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Journal of Horticultural Research 2020, vol. 28(2): 43–52
DOI: 10.2478/johr-2020-0024

MAXIMIZING HYBRID SEEDLINGS RECOVERY AND EARLY IDENTIFICATION OF HIGHLY POLYEMBRYONIC ACID LIME (*CITRUS AURANTIFOLIA* SWING.) × LEMON (*CITRUS LIMON* BURM.) HYBRIDS USING SSR MARKERS

Anil Kumar DUBEY*, Ankit GUPTA, Radha Mohan SHARMA, Nimisha SHARMA
ICAR-Indian Agricultural Research Institute, India

Received: June 2020; Accepted: November 2020

ABSTRACT

Nucellar embryony is the major obstacle in getting hybrid seedlings in interspecific crosses in citrus. Hence, the present study was conducted to standardize embryo age, culture media for maximizing germination, and subsequent identification of simple sequence repeat (SSR) markers to differentiate the hybrids. A factorial experiment was conducted with three embryo ages – 80–90, 110–120, and 130–140 days after pollination (DAP). The germination of the rescued immature embryos of acid lime × lemon crosses was initiated on three different growing media. The fruits harvested at 130–140 and 80–90 DAP had a higher number of functional seeds (90%). Older embryos (130–140 DAP) and middle-aged embryos (110–120 DAP) germinated in 100% on Murashige and Skoog (MS) medium supplemented with 500 $mg \cdot l^{-1}$ malt extract, 1.5 $mg \cdot l^{-1}$ gibberellic acid (GA_3), and 0.02 $mg \cdot l^{-1}$ naphthalene acetic acid (NAA). MS medium supplemented with 500 $mg \cdot l^{-1}$ malt extract proved better for the germination of embryos taken at 80–90 DAP. Plantlet survival was the highest in younger embryos (80–90 DAP) cultured on MS basal medium (84.21%) and the lowest in older embryos cultured on MS medium supplemented with 500 $mg \cdot l^{-1}$ malt extract plus 1.5 $mg \cdot l^{-1}$ GA_3 and 0.02 $mg \cdot l^{-1}$ NAA. The seedlings obtained from the culture 80–90 DAP had the highest root length (4.9 mm) and shoot length (5.3 mm) at 60 days after inoculation (DAI) on the above medium. SSR marker analysis revealed that CCSM-4 and CAC-33 markers expressed polymorphism between female and male parents, proving their ability to identify the hybrids of 'Kagzi' acid lime × 'Konkan Seedless' lemon.

Key words: embryo rescue, germination, nucellar, hybrid, polyembryonic, SSR

INTRODUCTION

Acid lime (*Citrus aurantifolia* Swing.) is one of the most important citrus fruits grown commercially in India (Dubey et al. 2016), but its susceptibility to citrus bacterial canker disease (CBCD) caused by *Xanthomonas axonopodis* pv. *citri* in the area of high rainfall and warm temperatures makes the cultivation of this crop unprofitable. On the other hand, lemon (*Citrus limon* (L.) Burm.), another acid citrus group species, is highly tolerant to CBCD, but produces fruit with the thicker rind and low acidity. The hybrids of these two species can be a viable option to produce canker-tolerant acid cultivars. Both these species produce seeds with more than one embryo derived from maternal somatic tissues. Development of these apomictic seedlings because of their polyembryonic nature is the main obstacle in the improvement of acid citrus fruit with conventional breeding approaches, as zygotic embryos fail to survive due to strong competition with apomictic embryos for nutrition and space (Soost & Roose 1996). The size and survival of sexual embryos have an inverse relationship with the number of embryos per seed (Soares Filho et al. 1992).

*Corresponding author:
e-mail: akd67@rediffmail.com

Production of nucellar embryos decreases the range of genetic variability, which further limits the extent of finding new genotypes through hybridization between polyembryonic citrus species. Accordingly, *in vitro* embryo culture has been proved to be a useful tool in citrus breeding, as it assures germination of intended embryos and their further development as hybrid seedlings (Ohta & Furusato 1957).

Embryo rescue is a very useful tool that is most commonly used to overcome the problem of embryo abortion in citrus sexual crosses, and helps to achieve the triploid seedlings through interploidy crosses (Jaskani et al. 2005; Viloria et al. 2005). However, in conventional breeding, few reports are available on the recovery of the zygotic embryos derived from the citrus species through the embryo rescue technique (Bastianel et al. 1998). To a large extent, the response of citrus embryos to tissue culture is determined by the stage of embryo rescue, the media composition, and the tested plant genotype (Yi et al. 2001).

Most often, the rescued citrus embryos are cultured on media developed by Murashige and Skoog in 1962 (MS) or Murashige and Tucker in 1969 (MT) with desired modifications to induce embryo germination with success. High germination rates of embryos obtained from monoembryonic seed progenitors have been induced on MS medium (Starrantino 1992) or MS supplemented with malt extract and adenine sulfate (Starrantino & Reforgiato-Recupero 1981). However, mandarin embryos do not germinate well on an identical basal medium supplemented with GA_3 (Khan et al. 1996). Increasing the sucrose concentration have shown to enhance the germination rates of mandarin embryos (Sykes & Lewis 1996).

A study of embryo developmental stage and medium composition, using polyembryonic diploid citrus species, revealed that pro-embryos required GA_3 and a high sucrose concentration in MT medium, while early cotyledonary embryos germinated well on MS with malt extract (Carimi et al. 1998). The present study was aimed to optimize the embryo age and media composition for high recovery of zygotic seedlings in the crosses of lime and lemon cultivars. The final purpose of this study was to enhance the hybrid seedling recovery from lime × lemon crosses, establish an efficient *in vitro* culture system for the germination and normal seedling growth of different crosses, and to identify the simple sequence repeat (SSR) markers for hybrid identification of this specific cross.

MATERIALS AND METHODS

Parents and hybridization

Controlled hand pollination was carried out using 'Kagzi' acid lime as the female and 'Konkan Seedless' lemon as the canker-tolerant male parent. Flower buds of the parents were enclosed with perforated butter paper bags 1 day before hybridization. Male flowers were collected in Petri plates on the day of hybridization in the morning, and after removing petals, they were kept for dehiscence in partial shade. Meanwhile, covers were removed from female flowers, and after discarding of opened flowers, the remaining flowers were emasculated and again covered with perforated bags. Pollination was carried out after anther dehiscence, and flower twig was rebagged and properly labeled. The coverings were removed 7 days after pollination (DAP), and the fruit set was recorded at 30 DAP.

In vivo seed development

Fruit setting was recorded at 30 DAP by counting the number of flowers cross-pollinated and flowers converted into fruits. After fruit cutting by a sharp blade and twisting, seeds were counted per fruit. Presumably functional embryos were sorted out by visual observation based on the shape of the embryos.

Collection of fruit and embryo excision

For embryo culture, developing immature fruits of acid lime × lemon cross-combination were harvested at three different terms (80–90, 110–120 and 130–140 DAP) for immature zygotic embryo isolation. The fruits harvested at different stages were disinfected with sodium hypochlorite solution for 10 min and washed with sterile water followed by 0.1% Teepol solution (15 min), and thereafter treated with a solution containing 0.1% each of Bavistin (carbendazim 50% WP) and Indofil M-45 (mancozeb 75% WP) along with 0.02% 8-hydroxyquinoline (8-HQC) for 3 h. Fruits were then rinsed with distilled water thrice and disinfected for the second time with $HgCl_2$ (0.1%) for 10 min,

followed by three to four washings with sterile double-distilled water. The treated fruits were then cut longitudinally using sterile scalpels into two parts after a sharp twist without damaging the embryo. Thereafter, seeds were counted in every fruit before embryo extraction. The zygotic embryos (single embryo at the micropylar end) were excised after removing the seed coat. In addition, 30 seeds obtained from mature fruits (180–190 DAP) of each cross-combination were sown *in vivo* in multipots to confirm the hybridity.

Induction of embryo germination

Isolated zygotic embryos were cultivated on MS media: pure MS (M1), MS supplemented with 500 mg·l^{-1} malt extract (M2), and MS supplemented with 500 mg·l^{-1} malt extract plus 1.5 mg·l^{-1} GA_3 and 0.02 mg·l^{-1} NAA (M3). Additionally, 30 g sucrose per liter was added in each medium. The pH of media was adjusted to 5.7 before autoclaving at 121 °C for 21 min. All cultures were incubated at 24 ± 2 °C with 16-h photoperiod (fluorescent light and 2500–3000 lx). Observations of germination of zygotic embryos, survival, and subsequent seedling growth were recorded.

***In vitro* embryo germination and development**

Cultured embryos were carefully observed every day starting from the next day after inoculation on the medium (DAI). The date on which embryo germination appeared was recorded for counting the days required for initiation of germination and for complete embryo germination.

Growth data in terms of root length, plantlet height, and the number of leaves were measured on 10 randomly selected plantlets in each treatment at 30th and 60th DAI. Plantlet height and length of primary root were measured from the joint of root and shoot to their apexes.

Isolation of genomic DNA

Total genomic DNA was extracted from the fresh leaves of both the parents, along with *in vitro* and *in vivo* raised seedlings by cetyltrimethylammonium bromide (CTAB, Ameresco) method (Doyle & Doyle 1987). Purification and quantification of DNA were done according to the method suggested by Shareefa et al. (2009). The quality of extracted DNA was estimated by comparison with the control ʎ phage DNA by visual assessment. Purified and quantified DNA was adjusted to a final concentration of 30 ng·µl^{-1}. Diluted samples were stored at 4 °C for immediate use, while the original concentrates were kept for long-term storage at −20 °C.

SSR analysis

A set of 37 SSR primers (Soni et al. 2019; Tan et al. 2007; Ruiz et al. 2000) with lengths of 20 or more bp were selected based on the polymorphism information content (PIC) observed in previous studies. Further, these markers were tested for optimization of the annealing temperature using genomic DNA of parents. The PCRs were performed in a 48-well PCR System (C1000 Touch Thermal cycler; Bio-Rad, USA). PCR amplification was done with 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 the primers. The volume was made up to 10 µl with sterile distilled water. The PCR cycling profile was 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in 4% MetaPhor agarose gels (Lonza, Rockland ME, USA) containing 0.1 µg·ml^{-1} ethidium bromide in 1 × TBE buffer at 120 V for 4 h. After electrophoresis, the PCR products were photographed using a gel documentation system Gel Doc (C200; Azure Biosystem, USA). The SSR profiles were scored manually; each allele was scored as present (1) or absent (0) for each of the SSR locus. The SSR markers giving consistent expected size products only were used for further analysis of variation.

Hybridity analysis of *in vitro* raised plantlets

Primers that generated polymorphic and reproducible band patterns for a specific cross-combination were selected for the final analysis. All the generated patterns were repeated twice with male and female parents. To confirm the reproducibility of the selected SSR markers, we analyzed female and male parents along with progenies obtained at different embryo ages through embryo rescue and *in vivo* raised seedlings of the same cross-combination to confirm reproducibility. To ascertain the hybridity of these *in vitro and in vivo* raised plantlets, banding pattern of each sapling was noticed and compared to their respective female and male parents.

Plantlets having two bands or a band similar to the male parent were regarded as hybrids, while plantlets having only one band similar to the maternal one were considered as nucellar seedlings.

Statistical analysis

The experiment was set up as a two-factor in a randomized complete design with five replicates. The first factor was embryo age (embryos of three age ranges), while the second factor was culture medium (three media). There were 77, 78, and 46 immature embryos cultured in each embryo age group, respectively. The analysis was performed using SAS software version SAS 9.3 (SAS Institute, Cary, NC, USA). All treatments were compared with each other at a confidence level of 0.05. In the tables, different letters have been assigned to the treatments, which present a significant difference at a $p \leq 0.05$ according to the Tukey's honest significant test.

RESULTS AND DISCUSSION

Fruit set, functional embryos, and embryo germination

In this study, 'Kagzi' lime (acid lime) × 'Konkan Seedless' (lemon) were crossed and a total of 78 flowers were cross-pollinated; from them, 12 fruits were harvested in all three embryo age groups. The number of seeds per fruit differed with the harvesting days (Table 1 & Fig. 1A–C). The highest seed number was present in fruits harvested in days 110–120 (32 seeds per fruit), while in those harvested at 80–90 DAP – 22 seeds per fruit and in those harvested at 130–140 DAP – 27 seeds per fruit. Furthermore, a total of 88, 96, and 53 seeds were obtained in fruits harvested in days 80–90, 110–120, and 130–140, respectively. Number of seeds with functional (germinating) embryos varied according to the fruit harvesting time (embryo age), and the highest (90%) number of seeds with functional embryos was found in the seeds obtained from fruits harvested in the days 130–140, while the lowest number (79%) was recorded in the seeds obtained from those harvested in the days 110–120. The effect of fruit harvesting stage on the growth and number of embryos in citrus has been reported earlier (Spiegel-Roy & Kochba 1980), and a single embryo (zygotic embryo) was noticed in the central position at the micropylar apex of the embryo sac at a more advanced developmental stage (Carimi et al. 1998).

Embryo age and culture media influenced embryo germination in lime × lemon crosses (Table 2). The highest embryo germination (on average 87%) was recorded among the embryos rescued at 130–140 DAP, while among those rescued after a shorter time, it was 84% and 81%. Considering the effect of media alone, the highest embryo germination (94%) was noticed on M3, the richest medium, whereas 71% on M1 and 88% on M2. It was also noticed that the oldest embryos took a minimum of 2 days to start and 7 days to complete germination, compared with 3 days to start and 8 days to complete for the youngest embryos. The type of medium did not influence the start of germination and its effect was not clear on germination. Without considering the effect of media, plantlet survival for 60 DAI was the highest (76%) among youngest embryos compared to 63% among the oldest embryos taken for culture (Table 2). The youngest embryos survived to a higher percentage on M1, the poorest medium.

Table 1. Effect of embryo age on number of seeds per fruit and seeds with and without embryos in 'Kagzi' (acid lime) × 'Konkan Seedless' (lemon) crosses

Embryo age (DAP)*	No. of seeds per fruit	No. of seeds obtained	No. of seeds containing functional embryos	Seeds with functional embryos (%)
80-90	22	88	77	88
110-120	32	96	76	79
130-140	27	53	46	90

*DAP – days after pollination

Table 2. Effect of embryo age and media composition on embryo germination, days taken for germination and survival of ‘Kagzi’ (acid lime) × ‘Konkan Seedless’ (lemon) seedlings

Medium composition	No. of embryo cultured	No. of germinated embryos	Days to initiate germination	Days to complete germination	Germination (%)	Seedling survival at 60 DAI** (%)
Embryo age (80-90 DAP)*						
M1	25	19	3	8	76 cd	84 a
M2	25	24	3	8	96 a	71 cb
M3	27	22	3	8	81 cb	73 cb
Embryo age (110-120 DAP)						
M1	26	16	2	9	62 e	75 b
M2	26	21	2	11	81 cb	67 c
M3	26	26	2	7	100 a	73 cb
Embryo age (130-140 DAP)						
M1	16	12	2	7	75 d	67 c
M2	15	13	2	7	87 b	69 cb
M3	15	15	2	7	100 a	53 d

*DAP – days after pollination, **DAI – days after inoculation

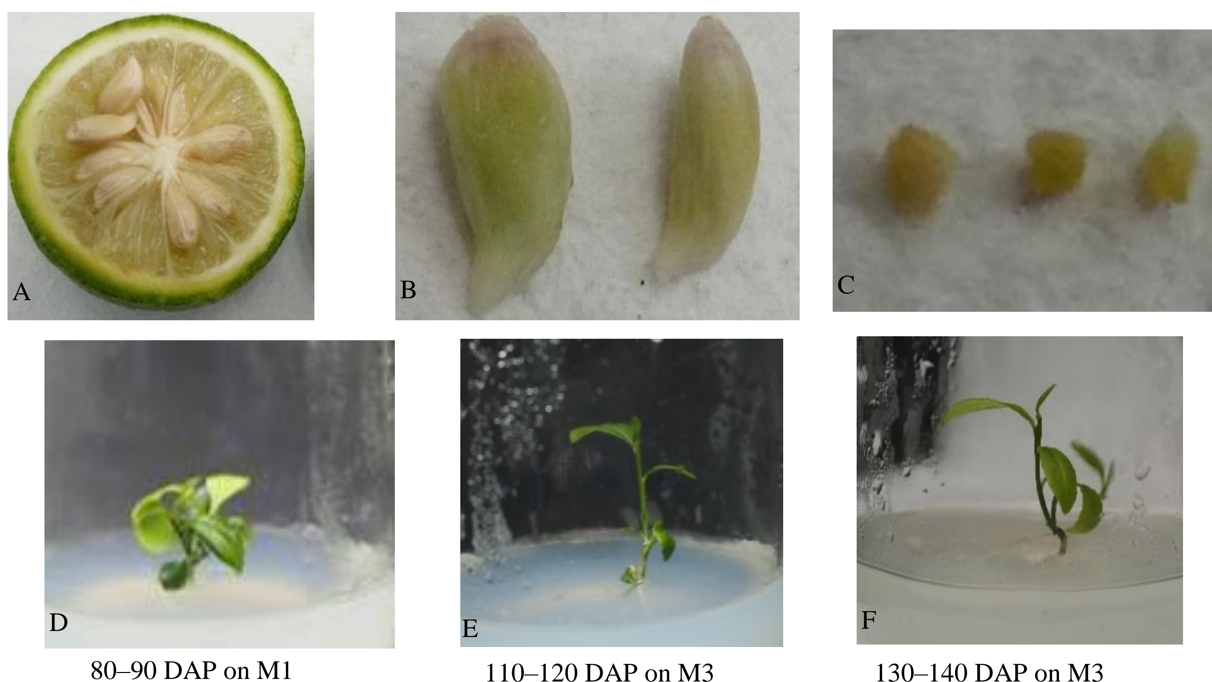

Figure 1. Fruit obtained from cross of ‘Kagzi’ acid lime × ‘Konkan Seedless’ lemon harvested at 80–90 DAP (A), seeds (B), embryo (C), hybrid seedling of embryo cultured on M2 medium (D), hybrid seedling obtained from embryo of 110–120 DAP cultured in M3 medium (E) and hybrid seedlings obtained from embryo of 130–140 DAP cultured in M3 medium (F)

Improvement of citrus functional features by traditional plant breeding methods has constraints because of their highly heterozygous and polyembryonic nature. Nucellar embryony is a major problem in citrus breeding, as it produces seedlings identical to the mother plant. Furthermore, it is often challenging to distinguish between nucellar and zygotic seedlings at an early stage (De Lange & Vincent 1977). In the present study, culture media did not influence the time required to initiate embryo germination within the same embryo age, though embryos obtained from the fruits harvested in 110–120 DAP and 130–140 DAP took fewer days to initiate germination on either medium than youngest embryos. Furthermore, M3 medium was also found to be promising to hasten the germination process in embryos obtained from fruits harvested in the days 110–120 and M2 medium for younger embryos (80–90 DAP). Earlier, Wang et al. (1999) reported *in vitro* embryo germinations after 50 days in different cross-combinations of citrus, while Tan et al. (2007) found embryo germination after 80 days in mandarin × trifoliate. Furthermore, variation in *in vitro* embryo germination due to harvesting stage was also reported in pomelo and tangerine (Deng et al. 1996), 'Femminello' (lemon cybrids) × 'Femminello' lemon hybrids (Scarano et al. 2003), the hybrid of Cleopatra mandarin × Carrizo citrange and Cleopatra mandarin × flying dragon (Turgutoglu et al. 2015) and sweet orange (Das et al. 2000; Kurt & Ulger 2014). *In vitro* embryo germination influenced by the composition of medium and embryo age has also been previously reported in oil palm (Pádua et al. 2014), sweet orange, and mandarin (Chagas et al. 2005) and mandarin × trifoliate orange hybrid (Tan et al. 2007). Turgutoglu et al. (2015) reported higher embryo germination on 2 $mg \cdot l^{-1}$ GA_3-containing medium. We observed variable results for plantlet survival in different embryo age groups. The M1 medium caused higher survival of plantlets in case of younger embryos, while both M1 and M2 media appeared to be promising for the survival of plantlets obtained from culturing of the oldest embryos.

Growth of plantlets

Embryo age and media composition to some extent the length of primary root, plantlet height, and leaf number at both 30 and 60 DAI (Table 3). Regardless of media, embryos harvested at 110–120 DAP produced the longest roots at 30 DAI. The increase in root length between 30 and 60 DAI was highest (100%) among seedlings of the youngest embryos (80–90 DAP) compared to seedlings from older embryos (57%). Considering the effect of media alone, it can be suggested that M3 medium promoted significantly higher length of primary root at both 30 and 60 DAI among seedlings of youngest (80–90 DAP) and oldest embryos (130–140 DAP).

Results revealed that embryo age and media, and their combined effect influenced seedling height significantly, but differences were difficult to explain (Table 3). One general result is that seedlings from youngest embryos (80–90 DAP) elongated more between 30 and 60 DAI (81% growth increase) than those from older embryos (70% and 51%) (Table 3 & Fig. 1D–F).

The number of leaves per plantlet was not affected by embryo age or media in a single time of observation (30 and 60 DAI; Table 3). Significant differences were found in increase in leaf number. Independent of media, the increase in the group of youngest embryo culture was 121%, whereas among embryos isolated at time 110–120 DAP, it was 93%. In the oldest embryos decrease in leaf number was noticed. The highest leaf number had seedlings obtained from the youngest embryos on media M1 and M2, while for older embryos, most leaves were formed on the medium M1, without additions. At large, more positive water potential is preferred as the embryos mature, and their nutritional requirements become less severe relative to those of the young embryos (Yeung et al. 2001). Earlier, it was reported that older embryos grown on medium containing 13% sucrose as an osmoticum tended to grow larger but had a lower percentage of conversion into plants relative to their younger counterparts (Yeung et al. 2001). In the present study, the media compositions used might be highly suitable for the growth of seedlings arising from younger than older embryos.

Previously, it was advocated that the sucrose concentration and medium strength impacted the growth and development of the aerial parts, as well as the plantlet root system in the culture of immature *Citrus* spp. embryos (Soni et al. 2019; Pasqual et al. 2003; Pérez-Tornero & Porras 2008).

Among the studied primers, only two SSRs: CCSM-4 (forward primer sequence TTCTCCTCATCTTCGACTCC and reverse primer sequence CCGATCTTACGACGTATCAA) and CAC-33 (forward primer sequence GGTGATGCTGCTACTGATGC and reverse primer sequence CAATTGTGAATTTGTGGATTCCG), produced markers that were polymorphic for parents (Fig. 2A–D). The bands sizes 200 and 230 bp were specific for P1 (female) and P2 (male), respectively. Then, these primers were used to confirm seedling hybridity when compared with parents' profiles. It was possible to reveal that all seedlings randomly selected from a population derived from embryos of different ages were of hybrid origin, and no nucellar seedlings were detected. Notwithstanding, all seedlings derived from embryos extracted from mature fruits (180–190 DAP) that germinated in the field showed nucellar origin (Fig. 2D).

Table 3. Effect of embryo age and media composition on plantlets growth obtained from 'Kagzi' (acid lime) × 'Konkan Seedless' (lemon) crosses at 30 and 60 DAI

Medium composition	Root length (mm)			Shoot length (mm)			Leaves per plantlet		
	30 DAI	60 DAI	Increase over 30 DAI (%)	30 DAI	60 DAI	Increase over 30 DAI (%)	30 DAI	60 DAI	Increase over 30 DAI (%)
Embryo age (80-900 DAP)									
M1	2.1 b	4.3 bc	102 a	2.8 ab	3.9 cd	37 b	2.0 ab	3.6 a	80 d
M2	2.2 b	4.3 bc	97 b	2.1 bc	4.8 ab	131 a	1.2 b	3.4 a	183 a
M3	2.5 b	4.9 a	100 ab	3.0 a	5.3 a	76 b	1.6 ab	3.2 a	100 c
Means			100			81			121
Embryo age (110-120 DAP)									
M1	2.5 b	4.7 ab	88 b	2.4 abc	4.2 bc	76 b	1.6 ab	4.0 a	150 cd
M2	2.7 ab	4.2 bc	57 c	2.5 ab	4.2 bc	687 b	2.0 ab	3.8 a	90 abc
M3	3.2 a	4.0 cd	25 d	2.2 abc	3.7 cd	66 b	2.6 a	3.6 a	38 e
Means			57			70			93
Embryo age (130-140 DAP)									
M1	1.3 c	2.5 e	914 b	1.5 c	2.6 e	73 b	2.6 a	3.6 a	38 ae
M2	2.5 b	3.5 d	40 cd	2.3 abc	3.6 cd	54 b	2.2 ab	1.6 a	-27 f
M3	2.7 ab	3.8 cd	40 cd	2.7 ab	3.4 d	27 c	2.6 a	2.0 a	-23 f
Means			57			51			-4
LSD ($p \leq 0.05$)									
Embryo age (EA)	0.21	0.21	8.03	0.31	0.24	9.50	0.41	0.88	4.11
Media (M)	0.21	0.21	8.03	0.31	0.24	9.50	0.41	0.88	4.11
EA × M	0.59	0.59	22.69	0.89	0.70	26.84	1.18	NS	11.61

*each data represents the mean value of five samples; values are representing different letters are significant at $p \leq 0.05$ (THST)

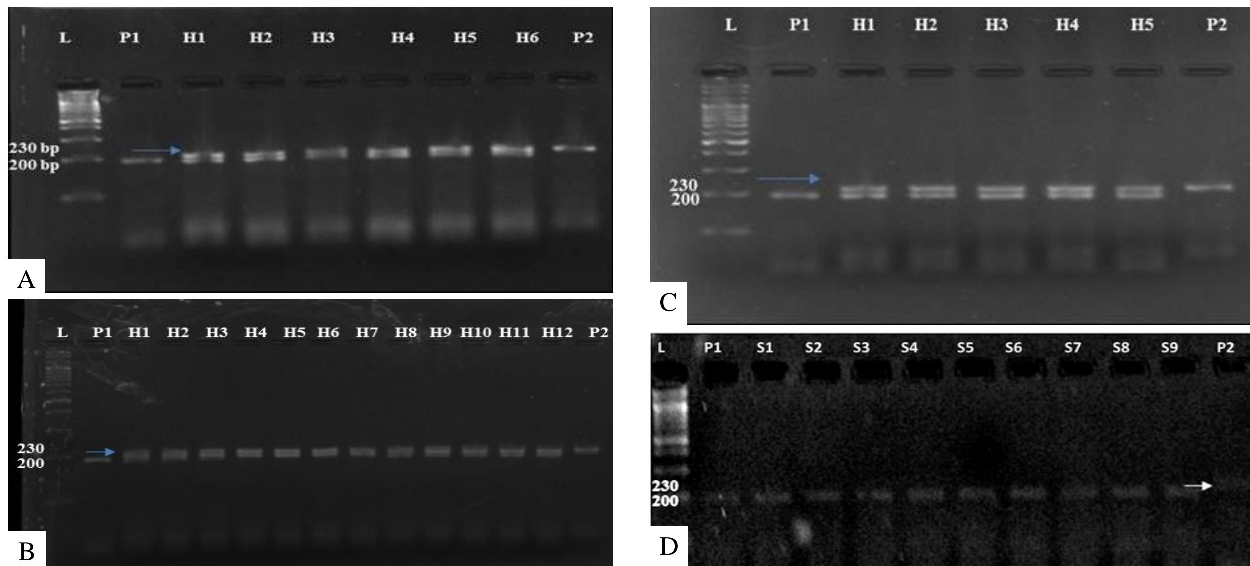

Figure 2. Agarose gel profiles of DNA amplification products obtained with SSR primer CCSM-4 of *in vitro* raised plantlets obtained from 80–90 DAP (A), 110–120 DAP (B), 130–140 DAP (C), and seedlings raised *in vivo* at maturity (180–190 DAP). In Fig. 2A to 2C, the lanes are as follows: P1, female parent; H1-H12 hybrids of 'Kagzi' acid lime × 'Konkan Seedless' lemon; P2, male parent. In Fig. 2D, S1-S9 seedlings raised in vivo from crosses of 'Kagzi' acid lime × 'Konkan Seedless' lemon, P1 female parent and P2 male parent

It can be very difficult to identify interspecific or intergeneric citrus hybrids on the basis of morphological traits due to high rate of nucellar embryony, which inhibits zygotic embryo development. On the other hand, microsatellites or SSR have been usually considered as reliable markers, since they are highly polymorphic and co-dominant (Brown et al. 1996; Liu et al. 2003), easy to use, transferable between laboratories, and not influenced by environmental conditions (Gupta et al. 1996). They are used for genetic mapping (Kijas et al. 1995), genetic diversity study (Herrero et al. 1996), and identification of hybrid zygotic citrus plants (Ruiz et al. 2000). Our study confirmed the usefulness of SSR markers for the identification of zygotic seedlings raised from 'Kagzi' lime (acid lime) × 'Konkan Seedless' (lemon) crosses, as both alleles were observed in the hybrids. Though the allelic profiles of these two SSR markers described in this study have already been published, however, our study is the first one that was conducted for confirming the hybrid origin of seedlings between 'Kagzi' lime (acid lime) and 'Konkan Seedless' (lemon) crosses. Its use for hybrid selection allows the early elimination of nucellar population, saving the labor, time, and resources. Earlier, Zhu et al. (2013) found single-nucleotide polymorphism-based allele-specific PCR (AS-PCR) to be useful for the successful confirmation and identification of citrus zygotic seedlings.

This study showed that zygotic embryos can be excised up to 140 DAP to select hybrid seedlings. The use of two SSR markers (CCSM-4 and CAC-33) was adequate to identify hybrids developed from 'Kagzi' lime (acid lime) × 'Konkan Seedless' (lemon) crosses.

Acknowledgments

Authors are thankful to the Department of Biotechnology, Ministry of Science and Technology, Government of India for providing fund for this study (Grant No. BT/PR12356/BPA/118/3/201).

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