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RESEARCH PAPER

HORTICULTURAL STUDIES

Improvement of Citrus Rootstock Hybrids Derived by 2x × 2x Intra Crosses with the Aid of Embryo Rescue and Ploidy Detection

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Abstract

Diploid *Citrus* × *Poncirus* hybrids have significantly contributed to citrus rootstock evaluation. In Türkiye, common sour orange rootstock is used in many different climates and soil conditions at almost 85% of citrus plantations, but this rootstock is susceptible to Citrus tristeza virus disease. This study was conducted to improve new rootstock genotypes by traditional hybridization. Common sour orange (CSO) and Common mandarin (*Citrus deliciosa* Ten.) (CM) were crossed with Troyer citrange (TC) while King mandarin (KM) was crossed with Carrizo citrange (CC). Embryos obtained from crosses were taken on 110, 120 and 130 days after artificial pollination (DAP), and were germinated on MT culture media. The embryos of 120 DAP of combinations had the highest germination rate within CSO×TC, 95.15%; CM×TC, 96.25%; KM×CC, 95.23%. The trifoliate rates for each combination at subculture (CSO×TC, 17.40%; CM×TC, 11.11%; KM×CC; 6.17%) were obtained from 110 DAP embryos. Survival rates of the genotypes were ranged between 72.13% and 90.28% in subculture and varied from 40.17% and 64.71% in the greenhouse. As a result of the ploidy analysis by flow cytometry, the nuclear DNA content of diploid genotypes were found between 0.78 pg/2C and 0.93 pg/2C. One of the genotypes derived from CM×TC hybridization on 120 DAP was determinated as a triploid plant.

1. Introduction

The rootstocks are the vital spot for the adaptation to different environments of citrus trees. In the Mediterranean basin, citrus tristeza virus (CTV) poses a great threat due to the widespread use of sour orange (*Citrus aurantium* L.) rootstock (Pestana et al., 2005). Sour orange rootstock has no incompatibility, yield, and quality problems with many other species and varieties. However, its susceptibility to CTV and its occurrence in some growing areas in Türkiye necessitate the improvement of this disease tolerant rootstocks that can be used instead of citrus rootstock (Tuzcu, 1978).

In order to achieve a more successful breeding program biotechnological methods have great importance in addition to classical breeding methods. *Poncirus* and its hybrids are the most important genetic resources to obtain new rootstocks, especially by combining with different oranges and mandarins (Castle, 2010).

In terms of nursery production, citrus rootstocks should have a high polyembryony level and high germination rate (Broadbent and Gollnow, 1993). The citrus are vegetatively propagated with generative material, and homogeneous genetically identical plants are obtained from their parents. Therefore, citrus breeders generally use at least one polyembryonic parent to receive polyembryonic hybrids. In polyembryonic citrus, the zygotic embryo has to compete with strong embryos derived from nucellar tissues for nutrient and expansion space (Soost and Roose, 1996). Since nucellar embryos

confine the formation of hybrid seeds by suppressing the development of zygotic embryos any variability in the population cannot be achieved (Pena et al., 2007). Most citrus cultivars are polyembryonic, and when used as the female parent in crosses, usually immature zygotic embryos are suppressed by nucellar embryos. Embryo rescue technique is carried out to capture the zygotic embryo and also to obtain hybrid plants from them after crosses (Cameron and Frost, 1968). The success of embryo culture depends on the plant growth regulators, nutrients, the embryo excision technique, and often the developmental stage of the extracted embryos (Raghavan, 1980; Xie et al., 2019). Most citrus varieties are diploid. However, polyploid plants such as triploid and tetraploid can be found among diploid populations (Aleza et al., 2011). Natural tetraploidization by folding the nucellar tissue as a result of mutation is rarely seen in citrus species. There is no natural or artificial tetraploid genotype that can be used commercial or in breeding programs in Türkiye even in plantations where preserved citrus genetic resources. In the present study, we have studied 2x × 2x intra spesific hybridizations applied between citrus species to obtain new hybrid rootstock genotypes. We have obtained different genotypes at different developmental stage embryos. We analysed the performance of *in vitro* embryo rescue conditions and then the ploidy levels of obtained genotypes by flow cytometry.

2. Materials and Methods

2.1. Hybridizations

Plant materials used in hybridizations studies were located at the genetic resource collection of Bati Akdeniz Agricultural Research Institute (BATEM), in Antalya (Türkiye). A total of 1215-controlled crosses were performed in all three hybridization combinations. The controlled hybridization studies were performed according to Batchelor (1943). Hybridization combinations for breeding new citrus rootstocks are showed in Table 1.

2.2. Embryo rescue and hybrids obtaining

Immature fruits obtained from articifical pollinations in specified combinations were harvested 110, 120 and 130 days after pollination (DAP). Fruits surface sterilization were performed by keeping them in 70% ethanol for 5 minutes and then immersed in 20% sodium hypochlorite for 30 minutes. Fruits were rinsed three times in a sterile flow cabinet. The fruits were cut horizontal using bistoury without damaging the embryo and the immature seeds were extracted with forceps. Immature embryos were removed under binocular microscope by carefully cutting the micropile end of the seeds taken at different developmental stages (Figure 1).

Table 1. Parents used in 2x × 2x crosses.

Female parent	Male parent
Common sour orange (CSO) (*Citrus aurantium* L.)	Troyer citrange (TC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)
Common mandarin (CM) (*Citrus deliciosa* Ten.)	Troyer citrange (TC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)
King mandarin (KM) (*Citrus nobilis* L.)	Carrizo citrange (CC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)

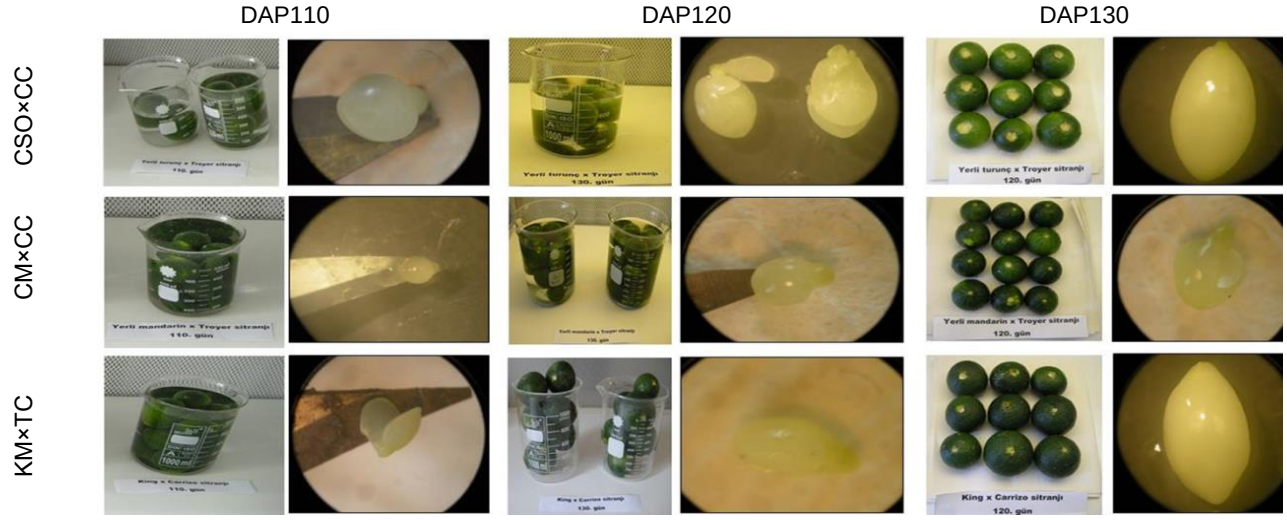

Figure 1. Harvested immature fruits at 110, 120, 130 DAP and extracted embryos under a binocular microscope (DAP: Days after artificial pollination, CSO: Common sour orange, CM: Common mandarin, TC: Troyer citrange, KM: King mandarin, CC: Carrizo citrange).

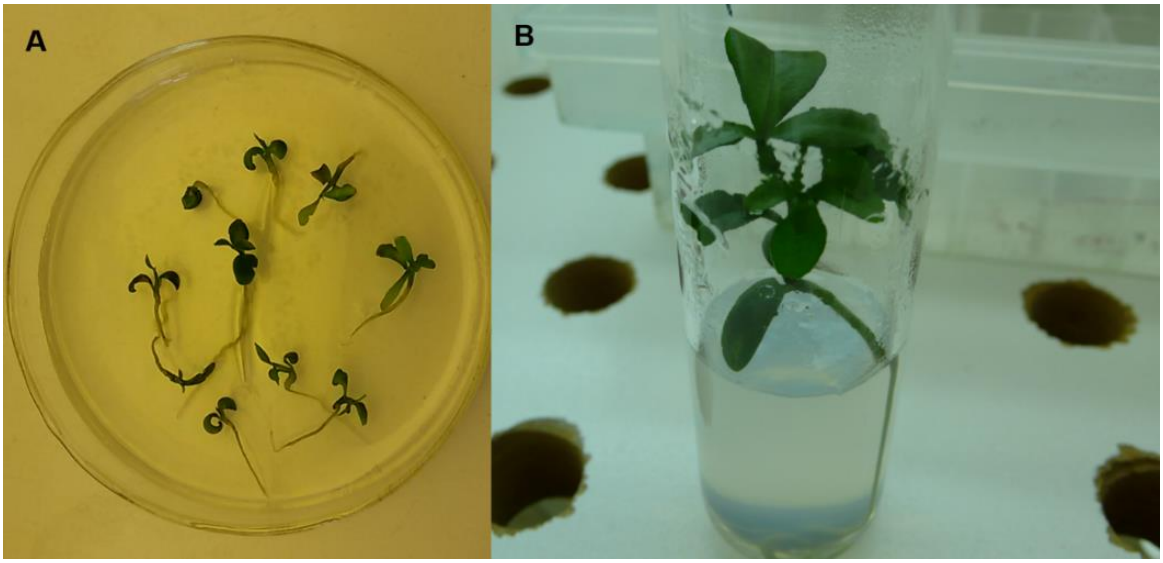

Figure 2. Germinated embryos (A) and subcultured trifoliate hybrids (B).

Modified Murashige and Tucker (1969) (MT) culture medium supplemented with 50 g l^{-1} sucrose, 25 mg l^{-1} adenine sulfate, 500 mg l^{-1} malt extract, 1.5 mg l^{-1} GA_3, and 8 g l^{-1} agar at pH 5.7 were used for embryo germination. Embryos excised at different developmental stages were placed with 10 embryos in each petri plate in a modified MT culture medium for germination and then petri plates were covered with parafilm. The embryos were kept in an incubation room at 26°C and in 16 h/8 h of photoperiod with 1000 lux light intensity.

According to their germination and development stage, embryos were transferred to culture tubes containing Murashige and Skoog (MS) (1962) culture media supplemented with 0.02 mg l^{-1} NAA, 20 g l^{-1} sucrose, and 8 g l^{-1} agar at pH 5.7 in order to increase plantlets development (Perez-Tornero and Porras, 2008) (Figure 2). At this stage, three different hybridization combinations were appointed for their effect on embryo rescue at three different embryo developmental stages and subcultured plantlets were determined trifoliate ratio and survival ratio.

After 60-80 days of subculture, in order to transfer the developed plantlets to *in vivo* conditions, polystyrene pots (250 ml) were filled with peat:perlite (3:1) mixture. To prevent water loss, they were covered with polyethylene and incubated in the growth rooms at 25-26°C temperature and 80-85% humidity for the high survival rates. Later, the seedlings were transferred to a greenhouse for acclimatization to *in vivo* conditions. Survival percentage of seedlings was determined after transferred to the mixture and also in the greenhouse.

Surviving seedlings in greenhouse were observed agro-morphologically such as stem diameter and height, leaf characteristics and thorn status. Morphological characteristics were examined by International Plant Genetic Resources Institute (IPGRI, 1999) for citrus features. Data on morphological characteristics were not considered in this study.

2.3. Ploidy analysis with flow cytometry

Ploidy levels of the genotypes were determined using a flow cytometer (Partec®). Ploidy analysis was carried out in 5 parents and 194 hybrid plants. Leaves were collected from each plants were kept between two layers of filter paper soaked in petri dishes and were kept cold during transportation. Samples were prepared using the ready kit of Partec and protocol of Partec®. The suspension of nuclei was filtered with 50 µl Partec® CellTrics filter and was transferred into the eppendorf tube, and was added 2 ml of 4.6 diamidino-2-phenylindole (DAPI, Partec®) staining solution (Aleza et al., 2009).

Histograms were analyzed using the Partec software, which determines peak position, coefficient of variation (CV), and the relative peak index of the samples. Nuclear DNA content was calculated according to the formula (Dolezel et al., 2007) below:

Sample 2C DNA content (pg/2C) = [Fluorescent density of hybrid sample (The value of the G1 peak) / Fluorescence intensity of the standard (The value of the G1 peak)] x Standard 2C DNA content.

2.4. Data analysis

The research was established in a randomized plot design. All data were subjected to analysis of variance with mean separation by Duncan's multiple range tests. Cultured embryos were observed and embryo germination ratio (%) was designated by rating the germinated embryo number to the total number of cultured embryos. Before comparing the percentage ratio with variance analyses, square root transformation was performed on the data.

3. Results and Discussion

3.1. Fruit set of controlled pollinations and seed formation

The number of pollinations (total 1215 crosess), number of obtained fruits, and fruit set ratio were presented in Table 2. In hybridization studies carried out with different citrus species and varieties until today, it has been shown that the effect of different pollinators on fruit set rate, fruit size, and number of seeds per fruit has shown a wide variation. Fruit setting in citrus can be affected by environmental conditions such as rootstock, climatic conditions and parents (Soost and Cameron, 1975). Aleza et al. (2012) reported that the fruit set rate was 36-39% in the crossbreeding study performed with different combinations in mandarins.

In the present study, the fruit set ratio in combinations in which mandarins are used as the female parent is close to the literature reports. It has been reported that the fruit set ratio of the crossbreeds in which the common sour orange was used as the female parent, the volkamer lemon, orange, lime, and mandarins as the male parents varied between 1.10% and 4.30% (Al-Naggar et al., 2009). In the combination in which we used the common sour orange as the female parent and Troyer citrange as the male parent, although the intergeneric hybridization, the fruit set was determined to be quite high, consistent with the literature reports. This might have been caused by the difference in pollen genotype, but the exact reason requires further study. The number of seeds may vary depending on the type of pollinator. However, there are studies reporting that the number of seeds is higher in more developed fruits (Yun et al., 2007).

The number of seeds obtained from fruits taken at different days after pollination (DAP) are presented in Table 3. The highest seed number was present in fruits harvested at 130 DAP from the CM×TC combination. Kim et al. (2020) indicated that the correlation coefficient between the number of seeds in the fruit and the average number of embryos per seed fluctuates from year to year, tree to tree, branch to branch and is not regular.

3.2. Germination rate of the embryos

Germination percentage of immature hybrid embryos under *in vitro* conditions was significantly affected by the embryo development stage, except for the KM×CC combination (Table 4). In embryo recovery studies, it is reported that different embryo development stages are appropriate depending on the species and variety. Raghavan (1980) stated that success in embryo rescue depends largely on the developmental stage of the isolated embryos, and Viloria et al. (2005) reported that the germination capacity of citrus embryos is affected by the genetic structure of the embryo and the embryo development stage. Regarding the time of embryo rescue in hybrids obtained from citrus; successful results were reported in studies conducted 50th (Wang et al., 1999), 80th (Tan et al., 2007) and 85th (Xie et al., 2019), 100th (Deng et al., 1996) days after pollination. Carimi et al. (1998), Tusa et al. (2002), and Ferrante et al. (2010) suggested that 105 days after pollination is suitable for embryo rescue time. Similar to our results, some researchers marked that immature embryos were removed 120 days after pollination for embryo culture in various citrus species and cultivars (Perez-Tornero et al., 2011; Kurt and Ulger, 2014). In addition, other studies showed that embryo rescue time was 118th (Chagas et al., 2005), 130-140th (Soni et al., 2019) and 145th (Kim et al., 2020) days after pollination for *in vitro* embryo culture.

The results of the study were consistent with the reports of the researchers and the highest germination rates were found on the 120 days after pollination in embryos obtained from both CSO×TC and CM×TC crosses. Although there was no statistically significant difference at KM×CC combination in terms of embryo germination rates, high germination was achieved with an average of 94.14% in all three stages. Our results clearly showed the species and varieties used in the

Table 2. Number of pollinations, number of fruits and fruit set ratio in 2x × 2x crosses.

Hybridizations	Number of pollinations	Number of fruits	Fruit set ratio (%)
CSO×TC	390	41	10.51
CM×TC	422	135	31.99
KM×CC	403	162	40.20

(CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).

Table 3. The number of fruits, total seeds number and seed number per fruit taken at different developmental stages after pollinations.

Parameters	Number of fruits			Total seeds number			Seed number per fruit		
DAP	110	120	130	110	120	130	110	120	130
CSO×TC	8	11	4	123	210	55	15.4	19.1	13.8
CM×TC	15	22	20	233	382	417	15.5	17.3	20.8
KM×CC	20	25	30	110	208	275	5.5	8.3	9.2

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).

Table 4. The effect of embryo recovery phase on germination in crosses and trifoliate plantlet ratio (%) of seedlings.

Parameters	DAP	Crosses		
		CSO×TC	CM×TC	KM×CC
Number of embryos cultured	110	350	350	320
	120	330	400	440
	130	230	250	320
Germination rate (%)	110	85.43 ± 1.76 b*	92.86 ± 1.27 a	94.38 ± 1.18^{Ns}
	120	95.15 ± 1.08 a	96.25 ± 1.06 a	95.23 ± 0.83
	130	93.91 ± 1.37 a	84.80 ± 1.31 b	92.81 ± 1.29
Number of seedlings	110	115	72	81
	120	292	122	218
	130	96	193	163
Trifoliate plantlet ratio (%)	110	17.40 ± 0.37 a	11.11 ± 0.37 a	6.17 ± 0.37^{Ns}
	120	13.70 ± 0.37 c	6.56 ± 0.37 b	5.50 ± 0.37
	130	15.63 ± 0.37 b	5.70 ± 1.38 b	6.13 ± 0.22

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).
* Significant at the $p < 0.05$ level, Ns: Not significant.
Mean followed by different letters within columns differ significantly ($p < 0.05$).

Table 5. Survival percentage of seedlings in subcultured (SPSS), in transplanted to mixture (SPSTM) and in transferred to the greenhouse (SPSTG).

Crosses	DAP	SPSS (%)	SPSTM (%)	SPSTG (%)
CSO×TC	110	80.00 ± 1.49^{Ns}	85.87 ± 1.49 a*	59.49 ± 1.49 a*
	120	84.60 ± 1.49	78.14 ± 1.49 b	49.20 ± 1.49 b
	130	80.21 ± 1.49	77.92 ± 1.49 b	58.30 ± 1.49 a
CM×TC	110	90.28 ± 1.49 a*	78.46 ± 1.49 ^{Ns}	64.71 ± 1.49 a*
	120	72.13 ± 1.49 b	81.82 ± 1.49	62.50 ± 1.49 a
	130	87.57 ± 1.49 a	79.88 ± 1.49	47.41 ± 1.49 b
KM×CC	110	85.19 ± 1.49 ab*	73.91 ± 1.49 a*	52.94 ± 1.49 a*
	120	89.90 ± 1.49 a	57.14 ± 1.49 b	40.17 ± 1.49 b
	130	84.05 ± 1.49 b	76.64 ± 1.49 a	41.90 ± 1.49 b

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).
* Significant at the $p < 0.05$ level, Ns: Not significant.
Mean followed by different letters within columns differ significantly ($p < 0.05$).

embryo recovery significantly affected the embryo development stage.

3.3. Trifoliate plant ratio

The trifoliate feature in citrus is controlled by two dominant genes (Cameron and Frost, 1968). For this reason, zygotic seedlings are obtained from controlled hybridization between *Citrus* and *Poncirus* have trifoliate characteristics (Ozsan and Cameron, 1963). In this study, based on this principle, among the individuals obtained from each combination, those showing trifoliate features were determined. Our results displayed that the embryo development stage has a significant effect on the ratio of trifoliate plantlets. The highest trifoliate ratio was observed in the seedlings obtained on the 110th day in CSO×TC and CM×TC combinations as 17.40% and 11.11%, respectively (Table 4).

Generally, the identification of hybrid embryos requires some additional analysis such as cytological, flow cytometry, isoenzyme or molecular analysis (Tusa et al., 2002). Since the trifoliate characteristic of the *Poncirus* genus is a dominant character, it is a powerful determinant used to define crossbred individuals morphologically and this is a useful tool that can be used to identify zygotic individuals (Rodríguez et al., 2004).

3.4. Survival percentage of seedlings

Survival percentage of seedlings was calculated over the total number of individuals transferred at each stage. The survival rates of seedlings were quantified for subcultured between 72.13% and 90.28%, for transplanted to mixture between 57.14% and 85.87%, for transferred to greenhouse between 40.17% and 64.71% (Table 5).

Regarding survival rates of seedlings after embryo rescue; successful results were reported in studies conducted 62.56% (Kurt, 2010) and 89.7% (Aleza et al., 2012) for subcultured; 59.0% - 89.3% (Jaskani et al., 2005) for transplanted to mixture; between 40.17% and 64.71 % (Jaskani et al., 2005), 68% (Singh et al., 2006) and 90% (Aleza et al., 2010) for transferred to greenhouse. When the study is evaluated together with the literature reports, survival rates in the study were close as well as higher or lower survival rates. These results indicated that combination selection as well as soil mix and environmental conditions had an impact on survival.

3.5. Ploidy analysis in hybrids

Nuclear DNA contents in parents and hybrids varied between 0.78 pg/2C and 0.93 pg/2C and

Table 6. Ploidy analysis results of hybrids with flow cytometry.

Parameters	DAP	Crosses		
		CSO×TC	CM×TC	KM×CC
Number of hybrids analyzed	110	22	21	17
	120	34	20	22
	130	17	20	21
Nuclear DNA content (pg/2C)	110	0.79 - 0.85	0.79 - 0.93	0.79 - 0.86
	120	0.78 - 0.85	0.79 - 1.16	0.79 - 0.87
	130	0.79 - 0.84	0.78 - 0.83	0.79 - 0.89
Ploidy level	110	Diploid	Diploid	Diploid
	120	Diploid	1 Triploid, Diploid	Diploid
	130	Diploid	Diploid	Diploid

(DAP: Days after artificial pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CC: Carrizo citrange).

were determined as diploid (Table 6). The nuclear DNA content was found 1.16 pg/2C in a hybrid of CM×TC (DAP 120) combination and this individual was evaluated to be triploid. In addition, this triploid hybrid in phenotypic observations had long thorns compared to the other hybrids. The results were compatible with the data of some researchers (Şeker et al., 2003; Ali et al., 2013). Small differences in values of nuclear DNA content obtained by flow cytometry may have resulted from sample preparation, staining, and analysis procedures.

4. Conclusion

In conclusion, we obtained 335 genotypes with the aid of embryo rescue technique from 1215 artificial pollinations of three different crosses. It was determined that parent combinations and embryo development stages have important effects on the germination rates of embryos. It can be said that this embryo development stage will be more appropriate in embryo culture studies, since germination rates are found to be the highest on the 120th days after pollination in hybridization combinations. However, it was found that 110th days embryos gave better results for all combinations in terms of trifoliate rate in hybrids. The survival rates after transfer to the greenhouse varied from 40.17% to 64.71%, and the effect of embryo development stages in all three combinations significantly differed. As a result of ploidy analysis, with the exception of the hybrid found as triploid, the nuclear DNA contents of the hybrids were found between 0.78 pg/2C and 0.93 pg/2C and it was determined that they were diploid. A genotype in CM×TC combination was determined to be triploid. It was also determined the triploid genotype had much longer thorns compared to the all hybrids based on morphological observations. Hybrids are now kept in the greenhouse to be studied for disease tolerance and rootstock properties.

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