharma D. R., Kaur R. and Kumar K. 1996. Embryo rescue in plants- a review. Euphytica, **89:** 325-337.

Siemonsma, I. S. 1982a. La culture du gombo (*Abelmoschus* spp) legume fruit. Thesis Univ Wageningen, the Netherlands.

Siemonsmo, J. S. 1982b. West African okra morphological and cytological indications for the existence of a natural amphiploid of *Abelmoschus esculentus* (L.) Moench and *A. Manihot* (L) Medikus. Euphytica, **31**(1): 241-52.

Singh H. B. and Bhatnagar A. 1975. Chromosome number in an okra from Ghana. Indian J. Genet. Plant Breed., **36:** 26-27.

Stelkens R. and Seehausen O. 2009. Genetic distance between species predicts novel trait expression in their hybrids. Evolution, **63:** 884–897.

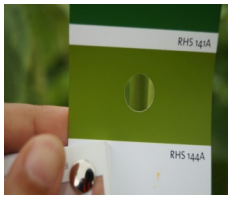
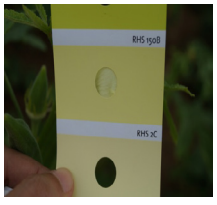
Udengwu O. S. 2009. Studies on heterosis in *Abelmoschus esculentus* (L.) Moench and *A. callei* (A. Chev) Stevels cultivars during shorter day photoperiods in south eastern Nigeria. Pak. J. Biol. Sci., **12**(21): 1388-1398.

Ulas S., Kesgin M. and Dilli Y. 2015. The success of *in vitro* embryo rescue technique in hybridization of seedless grape varieties. BIO Web of Conferences 5, 01008. DOI: 10.1051/bioconf/20150501008.

Wammanda D. T., Kadams A. M. and Jonah P. M. 2010. Combining ability analysis and heterosis in a diallel cross of okra (*Abelmoschus esculentus* L. Moench). Afr. J. Agric. Res., **5:** 2108-2115.

Yashitola J., Thirumurugan T., Sundaram R. M., Naseerullah M. K., Rhamesa M. S., Sarma N. P. and Sonti R. V. 2002. Assessment of purity of rice hybrids using microsatellite and STS marker. Crop Sci., **42:** 1369–1373.

Supplementary Table S 1. Morphological descriptions of Okra species

S. No.	Species	Specimen	Color/code	Descriptions
Parent 1. Cultivated species [Pusa Sawani (*Abelmoschus esculentus* L.)]				
1.		LEAF	Dark Green RHS 137A	**Leaf Shape**-Palm with highly lobed **Leaf Thickness**- Thick **Leaf Hair**- Less **Leaf Blade Lobe**- Deep **Color b/w veins of Leaf Blade**- Green **Intensity of color b/w veins of Leaf Blade**- Light **Leaf Major venation**- 7 **Leaf secondary venation**- 3 **Serration**-Single blunt dentate
2.		STEM	Dark Green RHS 144A	**Stem Color**- Green **Stem Color Intensity**- Light **Stem Diameter**- Large (≥1.5) **No. of Nodes at first flowering**- Few (≤5) **Stem branch pattern**-Alternate **Stem Hair**- No
3.		NODE	Dark Green RHS 144A	**Stipules on/near node**-Present
4.		POD	Yellow Green RHS 145A	**Fruit Length**- Long **Fruit Diameter**- Medium **Fruit Surface**- Flat **Fruit Pubescence**- Weak **Fruit Constriction of Basal part**-Weak **Fruit Shape of Apex**- Acute **Fruit Locules**- 5
5.		PEDICEL	Yellow Green RHS 145A	**Pedicel Length**- Short **Pedicel having Receptacle**- Present, Large
6.		PETAL	Light Yellow Green RHS 2C	**Petal Base color**- Purple both sides **Flower Length**- Small **Flower Diameter**- Small **Flower Bud**- Long and narrow **Epicalyx**- Medium **Petal Arrangement**- Imbricate **Petal shades**- No
7.		ANTHER	Yellow RHS 7D	**Antherquantity**- Dense **Anther Arrangement**- Covered the stigma **Stigma Receptor**-Arranged compactly

8.		LEAF RIM (ADAXIAL)	Purple RHS 58A	**Leaf rim color spread**- Only at the rim
9.		LEAF RIM (ABAXIAL)	Dark Purple Brown RHS 59A	**Leaf rim color spread**- Only at the rim
10.		PETIOLE	Dark Purple Red RHS 185B	**Petiole Length**-Long
11.		STEM SHADE	Purple RHS 58A	**Stem shade on entire plant**-Present
12.		POD STALK	Purple RHS 58A	**Pod Stalk Length**-Medium

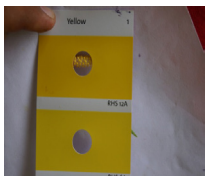
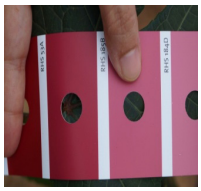
Parent 2. Wild species (*Abelmoschus moschatus* subsps. *tuberosus*)

13.		LEAF (MATURE)	Dark Green RHS 136A	**Leaf Shape**-Palm with less lobed **Leaf Thickness**- Medium **Leaf Hair**- Very Less **Leaf Blade Lobe**-Shallow **Color b/w veins of Leaf Blade**-Green **Intensity of color b/w veins of Leaf Blade**- Medium **Leaf Major venation**- 7 **Leaf secondary venation**- 3
14.		LEAF (YOUNG)	Dark Green RHS 144A	**Leaf Hair on both sid**e- Yes, very less **Leaf major margin point**- less pointed **Leaf rim color spread**- Absent at both side **Serration**-Single less pointed dentate

15.		STEM	Dark Green RHS 144A	**Stem Color**- Green **Stem Color Intensity**- Medium **Stem Diameter**- Small (0.5) **No. of Nodes at first flowering**- Many (≥8) **Stem branch pattern**-Whorled **Stem Hair**- Yes **Stipules on/near node**-Present
16.		POD	Dark Green RHS 144A	**Fruit Length**- Small **Fruit Diameter**- Small **Fruit Surface**- Deep concave **Fruit Pubescence**- Medium **Fruit Constriction of Basal part**- Absent **Fruit Shape of Apex**- Blunt **Fruit Locules**- 5
17.		PEDICEL	Dark Green RHS 144A	**Pedicel Length**- Long **Pedicel having Receptacle**- Absent
18.		ANTHER	Dark Orange Yellow RHS 21B	**Antherquantity**- Medium **Anther Arrangement**- Below the stigma **Stigma Receptor**-Arranged Loosely and bent towards anther
19.		PETAL (ADAXIAL)	Red RHS 47A	**Petal Base color**- White both sides **Flower Length**- Small **Flower Diameter**- Small **Petal Arrangement**- less Imbricate
20.		PETAL (ABAXIAL)	Red Pink RHS 52B	**Epicalyx**- Short **Petal shade**- White **Flower Bud**- Short and blunt

Parent 3. Hybrid (*Abelmoschus esculentus* L. x *Abelmoschus moschatus* subsps. *tuberosus*)

21.		LEAF (ADAXIAL)	Dark Green RHS 136A	**Leaf Shape**-wedge with medium lobed **Leaf Thickness**- Thin **Leaf Hair**- More **Leaf Blade Lobe**- Medium **Color b/w veins of Leaf Blade**-Green **Intensity of color b/w veins of Leaf Blade**- Light **Serration**-Single pointed dentate
22.		LEAF (ABAXIAL)	Green RHS 137C	**Leaf Hair on both sid**e- Yes, More **Leaf major margin point**-Pointed

23.		PETIOLE (ADAXIAL)	Green RHS 137C	**Petiole Length**-Medium
24.		PETIOLE (ABAXIAL)	Green RHS 146C	**Petiole Shade**- Absent
25.		LEAF VEIN	Green RHS 146C	**Leaf Major venation**- 7 **Leaf secondary venation**- 3
26.		STEM	Dark Green RHS 144A	**Stem Color**- Green **Stem Color Intensity**- Medium **Stem Diameter**- Small (0.5) **No. of Nodes at first flowering**- Medium (5) **Stem branch pattern**-Alternate **Stem Hair**- Yes **Stipules on/near node**-Present
27.		POD (CENTRAL)	Dark Green RHS 144A	**Fruit Length**- Medium **Fruit Diameter**- Medium **Fruit Surface**- Concave **Fruit Pubescence**- Strong **Fruit Shape of Apex**- Narrow **Fruit Locules**- 5
28.		POD (DISTAL)	Dark Green RHS 136A	**Fruit Constriction of Basal part**-Absent **Pod Stalk Length**-Medium
29.		PEDICEL	Yellow Green RHS 145A	**Pedicel Length**- Medium **Pedicel having Receptacle**- Present, Medium
30.		ANTHER	Yellow RHS 12A	**Antherquantity**- Scanty **Anther Arrangement**- Below the stigma **Stigma Receptor**-Arranged compactly The above reason resulted in sterility of flower.
31.		LEAF RIM (ADAXIAL)	Dark Purple Red RHS 185B	**Leaf rim color spread**- Only at the rim

32.		LEAF RIM (ABAXIAL)	Dark Purple Red RHS 53A	**Leaf rim color spread**- Only at the rim
33.		LEAF STALK SHADE	Dark Purple Red RHS 185B	**Leaf Stalk Shades**- Present at front side
34.		FLOWER	Light Red Pink RHS 38D	**Flower Length**- Large **Flower Diameter**- Large **Petal Arrangement**- Highly Imbricate
35.		PETAL VENATION	Pink RHS 68C	**Petal shade**- Pink Lines present on Adaxial side only
36.		PETAL (PROXIMAL + ADAXIAL)	Purple RHS 58A	**Petal Base color**- Purple both sides
37.		PETAL (PROXIMAL+ABAXIAL)	Purple RHS 58A	**Petal Base color**- Purple both sides **Epicalyx**- Long