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Ex vitro recovery of rough lemon (*Citrus jambhiri*) hybrids and identification with SSR markers

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ABSTRACT

Rough lemon (*Citrus jambhiri* Lush) is one of the leading citrus rootstock around the world besides sour orange. In the present studies, rough lemon was crossed with X 639 and Swingle citrumelo at Department of Fruit Science, PAU Ludhiana during 2015-16. Cent per cent seed germination was recorded for both the crosses under *in vitro* conditions but, the germination rates with growing media in plug trays were significantly lower (91.3 and 89.2% for RL× X and RL × SC, respectively). Higher proportion of multi-foliate seedlings (26.89%) was recovered by *in vitro* seed culture over the *ex vitro* seed germination (5.20%). All the multi-foliate seedlings were confirmed by SSR analysis using two primers (F 29 and F 87). The SSR analysis showed that only 85% multifoliate seedlings were zygotic while, the rest were not confirmed to be zygotic. The SSR analysis of the 50 unifoliate F_1 seedlings each from both the crosses with 5 SSR primers differentiated seedlings into two groups, viz. zygotic and nucellar seedlings. In RL × X cross, the most efficient SSR marker was F29, which identified 30% F_1 unifoliate seedlings to be zygotic followed by CCSME31, which identified 26% unifoliate seedlings as zygotic seedlings while the remaining seedlings were nucellar. In the cross RL × SC, the primer F29 was most efficient as it identified 28% zygotic seedlings and 72% nucellar seedlings whereas, the primer F87 identified 24 and 76% zygotic and nucellar seedlings, respectively.

Key words: Citrus breeding, Embryo rescue, Multi-foliate seedlings and rootstock.

In India, rough lemon is traditionally the most common citrus rootstock. It has deep root system, resistance against tristeza virus, suitable for high *p*H soils and results in high scion yields. However, it is susceptible to *Phytophthora* root rot, vigorous, and imparts poor fruit quality to the scion varieties. On the other hand, trifoliate rootstocks are resistant to *Phytophthora* root rot and results in better fruit quality. The trifoliate rootstocks also need improvement for adaptation to saline and calcareous soils (Medina-Urrutia 2008) which are common in the citrus growing regions of North-western India. The hybridization of rough lemon with trifoliate orange will resulted in amelioration of their limitations and development of better rootstocks. In citrus, competition between nucellar and zygotic embryos due to facultative apomixes (Kishore *et al.* 2012) leads to abortion of zygotic embryos during seed maturation (Kepiro and Roose 2010). Rescuing the embryos at an immature stage can prevent embryo abortion and results in high recovery of hybrid seedlings (Khan and Kender 2007, Tan *et al.* 2007). In citrus, the best fruit development stage for *in vitro* embryo rescue varied with the genotype (Tan *et al.* 2007, Perez-Tornero and Porras 2008, Kurt and Ulger 2014, Turgutoglu *et al.* 2015).

Early identification of hybrid seedlings is also important to eliminate unwanted nucellar embryos derived plantlets to save land and management expenditure due to large tree size and long juvenile period in citrus. Morphological and molecular markers have been developed and used for the identification of zygotic seedlings (Agarwal *et al.* 2008). SSR markers (Tan *et al.* 2007) and single nucleotide polymorphism (SNP) markers (Zhu *et al.* 2013, Caruso *et al.* 2014) have been used in citrus for the identification of zygotic seedlings in hybrids involving trifoliate orange but, they are expensive and not applicable in every laboratory (Dettori *et al.* 2015). Hence, the present studies were conducted with an objective to find an identifiable morphological trait based method for the early identification of the hybrid seedlings and its further validation using SSR markers.

MATERIALS AND METHODS

The rough lemon (RL) plants were crossed with X 639 (X; Cleopatra mandarin × trifoliate orange) and Swingle citrumelo (SC; Duncan grapefruit × trifoliate orange) plants at PAU, Ludhiana during 2015-2016. The RL fruits were harvested during September, 2015 at 190 DAP and seeds

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were extracted. One lot of seed was immersed in sodium hydroxide (1 mol/L) for 30 min to remove pectin from the seed coat. The seeds were washed 3 times with tap water and sterilized in sodium hypochlorite (2%) for 15-20 min. The embryos from each single seed were excised under 100x magnifications using a stereomicroscope (Carl Zeiss, Germany). All the embryos from a seed were cultured on a single petri dish in MS medium (HiMedia; Mumbai) supplemented with malt extract (ME; 500 mg/l), sucrose (50 g/l) and agar (7.5 g/l) to induce germination. The seedlings developed from the embryos were transferred on to the same medium in a tissue culture jar (470 ml) to induce further plant growth after four weeks. All the cultures were incubated at 25 ±2°C with 16 h of continuous fluorescent light (5000 lux) followed by 8 h of dark period.

The second lot of seed was washed with tap water and treated with 2% carbedazim (Bavistin® 50 DF, BASF) fungicide solution for 15–20 min. The treated seeds were sown in root trainer trays (300cc cell) filled with growing medium (nursery soil: FYM: cocopeat, 2:1:1) after removal of seed coat (Fig 1A, B). The seedlings with multifoliate leaves were transplanted in the black polythene bags (30 cm × 15 cm) filled with growing media and maintained under shade net house (50% shade) at 4–5-leaf stage. The survival rate of seedlings was recorded after 4 weeks of transfer into polybags.

A total of 1028 seedlings emerged from the 436 seeds (444 in RL × X and 584 in RL × SC). Up to 10 plants per seed were observed in RL × SC, up to 6 plants in RL × X seeds. All plants were visually screened for the presence of one or more multi-foliate (bi- or trifoliate) leaves for about 9 months. Twenty seven multifoliate plants were identified among RL × X and 33 in RL × SC progenies. Total DNA was isolated from young leaf using the modifed CTAB method. The DNA was amplified with SSR markers as per protocols described earlier (Singh *et al*. 2017). Five set of SSR primers (F29, F87, TAA1, CCSMEc4 and CCSME31) revealed the highest level of polymorphism out of the 55 SSR markers used for hybrid confirmation. All the multifoliate seedlings were screened with 2 SSR markers (F29 and F87) for confirmation of hybridity. About 160 unifoliate seedlings from 100 seeds from both the crosses were screened with 5 SSR markers (F29, F87, TAA1, CCSMEc4 and CCSME31) to find hybrid seedlings with unifoliate leaves.

The experiments of both *in vitro* embryo culture and *ex vitro* seed germination were carried out with three replications. Each replication had 25 seeds in embryo culture and 50 seeds in seed germination studies. The seedlings with multi-foliate leaves were treated as hybrids. The data regarding vegetative characters was recorded 80 days after culture or sowing. The data were analyzed with ANOVA (Analysis of Variation) using SAS (9.4 version) software at 5% level of significance.

RESULTS AND DISCUSSION

The seed germination (%) in the two crosses did not differ significantly under *in vitro* as well as *ex vitro* conditions. Cent per cent seed germination was recorded for both the crosses under *in vitro* conditions but, the germination rates in growing media were significantly lower

Fig 1 (A) RL × X seeds after removal of seed coat; (B) Multifoliate hybrids in root trainer trays.

(91.3 and 89.2% for RL×X and RL × SC, respectively). Higher average polyembryony (94.89%) was recorded under *in vitro* conditions in comparison to 76.11% in seeds raised in growing media (Table 1). Significantly, higher monomorphic (%) was recorded in the seeds sown in growing media (23.98%) in comparison to the 5.11% under *in vitro* conditions.

The Genotype had a significant effect on monomorphic per cent having 18.28% av. value for RL × X, which was followed by 10.81% in RL × SC. The interaction between genotype and seed sowing condition revealed the highest monoembryonic per cent (28.56%) in hybrid RL × X raised in growing media under *ex vitro* conditions followed by 19.40% in RL × SC. Significantly, lower monoembryonic % was recorded under *in vitro* conditions with 8% in RL × X, which was followed by 2.22% in RL × SC. Significantly, higher average survival rate (87%) of zygotic seedlings was recorded under *ex vitro* condition (Table 1) over *in vitro* conditions (79.67%). There was no significant effect of pollen parent on the survival % of the seedlings. The average number of seedlings per seed was significantly higher under *in vitro* conditions (3.41) over *ex vitro* seed germination (1.71) in growing medium (Table 1). The cross RL × SC had significantly higher number of seedlings per seed (2.80) over RL × X (2.33), irrespective of the seed germination conditions. There was no significant effect of growing conditions and pollen parent on the plant height of the hybrid seedlings. Furthermore, the interaction between the growing conditions and genotype was not significant for survival % and number of seedlings per seed.

Significantly higher proportion of multifoliate seedlings (26.89%) was recovered by *in vitro* over *ex vitro* conditions (5.20%) (Table 1). The pollen parent had no significant effect on the proportion of multifoliate seedlings. While studying the interaction between growing condition and genotype, higher proportion of multifoliate seedlings (31.11%) was recorded for RL × SC via *in vitro* culture followed by 22.66% multifoliate seedlings for RL × X by the same method. All the 60 multifoliate seedlings were confirmed by SSR analysis with two primers (F 29 and F 87). However, the SSR analysis showed that out of the 60 multi-foliate seedlings, only 51 were zygotic, while, rest of the 9 seedlings were not confirmed to be zygotic by the two SSR markers. The SSR analysis of the 50 unifoliate F_1 seedlings each from both the crosses with 5 SSR primers differentiated seedlings into two groups, viz. zygotic and nucellar seedlings. In RL × X, the most efficient SSR marker was F29, which identified 30% F_1unifoliate seedlings to be zygotic followed by CCSME31, which identified 26% unifoliate seedlings as zygotic seedlings while the remaining seedlings were nucellar.

In the cross RL × SC, the primer F29 was the most efficient as it identified 28% zygotic seedlings and 72% nucellar seedlings. Whereas, the primer F87 identified 24 and 76% zygotic and nucellar seedlings, respectively (Table 2).

In polyembryonic citrus genotypes, generally the nucellar embryos develop much earlier and are winners over the zygotic embryo for nutrients competition both for development and germination periods. Germination of hybrid embryos is affected by genotype and maturity stage of the fruit.

The embryo germination from mature fruits under *in vitro* conditions indicates that the embryos were viable till fruit maturity. The higher germination rates and number of seedlings per seed under *in vitro* conditions over *ex vitro* conditions may be due to separate culture of individual embryos, which prevented the completion between embryos during germination and development as suggested by Sykes (2011). Sykes (2011) also concluded that the insufficient nutrient supply during germination from the seed cotyledons

Table 1 Total number of seeds analyzed, germination rates and frequency (%) of monoembryonic and polyembryonic seeds from two crosses and number of seedlings per seed, survival per cent and plant height (cm) obtained from two culture conditions

Cross	No. of seeds cultured		Germination (%)			Polyembryonic (%)			Monoembryonic (%)		
	In vitro	In soil	*In vitro*	In soil	Mean	*In vitro*	In soil	Mean	*In vitro*	In soil	Mean
RL × X	75	150	100 ± 0.0a	91.3 ± 2.9a	95.67a	92.0 ± 5.1a	71.5 ± 2.4a	81.77a	8.0 ± 0.5a	28.6 ± 2.5a	18.28a
RL × SC	90	150	100 ± 0.0a	89.3 ± 2.9a	94.67a	97.7 ± 2.4a	80.7 ± r.1a	89.23a	2.2 ± 0.2a	19.4 ± 1.5a	10.81b
Mean			100.00a	90.33b		94.89a	76.11b		5.11b	23.98a	

	No. of seedlings per seed			*Survival (%)*			*Plant height (cm)*			*Zygotic (%) on the basis of multifoliate leaves*		
RL × X	3.10 ± 0.19a	1.55 ± 0.11a	2.33b	80.00 ± 2.07a	88.67 ± 2.15a	84.33a	22.82 ± 3.53a	21.33 ± 4.16a	22.08a	22.7 ± 1.8b	6.7 ± 0.5c	14.66a
RL × SC	3.72 ± 0.16a	1.87 ± 0.07a	2.80a	79.33 ± 2.62a	85.33 ± 2.09a	82.33a	29.55 ± 6.57a	19.89 ± 4.06a	24.72a	31.1 ± 2.3a	3.7 ± 0.3c	17.42a
Mean	3.41a	1.71b		79.67a	87.00a		25.44a	21.36a		26.89a	5.20b	

Data are means ± standard error, Mean followed by the same letter in each column are not significantly different based on the LSD tests (P=0.05).

Table 2 Level of polymorphism achieved with five SSR markers in RL × X and RL × SC crosses from unifoliate leaf seedlings

Primer	Sequence (5'-3')	G+C content %	Annealing temp (°C)	Zygotic seedlings (RL× X)	Zygotic seedlings (RL×SC)
F87	ATGAAGGCTTTTTAGAGCCGAGTT	41.67	60	8/50 seeds /75 seedlings	12/50 seeds /84 seedlings
F29	TTCACCACAAACGAAGACTCAGAC	41.67	60	15/50 seeds /75 seedlings	14/50 seeds /84 seedlings
TAA1	GAAAGGGTTACTTGACCAGGC	52.38	60	11/50 seeds /75 seedlings	10/50 seeds /84 seedlings
CCSMEc4	CTTGCTCGAGTCTACGCTCC	60.00	60	10/50 seeds /75 seedlings	12/50 seeds /84 seedlings
CCSME31	GGAATTCGAGTTGGAGGTCA	50.00	58	13/50 seeds /75 seedlings	9/50 seeds /84 seedlings

was the key factor restricting the germination of embryos. Similarly, Zhu *et al.* (2013) recovered hybrid seedlings by *in vitro* culture of mature embryos of *Citrus sunki* × *Poncirous trifoliate* hybrid seeds. However, in the present studies, 6.66 and 3.73% hybrid seedlings were recovered from the seed sown in growing media under net house from RL × X and RL × SC crosses, respectively, contrary to the results of Zhu *et al.* (2013). Higher number of seedlings per seed recovered under *in vitro* over the *ex vitro* conditions also substantiate the fact that competition between embryos might have hindered the germination of embryos under *ex vitro* condition. Pollen parent also influenced the number of seedlings with higher number of seedlings per seed in RL × SC over RL × X, irrespective to the germination conditions.

The cultivars in which the number of polyembryonic seeds is less than 7 are considered as monoembryonic (Cameron and Soost 1979). In both crosses (RL × X and RL × SC) higher polyembryony and lower monoembryony percentage was recorded under *in vitro* conditions over growing media. Genotype and germination condition had a significant effect on monomorphic % with higher monoembryonic % in RL × X over RL × SC. This may be due to reduction in completion and better nutrient supply to the individual embryos during germination as suggested above. This also confirms the fact that the zygotic embryo is usually unable to compete with nucellar ones during germination. Improper endosperm development also leads to degradation of nucellar embryos as the endosperm act as a channel for growth promotors and nutrients from the maternal tissue to the embryo (Gupta *et al.* 1996) and *in vitro* embryo culture may lead to embryo germination even under these conditions. Variation in polyembryony across the citrus species is due to the complex interrelation between genotype and environment (Kishore *et al.* 2012).

In general, the proportion of multi-foliate seedlings was very less (26.89% under *in vitro* and 5.20% under *ex vitro* conditions). The lower recovery of hybrid seedlings have been found in citrus and it depends on the seed parent, pollen origin and environmental influences (Moore and Castle, 1988). In our earlier unpublished studies involving rough lemon, higher proportions of hybrid seedlings (~57%) were recovered with early *in vitro* embryo rescue (95 days after pollination). Hence, the lower proportion of multifoliate seedlings in the present studies may be due to delayed culture of embryos as embryo rescue stage is an important factor for zygotic seedling recovery, which depends on the stage of embryo abortion or the time of nucellar embryo invasion (Tan *et al.* 2007). The higher recovery of multi-foliate seedlings by *in vitro* culture over *ex vitro* conditions may be due to prevention of completion between embryos during germination and development and proper nutrient supply as suggested by Sykes (2011) and Zhu *et al.* (2013). Under *in vitro* the higher proportion of multifoliate seedlings noted in RL × SC in comparison to RL × X. *In vitro* raised citrus seedlings lack cuticular waxes and are more prone to transpiration losses, which might have resulted in lower survival (79.67%) of the *in vitro* raised seedlings in comparison to *ex vitro* raised seedlings (87%).

The dominant 'trifoliate leaf' trait over the recessive unifoliate leaf trait makes it easy to identify the first generation hybrid seedlings in crosses between unifoliate citrus and trifoliate orange male parents (Sykes 2011). In view of the presence of unifoliate zygotic seedlings in the hybrids from the crosses between mandarins and hybrids of trifoliate orange (Swingle citrumelo) as male parent, multifoliate leaf trait cannot be considered as a reliable marker (Caruso *et al.* 2014). Further, the scarcity of morphological markers necessitates the use of other reliable techniques for identification of zygotic seedlings. DNA markers are very reliable tool to unmistakably identify the zygotic seedlings (Zhu *et al.* 2013). The multifoliate hybrid seedlings were confirmed using two SSR primers F29 and F 87. SSR analysis shows that out of the 60 multifoliate seedlings, only 51 were zygotic while, rest of the nine seedlings were not confirmed to be zygotic by the two SSR markers. The SSR analysis of unifoliate F_1 seedlings with five SSR primers differentiated seedlings into zygotic and nucellar seedlings. In both the crosses, the SSR primer F29 showed highest efficiency in identifying the unifoliate zygotic seedlings (15 and 14 hybrid seedlings per 50 seeds in RL × X and RL × SS, respectively). In RL × X cross, the primer CCSME31identified 13 hybrid unifoliate seedlings per 50 seeds and it was followed by 11 seedlings in TAA1. In RL × SC cross, 12 hybrid unifoliate seedlings per 50 seeds were identified by SSR pimer F 87 and CCSMEc4.

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