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Development of Interspecific Hybrids (*Abelmoschus esculentus* × *A. tetraphyllus)* in Okra using Embryo Rescue Approach

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Undomesticated related species are the rich stock of genes providing resistance to various diseases, pests and unfavourable environmental conditions. The sexual incompatibilities are bottleneck in introgression of such genes from wild species to popular varieties. The present study was executed to standardize a protocol of embryo rescue so to obtain the hybrids of *Abelmoschus esculentus* × *A. tetraphyllus* IC141017. Crosses were made between four (Arka Anamika, Pusa Makhmali, Parbhani Kranti, Jammu Okra-05) cultivated varieties of okra (*A. esculentus*) and a wild species (*A. tetraphyllus* IC141017) in reciprocal manner. Out of the four popular cultivars used in crossing, fruit set was recorded in Arka Anamika, Pusa Makhmali, Parbhani Kranti only when *A. tetraphyallus* IC141017 was used as a male parent. Fruit showed distal cracking after 25 d of pollination. The seeds appeared healthy for up to 15 d after pollination and later shrivelled and became pale yellow. Cross combinations, viz., Parbhani Kranti × *A. tetraphyllus* IC141017, Arka Anamika × *A. tetraphyllus* IC141017 and Pusa Makhmali × *A. tetraphyllus* IC 141017 showed the highest shoot regeneration on MS media containing 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} IBA. Greatest number of shoots per explant (0.92±0.12) as well as the highest frequency of shoot regeneration (86±0.12%) was reported in Parbhani Kranti × *A. tetraphyllus* IC141017. Arka Anamika × *A. tetraphyllus* IC 141017 (86±0.12%) and Pusa Makhmali × *A. tetraphyllus* IC 141017 (82±0.13%) resulted in high frequency of shoot regeneration on MS media containing 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} IBA. It can be inferred that MS media containing on 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} IBA showed positive results in regeneration of interspecific hybrids of okra.

Keywords: *Abelmoschus tetraphyllus*, embryo rescue, interspecific hybridization, Resistance breeding, YVMV.

Okra [*Abelmoschus esculentus* (L.) *Moench*] also called lady's finger or bindi is a polyploid vegetable with chromosome number 2n=130 belonging to family Malvaceae. A number of insect pests and viruses affect the production of this crop. Whitefly (*Bemisia tabaci*) transmitted virus complex consisting of a monopartite begomovirus, *bhendi yellow vein mosaic virus* (BYVMV) causing Yellow Vein Mosaic Virus (YVMV) is the most important and destructive viral disease in okra (Rana *et al*., 2006). The total loss due to YVMV has been reported to be 20-30%, which could rise to 80-90% if the carrier of the virus is not controlled (Richardson, 1997). The use of chemicals and culling off the infected plants is not practical and economical solution to

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control the virus, and therefore, development of resistant/tolerant varieties seems to be the best option to curtail the loss. Undomesticated related species are the genetic stocks of resistant genes for different pest, diseases and abiotic stresses (Rattan *et. al*., 2015). Lack of stable source of resistance to YVMV in cultivated species is the major constrain in developing stable resistant variety in okra. However, some of the wild species of okra have been reported to be stable and reliable sources of resistance to YVMV. One such wild species of okra, *A. tetraphyllus* is an important resistance sources to YVMV (Prabu, 2005), but the sterility problems and trouble in producing subsequent generations or even to carry out back crosses hampers the transfer of resistance from wild species. Hence for the development of YVMV resistant lines adoption of non-conventional methods of breeding like plant tissue culture mediated introgression is an urgent need. Embryo rescue technique can be efficiently used to overcome post-zygotic incompatibility which is found to operate between these species. In view of this, the investigation was undertaken with the objective to transfer resistance genes from wild species (*A. tetraphyllus* IC 141017) to popular okra varieties mediated through embryo rescue.

MATERIALS AND METHODS

Planting Material and Interspecific Hybridization

The planting materials used in the investigation comprised of four cultivated genotypes of okra [*A. esculentus* L. (Moench)], viz., Arka Anamika, Parbhani Kranti, Pusa Makhmali (procured from ICAR-NBPGR, New Delhi), Jammu okra-05 (procured from SKUAST, Jammu), and a wild species, *A. tetraphyllus* (IC 141017) procured from ICAR-Indian Institute of Vegetable Research, Varanasi. All these genotypes were sown in the Agriculture Farm of DAV University, Jalandhar in April, 2017. Reciprocal crosses were made between the cultivated and wild genotypes. Female flowers were selected at the balloon stage a day prior to pollination and their bagging was done to avoid undesirable crossing. Next day they were pollinated with the desirable pollen.

Embryo rescue and shoot and root regeneration

Fruits of okra at immature stage were harvested at 5, 10, 15 and 20 days after pollination (DAP). The immature seeds were extracted from these fruits and they were inoculated on media composed of Murashige and Skoog (MS) medium containing different concentrations of 6-Benzyl amino purine (BAP) for embryo emergence or callogenesis. The emerged embryos (4-5 cm) were further cultured on different treatment combinations so as to study their effect on shoot emergence. These treatment combinations were MS + 0.25 mg l^{-1} NAA + 0.5 mg l^{-1} IBA; MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA; MS + 1.0 mg l^{-1} NAA + 0.5 mg l^{-1} IBA; MS + 0.25 mg l^{-1} NAA + 1.0 mg l^{-1} IBA; MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} IBA; MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} IBA. Incubation of these cultures was done at 26±2^{0}C for 30 d after which they were sub-cultured on to freshly prepared medium for shoot multiplication. These shoots were transferred then transferred to root regeneration medium having composition of half-strength MS medium +0.25 mg l^{-1} IBA + 200 mg l^{-1} and activated charcoal, for root initiation.

Hardening of the plantlets

Well rooted plantlets were isolated from the test tube with utmost care so that the shoots do not get damaged. After washing them with distilled water to remove the adhering agar, the plantlets were transplanted in sterilized soil less media (cocopeat: vermiculite: perlite in ratio of 1:1:1) in the small pots. Watering of these plantlets was done at every 15 days with half-strength MS medium.

RESULTS AND DISCUSSION

Interspecific hybridization

The four cultivated varieties started flowering in the month of May 2017, whereas, the wild species (IC 141017) started flowering in the month of Sept. 2017. The main problem in the hybridization programme of the present study was non-synchronised flowering among cultivated and wild genotypes. The wild genotype started flowering by the Sept. 2017, however the cultivated genotypes which were sown in the April, 2017 stopped flowering by August, 2017. So, when the cultivated genotypes were in their peak period of flowering there was no flowering in the wild genotype. To overcome this problem, repeated sowings of cultivated varieties of okra

was done under controlled conditions so as to get a synchronous flowering.

A total of 50 crosses were made in each cross combination, of the four cultivated genotypes, fruit setting was observed only in Arka Anamika, Parbhani Kranti and Pusa Makhmali when cultivated genotypes were used as female parents. Fruit setting failed when *A. tetraphyllus* (IC 14101) was used as female parent (Table 1). Similar results were observed by Mamidwar et al. (1979), Meshram and Dhapake (1981), Sheela (1986). They observed that fruit set was maximum when *A. esculantus* was used as female parent in an interspecific cross between *A. esculentus* × *A. tetraphyllus*. Small fruits were obtained in the all the cross combinations. The fruits appeared normal till 25 d after pollination, but after that the fruits showed splitting/cracking from the distal end. The seeds looked healthy for up to 10-15 d after pollination, after that it shrivelled and became pale yellow (Fig. 2). The findings are in time with Mamidwar et al. (1979) who also obtained seed less fruits and shrivelled seeds when crossed *Abelmoschus esculentus* × *Abelmoschus tetraphyllus*.

Sureshbabu and Datta (1990) also observed the similar results and inferred that slow pollen tube growth, abnormal pollen tube, and abortion of fertilized ovules or scarcity of pollen grains could be the reason for no seed formation in interspecific hybrids. The findings are also in time with Sindhu (1993) who obtained shrivelled seeds in interspecific hybridization which may be attributed to the poor development of endosperm. Age at which embryo is extracted for inoculation affects a lot in regeneration of plant from embryo. The excision stage of embryo varies with crop. After 5, 10, 15 and 20 d of pollination, the fruits were harvested in the present study. This could be due to the fact that the embryo of the seeds which were harvested before 15 d of pollination were quite immature for pollination however, the embryo of the seeds which were harvested after 15 d of pollination were degenerated. Similar findings were reported by Rajamany et al. (2006), who obtained interspecific hybrids o *A. esculentus* × *A. moschatus* when excised embryos at 12 and 15 d after pollination through embryo rescue technique.

Effect of media of embryo emergence

The fruits of the all the cross combinations which were harvested at 5, 10 and 15 d, had seeds

Table 1. Cross compatibility between *Abelmoschus esculentus* L. (Moench) and *A. tetraphyllus* IC 141017

Cross combination	No. of cross attempted	Fruit setting	No. of fruits obtained after 25 DAP
Arka Anamika × *A. tetraphyllus* IC 141017	50	28	22
Parbhani Kranti × *A. tetraphyllus* IC 141017	50	32	26
Pusa Makhmali × *A. tetraphyllus* IC 141017	50	20	18
A. tetraphyllus IC 141017 × Arka Anamika	50	-	-
A. tetraphyllus IC 141017 × Parbhani Kranti	50	-	-
A. tetraphyllus IC 141017 × Pusa Makhmali	50	-	-

Table 2. Response of different interspecific hybrids on growth media

Cross combination	Different levels of BAP (mg l^{-1}) in MS Medium			
	0.25	0.5	1.0	1.5
Arka Anamika × *A. tetraphyllus* (IC 141017)	embryo emergence after 25 d of inoculation	embryo emergence after 45-50 d of inoculation	-*	-
Pusa Makhmali × *A. tetraphyllus* (IC 141017)	embryo emergence after 30-35 d of inoculation	embryo emergence after 45-50 d of inoculation	-	-
Parbhani Kranti × *A. tetraphyllus* (IC 141017)	embryo emergence after after 30-35 d of inoculation	embryo emergence after 45-50 d of inoculation	-	-

*: No response was noted.

Table 3. Shoot regeneration in different media combinations

Cross	MS medium containing 1 mg l^{-1} IBA			MS medium containing 0.5 mg l^{-1} IBA		
	1 mg l^{-1} NAA	0.5 mg l^{-1} NAA	0.2 5mg l^{-1} NAA	1.0 mg l^{-1} NAA	0.5 mg l^{-1} NAA	0.25 mg l^{-1} NAA
Arka Anamika × *A. tetraphyllus* (IC 141017)						
Callus formation	+++	+++	+++	+++	++	+
Avg. No. of shoots/explants	0.68 ± 0.108	0.89 ± 0.101	0.75 ± 0.113	0.62 ± 0.128	0.16 ± 0.114	0.08 ± 0.106
Shoot regeneration frequency (%)	52 ± 0.152	85 ± 0.113	70 ± 0.115	42 ± 0.110	22 ±0.156	12 ± 0.128
Parbhani Kranti × *A. tetraphyllus* (IC 141017)						
Callus formation	++	+++	++	++	+	+
Avg. No. of shoots/explants	0.58 ± 0.142	0.92 ± 0.126	0.64 ± 0.136	0.38 ± 0.142	0.28 ± 0.141	0.06 ± 0.128
Shoot regeneration frequency (%)	38 ± 0.126	86 ± 0.125	68 ± 0.129	29 ± 0.139	0.32 ± 0.100	0.14±0.132
Pusa Makhmali × *A. tetraphyllus* (IC 141017)						
Callus formation	++	+++	+++	+	+	+
Avg. No. of shoots/explants	0.72 ± 0.109	0.88 ± 0.132	0.72 ± 0.134	0.39 ± 0.112	0.39 ± 0.112	0.10 ± 0.109
Shoot regeneration frequency (%)	46 ± 0.132	82 ± 0.136	58 ± 0.128	44 ± 0.110	41 ± 0.108	26 ± 0.121

which were shiny and succulent yet small and white. As the number of days after pollination increased the seeds started appearing shrivelled and dull white (Fig. 2). However, the seeds remained succulent at 15 d after pollination. Inoculations of these harvested seeds were done on different treatment combinations viz., MS + 0.25 mg l^{-1} BAP; MS + 0.5 mg l^{-1} BAP; MS + 1.0 mg l^{-1} BAP; MS + 1.5 mg l^{-1} BAP. The results pertaining to this component is presented in Table 2. Cross combinations, Arka Anamika × *A. tetraphyllus* IC 141017 showed emergence of embryo 25 d after inoculation when inoculated media containing MS + 0.25 mg l^{-1} BAP. Cross combination, viz., Pusa Makhmali × *A. tetraphyllus* IC 141017 and Parbhani Kranti × *A. tetraphyllus* IC 141017 showed direct emergence of embryo 30-35 d after inoculation in the same media. Emergence of embryo was observed in all the cross combinations after 45-50 d of inoculation in the MS media containing BAP @0.5 mg l^{-1}. No cross combinations showed embryo emergence in media comprising of MS + 1.0 mg l^{-1} BAP; MS + 1.5 mg l^{-1} BAP.

Role of media is major in embryo rescue, as it acts as endosperm by providing nutrient to the excised embryo. The seeds of fruits harvested after 15 d of pollination were inoculated in MS media containing various concentrations of BAP. MS media containing 0.25 mg of BAP gave the best results in all cross combinations. These findings corroborates with the results of Kabir et al. (2008), who observed good regeneration with BAP. It was observed that lower the concentration of BAP greater were the results with early emergence of embryo. Negative effect on plant regeneration with higher concentration of BAP was also observed by earlier researchers *viz.*, Kashif Waseem *et al*. (2011) and Zayova *et al.* (2012).

Regeneration and Hardening plantlets

Shoot Regeneration

Different treatments such as MS + 0.25 mg l^{-1} NAA + 0.5 mg l^{-1} IBA; MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA; MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA; MS + 0.25 mg l^{-1} NAA + 1.0 mg l^{-1} IBA; MS + 0.5 mg l^{-1} NAA + 1.0 mg l^{-1}IBA; MS + 1.0 mg l^{-1} NAA + 1.0 mg l^{-1} IBA were used to subculture the two true leaf stage emerged embryos. MS media supplemented with 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} IBA resulted in the largest

number of shoots per explant (0.92±0.12%) with the maximum frequency of shoot regeneration (86±0.12%) in Parbhani Kranti × *A. tetraphyllus* IC 141017. The same media combination revealed maximum number of shoots/explants to the tune of 0.89±0.10 and 0.88±0.13 in cross combinations *viz*., Arka Anamika × *A. tetraphyllus* IC 141017 and Pusa Makhmali × *A. tetraphyllus* IC 141017 respectively. Similarly, the maximum frequency of shoot regeneration was observed in Arka Anamika × *A. tetraphyllus* IC 141017 (85±0.11%) and Pusa Makhmali × *A. tetraphyllus* IC 141017 (82±0.14%) on this media combination (Table 3). Kabir et al. (2008) also obtained shoot differentiation in okra when MS media was supplemented with NAA and Dhande et al. (2012) who observed that MS media supplemented with IBA and NAA gave good shoot regeneration in okra.

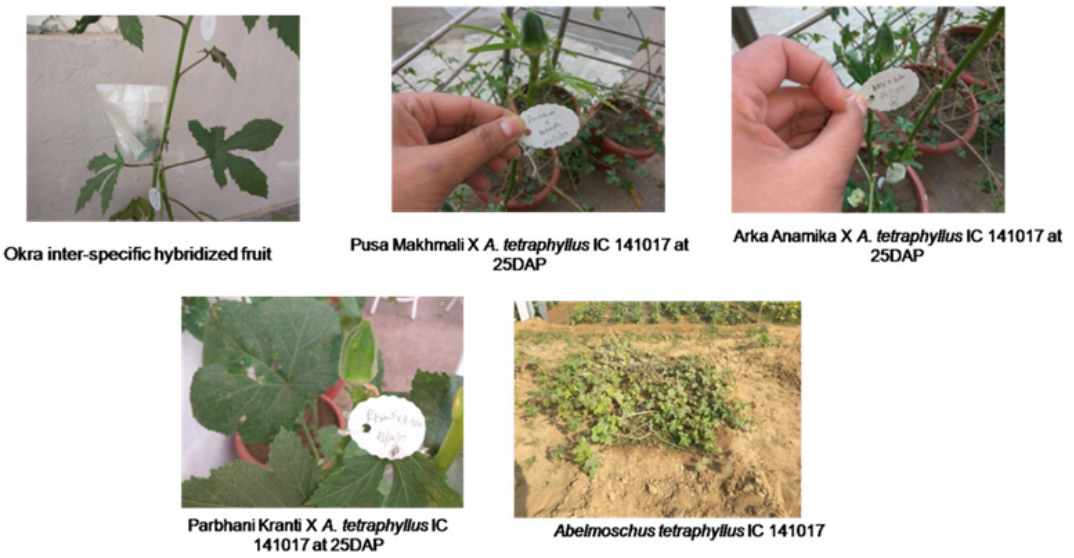

Fig. 1. Different interspecific cross combinations and wild genotype

Fig. 2. (a to d): a) Small interspecific hybrid fruit. b) Distal cracking in the fruits after 25DAP. c) Small while yet succulent seeds after 15 DAP. d) Degeneration of seeds after 25 DAP

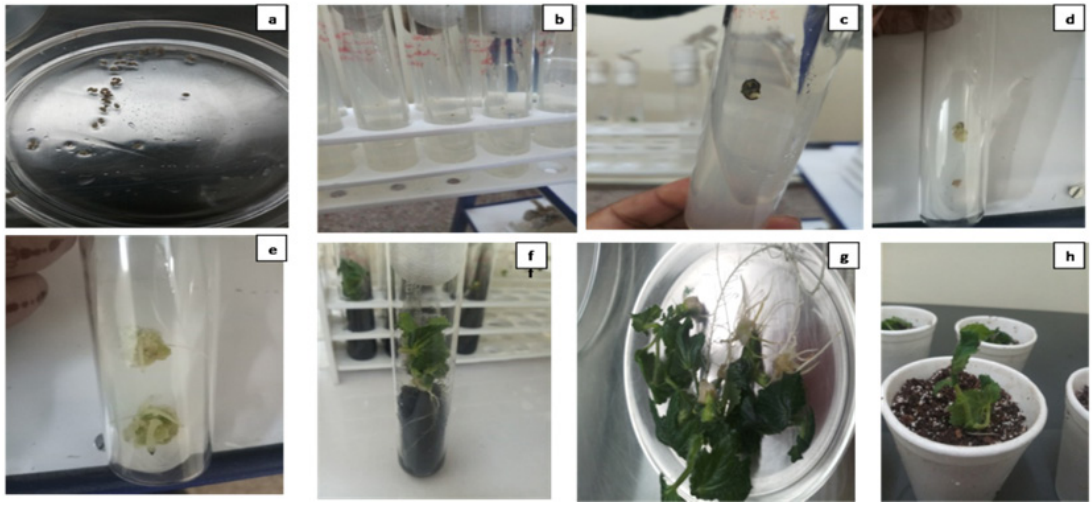

Fig. 3. (a to h): a) Immature seeds. b) Inoculation of immature seeds. c) Emergence of embryo from immature seed. d) Development of callus from embryo e) Development of embryonic callus. f) Shoot and root development. g) Invitro regenerated plantiets. h) Acclimatization of invitro developed plantiets

Root regeneration

The subculturing of regenerated shoots was done in the media containing different levels of auxins. After 10-15 days of subculturing there was initiation of roots in all cross combination and well developed roots were seen in 3 weeks. Arka Anamika × *A. tetraphyllus* IC 141017, Pusa Makhmali × *A. tetraphyllus* IC 141017, Parbhani Kranti × *A. Tetraphyllus* IC 141017 showed the maximum root regeneration to the tune of 56.08, 78.28, and 48.12%, respectively in half-strength MS media containing 0.25 mg l^{-1} IBA + 200mg l^{-1} and activated charcoal. Plantlets after proper rooting were transferred to sterilized soil less mixture (cocopeat: vermiculite: perlite in the ration of 1:1:1) in pots as presented in Fig. 3.

Introduction of roots on regenerated shoots is important for establishment of plantlets in soil Kabir et al (2008). Maximum roots were observed in MS media containing 0.25mg l^{-1} IBA + 200mg l^{-1} and activated charcoal in the present study. Kabir *et al.* (2008) also reported good root regeneration by supplementing the MS media with IBA. Activated charcoal was used as anti browning agent. Activated charcoal stimulates nitrogen uptake by shoots and induce a dark environment resulting invitro rooting (Thomas, 2008). However, Muhammad Isshad *et al*. 2017 suggested that the reduced salt concentration to be effective for *invitro* rooting due to reduced nitrogen content rather than reduced osmotic potential. The results also corroborates with Mohammad Irshad *et al.* (2017) who used 1/2 MS supplemented with IBA and AC (Activated Charcoal) for rooting in okra.

CONCLUSION

Interspecific hybridization using wild relatives through biotechnological interventions can be the best way to introduce desirable genes absent in the domesticated species. Present study was made with an aim to standardize a protocol of embryo rescue so to obtain the hybrids of *Abelmoschus esculentus* × *A. tetraphyllus* IC141017. Out of the four popular cultivars used in crossing, fruit set was observed only in Arka Anamika, Pusa Makhmali, Parbhani Kranti when *A. tetraphyallus* IC141017 was used as a male parent. Healthy seeds can only be obtained before 15 d after pollination. Ms media containing on 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} IBA resulted in highest shoot regeneration in cross combinations, viz., Parbhani Kranti × *A. tetraphyllus* IC141017, Arka Anamika × *A. tetraphyllus* IC141017 and Pusa Makhmali × *A. tetraphyllus* IC 141017. Among these Parbhani Kranti × *A. tetraphyllus* IC141017 showed greatest number of shoots per explant (0.92±0.12) as well as the maximum frequency of shoot regeneration (86±0.12%). On MS media supplemented with 0.5 mg l^{-1} NAA + 1.0 mg l^{-1}

IBA, the maximum frequency of shoot regeneration was recorded in Arka Anamika × *A. tetraphyllus* IC 141017 (86±0.12%) and Pusa Makhmali × *A. tetraphyllus* IC 141017 (82±0.13%)

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