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RESEARCH PAPER

HORTICULTURAL STUDIES

Assessing Autumn Cold Hardiness in Newly Planted Fruit Trees and Grapevines

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Abstract

Low-temperature damage is one of the key factors that limits the distribution of tree species in an area. This damage is not always the result of low temperatures in winter or during bloom. Actively growing trees or parts of trees do not harden, may be injured by lower temperatures or erratic temperature fluctuations in autumn. It is essential that the capability of each separate scion/rootstock combination to tolerate cold temperatures should be tested especially when the trees are young and a serious climate change is taking place. The overall goal of this study was to investigate the effect of early autumn temperature on fruit and grapevine species, including various cultivars and rootstocks, after plantings and to determine the cold hardiness. The autumn term of 2022 was one of the periods we have experienced notable temperature fluctuations was observed, particularly in September. The day-night temperature difference reached 21.5°C on September 24. Subsequent field observations revealed significant variation in autumn cold tolerance among species, cultivars, and rootstocks. In this study, cold injury was observed in fifteen of the 29 examined species in the autumn after planting. During unfavourable autumn conditions, young trees of fig, persimmon, walnut, and chestnut cultivars were classified as very susceptible. It is most likely that the hardening process in these four species was more affected by erratic temperature fluctuations in the early phase of hardening.

1. Introduction

Spring frosts represent a significant hindrance to the successful cultivation of fruit and grapevine species in most regions, frequently resulting in substantial economic losses (Ashworth and Wisniewski, 1991; Tromp, 2005). Therefore, considerable attention has been devoted to studying the prevention of freezing injury during bloom, particularly in various *Prunus* cultivars (Chmielewski et al., 2018; Dumanoglu et al., 2019; Demirsoy et al., 2022).

After grapevines have commenced growth, temperatures below 10°C can halt development, contingent upon the severity and duration of the cold spell; if temperatures near freezing (0°C) endure, developing shoots may suffer damage; in cold regions, early autumn frosts pose a risk, potentially harming grapevine shoots not yet fully matured (Çelik et al., 1998; Ren et al., 2023). Low-temperature damage in autumn or winter is relatively uncommon in temperate zones. However, in recent years, injuries caused by autumn or winter frost have been gradually increasing in extensive commercial production areas in Türkiye, especially in new plantings.

Various injuries caused by lower temperatures in grapevines and fruit species may occur in the

autumn or winter, including browning of xylem cells (blackheart), cambium injury, crotch injury, crown/collar injury, splitting of trunks, winter sunscald or bark splitting on the trunk or main branches, shoot death/dieback, killing of dormant flower buds, and rarely, root injury (Pearce, 2001; Tromp, 2005). The degree of injury depends on plant species/cultivar, stage of development, temperature, rate of temperature change, and the ability to tolerate low temperatures (Westwood, 1993; Rodrigo, 2000; Yu and Lee, 2020).

Soil conditions, management factors, the age of the plant, and the hardening phase can all influence the ability to tolerate freezing stress (Rochette et al., 2004). Some fully hardened woody plants are extremely tolerant to freezing stress in winter, reaching temperatures as low as -45°C and -35°C for fruit trees such as apple, pear, and peach (Tromp, 2005). In contrast, some young and nursery plants do not fully harden in autumn or early winter, as growth may continue during autumn, and they may be injured by lower temperatures or erratic temperature fluctuations (Walke, 2014; Wisniewski et al., 2018; Repo et al., 2021).

It has been predicted that climate change will alter the conditions affecting the phenology, frost hardiness of plants, and cold acclimation. However, the knowledge of how different fruit and grapevine species respond to changes in photoperiod and temperature in late summer and autumn is limited. To address this gap, it was conducted observations on autumn frost hardiness in fruits and grapevines, encompassing various cultivars and rootstocks, following plantings in the conditions of the Lake Region (Türkiye), renowned for its significant fruit production capacity.

2. Materials and Methods

The experiment was conducted at Burdur Mehmet Akif Ersoy University, Burdur, Türkiye (37°41′15″ N, 30°20′38″ E). The mean annual rainfall is 426.4 mm, mostly falling in autumn and winter (September to April), and the mean annual air temperature is 13.3°C. The average winter (December to February) and summer (June to August) temperatures are 3.5°C and 23.4°C, respectively.

Twenty-nine species/crosses from twenty genera were selected, considering their potential in the region. The rootstocks and rootstock-cultivar combinations used in the study are detailed in Table 1. Each genotype was represented by three plants, with one plant per plot in three replications. After lifting from the nursery, the plants were transplanted in January 2021.

Row and inter-row distances were designed according to commercial standards and the development characteristics of the genotypes. Management practices, including irrigation, nutrition, pest and disease control, as well as weed management, were conducted in accordance with local commercial orchards from 2021 to 2023.

Meteorological data were collected from a nearby meteorological station. The autumn frost hardiness of the plants was assessed in the field at the beginning of December 2022 through visual evaluation. Damages to plant tissues and organs were evaluated on a scale from 0 to 5, where 0 indicates no visible injury, and 5 indicates plants destroyed entirely by cold (Krasova et al., 2020). All figures were created using the ggplot2 package in R version 4.1.3 (R Core Team, 2022).

3. Results and Discussion

The autumn term of 2022 marked one of the periods with the highest temperature fluctuations in recent years, raising questions about the cold hardiness of our fruit and grapevine species. Daily air temperatures (minimum and maximum) and the difference in day-night temperatures were recorded at the study site from September 2022 to November 2022. Notably, the temperature difference between day and night exceeded 15°C on a total of 11 days in September, 7 days in October, and 6 days in November (Figure 1).

Significant temperature fluctuations, particularly in September, were observed. Through continuous field observations, we determined that autumn frost occurred on September 24, coinciding with the day when the temperature difference between day and night reached 21.5°C. It's noteworthy that the pictures in Figure 2 were taken on September 25.

Fruit trees and grapevines undergo gradual hardening as they are exposed to decreasing temperatures during the transition from late summer to fall and early winter, influenced by the shortening day length (Walke, 2014). Most fruit and grapevine species currently cultivated in the Lake Region are well adapted to these fall hardening conditions. Nevertheless, climate change, warm spells, and fluctuations from cold to warmer temperatures can disrupt this hardening process, thereby increasing the risk of frost injury (Rochette et al., 2004; Lenz et al., 2013; Vitasse et al., 2014). In this study, we observed this full range of situations in September 2022. The discoloration of freeze-injured tissues was visibly apparent at the end of September. The level of cold hardiness in plants can vary depending on when low temperatures occur, how quickly the temperature changes, and how long the low temperatures persist (Rodrigo, 2000; Pearce, 2001). A loss of hardiness due to erratic temperature fluctuations could increase plant vulnerability to freeze damage (Rochette et al., 2004; Wisniewski et al., 2018).

The fruit and grapevine species exhibited varying degrees of resistance to adverse autumn conditions in the Lake Region, as illustrated in Figure 3. Fig (Melli Yemişi), persimmon (Rojo Brillante), and walnut cultivars (Chandler, Iverto,

Table 1. List of the investigated fruit and grapevine species.

Genus	Species/Cross	Rootstock-Cultivar Combinations*	Rootstocks/Own-Rooted Cultivars
Castanea	Chestnut	Kemer/Seedling	-
Corylus	Hazelnut	-	Yomra*, Yağlı*
Crataegus	Hawtorn	Sarı Alıç/Seedling, Kırmızı Alıç/Seedling	-
Cydonia	Quince	Eşme/Quince A	Quince BA29*^{R}, Quince A*^{R}
Diospyrus	Persimmon	Rojo Brillante/Seedling	-
Ficus	Fig	-	Melli Yemişi*
Fragaria	Strawberry	-	Albion*, Rubygem*
Juglans	Walnut	Chandler/Seedling Iverto/Seedling, Pedro/Seedling	-
Lycium	Gojiberry	-	Orange*
Malus	Apple	Anna/MM111, Scarlet Spur/MM111	M9*^{R}, MM106*^{R}, MM111*^{R} Fuji (Seedling)^{R}
Mespilus	Medlar	İstanbul/Quince A	-
Morus	Mulberry	-	Beyaz Dut*
Olea	Olive	Gemlik/Delice (Seedling)	Delice (Seedling)^{R}
Pistacia	Pistachio	-	Siirt (Seedling)^{R}, Buttum (Seedling)^{R}
Prunus	Almond	Makako/GF677	-
	Almond × Peach	-	Garnem*^{R}, GF677*^{R}
	Mahalep × Mazzard	-	Maxma 14*^{R}
	Peach	Elegant Lady/GF677 Samantha/GF677, Monreo/GF677	-
	Plum	Stanley/Myrobolan 29C	Rootpac 20*^{R}, Marianna 2624*^{R} Pixy*^{R}, Myrobolan 29C*^{R}
	Plum × Almond	-	Rootpac R*^{R}
	Plum × Apricot	-	Kayısı Eriği*^{R}
	Sweet Cherry	Premier Giant/Maxma 14 0900 Ziraat/Maxma 14 Regina/Maxma 14, Staccato/Maxma 14	-
	Sour Cherry	Kütahya/Maxma 14	-
Pyrus	Pear	Triumph de Vienna/Quince BA29	-
Rubus	Raspberry	-	Willamette*
	Blackberry	-	Jumbo*
Vaccinium	Cranberry	-	Yalçınkaya77*
Vitis	Grapevine	-	41B*^{R}, 8B*^{R}, 110R*^{R}, 99R*^{R} 1103P*^{R}, 140Ru*^{R}, 5BB*^{R}
Ziziphus	Jujube	-	Gazipaşa*, Denizli*

*: Vegetatively propagated, ^{R}: Rootstock.

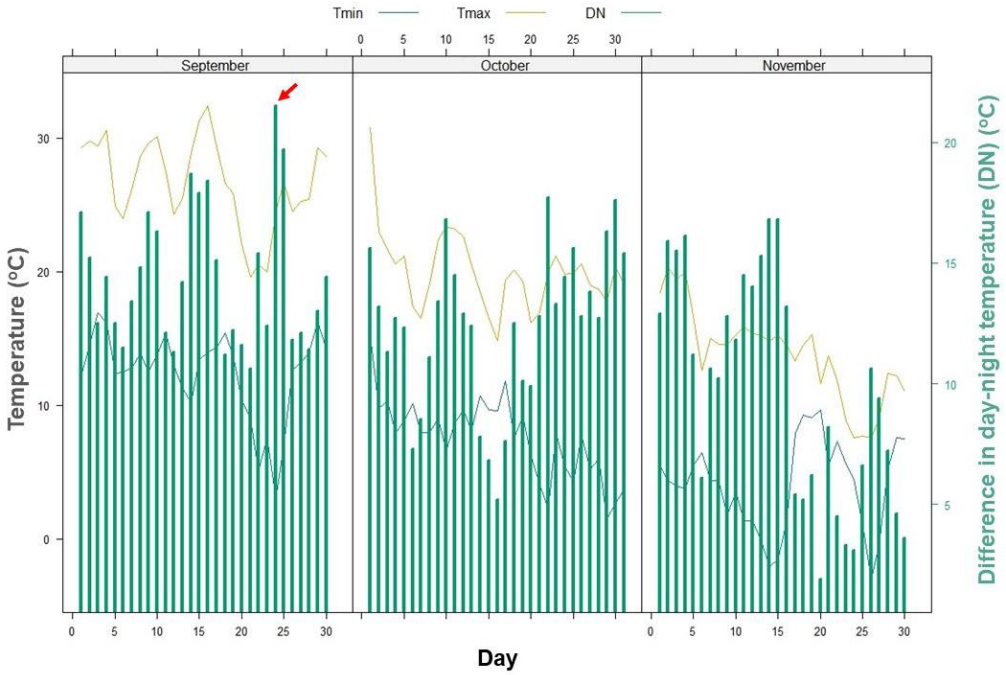

Figure 1. Air temperatures recorded at the research site during the autumn months of 2022 ('Tmin' represents the minimum temperature, while 'Tmax' represents the maximum temperature. The red arrow highlights the difference in day-night temperature “DN” observed on September 24, 2022).

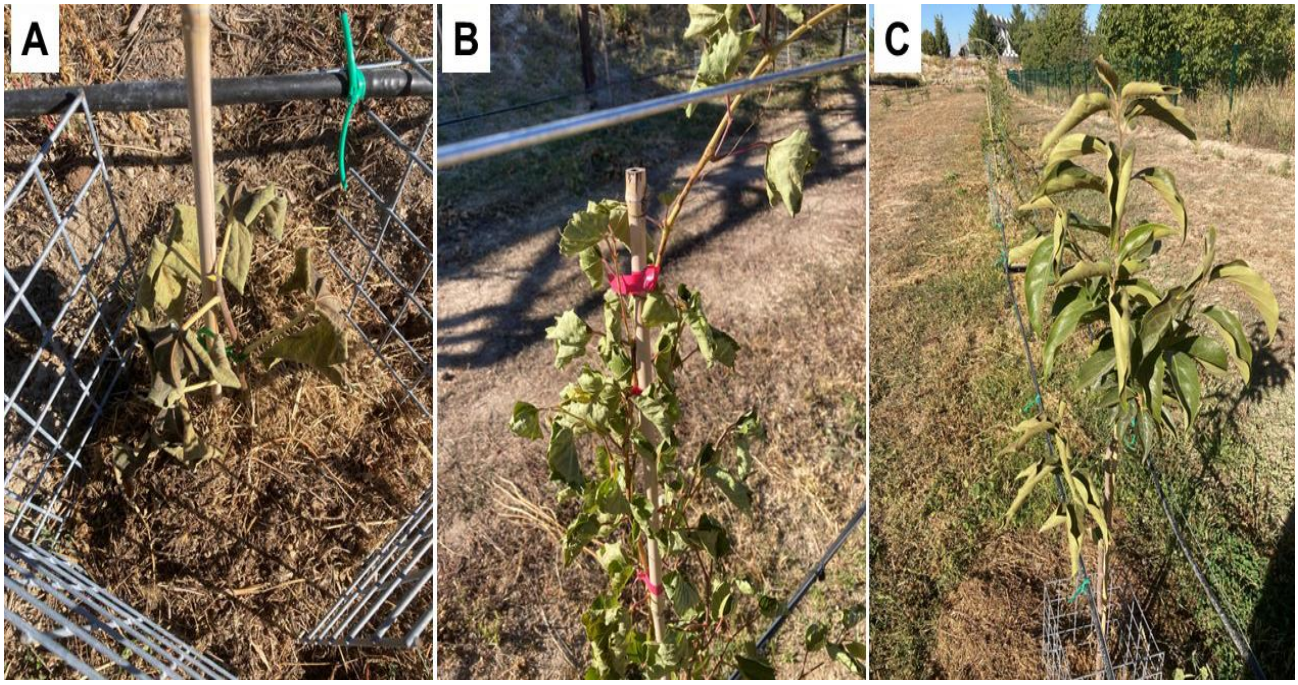

Figure 2. Views of damaged plants taken on September 25 following the frost event on September 24 (A: Melli Yemişi, B: Grapevine “140Ru”, and C: Persimmon “Rojo Brillante/Seedling”).

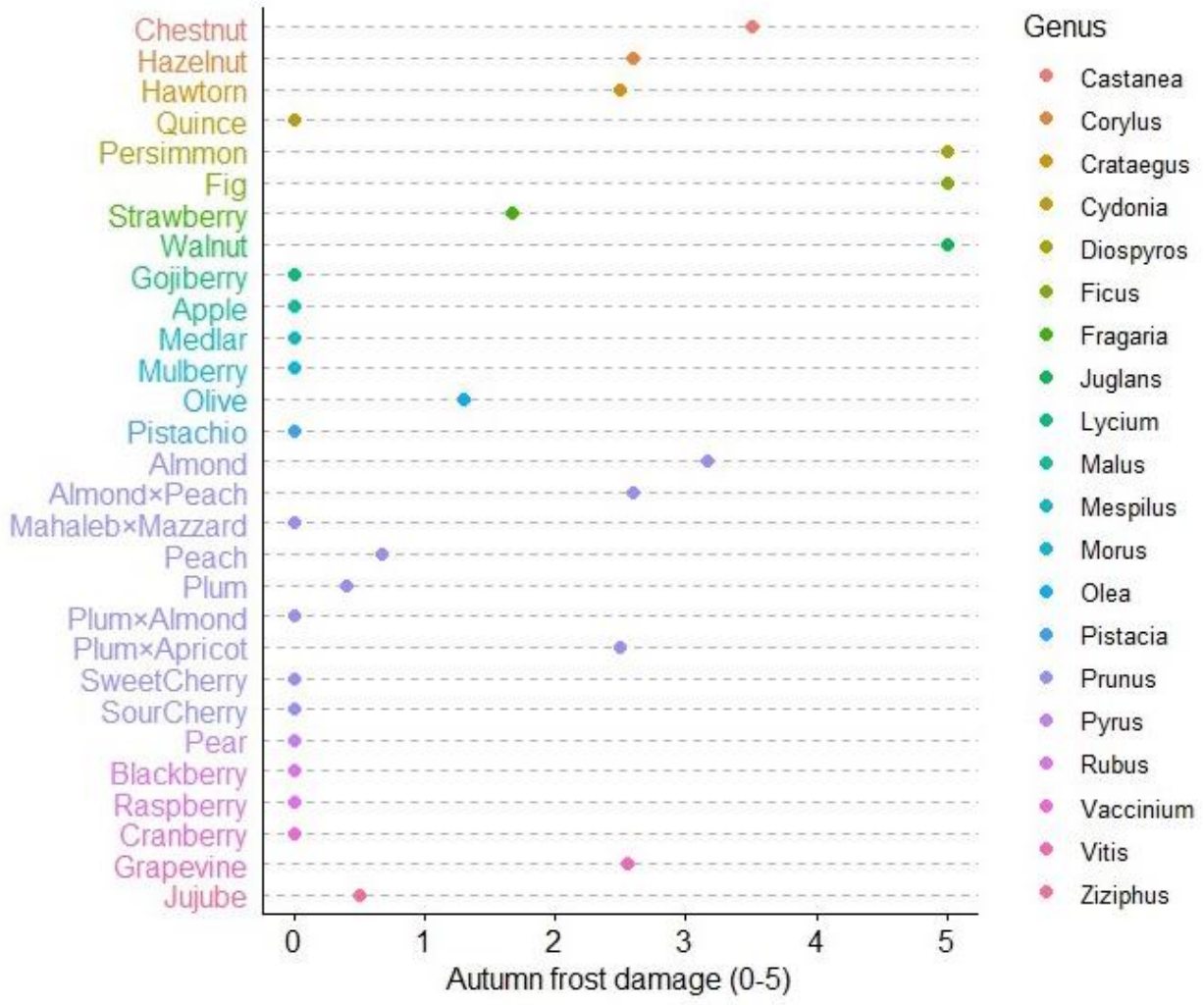

Figure 3. Damage degree of fruit and grapevine species and crosses after the autumn frost (The rating scale ranges from 0: no visible injury to 5: shoot system of plants damaged entirely by autumn cold).

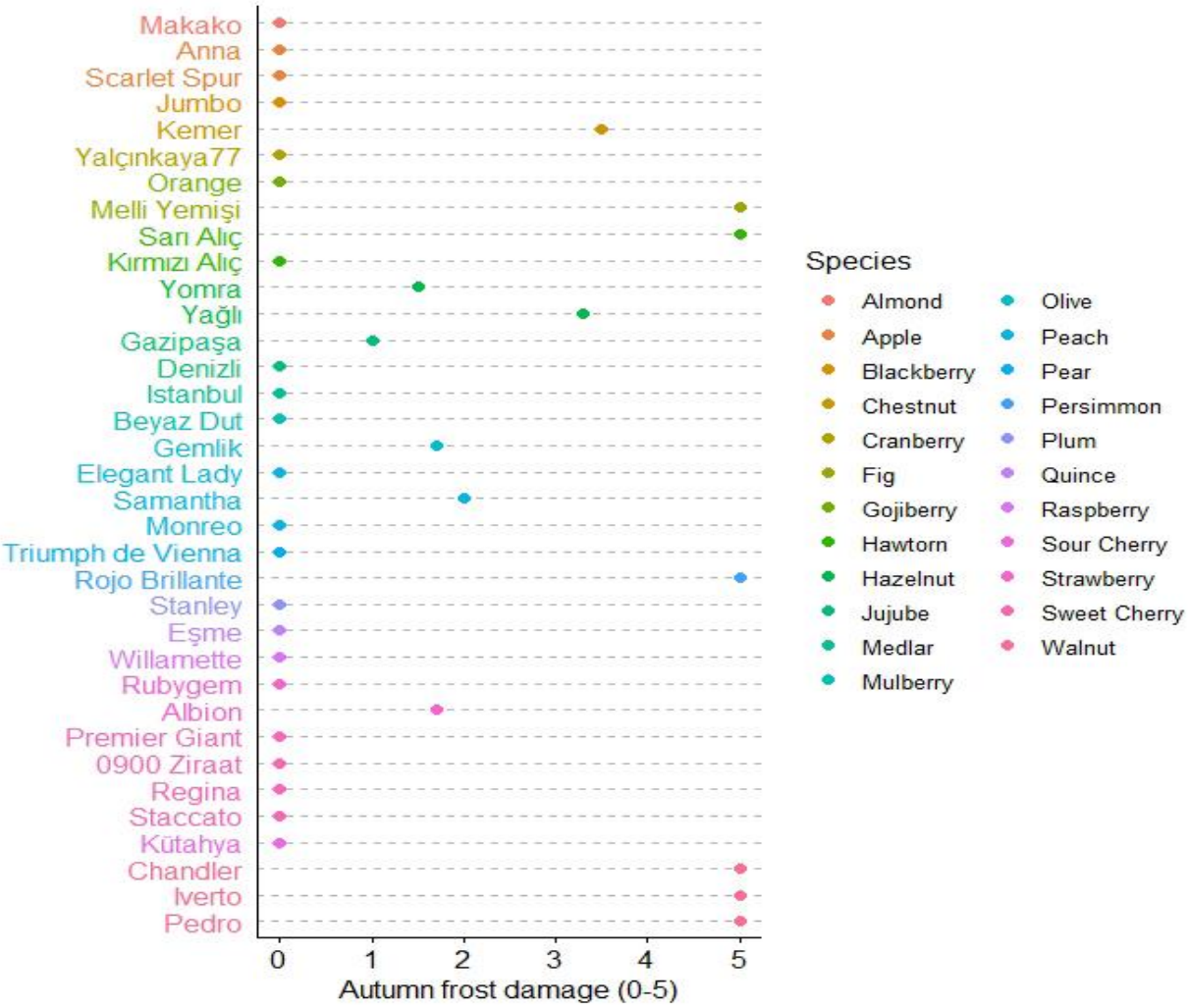

Figure 4. Damage degree of fruit and grapevine cultivars in field conditions after the autumn frost (The rating scale ranges from 0: no visible injury to 5: shoot system of plants damaged entirely by autumn cold).

Pedro) were classified as the most cold-susceptible, with average ratings of 5.0 points, resulting in almost all of their trees entirely dying. The above ground parts of fig, persimmon, and walnut varieties are completely dead. However, the following year, new shoots emerged from underground. Since the fig was propagated by cuttings, the genotype obtained the following year remained the same. On the other hand, in persimmon and walnut cultivars propagated by grafting, the plants obtained the following year were from seedling rootstocks. Chestnut and almond trees experienced cold damage, with freezing injury indexes recorded at 3.5 and 3.2, respectively. Almond cultivar Makako trees largely recovered within a year, producing new shoots. In contrast, trees of the chestnut cultivar 'Kemer' entirely died. The mean autumn frost damage values of the species hazelnut, grapevine, hawthorn, and the crosses Almond × Peach and Plum×Apricot were approximately 2.5 points, indicating intermediate hardiness (Figure 3).

There was significant variation in the autumn cold tolerance of the same species when comparing among cultivars and rootstocks for each of these species. Hazelnut cultivars showed injuries of 3.3 and 1.5 points for Yağlı and Yomra, respectively. Interestingly, Sarı Alıç was classified as the most autumn susceptible (5.0 points), and almost all of its trees were totally damaged, while there was no damage in Kırmızı Alıç. Among strawberry, peach, and jujube cultivars, only Albion (1.7), Samantha (2.0), and Gazipaşa (1.0) were partially damaged (Figure 4).

Damages of almond-peach hybrid rootstocks were scored as 1.5 and 3.3 for GF677 and Garnem, respectively. Grapevine rootstocks showed large variation in cold hardiness. The rootstocks 5BB (5 points), 140Ru (4.5 points), and 99R (4.3 points) were seriously injured, whereas it was observed that only minimal injury occurred in the rootstocks 1103P (1 point), 110R (1 point), 8B (0.6 points), and 41B (0.6 points). The rootstock Myrobolan 29C was the only plum rootstock that was damaged with an index value of 2.0. In olive, the freezing injury index of the Delice rootstock was 1 point, while this value was determined as 1.7 in the Gemlik cultivar grafted onto the same rootstock (Figure 5).

In our experiment, it was observed that there was no visible injury to trees of medlar, quince, cranberry, gojiberry, raspberry, blackberry,

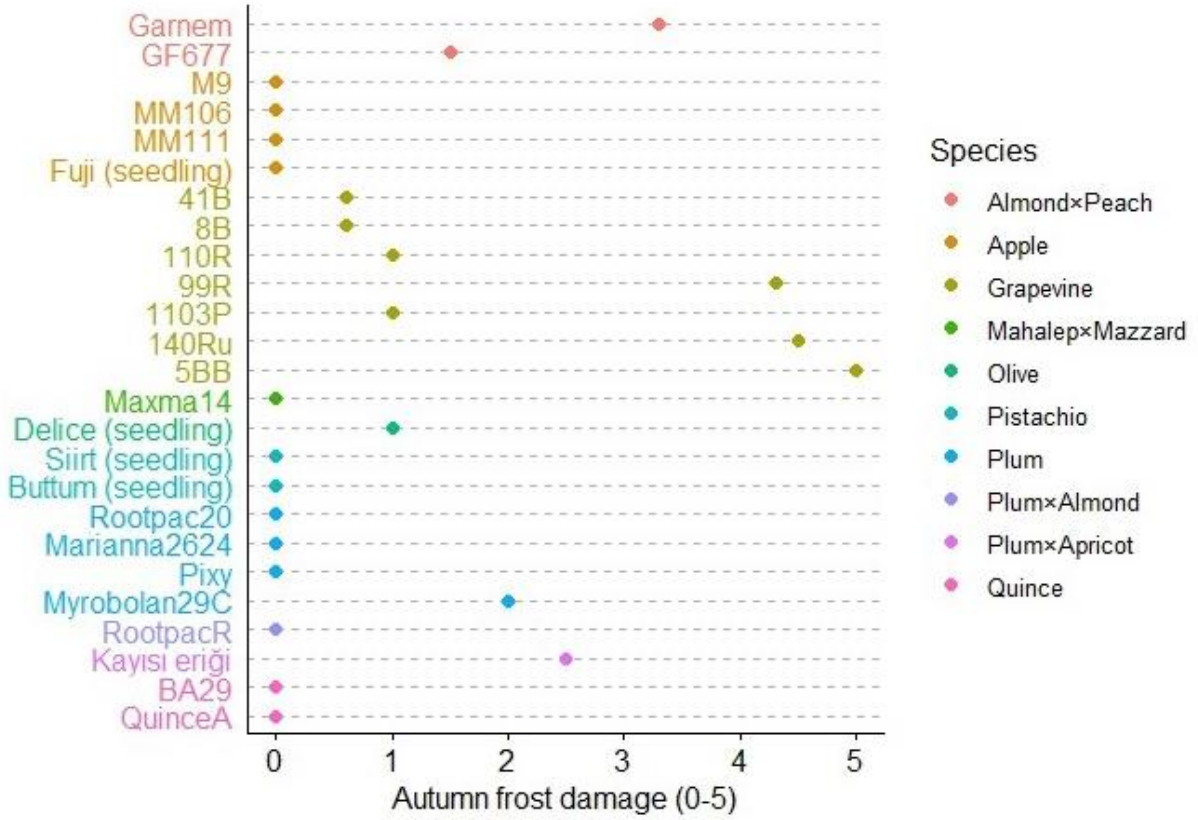

Figure 5. Damage degree of fruit and grapevine rootstocks after the autumn frost (The rating scale ranges from 0: no visible injury to 5: shoot system of plants damaged entirely by autumn cold).

mulberry, pistachio, pear, sweet cherry, sour cherry, apple, Plum × Almond, and Mahaleb × Mazzard cultivars and rootstocks (Figure 4 and Figure 5).

In consideration of the plant genotype's significance in determining potential frost resistance (Tromp, 2005), it is not surprising that differences in freezing tolerance can be substantial, not only between fruit and grapevine species but also within species among different cultivars and rootstocks. However, the survival of acclimatized woody tissues of fruit trees at low temperatures has received much less attention than flower buds, and limited information is available (Janes and Kahu, 2008; Neuner et al., 2019; Repo et al., 2021). Moreover, the situation in newly planted fruit and grapevine species is even more complicated since some parts of trees do not harden and may continue growth during the autumn. Mature plants acquire cold hardiness through cold acclimation, known as a seasonal increase in overwintering perennials' freezing tolerance (Arora and Taulavuori, 2016). Therefore, mature trees' responses to low temperatures may not accurately reflect the response of juvenile plants (Wolkovich et al., 2012; Vitasse, 2013; Lim et al., 2014). It is essential, therefore, that the capability of each separate scion/rootstock combination to harden and tolerate cold temperatures should be tested after planting in local environmental conditions.

In this study, cold injury was observed in fifteen of the 29 examined species during autumn. Among them, young trees of fig, persimmon, walnut, and chestnut cultivars were classified as very susceptible under unfavorable autumn conditions. Cold damage after planting is increasingly common in these species under our conditions. Factors such as water content, starch, and soluble carbohydrates at the onset of frost hardening could explain most of the temporal variability in frost resistance (Poirier et al., 2010; Charrier and Améglio, 2011), with their activity usually related to hardening (Améglio et al., 2004; Morin et al., 2007; Zuther et al., 2012). It is likely that the hardening process in these four species was more affected by erratic temperature fluctuations during the early phase of hardening, as the speed of hardening is closely related to temperature changes (Pogosyan and Sakai, 1969; Charrier et al., 2011).

Olive, the only evergreen species examined in this study, demonstrated robust autumn cold hardiness, surpassing that of most fruit and grapevine species. According to farmers' experiences, olive cultivars grafted on Delice seedling rootstock exhibit higher cold resistance, especially in the first 10 years after planting, compared to those grown on their own roots. Thus, the Gemlik olive cultivar used in our study is grafted onto Delice seedling rootstock. Additionally, higher leaf density and cell wall rigidity were observed in olive at lower temperatures (Arias et al., 2015), which may enable it to cope with sub-zero temperatures during winter.

Autumn frost damage, based on tissue browning, was determined for seven grapevine rootstocks with the order of cold resistance as follows: 41B > 8B > 110R > 1103P > 99R > 140Ru > 5BB (Figure 5). It was observed that grapevine rootstocks that continued their growth until the end of the season were more damaged by early autumn frosts. Although the mechanisms underlying grapevine cold tolerance remain largely unknown, phytohormones (e.g., ABA, ethylene, JA), metabolites (e.g., soluble sugars, proline, ascorbate), photosynthesis, and molecular changes associated with physiological changes play crucial roles in the cold response of grapevines (Ren et al., 2023).

No cold damage occurred in fourteen of the 29 examined species during unfavorable autumn conditions, and the examined cultivars and rootstocks belonging to these species were classified as cold-tolerant based on freezing injury indexes. The acquisition of plant cold tolerance results from highly complex physiological and biochemical processes, including the composition, structure, and function of the cell membrane (Dominguez et al., 2010; Arisz et al., 2013), hormone signal transduction, and the synthesis of soluble sugars, proteins, and other osmotic regulatory substances (Tauzin and Giardina, 2014; Shi et al., 2015). It also involves enhancements of antioxidative mechanisms and changes in lipid and protein composition (Gusta and Wisniewski, 2013). Despite significant advances in understanding the biochemical activities and molecular mechanisms of some plants, our knowledge of the cold response in fruit trees and grapevines remains fragmented, and the applicability of knowledge from different species to temperate species is yet to be determined (Mukhopadhyay and Roychoudhury, 2018; Ren et al., 2023). Identifying variations involved in cold tolerance would enhance our understanding of the complex mechanisms of plant adaptation to stress.

4. Conclusions

As a consequence, projected climate change is expected to significantly impact the cold hardiness of fruit and grapevine species in the Lake Region, presenting a substantial challenge for agriculture. The increasing frequency of temperature fluctuations and erratic episodes of unseasonal warming under changing climatic conditions have already led to a loss of hardiness, with autumn frost damage in our study being attributed to such events. Our findings suggest that freezing injury can be particularly detrimental, especially to newly planted fruit and grapevine species, during autumn temperature fluctuations. Young trees of fig, persimmon, walnut, chestnut, almond, hazelnut, and hawthorn cultivars, as well as almond × peach, plum × apricot, and grapevine rootstocks, experienced varying levels of cold damage. Integrating the evaluation of freezing injury with horticultural practices will be crucial to ensuring the successful cultivation of temperate fruit and grapevine species. We stress that the quality of nursery trees and improved hardening conditions in the fall could be key factors in reducing plant vulnerability to freezing injury. These results can inform orchard management practices and aid in assessing climate and weather risks in new production areas.

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RESEARCH PAPER

HORTICULTURAL STUDIES

Characterization of Citrus × Poncirus Embryo Rescued Hybrids as Rootstock Candidate using Morphological Markers

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Abstract

Generally, there are both nucellar and zygotic embryos in the citrus seeds after cross breeding. Since genetic variations are very important for the success of plant breeding, morphological traits of individuals are evaluated to identify diversity. The present study aimed to characterize of citrus hybrids for new rootstock genotypes based on their morphological characters at the seedling stage. A total of 335 putative interspecific hybrids, derived from 3 crosses [Common sour orange (*Citrus aurantium* L.) × Troyer citrange (*Citrus sinensis* L. × *Poncirus trifoliata* L.), Common mandarin (*Citrus deliciosa* Ten.) × Troyer citrange (*Citrus sinensis* L. × *Poncirus trifoliata* L.) and King mandarin (*Citrus nobilis* L.) × Carrizo citrange (*Citrus sinensis* L. × *Poncirus trifoliata* L.)], were observed by their plant morphology. The eight qualitative and five quantitative characteristics of hybrid plants such as seedling growth, leaf and thorniness characteristics were evaluated. The average plant height of the population was found between 70.2 cm and 133.2 cm. The average stem diameter varied between 5.9 mm and 8.0 mm. Hybrid seedlings were separated on the basis of dominant trifoliate leaf marker. There was wide diversity among the accessions with respect to quantitative leaf characters. In terms of leaf division, 268 genotypes have bifoliate and 67 were trifoliate in all combinations, and many intermediate forms were also observed. In addition 66 of the genotypes were thornless while 269 of the genotypes were thorny. The genotype No. 4, has been assessed as triploid, from Common mandarin × Troyer citrange combination, has the longest and dense spines. Morphological markers data were analyzed by clustering method to compare similarities of hybrids. The dissimilarity index was observed between 0.004 and 17.318 within three hybridization combinations. The hybrids obtained at 110 days after pollination were more distant relative to each other in all hybridization combination.

1. Introduction

The use of rootstock has become widespread due to the damages caused by *Phytophthora* and tristeza diseases, and the evaluation of citrus rootstock studies has accelerated considerably (Castle, 1983). Many different citrus rootstocks have been preferred in the various citrus production regions of the world (Syvertsen and Graham, 1985). Due to the sensitivity of present rootstocks to some diseases and rootstock-scion incompatibility, their use becomes limited. In order to provide uniformity, it is important that therootstock has many nucellar seeds (Soost and Cameron, 1975). Many of the rootstock used for citrus propagation are original species or old natural hybrids. However,

intergeneric hybrids (*Citrus* × *Poncirus*) such as citranges, citrumelos, and citrandarins are becoming increasingly important (Ollitrault and Navarro, 2012). *Citrus* genera is the origin of commercial citrus cultivars. *Poncirus* is a sexually compatible relative with *Citrus* in the Rutaceae family. Trifoliate (*P. trifoliata* [L.] Raf.) hybrids are important rootstock in certain countries and widely used as rootstock breeding materials (Swingle and Reece, 1967; Chen et al., 2008). *Poncirus* possesses several disease resistance or stress tolerance genes not found in *Citrus* (Frost, 1925; Krug, 1943). Complete or partial sexual compatibility is one common feature in citrus. Fertile hybrids among *Citrus*, *Poncirus*, and *Fortunella* can be quite easy for the selection of compatible rootstocks or desired scions (Soost and Roose, 1996). For example, Carrizo citrange and Swingle citrumelo, widely used rootstocks, were selected from the hybrids of *C. sinensis* cv. Washington Navel × *P. trifoliata*, and of *C. paradisi* cv. Duncan × *P. trifoliata*, respectively (Castle and Gmitter,1999). Hybridizations between mandarins and *Poncirus* appear promising to tolerance to abiotic and biotic stress conditions both by sexual breeding (Forner et al., 2003) or somatic hybridization (Grosser et al., 2000; Ollitrault et al., 2000).

Poliembryony, the formation of both nusellar and zygotic embryos, is a major problem in citrus breeding. Most of citrus varieties are polyembryonic, and generally produce vigorous and genetically identical nucellar embryos when used as seed parents because of suppressed, underdeveloped zygotic embryos may abort or die (Cameron and Frost,1968). Biotechnology has provided convenience and time savings in breeding and propagation studies. Embryo rescue has been used to isolate immature zygotic embryos at the early stage and *in vitro* to acquire seedlings (Rangan et al., 1969; Gmitter and Ling, 1991). Despite its polyembryonic nature, each seed usually produces one or several vigorous seedlings under *in vivo* conditions (Singh et al., 2020).

Leaf traits such as trifoliate character from the pollen parent will be exhibited in zygotic hybrids to assist early discrimination of zygotic from nucellar seedlings. The trifoliate of *Poncirus* versus the unifoliate of *Citrus* and others have been used the most widely in crosses between them (Ruiz and Asins, 2003; Gmitter et al., 2007). Morphological evaluation is the basic element for biodiversity and classification. Morphological characters have been used to identify and characterize a species (Susandarini et al., 2013; Sharma et al., 2016). Some studies have been conducted on the taxonomic definition of genotypes based on leaf shape, which is a morphological marker (Teich and Spiegel-Roy, 1972; Hearn, 1977; Chikaizumi and Matsumoto, 1978). Leaf shape has wide variations in genus *Citrus* and has been used as a marker of taxonomic character in classifying citrus species and varieties (Swingle and Reece, 1967; Tanaka, 1969; Handa and Oogaki, 1985). Although importance of leaf shape in taxonomy and breeding, knowledge on its inheritance is limited (Iwata et al., 2002). *Citrus* hybrids from crosses with *Poncirus* generally display multifoliate traits at early stages of growth (Chen et al., 2008; Caruso et al., 2014). In addition, leaf morphology is an important factor affecting fruit quality and quantity in citrus cultivation.

Phenotype of the hybrids is identified by observing specific morphological markers such as plant height, thorn status, leaf size and shapes (Dorji and Yapwattanaphun, 2011a; Roy et al., 2014). Individuals derived from a zygotic embryo in each hybrid seeds could be identified by the examination of their morphological characteristics but the selection could be made after the first fruit set (three to five years after planting) unless they have a distinctive character such as trifoliate (Rodriguez et al., 2004). Seedling height of citrus hybrid populations has been considered as an important criteria for distinguishing hybrid seedlings according to whether the seedlings are larger or smaller than normal (Moore and Castle, 1988).

Breeders are looking for new rootstocks that will solve the problems faced by citrus growers. However, it is imperative that new rootstocks or cultivars are properly selected and identified before introduced to the market. This information will be useful to breeders and geneticists working on citrus rootstock breeding programs. In addition to its convenience the morphological marker is useful for evaluating agronomic traits of research. Further, the technique is relatively cheaper and easier to conduct. The objectives of this study were to determine of leaf morphological characters and plant growth traits to observe developmental differences in hybrid populations generated via embryo rescue for rootstock breeding.

2. Materials and Methods

2.1. Plant materials

This research was conducted at the genetic resource collection parcels and greenhouse of Batı Akdeniz Agricultural Research Institute (BATEM) (36°55′ 32.40″ N and 35°00′ 35.75″ E), in Antalya, Türkiye. A total of 1215-controlled crosses were performed in all three hybridization combinations according to Batchelor (1943). The embryo rescue is the most important part of this study. Embryos were cultured at different developmental stages (110, 120, and 130 days after pollination 'DAP') with modified Murashige and Tucker (MT) (1969) in *in vitro.* Embryos germinated in MT medium culture were transferred to culture tubes containing Murashige and Skoog (MS) (1962), and survival rates and trifoliate rates were also determined. The peat and perlite (3:1) growth medium were used for

these hybrids which were transferred to the greenhouse in late October-Early November. The irrigation and fertilization were applied with octopus drip irrigation system. Morphological observations on the 335 survived individuals obtained for breeding new citrus rootstocks were evaluated in the greenhouse (Figure 1). Hybridization combinations are presented in Table 1.

2.2. Morphological characteristics of hybrids

Plant height and stem diameter were measured during a year to observe the development of hybrid plants transferred to the greenhouse after embryo recovery and then survived. Plant height measurement was made in every month, plant stem diameter was carried out in two periods. The only part is the vegetative shoot that could be morphologically identified at seedling stage. Hybrid seedlings were classified on the dominant trifoliate leaf marker. The dominant trifoliate property over the recessive unifoliate trait makes it easy to determine the first-generation hybrid rootstock seedlings in crosses between *Citrus* and *Poncirus* male parents. Visual observations were also made with regard to leaf morphology, particularly leaf shape and colour (Figure 2). Hybrid rootstock populations were separated by size and other morphological criteria, such as abnormal growth habits or leaf characteristics. Using the morphological descriptors recommended by the International Plant Genetic Resources Institute (IPGRI, 1999) for citrus, features such as leaf type, color or shape, and thorn status were defined (Table 2).

2.3. Data analysis

The research was established in a randomized plot design. Data were subjected to analysis of variance with mean separation by least significant difference (LSD) test. These 8 qualitative and 5 quantitative characteristics were evaluated on the hybrid seedlings. The quantitative data obtained from morphological characterization studies were presented with descriptive as minimum, maximum and average values. The data on the difference of leaf lenght, leaf width, leaf length/width, plant height and plant stem diameter each combination were compared by the ANOVA in the SAS package program (SAS Institute, Cary, NC, USA). In addition, the LSD multiple comparison test ($P<0.05$)

Figure 1. Morphological observations on the hybrid plants in greenhouse.

Figure 2. Differences in leaves in terms of color, size and shape and thorn state of hybrid plants.

Table 1. Combinations of hybridization.

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

was used to compare the averages. Data obtained by morphological characterization were subjected to the Ward Hierarchical Clustering Method in the MINITAB package program to demonstrate the overall phenotypic relationships among these genotypes.

3. Results and Discussion

3.1. Hybrid plant height and stem diameter

The hybrid plants showed linear growth in general as a result of plant height and stem diameter measurements made for 1 year after transfer to the greenhouse (Table 3). There was a high variation within the population in terms of growth parameters. The end of the growth season, the height of the plants of hybrid population varied between 8.0 cm and 205.0 cm. The average plant height of the population was found between 70.2 cm and 133.2 cm depending on the hybridization combination. The genotypes stem diameter varied between 2.4 mm and 12.7 mm. Viloria et al. (2005) observed the plant height of seedlings obtained from embryos at different stages of development in hybridization combinations. They reported that seedlings from smaller embryos grew more slowly. The effect of embryo development stage on the stem diameter was less in combinations that the female parent was mandarin. It is thought that the variation seen in plant heights is due to the fact that hybrid individuals have different genetic backgrounds. The selection of the hybrid population

Table 2. Traits identified in hybrid plants for morphological evaluation.

Traits	Classes
Plant height	From the root collar of the plant by a meter (cm)
Plant stem diameter	Measured above the root collar by a digital caliper (mm)
Leaf division (LD)	Simple (S), Bifoliate (B), Trifoliate (T)
Leaf color (LC)	Light Green (LG), Green (G), Dark Green (DG)
Leaf length (LE)	By digital caliper (mm)
Leaf width (LW)	By digital caliper (mm)
Ratio length/width (RTW)	By calculation (mm)
Leaf stalk (LS)	Absent (A), Short (Sh), Long (Lo)
Petiole wings state (PW)	Absent (A), Narrow (N), Large (L)
Petiole wings shape (PWS)	Obcordate (Oc), Obdeltate (Od), Obovate (Oo), Linear (L)
Leaf lamina shape (LLS)	Elliptic (E), Ovate(O), Obovate (Oo), Lanceolate (L), Orbicular (Or), Obcordate (Oc)
Shape of the leaf edges (SE)	Crenate (C), Dentate (D), Entire (E), Sinuate (Si)
Thorn status (TS)	Short (Sh), Medium (M), Long (Lo)

Table 3. Hybrid plants height (cm) and stem diameter (mm) obtained from embryos at different stage of combinations.

CH	Hybrid plant height (cm)								
	110 DAP			120 DAP			130 DAP		
	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
CSO×TC	8.0	194.0	79.0 ± 10.95 b*	45.0	199.0	127.7 ± 11.07 a	41.0	205.0	118.4 ± 10.29 ab
CM×TC	81.0	171.0	133.2 ± 6.59 ^{NS}	60.5	172.0	133.0 ± 6.60	99.0	196.0	130.1 ± 6.29
KM×CC	27.0	143.0	70.2 ± 7.08	29.5	129.0	77.5 ± 5.90	9.0	129.0	73.5 ± 7.26
CH	Hybrid plant stem diameter (mm)								
CSO×TC	2.4	9.8	5.9 ± 0.47	4.5	12.7	8.0 ± 0.56	2.4	10.0	7.0 ± 0.43
CM×TC	5.6	9.5	8.0 ± 0.26	4.0	9.4	7.7 ± 0.32	5.8	9.2	7.6 ± 0.20
KM×CC	3.4	8.5	6.0 ± 0.32	4.6	12.1	6.6 ± 0.41	3.5	11.9	6.5 ± 0.43

CH: Combinations of hybridization, DAP: Days after pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CS: Carrizo citrange; * Different letters indicate significant differences (P<0.05) according to the Least Significant Difference test, LSD: (hybrid plant height: 40.612), NS; Non significant.

Table 4. Quantitative leaf traits of genotypes obtained from embryos at different stage of three hybridization combinations.

CH		Leaf characteristics								
		Leaf length (mm)			Leaf width (mm)			Leaf length / width		
	DAP	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
CSO×TC	110	40.3	161.7	92.1 ± 5.6 a *	13.6	54.6	36.4 ± 2.0 b	0.33	0.50	0.41 ± 0.02
	120	54.7	176.8	107.9 ± 5.0 ab	17.1	77.8	45.3 ± 1.8 a	0.24	0.62	0.43 ± 0.01
	130	26.0	165.9	120.0 ± 7.03 b	18.1	65.1	48.0 ± 2.3 a	0.32	1.04	0.42 ± 0.02
CM×TC	110	45.5	114.8	84.9 ± 3.2 ^{NS}	20.1	56.9	37.1 ± 1.3	0.33	0.63	0.44 ± 0.01
	120	67.4	117.7	90.8 ± 2.3	23.1	57.9	39.0 ± 1.5	0.32	0.57	0.43 ± 0.01
	130	54.4	124.9	82.4 ± 2.3	19.3	51.1	35.2 ± 1.1	0.33	0.60	0.43 ± 0.01
KM×CC	110	63.7	151.5	122.8 ± 4.8	29.1	66.4	53.1 ± 2.0	0.37	0.54	0.43 ± 0.01 b
	120	46.7	160.1	128.4 ± 4.0	18.8	74.7	58.9 ± 1.9	0.40	0.58	0.46 ± 0.01 a
	130	27.1	166.2	119.8 ± 4.9	12.2	73.5	54.6 ± 2.2	0.40	0.54	0.46 ± 0.01 ab

CH: Combinations of hybridization, DAP: Days after pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CS: Carrizo citrange; * Different letters indicate significant differences (P<0.05) according to the Least Significant Difference test, LSD: (leaf length: 22.294), (leaf width: 7.856), (leaf lenght/ width: 0.023), NS; Non significant.

is possible by examining the morphological characteristics.

3.2. Morphological analysis of hybrid populations

Leaf length, width, and index were evaluated to hybrid plants (Table 4). The leaf morphological characters were observed among the progenies either all three hybridizations or each hybridization combination. As a result of observations, leaf division was detected from completely unifoliate to completely trifoliate, also many intermediate forms were also observed. This situation showed that the leaf division character is expressed non-uniformly in hybrid seedlings. In citrus, various methods have been used to assess of diversity and genetic relationships among the genotypes. Researchers assessed morphological analysis as a tool to study variation between Kinnow mandarin and Rough lemon (Jaskani et al., 2006; Altaf and Khan, 2008). In the current study, leaf length, width and index among the combinations was found to be similar to each other, contrary to previous reports (Teich and Spiegel-Roy,1972; Stitou et al., 2020). Different morphological markers have successfully been utilized for separating nucellar and zygotic citrus seedlings among polyploid parents including characters like shape of the leaf edges (Jaskani and Khan, 2000), ratio leaf length and width, petiole size (Donadio, 1981), petiole wing (Ballve et al., 1997) and stem diameter (Khan et al., 1992).

In the study, leaf characteristics such as leaf length, width, leaf length/width index, leaf division, leaf color, petiole wings state, petiole wings shape, leaf lamina shape of the leaf edges, as well as thorn state were also examined. Width of petiole wing is a morphological marker for screening of hybrids in citrus (Blanco et al., 1998). Similarly, Ballve et al. (1997) found that the broadness of leaf petiole wing is a good indicator for the identification of hybrids of sour orange (*C. aurantium*) and sweet orange (*C. sinensis*) in crosses with very narrow-winged species such as *C. sunki* and *C. limonia*. Furthermore, they reported that 90% of the hybrids were visually identified.

There was significant diversity in terms of leaf traits among hybridization combinations. In CSO × TC combination, 76 bifoliate, 46 trifoliate, 109 thorny and 13 accessions as thornless were observed, while 102 bifoliate, 12 trifoliate genotypes, all of were as thorny in the CM × TC combination. Because of ploidy analysis, one of these genotypes was determined as triploid (Kurt and Koyuncu, 2023). The thorn status of this genotype, which has long and dense spines, is compatible with the article of Padoan et al. (2013). In the KM × CS combination, these traits were assessed as; 90 bifoliates, 9 trifoliates and 53 thornless genotypes. Leaf properties are one of the basic characters within morphological evaluation of *Citrus*. Das et al. (1998) reported that Rangpur lime and Rough lemon were crossed with Troyer citrange and trifoliate orange, leaf characteristics varied from fully unifoliate to fully trifoliate. Similarly, Singh (2006) reported that leaves in both Indian wild orange and sour pummelo have simple leaf characteristics. Furthermore, in the study on the inheritance of agronomic traits in citrus, it was stated that these traits are controlled by multiple genes, which are assessed through morphological evaluation (Liu and Deng, 2007).

3.3. Cluster dendrograms of hybridization combinations

Morphological characterization data were evaluated using cluster analysis to compare similarities between hybrids. The morphological characteristics of the 122 hybrids in the CSO × TC population were analyzed and represented in a dendrogram (Figure 3). The similarities were obtained between 0.023 and 17.318 as two main groups. In the resulting dendogram, No.35 (120 DAP) and No.9 hybrids (130 DAP) were found the most similar to each other, while No.1 (110 DAP) and No.3 hybrids (110 DAP) were the most distant relatives.

The dendrogram, made from morphological characteristics of 114 hybrids obtained crossbreeding Common mandarin with Troyer citrange, a genetic difference between 0.004-12.861 was obtained (Figure 4). According to dendrogram, No.4 (130 DAP) and No.25 hybrids (130 DAP) were similar to each other, while No.1 (110 DAP) and No.7 hybrids (110 DAP) were the most distant relatives.

The dendrogram obtained by analyzing the morphological characters of the KM × CC hybrid populations is presented in Figure 5. Among the 99 hybrids, No.11 (110 DAP) and No.14 hybrids (120 DAP) were similar to each other in terms of morphological features, with a similarity ratio of 0.015. With a similarity ratio of 16.862, No.1 (110 DAP) and No.5 hybrids (110 DAP) were found as the most distant relatives.

The citrus hybrids of could selected through morphological identification (Oliveira et al., 2002; Koehler-Santos et al., 2003; Malik et al., 2012). Moreover, this method is relatively simple, easy, cost-and time-saving (Dorji and Yapwattanaphun, 2011b). Reece (1969) stated that Tanaka (1969) accepted 35 species within mandarins and the key to distinguishing them was the differences in leaf and fruit sizes. Many previous authors (Koehler-Santos et al., 2003; Campos et al., 2005) reported that molecular and morphological diversity is independent and rather complementary to genetic diversity in citrus. Budiarto et al. (2021) stated that morphological observations on the 21 citrus genotypes at the seedling stage confirmed the similar grouping pattern because of both cluster analysis and principal component analysis (PCA). Traband et al. (2023) reported, the analysis of the

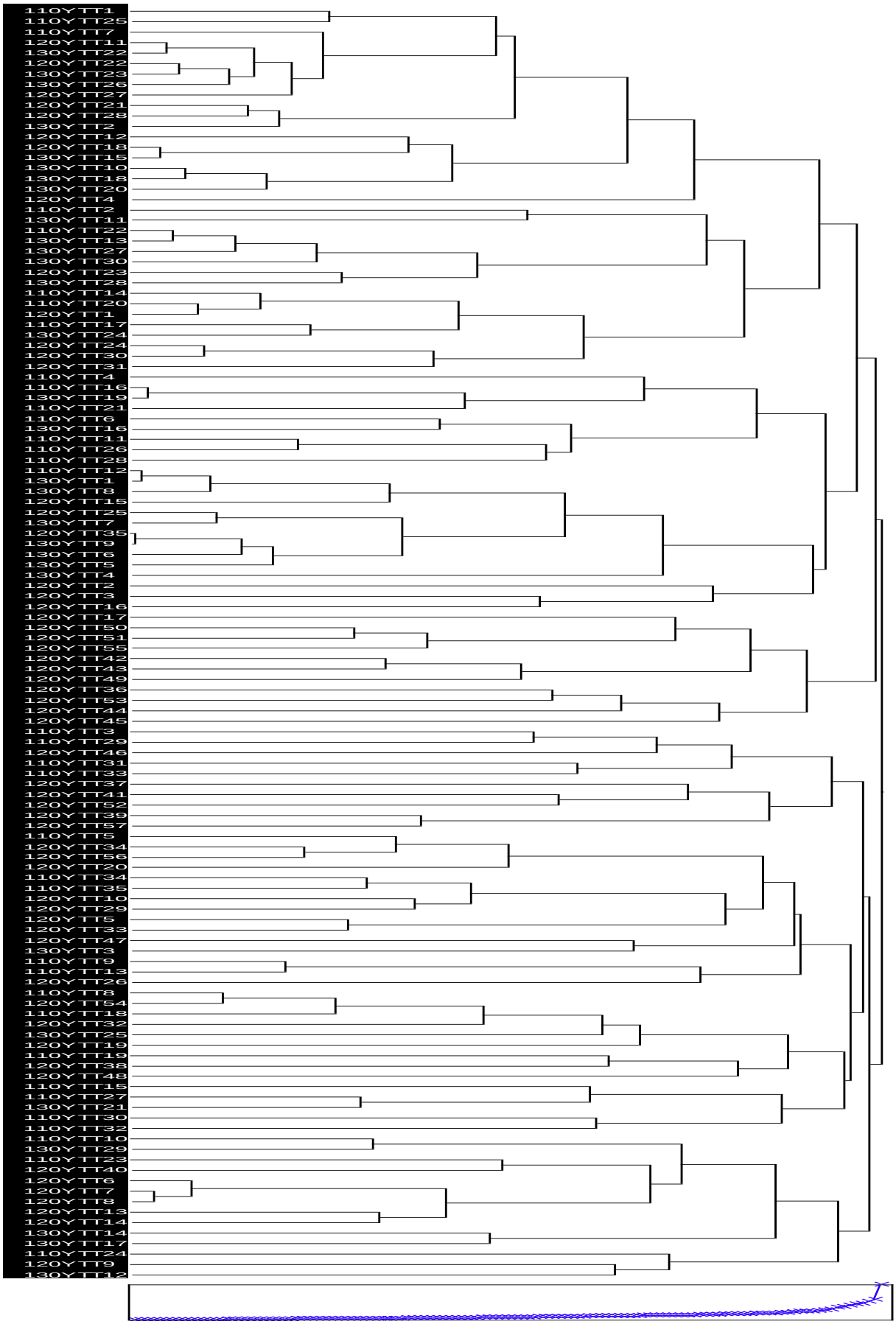

Figure 3. Dendrogram illustrating morphological dissimilarities of 122 hybrids of CSO × TC at seedling stage.

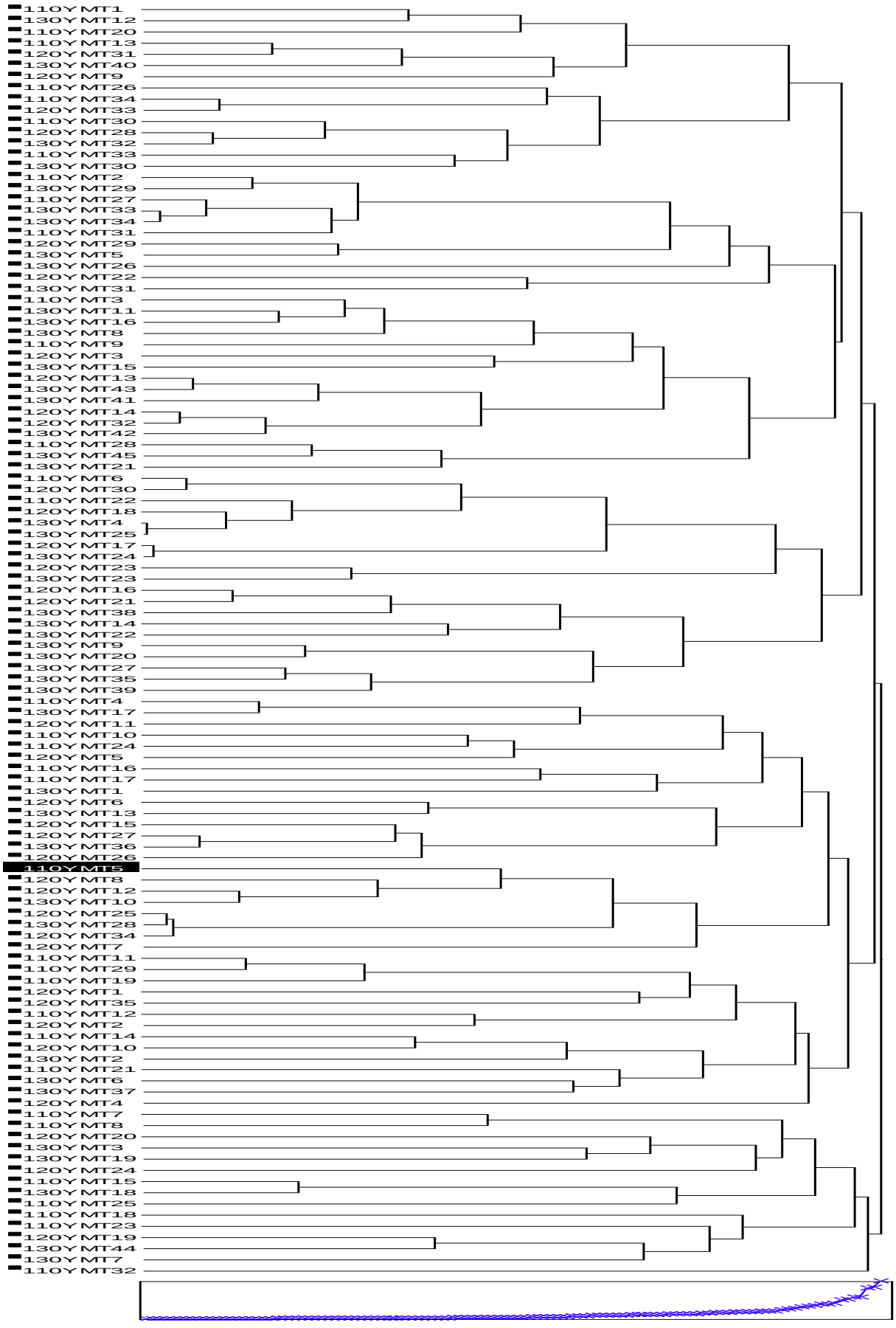

Figure 4. Dendrogram illustrating morphological dissimilarities of 114 hybrids of CM × TC at seedling stage.

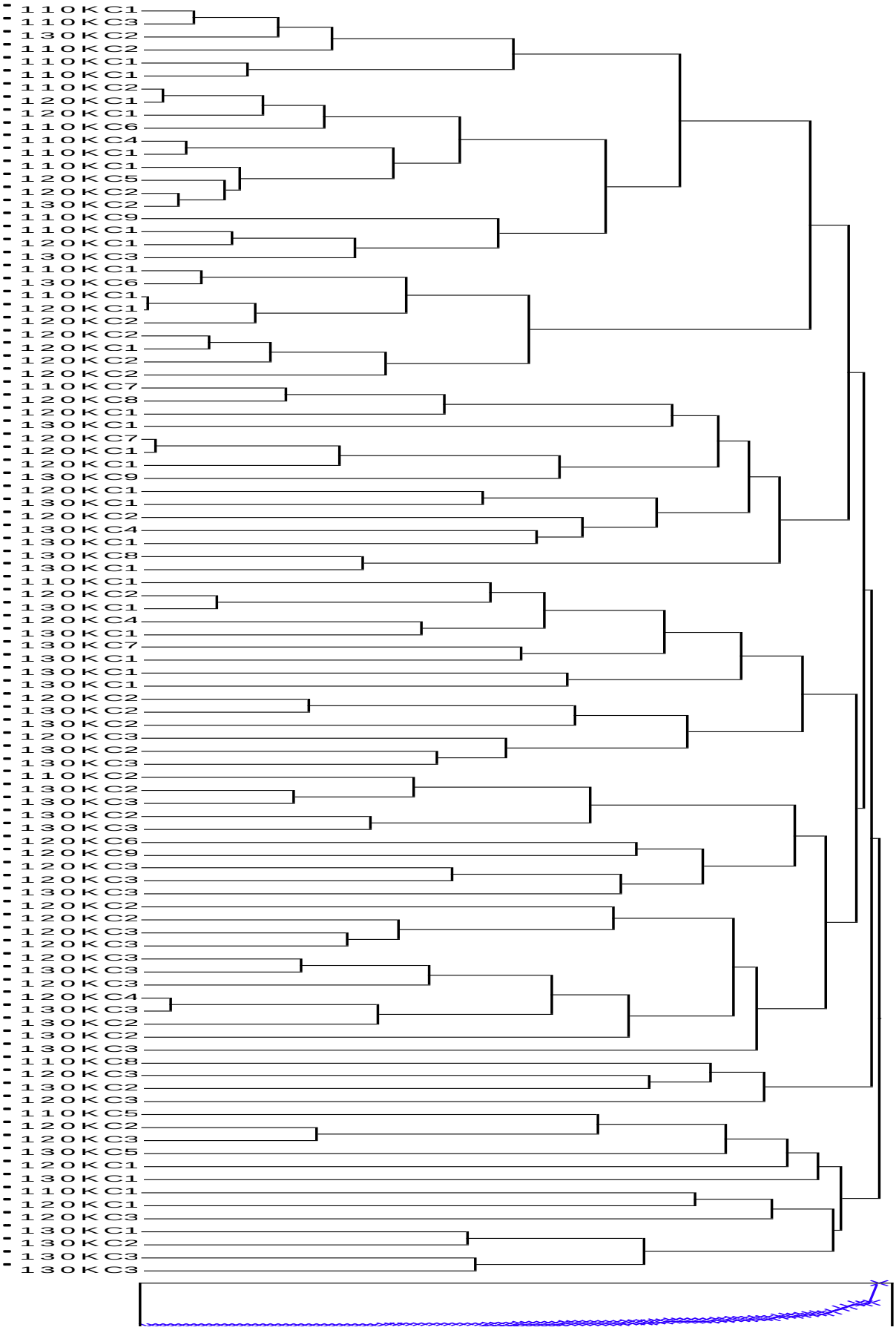

Figure 5. Dendrogram illustrating morphological dissimilarities of 99 hybrids of KM × CC at seedling stage.

morphology of more than 4000 leaves in more than 190 varieties were found significant differences in leaf morphology among the main citrus species groups, and hybrid varieties obtained through breeding exhibited intermediate leaf morphology compared to the parent citrus species. Çimen et al. (2016) evaluated 17 different morphologic characters such as shoot growth, leaf structure and thorniness in their studies.

In a cluster analysis based on morphological traits of 335 hybrids, it was seen that the hybrids obtained at 110 DAP were more distant relative to each other in all hybridization combinations. The diversity among genotypes also varied for all the leaf characters. Morphological analysis showed variation among hybrids. This variability of genotypes could be attributed to cross-pollination and it is very promising for breeders and growers.

4. Conclusions

The present study aimed to identify the diversity of citrus hybrids based on its morphological characters at seedling stage. A total of 335 putative interspecies hybrids, derived from 3 crosses were observed by their leaf morphology. According to our results, it can be concluded that wide diversity existed among the accessions with respect to quantitative leaf characters. In terms of leaf division, 268 genotypes have bifoliate and 67 were trifoliate in all combinations. In addition, 66 of the genotypes were thornless while 269 of the genotypes were thorny. The No. 4 genotype (120 DAP), found as triploid in CM × TC combination, has the longest and dense spines. The dissimilarity index in clustering analysis conclusion was between 0.004 and 17.318 within three hybridization combinations.

The morphological descriptions are very important regarding identification of citrus rootstocks and evaluating their characteristics in breeding programs and germplasm. However, selecting the hybrids only by leaf morphology, size, and growth habit is not always reliable. Because of the difficulty to visuall identification of some hybrids, modern techniques such as isoenzyme analysis and molecular analysis could be reliable.

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RESEARCH PAPER

Gene Expression Analysis of the Early Flowering 6 Homologues in Apricot Reveals Their Potential Role in Developmental Stages

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Abstract

In higher plants, regulation of gene expression and chromatin formation occurs by histone methylation and demethylation. Genes encoding JmjC-JmjN domains belong to the histone demethylase family and have an important role in the regulation of plant growth and development. *Early Flowering 6* (*AtELF6*), which encodes the JmjC-JmjN domain in *Arabidopsis thaliana*, is a demethylase that regulates growth and development as well as the transition to flowering, but it has not been identified in apricot so far. In this study, two genes homologous to *AtELF6* were identified for the first time in apricot. Gene expression analysis by RT-qPCR revealed that both *ELF6* homologs were expressed in 12 different developmental stages of three different tissues. The fact that both homologues were expressed, especially in the flower bud, suggested that they play a role in the transition to flowering, similar to *Arabidopsis thaliana*. In summary, the information obtained from this study will provide a unique resource for understanding the role of *ELF 6* in apricot growth and development, as well as for future functional characterization studies for the manipulation of the flowering transition.

1. Introduction

Epigenetic is change that represent enzymatically reversible modifications of gene expression without any changes in DNA sequences and can be passed on to future generations (Akimoto et al., 2007). Epigenetic mechanisms are classified as non-coding RNA editing, covalent modification of histones, nucleosome restructuring and DNA methylation (Allis and Jenuwein, 2016). The epigenetic mechanism activates or inhibits transcription by modifying histone proteins, regulating chromatin state, or directly recruiting specific effector proteins (Stricker et al., 2017). In plants, histone proteins undergo eight different epigenetic changes: methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, deamination and proline isomerization (Berger, 2007). Plants control their growth and development by spatiotemporally regulating gene expression with these epigenetic mechanisms (Yamaguchi, 2022).

Histone demethylation is one of the most complex epigenetic mechanisms that regulate plant development from flowering to fruiting and resistance to biotic and abiotic stresses (Klose and Zhang, 2007). Histone demethylation is a reversible process organized by demethylase (He et al., 2021). There are two classes of histone lysine demethylases in eukaryotes, Lysine-specific demethylase 1 (LSD1) and Jumonji C domain-containing demethylases (JMJ-C), and they are highly conserved in plants, yeast, and humans (Lan et al., 2008). Histone lysine demethylases (“erasers”) dynamically regulate methylation levels at Lys4 (K4), Lys9 (K9), Lys27 (K27), and Lys36 (K36) of histone H3 in *Arabidopsis thaliana* (Liu et al., 2010). In general, histone H3K9 and H3K27

methylation is characterized by transcriptionally silenced regions, and H3K4 and H3K36 methylation is characterized by active genes (Berger, 2007). Different studies have shown that H3K27me3 represses flower development genes in *A. thaliana* seedlings and must be reactivated by the addition of active chromatin marks for mature *Arabidopsis* plants to switch to flowering (Wang et al., 2016; Pfluger and Wagner, 2007). Among the H3K27me3 demethylases identified to date, three genes that have been shown to regulate floral development stand out: *Early Flowering 6* (*ELF6*)/*JMJ11*, *Relative of ELF6* (*REF6*)/*JMJ12* and *JMJ13* (Keyzor et al., 2021; Yamaguchi, 2022). Although changes in flower morphology have been observed in *elf6 jmj13 ref6* triple mutants in *A. thaliana*, the function of each demethylase in the control of flower development and self-pollination is not yet known (Yan et al., 2018). *AtELF6* and its closest homolog *AtREF6* encode nuclear proteins with JmjC, JmjN and zinc finger (ZnF) domains and are critical regulators of flowering time in *A. thaliana* (Lee et al., 2005; Metzger et al., 2005). In *A. thaliana*, ELF6 and JMJ13 are in an antagonistic relationship during the flower development stage. Compared to the wild type, increased self-fertility was observed in *elf6* mutants, while self-fertility was decreased in *jmj13* mutants (Keyzor et al., 2021). In addition, JMJ13 promotes stamen growth by activating the expression of *SAUR26*, while jasmonic acid suppresses carpel growth by activating signaling. Based on transcription data in *A. thaliana*, *ELF6* has been shown to promote carpel elongation by activating expansin genes (Yamaguchi, 2022; Keyzor et al., 2021). In angiosperms, *FLOWERING LOCUS T* (*FT*) is the key gene for the transition to flowering (Kobayashi et al., 1999), and *ELF6* delays flowering by demethylation of histones in the region where *FT* initiates transcription (Jeong et al., 2009). *BraELF6*, an *AtELF6* homolog in *Brassica rapa*, was cloned and functionally characterized by transferring it into *Arabidopsis elf6-4* mutants. The expression of *BraELF6* in T1 plants was relatively higher in transgenic plants, and later flowering was observed than in *elf6-4* mutants (Li et al., 2019).

Apricot, a member of the *Prunus* genus within the highly diverse *Rosaceae* family, is one of the most delicious and commercially traded fruits in the world (Erdogan-Orhan and Kartal, 2011). Based on data from the Food and Agriculture Organization Institutional Statistics Database, global apricot production in 2022 was determined to be 3,863,180 metric tons, up from 3,622,553 tons in 2021, with an increase of 6.6% (FAOSTAT, 2024). In addition to being consumed as a fruit, this type of drupe is also widely used for edible, cosmetic and medicinal purposes (Shi et al., 2023). However, late spring frosts severely damage the plant due to its short dormancy period and early flowering. In recent years, in order to solve this problem, the strategy of investigating key genes related to flower bud dormancy, control of flowering time and cold tolerance and determining their expression patterns has come to the fore. To date, although *ELF6* has been identified in model plants, especially *Arabidopsis*, and its relationship with flowering has been demonstrated, it has not yet been identified in the apricot and its possible roles have not been revealed. In this study, apricot homologues of *AtELF6*, which were shown to be involved in the epigenetic control of flowering, were identified by bioinformatics methods, and their expression patterns in 12 different developmental stages were analyzed. In addition to understand the possible roles of *ELF6* in apricot, this study will be a unique resource for future functional characterization studies to create later and/or earlier flowering apricot lines with genome editing techniques.

2. Material and Methods

2.1. Identification of *ELF6* genes in apricot

To identify ELF6 homologs in apricot, JmjC (PFAM02373) and JmjN (PFAM02375) domains were downloaded from the InterPro database (https://www.ebi.ac.uk/interpro/). Then, using the amino acid sequences of the JmjC and JmjN domains as queries, putative PaELF6 proteins were identified from the *Prunus armeniaca* genome database (https://www.rosaceae.org/) (Jung et al., 2019) by BLAST (Camacho et al., 2009). Additionally, the obtained proteins were re-examined using InterPro (https://www.ebi.ac.uk/interpro/) and SMART (http://smart.embl-heidelberg.de/) with an E value cut-off level of 1.0 and 5.0, respectively. After eliminating low similarity sequences, PARG18718m01 and PARG18718m02 sequences were identified AtELF6 homologues. These identified homologs were named *ParELF6-1* and *ParELF6-2*, respectively.

2.2. Plant materials and sample collection

In this study, 12-year-old apricot trees (*Prunus armeniaca* L. cultivar Sekerpare) that were fully productive and grown within the scope of standard horticultural practices in the garden of Burdur Mehmet Akif Ersoy University (Burdur, Türkiye) (latitude: 37° 41′ 27.4″; longitude: 30° 20′ 35.42″) were used. Tissues used in gene expression studies were collected in the following order in 2023; flower bud, leaf bud, young leaf (2 cm diameter), mature leaf (5 cm diameter), flower organs such as sepals, petals, stamens, carpels, young fruit (30 DAB), large green fruit (45 DAB), breaker fruit (60 DAB), mature fruit (75 DAB) and separated and frozen immediately in liquid nitrogen, and stored at -80°C until used. Each sample consisted of three biological replicates from three different trees, and each biological replicate contained at least three samples collected from one tree.

2.3. RNA extraction and RT-qPCR analysis

In this study, total RNA of developmental stages was isolated by a Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturers' instructions. Tissues from which total RNA was isolated were treated with RNase-Free DNase I (Norgen Biotek Corp., Thorold, ON, Canada) to eliminate DNA contamination. Total RNA quality was measured with microplate spectrophotometer (Epoch Microplate Spectrophotometer, Biotek Instruments, Inc.) for A260/A280 ratio and visualized by agarose gel electrophoresis. For cDNA synthesis, 1 µg RNA was reverse-transcribed using the VitaScript™ FirstStrand cDNA Synthesis Kit (Procomcure Biotech) according to the manufacturer's procedure. The RT-qPCR system contained 5 µl iTaq Universal SYBR Green Super Mix (Bio-Rad Laboratories, Hercules, CA, USA), 1 µl forward and reverse primers, 4.0 µl cDNA and 4.0 µl deionized water in a final volume of 10 µl.

The RT-qPCR procedure was devised as follows: 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 40 s. The apricot *Translation Elongation Factor 2* (*TEF2*) (Tong et al., 2009) and *Actin* (*ACT*) (Niu et al., 2014) genes were employed as internal controls for normalizing the transcript level of the target gene among different samples. Three biological replicates were performed for each gene, with each biological repeat having three technical replicates. The $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) was employed to calculate the relative expression levels of *ParELF6* genes. The primers for RT-qPCR analysis were designed using Primer-BLAST and showed Table 1. (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)

2.4. Statistical analysis

In this study, samples were taken at different developmental stages, following a randomized plot design involving three replications, and were examined using a general linear model. In significant cases, comparisons were made using the Fisher (LSD) test ($p < 0.05$). The error bars in the figures represents the standard error. Variance analysis and comparison tests were conducted using the statistical package program XLSTAT (v.2016.02.28451, Addinsoft, France). The graphs were created using the ggplot2 (Gómez-Rubio, 2017) and openxlsx packages in the R programming language, utilizing the RStudio platform (v.2023.12.1.402, Posit Software, PBC, Boston, MA) (Allaire, 2011).

3. Results and Discussion

RT-qPCR is a technique developed by combining PCR with fluorescence techniques and is widely used to analyze gene expression (Gibson et al., 1996; Tichopad et al., 2003). It is used to examine transcript levels in basic research due to its sensitivity, specificity, wide dynamic range and high throughput capacity (Karuppaiya et al., 2017). In this study, to reveal the possible functional roles of *ELF6* homologues in apricot, they were analyzed via RT-qPCR in 12 different developmental stages of leaves, flowers and fruits. As shown in Figure 1, it has been determined that the expression of *ParELF6* genes varies at different levels in leaves, flowers and fruits. Two of the three different leaves developmental stages showed similarities in *ParELF6* genes, but at the mature leaf stage, *ParELF6-2* showed higher expression. In flower bud and floral organs, both genes showed a decreasing pattern, except that *ParELF6-2* was higher in stamens. It was found that the expression of *ParELF6* genes gradually decreased in contrast to fruit ripening. Expression data analysis indicated that great differences in expression of *PaELF6* homologs in leaf, flower and fruit developmental stages, indicating that these genes participate in different developmental stages and may have important effects in adapting to physiological processes. To summarize, the high variance in the expression levels of these two *ELF6* homologs identified in apricot at 12 different developmental stages implied that these genes have more than one potential function in apricot development (Figure 1).

Li et al. (2019) measured the expression of the BraELF6 gene in four different organs at three different developmental stages (four-leaf seedling plant, bud stage, and flowering stage) in *Brassica rapa*. Accordingly, the expression of the BraELF6 gene gradually increased in the stem, leaf and flower during three developmental stages, while it remained constant in the roots. While BraELF6 was expressed three times higher in flowers than in roots

Table 1. Primer sequences specific to the *ParELF6* genes used in this study.

Primer name	Primer sequence (5'-3')
ParELF6-1-F	GAGGCTCAGAGTCCCCCATC
ParELF6-1-R	AGTGATCAGGGCTTTGGAGAGG
ParELF6-2-F	GACCGCCTAGGTCCAGTTTC
ParELF6-2-R	TGGCAGCTTCCCCACAGTTA
TRANSLATION ELONGATION FACTOR 2-F	GGTGTGACGATGAAGAGTGATG
TRANSLATION ELONGATION FACTOR 2-R	TGAAGGAGAGGGAAGGTGAAAG
ACTIN-F	GTTATTCTTCATCGGCGTCTTCG
ACTIN-R	CTTCACCATTCCAGTTCCATTGTC

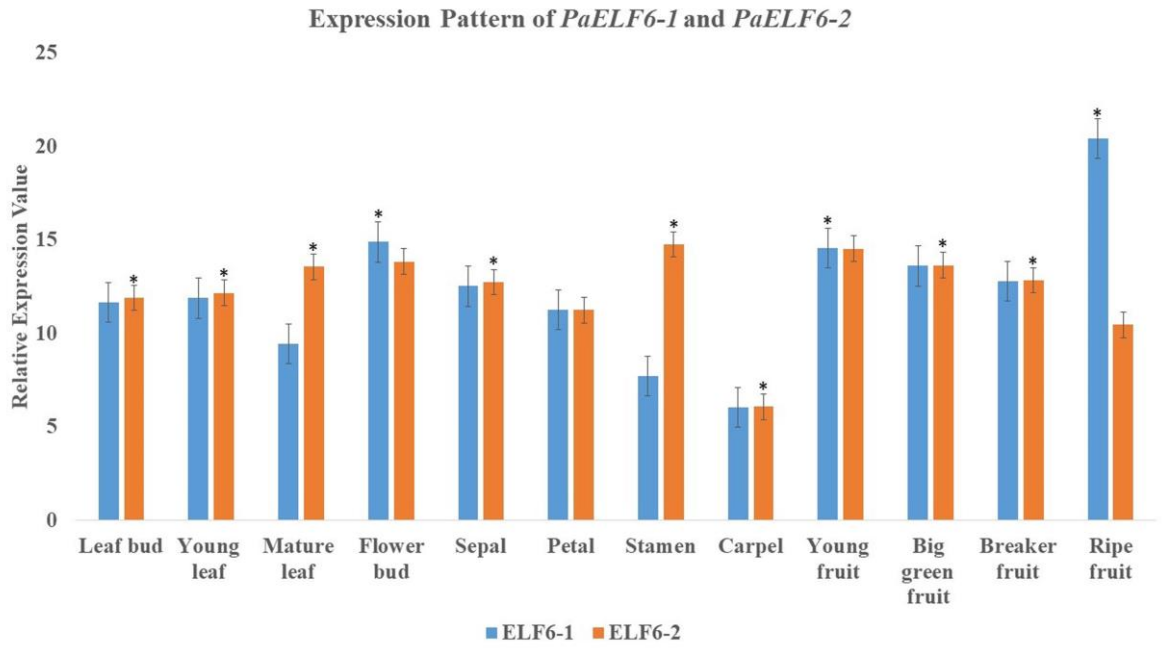

Figure 1. The expression profiles of *ParELF6* genes at different developmental stages analyzed by RT-qPCR.
Bars represent the mean of replicates ± standard error and indicate a significant difference at $P < 0.05$, determined by Fisher (LSD) test. Leaf, flower and fruit development stages are shown with green, red and orange bars, respectively.

at the flowering stage, its expression in stems was found to be similar to that in leaves. It was observed that the expression of BraELF6 was at its highest level in the floral tissue at three growth stages and increased gradually. There was an 11-fold higher expression of BraELF6 in floral tissue at the flowering stage than in roots, which suggested by the authors that the BraELF6 gene in *Brassica rapa* is associated with flowering. These differences in expression between apricot and *Brassica rapa* can be explained by the fact that both species belong to different families and have different growth patterns.

Transcriptomic is defined as a snapshot of gene expression in a specific tissue, provided by capturing the total RNA within that tissue at a specific moment in time, and is an important analysis for understanding plant growth and development (Ward et al., 2012). According to transcriptome data, ELF6 is expressed at 47 different developmental stages in *A. thaliana*, with the highest expression in the dry seed and the lowest in the vegetative rosette stages (Winter et al., 2007). In *Solanum lycopersicum* cv. Heinz, the expression of ELF6 peaked in the root and fully opened flower. Conversely, in *Solanum pimpinellifolium*, the expression is the highest on the fifth day of breaker fruits and in the leaf (Shi et al., 2012). In heterozygous diploid *Solanum tuberosum*, ELF6 is expressed in the root, tuber and shoot apex, from high to low, respectively (Massa et al., 2011). In *Oryza sativa*, ELF6 is expressed highest in the young flowering stage and lowest in the flowering stage (Jain et al., 2007). To summarize, information from both RT-qPCR and transcriptome data reveals that ELF6 is an important actor of growth and development in the plant kingdom. However, further functional characterization studies, such as mutant or gene editing, are needed to validate the information obtained from both RT-qPCR and transcriptome studies.

4. Conclusion

In this study, ELF6 gene homologues, which play a critical role in the epigenetic regulation of the flowering transition in *Arabidopsis thaliana*, were identified for the first time in apricot. Expression patterns were revealed by RT-qPCR in 12 different developmental stages of three different organs of apricot. According to the analysis, both ELF6 homologs, ParELF6-1 and ParELF6-2, showed strong expression at all developmental stages. The information obtained from this study will be a unique resource for understanding the epigenetic regulation of apricot flowering and for obtaining early or late flowering cultivars in the future by genome editing methods such as CRISPR.

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RESEARCH PAPER

HORTICULTURAL STUDIES

QTL-Seq: Rapid, Cost-Effective, and Reliable Method for QTL Identification

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Abstract

QTL-seq is a powerful method that integrates whole-genome sequencing (WGS) with bulk-segregant analysis to rapidly and reliably identify quantitative trait loci (QTLs) associated with specific traits. This approach significantly advances traditional QTL mapping by eliminating the need for genome wide DNA markers such as SSR, RFLP, and INDELs, which are typically used in linkage-based QTL mapping. Instead, QTL-seq leverages WGS to detect all genetic variations such as SNPs, Indels, and Structural Variants across the entire genome, providing a comprehensive resource for marker development in marker-assisted selection. The QTL-seq process begins with the creation of genetically diverse mapping populations, such as F_2 or RILs, followed by detailed phenotypic characterization. DNA from plants exhibiting similar phenotypes is pooled into bulk groups and sequenced, allowing for cost-effective and efficient QTL identification. Identified QTLs can be further validated through fine mapping using recombinant screenings and progeny testing, leading to the identification of candidate genes associated with traits of interest. In this study, we outline a user-friendly QTL-seq pipeline, from sequencing to data visualization to demonstrate its practical application. While the manuscript primarily focuses on describing the pipeline, we also conducted a case study analysis with real data to showcase its effectiveness. Our work contributes to the broader understanding of QTL-seq applications and offers practical recommendations for optimizing this method in future breeding programs.

1. Introduction

The current world population of 8.1 billion people as of May 2024 is estimated to reach 9.8 billion by 2050, hence humanity has to find sustainable ways to feed an extra 1.8 billion mouths (UN DESA, 2017). This situation underscores the urgent need for innovative agricultural practices, improved crop varieties with superior yield and resistant to biotic and abiotic stresses. Moreover, the issue is compounded by the gradual reduction in the amount of land available for agriculture (Godfray et al., 2010). In crop plants, many agronomically important traits such as yield, grain size, fruit weight, and plant height are governed by the collective effects of several genes with smaller effects called as quantitative trait loci or QTLs (Falconer, 1996). The QTL-identification is an arduous task yet of paramount importance for genetic enhancement of many important crops. Once these QTLs are identified, the next step is the integration of favorable alleles of QTLs into elite germplasm

mostly via backcrossing with the help of marker assistant selection (Collard and Mackill, 2008; Ribaut and Hoisington, 1998). One of the oldest yet reliable QTL-mapping approaches was linkage-based QTL mapping, in which DNA markers are tightly linked to targeted QTL. However, limitations in linkage mapping such as a restricted number of DNA markers, low marker density across the entire genome, long duration required for developing mapping populations, and difficulty in capturing all the recombination events, presence of heterogeneity in early generations (Abdurakhmonov and Abdukarimov, 2008; Madhusudhana, 2015) prompted researchers to seek alternative rapid, cost-effective, and reliable methods. QTL-seq was introduced by Takagi et al. (2013) more than a decade ago and offered as an alternative tool that may overcome these above-mentioned hurdles. This method simply relies on the advantages of next generation sequencing and bulk segregant analysis (BSA). The BSA method involves selecting individuals with extreme phenotypes from a segregating population, after which the DNA from these selected plants is pooled together into two separate bulks based on the phenotype. Each bulk is expected to be genetically identical within the regions linked to the target trait but different from the other bulk in these regions. This genetic difference between the two bulks is used to identify markers associated with the trait of interest. Essentially, the two pooled DNA samples are genetically identical (monomorphic) except for the regions linked to the trait, where they exhibit genetic dissimilarities (heterozygosity). The advances in whole-genome sequencing have opened a new era for plant breeders. This is mostly because several accessions have been re-sequenced and high-quality reference genomes for many crops such as tomato (Tomato Genome Consortium, 2012), maize (Jiao et al., 2017), rice (Kawahara et al., 2013), soybean (Schmutz et al., 2010), arabidopsis (Cheng et al., 2017) have become available over the past years. Another key component of the QTL-seq is BSA, which is introduced early in 1990s to map a downy mildew resistance in lettuce (Michelmore et al., 1991). In this method, individuals displaying extreme phenotypes are selected from a segregating population, after which the DNAs from these plants are bulked together. Within each pool, the plants are assumed to be genetically identical for a target region, but the pools themselves are dissimilar, variants used for developing markers are polymorphic and highly associated with the trait of interest (Takagi et al., 2013; Wang and Wang, 2023). In other words, two pooled DNA samples exhibit genetic dissimilarities solely within the targeted region, appearing heterozygous and monomorphic for all other regions. Even though BSA offers numerous advantages, genotyping of each marker mostly based on restriction fragment length polymorphism (RFLP) or simple sequence repeat (SSR) for the two bulked DNAs is still a laborious and limiting factor. In contrast to RFLP and SSR commonly used in the past, single nucleotide polymorphisms (SNPs) have numerous advantages due to their abundance, high-throughput genotyping capabilities, cost-effectiveness, and genome-wide distribution (International Rice Genome Sequencing Project, 2005; Nelson et al., 2004; Seeb et al., 2011; Singh et al., 2013). Therefore, BSA equipped with next-generation sequencing is capable of rapid, cost-effective, and reliable QTL mapping in various crops. To date, numerous traits have been mapped and utilized in plant breeding studies. Some of these traits were summarized in Table 1.

The main goal of this research is to present a comprehensive and user-friendly QTL-seq pipeline that encompasses all stages from sequencing to data visualization. By leveraging the methodology and data from Takagi et al. (2013), we aim to provide a clear and practical framework for implementing QTL-seq in plant breeding. Through a detailed case study analysis, we demonstrate the pipeline's effectiveness and offer insights for optimizing this approach, thereby advancing the application of QTL-seq in future breeding programs.

2. Material and Method

2.1. DNA extraction procedures and library preparation for sequencing

The DNA isolation and library preparation determines the success of the following steps. Hence, a high-quality DNA (high molecular weight and contaminant-free such as polysaccharides or phenolics) must be extracted with kits such as DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA), Genomic DNA Purification Kit (Thermo Scientific™ Waltham, Massachusetts, USA), and Quick-DNA Plant/Seed 96 Kit (Zymo Research, Irvine, California, USA). Before NGS library preparation, it is essential to quantify both the quality and quantity of DNA from the selected individuals using NanoDrop ND-1000 spectrophotometer (Thermo Scientific) to ensure that the UV absorbance A260/A280 ratio falls within the range of 1.8 and 2.0 and A260/A230 ratio ≥ 1.5). Moreover, Qubit 2.0 Fluorimeter (Invitrogen, Carlsbad, CA, USA) could also be employed for the same reason. With respect to library preparation, NEBNext Ultra™ II DNA Library Prep Kit (New England Biolabs, USA) in conjunction with barcoded primers from the NEBNext® Multiplex Oligos obtained from Illumina kits (New England Biolabs, USA) could be used.

2.2. Comparative variant analysis

Whole genome sequencing can be performed using platforms such as the Illumina NextSeq 550,

Table 1. Summary of QTL-seq studies.

Crop	Trait of interest	Population size	Generations	QTL interval Mb	Reference
Rice	*Magnaporthe oryzae* (rice blast) resistance	n=241	RILs	Chr 6 2.39 to 4.39	Takagi et al. (2013)
	Seedling vigor	n=531	F_2	Chr 3 36.21 to 37.31	Takagi et al. (2013)
	Salt tolerance	n=199	$F_{2:3}$	Chr 7 20.16 to 24.33	Lei et al. (2020)
	Grain length and weight	n=176	NIL-F_2	Chr 5 15.00 to 20.00	Yaobin et al. (2018)
Cucumber	Early flowering	n=232	F_2	Chr 1 22.86 to 26.31	Lu et al. (2014)
	Pre-harvest sprouting	n=298	F_2	Chr 4 7.30 Mb[a]	Cao et al. (2021)
				Chr 5 0.15 Mb	
Tomato	Heat-tolerance	n=200	F_2	Chr 1 23.80 to 63.52	Wen et al. (2019)
				Chr 2 38.98 to 40.85	
				Chr 7 10.08 to 52.20	
	Fruit weight	n=100	F_2	Chr 1 12.48 to 51.58	Illa-Berenguer et al. (2015)
	Fruit weight	n=100	F_2	Chr 11 49.73 to 51.35	
	Fruit weight	n=200	F_2	Chr 03 60.86 to 61.72	
	Locule number			Chr 2 33.67 to 35.30	
	Locule number			Chr 5 3.25 to 3.98	
	Locule number			Chr 6 41.16 to 43.93	
	Blossom-end rot	n=192	F_2	Chr 3 54.21 to 59.89	Topcu et al. (2021)
				Chr 11 48.13 to 52.12	
	Yellow shoulder disorder	n=192	F_2	Chr 1 21.36 to 55.92	Topcu (2024)
				Chr 4 30.57 to 53.50	
				Chr 11 51.33 to 53.26	
Chickpea	Seed weight	n=221	F_4	Chr 1 0.84 to 0.87	Das et al. (2015)
Groundnut	Rust resistance	n=268	RIL	Chr A03 131.60 to 134.66 Mb	Pandey et al. (2017)
	Late leaf spot resistance	n=268	RIL	Chr A03 131.67–134.65 Mb	
Melon	Stigma color	n=150	F_2	Chr 6 141.48–152.83 cM	Qiao et al. (2021)
				Chr 8 19.71–57.33 cM	
Peanut	Seed weight	n=242	RIL	Chr A05 101.70−111.64 Mb	Wang et al. (2022)
				Chr B02 103.90−111.75 Mb	
				Chr B06 0.30−50.22 Mb	
Maize	Semi-dwarfism	n=533	F_2	Chr 9 111.07 to 124.56 Mb	Chen et al. (2018)
Soybean	Two-seed pod length		BC_3F_{2-n}	Chr03 0.50 to 4.76 Mb	Xie et al. (2021)
				Chr11 3.38 to 7.06 Mb	
				Chr12 9.72 to 11.25 Mb	

[a]Results were given as interval.

1000, and 2000, which utilize paired-end 150 base pairs (bp) (PE150) flow cells. Once sequencing procedure is finished, the raw fastq.gz files can be downloaded directly from the sequencing webpage using the "*wget [option] [URL]*". Before proceeding with further analysis, the FASTQ files are suggested to be filtered and trimmed, which can be done using Trim Galore (version 0.6.5, https://github.com/FelixKrueger/TrimGalore) to ensure a minimum quality value of 28. For this purpose, the following command "trim_galore --paired file_R1.fastq.gz file_R2.fastq.gz --quality 28 --fastqc --stringency 3 --length 60 --illumina" could be used, in which "--quality 28" removes low-quality ends from reads based on the phred score threshold of 28, "--fastqc" runs the FastQC in the default mode on the FastQ files once trimming is completed, "--paired" specifies the paired sequencing files, "--illumina" trims the first 13bp of the Illumina universal adapter 'AGATCGGAAGAGC', "--length 60" discards reads that became shorter than 60bp, "--stringency 3" enables that a minimum of 3 base pairs of the adapter must be present for it to be trimmed. The next step involves aligning the remaining high-quality reads to the reference genome which can be downloaded from public databases using "wget". This reference genome can either be one of the parental accessions to be sequenced along with the bulks or a high-quality reference genome. Before aligning with the bowtie2 (Version 2.4.1) (Langmead and Salzberg, 2012), or SpeedSeq (Chiang et al., 2015), reference genome should be indexed using the "bowtie2-build reference_sequence.fasta index_name" where reference_sequence.fasta is the reference genome fasta file to be indexed, and index_name is the output name. After indexing is done, the aligning can be performed using the following command line "bowtie2 -p 8 n -x index_name -1 file_R1.fastq.gz -

2 file_R2.fastq.gz -S output.sam*"*. In this command line, "-p" is the number (8) of processors/threads used, "-x" is the genome index, "-1 file_R1.fastq.gz" is the file of first paired end read, "-2 file_R2.fastq.gz" is the file of second paired end read, and "-S output.sam" is the output alignment in sam format. Next, the "output.sam" files need to be converted to BAM files using samtools (version 1.16.1) (Li and Durbin, 2009). To achieve this step, the following command line "samtools view -@ 10 -bS output.sam > output.bam" can be utilized. While "-@ 10" defines the number of threads which in this case is 10, -bS defines the output in the BAM format and ignores the compatibility with previous samtools versions. This step is followed by sorting of the bam files using "samtools sort -@ 10 -m 3G output.bam -o output_sorted.bam", in which "-m*"* defines the maximum required memory per thread to be used and "*-o" writes* the final sorted output. Upon indexing the sorted bam files with following command "samtools index output_sorted.bam" Picard tools (Picard version 2.27.5) (https://broadinstitute.github.io/picard/) will be employed to replace read groups and identify duplicate reads. To achieve this step, the following command "java -jar $EBROOTPICARD/picard.jar AddOrReplaceReadGroups --INPUT= output_ sorted.bam --OUTPUT=output_sorted.RG.bam --RGID=4 --RGSM=output --RGLB=output --RGPL=ILLUMINA --RGPU=ignore" and "java -jar $EBROOTPICARD/picard.jar MarkDuplicates INPUT= output_sorted.RG.bam OUTPUT= output_sorted_mkdupl.RG.bam METRICS_FILE= output_sorted_mkduplMetrics.txt*"* can be used. While *"*AddOrReplaceReadGroups" consolidates all the reads in a file under a singular new read-group, "MarkDuplicates" locates, and tags duplicate reads in a BAM-files. The command "*java -jar $EBROOTPICARD/picard.jar*" utilizes Java to run a JAR file named picard.jar, which is located in the directory specified by the environment variable $EBROOTPICARD. In the command lines, "*--INPUT*" shows Input file, "*--OUTPUT*" designates Output file, "*--RGID*" defines Read-Group ID, "*--RGSM*" displays Read-Group sample name, "*--RGLB*" denotes Read-Group library, "*--RGPL*" illustrates Read-Group platform (such as ILLUMINA and SOLID) and finally "*--METRICS_FILE*" specifies the file where metrics about the duplicates will be written. These metrics may contain data such as the count of identified duplicates, their respective locations, and other pertinent statistical information. After completing the previous step, the next step involves indexing the sorted and marked BAM file. This is accomplished by executing the command "*samtools index output_sorted_mkdupl.RG.bam*".

2.3. Variant calling

The variant calling is of utmost importance since QTL-seq heavily depends on the variance between created bulks. Hence, to get reliable results and enhance the accuracy, we must annotate potential insertions/deletions (INDELs) or misalignments accurately. The first step in variant calling pipeline begins with reference genome indexing. The reference genome can be indexed with "SAMtools" developed by Li and Durbin (2009) using the "*Samtools faidx reference_sequence.fa*" command. The INDEL realignment is performed utilizing the Genome Analysis Toolkit (GATK, Version 3.8-1) (McKenna et al., 2010) by following the commands " -T RealignerTargetCreator" which identifies what regions need to be realigned and "-T IndelRealigner" that performs the actual realignment. Both determine false positive SNPs and perform a local realignment in a sequencing dataset. While the first command, "*java -Xmx150g -jar $EBROOTGATK/GenomeAnalysisTK.jar -T RealignerTargetCreator -R reference_sequence.fa -I output_sorted_mkdupl.RG.bam -o output _intervals.list*" creates a list of target intervals for the following step, the second command "*java -Xmx150g –jar$EBROOTGATK/GenomeAnalysis TK.jar -T IndelRealigner -R reference_sequence.fa -I output_sorted_mkdupl.RG.bam -targetIntervals output_intervals.list -o output_realigned_reads .bam*" executes the real realignment of reads based on the target intervals. In both commands abovementioned, "*-Xmx*" defines the memory to be allocated, "*-R"* designates the reference genome to be used, "*-I*" describes the input BAM file containing aligned reads, "*-targetIntervals"* designates the interval file generated from the RealignerTargetCreator step and finally "*-o"* specifies the output file where the information about potential realignment sites will be stored. Before proceeding to the final step of variant calling, the output of the previous command (*output_realigned_reads.bam*) needs to be indexed. The final command in variant calling step utilizes GATK to call haplotypes from aligned reads in the "*output_realigned_reads"* BAM file. The command is "*java -Xmx150g -jar $EBROOTGATK/GenomeAnalysisTK.jar -T HaplotypeCaller -nct 10 -R reference_sequence.fa -I output_realigned_reads.bam –emitRef Confidence GVCF --variant_index_type LINEAR --variant_index_parameter 128000 -o raw_ variants_gvcf.vcf*". In the command line, "*-T HaplotypeCaller"* specifies the tool as HaplotypeCaller, which identifies potential variants. Furthermore, "*-nct 10"* indicates the number of CPU threads to use for parallel execution, "*-R"* refers to reference genome fasta sequence, "*-I*" designates Input BAM file, the "emitRefConfidence" option emits reference confidence scores for each site in the (Genomic Variant Call Format) *GVCF* file, providing information about the likelihood that a particular reference allele is actually present at a given genomic position. "Variant_index_type *LINEAR*" parameter specifies the indexing strategy as LINEAR, meaning that variants are indexed sequentially according to their genomic position for

the output GVCF. The final parameter required in GATK versions older than 3.4 is "*variant_index_parameter 128000*" indicating the size of the bins used in the linear indexing strategy.

2.4. Combining variant callings and filtering

In QTL-seq studies, two representative bulks are typically created to identify genomic regions associated with the trait. In the following command, the variant calls that were previously done for each bulk separately are merged into a single VCF file for the downstream analysis. The command is "*java -Xmx150g -jar $EBROOTGATK/GenomeAnalysisTK.jar -T GenotypeGVCFs -R reference_sequence.fa --variant raw1_variants_gvcf.vcf --variant raw2_variants_gvcf.vcf -nt 10 -o merged.vcf".* While *"-T GenotypeGVCFs"* parameter specifies the tool in GATK being used to perform joint genotyping that involves combining variant calls from multiple samples on GVCF files generated by HaplotypeCaller, "*--variant"* parameter designates which files need to be merged. The following command "*java -Xmx150g -jar $EBROOTGATK/GenomeAnalysisTK.jar -T SelectVariants -R reference_sequence.fa -V merged.vcf -selectType SNP -o SNPs.vcf*" is used to extract SNPs from merged variant calling VCFs, in which "*-T SelectVariants*" indicates the tool being used in GATK that allows selection of specific variants whereas "*-selectType SNP" or "--select-type-to-include SNP"* selects SNP variant from the supplied VCF file, designated by "-V". Once SNPs have been selected, the subsequent steps involve identifying and flagging SNPs with poor quality based on genotype quality, read depth, allele frequency, and various annotation scores, and then filtering them out. This filtering step is crucial to identify high quality SNPs that can be converted into genotyping markers such as KASP (Kompetitive Allele-Specific PCR). To tag low quality SNPs, the following command can be used "*java -Xmx150g -jar $EBROOTGATK/GenomeAnalysisTK.jar -T VariantFiltration -R reference_sequence.fa -V SNPs.vcf --filterExpression "QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0" --filterName "Default_recommended" -o Filtered_snps.vcf".* In the command line, "*-T VariantFiltration*" indicates the tool in GATK being used to filter variants, *"-V SNPs.vcf"* shows the input VCF file containing SNPs that need to be filtered, "*--filterExpression "QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0"* defines the filtering criteria based on QD < 2.0: Variant Quality by Depth (QD) less than 2.0, FS > 60.0: FisherStrand (FS) greater than 60.0, MQ < 40.0: Mapping Quality (MQ) less than 40.0, MQRankSum < -12.5: Mapping Quality Rank Sum Test less than -12.5, ReadPosRankSum < -8.0: Read Position Rank Sum Test less than -8.0. Furthermore, "--filterName "Default_recommended" defines the name of the filter to be applied to variants. The next step involves filtering using VCFtools (version 0.1.16) (Danecek et al., 2011). To keep only high quality SNPs and the following command "vcftools --vcf *Filtered_snps.vcf* --remove-filtered-all --recode --max-missing 1 -c > Filtered_passed_snps.vcf" is performed, in which "vcftools" defines which tools to be used in VCFtools, *"--vcf Filtered_snps.vcf"* specifies the input VCF file containing variants that need to be filtered, *"--remove-filtered-all"* removes all variants that have been flagged as filtered by previous filtering steps, *"--recode"* forces VCFtools to output the filtered variants into a new VCF file as *"Filtered_passed_snps.vcf*" designated in the command line. The last criteria are "*-max-missing 1*" that filters variants where more than one sample has missing data, and "-c" defines the output as compressed VCF files. The steps described above are summarized in Figure 1.

Additionally, a master script detailing each step is provided in Supplemental File 1. Using this script we re-analyzed the QTL-seq data (Takagi et al., 2013) which identified a QTL located in the 2.39 to 4.39 Mb region on chromosome 6, associated with partial resistance to *Magnaporthe oryzae,* the causal agent of rice blast disease in the rice. The final VCF file that shows the SNPs and INDELs between R-bulk (Mainly Nortai-type genomic segments) and S-bulk (Mainly Hitomebore-type genomic segments) was given in (Supplemental File 2).

3. Results and Discussion

The last step in the QTL-seq pipeline is visualizing the SNP allele frequencies or SNP-indexes along the genome and identify QTL regions associated with the trait of interest. This visualization can be done using an R package called QTLseqr (Mansfeld and Grumet, 2018). Since, the R package requires a tabular file format, we need to convert VCF file that has the SNP variants identified between two bulks into tabular format using following command "*java -jar $EBROOTGATK/GenomeAnalysisTK.jar -T VariantsToTable -R reference_sequence.fa -V Filtered_passed_snps.vcf -F CHROM -F POS -F REF -F ALT -GF AD -GF DP -GF GQ -GF PL -o QTL-seqr.table*". While *"-T VariantsToTable"* in the command line designates the tool that converts the variant information from VCF format to a tabular format, "*-R"* defines the reference fasta, "*-V*" specifies the input VCF file containing the filtered SNP variants. Further, *"-F CHROM -F POS -F REF -F ALT"* specifies the components such as chromosome, position, reference allele, and alternate allele of each variant to be included in the output table. Finally, *"-GF AD -GF DP -GF GQ -GF PL"* defines the genotype fields (GF) to be included in the output table such as allelic depths (AD), total read depths (DP), genotype quality (GQ), and

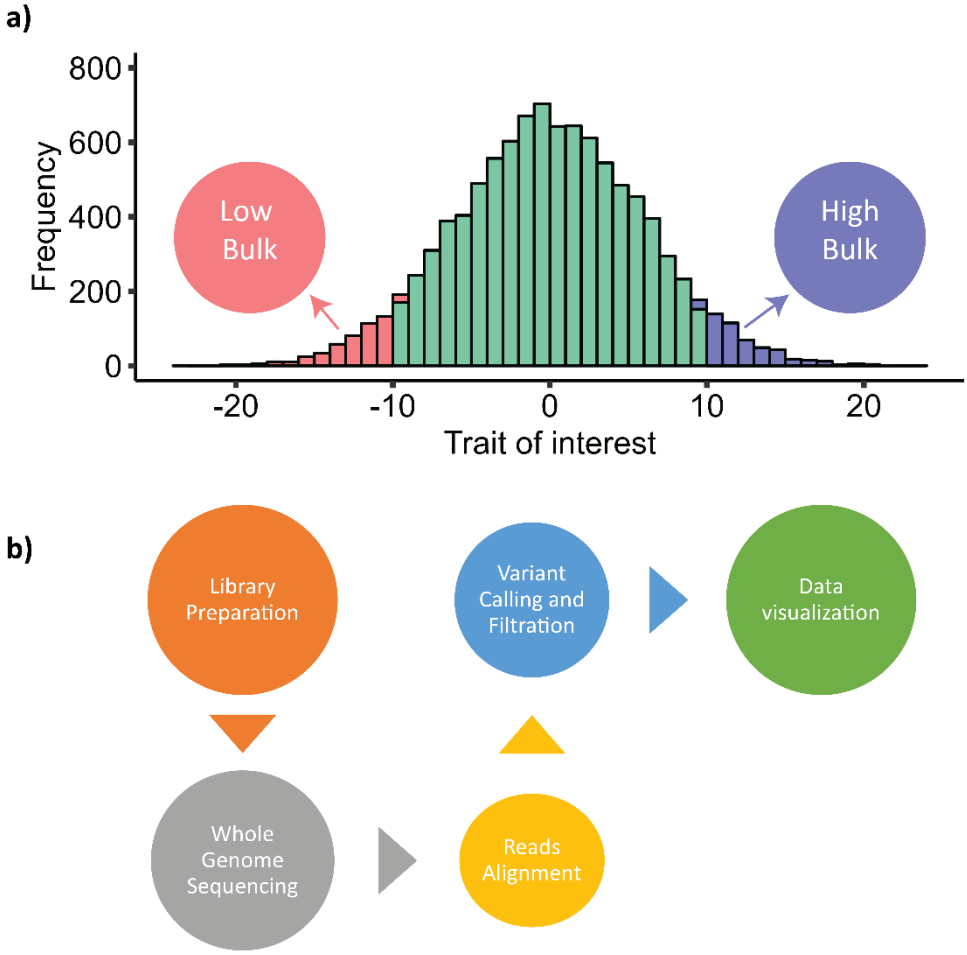

Figure 1. General outline of a QTL-seq script. a) The phenotypic distribution of a hypothetical mapping population. A dataset of 10,000 continuous values was generated using a normal distribution with a mean of 0 and a standard deviation of 6. A seed (set.seed (123)) was used to ensure reproducibility. The 5th percentile of the data defined the lower extreme values (low bulk), while the 95th percentile defined the upper extreme values (high bulk). b) The workflow began with library preparation for each bulk, followed by whole genome sequencing to generate raw reads. These reads were then aligned to a reference genome, and variant calling was used to identify genetic variants (SNPs). The process concluded with data visualization for the analysis and presentation of the results.

phred-scaled likelihoods (PL) for each genotype. The corresponding "*QTL-seqr.table"* file for the rice data was also given as Supplemental File 3. In the QTL-seqr package, further filtering steps can be used based on reference allele frequency, maximum total depth, minimum total depth, sample depth and genotype quality. After desired filtering criteria are met, the "runQTLseqAnalysis() function "can be implemented with some minor changes to original pipeline of (Takagi et al., 2013). The modified "R" script that contains further filtering and QTL-visualization steps was given in Supplemental File 4. We successfully mapped the fungal rice blast disease QTL, *qPi-nor1(t)*, with our script and validated the results obtained by Takagi et al. (2013). The rice blast disease trait, which was used to test our QTL-seq analysis, was estimated to exhibit moderate broad-sense heritability (54.16%) previously (Salleh et al., 2022), underscoring the genetic basis of this trait. The corresponding QTL-seq results were given in Supplemental Figure 1. We identified two QTLs associated with the blast resistance Figure 2. Although the previously identified QTL on chr 6, *qPi-nor1(t)*, was located between 2.39–4.39 Mb ($P < 0.01$), we defined the border of *qPi-nor1(t)* as 2.50- 5.39 ($P < 0.01$) Figure 2a. In addition, we identified another QTL (named as *blast9.1)* on chr9, which locates between 9.28-10.20 ($P < 0.05$) Figure 2b.

The power of next generation sequencing, especially the advances in long and short read sequencing with reduced costs, has opened a new era for QTL mapping and dramatically changed the way of crop breeding practices and genetic studies in various organisms (Varshney et al., 2009; Kim et al., 2016; Varshney et al., 2020). Once more plant genome assemblies along with complete annotations are readily available in plant science, numerous QTL mapping methods have been proposed, and several innovative concepts have been introduced to map QTLs (Bazakos et al., 2017; Wang & Han, 2022). SHOREmap, introduced by Schneeberger et al. (2009) can be seen a corner stone as it was one of the original approaches that

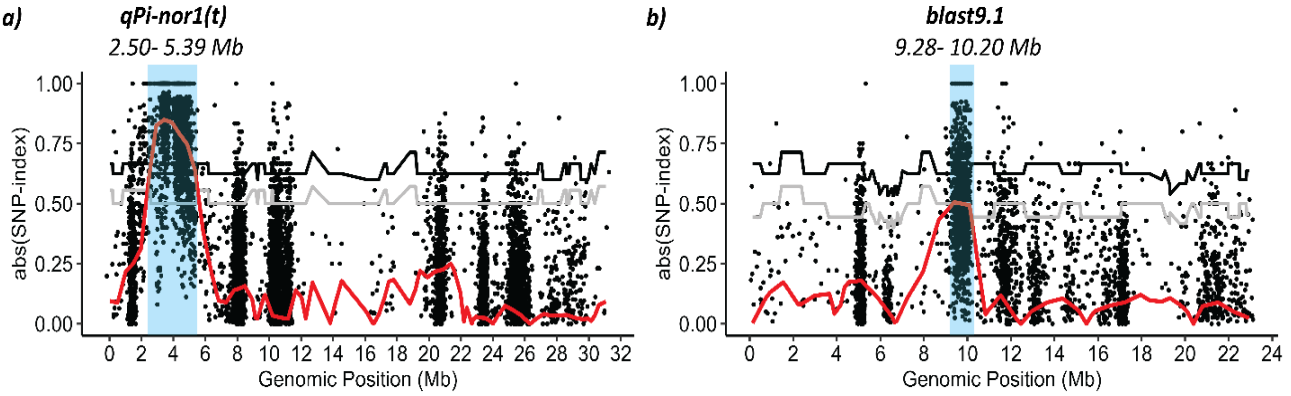

Figure 2. QTL-seq identifies qPi-nor1(t) and blast9.1 QTLs associated with Magnaporthe oryzae (rice blast disease) resistance on a) chr 6 and b) chr 9, respectively. The tricube-smoothed absolute Δ(SNP-index) is shown in red, while confidence intervals of $P < 0.05$ and $P < 0.01$ are depicted in grey and black lines, respectively. The X-axis represents the genomic position in megabases (Mb), and the Y-axis shows the absolute Δ(SNP-index) values. The blue shaded areas on chr6 and chr9 show the QTL-intervals for qPi-nor1(t), and blast9.1 responsible for the rice blast disease.

integrates whole genome resequencing and phenotyping in a large pool of recombinants. Moreover, BSA equipped with genome analysis using microarray-based genotyping or massively parallel sequencing was another pioneering approach that was focusing on mapping of QTLs with minor effects (Ehrenreich et al., 2010). This method, called as Extreme QTL mapping (X-QTL), has three main components. Creating of a large segregating population and selecting progenies from this large mapping population with extreme trait values for comprehensive analysis are of foremost importance for the method (Ehrenreich et al., 2010). The last component is microarray-based genotyping or massively parallel sequencing of pooled allele frequencies. In a similar manner, Next Generation Mapping (NGM) approach, introduced by Austin et al. (2011), detects mutations by sequencing a small pooled F_2 population, without prior knowledge of genetic analysis. Following these ideas, Abe et al. (2012) developed MutMap, a method based on whole-genome resequencing of pooled DNA from a segregating plant population. While MutMap offers significant utility, crop breeding has predominantly relied on QTL breeding, leveraging genetic variations among diverse cultivars and species. Hence, examining QTL variations in natural variants is highly essential for identifying important alleles of genes controlling essential agronomic traits and enhancing breeding efforts. By combining the power of next-generation sequencing with BSA, Takagi et al. (2013) proposed the QTL-seq method as reliable, quick and most importantly cost-effective approach to QTL mapping, leading the way for significant enhancements in crop improvements and sustainable agriculture. Until now, numerous agronomically important traits have been successfully mapped using QTL-seq, and researchers were able to rapidly fine-map and ultimately identify candidate genes in many agronomically important crops (Table 1).

The effectiveness of QTL-seq is mostly determined by the population size, the heritability of the trait, the percentage of plants chosen for each bulk and population structure (e.g., F_2, F_5, NILs or RIL). In addition to these factors, the nature of the trait whether it is governed by single major QTL or many QTLs with minor effects plays a crucial role. Moreover, read depth of the sequencing along with recombination frequency are also important factors. Furthermore, and more importantly, the inheritance of traits, including various forms such as complete dominance, incomplete dominance, overdominance, additive effects, recessive effects, and epistasis, plays a critical role in determining the success of QTL-seq (Takagi et al., 2013). The way these inheritance patterns exhibits in a given population can significantly impact the identification and mapping of QTLs. For example, additive effects allow for a more straightforward association between genotype and phenotype, while dominance and epistasis can complicate QTL detection. Additionally, gene-by-environment interactions (GxE) further influence trait expression, adding another layer of complexity to QTL-seq analysis. These genetic factors, along with the heritability of the traits, precision and depth of sequencing, size of the mapping population, and accuracy in phenotyping, are all crucial components that contribute to the identification of significant QTLs and understanding their effects across various genetic backgrounds and environmental conditions. Based on the previous studies, a minimum population size of 200 is mostly used for QTL mapping, although successful QTL identification has been achieved even with population sizes as small as 100 in tomato (Table 1). The second consideration is the percentage of individuals included in each bulk. Based on a study conducted by Takagi et al. (2013), it was recommended to bulk 10-15% of the population. Furthermore, the appropriate read depth for sequencing largely depends on factors such as the

generation of the population (F_2 vs F_7), genome size of the crop, and the genetic effects under consideration, such as dominance versus complete dominance. For F_2 populations, a minimum read depth of 10x to 20x is recommended, whereas even 5x read depth may suffice in the F_7 generation to detect codominant QTL. However, for QTLs exhibiting a dominance effect, it is advisable to have a read depth of at least 20x or higher in F_2 populations to ensure successful QTL identification (Takagi et al., 2013). Since its conceptualization and widespread adoption of the QTL-seq, several modifications or improvements have been implemented. To accelerate genetic mapping process, Wang et al. (2019) introduced "GradedPool-Seq" approach, in which individuals from F_2 population are assigned into three or more graded groups based on their phenotypic values. Once GradedPool-Seq is compared with the previous methods like MutMap, SHOREmap, Next-Generation Mapping, and QTL-seq, it has several advantages such as high-resolution genetic mapping (~400-kb) and detecting multiple QTLs along with the ability of evaluating multiple phenotypic characters in a single F_2 population. (Wang et al., 2019). "Modified QTL-seq," which is a novel strategy of NGS-BSA application, was introduced by Wang and Wang (2023). The main advantage of this method is multiple comparison analysis, which can effectively speed up QTL mapping for complex traits, thereby accelerating the breeding process in crops (Wang and Wang, 2023). Although QTL-seq and other modified approaches have various advantages, there are still concerns that may hinder successful QTL mapping using these methods (Ott et al., 2011; Slate, 2013; Ashton et al., 2017; Bazakos et al., 2017). These constraints encompass genetic basis of complex traits like epigenetic and epistatic factors, family based experimental designs, pooling errors in BSA, the potential omission of minor QTLs, the influence of environmental interactions, the prevalence of high rates of false positive SNP detection (Flint and Mott, 2001; Mackay, 2001; Clevenger et al., 2018). To address many of these challenges, the size of the mapping population plays a pivotal role as it is related to allele frequency and statistical power (Hamblin et al., 2011; Hong and Park, 2012). Previous studies employing QTL-seq have indicated an average population size of 241, suggesting a reasonable benchmark for future QTL investigations. However, adjustments to the population size should be made based on the specific trait under scrutiny especially to avoid Beavis effect and capture the minor QTL effects (Slate, 2013). Traits with high heritability may tolerate smaller population sizes, whereas traits with lower heritability may benefit from larger population sizes to enhance the detection of minor QTLs and narrow down QTL intervals early in the mapping process (Topcu et al., 2021). To minimize the errors in pooling, the phenotyping should be evaluated in controlled conditions and if it is possible in different environments to minimize the environment effects. Nevertheless, it's important to note that many of these concerns are relevant to other QTL mapping methods as well.

4. Conclusion

In conclusion, the QTL-seq method has demonstrated its effectiveness as a rapid, cost-effective, and reliable approach to QTL mapping across various contexts. This study provides a comprehensive overview of the entire process, from initial DNA isolation to data visualization, offering a valuable pipeline for researchers, particularly in the field of plant breeding.

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HORTICULTURAL STUDIES

RESEARCH PAPER

Extending the Vase Life of Gerberas with Organic Compounds

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Abstract

The popular cut flower gerbera (*Gerbera jamesonii*) has a limited vase life at the end request of user because proper postharvest treatments are not used. Vulnerable to microbial contamination. The purpose of this study was to ascertain how various preservation solutions affected the cut *Gerbera jamesonii* flower (cv. Yeliz) quality and vase life. Cut flowers were placed in glass jars containing solutions in six different treatments: 100, 150, and 250 mg L^{-1} of citric acid; 100, 150, and 200 mg L^{-1} of thymol; and distilled water as a control. Fresh flower weight, water uptake, vase life, pH, EC, and pigment color assessment were among the parameters that were noted based on 0, 3, 6, 9, 12, and 15 days of storage. According to the results, the solution containing 250 mg L^{-1} of citric acid produced the highest values for fresh weight, water uptake, and flower vase life and least color change of the flowers. This was followed by the solution containing 100 mg L^{-1} of thymol. Flowers treated with 200 mg L^{-1} of thymol had the lowest fresh weight and vase life performance. The quality and vase life of cut *Gerbera jamesonii* flowers were found to be significantly affected by the use of 250 mg L^{-1} citric acid in preservation solutions. In comparison to other treatments, high dosages of tyhmol (150 and 200 mg L^{-1}) had a negative impact on floral quality and vase life.

1. Introduction

Gerbera (*Gerbera jamesonii*), sometimes referred to as the Transvaal daisy, is fourth among the top ten commercially cut flowers worldwide (Flora Cultural International, 2022). The appeal of gerbera is attributed to its attractive aesthetics, broad range of color, and adaptability to various environmental circumstances (Hema et al., 2018). As is the case all other cut flowers, gerberas have a very short vase life and are highly perishable. Their commercial value is largely determined by their ability to bend their stems, also known as scape-bending or bent neck (Perik et al., 2014; Naing et al., 2017; Muraleedharan et al., 2019; Shabanian et al., 2019).

Vase treatments are used to reduce cut flower postharvest challenges. Vase conditions also influence the development of microbes on the stem that can obstruct the water uptake and cause dehydration in delicate flowers by influencing their production of ethylene (Hema et al., 2018; Muraleedharan et al., 2019).

Vase solutions contain active ingredients that are categorized based on their function. These include antioxidants like citric acid and salicylic acid, antibacterial agents like 8-hydroxyquinoline, silver nitrate, and silver nanoparticles, and ethylene inhibitors like silver thiosulfate (Li et al., 2018). Many organic acids, like ascorbic acid, citric acid (CA), malic acid, and salicylic acid, are crucial in increasing the postharvest life of cut flowers. Organic acids are utilized in the respiratory cycle and other metabolic pathways, providing cells with energy and carbon (da Silva, 2003; Darandeh and Hadavi, 2012). Like other organic acids, citric acid

may impact the duration that cut flowers last in a vase. According to van Doorn (1997), citric acid enhances the water conductivity in the xylem of cut flowers and decreases the number of bacteria in vase solution. Since citric acid is one of the iron forms that are mobile in plants, it is crucial to the transfer of iron (Hell and Stephan, 2003; Darandeh and Hadavi, 2012). By decreasing the pH of the water and encouraging the growth of bacteria, citric acid blocks the xylem vessels in the area that has been cut (Nowak and Rudnicki, 1990). Citric acid has been shown to improve the postharvest longevity of several cut flowers, such as tuberose and lilies (Eidyan, 2010; Darandeh and Hadavi, 2012). Essential oils are healthy, organic natural compounds that are not damaging to the environment. Due to their high concentrations of phenolic compounds including eugenol, thymol, and carvacrol, essential oils exhibit potent antibacterial effects against a variety of diseases (Bounatirou et al., 2007; Sharififar et al., 2007). Antimicrobial activity of carvacrol against certain bacteria and fungi was recently discovered (Botelho et al., 2007; Martinez-Romero et al., 2007; Yahyazadeh et al., 2008). Other essential oils that have been found to be effective against some bacteria and fungi include thymol, thyme oil, and zataria oil. These oils are used to prevent plant diseases, especially those that affect fruit (Svircev et al., 2007; Braga et al., 2008; Yahyazadeh et al., 2008). On the other hand, insufficient data is currently accessible on the application of thymol to reduce microbial contamination and prolong the vase life of cut flowers, including gerberas. This study investigated to determine whether different concentrations of thymol and citric acid affected the vase life, post-harvest quality, and other characteristics of cut gerbera (*Gerbera jamesonii* cv 'Yeliz') flowers.

2. Material and Method

2.1. Plant material

The experiments were conducted at Bingol University, Department of Horticulture (38°53'59.34"N, 40°29'15.95"E). *Gerbera jamesonii cv.* 'Yeliz' was obtained from a commercial farm in Antalya, Türkiye, in May 2024. The handling and harvesting of the gerbera flowers followed the procedure described by Tonooka et al. (2023a). The cut flowers were pre-cooled for six hours at 4°C to lessen the impact of the high field temperature. Afterward, they were carried in dry conditions (12 hours at room temperature) to the vase life room at Bingol University, Department of Horticulture The blooming stems were clipped to a length of 40 cm.

2.2. Treatments and design of the experiment

There were six treatments in the randomized complete blocks design (RCBD) trial. Treatments included thymol at doses of 100, 150, and 200 mg L^{-1}, citric acid at doses of 100, 150 and 250 mg L^{-1} in addition to the control. A glass (1000 ml) containing 750 ml of vase solutions was filled with the cut flowers. Each treatment had five replicates, with three blossoms in each. As a control, distilled water was utilized. Every solution was made from scratch at the start of the experiment. The temperature in the vase life room was 21±2°C, the relative humidity was 60±5%, and there was 1000 lux of light.

2.3. Vase life, relative fresh weight (RFW), and water uptake

Vase life was measured from the conclusion of pretreatment until one of the following symptoms appeared: a flower stem bent more than 90°, a single ray petal abscission, a break right below the flower head, or withering of the petals. For each treatment, fifteen flowers were utilized. Once every three days, the fresh weight of the cut flowers and the amount of water absorbed were measured. The method described by He et al. (2006) was used for calculating the relative fresh weight (RFW).

2.4. pH and EC measurements in the solutions

pH of solutions were measured by a pH/ORP meter (HI 2211 HANNA Instruments RI/USA) and Electrical conductivity (EC) was measured by a Conductivity Benchtop (Orion 3-Star, Thermo Scientific).

2.5. Color measurements of the pigment

Colorimeter (Lovibond; Spectrophotometer a sphere, Serie SP60) was used to measure the pigment color. CIELAB values were obtained, and color variations were observed once every three days.

2.6. Statistical analysis

A completely randomized design was used to evaluate the obtained data in the study. Fifteen flowers were utilized for each treatment. The data were subjected to a one-way analysis of variance (ANOVA) using IBM SPSS Statistic 20.0. The means were compared at ($P \leq 0.05$) using the Duncan's test.

3. Results and Discussion

3.1. Vase life, relative fresh weight (RFW), and water uptake

Statistically significant differences were observed in the vase life of cut gerbera flowers of 'Yeliz' ($P \leq 0.05$). The citric acid 250 mg L^{-1} had the longest vase life (12.87 days), which was 1.8 days longer than the control group (11.07 days). The

second longest vase life was determined 100 mg L^{-1} (12.80 days), despite the fact that thymol 200 mg L^{-1} had the shortest vase life (10.20 days) (Figure 1).

The RFW of the cut flowers increased in all treatments for the first three or four days and thereafter decreased. Although there is an increase in the RFW ratio between the 6th and 9th day, a decrease is observed in all treatments after the 6th day (Figure 2).

Although in terms of total water uptake, there was no statistically significant variation, the highest total solution uptake was determined in Citric acid 250 mg L^{-1} with 23.02 ml, followed by Citric acid 150 mg L^{-1} with 22.53 ml and Thymol 100 mg L^{-1} with 22.49 ml (Figure 3).

Water stress affects cut gerbera flowers, and a deficiency in water causes the water balance of calyxes to become negative. Water stress is mostly caused by vascular blockage at the stem tip and disruption of the water supply (Tonooka et al., 2023b). The majority of cut flowers, including cut gerbera flowers, have a high rate of initial solution uptake; however, with time, this rate decreases mostly because of air embolism and microbial vascular blockage (Mashhadian et al., 2012). It is believed that cut gerbera are susceptible to water shortages brought on by upsetting the postharvest water balance is the basis for the impact of maintained water relations on prolonging the vase life (Mashhadian et al., 2012; Rafi et al., 2013; Kazaz et al., 2019). The benchmark for the end of the vase life is when the flowers wither and the quality of the cut flowers deteriorates if these causes continue (Shabanian et al., 2018). Similar to our comparison results of preservative treatment, solution uptake through preservative treatment increased the water balance and freshness of flowers and decreased early wilting, consequently improving the vase life of cut flowers (Ichimura and Goto, 2000). In order to increase the marketability of cut gerbera flowers, we observed in our study the vase life of the flowers based on the combination of a preservation solution at the consumer stage and solution combination for each postharvest treatment stage after harvest. The antibacterial agents thymol and citric acid were utilized in this investigation (Yagi and Elgimabi, 2014; Memar et al., 2017). Thymol and citric acid are useful in prolonging the vase life of certain cut flowers, according to numerous studies on the subject (Asrar, 2012; Babarabie et al., 2015; Kazaz et al., 2020; Alkaç et al., 2023). According to our results, citric acid 250 mg L^{-1} (12.87 days) and thymol 100 mg L^{-1} (12.80 days) extended the vase life of cut gerberas. These results might be explained by function of citric acid and thymol as an antibacterial agent, which may have decreased stem plugging. However, when thymol dosages increased, a decrease in floral vase life was seen. Pourianejad et al. (2014) achieved similar results. Due to their herbicidal activity in a variety of plant systems, essential oils like thymol have a phytotoxic effect that can lead to electrolyte leakage and subsequent cell death (Kordali et al., 2008). High thymol treatment concentrations in this study may be toxic and cause cell death, which would explain why the vase life of the treatments was shorter than that of the control.

As a result of their potent antibacterial properties, citric acid and thymol preserve solution absorption by inhibiting the growth of microbes in the xylem vessels of cut flower stems (Salehi Sardoei et al., 2014; Elgimabi and Yagi, 2016). Our results showed that all treatments of citric acid and thymol 100 mg L^{-1} enhanced solution absorption. This is assumed to be caused by antibacterial properties of citric acid. Citric acid-like acidic

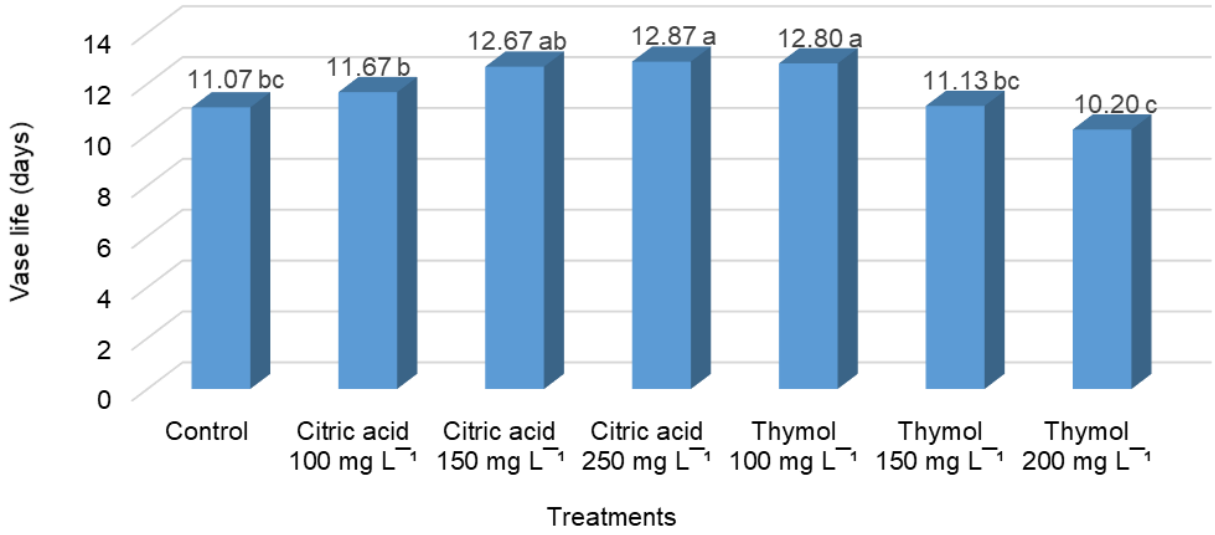

Figure 1. Effect of some organic acid compounds on the vase life of cut *Gerbera jamesonii* cv. 'Yeliz'. (Different letters at each time point indicate significant differences "P≤0.05" using the Duncan's test).

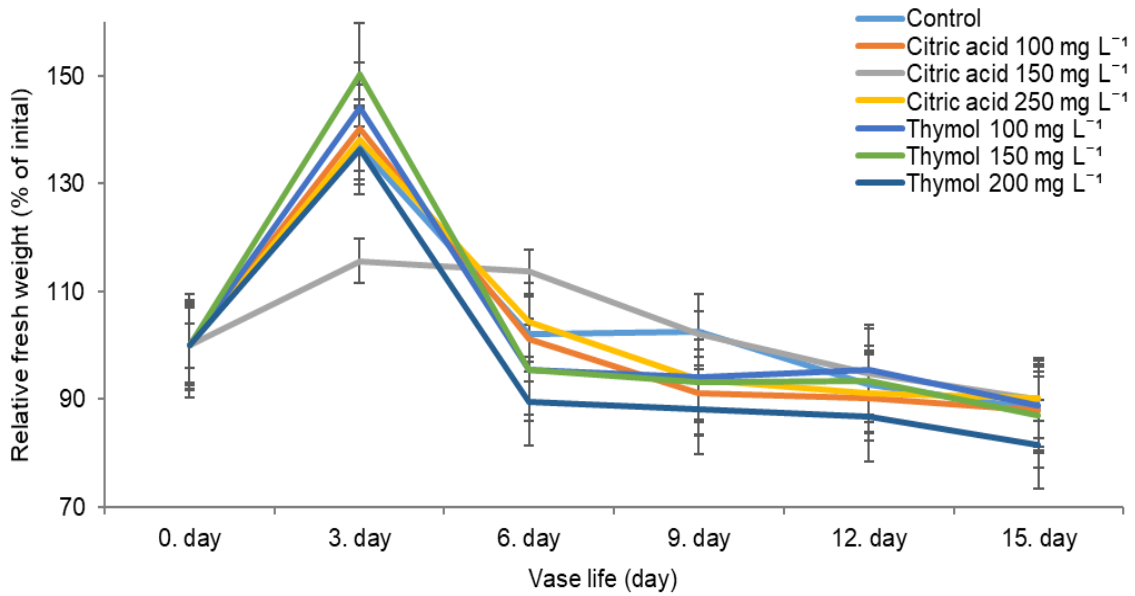

Figure 2. Effect of some organic acid compounds on the relative fresh weight (%) of cut *Gerbera jamesonii* cv. 'Yeliz'

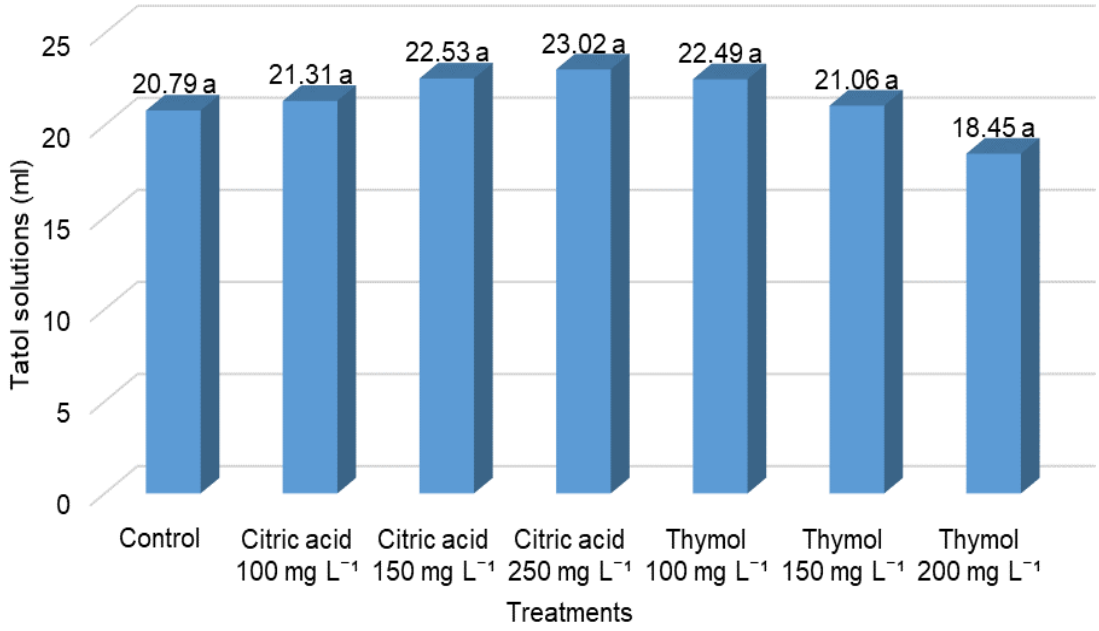

Figure 3. Effect of some organic acid compounds on the total solution cut *Gerbera jamesonii* cv. 'Yeliz' (Different letters at each time point indicate significant differences "P≤0.05" using the Duncan's test).

components have been found to improve the water intake of cut flowers by preventing bacteria development and accumulation in vase solutions, similar to the current findings (Alaey et al., 2011; Mansouri, 2012). Additionally, it was noted that treatments with citric acid increased plants of *Acacia holosericea* stem water uptake from vase solution (MohdRafdi et al., 2018). Higher thymol concentrations, on the other hand, reduced solution absorption. Prior research has indicated that high thymol concentrations have detrimental effects on solution absorption (Bazaz and Tehranifar, 2011; Salehi Sardoei et al., 2014). Additionally, it was said that essential oil concentrations, such as thymol, are extremely important and require more research (Mirdehghan and Aghamolayi, 2016).

Citric acid 250 mg L^{-1}, citric acid 150 mg L^{-1} and thymol 100 mg L^{-1} treatments decreased the fresh weight loss compared to other treatments, based on our findings about relative fresh weight. Treatments with 150 mg L^{-1} and 200 mg L^{-1} of thymol hadn't worked to prevent relative weight loss. Increased solution uptake and decreased transpiration rate prevent cut flowers from losing water, which may have had a good impact on fresh weight. Prior research has indicated a connection between fresh weight and solution uptake (Alaey et al., 2011; Amin, 2017). In addition, in parallel with the study by Alkaç et al. (2023) citric acid applications reduced the bacterial population, prevented clogging of vascular tissues and promoted water uptake. Thus, the best results in terms of vase life, total vase

Table 1. Effect of some organic acid compounds on the pH and EC (dS m^{-1}) variation during the vase life of cut *Gerbera jamesonii* cv. 'Yeliz'.

Treatment	0. day		3. day		6.day		9. day		12. day		15. day		Change in pH	Change in EC
	pH	EC	pH	EC	pH	EC	pH	EC	pH	EC	pH	EC		
Control	6.68 a	6.80 e	5.54 b	24.00 e	5.29 b	30.00 d	5.82 bc	32.00 c	5.87 b	40.30 d	5.95 a	40.4 d	0.70 a	-33.57 c
Citric acid 100 mg L^{-1}	3.71 d	253.90 a	3.73 d	208.90 b	3.84 d	199.74 b	4.12 d	198.30 a	4.32 d	199.10 a	4.39 b	199.97 a	0.68 b	-58.43 b
Citric acid 150 mg L^{-1}	3.78 d	177.00 d	4.04 c	137.60 d	4.26 d	142.1 c	5.43 c	125.40 b	6.54 ab	108.50 c	6.67 a	105.80 c	-2.89 d	71.19 b
Citric acid 250 mg L^{-1}	3.75 d	216.00 b	3.82 d	168.70 cd	3.97 d	134.30 c	4.45 d	123.37 b	5.17 c	116.40 bc	5.77 a	115. 0 bc	-2.03 cd	100.10 b
Thymol 100 mg L^{-1}	5.83 c	199.60 c	6.35 a	180.80 bc	6.03 ab	166.90 bc	6.26 ab	138.90 b	6.77 a	133.00 bc	6.88 a	132.50 bc	-1.05 bc	67.09 b
Thymol 150 mg L^{-1}	6.36 b	217.40 b	6.39 a	191.00 bc	6.30 ab	180.30 b	6.36 a	157.50 ab	6.53 ab	148.80 bc	6.71 a	140.80 bc	-0.35 b	76.64 b
Thymol 200 mg L^{-1}	6.44 b	311.00 a	6.60 a	249.80 a	6.61 a	240.50 a	6.61 a	193.00 a	6.61 ab	157.10 ab	6.63 a	153.10 b	-0.19 ab	157.93 a

($P \leq 0.05$).

solution uptake and relative fresh weights were obtained with this treatment.

3.2. pH and EC measurements in the solutions

The pH and EC of the vase solutions of gerbera flowers varied at certain rates for 15 days and showed statistically significant differences ($P \leq 0.05$). During the vase life, pH value of contol and citric acid 100 mg L^{-1} increased (0.70 and 0.68, respectively) and EC value decreased (-33.57, -58.43, respectively). Except for these two treatments, pH value of the other five treatments decreased and EC value increased (Table 1).

Due to their increased H^+ ion release, citric acid combinations have a lower pH than other preservative solutions. The pH fluctuations observed during the vase's longevity could potentially be attributed to the composition of the liquids within, the transport physiology of the plant, and the quantity of microorganisms involved in its metabolism. Shanan (2017) and Paul et al. (2021) have discovered similar results in their studies. EC values decreased during the vase life except for the control group. This is because flowers can absorb minerals and nutrients, which reduces the amount of dissolved matter in the vase water.

In addition, some substances can precipitate and settle to the bottom of the vase, lowering the concentrations in the water. In the control group, dissolved minerals and salts in the water may accumulate in the vase solution as the flowers absorb water, which may cause an increase in electrical conductivity, and cell fluids released from plant stems may also increase the EC value. In addition, since bacteria grow in the vase solution, organic and inorganic substances may be released as a result of the metabolic activities of these microorganisms, which may increase the EC value of the water.

3.3. Color measurements of the pigment

Different variations in L, a, and b values were seen in the study throughout the red gerberas' vase life. The fresh gerbera color exhibits high luminosity, or brightness, which is why the 'Yeliz' cultivar of gerbera flowers had a high L rating on the first day. The color turned dull and the L value dropped in all treatments-including the control-when the blossom had fully faded (Figure 4). In general, the value of the control treatment increased as the flower withered and turned brown, orange, pink, or yellow in the latter days of its vase life. (Figure 5). The b value of "Yeliz" in gerberas is positive during the vase life. Aging may cause a modest browning or yellowing of the plants. The maximum color change was determined at 150 mg L^{-1} of thymol (Figure 6).

There are several possible reasons why the L value (lightness or brightness value) of red gerberas gradually decreases during their vase life. The first is that flowers naturally lose their colour over time. Secondly, as microorganisms such as bacteria and fungi may accumulate in the vase water, they may prevent the water uptake of the flowers and cause faster fading and discolouration. In our study, the fading rate of the colours progressed in parallel with the vase life and in parallel with the development of microorganisms, the least change was seen in thymol 100 mg L^{-1} and citric acid 250 mg L^{-1} in all treatments.

4. Conclusions

The impact of various preservation treatments on vase life of *Gerbera jamesonii* cv. 'Yeliz' was examined in the present study. Cut gerberas and adding 250 mg L^{-1} of citric acid and 100 mg L^{-1} of thymol prolonged their vase life although increased thymol concentration did not prolong the vase life of cut gerbera flowers. The two most crucial recommendations for professional growers and contributors are as follows: firstly, for practical use, organic acid must be formulated in the right amounts, and research on how to apply them should be expanded upon and secondly using citric acid to prolong the vase life of cut gerbera flowers and treating with thymol solution to inhibit the growth of germs are both beneficial. Nonetheless,

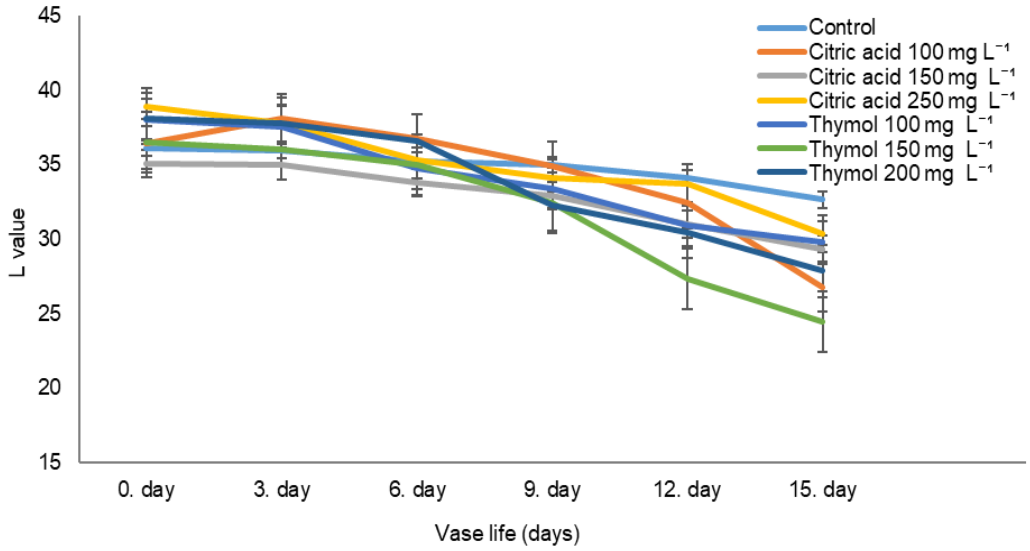

Figure 4. Variances in '*Gerbera jamesonii* cv. 'Yeliz' flower color and L value (brightness) during the vase life.

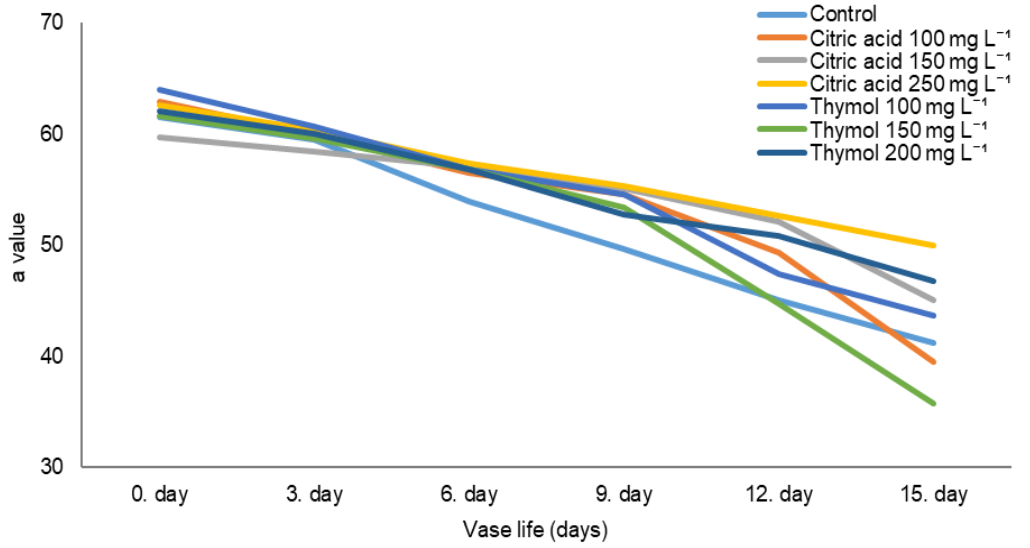

Figure 5. Variances in *a* value color of '*Gerbera jamesonii* cv. 'Yeliz' flowers during vase life.

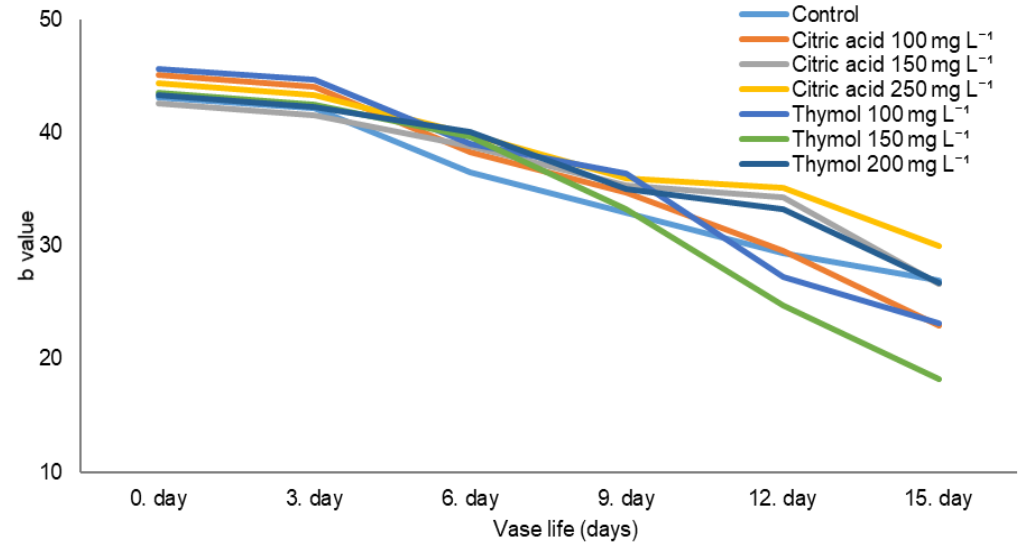

Figure 6. Variances in *b* value color of '*Gerbera jamesonii* cv. 'Yeliz' flowers during vase life.

further research is required to ascertain the precise concentration of thymol and citric acid as well as the vase life of cut gerbera flowers.

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HORTICULTURAL STUDIES

RESEARCH PAPER

Two-Sex Life Table Analysis of *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae) on Lettuce, Parsley, and Rocket

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Abstract

Phenacoccus solenopsis Tinsley (Hemiptera: Pseudococcidae), commonly known as the cotton mealybug, is a polyphagous pest causes significant economic losses worldwide. This study aimed to determine two-sex life table analysis of *P. solenopsis* on lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*), and rocket (*Eruca sativa*). Experiments were conducted under controlled conditions at 26°C, 65 ± 10% relative humidity, and a 16:8 light-dark cycle. The results indicated that lettuce was more suitable for *P.* solenopsis development compared to parsley and rocket. Total fecundity of *P. solenopsis* was recorded as 221.45, 158.00, and 121.64, on lettuce, parsley, and rocket respectively. In addition, oviposition period of *P. solenopsis* was 14.00, 9.73, and 8.45 days on lettuce, parsley, and rocket respectively. The highest values of net reproductive rate (R_0), intrinsic rate of increase (r), and finite rate of increase (λ) were obtained as 97.44 offspring female^{-1}, 0.14 day^{-1}, 1.16 day^{-1}, respectively from lettuce in this present research. The results showed that lettuce was the better host plant for *P. soleneopsis* than parsley and rocket. However, further studies should be done about host plant pest interactions to provide successful control measures against *P. solenopsis.*

1. Introduction

Although the origin of *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) is in the U.S.A (Tinsley, 1898; Abbas et al., 2010), this mealybug species was detected in different regions worldwide, such as Neaarctic, Neotropical, Oriental regions, around the World (Ben-Dov et al., 2015; Fand and Suroshe, 2015). This pest is a polyphagous species and determined on 226 host plants from 66 families (Garcia et al., 2016). Especially, in India and Pakistan cotton is main host for this pest and causing up to 60% damage to cotton crops (Fand and Suroshe, 2015). Additionally, several economically important vegetables and ornamentals serve as host plants for this mealybug (Fand and Suroshe, 2015). This pest was first detected on ornamental plants in 2012 in Adana province of Türkiye (Kaydan et al., 2013). Since then, this pest has continued to spread across in Türkiye and detected on 72 host plants from 55 families between 2012 and 2017 (Çalışkan and Ulusoy, 2018). *Phenacoccus solenopsis* can feed on all green parts of plants, and easily spread to uninfected areas by wind, water, agricultural activities and especially via international trade (Fand and Suroshe, 2015). Due to its high reproductive capability, cotton mealybug reaches higher population density on its host plants and causing significant economic damage to both agricultural and ornamental crops (Joshi et al., 2010).

The host plant is a significant factor in developing effective control strategies against *P. solenopsis*, with life table studies providing essential insights into its biology (Arif et al., 2013).

A life table is a crucial factor for studying population dynamics, as it offers comprehensive details for individuals within a cohort (Carey, 1993). Examining life table parameters can help to calculate reproductive and mortality patterns of organisms across various hosts (Price et al., 1980). The age-stage, two-sex life table analysis, first developed by Chi and Liu (1985) and Chi (1988). This approach allows for the inclusion of both sexes in the calculation of life table parameters, thereby providing a more comprehensive and accurate assessment of the biological and demographic characteristics of populations.

There were many studies carried about biology of cotton mealybug on many host plants by different researchers. Keçe (2019), Çalışkan et al. (2016), El Aalaoui and Sbaghi (2022), and Cai-Lang et al. (2017) have studied biology of *P. solenopsis* on varied host plants, such as different vegetables and ornamental species. In addition, some studies were carried out on different cotton varieties and other host plants for *P. solenopsis* (Prasad et al., 2012; Kumar et al., 2013; Ata, 2019). Above studies illustrated that, the two-sex life table parameters of *P. solenopsis* has been examined on various vegetables and ornamental plants. However, there is no study conducted about the biological parameters of *P. solenopsis* on lettuce, parsley, and rocket. This study aimed to determine the life table parameters of *P. solenopsis* on these three host plants (lettuce, parsley, and rocket) under controlled conditions.

2. Material and Methods

2.1. Plant materials

Parsley (*Petroselinum crispum*), lettuce (*Lactuca sativa*), and rocket (*Eruca sativa*) have been used as a host plant of *P. solenopsis* in this study. The plants used in the experiments were obtained pre-grown from external sources, and their leaves were washed daily before being used in the experiments. This study was carried out biological control laboratory of Plant Protection Department in Eskişehir Osmangazi University between 2023 and 2024.

2.1. Mealybug culture

Phenacoccus solenopsis culture has been grown in the controlled condition in climate cabinets at 26°C, 65 ± 10% relative humidity (RH), 16:8 light-dark (L:D) cycle. Sprouted potatoes were used for the maintenance of *P. solenopsis* culture.

2.3. Experimental design

Mated female individuals were transferred to 9 mm petri dishes containing agar for each host plant separately. Twenty four hour's later, mated mealybugs started to lay their eggs and hatched in few hours. First nymphal stages of individuals were transferred to 6 mm petri dishes with agar for each host plant (parsley, rocket, and lettuce) separately. Daily control has been done to detect immature stages of *P. solenopsis* on lettuce, parsley and rocket separately and host plant leaves were changed when its dried.

After pre-adult stage completed, one male mated with female in the same petri dishes and both female and male longevity were recorded daily. In addition, female was observed with pre-oviposition, oviposition and post-oviposition durations for individuals. Daily and total laid eggs were recorded for each host plant (parsley, lettuce and rocket) in this study. The experiments were conducted with 50 replications for each host plant at 26°C, 65 ± 10% RH, 16:8 L:D in the climate cabinet.

2.4. Statistical analysis

The life table analysis of *P. solenopsis* on lettuce, parsley, and rocket were performed with TWOSEX-MS Chart program developed by Chi and Liu (1985), Chi (1988), and Chi (2014). Age-specific survival rate (l_x), age-stage-specific fecundity (f_{xj}), age-specific fecundity (m_x), and age-stage-specific survival rate (s_{xj}) were determined with daily records of all individuals for each host plant separately (Wei et al., 2020; Chi et al., 2022). In addition, life table parameters were calculated for each host plant in this study

One hundred thousand (100.000) bootstraps were applied to calculate means and standard errors. The paired bootstrap method ($p<0.05$) was used to determine statistically significant differences between each host plant for the life table parameters of *P. solenopsis*. (Chi et al., 2022)

3. Results and Discussion

Total preadult periods for female *P. solenopsis* were 18.27±0.36, 19.82±0.30, 18.82±0.30 days and for male individuals 19.40±0.16, 20.60±0.37, 19.50±0.17 days on lettuce, rocket and parsley respectively. The highest preadult periods were detected on rocket for both male and female individuals. The immature stages of *P. solenopsis* showed significant differences for three different host plants (lettuce, rocket, and parsley) statistically ($p<0.005$) (Table 1).

The lowest adult preoviposition period (APOP), and total preoviposition period (TPOP) for *P. solenopsis* were detected as 7.73±0.56, and 26±0.65 on lettuce. In addition, the highest oviposition period was recorded as 14±1.18 days on lettuce as well in this study. Regarding total fecundity, the highest values were detected on lettuce and the lowest values were obtained from rocket (Table 2). There were statistical differences determined between host plants ($p<0.05$). However,

Table 1. The preadult stages of *Phenacoccus solenopsis* on different host plants.

Host plant	First nymphal stage (Mean±SE)		Second nymphal stage (Mean±SE)		Third nymphal stage (PP) (Mean±SE)		Total preadult (Mean±SE)	
	Female	Male	Female	Male	Female	Male	Female	Male
Lettuce	6.09±0.16a*	7.1±0.23b	4.73±0.19a	6.1±0.1b	7.45±0.25b	6.2±0.20a	18.27±0.36a	19.40±0.16a
Rocket	6.64±0.15b	6.50±0.17a	5.36±0.24b	5.90±0.28a	7.82±0.26b	8.2±0.25c	19.82±0.30b	20.60±0.37b
Parsley	6.55±0.21b	6.60±0.22a	5.91±0.16b	6.20±0.13b	6.36±0.31a	6.70±0.30b	18.82±0.30a	19.50±0.17a

*Same letters within the column are not statistically important in accordance with paired bootstrap test (p<0.05)

Table 2. Biological parameters of *Phenacoccus solenopsis* on different host plants.

Host plant	APOP (Mean±SE)	TPOP (Mean±SE)	Oviposition period (Mean±SE)	Post-oviposition period (Mean±SE)	Total fecundity (Mean±SE)	Adult longevity (Mean±SE)	
						Female	Male
Lettuce	7.73±0.56a*	26±0.65a	14±1.18b	3.09±0.25b	221.45±18.33b	24.82±1.22c	4.3±0.15a
Rocket	9.45±0.51b	29.27±0.52b	8.45±1.34a	2.63±0.20a	121.64±19.31a	20.54±1.15a	4.8±0.40b
Parsley	10.36±0.39b	29.18±0.39b	9.73±1.19a	2.27±0.23ab	158±17.12a	22.36±1.06b	4.3±0.29a

APOP: Adult pre-oviposition period, TPOP: Total pre-oviposition period.
*Same letters within the column are not statistically important in accordance with paired bootstrap test (p<0.05).

Table 3. Life table parameters of *Phenacoccus solenopsis* on lettuce, rocket, and parsley.

Host plant	r (day^{-1}) (Mean±SE)	R_0 (offspring) (Mean±SE)	λ (day^{-1}) (Mean±SE)	GRR (offspring) (Mean±SE)	T (day) (Mean±SE)
Lettuce	0.148±0.008b*	97.44±23.33b	1.160±0.009b	238.97±24.12b	30.74±0.67a
Rocket	0.121±0.008a	53.52±14.52a	1.129±0.009a	144.03±22.96a	32.739±0.51b
Parsley	0.128±0.008a	69.52±17.31b	1.137±0.009a	180.59±19.39a	32.96±0.56b

r: intrinsic rate of increase, R_0: Net reproduction rate, λ: finite rate of increase, GRR: Gross reproductive rate.
*Same letters within the column are not statistically important in accordance with paired bootstrap test (p<0.05).

there were differences detected between host plants in terms of female and male longevity in this study (p<0.05) (Table 2).

Life table parameters of *P. solenopsis* on lettuce, rocket, and parsley was found statistically significant in this study (p<0.05). The highest R_0 was found on lettuce (97.44±23.33) and lowest result were obtained from rocket (53.52±14.52) in this study (Table 3). λ and r values were highest for lettuce and were lowest on rocket. In addition, GRR (Gross reproductive rate) was highest for lettuce (238.97±24.12) and the lowest for rocket (144.03±22.96) in this study (Table 3). Regarding life table parameters, lettuce showed better performance as a host plant than parsley and rocket. Although lettuce may be better host plant for *P. solenopsis,* this pest can be completed its generation and may cause damage on parsley and rocket. Results indicated that three host plants are suitable for *P. solenopsis* but lettuce may be more suitable than parsley and rocket in this study. In addition, Figures 1, 2, and 3 have demonstrated the population parameters of *P. solenopsis* on lettuce, parsley, and rocket, respectively.

Host plants choice is significant to determine biology of insect pests. Biological parameters of pests may change on different host plants. Studies conducted for life table parameters may help to detect reproductive rates and other parameters of insects under laboratory conditions on various host plants (Awmack and Leather, 2002; Umbanhowar and Hasthings, 2002; Saeed et al., 2010). Cotton is known as the main host plant for *P. solenopsis* but host plant range has been spreading to non-origin geographical areas in recent years due to climate change (Fand et al., 2014). In life table studies some parameters helps to comment properly about suitability of host plants such as shorter TPOP and APOP, and higher fecundity, net reproductive rate (Abbes et al., 2024).

Some researchers have been carried out studies about the biology of cotton mealybug on varied host plants. Abbes et al. (2024) investigated the host suitability of three economically important crops (tomato, potato, and eggplant) for the invasive polyphagous pest *P. solenopsis* using age-stage two-sex life tables to assess pest fitness, life table parameters, and eggplant notably induced the highest R_0 (243.32), λ (1.18), and fecundity (276.50), alongside extended longevity of adult (males: 6.50 days, females: 24.15 days). Cai-Liang et al. (2017) assessed the impact of various vegetable host plants (potato, purple sweet potato, cucumber, lettuce, and cabbage) on the growth, development, and reproduction of *P. solenopsis* under controlled laboratory conditions (25±1°C, 60%±5% RH, 14:10 L:D) and results indicated that potato demostrated the shortest developmental generation duration and highest average survival rate, whereas cucumber resulted in the longest developmental duration and lowest survival rate. Female longevity peaked on lettuce, while male longevity was highest on potato. These findings demonstrated that different vegetable hosts significantly influence the population growth parameters of *P. solenopsis*, with potato emerging as the most conducive to the pest's growth and reproduction among the vegetables tested in that

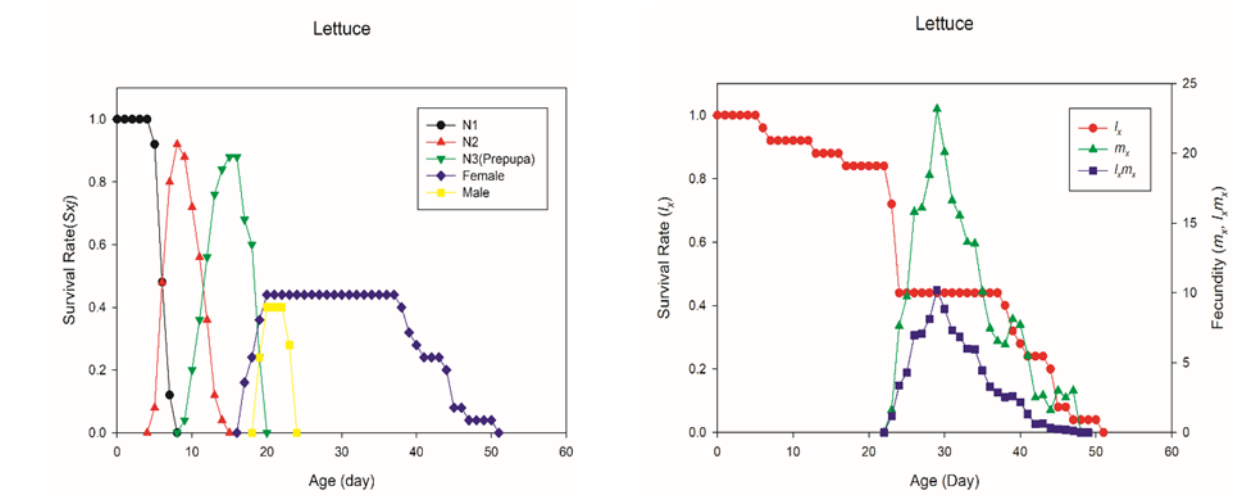

Figure 1. Age-stage survival rate (*Sxj*), age-specific survival rate (l_x) and fecundity (*mx, lxmx)* of *P. Solenopsis* on lettuce.

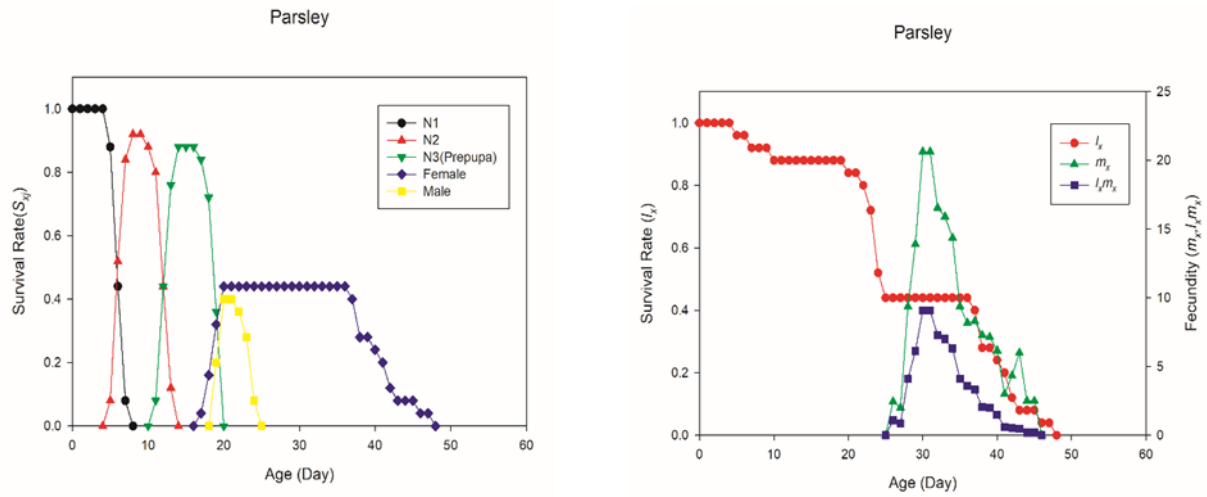

Figure 2. Age-stage survival rate (*Sxj*), age-specific survival rate (l_x) and fecundity (*mx, lxmx)* of *P. Solenopsis* on parsley.

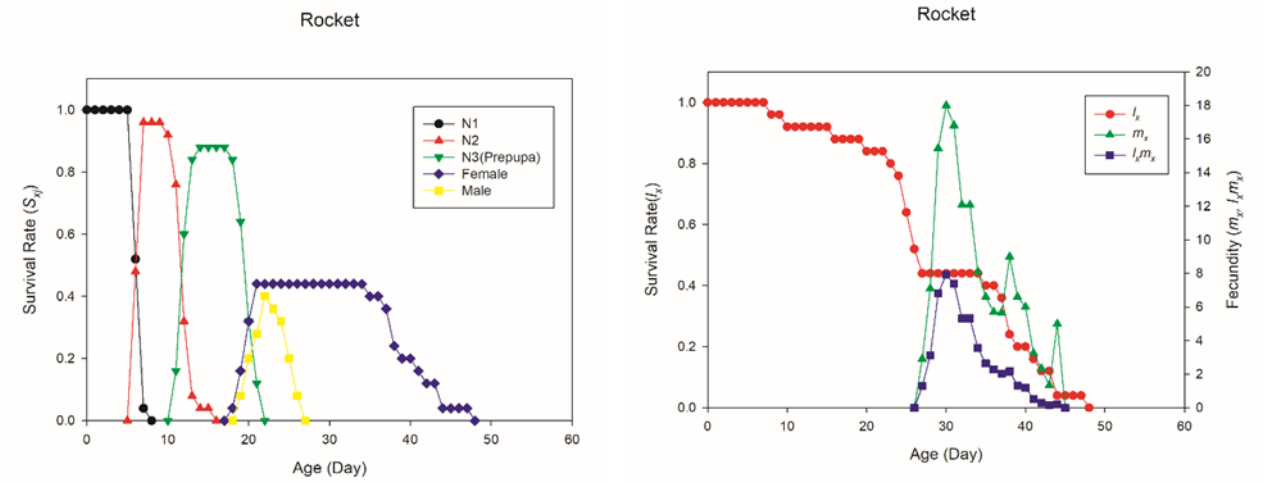

Figure 3. Age-stage survival rate (*Sxj*), age-specific survival rate (l_x) and fecundity (*mx, lxmx)* of *P. Solenopsis* on rocket.

study (Cai-Liang et al., 2017). Another study conducted for *P. solenopsis* on pepper, tomato, eggplant, cotton under laboratory conditions in 2018-2019 and the results indicated eggplant as the most suitable host, exhibiting the highest values for key life table parameters, including (R_0) of 184 nymphs per female, (r_m) of 0.269 per day, (λ) of 1.31 per day, and (GRR) of 264 nymphs per female in that study (Keçe, 2019). Nagrare et al. (2018) assessed biology of the invasive cotton mealybug on five preferred host plants (China rose, okra, cotton, congress grass, tomato) under controlled laboratory conditions, revealing that the mean development time for females varied from 13.57

days on congress grass to 16.61 days on cotton, which was identified as the most favorable host due to its longest ovipositional period (15.20 days), highest female longevity (27.40 days),highest fecundity (328.30 eggs), and highest intrinsic rate of increase (0.19 day−1), net reproductive rate (284.3), thereby highlighting the significant impact of host plant species on the population dynamics of *P. solenopsis*. Dogar et al. (2018) studied the effect of various host plants (*Jasminum sambac*, *Anthurium andraeanum*, *Plumeria rubra*, *Hibiscus rosasinensis, Caesalpinia pulcherrima*) on *P. solenopsis* and the findings indicated that *H. rosasinensis* shortened pupal and generation times and reduced adult longevity, whereas *C. pulcherrima* and *A. andraeanum* led to lower fecundity and hatchability, highlighting the importance of host plants in *P. solenopsis* population dynamics and informing potential cultural management strategies. Another study investigated population growth parameters of the cotton mealybug, on *B. aurea*, *H. syriacus, C. nocturnum,* and *H. rosa-sinensis* under controlled conditions, revealing that rearing on *H. syriacus* resulted in the highest GRR (342.6), R_0 (258.0), λ (1.3380), r (0.2911), and shortest (T) (19.1 days), highlighting the significant impact of host plant species on the population dynamics and potential management strategies of *P. solenopsis* (Çalışkan et al., 2016). As observed in studies conducted by various researchers, some vegetable and ornamental species can be as suitable host plants for *P. solenopsis*. Particularly, eggplant was identified as the most suitable host plant by Abbes et al. (2024) and Keçe (2019). Conversely, Nagrare et al. (2018) found cotton to be the most suitable host plant. Additionally, Dogar et al. (2018) and Çalışkan et al. (2016) generally focused on ornamental plants. Cai-Liang et al. (2017) studied various host plants and found that lettuce was a partially better host plant, which supports with the findings of this study. In the present study, lettuce, parsley, and rocket were investigated. The results indicated that lettuce may be the most suitable host plant for *P. solenopsis.* However, parsley and rocket did not show as favourable results, suggesting that further detailed studies should be conducted under different conditions for these plants.

4. Conclusion

In conclusion, the present study investigated two-sex life table parameters of *Phenacoccus solenopsis* on three different host plants: lettuce, parsley, and rocket. The findings revealed significant variations in several key parameters among these hosts. Lettuce demonstrated better suitability as a host for *P. solenopsis* compared to parsley and rocket, as evidenced by shorter pre-adult development periods, higher fecundity rates, and greater reproductive success. Specifically, lettuce exhibited the shortest adult pre-oviposition period (APOP) and total pre-oviposition period (TPOP), highest oviposition period, and net reproduction rate (R_0), indicating it as the most conducive host for sustaining *P. solenopsis* populations under laboratory conditions. Conversely, parsley and rocket showed less favourable results in terms of life table parameters such as pre-adult development times and reproductive output. However, this pest can complete its life cycle and reproduction on both parsley and rocket, this situation showed that, their potential as secondary hosts for *P. solenopsis*. These findings contribute valuable insights into the host plant preferences and reproductive capabilities of *P. solenopsis*, crucial for developing targeted pest management strategies in agricultural crops and ornamental plants. Further research could explore additional factors influencing *P. solenopsis* dynamics on various hosts under different environmental conditions to enhance pest control effectively and successfully.

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