



## Embryo rescue and hybridity establishment in hybrid progenies derived from polyembryonic *Citrus* species

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### ABSTRACT

Due to highly polyembryonic nature of Rangpur lime and sour orange, development of hybrid is difficult through conventional breeding. Therefore, the present study was carried out at the Division of Fruits & Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi during 2017–19 to improve hybrid seedling recovery among the crosses made between these species. Germination was induced in rescued immature embryos ages (90-100, 110-120, 130-140, 150-160 and 170-180 days after pollination) on two culture media (MS medium supplemented with 500 mg/l malt extract + 1.5 mg/l GA<sub>3</sub> + 0.02 mg/l NAA (M1) and DKW medium added with 1.5 mg/l GA<sub>3</sub> + 0.02 mg/l NAA (M2). Fruits harvested at younger stage (90-120 DAP) had higher number of functional seeds. Older embryos (>150 days old) germinated earlier on either medium. Embryos rescued at early stage (90-100 DAP), middle age embryos (130-140 DAP) and oldest embryos (170-180 DAP) germinated efficiently (88.85–100%) on M2 medium. The seedling obtained from 170-180 days old embryos grown on M2 medium produced longest primary root and tallest plantlets (7.52 and 4.48 cm) at 60 days after inoculation (DAI). SSR marker analysis revealed that GT03 loci exhibited the polymorphism between male and female parents, proving their ability to detect the hybrids of Rangpur lime (*C. limonia*) × sour orange (*C. aurantium*).

**Keywords:** Embryo rescue, Germination, Hybrid, Nucellar, Polyembryonic, SSR

Rangpur lime is tolerant to citrus tristeza virus (CTV) and drought with Cl<sup>-</sup> excluding property but susceptible to cold and Phytophthora rot, yielding poor quality fruits. On the other hand, sour orange is the important rootstock of citrus worldwide due to its adaptability to the calcareous soils having high pH with Cl<sup>-</sup> exclusion property. It is also tolerant against Phytophthora root rot, citrus blight, drought and cold; resulting in higher yield of superior quality fruits. However, it is not recommended in CTV affected areas (Bar Joseph *et al.* 1989). The polyembryonic behaviour in these species is the main impediments in the conventional rootstock breeding. Accordingly, *in vitro* embryo culture may be suitable to overcome the related breeding obstacles. To a large extent, the response of citrus embryos germination under *in vitro* culture conditions depends on embryo development stage, media composition and genotype constitution (Yi *et al.* 2001). Hence, identification of appropriate embryo developmental stage for embryo rescue and its subsequent culture in a specific cross combination offers opportunities for increasing the hybrid seedling recovery.

Furthermore, the composition of the culture medium has a direct influence on embryo germination and seedling growth. Liu

*et al.* (2003) reported better ovule growth and embryo production under *in vitro* conditions using Bouquet & Davis and Nitsch & Nitsch media. Carimi *et al.* (1998) found better early cotyledonary embryos germination on MS medium added with malt extract; while, pro-embryos did better on MT medium supplemented with GA<sub>3</sub> with high levels of sucrose concentration. The SSR markers are important for rapid assessment of hybrid, more polymorphic, co-dominant and easily reproducible. Recently, Soni *et al.* (2019) have reported three SSR markers (TAA45, CAC15 and CAC39) to be polymorphic between female and male parents of sour orange and Sacaton citrumelo, showing ability to identify their hybrids. Hence, available user-friendly molecular markers need to be selected for early identification of hybrids of Rangpur lime × sour orange crosses. Therefore, the objectives of the present study were to standardize the embryo age and suitable media composition for higher embryo germination and plantlet growth and to select SSR loci to distinguish between nucellar and hybrid seedlings.

### MATERIALS AND METHODS

The hybridization was carried out in the hybridization block of the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi during 2017–19. Controlled hand pollination was carried out using Rangpur lime (*C. limonia*) as female and sour orange (*C. aurantium*) as the male parent. Hand pollination was carried out with a smooth brush loaded with pollen while

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the stigma was highly receptive (oozing out of sticking substance), followed by bagging of the pollinated flowers. Developing immature fruits of Rangpur lime × sour orange cross combinations were harvested at five intervals (90-100, 110-120, 130-140, 150-160, 170-180 days after pollination) for *in vitro* culture. The harvested fruits were immersed in sodium hypochlorite solution for 10 min and rinsed with distilled water followed by 0.1% teepol solution (15 min) for disinfection, subsequently treated with 0.1% solution of Bavistin and Indofil along with 0.02% 8-HQC for 3 h. Fruits were then rinsed thrice with distilled water and shifted to laminar flow hood. Surface decontamination was done for 10 min using HgCl<sub>2</sub> (0.1%) followed by 3-4 rinsing with sterile double distilled water. The treated fruits were cut and seeds were counted in every fruit before embryo extraction. The zygotic embryos were excised from micropylar end after eliminating the seed coat. Isolated zygotic embryos were *in vitro* cultured on MS media (Murashige and Skoog 1962) added with malt extract (500 mg/l) + GA<sub>3</sub> (1.5 mg/l) + 0.02 mg/l NAA (M1 medium) and DKW medium (Driver and Kuniyuki 1984) added with GA<sub>3</sub> (1.5 mg/l) + 0.02 mg/l NAA (M2 medium). Additionally, 30 g/l sucrose was added in each medium. Fruits were cut using a sharp blade and thereafter total seeds in fruit were counted. Observation on cultured embryos was recorded daily starting from the following day of embryo culture. The data on growth in respect to plantlet and root length and the leaves were recorded on 12 randomly selected plantlets per treatment at 30<sup>th</sup> and 60<sup>th</sup> day of inoculation (DOI). Isolated, whole genomic DNA from fresh leaves of randomly selected *in vitro* raised hybrid plantlets as well as both the parents using Cetyltrimethylammonium bromide (CTAB, Ameresco) method (Doyle and Doyle 1987). The method suggested by Shareefa *et al.* (2009) was employed for purification and quantification of DNA. Randomly selected 37 SSR loci from the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3810390>) were utilized to test the polymorphism between the parents. These primers were synthesized by Microgen. PCR amplification was done using 50 ng of genomic DNA, 0.5 µl Taq DNA polymerase, 5.0 µl of 1× PCR master mix (Bioscience) and 0.4 µl of each of primers with a final volume of 10 µl in a 48-well PCR System C1000 Touch TM Thermal cycler (Bio Rad, USA).

Primers those exhibited polymorphic and reproducible banding patterns for Rangpur lime and sour orange cross combination were picked for final analysis. All the reactions were repeated twice with both maternal and paternal parents. Subsequently, female and male parents were analysed along with progenies obtained from the culture of the embryo of different ages to confirm the reproducibility. One embryo per test tube was cultured, and 48 tubes were utilized in each main treatment (embryo age). For embryo germination, plantlets survival and growth observations, treatments were arranged in a factorial experiment in complete randomized design with three replications. For all observations, each replication consisted of four embryos plantlets. Data were subjected for statistical analysis using SAS software version

SAS 9.3 (SAS Institute, CARY, NC, USA). P value of ≤0.05 was considered significant.

## RESULTS AND DISCUSSION

The numbers of seeds/fruit did not follow any systematic pattern and seeds/fruit varied from 9.75 (fruits harvested at 150-160 DAP) to 14.75 (fruits harvested at 130-140 DAP). Contrary to this, the number of seeds containing functional embryos decreased with increasing stage of fruit harvesting and lowest number of seeds with functional embryos (83.33%) was found in the fruits harvested at 170-180 DAP; however, vice versa trend was noticed for seeds contained non-functional embryos .

Embryo age and culture media in general influenced days required for initiation of germination and completion of the end of the germination in Rangpur lime × sour orange crosses (Table 1). Days needed to start embryo germination reduced with increased days of fruit harvesting. Embryo germination began earliest (2 days) while embryos excised from the fruit harvested at 150-160 DAP and 170-180 DAP. Embryos extracted from the fruits harvested at earliest took

Table 1 Effect of embryo age and media on days taken for embryo germination, embryo germination and survival of Rangpur lime × sour orange crosses

Treatment	Days required for initiation of germination	Days taken for complete germination	Germination (%)	Plantlets survival 30 DAI** (%)
<i>Embryo age (90-100 DAP*)</i>				
M1 medium	4	11	85.05bcd	75.00ab
M2 medium	4	11	100.00a	72.77ab
<i>Embryo age (110-120 DAP)</i>				
M1 medium	4	9	72.07de	78.61a
M2 medium	3	8	72.53de	72.36ab
<i>Embryo age (130-140 DAP)</i>				
M1 medium	3	9	79.29cde	73.88ab
M2 medium	3	9	88.85abc	69.30b
<i>Embryo age (150-160 DAP)</i>				
M1 medium	2	5	76.19cde	57.27c
M2 medium	2	6	85.86bcd	76.50ab
<i>Embryo age (170-180 DAP)</i>				
M1 medium	2	6	65.66e	69.99b
M2 medium	2	6	95.05ab	78.71a
LSD (P≤0.05)				
DAP			5.85	3.55
Media			3.70	3.24
DAP × media			14.13	8.57

Each data represents the mean value of three samples. Values are representing different letters are significant at P≤0.05 (THST). \*days after pollination; \*\*days after inoculation

the longest duration to start germination (4 days). Culture media failed to influence the days required to begins embryo germination. Almost pattern similar to days required to start germination was observed in case of days needed to complete germination. Embryos obtained from earliest harvesting of fruits took the longest duration to complete the germination process (11 days) on either medium, followed by the embryos of fruits harvested at 110-120 DAP and 130-140 DAP (8-9 days) on either medium. Embryos obtained either from fruit harvested at 150-160 DAP or 170-180 DAP, took least days (5-6 days) to complete the germination on either medium. Combined results of embryo age and media indicated 100% embryo germination of youngest embryos (90-100 DAP) was cultured on M2 medium followed by oldest embryos while cultured on alike medium (95.05%). Lowest embryo germination was recorded in the oldest embryos (170-180 DAP) when inoculated on M1 medium.

Plantlets survival at 30 days after inoculation indicated a significant effect of embryo age and media (Table 1). The effect of media and embryo age showed highest plantlet survival in seedlings obtained from the culture of oldest embryos (170-180 DAP) inoculated on M2 medium which showed statistical parity with the progenies of younger embryos obtained from the fruits harvested at 110-120 DAP and cultured on M1 medium. The plantlets survival was found to be higher in progenies of embryos obtained from fruits harvested at 150-160 DAP, while cultured on M1 medium. To date, many studies have established the effect of the embryo age on success of *in vitro* culture including citrus (Spiegel-Roy and Kochba 1980) and grapevine (Ji *et al.* 2013). In our study, fruits harvested either at 80-90 DAP or 110-120 DAP had a higher number of fully developed seeds with functional embryos and no reduction in the number of seeds with function embryos were noticed from 130-140 DAP to 170-180 DAP. The similar type of impact of fruit harvesting phase on growth and number of embryos has been reported in grapevine (Liu *et al.* 2003). It was argued that the success of *in vitro* culture in crops is mainly dependent on the developmental stage of embryos/ovules; hence, sampling embryos at a relatively later stage can boost the success rate in citrus (Turgutoglu *et al.* 2015) and grapevine (Liu *et al.* 2003). As embryos of different developmental stages vary in their food storage and physiological conditions, thus affecting the germination success, as also has been observed in the present study. Effect of culture media on embryo germination is very well documented in different crops such as Gamborg B-5 medium for sour orange × Sacaton citrumelo hybrid (Soni *et al.* 2019), Mandarin × trifoliolate hybrid (Tan *et al.* 2007). On the other hand, Liu *et al.* (2003) reported higher embryo recovery rates in either BD or NN media. Both media and embryo age jointly contributed significantly for the formation of longest primary root in progenies of oldest embryos (170-180 DAP) cultured on M2 medium at both stage of growth followed by progenies obtained from 150-160 days old embryos on M1 medium at both growth stages, though it was non-significant with plantlets

of 90-100 DAP embryos while cultured on M2 medium.

Further highest enhancement in root length was observed at 60 DAI over 30 DAI in seedlings of 110-120 days old embryos cultured on M1 medium (Table 2). Differential response of culture media and phytohormone have also been reported in the root and shoot growth of *in vitro* raised progenies of sour orange × Sacaton citrumelo (Soni *et al.* 2019) and grape (Liu *et al.* 2003). We did not observe the difference in the number of leaves at either stage due to media and embryo age in most of the cases, except progenies obtained from the oldest embryos, where M2 medium proved effective. Furthermore, the shared effect of embryo age and media presented highest plantlet height at 30 DAI (3.82 cm) and 60 DAI (4.48 cm) in seedlings of oldest embryos cultured on M2 medium having similarity statistically with seedlings obtained from the culture of 130-140 days old embryos on either medium. However, a record increase in plantlets height at 60 DAI over 30 DAI was found when 110-120 days old embryos were inoculated on M2 medium (115.31%) without any significant differences which tended to show statistical similarity with those obtained from the culture of oldest embryos (170-180

Table 2 Effect of embryo age and media on growth of plantlets obtained from Rangpur lime × sour orange crosses at 30 and 60 days after inoculation (DAI)

Treatment	Root length (cm)		Shoot length (cm)		Leaves/plantlets	
	30 DAI	60 DAI	30 DAI	60 DAI	30 DAI	60 DAI
<i>Embryo age (90-100 DAP)</i>						
M1	3.08e	3.56e	2.10c	2.66c	1.60c	2.2d
M2	4.64c	6.38cb	2.02cd	2.72c	1.20c	2.6cd
<i>Embryo age (110-120 DAP)</i>						
M1	1.16g	2.78f	1.30f	2.34c	4.20a	4.4a
M2	1.68f	3.72e	1.76cde	3.64b	3.40ab	4.4a
<i>Embryo age (130-140 DAP)</i>						
M1	5.92b	6.8b	3.40b	4.52a	2.20bc	4ab
M2	4.52c	5.92c	3.32b	5.08a	2.00bc	3.4abc
<i>Embryo age (150-160 DAP)</i>						
M1	1.94f	3.24ef	1.40ef	2.74c	1.20c	3.2bcd
M2	2.66e	3.5e	1.40ef	2.28c	1.40c	2.8cd
<i>Embryo age (170-180 DAP)</i>						
M1	3.68d	4.56d	1.70de	3.52b	1.80c	2.6cd
M2	6.64a	7.52a	3.82a	4.48a	4.00a	4.2ab
LSD (P≤0.05)						
DAP	0.19	0.24	0.16	0.26	0.67	0.50
Media	0.12	0.15	0.10	0.17	0.42	0.31
DAP × Media	0.46	0.58	0.38	0.63	1.58	1.18

Each data represents the mean value of 12 samples. Values represented different letters are significant at P ≤ 0.05 (THST).

\*Days after inoculation

DAP) on M1 medium. The combined effect of embryo age and media exhibited highest leaves per plant in plantlets obtained from the culture of 110-120 days old embryos on either medium at both 30 and 60 DAI, which was found statistically similar to the progenies of oldest embryos while cultured on M2 medium at both growth stages (Table 2).

For verifying the hybrid origin of progenies in the studied population, 37 SSR loci were assessed to test the polymorphism between female (Rangpur lime) and male (sour orange) parents. Out of these, one SSR loci (GT03) tended to show the polymorphism between these parents due to their different allelic patterns. The allele size 1000 bp and 950 bp were found to be specific for parent P1 (female) and P2 (male), respectively. To further examine the validity of this primer, hybrids were screened beside their parents, having both the alleles (1000 bp and 950 bp), indicating their hybrid origin. It is also confirmed by this polymorphic marker that all plantlets randomly selected from a lot of plantlets obtained from the culture of different aged embryos, were of hybrid origin, and no nucellar seedling was found. Moreover, the resolution power of the ultra-pure agarose gel was enough for both the primers tested. During the present study, SSR markers proved to be able to identify zygotic seedlings grown *in vitro* from Rangpur lime (*Citrus limonia*) × sour orange (*C. sinensis*) progenies. Nevertheless, the allelic profile of GT03 primer explained in this study have already been documented, but our study is first to use this for confirming the hybrid origin of seedlings between Rangpur lime × sour orange progenies. Both Rangpur lime × sour orange are important rootstock genotypes, and the SSR marker identified for conforming the zygotic progenies of these two parents, allowed the early distinction of hybrid population, thus helping in generating considerable hybrids, and saving the manpower, time and resources. It had been confirmed that 36 standard SSR markers were unable to distinguish between Rangpur lime (*Citrus limonia*) × sour orange (*C. sinensis*) and their hybrids.

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