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Effect of harvest time on seed germination and seedlings growth of Sour orange and Mexican lime under *in vitro* conditions

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Abstract: The aim of this research was to determine the best time to harvest the fruits for seed production which would ultimately lead to the production of citrus rootstocks of optimum quality. The sour orange and Mexican lime fruits were harvested on 7 and 5 occasions, respectively. The very first fruits were harvested 80 days after flowering and subsequent harvests were gathered every 30 days. An *in vitro* experiment was carried out in a completely randomized design, with four replications and 20 seeds in each replication. Based on fruit growth curve the time of fruit harvest affected seed germination (percentage and rate) and seedling growth (stem and root length, fresh and dry weight of stems, roots and leaves). The results showed that the best time to harvest the fruits of sour orange and Mexican lime was 230 and 170 days after flowering, respectively, which led to maximum seed germination (Mexican lime 100% and sour orange 85%) and seedling growth. The highest root, stem and leaf fresh and dry weight was also obtained at 230 and 170 days after flowering in sour orange and Mexican lime respectively.

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

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1. Introduction

Citrus is an important genus of subtropical fruit trees, with substantial roles in the economy of many countries (Iglesias *et al*., 2007; Tercan and Dereli, 2020). Among different citrus species, the fruits reach maturity at different times of the season and as a result, the harvest time of fruits usually lasts several months (Orbović *et al*., 2011; Deterre *et al.,* 2021). It has been reported that a variety of physiological factors in citrus fruits, including fruit color change, sugar concentration and acid content affect the quality and marketability of fruits. However, there is insufficient information about the effects of seasonal changes on the fruit seeds, their germination potential and seedling vigor (Moulehi *et al.,* 2012; Orbović *et al.,* 2013). Many citrus cultivars that are selected for the production of high quality fruits do not have suitable root systems and, thus, it is highly recommended that these cultivars be grafted onto desirable rootstocks (Zhu *et al.,* 2020). Previous reports suggest that more than 20 traits of a grafted plant are affected by the rootstock, including drought tolerance, nutrient uptake, growth vigor, tree size, root penetration depth, tolerance to disease, amount of yield, fruit size and quality (Zhu *et al.,* 2013; Khoshbakht *et al.,* 2015). In southern regions of Iran, among the various citrus rootstocks, Mexican lime (*Citrus aurantifolia* L.) and sour orange *(Citrus aurantium* L.) are the most widely used due to their special characteristics. Mexican lime has high growth vigor and yield (Haji Vand and Lee Abdullah, 2012) and Sour orange is resistance to root rot, tolerance to calcareous and salinity soils and has deep root system (Louzada *et al.,* 2008; Etehadpour *et al.,* 2020). Citrus rootstocks are mostly propagated by seed.

Seed germination is a vital stage in the plant life cycle (Bakhshandeh *et al.,* 2017). Citrus growers often face many problems such as poor seed germination and great mortality rate of seedlings during the nursery stage (Dilip *et al.,* 2017; Chaudhary *et al.,* 2019). Citrus seeds usually do not have dormancy, they can germinate quickly and are considered as short-lived seeds (Khopkar *et al.,* 2017), their germination rate decreases as the seeds lose moisture (Hassanein and Azooz, 2003). Therefore, the seeds have low capacity of storability and should be sown quickly after extraction from the fruit (Khopkar *et al.,* 2017). Nursery operations to establishment of plant are very dependent on the high germination rate and growth of seedlings (Alouani and Bani-Aameur, 2004) so that they will reach the proper size for a short time and be ready for grafting, which can ultimately reduce the cost of growing grafted citrus plants (Girardi *et al.,* 2005).

An important factor that determines seed quality is the physiological maturity of seeds. Maximum germination (%) is reached when seeds are at their physiological maturity stage, which is associated with an optimum presence of nutrients in seeds to support the growth of seedlings with good vigor (Murrinie *et al.,* 2019). It has been also reported that the growth and maturity of fruits can affect seed germination percentage (Abbasi and Heidari, 2011; Mombeini *et al.,* 2011; Bareke, 2018). According to our knowledge, there is a lack of information about the best stage and time to harvest the fruits of sour orange and Mexican lime for producing seedling rootstocks. The aim of this research was to assess the effects of fruit harvest time, taking into account the fruit growth curve of sour orange and Mexican lime, on seed germination and seedling growth vigor under *in vitro* condition.

2. Materials and Methods

Plant materials and research site

This research was carried out in the laboratory of plant tissue culture and biotechnology, Faculty of Agriculture, Shiraz University. The seeds of two *citrus* species Mexican lime and sour orange were collected from an orchard belonging to the Citrus Research Institute, Larestan (27.66° N, 54.38° E, altitude 900 meters above sea level). The maximum and minimum temperatures and rainfall on an average of ten years in the region are 43.5°C, 4°C and 203 mm, respectively. The climate is characterized by mild winters and warm summers.

Seed samples were taken from fruits harvested at different times during the growing season. Sampling began from 80 days after flowering (June 1) when the seeds formed in fruits. Subsequent samples were taken on a monthly interval. The total time span considered for the harvest of sour orange and Mexican lime were 260 and 200 days after flowering, respectively. All sour orange fruits were harvested from a 10-year-old tree and all Mexican lime fruits were from an 8-year-old tree. The fruits were harvested from the same tree throughout the experiment. After harvesting the fruits, they were transferred to the laboratory in order to measure their diameter, length, and weight. Data were reported as the mean value of 10 fruits.

Effect of harvest time on in vitro seed germination

The treatments included 7 and 5 harvest times for sour orange (80, 110, 140, 170, 200, 230 and 260 days after flowering) and Mexican lime (80, 110, 140, 170 and 200 days after flowering) species, respectively. After separating from the fruits, the seeds were soaked in water for 12 hours and then washed with water and a few drops of dishwashing liquid for a few minutes to remove the gelatin-like material around the seeds. Then, the protective layer of the seeds was removed. The seeds were placed in vials containing water and a few drops of dishwashing liquid for 15 minutes to remove surface contaminants. After that, they were disinfected under sterile conditions by immersing in 70% alcohol for 30 seconds and then in 15% common bleach (containing 5.25% sodium hypochlorite) for 15 minutes. Then, the seeds were washed three times in sterile distilled water. The seeds were then cultured on liquid MS medium (Murashige and Skoog, 1962) without any plant growth regulator and a filter paper was used to prevent them from being submerged. The mentioned medium was fortified with 30 g/l of sucrose, and the pH of the medium, before autoclaving (at 121 ° C and 15 psi) was regulated on 5.8.

The jars containing the cultured seeds were taken to the growth chamber in dark conditions (25±2°C). Within an initial period of 30 days, the percentage and rate of germination were measured by the Maguire (1962) method.

$$\text{Germination (\%)} = \frac{\Sigma\text{number of germinated seeds}}{\text{Total number of seeds)}} \times 100$$

$$\text{Germination rate} = \frac{\text{number of seeds until n-1 day}}{\text{number of days}}$$

In vitro plantlets growth

After 30 days in darkness, all cultures were maintained in a growth room at 25±1°C under a 16/8 h (light/dark) photoperiod of 45-50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes and with 55-60% relative humidity. After 4 weeks, we measured growth indices such as stem and root length, number of leaves, fresh and dry weight of stems, roots and leaves

Experimental design and data analysis

The experiment was performed in a completely randomized design with 4 replications. In each replication, 20 seeds were checked for the percentage and rate of germination. Then, within the germinated seeds, 5 seedlings per replication were used for measuring growth characteristics. Statistical analyzes of the data were carried out using SAS 9.4 software and mean comparison was performed using LSD (P≤ 0.05). Microsoft Excel 2013 was used to draw the figures.

3. Results

The analysis of variance (Tables 1 and 2) showed that the fruit harvest stage significantly affected seed germination indices (germination rate and percentage) and seedling growth (root and stem length, number of leaves, fresh and dry weight of leaves,

Table 1 - Analysis of variance of the effect of harvest time on sour orange and Mexican lime on seed germination and seedling growth *in vitro* condition

	Source of variance	df	Germination percentage	Germination rate	Root length	Stem length	Number of leaves
Sour orange	Harvest time	6	5167 **	0.01 **	84.17 **	40.62 **	30.12 **
	Error	21	72.32	0.00006	3.82	1.49	0.87
	CV (%)	-	10.18	17.34	15.98	17.15	6.76
Mexican lime	Harvest time	4	3870 **	0.004 **	30.62 **	41.82 **	19.17 **
	Error	15	120	0.00008	3.55	1.17	1.88
	CV	-	10.12	10.86	9.32	14.59	11.28

**, significant at the level of 1 % probability using LSD.

Table 2 - Analysis of variance of the effect of harvest time on growth characteristics of sour orange and Mexican lime seedlings *in vitro* condition

	Source of variance	df	Root Fresh Weight	Stem Fresh weight	Leaf Fresh weight	Root dry weight	Stem dry weight	Leaf dry weight
Sour orange	Harvest time	6	1.04**	0.214**	0.926**	0.0796**	0.102**	0.100**
	Error	21	0.0003	0.0002	0.0004	0.00009	0.00005	0.0002
	CV (%)	-	10.21	2.4	5.85	12.95	3.38	4.25
Mexican lime	Harvest time	4	1.736**	0.268**	1.176**	0.141**	0.031**	0.055**
	Error	15	0.0004	0.0004	0.0005	0.0002	0.0002	0.0002
	CV	-	8.19	7.82	7.37	4.35	5.48	7.01

**, significant at the level of 1 % probability using LSD.

roots and stems) in both studied species (P≤0.01).

Fruit growth characteristics

Both citrus fruits species exhibited a simple sigmoid growth curve based on fruit dimensions and weight (Fig 1 A and B respectively). The growth curve in the first stage, i.e. 140 and 110 days after flowering showed a slow growth in sour orange and Mexican lime, respectively. Fruit growth at this stage is mostly a manifestation of cell division. The fruits in the second stage, from 140 to 200 days after flowering in sour orange and from 110 to 170 days after flowering in Mexican lime showed rapid growth and cell enlargement and water accumulation in fruit tissues. In the third stage, from 200 to 260 after flowering in sour orange and from 170 to 200 after flowering in Mexican lime, fruit growth had reduced growth rate and, accordingly, the process of non-climacteric ripening began in fruits.

In vitro germination characteristics

The highest percentage of seed germination was observed when sour orange harvested at 230 and Mexican lime at 170 days after flowering (Fig. 2 A and D), both of which are significantly higher than final stage of harvest. The lowest germination percentage of sour orange and Mexican lime was observed in the seeds of fruits harvested at 170 and 80 days after

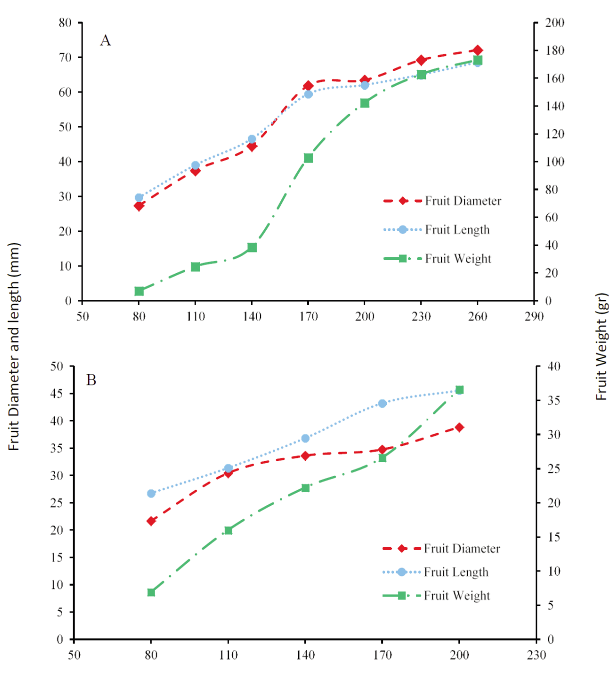

Fig. 1 - Fruit growth curve based on fruit weight, diameter and length of sour orange (A) and Mexican lime (B).

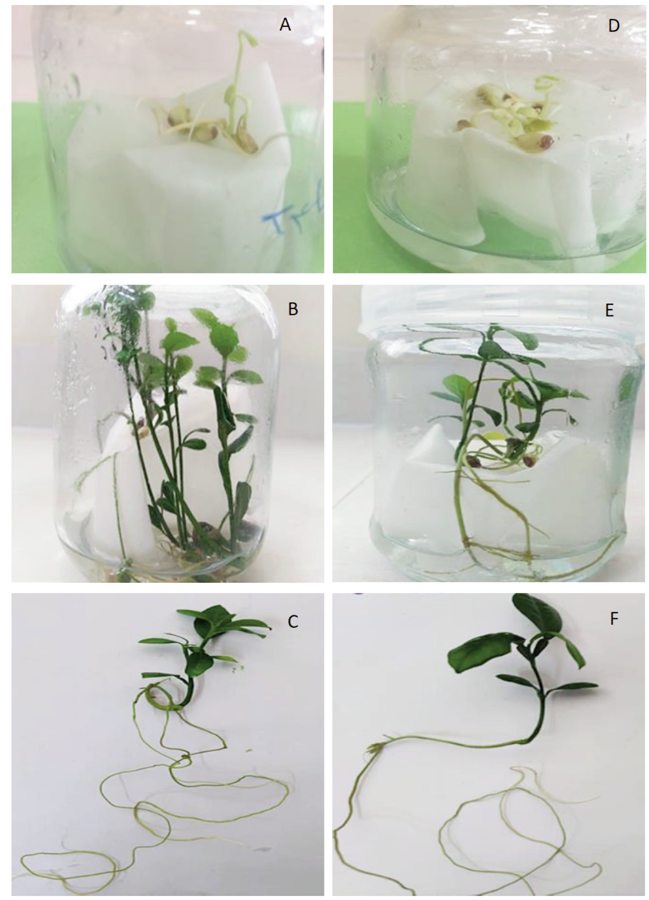

Fig. 2 - *In vitro* seed germination and plantlet growth. Germinated seeds of Mexican lime (A) and Sour orange (D) in darkness, 170 and 230 days after flowering respectively. Plantlets growth of Mexican lime (B, C) and Sour orange (E, F) after 4 weeks in light, 140 and 230 days after flowering respectively.

flowering, respectively (Fig. 3 A and C). It is also noteworthy that the seeds obtained from sour orange in the first three stages, namely 80, 110 and 140 days after flowering, were not able to germinate.

By increasing the harvest time, the germination rate increased in both species. In sour orange, the highest germination rate occurred at 230 days which was significantly 3.13 times higher than 170 days after flowering. In Mexican limes, the maximum seed germination rate occurred at 170 days which was significantly increased 1.57 times compared to 80 days after flowering. The lowest germination rate was observed in seeds of fruits harvested 170 days after flowering in sour orange and 80 days after flowering in Mexican limes (Fig. 3 B and D)

In vitro seedling growth indices

Fruit harvest time in both citrus species had a significant effect on seed growth indices (stem length,

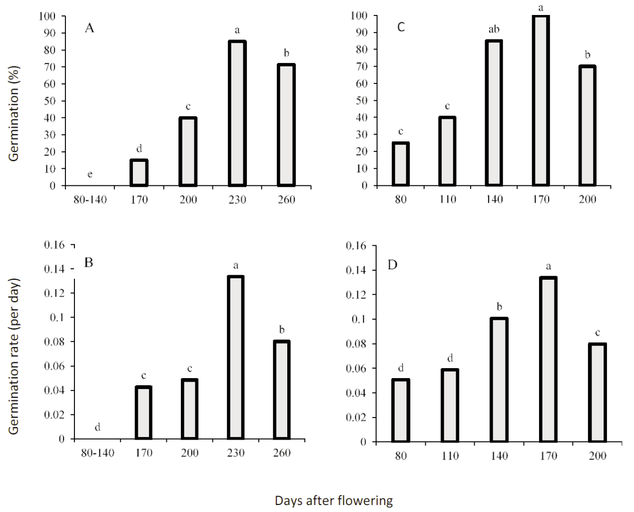

Fig. 3 - Effect of harvest time on germination percentage and germination rate of sour orange (A, B and Mexican lime (C D), under *in vitro* conditions. Means with the same letter are not significantly different at 1% probability using LSD test.

root length and number of leaves) (Fig. 4). In sour orang, the uppermost number of leaves were observed in the produced seedlings from the seeds of fruits that had been harvested 260 days after flowering which was significantly different from the 170 and 200 days (Fig. 4 C). However, there was no significant difference between the two treatments of 260 and 230 days after flowering.

In Mexican lime, the produced seedlings from the seeds of the fruits harvested 170 days after flowering indicated the higher number of leaves (9 leaves/seedling) which was significantly different compared to the produced seedlings from 80 (3.75 leaves /seedling) and 200 (6.75 leaves/seedling) days after flowering (Fig 4 F).

In sour orange, the *in vitro* seedlings of the 230 day after flowering showed the highest root length (10.75 cm) which did not show significant different compared to 260 days after flowering. Regarding Mexican lime, the highest root length occurred in the seedlings of 170 days after flowering (12.5 cm) which was significantly higher than those from 80 days after flowering. (Fig. 4 A and D). In both sour orange and Mexican lime seedlings, stem length increased with increasing the harvest time. In sour orange, stems of the seedlings related to 260 days after flowering showed the highest length of 7.25 cm which was not significantly different compared to 230 days after flowering (Fig 2 B and C). In Mexican lime, the seedlings of the 170-days harvest treatment indicated the maximum stem length of 11.75 cm which was significantly higher than the other harvest times (Fig 2 E and F) (Fig. 4 B and E).

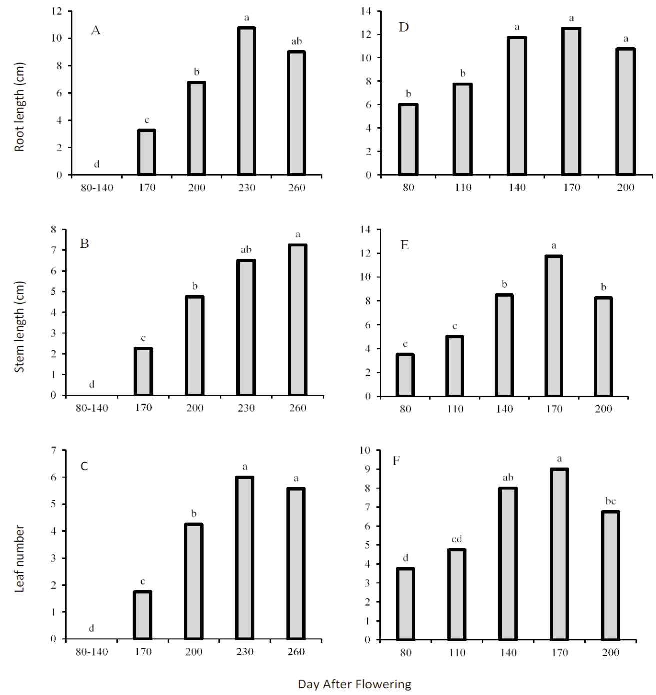

Fig. 4 - Effect of harvest time on seedlings root length, stem length and leaf number of sour orange (A-C) and Mexican lime D-F) under *in vitro* conditions. Means with the same letter are not significantly different at 1% probability using LSD test.

The results showed that a significant increase occurred in the fresh and dry weight of seedlings by increasing the harvest time. In sour orange, the produced seedlings in 230-day harvest treatment time indicated the highest fresh and dry weights of roots, stems and leaves which were significantly greater than other treatments (except in root fresh weight and leaf dry weight that was not significant different between 230 and 260 days after flowering) (Fig. 5 A-F). Regarding Mexican lime seedlings, with increasing the harvest time the fresh and dry weight of all organs increased. The highest fresh and dry weights of roots, stems and leaves were occurred in the seedlings of the 170-day harvest period, however with increasing harvest time to 200 days all measured traits decreased (Fig. 6 A-F).

4. Discussion and Conclusions

In this research, different stages of fruit harvest affected seed and rate of germination and seedling

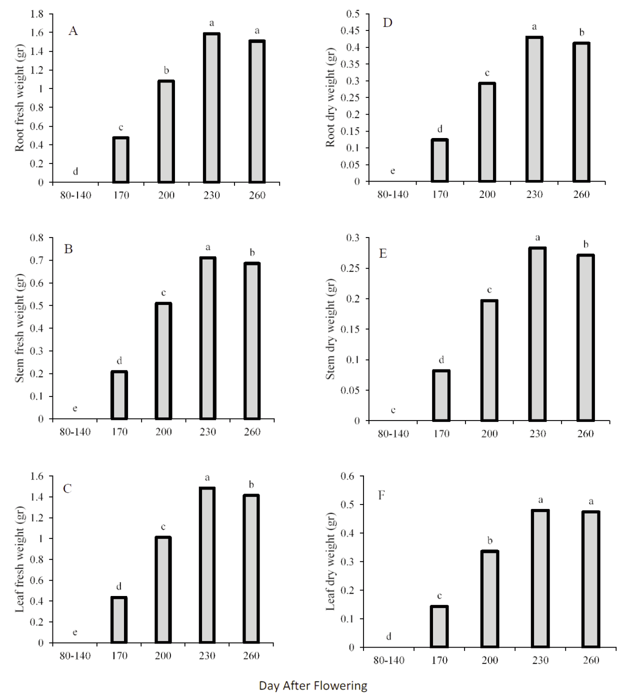

Fig. 5 - Effect of harvest time on seedlings root, stem and leaf fresh weight (A-C) and dry weight (D-F) of sour orange under *in vitro* conditions. Means with the same letter are not significantly different at 1% probability using LSD.

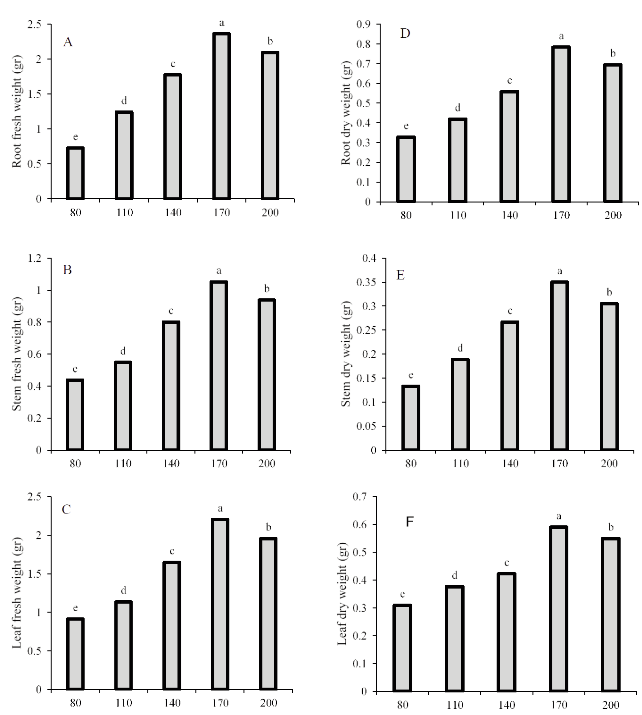

Fig. 6 - Effect of harvest time on seedlings root, stem, and leaf fresh weight A-C and dry weight (D-F) of Mexican lime. Means with the same letter are not significantly different at 1% probability using LSD test.

growth in both species (sour orange and Mexican lime). It has been demonstrated that by increasing the number of days after flowering to 230 days in sour orange and 170 days in Mexican lime, germination and seedling growth factors improved (Murti and Upreti, 2003; Kondo *et al*., 2004).

The results showed that the fruit growth of sour orange and Mexican lime based on fruit weight and dimensions have a single sigmoid growth, which is divided into three stages (Tadeo *et al.,* 2008). In the first phase of fruit growth, which is approximately between the flowering onset and June-drop, the rate of fruit growth is slow but cell division is high. The second is a period of rapid growth in which the size of the fruit increases through cell enlargement and accumulation of water. As these two growth stages end, the growing fruits change from the consumption phase to the storage stage (Mehouachi *et al.,* 1995). In the third stage, growth stops and the fruits undergo a non-climacteric ripening process (Mehouachi *et al.,* 1995; Tadeo *et al.,* 2008). Regarding fruit growth curve, our results showed that in order to achieve quality seeds and produce strong seedlings, the best time to harvest sour orange and Mexican lime fruits was in the beginning of third stage of fruit growth, about 230 and 170 days after flowering, respectively. Early harvest may reduce seed quality due to the partial development of basic seed structures, while late harvest may lead to reduced seed quality because of aging. Mombeini *et al.* (2011) reported that the highest seed germination occurred in sour orange at 250 days after flowering before full fruit ripening and then reduced until ripening, which is in line with our results.

It has been documented that one of the possible reasons for the differences in the physiological potential of seeds is related to changes in the embryo and endosperm of seeds at different stages of growth and development (Tekrony, 2003). The seed reaches its maximum potential for germination at the stage of physiological maturity, when more nutrients are available to support seedling growth and vigor (Murniati *et al.,* 2008). However, Murniati *et al.* (2008) also reported that papaya seeds extracted from fruits before full ripening (30-40% yellow color of the fruit skin) had maximum germination and growth.

Another study indicated that physiological maturity is a genotypic trait that is influenced by environmental factors. Environmental conditions during seed growth and maturity, including temperature, environmental stresses, and nutrient deficiencies, affect seed quality (Mahesha *et al.,* 2001). In the process of seed development, various mechanisms occur from fertilization to physiological maturity and into the phases of cell division, development and then the phase of nutritional storage in seeds. There is usually an increase in the dry weight of seeds and finally a decrease in seed moisture due to changes in cell membrane structure and enhanced levels of enzyme synthesis, necessary for successful seed germination (Bareke, 2018). Theoretically, it can be said that during physiological maturity, the germination percentage of seeds increases and reaches a maximum when the seeds reach their maximum dry weight (Orbović *et al.,* 2013). In another study, the relationship between the germination percentage of grapefruit seeds and sour orange were evaluated from fruits harvested at the beginning of the season. Studies have shown that when a seed reaches physiological maturity, the seed vigor becomes consistently high throughout the harvest season (Fucik, 1978).

The produced seedlings from the seeds extracted from fruits at 230 and 170 days after flowering in sour orange and Mexican lime, respectively, had better growth than the seedlings produced from the harvested seeds at the final stage of fruit maturity. Accordingly, seedling growth indices such as fresh and dry weight stems, root and leaves were significantly higher at a stage before the last stage of harvest. It has also been reported that structural and chemical changes in fruits and seeds are associated with germination vigor and seedling growth indices (Abbasi and Heidari, 2010; Mombeini *et al.,* 2011). Orbović *et al.* (2013) stated that the ability of grapefruit seeds to germinate at the end of the season is a physiological manifestation of a change in hormonal balance in the fruits, which is largely associated with a slight decrease in abscisic acid levels in the seed. It has been also reported that Valencia orange seeds showed a great peak in ABA (Abscisic Acid) concentration at 150 days after flowering (in stage II) and this increment obviously decreased at 188 days after flowering while, amount of IAA (Indole-3-acetic acid) increased (Kojima 1995). It is possible the better seedlings growth in the beginning of third stage of growth be due to the increase of IAA content of the seeds.

In conclusion, we found that the fruit harvest stage can have a significant effect on seed quality for rootstock production in citrus. Fruit growth (length, diameter and weight) was affected by harvest time in sour oranges and Mexican lime. The best seed germination characteristics were obtained at the onset of the maturation stage in both citrus species (230 and 170 days after flowering, in sour oranges and Mexican lime, respectively), followed by the highest seedling growth. But, seed germination and seedling growth parameters were significantly reduced in 30 days after maturation.

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