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Chapter 23

Rootstock Breeding for Abiotic Stress Tolerance in Citrus

Berken Cimen and Turgut Yesiloglu

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62047

Abstract

Citrus species are the most widely produced fruit crops in the world. Citrus fruits are mainly produced in coastal areas in several countries as well as Mediterranean region, and production in these regions is affected by both biotic and abiotic stresses, including drought, extreme temperature, salinity, citrus canker, citrus tristeza virus, citrus greening, and others. The use of rootstocks in fruit production includes not only stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metals, nutrient stress, water stress, and alkalinity. There is extensive genetic diversity in citrus which provides several materials to be used as rootstocks against abiotic stress. In this work, we tried to provide an overview of the abiotic stresses in citrus by combining literature with our studies, role of citrus rootstocks commercially used against abiotic stresses and rootstock breeding in citrus.

Keywords: Citrus, rootstock, salinity, alkalinity, drought, breeding

1. Introduction

Citrus is the most important tree fruit crop in the world, and citrus fruits are regarded as major household items in more than 100 countries around the world as well as the world juice industry which is also led by citrus juices. Citrus industry is regarded as a leading industry in some regions, such as the mountainous regions of China and coastal plains in several countries, such as California and Florida in USA, Valencia in Spain, and Adana in Turkey. According to the data published in 2013, the world's total citrus fruit production is 135.761.181 tons [1], which consists of 71.445.352 tons of oranges (*Citrus sinensis* (L.) Osb.), 28.678.213 tons of mandarins (*Citrus reticulata* Blanco), 15.191.482 tons of lemons (*Citrus limon* Burm. F.) and limes (*Citrus latifolia* Tan. and *Citrus aurantifolia Swingle)*, 8.453.446 tons of grapefruits (*Citrus paradisi* Macf.) and pummelos [*Citrus maxima* (Burm.) Merr.], and 11.992.686 tons of other citrus fruits (Figure 1).

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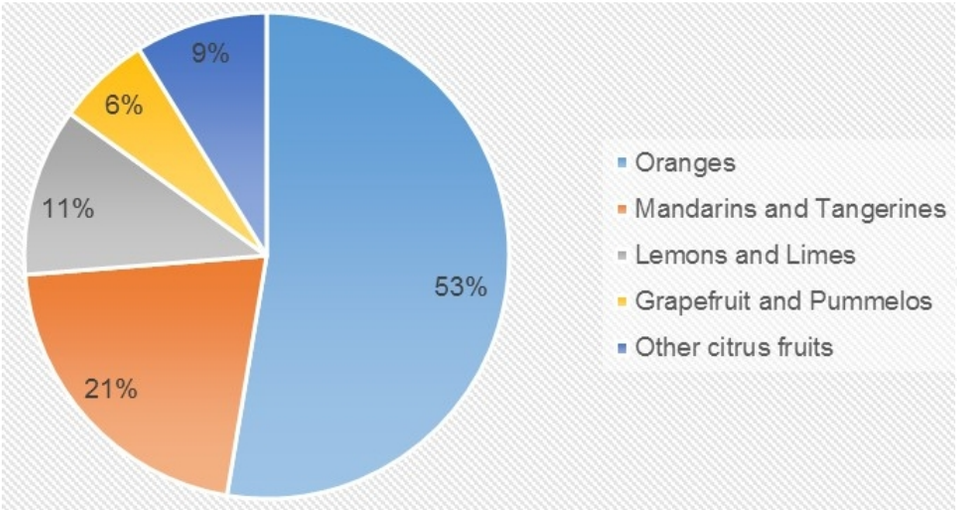

Figure 1. World's citrus production quantity (tons)

The role of citrus fruits in providing nutrients and medicinal value has been recognized since ancient times. Citrus fruits, belonging to the genus Citrus of the family Rutaceae, are well known for their refreshing fragrance, thirst-quenching ability, and providing adequate vitamin C as per recommended dietary allowance. In addition to ascorbic acid, these fruits contain several phytochemicals, which play the role of nutraceuticals, such as carotenoids (lycopene and β-carotene), limonoids, flavanones (naringins and rutinoside), and vitamin B complex and related nutrients (thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, folic acid, biotin, choline, and inositol). These substances greatly contribute to the supply of anticancer agents and other nutraceutical compounds with antioxidant, inflammatory, cholesterol, and allergic activities, all of them essential to prevent cardiovascular and degenerative diseases, thrombosis, cancer, atherosclerosis, and obesity. In spite of these beneficial traits, there is still a major need to improve fruit quality to meet current consumers' demands [2, 3].

Being vegetatively propagated, a citrus tree is normally composed of the rootstock and scion. Rootstocks play an important role in the rapid development of citrus in the world as well as breeding new cultivars. The necessity of using rootstocks for citrus fruits is to have a profitable production against some limiting factors such as climate, bad soil conditions, and diseases. Besides these factors, the use of citrus rootstocks provides a large number of choice to the growers to increase fruit quality and yield, obtain early fruiting and uniform cropping, avoid juvenility, controlling the tree size, have the opportunity for high-density planting, etc. These factors give many economic important advantages to the growers and, as a result, the citrus fruits are the most produced fresh fruits in the world for several decades [4]. Choosing a rootstock is an important decision, and local climatic and soil conditions are important factors in rootstock selection. Although any citrus variety can be used as a rootstock, some of them are better suited to specific conditions than the others [5, 6]. Some characteristics in a desirable citrus rootstock should be listed such as a good adaptation to all kinds of soils, tolerance to salinity, iron chlorosis, flooding, drought, high affinity with commercial species/cultivars, high yields of good fruit quality, reduced tree size, resistance to citrus tristeza virus (CTV), resist-

ance to citrus blight, resistance to fungal diseases affecting citrus (*Phytophthora* spp., *Armillaria mellea,* etc.), and resistance to nematodes.

Citrus production is affected by both biotic and abiotic stresses, including drought, extreme temperature, salinity, citrus canker, citrus tristeza virus, citrus greening, and others. These stresses can severely influence growth and development of both rootstocks and/or scions of citrus trees, thus reducing both fruit production and fruit quality. The use of rootstocks in fruit production includes not only stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metals, nutrient stress, water stress, and alkalinity [7]. Soil salinity is a major factor reducing crop production among the world as well as citrus production. Responses to salinity in terms of citrus production are affected by the amount of irrigation, climate, soil type, and fertilization [8]. Citrus rootstocks differ in terms of their tolerance to salinity conditions; many soils and sources of water contain high amounts of salts that can inhibit the growth and yield salt-sensitive citrus [9]. The high level of bicarbonate ions in the soil affects metabolic processes in roots and leaves, decreasing soil and plant Fe availability, leading to the condition known as lime-induced iron chlorosis. The most evident effect of Fe chlorosis is a decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls, and production of yellow, chlorotic leaves resulting as a decrease in fruit yield and quality [10, 11, 12]. Drought is considered as the principal factor that limits global agricultural production, among environmental constraints. Species greatly differ in the ability to overcome water deficiency. Drought in citrus trees causes reductions in stomatal conductance (g_S), leaf transpiration rate (E), and net CO_2 assimilation; decreases fruit quality and yield in long-term periods of stress; and increases fruit abscission [13].

Although the sour orange has many excellent horticultural advantages in terms of abiotic stress, it has a very important disadvantage for its susceptibility to citrus tristeza virus. This problem has severely reduced the use of this rootstock in many places especially the Western Mediterranean. Castle and Gmitter [14] reported that sour orange no longer has a secure place in today's rootstock portfolios because of its susceptibility to CTV. Castle [15] indicated that sour orange is an excellent rootstock for areas free of CTV. Ollitrault et al. [16] reported that the arrival of tristeza radically called into question of using sour orange in the Mediterranean area, whereas it had been almost the only rootstock in the region.

The need to produce stress-tolerant crops was evident even in ancient times [17]. Searching rootstock alternative to sour orange keeps on all over the world. In addition, it is a known fact that every rootstock cannot be used in every ecology. Due to the limiting effects of different ecological factors and diseases, a rootstock which is suitable for a country could not succeed in another one. Accordingly, the rootstocks that will be offered as alternative to sour orange is supposed to show some characteristics such as tolerance to lime-induced iron chlorosis in calcareous soils, tolerance or resistance to *Phytophthora citrophthora.* In addition to these, the alternative rootstocks should have CTV tolerance which does not exist in sour orange. On the contrary, all the required traits are present in the citrus germplasm. For instance, *Poncirus trifoliata* has tolerance to mainly biotic stress and cold hardness and some *Citrus* species have

adaptation to both abiotic and biotic stresses. However, the complexity of citrus biology and genetics makes it difficult to combine them through traditional breeding.

Consequently, in this chapter, we try to provide an overview of the abiotic stresses in citrus, role of citrus rootstocks commercially used against abiotic stresses, and rootstock breeding for tolerance to abiotic stress.

2. Types of abiotic stresses in citriculture

Many different citrus genotypes are commercially grown in a wide diversity of soil and climatic conditions; therefore, trees are subjected to important abiotic and biotic stresses that limit the production and, in some instances, the use of certain rootstocks and varieties [18]. Citrus trees are subjected to several abiotic constraints such as acid, alkaline, and salty soils; flooding and drought; and freezing and high temperatures.

Related to the global warming, drought problems have occurred in many countries. In addition, salinity became a major cause for citrus production in the coastal regions of Mediterranean by increasing the use of fertilizers and decreasing precipitations. Salinity and drought in the calcareous soil of Mediterranean region can lead to major problems in citriculture in terms of fruit yield and quality. Thus it is necessary to breed new rootstocks that are genetically tolerant to abiotic stress conditions and alternatives to existing rootstocks.

In a recent work, we have tried to handle three common abiotic stresses (salinity, drought, and alkalinity) occurring in citriculture especially in the coastal Mediterranean region by combining the literature with the studies conducted at Çukurova University.

2.1. Salinity

Salinity is a major environmental factor affecting the performance of many crop plants and reducing agricultural productivity [19, 20]. It is estimated that more than a third of all of the irrigated soils in the world is affected by salinity. The loss of farmable soils due to salinization is directly in conflict with the needs of the world population which is increasing continuously. Salt stress is a major stress problem in arid and semiarid regions and irrigated areas. Almost 7% of the world land area, 20% of the cultivated land, and nearly half of the irrigated land are affected by high salt concentrations [21].

Salinity affects the crop during both the vegetative and the reproductive stage and therefore causes reduction in plant growth and development with low water potential in the root medium (osmotic effect), too high internal ion concentration (ion excess/toxicity), and nutritional imbalance by depression in uptake and/or shoot transport (ion deficiency). Most of the salt stress in nature is due to sodium salts, particularly NaCl [22, 23]. High concentrations of Na^+ and Cl^- in the root medium saturation depress nutrient–ion activities and produce extreme rations of Na^+/Ca^{2+}, Na^+/K^+, Ca^{2+}/Mg^{2+}, and Cl^-/NO_3^- [24]. As a result, plants become susceptible to osmotic and specific ion injury as well as nutritional disorders that may result in reduced yield and quality. These processes may be occurring at the same time, but whether they

ultimately affect crop yield and quality depends on the salinity level, composition of salt, exposed period to salinity, the crop species and cultivars, the growth stage of plants, and a number of environmental factors [25, 26, 27, 28]. When the salt concentration reaches a harmful level for plant growth, a salinity condition is said to have developed. The degree to which growth and normal metabolism can be maintained is described as salt tolerance. Salt tolerance of vegetable crops varies considerably among species and depends upon the cultural conditions under which the crops are grown. Soil, water, plant, and environment can affect the salt tolerance of a plant. Therefore, plant response to a given salt concentration cannot be predicted on an absolute basis but on relative performance [29].

The effects of salinity on plants are evidenced by a severe reduction in plant growth and yield and, if the saline conditions persist, plant death can occur [30]. Salinity causes a deficiency of water in plant tissue, and low water potential reduces growth by inhibiting cell division and cell expansion [31]. The reduction in growth is mainly due to an osmotic effect of the accumulation of salts near the root zone, whereas the buildup of toxic saline ions in plant tissues is responsible for the progressive impairment of several physiological processes [20].

Osmotic effects resulting from salinity may cause disturbances in the water balance of the plant, including a reduction of turgor and an inhibition of growth, as well as stomatal closure and reduction of photosynthesis [32, 33]. The primary effect of high salt concentration in plants is stomatal closure. This causes a low transpiration rate and reduces the CO_2 availability for photosynthesis [34]. Hussain et al. [35] indicated that salinity reduced the photosynthetic availability of some citrus species and genera. As a result, plants become susceptible to osmotic and specific ion injury as well as to nutritional disorders that may result in reduced yield and quality.

Many researchers so far have reported citrus trees as salt-sensitive plants [30, 36, 37]. Salinity reduces citrus tree growth and fruit yield [9]. Growth reduction and some physiological and biochemical disturbances due to excessive concentrations of Cl^- and Na^+ in leaves are the main problems that are caused by salinity stress [37]. Also, salt stress has a dramatic impact on the citrus industry by decreasing the growth of trees and fruit yield and quality. Salinity may also cause nutrient deficiencies or imbalances, due to the competition of Na^+ and Cl^- with nutrients such as K^+, Ca^{2+}, Mg^{2+} and NO_3^-. In addition to osmotic effect, high K^+ concentration in a salinized nutrient solution increased the absorption of Cl^- citrus roots. Salt tolerance in citrus has been linked to the exclusion of toxic ions from the shoot [38]. Thus, citrus rootstocks have a great influence on the amount of Cl^- and/or Na^+ accumulated in the foliage of grafted trees [30]. For instance, in Fino lemon trees, sour orange rootstock is considered a good Cl^- and Na^+ excluder, whereas the *Citrus macrophylla* rootstock is a Cl^- and Na^+ accumulator [39].

Citrus trees, under salinity stress, suffer growth reduction and some physiological and biochemical disturbances due to excessive concentrations of Cl^- and Na^+ in leaves [37]. Depending on the soil type, irrigation method, and frequency, the soil solution salinity might also rise several fold between irrigations. Continual improvement of rootstocks and/or scions will be necessary to sustain irrigated citrus in increasingly salinized environments [30].

Sour orange is one of the most frequently used rootstocks in Mediterranean countries. It is known to be tolerant to salinity and calcareous soil among citrus rootstocks. However, it is highly susceptible to tristeza disease [37], that the disease should take into consideration the citriculture in Turkey and other countries which use sour orange as rootstock owing to be threatened by it. Genetically improved with favorable agronomical characteristics, such as resistance to pests and diseases such as the citrus tristeza virus, and salinity tolerance, rootstocks may be a long-term approach. Hence, screening studies based on physiological responses of genotypes to salinity stress should be used. In addition, continuous improvement of rootstocks is necessary to sustain cultivating citrus trees under salinized environments.

Yesiloglu et al. [40] established a screening study for the physiological evaluation of global tolerance to salinity rootstock collection of Çukurova University in the frame of the CIBEWU project, No: 015453. They screened 29 different genotypes that can be used as citrus rootstock under salinity stress assessed by several growth and physiological parameters such as fresh and dry weights of shoot and root; leaf chlorophyll concentration; and fluorescence, chloride, and Na content. High concentration of Cl and/or Na in the leaves of Citrus has been frequently related to disturbances in nutrition, gas exchange, and water relations. Unpublished data of the project regarding the genotypes used in the screening study and Cl^- and Na^+ concentrations in root and leaves of the genotypes are presented below. 34-12 N citremon, 4475 SRA citrumelo, CRC 4475 Swingle citrumelo, *Severinia buxifolia* have accumulated higher chloride in leaves, while Tuzcu Cleopatra mandarin, Gou Tou, Rangpur lime, CRC 02 Volkameriana, Tuzcu 31-31 sour orange have lower chloride in leaves. Rubidoux trifoliate with trifoliate hybrids and *Severinia buxifolia* were poor Cl^- excluder. It was found that Tuzcu Cleopatra mandarin and Rangpur lime are the best Cl^- excluders. Gou Tou, Antalya Cleopatra mandarin have the lowest amount chloride in the roots, whereas *Citrus ichangensis*, Benecke trifoliata, Volkameriana, 08 A 3015 Rubidoux, and SRA Pomeroy have the highest values. According to the results, Tuzcu Cleopatra mandarin, Rangpur lime, Gou Tou, and Antalya Cleopatra mandarin were found to be the most tolerant to salt stress. *Severinia buxifolia*, CRC-4475 Swingle citrumelo, Local trifoliata, and Benecke trifoliata were the most sensitive group to salt stress. Data were evaluated by using modified "weighted-ranking" method based on the parameters of chlorophyll fluorescence (*F*v'/*F*m'), leaf chlorophyll concentration by SPAD readings, leaf Cl and Na concentrations, leaf K and Ca concentrations, root K and Ca concentrations, growth parameters (shoot and root fresh and dry weights, shoot length, and leaf number), and visual ratings of leaf chlorosis. A classification which belongs to the screening study for salinity stress was performed as a result of the work and reported as follows in Table 1 (unpublished data). Genotypes were classified as very sensitive, sensitive, acceptable, tolerant, and very tolerant to salinity.

Khoshbakht et al. [41] reported that the effects of salinity on photosynthesis range from the restriction of CO_2 diffusion into the chloroplast, via limitations on stomatal opening mediated by shoot- and root-generated hormones, and on the mesophyll transport of CO_2, to alterations in leaf photochemistry and carbon metabolism. The authors conducted a study and investigated the NaCl effects on gas exchange parameters of nine citrus rootstocks and reported that sour orange and Cleopatra mandarin were the rootstocks most tolerant to salinity of all the

Genotypes	Tolerance to salt stress
Rubidoux trifoliate 08A 30.15	2
Citremon 34 12 N	1
Citrumelo 4475 SRA	1
AREC Swingle citrumelo	3
Antalya Cleopatra mandarin	3
Benecke trifoliate	2
C-35 Citrange	4
Swingle citrumelo 4475 CRC	1
Volkameriana CRC 01	4
Volkameriana CRC 02	4
Citrumelo CRC 1452	3
Carrizo citrange	3
Citrus ichangensis CRC	3
Citrus sulcata	3
Citrus sunki	3
Macrophylla	2
Rangpur lime	5
Gou Tou sour orange SRA 506	4
Pomeroy trifoliate SRA	1
Sacaton citrumelo	4
Severinia buxifolia SRA	1
Smooth Seville sour orange	4
Taiwanica	4
Troyer citrange	3
Tuzcu 31-31 sour orange	4
Tuzcu 891 sour orange	4
Tuzcu Cleopatra mandarin	5
Volkameriana	4
Local trifoliate	2

*1: very sensitive, 2: sensitive, 3: acceptable, 4: tolerant, 5: very tolerant

Table 1. Classification of rootstocks in collection of Çukurova University in respect of salinity tolerance

nine citrus rootstocks studied. Also, Cimen et al. [42] determined that the tolerances of Sarawak bintangor, Shekwasha, Fuzhu and Cleopatra mandarin to salt stress were determined by investigating the photosynthetic parameters and significant salinity effects on the photosynthetic performances of these rootstocks were reported (Figure 2).

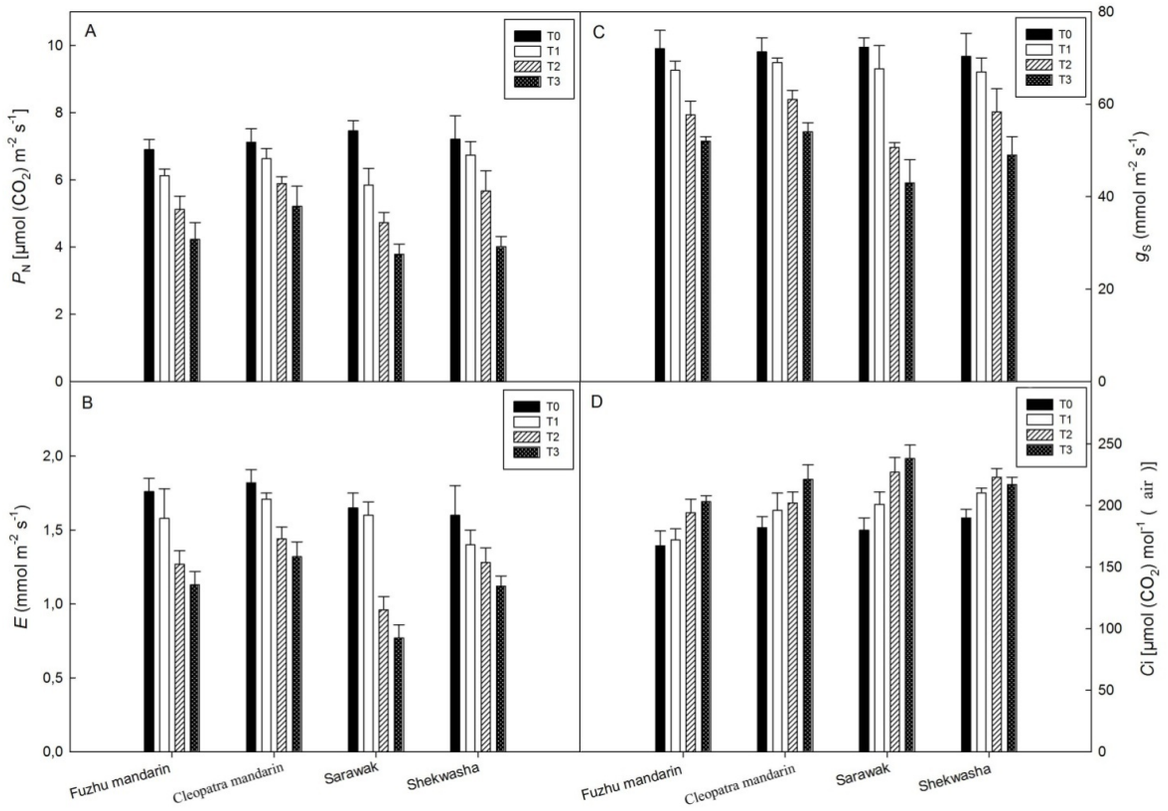

Figure 2. Effects of different salinity levels on photosynthetic rate (A), transpiration rate (B), stomatal conductance (C), and initial CO_2 concentrations (D) of four genotypes. The bars indicate the standard deviation. (T0 = 0 mM NaCl, T1 = 50 mM NaCl, T2 = 75 mM NaCl, T4 = 100 mM NaCl). Data presented from Cimen et al. [42].

2.2. Alkalinity

It is well known that iron is an essential micronutrient for all higher plants including citrus. Most of the iron existing in rhizosphere cannot be taken up by plants because iron is highly insoluble. Two main working hypotheses have been put forward for this chlorosis. In the first working hypothesis, the main cause of Fe deficiency chlorosis is thought to be the inhibition of Fe acquisition by HCO_3^- in the rhizosphere. For the second hypothesis, Fe inactivation in the leaf apoplast by an alkalization process properly noted by the "distant effect" of HCO_3^- is thought to be the main trigger for Fe deficiency chlorosis in leaves [43]. The Mediterranean Basin is characterized by the prevalence of calcareous surface horizon. In these soils, iron (Fe) chlorosis can lead to diminished yields and even plant death, particularly in semiarid areas where irrigation water has high bicarbonate contents, soil pH is high (7.0–9.0), and organic matter content is low [44]. Citrus production is increasing throughout the Mediterranean countries and more and more citrus orchards are being planted on marginal soils. Mediterranean countries have a suitable climate for citrus production, but it is estimated that 20–50% of fruit trees grown in the Mediterranean basin suffer from iron (Fe) deficiency. The most prevalent cause of Fe deficiency in this region is the presence of high levels of carbonate ions in calcareous soils, characterized by a high pH [45]. These soils often have more than 20% of calcium and magnesium carbonates and are strongly buffered, with a pH between 7.5 and 8.5

http://dx.doi.org/10.5772/62047

[11]. Fe uptake is highly dependent on soil pH, and Fe activity in solution decreases 1000-fold for each pH unit rise to reach a minimum within the range from 7.4 to 8.5 [46]. Leaf Fe chlorosis in plants is an old problem occurring in areas of calcareous and/or alkaline soils. Yield reductions from Fe-induced leaf chlorosis have been found in tomato, raspberry, kiwifruit, pineapple, vines, and citrus [47]. Moreover, the severity of leaf chlorosis and the differential behavior of genotypes can be determined by the chlorophyll concentration in leaves [11, 46, 48]. The high level of bicarbonate ions in the soil affects metabolic processes in roots and leaves, decreasing soil and plant Fe availability, leading to the condition known as lime-induced iron chlorosis. The most evident effect of Fe chlorosis is a decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls and producing yellow, chlorotic leaves. The loss of pigmentation is caused by decreased chlorophyll content in chloroplasts. This negatively affects the rate of photosynthesis and, therefore, the development of biomass. Fe deficiency affects the physiology and biochemistry of the whole plant, as Fe is an important cofactor of many enzymes, including those involved in the biosynthetic pathway of chlorophylls [10, 11].

The use of rootstocks in fruit production includes not only a stronger resistance against pathogens but also a higher tolerance to abiotic stress conditions such as salinity, heavy metal, nutrient stress, water stress, and alkalinity [7]. Recent studies showed that citrus rootstocks had different tolerance levels to iron deficiency [48, 49]. Studies emphasized that high pH conditions reduced iron uptake in citrus rootstocks [48, 49, 50, 51, 49]. Also, rootstocks affect tree growth, fruit quality, and yield [15, 48, 52]. Moreover, scion behavior depends in part on the rootstock-induced effects on leaf gas exchange [53]. González-Mas et al. [53] indicated that in calcareous soils, citrus production depends on the availability of suitable rootstocks that are tolerant to low Fe soil conditions. Studies have found that "Volkameriana,"' and "sour orange" plants were tolerant; "Carrizo and Troyer" citranges were intermediate, whereas the "*Poncirus trifoliata"* rootstock was more sensitive to iron chlorosis [46, 48, 49, 51, 54]. In addition, Cimen et al. [55] indicated that Young "Navelina" orange trees budded on Tuzcu 31-31 sour orange, and Gou Tou sour oranges performed best under Fe deprived conditions in plant growth chamber. Navelina on Volkameriana and Cleopatra mandarin was moderate; C-35 citrange and local trifoliate were poorly adapted to lime-induced Fe deficiency (Figure 3).

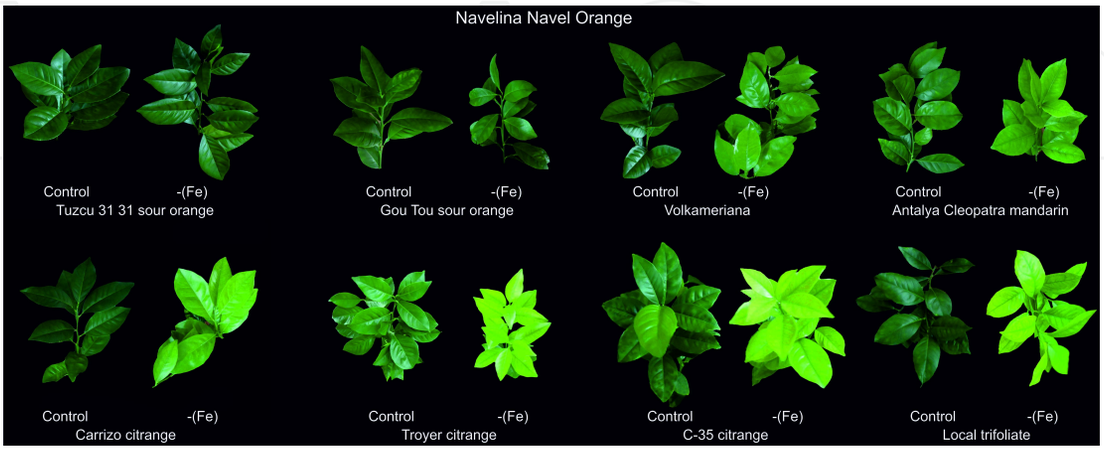

Figure 3. Response of Navelina Nave orange budded on eight different rootstocks in response to iron deficiency. Data presented from Cimen et al. [55].

Among physiological processes, photosynthesis is the basic determinant of plant growth and productivity, and the ability to maintain the rate of carbon assimilation under environmental stress is of fundamental importance to plant production [56]. Since Fe catalyzes chlorophyll biosynthesis [57], it would be expected to promote the photosynthetic rate (*P*n) while Fe deficiency to reduce it [58, 59]. Most of the knowledge concerning the effect of Fe deficiency on the photosynthetic parameters has been obtained with annual plants. However, relatively few studies have focused on the consequences of induced Fe deficiency on photosynthesis in evergreen fruit trees and especially in citrus. On the contrary, Fe is a component of several metalloenzymes, including peroxidase and catalase. Although both enzymes could be used as biochemical indicators of Fe availability in citrus [36], there are contradictory reports concerning the effect of Fe deficiency on catalase and peroxidase activity [60]. Cimen et al. [55] reported that at sufficient Fe supply, plants had higher activity of catalase (CAT) than the plants with Fe deprived conditions. Slight decreases were recorded on the Navelina orange leaves of Tuzcu 31-31 and Gou Tou sour oranges, while decreases were remarkable in the leaves of C-35 citrange and TGK0633 (obtained by a selection of trifoliate orange in Turkey) under short supply of Fe. In addition, Navelina leaves of C-35 citrange and TGK0633 displayed maximum decreases in APX (ascorbate peroxidase) activity, similarly CAT activity. There were no significant APX activity decreases in the leaves of Tuzcu 31 31, Gou Tou sour oranges, Volkameriana, and Antalya Cleopatra mandarin (Figure 4).

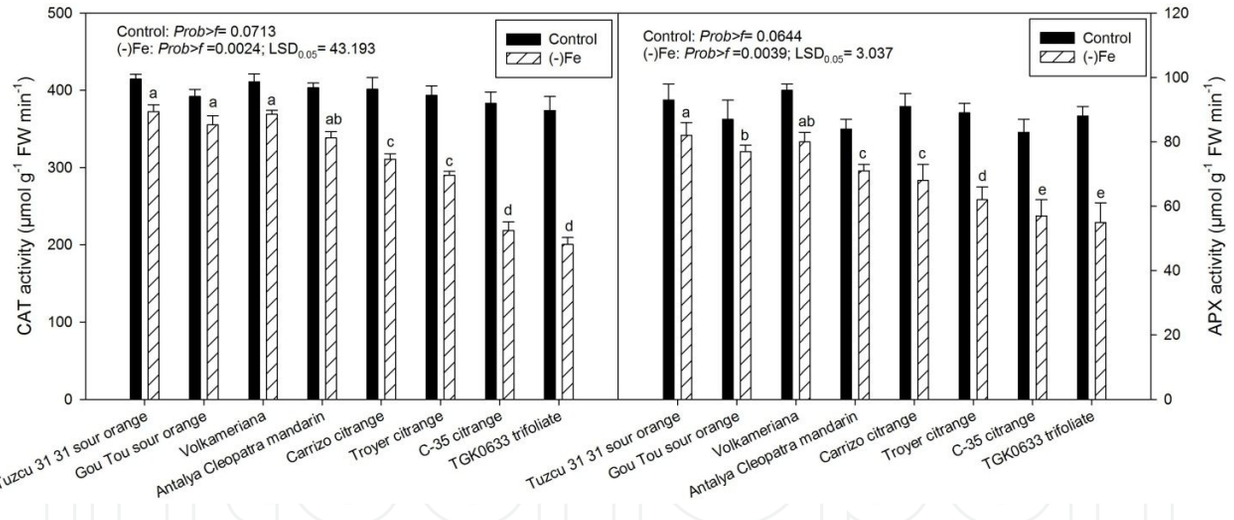

Figure 4. Catalase and ascorbate peroxidase activities of Navelina leaves of different rootstocks under Fe sufficient and deprived conditions. The bars show the standard deviation. Data presented from Cimen et al. [55].

The intensity of iron chlorosis can be quantified by total Fe, active Fe, leaf chlorophyll meter, photosynthetic parameters, enzymes, plant growth parameters, and visual ratings of leaf chlorosis. One of the distinctive characteristics of iron deficiency in field crops is the lack of correlation between leaf iron content and chlorosis. This has been termed the "chlorosis paradox." Therefore, leaf chlorophyll contents are generally used to monitor iron chlorosis [61]. The use of visual ratings and readings of a portable chlorophyll meter are the most efficient approaches to define iron chlorosis in citrus [51]. Yesiloglu et al. [40] established a screening

study for the physiological evaluation of global tolerance to lime-induced Fe chlorosis in rootstock collection of Çukurova University in the frame of the project CIBEWU, No: 015453 and evaluated by using modified "weighted-ranking" method based on the parameters of total Fe, active Fe, chlorophyll fluorescence (*F*v'/*F*m'), leaf chlorophyll concentration by SPAD readings, visual ratings of leaf chlorosis, and shoot and root weight.

Local trifoliate, Rubidoux trifoliate, and Benecke trifoliate exhibited severe chlorosis and were more chlorotic than other genotypes. Cleopatra mandarins among mandarin and its hybrids were more tolerant than Sunki and Calamondins. Macrophylla was the best in lemon and lemon hybrids group. Campbell [50] reported that Macrophylla was well adapted to soils of high pH. Volkameriana and Rangpur were almost the same. Gou Tou was really so tolerant to iron deficiency. All sour oranges were similar to each other. Alanya Dilimli sweet orange is a variety selected in south of Turkey. It is known to be very resistant to high pH conditions. The results confirm that Alanya Dilimli is very tolerant to high pH. According to the results, Carrizo citrange, Flhorag1, Macrophylla, Antalya Cleopatra mandarin, Tuzcu 31-31 sour orange, Gou Tou sour orange, and Alanya Dilimli sweet orange were more tolerant than others in citrus rootstock collection of Çukurova University (Table 2 – unpublished data).

Genotypes	**Tolerance to iron chlorosis**
Rubidoux trifoliate 08A 30.15	2
Citremon 34 12 N	3
Citrumelo 4475 SRA	3
Arec Rubidoux trifoliate	3
Arec Swingle citrumelo	2
Benecke trifoliate	2
Swingle citrumelo 4475 CRC	2
Carrizo citrange	4
Flhorag1	5
Pomeroy trifoliate SRA	2
Sacaton citrumelo I	2
Sacaton citrumelo	2
Citrumelo 1452 CRC	2
Local trifoliate	1
Troyer citrange	3
Antalya Cleopatra mandarin	4
Citrus sunki	4
Calamondin CRC	2

Genotypes	Tolerance to iron chlorosis
Calamondin 108 USDA	1
Tuzcu Cleopatra mandarin	2
Volkameriana CRC 01	3
Volkameriana CRC 02	3
Macrophylla	4
Rangpur lime	3
Volkameriana	3
Citrus ichangensis CRC	3
Citrus sulcata	2
Severinia buxifolia SRA	2
Alanya Dilimli sweet orange	5
Gou Tou sour orange SRA 506	5
Smooth Seville sour orange	3
Taiwanica	2
Tuzcu 31-31 sour orange	5
Tuzcu 891 sour orange	4

*1: very sensitive, 2: sensitive, 3: acceptable, 4: tolerant, 5: very tolerant

Table 2. Classification of the rootstocks in collection of Çukurova University in terms of tolerance of iron deficiency.

Although trifoliate orange has many advantages in terms of tolerance to abiotic and biotic stresses, it is susceptible to calcareous soil conditions. However, there are some superior genotypes to improve the tolerance to high pH of present rootstocks by hybridization and crossing [44].

Several rootstock breeding programs have been carried out by different countries leading in citriculture. Forner et al. [62] reported two new citrus rootstocks named F-A 5 and F-A 13, released in Spain. These rootstocks are hybrids of Cleopatra mandarin × Rubidoux trifoliate crosses with a high level of tolerance to lime-induced iron chlorosis. Besides, Bowman and Rouse [63] mentioned a new citrus rootstock named as US-812 which is a hybrid obtained by a cross between Sunki mandarin (*Citrus reticulata*) and Benecke trifoliate orange in USDA Indio Research Station, California. They have reported that using Valencia trees budded on to US-812 resulted in some tolerance to high alkalinity under pH conditions 8.1– 8.3. Moreover, Federici et al. [64] indicated that three citrus rootstocks released in August 2009 by the University of California named as ”Bitters,” ”Carpenter,” and “Furr” trifoliate hybrids, tested as C22, C54, and C57, respectively, by crossing Sunki mandarin × Swingle trifoliate orange. Bitters were found to be very tolerant to calcareous soil, whereas Carpenter and Furr were found to be moderately tolerant.

http://dx.doi.org/10.5772/62047

2.3. Drought

Mediterranean region has a subtropical climate and is an important region for citriculture. A significant amount of high-quality citrus fruits is produced in Mediterranean countries such as Spain, Turkey, Italy, Greece, Egypt, Morocco, and Tunisia. Fruit yield and quality are affected by genetic traits together with environmental factors. Fruit crops are frequently exposed to environmental stresses spontaneously or by conventional agronomic conditions. Some of these conditions such as high temperature may last only for a short period of time, whereas lack of water in soil may last for longer periods. Global warming is a type of greenhouse effect which is defined as the increase of Earth's average surface temperature due to the effect of greenhouse gases, such as carbon dioxide emissions from burning fossil fuels or from deforestation, which trap heat that would otherwise escape from Earth.

Yaacoubi et al. [65] indicated that Mediterranean fruit tree production is facing major changes that have environmental and socioeconomic consequences. Climatic changes related to temperature warming have been reported worldwide.

Drought stress, as one of the most ominous abiotic factors limiting the productivity of horticultural crops, is increasingly growing in dimension of severity in many regions of the world [66]. In general, the mechanism of drought resistance in plants can be explained as drought escape, drought avoidance, and drought tolerance. These traits consist of osmotic adjustments, cell membrane stability, epicuticular wax, partitioning and stem reserve mobilization, manipulation and stability of flowering processes, and seedling drought traits.

Drought tolerance is a complex trait that is important at different growth stages and involves multiple adaptations. Fundamental to this is the ability to maximize the extraction of water from the soil while minimizing loss from the leaves. Morphological adaptations include the development of deep roots and alterations in leaf morphology and cuticle structure, while physiological adaptations involve changes in stomatal density to maximize water uptake and retention [67, 68, 69].

Drought stress effects on the plant may range from slight suppression of growth and yield to temporary wilting, in which leaves flag but recover after transpiration demands decrease, to permanent wilting in which the plant suffers injury and death [70]. A plant responds to a lack of water by halting growth and reducing photosynthesis and other plant processes in order to reduce water use. As water loss progresses, leaves of some species may appear to change color, usually to blue-green. Foliage begins to wilt and, if the plant is not irrigated, leaves will fall off and the plant will eventually die. Drought lowers the water potential of a plant's root and upon extended exposure, abscisic acid is accumulated, and as a result stomatal closure occurs. This reduces a plant's leaf relative water content. The time required for drought stress to occur depends on the water-holding capacity of the soil, environmental conditions, stage of plant growth, and plant species [71]. Plants growing in sandy soils with low water-holding capacity are more susceptible to drought stress than plants growing in clay soils. A limited root system will accelerate the rate at which drought stress develops. A plant with a large mass of leaves in relation to the root system is prone to drought stress because the leaves may lose water faster than the roots can supply it. The root system has a great importance when the plant faces

drought. For instance, Rough lemon rootstocks are very drought tolerant because of their extensive, deep root systems. Newly installed plants and poorly established plants may be especially susceptible to drought stress because of the limited root system or the large mass of stems and leaves in comparison to roots.

Citrus, a perennial crop with a long orchard life, is likewise a globally important fruit crop responsible for world trade and often exposed to the vagaries of soil and atmospheric drought stress [72]. Drought stress is known to restrict the vegetative growth and yield of citrus, in addition to adversely affecting fruit quality and incurring huge economic loss to the citrus growers [73]. Therefore, screening and selection of germplasm are of great importance in terms of drought tolerance.

Pedrosoa et al. [74] reported that citrus rootstocks have differential capacities for supplying shoot tissues with water and carbon, improving the resistance to biotic and abiotic stresses and affecting plant water status and photosynthesis. Water relations have been well studied in citrus trees, showing that rootstocks alter the physiological performance under water deficit through variations in plant hydraulic conductance, leaf water potential, and stomatal conductance [75, 76, 77]. In addition, several studies have found that citrus rootstocks showed different performances when they are exposed to drought [13, 73].

Treeby et al. [78] investigated irrigation management and rootstock effects on navel orange and reported that irrigation management is far more critical for external fruit quality for trees on sweet orange and, to a lesser extent, trees on the citranges compared to trees on trifoliate orange and Cleopatra mandarin.

Some studies also indicate that using tetraploid rootstocks increases the drought tolerance in comparison to their diploid clones in citrus. Allario et al. [79] reported that polyploidy is common in many plant species and often leads to better adaptation to adverse environmental conditions. The authors examined the drought tolerance in diploid (2x) and autotetraploid (4x) clones of Rangpur lime (*Citrus limonia*) rootstocks grafted with 2x Valencia Delta sweet orange (*Citrus sinensis*) scions, named V/2xRL and V/4xRL, respectively. The results of the authors showed that using tetraploid clones of Rangpur lime had increased the drought tolerance in grafted sweet orange.

3. Germplasm and genetic variability

The origin of citrus is believed to be southeast Asia, including south China, northeastern India, and Burma. Commercial citrus species and related genera belong to the order Geraniales, family Rutaceae, and subfamily Aurantoidea. Fruit crops is a very heterogeneous group of plants including trees, shrubs, climbing vines, and perennial herbs. They inhabit different climates, ranging from tropical to subarctic zones and altitudes from sea level to higher mountains. Fruit crops also differ considerably with respect to their origin, taxonomy, and breeding systems. The biodiversity in plants has been progressed by natural and artificial hybridization and mutation which are the basic resources of biological evolution. The deteri-

oration of ecosystem directly or indirectly by human beings has always been causing the destruction of biodiversity and many advanced genotypes [80].

There is extensive genetic diversity in citrus. Aydin and Yesiloglu [81] reported that the genus *Citrus* L. belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family [82]. The Aurantioideae is one of seven subfamilies of Rutaceae which consists of two tribes and 33 genera. Each of tribes Clauseneae and Citreae is composed of three subtribes. Clauseneae includes Micromelinae, Clauseninae, and Merrillinae, and Citreae has Triphasiinae, Citrinae, and Balsamocitrinae. The Citrinae is distinct from all the other subtribes in the subfamily by having pulp vesicles in the fruit. This subtribe contains three groups: primitive citrus fruit, near citrus fruit, and true citrus fruit trees. True citrus fruits have six genera: Clymenia, Eremocitrus, Microcitrus, Poncirus, Fortunella, and Citrus [83].

All rootstocks and varieties used are included in the genus Citrus, except for kumquats (*Fortunella* spp.) and trifoliate orange (*Poncirus trifoliata* L. Raf.), the latter is used exclusively as a rootstock. Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] is an important citrus relative for breeding new rootstocks. In addition to its tolerance to citrus tristeza virus, citrus nematodes, and cold weather and edaphic conditions, the dominant nature of the "trifoliate" leaves of trifoliate orange is a useful morphological marker in the visual identification of hybrids from crosses using trifoliate orange as male parent. Some hybrids of commercial interest, including citranges (sweet orange × trifoliate orange) and citrumelos (grapefruit × trifoliate orange), are used as rootstocks.

In general, the diversity of genetic structures in the subfamily of Aurantioideae, which occurred within the steps of biological evolution, is the genetic resource that has been lost before the determination of their characteristics during the rapidly vanishing process. Preservation of this kind of material is a challenge particularly for "conservation breeding" branch of plant breeding [80]. The determination of citrus genus and close relative genus and species collections with *in situ* and *ex situ* structure in the selected countries and regions, describing and establishing international legal tender for them, are very important.

The richness of germplasm has benefited genetics and breeding research in the countries that have a long history of citriculture. China is the most important place of origin for citrus. The long history and diversified climates enable China to harbor the most citrus varieties. Southern China is one of the centers of diversity for *Citrus* and related genera such as *Fortunella.* A National Citrus Germplasm Repository was established in China in the early 1980s, and a record in 1996 reported 1041 accessions [84, 85]. In India, there is an *in situ* germplasm including 627 accessions as it was reported by Singh [86], and eight *ex situ* citrus conservation collections have been established. Three *in situ* collections were established in Malaysia. In addition, there are three collections in Indonesia and Thailand, two collections in Philippines. The orchards of The Federal Fruit Crops Research Station in Tsukuba, Okitsu, and Kuchinotsu have the widest diversity collection of citrus and the relative types. It is declared that there are totally more than 1300 accessions in these three stations [84]. The original natural dispersion areas of Microcitrus and Eremocitrus as well as many relative types of citrus are located in Australia and the main collection in Australia is located at the Biological and Chemical Research Institute under the NSW Agriculture & Fisheries Depart-

ment in Rydalmere in New South Wales [84]. Tuzcu [80] reported that the collection of citrus in California began in the 1890s, and the conservation and utilization system of these were structured in 1910, shortly after the establishment of Citrus Experiment Station (CES) in Riverside [87]. H.J. Webber, the first manager of the station, ensured the establishment of Citrus Variety Collection in 1917 in the field which is currently in the Riverside Campus Area of California University. United States Department of Agriculture (USDA) National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) was established as a top organization for the coordination of citrus genetic resources studies in 1974. It is working in close cooperation with other two establishments. There are about 350 virus-free accessions under its conservation [84]. The most important collection is located at National Research Center for Cassava and Fruit Crops, CNPMF in Cruz das Almas/Bahaia, which includes 1858 accessions in Brazil. Valencia Agricultural Research Institute [Instituto Valenciano de Investigaciones Agrarias (IVIA)] is responsible for all the actions regarding citrus genetic resources. According to the last records, there are 478 elite accessions in total including 13 genera (16 accessions) of the Aurantioidea subfamily in addition to Citrus genus in IVIA [84, 88]. In France, 1300 accessions at Agricultural Research Station SRA tied to National Agricultural Research Institute (Institut National de la Recherches Agronomiques – The National Agronomic Research Institute (INRA) at San Nicola in Corsica Island exist in "SRA Citrus Collection" [84]. In terms of citrus genetic resources, mainly, there is one established citrus germplasm in Turkey. This establishment is named as Tuzcu Citrus Collection (TCC) and consists of 964 accessions in Çukurova University Faculty of Agriculture [80].

4. Some developed citrus rootstocks and their tolerance to abiotic stress

The first use of rootstocks in citriculture was in 1842 against Phytophthora in Azores Islands through the use of resistant rootstocks. Since then, commercial citrus orchards are established by combining scion and rootstocks in order to achieve the highest quality for the scion. The use of rootstocks in citrus decreases the long juvenility period and allows the cultivation of citrus under several abiotic and biotic stress conditions. The physiology of the whole tree is affected by rootstock, including traits of economic relevance such as fruit yield, fruit size, juice quality, tree vigor, and resistance against biotic and abiotic stresses [89]. Fruit maturation, fruit holding on tree, and postharvest preservation are also affected by rootstock [90].

There is no ideal rootstock in order to manage all abiotic and biotic stress conditions. For instance, sour orange (*Citrus aurantium* L.) which has a high adaptation capability to different soil conditions has been the most commonly used rootstock in commercial citrus trees. Also, sour orange *(Citrus aurantium* L.) is tolerant to root rot, citrus blight disease, calcareous soils, water deficit, and cold, inducing high yield and high fruit quality. However, sour orange is susceptible to citrus tristeza virus, and the usage of this rootstock is decreasing in some countries due to the existence of CTV. On the contrary, Rangpur lime (C. *limonia*), Volkamer lemon (*C. volkameriana* V. Ten. and Pasq.), and rough lemon (C. *jambhiri* Lush.) are drought resistant and increase the fruit yield of the grafted scion. Besides, they reduce the fruit quality

as compared to fruits obtained from sour orange, trifoliate orange, Carrizo citrange, and Troyer citrange. However, they are sensitive to cold.

The search for new rootstock in citrus production as well as many fruit species is necessary to sustain production under the inevitable abiotic stresses in many different ecological conditions of the citrus-growing areas of the world. Also, new diseases, the spread of the known diseases, and different environmental conditions affected by the climatic change force the demand for developing new citrus rootstocks. Below, we have tried to cover and explain some of the newly released citrus rootstocks obtained by breeding programs carried out in several countries by several researchers.

Swingle citrumelo is a hybrid of Duncan grapefruit and trifoliate orange produced in 1907 by Swingle and released by the United States Department of Agriculture in 1974. Since then, it has been used successfully as a rootstock in many countries. Most varieties produce very satisfactory yields of good to excellent quality fruit on Swingle citrumelo. Fruits produced on Swingle citrumelo are similar to sour orange, Carrizo, and Troyer citranges in terms of fruit quality. The trees have good cold-hardiness only slightly inferior to that of trees on trifoliate oranges. Scions on Swingle citrumelo are very tolerant to CTV, blight, and root rot as well as being resistant to citrus nematodes. They also have good exocortis and xyloporosis tolerance. The trees grow well on most soils and are reportedly especially tolerant of waterlogged conditions. In contrast, Swingle citrumelo is an unacceptable choice in heavy clay, calcareous soils, and high pH soils. Swingle is sensitive to high pH soils and is unsuitable for highly calcareous soils. Therefore, it is not so popular in many Mediterranean countries. Tolerant to environmental and soil conditions, Swingle citrumelo is sensitive to high chloride levels in soil and irrigation water but is more salt tolerant than other trifoliate hybrids such as Carrizo and Troyer citranges. Swingle has moderate drought tolerance [91].

Citranges are known as hybrids of sweet orange and trifoliate orange. The main purpose of the citrange development in Florida was to combine good traits of sweet orange with the cold-hardiness of the trifoliate orange in order to create cold hardy scions. Although unsuccessful, a most significant source of new rootstocks was produced instead. There are several named selections, the more important of which are Carrizo and Troyer citranges. These are hybrids of Washington navel orange and *Poncirus trifoliata*. The original crosses were made in the early 1900s by the United States Department of Agriculture with the intention of producing cold-tolerant scion varieties. They were later identified as being suitable for use as rootstocks. Fruit quality of the scion on Carrizo and Troyer is excellent. Trees on both grow moderately vigorous on a range of soil types but have poor salt tolerance and are sensitive to calcareous soils and exocortis virus. They have intermediate frost tolerance but are less cold-hardy than those on Cleopatra mandarin and trifoliate orange. In Turkey, Troyer citrange and trifoliate orange show superior fruit quality for Satsuma mandarins, and they are the mainly used rootstock in Aegean region of Turkey. However, using Carrizo citrange as a rootstock to especially mandarins and oranges is more common due to high soil pH levels in the Mediterranean region of Turkey. In general, the performance of Carrizo citrange is slightly better than Troyer in cancerous soils of Çukurova. However, sour orange is the main rootstock in Turkey. Ninety percent of citrus varieties in Turkey are grafted on the sour orange rootstock.

Benton citrange is a hybrid of Ruby Blood orange and trifoliate orange. It was bred in the late 1940s by the Department of Agriculture, New South Wales, Australia. The seed was first released to the industry in 1984. There are some commercial plantings using this rootstock which were established in 1990, but poor seed production in the seed source trees has been an impediment to its widespread usage. Because of its erratic performance under orange and mandarin scions, it is only recommended for Eureka lemons in Australia and only replant situations in Queensland. In Florida, Benton citrange is recommended for small-scale commercial trials with oranges and grapefruit. Trees on Benton rootstock are reported to be moderately cold tolerant and higher yielding.

C-35 citrange was bred by the University of California and released in 1987 and is a hybrid obtained by crossing Ruby Blood orange × Webber-Fawcett trifoliate. C-35 is tolerant to *Phytophthora* and CTV and resistant to citrus nematodes. Frost tolerance is good as or slightly better than Carrizo. Trees grow 25% smaller than Carrizo, making C-35 a candidate for closer spacing plantings. Trees grown in sandy, loam, and clay soils are satisfactory, but they are more sensitive to calcareous soils than Carrizo. C-32 has the same parentage as C-35 citrange and is a hybrid between Ruby orange and Webber-Fawcett trifoliate. Its very low seed production makes this citrange's seedling propagation difficult in order to use as rootstock [91].

Several rootstock breeding programs have been carried out by different leading countries in citrus industry in order to handle increasing problematic issues by abiotic and biotic stress factors.

Forner et al. [62] reported two new rootstocks released in Spain. Forner-Alcaide 5 (F-A 5) and Forner-Alcaide 13 (F-A 13) are two interspecific hybrids obtained through traditional hybridization by a senior author in a program for breeding citrus rootstocks at the IVIA in Moncada (Valencia), Spain. The researchers aimed to obtain new rootstocks tolerant to CTV, salinity, and lime-induced chlorosis and resistant to Phytophthora. They reported the resistance of F-A 5 and F-A 13 to CTV. In addition, F-A 5 was found to be more tolerant to lime-induced chlorosis than Carrrizo citrange, whereas F-A 13 is less tolerant [92]. Besides, both rootstocks have good tolerance to salinity and an excellent tolerance to flooding, as reported. Gonzalez-Mas et al. [53] conducted a rootstock field study in order to investigate rootstock effects on leaf photosynthesis in "Navelina" trees grown in calcareous soil. Authors have used seven new citrus rootstocks with Carrizo citrange obtained by J. Forner at the IVIA: F-A 5, F-A 13, F-A 418, F-A 517, 030116 (Cleopatra mandarin × *P. trifoliata*), 020324 (Troyer citrange × Cleopatra mandarin), and 230164 (*C. volkameriana* Ten. and Pasq. × *P. trifoliata*). Trees grafted on F-A 5 performed best under these calcareous soil conditions, whereas those on Carrizo citrange were poorly adapted, regarding the parameters investigated.

Bowman and Rouse [63] reported the release of US-812 citrus rootstock in May 2001 by the Agricultural Research Service of the USDA and is the result of a cross between Sunki mandarin and Benecke trifoliate. The rootstock was found to be highly productive of good quality fruit with a moderate vigor (standard medium tree size) as it was reported. The US-812 shows tolerance or resistance to CTV and citrus blight. It was reported that US-812 has good soil adaptability and disease resistance. Valencia orange grafted on US-812 performed well under high pH conditions in calcareous soils. Bowman [93] also introduced US-802 and US-897 and

reported high productive per tree size, good soil adaptability and disease resistance, tolerance of Diaprepes and Phytophthora complex, large contrast in vigor, and tree size as the forthcoming features of the rootstocks. Besides, US-942 rootstock is very highly productive with good fruit quality and soil adaptability.

According to the report of Federici et al. [64], three new citrus rootstocks were released in 2009 by the University of California, Riverside. These three rootstocks were named as "Bitters," "Carpenter," and "Furr" trifoliate hybrids, tested as C22, C54, and C57, respectively, and obtained by sexual hybridization of Sunki mandarin × Swingle trifoliate orange. The main character of these three hybrids is they all show good tolerance to citrus tristeza virus.

As reported by Federici et al. [64], "Bitters" showed good tolerance to freezing. It is tolerant to CTV, moderately tolerant to *Phytophthora parasitica*, not very tolerant of citrus nematode, and very tolerant of calcareous soil. "Carpenter" showed moderate tolerance to freezing. It is tolerant to CTV, moderately tolerant to *P. parasitica*, very tolerant of citrus nematode, and moderately tolerant of calcareous soil. "Furr" also showed good tolerance to freezing, and it is tolerant to CTV, very tolerant to *P. parasitica*, very tolerant of citrus nematode, and moderately tolerant of calcareous soil [64].

5. Breeding techniques for tolerance to abiotic stress: Traditional and biotechnological approaches

Nearly all commercial citrus in the world are grown as grafted trees, with the scion cultivar budded on a selected rootstock cultivar. A good scion and rootstock combination supports the development of trees that bear large quantities of high-quality fruit. However, many available rootstocks are inadequate to meet the emerging needs and challenges. A large proportion of the problems faced by the citrus industry could be overcome by the use of improved rootstocks [93, 94]. However, developing an improved rootstock is a long-term approach because of the several difficulties, mainly the complexity of citrus biology. Typically, it takes at least 15 years from the begging of a cross-hybridization program until a new selected rootstock is released to the industry for a commercial use.

5.1. Traditional breeding

Although somatic hybridizations via *in vitro* culture methods and genetic transformation via the regeneration process of plant tissues have opened new enthusiastic prospects for citrus genetic improvement, classical breeding techniques still remain important for citrus breeding. Genetic variations have a great importance in terms of plant breeding. These variations can be obtained either spontaneously or artificially by mutations and sexual hybridizations in order to achieve specific breeding objectives. Planned or unplanned sexual hybridizations have been responsible for the evolution of the new genotypes for using either rootstock or scion. Many intergenic hybrids were produced by controlled pollination. For example, citranges (*C. sinensis* × *P. trifoliate*), citrumelos (*C. paradisi* × *P. trifoliata*), citremons (*C. lemon* × *P. trifoliate*),

citradia (*C. aurantium* × *P. trifoliate*), citrumquat (*Fortunella* spp. × *P. trifoliate*), and Eremoradia (*Eremocitrus glauca* × *C. aurantium*).

Citrus flowers usually bloom in the spring in one great flush, except acid limes and lemons which are noted for flowering throughout the year in cold subtropical climates. In tropics, flowering may occur more than once throughout the year. In addition, drought and excessive fertilizing may induce flowering. Citrus flowers are mostly hermaphrodite and release pollen when the stigma is receptive. However, there are some exceptions such as staminate and pistillate flowers occurring in lemons Satsumas, Shamouti, and sour orange. Besides, late harvest of the fruits instead of optimum harvest time and fertilization deficiencies can increase the ratio of staminate and pistillate flowers on trees. Besides, W. Navels are known to have the ability to set parthenocarpic fruits due to their pollenless flowers. The flowers never close; the petals merely shed a few days later. The stigma becomes receptive just before the bud breaks open, but the stamens usually do not release pollen until several hours later, after the flower is fully open. This should be considered in terms of collecting flowers for pollens to be used as male parents. Most pollination in citrus is done by insects except for varieties showing parthenocarpy that no pollination is required for fruit development.

Many citrus cultivars are known to be self-incompatible and, in some cases, cross-incompatible. With such cultivars, an appropriate pollen supply and pollinating agents is needed. Pollination requirements vary among the species and cultivars. For example, open pollinated flowers of grapefruit result in significant increases in both fruit number and seed numbers. When lemons are protected from insect visitations, a set of fruits decreases. Pummelos are known to be self-incompatible as well as Clementines, Lee, Page, Nova, and Robinson. In contrast, no pollination problems have been observed in citron, kumquat, Meyer lemon, and trifoliate orange, but there have been problems of seed set in ”Morton” end “Troyer” citrange [95].

We consider the apomixes as one of the major problems in citrus rootstock breeding. Nucellar embryony is the most unusual feature that exists in the reproductive biology of citrus. This mechanism limits crossing and selfing in many varieties. Most of the genotypes that can play important roles as female parents in traditional crossing studies are highly apomictic (for example, see Table 3, unpublished data recorded at the Çukurova University, Faculty of Agriculture, Department of Horticulture, Citrus Germplasm Orchards). Hence, citrus breeding is limited by nucellar embryony of most diploid genotypes [96, 97]. The nucellar tissue which surrounds the megagametophyte can produce additional embryos (polyembryony) which are genetically identical to the parent plant. In contrast, zygotic seedlings are sexually produced and inherit genetic material from both parents. Zygotic and nucellar embryos can occur in the same seed. Not all citrus species exhibit the characteristic of polyembryony, a some produce only zygotic embryos (pummelo, citron, Clementine, Temple, and Persian lime, for example). Others produce only nucellar embryos. Many citron and lime varieties produce a significant percentage of zygotic seedlings but oranges, grapefruit, and many mandarins usually have a low percentage.

Genotype	Polyembryony (%)
Tuzcu 01-21 sour orange	60
Carrizo citrange	92
Troyer citrange	71
Swingle citrumelo	50
Local trifoliate *(P.trifoliata)*	80
Cleopatra mandarin	95
Volkameriana	60
Rough lemon	56
Florida Rough lemon	93
Sunki mandarin	50
Yuzu	60
Taiwanica	50
King mandarin	81
C. myrtifolia	85
C. obovidea	76

Table 3. Polyembryony ratio of some potential genotypes in CU Citrus Germplasm Orchards to be used in breeding studies.

In addition, sexual hybridization faces some constrains in citrus, due to high heterozygosity, long juvenility, and polyembryony of most citrus cultivars. Moreover, it is difficult to identify sexual hybrid embryos in their early stage. In this case, using trifoliate oranges, which are valuable rootstocks due to their characteristics such as cold-hardiness and resistance to root rot, CTV, and nematodes, gains another importance for citrus rootstock breeding against polyembryony. Since the trifoliate character is dominant, progenies exhibiting the trifoliate phenotype of the pollen parent can be considered as putative hybrids. So using trifoliate trait as a morphological marker is useful for early separation and characterization in citrus rootstock breeding studies. In contrast, progenies obtained by crossing combinations using polyembryonic genotypes as females have to be identified via molecular markers systems (RAPD and SSR) in order to speed up the separation of zygotic hybrids from nucellar seedlings.

Controlled cross-pollination in citrus is mainly performed for combing desirable traits from different genotypes or species and inducing heterosis. Based on this method, many hybrid rootstocks between citrus and *Poncirus* have been developed (see Section 4).

Controlled pollination is relatively easy in citrus. Seed parent and pollen parent flowers should be protected against contamination. Emasculation is generally easy and less effortless at the flowers that are nearly ready to open. Emasculation is accomplished by gently separating the petals, pulling off the anthers while avoiding contact with stigma. Pollination should be carried

out immediately after emasculation. A special storage of the pollen is seldom necessary while crossing within the genus *Citrus*. The genera *Poncirus* and *Fortunella* can be crossed with *Citrus*. Trifoliate orange naturally bloom earlier than citrus, so the pollens must be stored until the flowering time of Citrus. Pollens should be collected from unopened flowers from the branches of trees. After a waiting period of 24 h in the room temperature, a high quantity of pollen grains can be collected from anthers. Calcium chloride can be used as a drying agent just before storage of the pollen grains in a cold condition. On the contrary, *Fortunella* bloom much later than citrus in many areas. Figure 5 presents a traditional breeding procedure in citrus at Çukurova University, Citrus Germplasm Orchards.



Figure 5. Traditional cross-hybridization in citrus. (A) a large unopened bud, (B) emasculation, (C) pollination of the emasculated flower, (D) cotton pad wrapping around the twig, (E) bagged twig, (F) general view of the seed parent after crossing.

Fruit breeding, especially using classical breeding methods, is a difficult work taking a lot of time. In terms of citrus, chance seedlings were the main source for the cultivars, and spontaneous mutations on branches were used to select new cultivars. Current breeding projects in the present day is crossing superior selections and inducing mutations for seedlessness as well as crossing at different ploidy levels for seedless triploids.

5.2. Current biotechnologies applied in rootstock breeding

Genetic improvement of citrus through conventional breeding is limited by their genetic and reproductive characteristics. Citrus species have a complex reproductive biology, with many cases of cross- and self-incompatibility, apomixis, and high heterozygosity, and most of them have very long juvenile periods. Most species are highly heterozygous and produce progeny segregating widely many characters when crosses are made. In addition, juvenile periods are often extensive and most significantly, the presence of adventitious embryos in the nucellus of developing ovules of most citrus types greatly inhibits hybrid production [97, 98].

Plant somatic hybridization via protoplast fusion has become an important tool in plant improvement, allowing researchers to combine somatic cells (whole or partial) from different cultivars, species, or genera resulting in novel genetic combinations including symmetric allotetraploid somatic hybrids, asymmetric somatic hybrids, or somatic cybrids [99].

Briefly, the development of hybrid plants through the fusion of somatic protoplast derived from different sources of two different plant cultivars, species, and genera is called somatic hybridization. The technique of somatic hybridization involves the following steps: (1) isolation of protoplasts, (2) fusion of the protoplasts obtained from desired genotype, (3) culturing the hybrid cells, and (4) regeneration of hybrid plant. Mechanical or enzymatic methods can be used for the separation of protoplasts from plant tissue. However, the mechanical method is a laborious process that has some disadvantages such as low yield of protoplast and low protoplast viability. A plant cell consists of cell wall which has to be degraded if the protoplasts of the cell have to be manipulated as required. For this purpose, the plant cell is treated with enzymes, such as pectinase, macerozyme, cellulase, etc., that hydrolyze the plant cell wall. Since protoplasts are present in every plant cell, it can be theoretically isolated from all parts of the plant. But most successful isolations are made possible from the leaf of the plants.

Once purified protoplasts have been obtained from two different plant or tissue sources, various treatments can be given to induce them to fuse together. Generally, chemical agents or electrical manipulation is necessary to induce membrane instability that leads to protoplast fusion.

Polyethylene glycol (PEG) is used most frequently in conjunction with alkaline pH and high calcium concentrations. There are a number of steps in the fusion of plant protoplasts using PEG as a chemical facilitator. Another type of cell fusion that has emerged in recent years involves the manipulation of cell membranes by electrical currents. This process involves passing low-voltage electric pulses in a solution of protoplasts to be fused so that they line up for fusion. The protoplasts can be fused by subjecting it to brief exposure to high-voltage electric current which leads to alteration of membrane so that the adjacent protoplasts fuse. Electrofusion of plant protoplasts is often preferred over PEG fusion because it does not employ reagents that are toxic to the cells being fused. As with all other procedures, the conditions for electrofusion must be optimized for specific cell types to achieve maximum effectiveness. Typically, a yield of 20% or greater fusion products can be obtained by electrofusion of protoplasts compared to less than 1% fusion products with PEG [100].

Somatic cell fusion could overcome sexual incompatibility and long juvenility and may play a potential role in citrus genetic improvement, including producing directly or indirectly superior varieties, improving citrus scion and rootstock, or creating allopolyploids for triploid breeding [101, 102]. As Grosser and Gmitter [103] reported, this technique can facilitate conventional breeding, gene transfer, and cultivar development by bypassing some problems associated with the conventional sexual hybridization including sexual incompatibility, nucellar embryogenesis, and male or female sterility. Conversely, somatic hybridization is very promising for citrus rootstock breeding for combining genotypes having different tolerance to abiotic stress [104]. Citrus rootstock differs in terms of tolerance/resistance to abiotic stress

conditions. Incompatibility between some genotypes that have high level of tolerance to abiotic stress conditions limits the usage of traditional hybridization [105]. Oigawara et al. [106] reported the first intergeneric citrus hybrids obtained by combining embryonic callus of sweet orange and *Poncirus trifoliate* leaves via protoplast fusion. Grosser et al. [107] indicated that the regeneration of more than 300 plants obtained by protoplast fusion of Hamlin sweet orange and Flying Dragon trifoliate. The regenerated plants were determined as tetraploids. Kobayashi and Ohgawara [108] recovered tetraploid somatic hybrids by fusing the protoplast obtained from the embryonic callus of Trovita orange and leaf mesophyll protoplasts of Troyer citrange. Grosser et al. [109] reported tetraploid somatic hybrids obtained by fusing the protoplast via PEG method. They used several manipulations such as Cleopatra mandarin (*Citrus reshni*) + trifoliate orange (*Poncirus trifoliata* (L) Raf.), Acidless orange (*Citrus sinensis* (L) Osb.) + trifoliate orange (*Poncirus trifoliata* (L) Raf.), sour orange (*Citrus aurantium* L) + Flying Dragon trifoliate (*Poncirus trifoliata*), sour orange (*Citrus aurantium* L) + Rangpur lime (*Citrus limonia* Osb.), and Milam lemon + Sun Chu Sha mandarin (*Citrus reticulata* Blanco). Tetraploid plants were identified and propagated for further rootstock experiments. Ollitrault et al. [104] had reported the first intergeneric somatic hybrid obtained from protoplast fusion between *Citrus reticulata* + *Fortunella japonica* in France. The authors have regenerated approximately 100 plantlets by several manipulations (*C. reticulata* + *C. sinensis*, *C. reticulata* + *C. paradisi*, *C. reticulata* + *C. limon*, *C. reticulata* + *C. aurantifolia*, *C. reticulata* + *Poncirus trifoliata*, and *Citrus aurantium* + *Eremocitrus glauca*) and reported the possible use of these population as parental germplasm for both scion and rootstock breeding programs in citrus. Grosser et al. [99] indicated that the somatic hybrids obtained by *Citrus* + *Severinia* and *Citrus* + *Fortunella crassifolia* had lower performance as rootstocks, whereas promising performance was recorded from the scion grafted on somatic hybrids obtained from the manipulations of Acidless orange + *Atalantia ceylanica* and Nova mandarin + *Citropsis gilletiana*. Also, the researchers reported the dwarfing effects of somatic hybrids obtained by fusing the protoplast of sour orange + Flaying Dragon and Cleopatra mandarin + Flying Dragon. Ollitrault et al. [16] had selected 11 allotetraploid somatic hybrids by using flow cytometry and molecular markers and propagated them for rootstock trials in order to investigate their tolerance to abiotic and biotic stress. Mourão Filho et al. [110] reported the root rot tolerance of the somatic hybrids ("Cleopatra" mandarin + "Volkamer" lemon, "Cleopatra" mandarin + sour orange, "Caipira" sweet orange + "Volkamer" lemon, and "Caipira" sweet orange + "Rangpur" lime). Somatic hybrid combinations involving sour orange or *Fortunella obovata* as one of the progenitors were intolerant to CTV. They suggested future filed evaluations with somatic hybrids, especially those with tolerance to CTV.

In addition to these findings, tetraploid rootstocks usually have a built-in tree-size control mechanism due to some unknown physiological reaction with the diploid scion. Mourão Filho et al. [110] indicated that plants budded on tetraploid rootstocks are generally smaller, which could lead to reduced harvest costs and greater production efficiency. In Florida, more than 70 somatic hybrids that can potentially be used as rootstocks have already entered into commercial field trials. Preliminary results from these trials have shown that somatic hybrid rootstocks can produce adequate yields of high-quality sweet oranges (*Citrus sinensis* L. Osbeck) on small trees [111]. Ollitrault et al. [112] reported an intergeneric somatic hybrid

between Willow leaf mandarin and Pomeroy trifoliate named as ”Flhorag1.” Dambier et al. [44] reported the agronomic evaluation of the Flhorag1 in Morocco. Valencia orange trees on Flhorag1 displayed the lowest growth followed by Carrizo citrange and Volkamer lemon in an agreement regarding tetraploid rootstocks controlling the tree size [109]. Flhorag1 also proved highly tolerant to iron deficiency (unpublished data of Çukurova University obtained within the framework of the INCO ”CIBEWU” project).

Genetic transformation is also an attractive alternative technique for citrus genetic improvement. Almeida et al. reported that genetic transformation in Citrus has been obtained mainly from juvenile material such as embryogenic cells, epicotyl segments from *in vitro* germinated seedlings, and internodal segments from plants cultivated in the greenhouse due to a higher morphogenic ability compared to that of mature tissues [113, 114, 115, 116]. Peña et al. [18] concluded that the transformation efficiencies are generally low, and protocols are dependent on species, or even cultivar dependent. One of the limitations within this technology is low plant regeneration frequencies especially for many of the economically important citrus species [117, 118].

Another big area of biotechnology is DNA marker technology, derived from research in molecular genetics and genomics, which offers great promise for plant breeding. Owing to genetic linkage, DNA markers can be used to detect the presence of allelic variation in the genes underlying these traits. By using DNA markers to assist in plant breeding, efficiency and precision could be greatly increased. The use of DNA markers in plant breeding is called marker-assisted selection (MAS) and is a component of the new discipline of ”molecular breeding” [119].

Genomic research in recent years led to the development of screening tools via marker-assisted selection, which enables much more efficient selection of superior recombinants improved for multiple traits from conventional breeding efforts. MAS can increase the efficiency of citrus breeding and may speed the release of new cultivars. In this section, the possibilities of using MAS method for early selection in citrus rootstock breeding programs will be discussed.

MAS can be very useful to efficiently select for traits that are difficult or expensive to measure, exhibit low heritability, and are expressed late in development. However, it is usually essential to confirm at certain points in the breeding process that the selected individuals or their progeny do in fact express the desired phenotype or trait. Marker types can be classified as morphological, biochemical, cytological, and DNA based (molecular). The successful application of MAS relies on the tight association between the marker and the major gene or quantitative trait locus (QTL) analysis responsible for the trait [120].

Carillo et al. [121] reported that many studies have focused on mapping QTLs for salt tolerance-related traits in rice because of its requirement for irrigation for maximum yield, its sensitivity to salinity, and its relatively small genome. Gmitter et al. [122] reported that a localized genetic linkage map of the region surrounding the citrus tristeza virus resistance gene was developed from *P. trifoliate.* The authors indicated that the identification of markers tightly linked to CTV will enable citrus breeders to identify plants likely to be CTV resistant by indirect, marker-assisted selection, rather than by labor-intensive direct challenge with the pathogen. For early

selection in rootstock breeding program in citrus, Xu et al. [123] suggested that the feasible application of MAS in citrus rootstock breeding for citrus nematode resistance needs at least two genetic markers, each corresponding to related locus, in order to pyramiding the multi-genes associated or cofunctioned in controlling the citrus nematode resistance.

6. Conclusions

Citrus species are the most produced fruit crops and one of the most imported fruit groups subjected in both domestic and export markets. Citrus fruits can be produced in the tropical, semi-tropical, and subtropical climates with such a great market value. Factors limiting citrus growth in these climates significantly vary. Epidemic levels of disease and pest regarding the ecological conditions (temperature, relative humidity, and solar radiation) also remarkably differ. On the contrary, the existence of common abiotic stresses such as drought, salinity, and iron chlorosis in citrus-producing countries differs depending on the countries and the production areas of the countries. Rootstock is used for a successful citrus production against the abiotic stresses discussed.

In addition, searching for dwarfing rootstock, which enables high-density planting, has gained importance in terms of increasing the fruit yield per area, fruit quality, use of mechanical pruning, harvesting and decreasing pest, and disease management in citriculture. With this knowledge, we can say that there is no ideal rootstock which can combine all these traits. Hence, countries with different ecological conditions have to manage individual rootstock programs. In contrast, abiotic and biotic factors as well as the producer demands change depending on market conditions and new trends. Thus, breeding studies have to be persistent and managed by the current production situation and future demands.

In several breeding programs, existing rootstocks or genotypes that can be used as rootstocks in citrus production have been screened for abiotic stress conditions, and their current tolerance/resistance levels had been reported. These rootstocks currently have been used in citriculture depending on the ecological conditions of the producer countries. In addition, traditional breeding studies were carried out with these genotypes in order to combine their different tolerance/resistance characteristics in one genotype. Several successful rootstocks have been obtained so far by breeding programs. But traditional breeding takes at least 15 years from the begging of a cross-hybridization program until a new selected rootstock is released to the industry for a commercial use and limited by the complex reproductive biology of citrus. Therefore, rootstock breeders have begun to benefit using biotechnological methods in citrus breeding such as somatic hybridization, genetic transformation, and marker-assisted selection in recent years.

Genetic capacities of the parents or genotypes have great importance in breeding studies whichever method (traditional or biotechnological) is used, since the aim of breeding is to expose or combine the existing genetic capacity of parents. Therefore, screening both *ex situ* and *in situ* germplasm in the world regarding problems of citrus producer is necessary. Using promising genotypes obtained in screening studies carried out in germplasms in addition to

current citrus rootstock will result in an increase in breeding programs. As mentioned before, stress conditions are not stable and differ regarding the ecological conditions. Therefore, the sustainability of germplasm is necessary as long as human beings and citriculture exist.

Practical experience shows that abiotic stresses occur at high or low intensity in about all citricultural growing areas around the world. In this study, we have tried to explain the current situation of using citrus rootstocks for tolerance to abiotic stresses and breeding studies resulted and ongoing against abiotic stress in citrus production.

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