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Hybrid embryo rescue: a non-conventional breeding strategy in horticultural crops

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ABSTRACT

Production of interspecific and intergeneric hybrids is useful for transfer of desirable genes from wild species into cultivated species. In many instances, progeny from wide crosses is difficult to produce owing to several barriers. Post-zygotic barriers such as endosperm abortion and, at later stages, embryo degeneration are of common occurrence, leading to low fertility; but these have been overcome through the use of embryo rescue and several hybrids have been developed. This approach is especially useful in horticultural crops, more so in fruit crops. In our laboratory, we have developed protocols for hybrid embryo rescue in several top-of-the-line fruit crops that suffer from an inability to cross naturally (e.g., distant crosses, use of seedless parent/s) or instances where initial fruit drop is very high. Thus, interspecific, intergeneric and intervarietal hybrids have been generated in mango, banana, seedless grape, papaya and seedless citrus using embryo rescue. Culture of embryos has also been demonstrated in rose, capsicum, hot pepper, onion and tomato. Among the very important strategies under non-GM biotechnologies figure techniques of hybrid embryo rescue, and related applications like ovule / ovary / placental cultures through sequential embryo culture. Embryo culture applied to practical problems is a tissue culture technique that has proven to be of greatest value to breeders.

Key words: Hybrid embryo rescue, horticultural crops, *in vitro* culture, non-conventional breeding

INTRODUCTION

Successful sexual hybridization involves a series of events including pollen germination, pollen-tube growth, fertilization, embryo and endosperm development and seed maturity, leading to germination (Debbarama *et al*, 2013). These events can be hampered by somatoplastic sterility, cytoplasmic-genic male sterility and structural differences in chromosomes. A very important and useful biotechnological tool for raising hybrid progeny in intractable crosses is embryo rescue. A given cross may fail for any of a number of reasons: seedlessness in either or both parents, inimical influence of endosperm resulting in inhibited embryo development, poor fruit-set / heavy fruit-drop at initial stages of the cross, distant crossing (interspecific / intergeneric / intervarietal breeding resulting in embryo abortion), genetic incongruity of parental genomes, mismatch in ploidy levels, etc. Embryo culture is one of the earliest forms of *in vitro* culture applied to practical problems and is probably the tissue culture technique that has proven of greatest value to breeders (Dunwell, 1986). At the Indian Institute of Horticultural Research, Bangalore, the authors have successfully developed technologies using excised hybrid embryo / sequential embryo culture in a number of horticultural crops (Leela Sahijram, 2007, 2009, 2011). The authors have raised hybrids in seedless grape & lime, in difficult crosses of papaya, and in mango, banana, rose, etc. The underlying principle of embryo rescue technique is aseptic isolation of the embryo and its transfer to a suitable medium for facilitating its development under optimal conditions. The potential these techniques hold is a viable alternative to parasexual hybridization and somatic embryogenesis (Stewart, 1981).

Failure to produce a hybrid may be due to either pre- or post-fertilization incompatibility. If fertilization is possible between two species or genera, the hybrid embryo may abort before maturation. If fertilization does occur, the embryo resulting from an interspecific or intergeneric cross can oftentimes be rescued and cultured to produce a whole plant. Such a method is referred to as *embryo rescue*. It is a tissue culture technique used in various breeding programs engaged in creating new genotypes. The procedure involves dissection of the developing embryo out from its environment

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and culturing it on a synthetic medium. In certain instances, ovule culture may need to be applied preceding excised embryo culture.

Plant breeders usually rescue inherently weak, immature or hybrid embryos to prevent their degeneration. Successful production of plants from cultured embryos depends largely upon maturation stage and composition of the medium besides, of course, the genotype. Abortion of embryos at one or the other stage of development is a characteristic feature of distant hybridization. Hybrid embryo rescue is a popular approach for raising hybrids. Currently, embryo rescue holds great promise for not only effecting wide crosses but also for obtaining haploid plants, and for shortening breeding cycle in presence or absence of a protracted dormancy.

THE TECHNIQUE OF EMBRYO CULTURE

Embryo culture is the sterile isolation and growth of an immature or mature embryo *in vitro*, with the goal of obtaining a viable plant. The first attempt to grow embryos of angiosperms was made by Hannig in 1904 (Bridgen, 1994) who obtained viable plants from *in vitro* isolated embryos of two crucifers *Cochleria* and *Raphanus*. In 1924, Dietrich grew embryos of different plant species and established that mature embryos grew normally but those excised from immature seeds failed to achieve the organization of a mature embryo. They grew into seedlings, skipping the stages of normal embryogenesis and without completion of the dormancy period. Laibach (1925, 1929) demonstrated practical application of this technique by isolating and growing the interspecific cross *Linum perenne* x *L. austriacum* that otherwise aborted *in vivo*. Embryo culture is now a well-established and useful branch of plant tissue culture and the subject has been reviewed by researchers (Sharma *et al*, 1996; Reed, 2004; Raghavan, 2003; Taji *et a*l, 2002; Leela Sahijram, 2007, 2010; Bhojwani *et al*, 1999; Bhojwani and Razdan, 1996; Chadha *et al*, 2000; Chawla, 2002).

There are broadly two types of embryo culture:

(i) Mature embryo culture is the culture of mature embryos derived from ripe seeds. This type of culture is done when embryos do not survive *in vivo* or become dormant for long periods of time, or, is done to eliminate inhibition of seed germination. Seed dormancy of many species is due to chemical inhibitors or mechanical resistance present in structures covering the embryo (rather than dormancy of the embryonic tissue). Excision of embryos from the testa and culturing them in nutrient media may bypass such seed dormancy. Some species produce sterile seeds (which may be due to incomplete embryo development). Embryo culture procedures may yield viable seedlings. Embryos excised from the developing seed at or near the mature stage are autotrophic and can be grown on a simple inorganic medium with a supplemental energy source. Seeds having hard seed coats are sterilized and soaked in water for a few hours to a few days. Sterile seeds are then split open and the embryos excised out.

(ii) Immature embryo culture, also known as **embryo rescue**, is the culture of immature embryos to rescue embryos from wide crosses, crosses involving seedless parent/s, or where fruit fall is heavy in early stages of embryo development. This is mainly used to avoid embryo abortion, with the purpose of producing a viable (hybrid) plant. Wide hybridization, where individuals from two different species of the same genus or different genera are crossed, often leads to failure of the cross. There are several barriers which operate at pre- and post-fertilization levels to prevent successful gene transfer from wild into cultivated species. Pre-fertilization barriers include all factors that hinder effective fertilization, which is usually due to inhibition of pollen-tube growth by the stigma or upper style. Post-fertilization barriers hinder or retard development of the zygote after fertilization and inhibit normal development of the seed. This frequently results from failure of the hybrid endosperm to develop properly, leading to starvation of the hybrid embryo or results from embryo-endosperm incompatibility where the endosperm produces toxins that kill the embryo.

Raghavan (1976) discussed evidence suggesting that embryos of inviable hybrids possessed a potential for initiating development, but were inhibited from reaching adult size with normal differentiation. Endosperm failure generally results in eventual starvation of the abnormal embryo. Isolation and culture of hybrid embryos prior to abortion circumvents these strong post-zygotic barriers. Production of interspecific and intergeneric hybrids is the most impressive and conspicuous application of embryo rescue and culture technique, particularly, for subsequent valuable gene transfer from wild species. There are normally no problems of disinfection of embryos in such cultures. Florets are removed at the proper time, and, either the florets or the ovaries are sterilized. Ovules can then be removed from the ovaries. The tissue within the ovule in which the ovule is embedded is already sterile. For mature-embryo culture,

either single, mature seeds are disinfected or, if the seeds are still unripe, then the still-closed fruit is disinfected. The embryos can then be aseptically removed from the ovules. The most important aspect of embryo culture work is selection of the medium necessary to sustain continued growth of the embryo. Younger the embryo, more stringent is its requirement. Although mature embryos can be grown on basal salt-media with a carbon-energy source such as sucrose, young embryos, in addition, require various vitamins, amino acids and growth regulators and, in some cases, natural endosperm extracts. Young embryos should be transferred to a medium with high sucrose concentration (8-12%) which approximates the high osmotic potential of intracellular environment of the young embryo-sac, and, a combination of hormones that supports growth of heart-stage embryo (i.e., a moderate level of auxin and a low level of cytokinin). Reduced organic nitrogen in the form of aspargine, glutamine or casein hydrolysate is always beneficial. Malic acid is often added to the culture medium. After 1-2 weeks (when the embryo ceases to grow), it must be transferred to a second medium with normal sucrose concentration, low levels of auxin and a moderate level of cytokinin, which allows for renewed embryo growth, with direct shoot growth in many cases. Where the embryo does not show direct shoot formation, it can be transferred to a medium for callus induction, followed by shoot induction. When the embryos have grown into plantlets *in vitro*, they are generally transferred to sterile soil and grown to maturity under greenhouse conditions.

Table 1: Resistance traits transferred to a variety of agricultural crops using embryo rescue

S.No.	Species crossed	Resistance trait/s
1	*Lycopersicon esculentum* x *L. peruvianum*	Virus, fungi, nematodes
2	*Solanum melongena* x *S. khasianum*	Brinjal fruit & shoot borer (*Leucinodes arbonlis*)
3	*Solanum tuberosum* x *S. etuberosum*	Potato leaf-roll virus
4	*Brassica oleracea* x *B. napus*	Triazene resistance
5	*Brassica napus* x *B. oleracea* (kale)	Cabbage aphid
6	*Triticum aestivum* x *Thinopyrum scirpeum*	Salt-tolerance
7	*Hordeum sativum* x *H. vulgare*	Powdery mildew and Spot-blotch
8	*Hordeum vulgare x H. bulbosum*	Powdery mildew
9	*Oryza sativa* x *O. minuta*	Blast (*Pyricularia grisea*); Bacterial blight (*Xanthomonas oryzae*)

Applications of embryo culture:

Prevention of abortion in wide crosses: Development of intergeneric hybrids is possible: *Raphanus sativus* x *Brassica napus*; *Actinidia deliciosa* x *A. eriantha*; *Actinidia de*liciosa x *A. ar*guata. *Carica* and *Citrus* also produced distant hybrids from their relatives using embryo rescue. In peach, a cross between an early Japanese cultivar Sunago Wase and a Chinese cultivar Yuhualu resulted in 'Zaoxialu' – an extra-early maturing hybrid (55 days to maturity, in Hanzhou, China). Some hybrids obtained through embryo rescue have recombined desirable genes like disease- and pest-resistance in various agricultural crop species (Table 1), besides inducing earliness. Embryo rescue is also used to recover crosses between diploids and tetraploids.

Production of haploids: Embryo culture can be used for production of haploids or monoploids. Kasha & Rao (1970) developed a technique to produce barley monoploids. Interspecific crosses are made with *Hordeum bulbosum* as the pollen parent, and the resulting hybrid embryos are cultured. But, these exhibit *H. bulbosum* chromosome-elimination, resulting in monoploids of the female parent *H. vulgare*.

Overcoming or breaking seed-dormancy: Seed dormancy can be caused by numerous factors, including endogenous inhibitors, specific light-requirements, low temperature, dry-storage requirements and embryo immaturity. Seeds of many species exhibit orthodox dormancy which, is, inhibition of germination by presence of certain plant hormones in tissues surrounding the embryo within a seed. Examples of such species include apples, pears, peaches, cedars, hemlocks, pines, firs, maples and roses. Dormancy can be a vexatious problem for growers and hybridizers alike. Embryo culture allows rapid testing of seed viability where seed dormancy can be circumvented.

Removal of pericarp and testa from around the embryo removes the source of hormones that inhibit germination and, if all else is in place, allows the embryo to germinate and grow. It is a well-established technique, but, many previously-published protocols for embryo culture have complex and expensive culture requirements.

Seed dormancy in *Iris* is due to the presence of a stable chemical inhibitor in the endosperm. In American basswood (*Tilia americana*), the seed is borne within a tough, indehiscent pericarp where the resistance is mechanical. Naturally-vegetatively propagated plants like bananas and *Colocasia* produce seeds that do not germinate

in nature, probably due to recalcitrant dormancy. These stand to benefit from embryo culture (Leela Sahijram and Doreswamy, 2000).

Shortening the breeding cycle: Species that exhibit seed dormancy factors localized in the seed-coat, pericarp, endosperm, or all three benefit from removal of these inhibitors to promote seed germination, eg., Brussels sprout, rose, apple, oil palm and *Iris*. Hollies (*Ilex*) embryos remain in the immature heart stage even though the fruits may have reached maturity. It takes three years for *Ilex opaca* seeds from mature berries to complete their embryonic development and to begin germination. Through embryo rescue, hybrid plants from this can be obtained in 2-3 weeks. *Rosa* normally takes a year to come to flowering; through embryo culture it was shown that it was possible to produce two generations in a year.

Prevention of embryo abortion with early-ripening stone fruits: Early-ripening varieties of peach, cherry, apricot and plum produce sterile seeds (Ramming, 1985) which do not germinate under natural conditions and, eventually, decay in the soil. Seed-sterility here may be due to incomplete embryo-development. In early-ripening stone fruits, transport of water and nutrients to the immature embryo is cut off too soon sometimes, resulting in embryo abortion. Precocious germination has been prevented in *Prunus* through ovule culture, where the integument acts as a natural inhibitor (Ramming, 1985). 'Makapuno' coconuts are prized for their characteristic soft endosperm which fills the whole nut. These nuts fail completely to germinate because the endosperm invariably rots before the germinating embryo can emerge out of the shell. Embryo culture has been practiced as a general method in horticultural crops including avocado, peach, nectarine and plum. Two cultivars, 'Goldcrest peach' and 'Mayfire nectarine' have resulted from embryo culture and are now commercially grown.

Embryos for propagation/ conservation/ germplasm exchange: Embryos are excellent material for *in vitro* preservation (Leela Sahijram and Rajasekharan, 1998) and propagation (Leela Sahijram and Doreswamy, 1989; 2000; 2004a), especially in conifers and in Gramineae family. As per IPGRI, Rome, it is compulsory to exchange germplasm of coconut internationally using embryo cultures (otherwise, shipping of whole nuts is cumbersome besides requiring huge cargo-space on flights or ships).

Germination of seeds of obligatory parasites: Parasitic plants produce seeds that can be germinated to produce plantlets only on their chosen host-plant. Germination of seeds of obligate parasites *in vivo* without the host plant is impossible, but is achievable with embryo culture. Insectivorous plants have also been shown to propagate successfully on a synthetic medium using these techniques.

EMBRYO RESCUE IN FRUIT CROPS

Embryo rescue techniques in fruit crops have played an important role in breeding new, early-maturing seedless triploid types as well as obtaining distant hybrids preventing embryo degeneration at early development stage/s and shortening breeding cycle. Application and progress of embryo rescue in fruit crop breeding was reviewed and several problems, e.g., suitable culture-period, nutrient and environment conditions studied (Yi Hualin *et al*, 2001). With interspecific crosses, intergeneric crosses, and crosses between diploids and tetraploids, the endosperm often develops poorly or not at all. A synthetic medium can compensate for lack of support from endosperm.

Mango

Mango (*Mangifera indica* L.) is the leading fruit crop of India. In controlled crosses in breeding programmes of this crop, fruit-set in relation to the original number of flowers pollinated is extremely low, sometimes as low as 0.01% (Iyer, 1991). Therefore, our aim was to develop an embryo rescue based technology for improving breeding success.

The mango has long been known to be a difficult system in traditional breeding programmes (Brooks, 1912; Mukherjee *et al*, 1968; Singh *et al*, 1980) because of certain of its inherent characteristics which include:

1. Long juvenile phase
2. High level of heterozygosity resulting in unpredictable outcome in hybridization
3. Heavy fruit-drop leading to low retention of crossed fruits
4. One seed per fruit
5. Polyembryony in some cultivars
6. Large acreage required for meaningful assessment of hybrids

Classically, barring a few hybrid varieties resulting from planned hybridization programmes, almost all known cultivars have resulted from selection of chance seedlings from natural cross pollination. In Florida and South Africa, for example, none of the cultivars developed has come from a breeding programme.

Modern horticultural and industrial requirements in mango breeding (Iyer and Degani, 1997) emphasize:

1. Precocious bearing
2. Dwarf tree habit
3. Heavy and regular bearing
4. Freedom from physiological disorders
5. Resistance to major pests and diseases
6. Good shipping qualities and shelf-life
7. Ideal tree architecture

Floral biology and pollination in mango

The total number of flowers in a panicle may vary from 1000 to 6000 (Mukherjee, 1953). Initial fruit-set in mango is directly related to the proportion of perfect (hermaphrodite) flowers to staminate flowers, although, the final fruit-set does not necessarily depend on this ratio. This proportion becomes critical for optimum fruit-set in a cultivar when the proportion drops to 1%.

In self-pollinated cultivars like 'Dashehari', 'Langra', 'Chausa' and 'Bombay Green', embryological studies have shown that although fertilization takes place after self-pollination, degeneration of endosperm occurs 15 days post-pollination in the self-incompatible parent. The self-incompatibility system operating in mango therefore appears to be of the sporophytic type. Leela Sahijram and Doreswamy (1987, 1988) initiated work on hybrid embryo culture and nucellar embryogeny in mango. *In vitro* hybrid embryo rescue was demonstrated successfully in mango (*Mangifera indica* L.) breeding by Leela Sahijram and Doreswamy (1990, 1991, 1992) and Leela Sahijram *et al* (2005). Immature hybrid embryos from controlled crosses involving cvs. Amrapali, Alphonso and Arka Anmol as the female parent and 'Kerala Dwarf' as the male parent were successfully rescued using excised embryo culture. Leela Sahijram *et al* (2000) showed that determination of hybrid embryo success rate in mango was genotype-dependent while Leela Sahijram and Ravindra (1997) worked out protocols for maturation of immature zygotic embryos from an intervarietal cross involving cvs. Amrapali and Kerala Dwarf.

Breeding objectives in mango:

Developing cultivars with

(i) Regular bearing

(ii) Dwarf tree habit with precocity in bearing

(iii) Attractive, good-sized (300-500g), shape, good quality fruits (taste, flavour and firm flesh without fibre), high pulp:stone ratio

With regard to improvement of rootstocks by breeding, the main desirable features are:

1. Polyembryony (a recessive trait)
2. Dwarfing influence on the scion
3. Tolerance to adverse soil conditions (pH, calcareous soil, etc.)
4. Good scion-compatibility

In the late 1980s, the authors initiated embryo rescue work the on controlled cross 'Mulgoa' X 'Neelum' and successfully obtained hybrid plantlets *in vitro*. However, the hybrids on transfer to soil could not survive for more than four months. With this experience, another strategy was applied to the further crosses carried out by the group. The delicate and vulnerable hybrid vitroplantlets were micrografted *ex vitro* onto the rootstock seedling as has been elaborated later in this paper.

Breeding for special objectives

Dwarfness - for orchard management and fruit quality: Owing to the obvious benefits of comparatively dwarf trees for orchard management and fruit quality, attempts were focused on obtaining hybrids with a dwarf tree framework. Breeding for dwarfness is important in mango, since, a consistent dwarfing effect of any rootstock has not been established to date. Indian cultivars found to be useful as a source of imparting dwarfness include 'Kerala Dwarf', 'Amrapali', 'Janardhan Pasand' and 'Nileshwar Dwarf' (Singh, 1990; Iyer and Subramanyam, 1986).

Controlled crossing was effected initially for three consecutive years by the authors, using following mango varieties identified on the basis of breeding priority:

S. No.	Cross-combination
1.	'Amrapali' X 'Kerala Dwarf' – (AKD)
2.	'Alphonso' X 'Kerala Dwarf' – (ALK)
3.	'Arka Anmol' X 'Kerala Dwarf' – (AnKD)
4.	Open-pollinated Totapuri (OpTp)

Young fruitlets from these crosses involving monoembryonate cultivars were harvested at 4-6 weeks post-pollination and excised embryos cultured *in vitro*. In AKD, 67.24% (39 out of 58) excised embryos cultured on defined media formed normal plantlets *in vitro*, while, in OpTp 15 out of 20 (75%) embryos cultured formed plantlets. Season I data on in vitro culture and regeneration response is presented in Table 2. Type of initial response in 'Alphonso'

X 'Kerala Dwarf' (ALK) is shown in Table 3 which draws attention to the fact that nearly 93% of fruitlets from a controlled cross may be lost to unforeseen circumstances. Table 4 shows the same cross in Year II up to transfer of hybrid plantlets into mini-pots.

Table 2: Data on controlled crossing in 'Alphonso' X 'Kerala Dwarf' in Year I

Parameter	Result
Total no. of panicles used for crossing	487
Total no. of flowers used as female parent	2646
Fruit set (No.)	208
% fruit set	7.86%
No. of fruits lost to leaf hopper infestation/ sooty mold	193 (92.8%)
No. of exciseable hybrid embryos	15
Ratio of panicle to fruit set	1:0.42
Ratio of flowers crossed to fruit set	176.4:1

Table 3: Data on culture and regeneration response of hybrid embryos in mango in Season I

Parameter		AnKD*		OpTp**	
		No.	%	No.	%
a.	No. of embryos inoculated	243	NA	232	NA
b.	No. of embryos forming plantlets in vitro	185	76.13	170	73.28
c.	No. of plants transferred from culture vessel into minipot	206	84.77	134	57.76
d.	No. of potted plants surviving primary transplantation	48	25.95	24	14.12
e.	No. of potted plants surviving secondary transplantation	10	4.11	2	0.86

*AnKD: 'Arka Anmol' X 'Kerala Dwarf'; **OpTp: Open-pollinated 'Totapuri'; NA: Not Applicable

Table 4: Data on controlled crossing and hybrid plants transferred *ex vitro* in 'Alphonso' X 'Kerala Dwarf' cross in Year II

Parameter	Result
No. of panicles used for crossing	221
No. of flowers used as female parent	857
Average no. of flowers crossed/panicle	3.878
Initial fruit-set (no.)	57
No. of fruits lost to fruit drop	7
% Fruit-set	6.65%
No. of fruits used for embryo excision	50
No. of cultures contaminated in vitrovent vessels	7
No. of cultures showing no response	4
No. of plants transferred to mini-pots	39

Comparative analysis of reproduction parameters for three years on controlled crossing in this cross is presented in Table 5.

Table 5. Comparative analysis of reproduction parameters for three years on controlled crossing in 'Alphonso' X 'Kerala Dwarf'

Parameter	Year I	Year II	Year III
No. of panicles used for crossing	496	221	487
No. of flowers used as female parent	3531	857	2646
Fruit-set (no.)	164	57	208
% Fruit-set	4.64%	6.65%	7.86%
Ratio of flowers crossed to fruit-set	22:1	15:1	13:1
No. of fruits used for embryo excision	109	50	15

A comparison was made between survival rate of hybrids of 'Amrapali' X 'Kerala Dwarf' and open-pollinated 'Totapuri' (Table 6).

Table 6: Comparative survival rate of plantlets at different stages of development *in vitro* and *ex vitro*

Particulars	'Amrapali' X 'Kerala Dwarf'	Open-pollinated 'Totapuri'
% of embryos forming plantlets *in vitro*	67.24	75.1
% Survival of plants from test-tube to pot	56.40	51.0
Success % in survival from pot stage to field	100.00	80.3

Although the rate of 'normal plantlet' formation *in vitro* in AKD during the first season (Fig.1) was very high (67.24%), mortality from the stage of culture vessel to field-planting (through mini-pots and later, into large pots) was very high too (48.7%), ie., only 20 hybrid plantlets out of 39 AKD survived to the field-planting stage (survival rate 51.3%). In OpTp, only 20% of the plantlets survived field-planting.

In the following season, in ALK, 88 hybrid embryos out of a total of 109 from the controlled cross formed plantlets *in vitro* (ie., 80.73%). Of the 88 hybrid plantlets transferred *extra vitrum*, only 2 survived to the field-establishment stage (ie., 2.27% survival rate). In the same year (1999), 105 embryos of OpTp formed plantlets *in vitro* out of 127 embryos cultured (ie., 82.68%). Four OpTp plantlets survived upto the final field-establishment stage (ie., 3.81% survival rate).

In the third season, in AnKD, 244 hybrid embryos were excised and cultured *in vitro* of which 177 formed normal plantlets (72.5%). In OpTp, of the 232 embryos, 166 formed plantlets *in vitro* ie., 79.55%. Plantlets were transplanted into soil in minipots and subsequently, into large pots.

In 'Amrapali' x 'Kerala' Dwarf (AKD), 69.23% cultures responded, with shoot and root induction. Upon

normal plantlet development, the hybrid vitroplants were transplanted *extra vitrum* in a phased manner into mini-pots. Eight weeks later, these hybrid plants were transferred to larger pots and finally, into the field. Field-established plants showed overall survival percentage of 34.4% in AKD and 20.0% in OpTp. In the controlled cross Alphonso X Kerala Dwarf (ALK) and open-pollinated Totapuri (OpTp), induction of rooting was achieved in 70.9% and 72.4% cultures, respectively, in Season II. Performance of ALK and OpTp was comparable (77.8 and 77.5%, respectively) on medium containing 1500 mg/l casein hydrolysate (CH) and 6% sucrose (w/v). OpTp responded best (87.5%) on medium containing 1000 mg/l CH + 3% sucrose (w/v).

Cultures showing both shoot and root induction were transplanted *extra vitrum* into large, polyvinyl pots. In all, 80.7% cultures of ALK and 82.7% of OpTp were transferred to soil. After 4 – 5 months, the plants were transferred to large earthen pots. Finally, an overall survival percentage of 4.54% and 3.81%, respectively, of ALK and OpTp plants was obtained. In season III (year 2000), 84.77% of AnKD cultures and 57.76% of OpTp cultures developed into plantlets *in vitro*. Overall percentage survival in AnKD was found to be 4.11, and 0.86 in OpTp.

Controlled crosses between the parental lines 'Alphonso' X 'Kerala Dwarf' (ALK) were repeated owing to poor survival in the earlier cross. Embryo excision was carried out at 6-8 weeks post-pollination (WPP). Of these, 78% of vitroplants were transferred to mini-pots (Leela Sahijram *et al*, 1999, 2009). Direct crossing between these parental lines (ALK) was repeated. Zygotic embryos here too were excised at 6 – 8 weeks post-pollination.

In all, during five seasons of explanting, various controlled crosses were made with 'Kerala Dwarf' as the male parent. Cultivars Amrapali, Alphonso, Arka Anmol and Ratnagiri Alphonso were used as the female parent. Open-pollinated Totapuri, Alphonso and Ratnagiri Alphonso were also used in the study. Zygotic embryos were excised aseptically at 5 to 8 weeks post-pollination and inoculated onto modified semisolid MS medium with complex growth addenda. Factors detrimental to initial embryo growth and development were found to be fungal/ bacterial contamination, excessive exudation of phenolics into the medium, and failure of the embryo to respond.

A protocol was earlier also worked out for the cross 'Mulgoa' X 'Neelum'. Using immature zygotic embryos as starter explants, Leela Sahijram *et al* (1996) could also induce somatic embryogenesis in mango embryos / nucellus and elucidate factors influencing the same. These plantlets did not survive beyond four months of *ex vitro* transfer.

Ex vitro shoot-tip grafting (*Ex vitro* STG)

In the earlier seasons, transfer of vitroplants to soil resulted in very high mortality despite a healthy status of these plants at transplanting. Hence, a novel technique of *ex vitro* shoot-tip grafting (*ex vitro* STG) was devised by the authors (Leela Sahijram, 2009; Anon., 2002) using 'Totapuri' as the rootstock. The hybrids were grown to maturity and evaluated. Further multiplication of these hybrids was done onvarious rootstocks.

'Totapuri' (rootstock) stones were germinated in the traditional manner in seed pans on a sand medium. Shoot apices of the hybrid plantlets were excised and grafted *ex vitro* onto the rootstock using the epicotyl grafting technique. Diameter of the scion shoot apex at the cut surface was 2 - 4 mm. Graft-union established successfully in 3 weeks

Table 7. Comparison between cross-combinations in crosses effected during five years

Season	Cross combination*	No. of embryos excised for	% embryos forming plantlets *in vitro* culture	% cultures surviving transplant into mini-pot	Survival % from mini-pot stage to larger pots/field
1998	AKD	58	67.24	56.80	34.48
	OpTp	20	75.00	50.00	20.00
1999	ALK	109	80.73	80.73	1.83
	OpTp	127	82.67	82.67	2.36
2000	AnKD	243	76.13	84.77	4.11
	OpTp	232	73.28	57.76	0.86
2001	ALK	50	70.80	78.00	0.00
2002	ALK	15	73.33	60.00	66.70
	OpRA	63	63.49	45.00	72.22
	OpTp	23	60.86	92.85	92.31
	OpA	17	35.29	66.70	100.00

*AKD= Amrapali x Kerala Dwarf; OpTp= Open-pollinated 'Totapuri'; ALK= 'Alphonso' X 'Kerala Dwarf'; AnKD= 'Arka Anmol' X 'Kerala Dwarf'; OpRA= Open-pollinated Rathnagiri Alphonso; OpA= Open-pollinated Alphonso

Fig 1. Hybrid embryo rescue in mango cross 'Amrapali' X 'Kerala Dwarf': A. *In vitro* growth of embryo; B. Secondary root induction; C. Hybrid vitroplantlets ready for transfer; D. Etiolated seedling of cv. Totapuri for receipt of graft; E. *Ex vitro* shoot-tip grafting; F. Graft-union secured: G. Polybagged graft; H. Development in minipots; I. Field-establishment of hybrid; J. Flowering hybrid; K. Fruit induction; L. Mature fruits

and, at the end of 8 weeks, the scions showed vigorous growth and development. Overall graft success rate was 79.54%. *Ex vitro* STG was carried out using vitroplantlets of Alphonso X Kerala Dwarf, open-pollinated Alphonso (OpA), Ratnagiri Alphonso (OpRA) and Totapuri (OpTp) with a success rate of 66.7%, 100%, 72.2% and 92.31%, respectively.

These grafts were transferred to polybags of size 9" height X 7" diameter containing sand, soil and vermicompost @ 1:1:1 at 10-12 weeks. Further, the grafts were transferred to larger pots containing sand, soil and farmyard manure @ 1:1:1, at 6 months. These grafts were further field-planted and hybrids evaluated for desirable traits (Fig. 1). Results of crosses effected during five years are compared and summarized in Table 7.

Molecular studies on putative mango hybrids obtained through embryo culture

Morphological parameters of the two parents in a cross and their hybrid progeny can be compared to look for clear-cut differences. Morphological traits, however, are subject to environmental influences and can be misleading. Besides, these lack power of resolution to identify hybrids at juvenile stages, for which it becomes necessary to wait until plant maturity (Debbarama *et al*, 2013). Distant crosses rescued through embryo rescue can be confirmed for hybridity using molecular markers such as RAPD, AFLP, etc. Therefore, testing of hybridity using molecular markers is advantageous, as, it obviates these hindrances.

Molecular markers

Mini- and micro-satellite DNA markers are highly polymorphic in many species due to a variable number of tandem repeats. DNA fingerprints obtained by hybridization of mini- and micro-satellite probes with genomic DNA were shown to be useful for cultivar identification. DNA fingerprint information for identification of nucellar and zygotic seedlings was attempted. For this purpose, DNA was isolated using CTAB method from fully mature leaves of 6 month old seedlings (stones with single-plants, as well as polyembryonic seedlings) and from field-grown mother trees. DNA samples were quantified using spectrophotometer and confirmed for presence of DNA using agarose gel electrophoresis.

To multiply Jeffrey's minisatellite probe (33.6 plasmid DNA was used as the probe), the plasmid was incorporated into DH5a *E. coli* competent cells and cultured on LB Agar medium with Ampicillin @ 50mg/ml. Single colonies from the transformed ones were isolated and inoculated onto the same medium. After adequate growth of the culture, the plasmid was isolated and checked for presence of minisatellite 33.6 (700 bp DNA) on 0.8% agarose gel, using restriction enzymes *Bam H1* and *EcoR1*. Further development of the non-radioactive labelled probe (33.6 minisatellite), restriction digestion of the plant genomic DNA and Southern transfer/hybridization was worked out.

PCR-amplification using ISSR primers

ISSR primers UBC 814, 835, 841, 844, 848, 868, 873, 881, 898, 901 were used. The reaction mixture (20µl) contained 10mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM mixed dNTP, 1 mM primer, 0.5 unit Taq polymerase and 25 ng/µl of genomic DNA. DNA amplification was obtained through 40 cycles (92° C for 1 min, 42° C for 1 min 30 sec and 72° C for 1min 30 sec), followed by extension at 72° C for 8 min. Of the ten ISSR primers used, only six (UBC 835, 841, 848, 868, 873, 898) showed amplification. Bands amplified in the hybrids were similar to that in the parents suggesting that these were true hybrids. Forward and reverse primers, viz., 1F1R, 5F5R, 6F6R and 9F9R were also used, of which 1F1R, 6F6R and 9F9R showed amplification.

Seedless grapes

The second food crop to be fully sequenced genomically after rice is the grape. Improvement in grape is slow and time-consuming since the crop is heterozygous and exhibits pronounced inbreeding depression (Leela Sahijram, 2011). Thompson Seedless is the ruling variety of grape in India and other parts of the world, but, is susceptible to Downy mildew disease caused by the fungal pathogen *Plasmopara viticola*. Obtaining a downy mildew resistant Thompson Seedless would be a boon to the global viticulture industry, resulting in massive savings in pesticide-use while providing a cleaner, safer environment. Seedless grapes are stenospermocarpic, in that, these form embryos successfully, but the embryo does not develop, and aborts due to inability of the ovular wall to expand. These embryos can, however, be rescued before they abort by initially culturing the whole ovule; after about eight weeks, the developing embryo can be dissected out from the ovule and grown on a different medium. This is termed 'sequential embryo culture'.

Ramming (1990) pioneered hybrid embryo rescue in seedless grape breeding. Sequential hybrid embryo rescue was carried out by the authors using 'Thompson Seedless' as the maternal parent and 15 different pollen-donor parents

Fig 2. Sequential embryo culture in Thompson Seedless grape breeding: A. Grape bunch at six weeks post-pollination with seed traces; B. Enlarged longitudinal section of fruitlet; C. *In ovulo* embryo culture; D. Excised embryo under high magnification; E. Germination of hybrid embryos; F. Transfer of plantlets to liquid medium; G. Hybrids transplanted into polybags; H. Plants ready for shifting to glasshouse; I. Acclimatization in glasshouse; J. Field-establishment of hybrids

Table 8. Data for Year I on crossing 'Thompson Seedless' (female parent) with 14 Downy mildew resistant pollen-donor parents, and, open-pollinated 'Thompson Seedless'

Sl. No.	Pollen-donor parent used	Total no. of ovules cultured from the cross	No. of fully developed hybrid plantlets recovered	% hybrid plants recovered
1	*Vitis tilifolia*	165	28	16.97
2	'Madras Field Court'	134	57	42.54
3	*Fruehort veltliner*	110	48	43.64
4	*Vitis candicans*	39	3	7.69
5	'SV 23501'	5	0	0.00
6	'SV 18315'	172	14	8.14
7	'SV 12309'	58	33	56.90
8	'Catawaba'	60	23	38.33
9	'James'	117	19	16.241
10	*'Riparia X Rupestris'*	134	25	18.66
11	'SV 12364'	189	64	33.86
12	*Vitis assamica*	111	26	23.42
13	'SV 18402'	51	11	21.57
14	*Vitis lanata*	8	0	0.00
15	Open-pollinated Thompson Seedless	37	13	35.14
	Total	1390	364	26.19

(Tables 8) variously resistant to Downy mildew disease caused by the Deuteromycetan fungal pathogen (Leela Sahijram, 2005), as mentioned above. Open-pollinated 'Thompson Seedless' was also used by the authors who used a 2-step *in vitro* procedure: *in ovulo* embryo culture followed by *ex ovulo* embryo culture (sequential embryo transfer) to rescue immature embryos of these conventionally intractable crosses (Kanamadi *et al*, 1996, 1999a,b,c; Leela Sahijram and Kanamadi, 2004; Leela Sahijram *et al*, 2005a,b; Anon., 2005).

In our studies (Leela Sahijram, 2004b), we found that pollen-donor parent overwhelmingly determined the outcome of crossing and success rate thereof (Table 9). From the directed crosses, of the 1500 hybrids generated and grown in the glasshouse, about 700 were transferred to the field (Fig. 2), established to maturity and evaluated for downy mildew resistance while retaining desirable pomological qualities of the seed-parent. In some crosses involving Seedless X Seedless parents, the authors have demonstrated through histological studies that the zygote does not develop beyond first cell division (i.e., 2-cell stage).

Table 9. Comprehensive data on crossing 'Thompson Seedless' (female parent) and 15 pollen-donor parents resistant to Downy mildew

Sl. No.	Male Parent	No. of panicles used	Total no.of buds used	No. of ovules obtained 6-11 weeks post-pollination					Total no. of berries dissected	Total no. of ovules recovered in a cross	Total no. of apparently viable embryos	No. of fully developed plants regenerated
				6	7	8	9	10				
1	*V. tilifolia*	5	700	31	53	-	-	81	289	165	28	24
2	Madras Field Court	4	1070	21	41	-	24	48	303	134	38*	54
3	*F. veltiner*	4	630	8	26	-	76	0	276	110	36*	39
4	*V. candicans*	6	1230	0	0	-	29	10	446	39	2*	3
5	S. V. 12375	1	80	na	-	-	-	-	0	0	0	0
6	S. V. 23501	2	230	0	5	-	-	-	123	5	0	2
7	S.V. 18315	4	460	55	103	-	-	14	247	172	20	11
8	S. V. 12309	3	360	10	30	18	-	-	146	58	21*	27
9	Catawaba	3	360	12	1	-	-	47	192	60	7*	18
10	James	3	210	23	51	43	-	-	195	117	16	15
11	*Riparia X Rupestris*	3	180	-	114	-	20	-	161	134	21	20
12	S. V. 12364	6	415	41	44	104	-	-	269	189	35*	48
13	*V. assamica*	3	210	19	-	92	-	-	191	111	20*	26
14	S. V. 18402	3	220	17	-	34	-	-	225	51	8	7
15	*V. lanata*	1	100	-	-	-	-	8	44	8	3	0
	Total	51	6455						3107	1353	255*	294
	OpTS[#]	52	37	11	8							

Total no. of crosses = 15 Source: Leela Sahijram *et al*, 2005b

Average no. of buds used per panicle = 127

Ovules harvested at an age ranging from 6-11 weeks post-pollination

*No. of apparently viable embryos but some of the apparently non-viable embryos also developed into plantlets

[#]Open-pollinated Thompson Seedless

Bharathy *et al* (2003, 2005) in their study on 'Thompson Seedless', 'Flame Seedless' and eight other varieties of grape found embryo recovery to increase significantly with application of benzyladenine - a cytokinin - at pre-flowering and flowering stages. Maximum embryo recovery (47.57%) was obtained with 'Thompson Seedless' (TS) × 'Concord', followed by TS × SV18402 (29.75%). Murthy *et al* (2006) achieved field-establishment and screened embryo-rescued hybrid seedlings of grape with special reference to downy mildew.

Molecular markers: Leela Sahijram and Raghavendra (2005), Raghavendra *et al* (2006) and Leela Sahijram and Bhaskara Reddy (2009) from the authors' laboratory carried out molecular studies using ISSR markers in seedless grape hybrids generated by the authors and confirmed their hybridity.

Papaya

Leela Sahijram and Doreswamy (1993) tested the efficacy of using placental cultures for rescuing hybrid embryos from controlled crosses involving *Carica papaya* and *Vasconcellia cauliflora*. They were able to induce transient expression of anthocyanin gene (inky-blue in colour) in placental cultures of hybrid papaya. Intergeneric and interspecific crosses were used for hybrid embryo rescue and hybrid plants were recovered. Vitroplantlets were transplanted into soil *ex vitro* (Fig. 3).

Fig 3. Intergeneric breeding strategy in papaya: Hybrid plantlets *in vitro* (top) and transfer to soil (bottom)

Seedless lime

Prasad *et al* (1996) emphasized the importance of embryo rescue in improving seedless lime quality. Controlled crossing between 'Seedless lime' and Acid lime resulted in immature zygotic embryos that were rescued *in vitro* (Fig. 4) and grown to maturity in the field. The objective here was to develop a hybrid of acid lime with resistance to citrus canker.

Fig 4. Breeding Seedless Lime X Acid Lime: Hybrid embryos rescued *in vitro* (Left) and potted hybrids (Right)

Role of embryo culture techniques in improvement of fruit quality in seedless lime was elucidated by Prasad *et al* (1996). Rao *et al* (2011) highlighted the importance of genetic resources in citrus fruits and their exploitation in citrus improvement programmes using various in vitro techniques such as embryo rescue, nucellus culture, etc.

Banana

Commercially popular varieties are seedless owing to their triploid status. Protocols have been developed for rescuing excised hybrid embryos from crosses involving *Musa acuminata* and *M. balbisiana* (Fig. 5). Hybrid plants were raised to maturity in the field and evaluated (Doreswamy and Leela Sahijram, 1991, 1993; Chadha and Leela Sahijram, 2000). The hybrids were added to the germplasm pool in the Institute for further use in breeding.

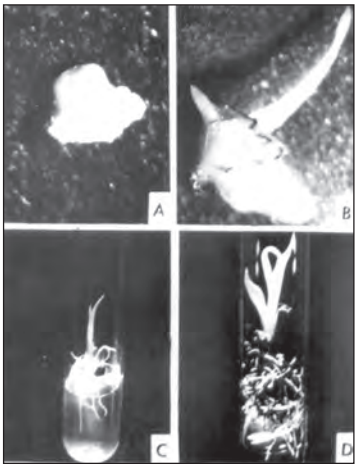

Fig 5. Rescued interspecific cross *Musa acuminata* X *M. balbisiana*: A. Excised embryo on semisolid medium; B. Germination; C. Plantlet development; D. Profuse rooting in the hybrid banana

Olive

Acebedo *et al* (1997) found the growth of plantlets derived from *in vitro* germinated embryos in the greenhouse to be normal. Thus, embryo culture can increase the efficiency and shorten the time for starting initial progeny evaluation and thereby speeding up seedling development in olive breeding programmes.

VEGETABLE CROPS

Wide-hybridization to a vegetable breeder and cytogeneticist is the first step in transferring genes from

wild species into cultivated ones. Embryo rescue technique has been successfully demonstrated in tomato, capsicum, chillies, okra and radish. In India, embryo culture work has been initiated in okra for rescuing interspecific hybrids. The following literature is by no means an exhaustive account of work done.

Tomato

Smith (1944) and Demirel & Seniz (1997) made observations on the possibility of utilizing embryo culture for improvement of tomato. For *in vitro* breeding in tomato, Aragao *et al* (2002) used three culture media in combination with distinct accessions, crossing generations, and periods of time after artificial pollination, and evaluated them to identify more efficient protocols to recover interspecific hybrids between *Lycopersicon esculentum* and *L. esculentum*. Bhattarai *et al* (2009) found germination of immature seeds to be a better alternative to culture of excised immature embryos in hastening tomato breeding programmes.

Capsicum / Chilli

In the state of Meghalaya in India, chilli is the third most important spice crop after ginger and turmeric. However, diseases namely, tobacco mosaic virus, root rot, tomato spotted wilt virus, etc. lead to considerable decline in yield. *Capsicum chinense, C. annuum* and *C. frutescens* were crossed with each other and embryos rescued between 27-33 days after pollination. Highest percentage of embryo growth was observed on MS medium with 0.5 mg/l GA_3 and 0.05 mg/l NAA. Hybrid plants were obtained and their hybridity confirmed using morphological as well as RAPD markers (Debbarama *et al*., 2013).

Radish

Intergeneric herbicide-resistance transfer to radish has been recently accomplished using embryo rescue (Mithila and Hall, 2012). For introgressing auxinic herbicide (Dicamba) resistance from wild mustard (*Sinapis arvensis*) into radish (*Raphanus sativus*; 2n=18), embryo regeneration and hybrid plant production was achieved involving several hundred reciprocal crosses performed between these two species. Upon altering cultural conditions and media composition, a high frequency of embryo regeneration and hybrid plant establishment was achieved.

Okra

As resistance is not available in cultivated species of okra [*Abelmoschus esculentus* (L.) Moench.], interspecific crosses were made between *A. esculentus* and *A. moschatus* (resistant wild species) to develop resistant varieties. Post-zygotic incompatibility was found to operate between the species. Crossed seeds were shriveled and non-viable. *In vitro* embryo rescue to overcome the incompatibility revealed that culturing 12 and 15 day old embryos of *A. esculentus* var. Kiran (a high yielding line) x *A. moschatus* and *A. esculentus* var. Anakomban (a landrace) x *A. moschatus* on MS medium supplemented with BA 0.5 mg l^{-1} and CW 150 ml l^{-1} yielded transplantable hybrids. Embryos of crosses *A. moschatus* x *A. esculentus* (i.e., reciprocal cross) turned brown by the 11th day of pollination and could not be cultured *in vitro* (Rajamony *et al*, 2006).

The authors' laboratory has initiated work on *in ovulo* and *ex ovulo* culture of okra embryos (Fig. 6) with a view to generating interspecific hybrids in the long run. In another study (Fatokun, 1987), crosses were made between members of two West African okra types, 'Soudanien' and 'Guineen'. All crosses succeeded in both directions but F_1 plants that showed hybrid vigour for plant stature were partially sterile. Cytological observations of these F_1 plants revealed abnormal meiosis which resulted in production of microspores of variable size. Frequency of viable pollen (indicated by acetocarmine staining) was low in the hybrids: 35.80% (U.I.92× U.I.313) and 39.41% (1bk-1×U.I.215). Number of seeds produced per fruit was also low in the hybrids and only a few of these seeds were viable.

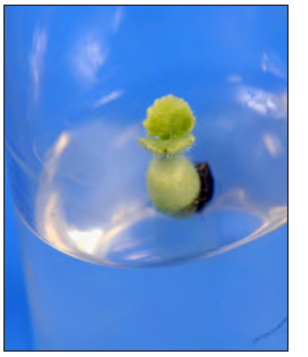

Fig 6. *In ovulo* embryo culture in okra

Brinjal

Incompatibility barrier was overcome in brinjal in F1 hybrids of the interspecific cross involving *Solanum melongena* and *S. macrocarpon*. Reciprocal crosses made by the breeder resulted in F2 progeny. BC1 - Backcross 1, [BC(H1 x M) Pl. 3] (Fig. 7), was rescued and has been transferred to soil for evaluation (Leela Sahijram and Padmini, unpublished data 2013).

Fig 7. Hybrid plantlet of brinjal rescued from an interspecific cross

Artichoke

Globe artichoke breeders have two important problems: lengthy seed-to-seed cycle, and seed-borne diseases caused by fungal pathogens. Both problems can be solved by embryo rescue. Embryos were collected from five cultivars in different post-pollination stages (Cravero and Crointy, 2007). Root induction was poor, but shoot development per explant was better. Twenty days were required as the optimal time for embryo rescue.

ORNAMENTAL CROPS

Van Tuyl and Lim (2003) reviewed interspecific hybridization and polyploidization as tools in ornamental plant breeding. Several crops in floriculture have benefitted from *in vitro* interventions in breeding programmes. Some of the successes are reported herein.

Rose

Marchant *et al* (1994) used embryo culture for production of F1 hybrids in English rose. Rout *et al* (1999) reviewed advances made in the biotechnology of rose and Holeman (2009) outlined a simplified method of embryo culture in rose. Hybrid / zygotic embryos were rescued form controlled crosses and open-pollination, respectively, in rose cultivars. Mohapatra and Rout (2005) in their study on embryo rescue in floribunda roses rescued immature embryos from "Arunima' and "Shocking Blue' roses. Time required for breeding roses through conventional breeding, for example, is very long. It is occasionally hampered by premature abortion of the developing embryo, resulting in few or no viable seeds (Rout et al., 1999). Rose is highly heterozygous. Lack of germination is due to mechanical restriction of embryo-expansion by presence of a thick, hard pericarp or due to dormancy regulated by growth inhibitors present within the achene. There are also a few reports of culture of mature embryos *in vitro*. Embryo rescue in *Rosa hybrida* and English roses is also reported from elsewhere. Gudin (1994) also demonstrated the usefulness of embryo rescue in *Rosa hybrida* L.

Lilies / Liliums

In order to introduce new traits such as disease resistances, flower shape and colour from wild species into the cultivar assortment of lily, it is necessary to overcome interspecific crossing barriers. Several techniques like the cut-style method, the grafted-style method and *in vitro* isolated ovule pollination technique, have been developed to overcome pre-fertilization barriers. Post-fertilization barriers can be circumvented by *in vitro* pollination and/or rescue methods as embryo, ovary-slice and ovule culture. Using these techniques, wide interspecific lily crosses with species and cultivars from the different sections of the genus *Lilium* (*L. longiflorum*, *L. henryi*, *L. canadense*, *L. pardalinum*, *L. concolor*, *L. dauricum*, *L. candidum*, *L. rubellum*, *L. martagon*, Asiatic and Oriental hybrids) could be made (Van Tuyl *et al*, 2000; Van Tuyl *et al*, 2002) and breakthroughs achieved.

Interspecific hybrids have been developed in liliums using early-stage ovule culture in vitro (Wang *et al*, 2009). Yuan et al (2003) demonstrated shortening of breeding cycle in spider lilies (*Lycoris* spp.) through embryo culture.

Differential ovule development following self and cross pollination and the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae) was shown by Sage *et al*, 1999. Earlier, Hayashi *et al* (1986) demonstrated ovary-slice culture in *Lilium formosanum* Wallace and Ikeda *et al* in 2003 produced seedlings from ovules excised at a time when they contained zygote stage of the hybrid product and cultured them in *Lilium* spp.

Pelargonium

As a means of integration of *in vitro* techniques in ornamental plant breeding, Bentvelsen *et al* (1990) made interspecific crosses in *Pelargonium* by applying embryo rescue methods. Some reports of artificial hybrids are available but no evidence of natural hybridization is seen.

Alstroemeria

Interspecific hybridization in the genus *Alstroemeria* is hindered by post-fertilization barriers (Buitendijk *et a*l, 1995). Histological analysis revealed poor endosperm development from 18 days after pollination onwards, followed by malformation and abortion of embryos. To create interspecific hybrids between *Alstroemeria aurea*, *A. pelegrina*, *A. magnifica*, *A. inodora* and *A. psittacina* in diallelic combinations, an ovule culture technique was developed. Influence of age of ovules, sucrose concentration of medium and temperature and light during culture were tested. Harvesting ovules before the onset of endosperm degeneration, i.e. at 14 days after pollination, cutting them into halves and culturing the micropylar halves in a rotating liquid culture medium containing 6% sucrose at 21 °C in the dark, led to successful embryo rescue. Germinated embryos were sub-cultured in vitro until rhizomes were formed, a prerequisite for successful transfer to the greenhouse. Full grown plants all showed interspecific morphological traits

and analysis of chromosome complement confirmed their hybrid nature. Diploid hybrid plants were obtained in all the 20 interspecific 2*x*-2*x* combinations. A total of 260 interspecific hybrid plants were produced. Half-ovule culture of 2*x*-4*x* and 4*x*-2*x* crosses resulted in 43 triploid hybrid plants. Because interspecific hybrids were obtained in 100% of the interspecific combinations, it is expected that the described technique can be applied to overcome post-fertilization barriers in most crosses within the genus *Alstroemeria*.

Chunsheng and Bridgen (1996) studied the effect of genotype, culture medium and developmental stage of the embryo on *in vitro* responses of ovule cultures in the interspecific hybrids of *Alstromeria*.

Cacti

Embryo rescue and plant regeneration has been successfully demonstrated in cacti following interspecific crosses in the genus *Hylocereus* (Aroldo and Noemi, 2010). Gynogenesis has also been shown to be possible in the vine cacti *Hylocereus* and *Selenicereus* by Benega-Garcia *et al* (2009).

Other ornamental crops

In primulas, Kato *et al* (2001) obtained different genomic combinations in inter-section hybrids from crosses in primulas through embryo rescue. Two types of triploids with different genome combinations were generated. Interspecific hybridization in rhododendron has been shown to be possible using embryo rescue in the genus *Rhododendron* (Tom Eeckhaut *et a*l, 2007).

Other attempts at embryo rescue include that in Narcissus and Zinnia (Miyajima, 2006) and *Ornithogalum* (Josephina, 1990). Kasten and Kunert (1991) devised a method for culture of isolated immature embryos of various lupins (*Lupinus* species); Custers (1995) overcame interspecific crossing barriers in tulips using embryo rescue by successful direct transfer of *Tulipa kaufmanniana* Regel germplasm into that of *T. gesneriana* L.

Tuber crops: Cassava

Cassava is one of the most-planted tuber crops in the tropical world. Importance of cassava is growing as a food security crop in sub-Saharan Africa, where malnutrition is a menace. However, a major hindrance in fast improvement of the crop is a long generational-cycle in this crop, poor germination of the seeds, and low multiplication-rate of the stem cuttings. *In vitro* germination of 495 seeds from a backcross population was done. Each genotype was multiplied for sufficient planting material, hardened in the greenhouse and transplanted to the field. Percentage germination of the seeds in embryo culture was high (66%). Raising plantlets in the greenhouse was found to be useful to select healthy plants and, thus, obtain a uniform stand in the field. The genotypes were planted in a single row trial and harvested eight months after planting. Transplanted plantlets gave 98.89% establishment. Yield-related traits were significantly high compared to results from past experiments. The high percentage of plant recovery from seed through to the field, is a means of overcoming some problems associated with traditional methods of cassava breeding through direct seed planting, to generate planting material (Akinbo *et al*, 2010).

BASIC STUDIES CARRIED OUT USING EMBRYO CULTURE:

In addition to applied uses of embryo culture, the procedure is useful in basic studies. Growing embryos outside the ovule (*ex ovulo*) is an excellent way to study nutrition and metabolism of the embryo at various stages of its development. The technique can also be used to examine growth requirements of embryos, effects of phytohormones and environmental conditions on zygotic embryogenesis, and the regeneration potential of whole embryos or their segments (Bridgen, 1994). Embryo culture can be used to localize sites of germination promoters and inhibitors, for studies on embryogenesis, and for cryopreservation. Embryo culture is useful in understanding precocious germination. Studies have shown that seed tissues play an important role in regulating development of embryos *in situ*. Cotyledon growth stops almost immediately upon excision of immature embryo, indicating probable cessation of cell division, as seen in cotton. An embryo is programmed to germinate even when it is very small.

In situ the embryo may not germinate because it lacks the germination-mRNA. But, removal of the seed from its environment activates the required machinery of embryo cells to synthesize the information necessary for germination. It has been shown in cotton that germination-specific mRNA is normally transcribed when cotyledons are about 3/5th their final size. However, to check precocious germination that may lead to vivipary (a lethal development), translation of mRNA is prevented by simultaneous appearance of abscisic acid (ABA) in the seed. Embryo excised at this stage undergoes precocious germination which suggests, that, ABA is contained in tissues surrounding the embryo rather than

in the embryo itself. This conclusion is also supported by the fact that exogenous application of ABA to excised embryos also prevents their precocious germination. Accumulation of ABA during late stages of seed development has been shown to occur in a number of plants.

Other non-conventional approaches to breeding crop plants include:

1. Induction of haploidy through androgenesis or gynogenesis and subsequent diploidization, as a short-cut to breeding
2. Generation of triploids through endosperm culture
3. *In vitro* mutation breeding
4. Development of biotic/abiotic stress-resistant plants by challenging plant tissues *in vitro* with the specific stressant
5. Somatic hybridization using protoplast isolation, culture and fusion
6. Production of transgenics using one or more of the following technologies:
   (i) Microinjecting foreign DNA
   (ii) Use of biolistics (gene gun/ helium gun)
   (iii) Vector-mediated transformation

CONCLUSION

A very important and valuable biotechnological tool for raising hybrid progeny in intractable crosses is embryo rescue. It is most often used to rescue embryos from interspecific and intergeneric crosses and from embryos that do not fully develop naturally (as in early-ripening and seedless fruit crops where the embryo aborts). Distantly related plant species in a breeding programme ('wide hybridization') may result in no/aborted hybrid embryos. Seedless parents may be unable to produce seeds in a cross. Alternately, breeding may be hindered owing to heavy fruit-drop in the early stages of fruit development. In all such cases, hybrid embryo rescue is a very powerful and useful tool and an indispensable technique. The method also can be used to rescue seedless triploid embryos, produce haploids, overcome seed dormancy, or determine seed viability. It is useful in understanding embryo morphogenesis and precocious germination. As research continues with this technique, new and valuable uses will be developed to assist biotechnological breeding of plants.

Embryo rescue / culture as a technique has found wide acceptance and utilization. Many interspecific and intergenetic hybrids have been successfully produced by culturing immature embryos that normally do not survive to maturity *in situ*. Embryo culture techniques are also used to rescue embryos from early maturing fruit varieties, to hasten maturation in some species and to overcome dormancy requirements in others. *In ovulo* embryo culture facilitates embryo development from the zygote stage to maturity when the ovule is cultured. A few interspecific and intergeneric hybrids have been made by first accomplishing fertilization *in vitro*, then culturing the ovules to maturity. Self-incompatibility in some species can be circumvented with these techniques. The potential of these may be a viable alternative to parasexual hybridization and somatic embryogenesis.

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