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*(Original Article)*

# Impact of PEG Induced Drought Stress on Three Citrus Rootstocks

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## Abstract

Citrus is one of the most popular fruit crops worldwide. However, citrus growth and productivity are widely influenced by unlimited biotic and abiotic stresses. Among stresses, drought is one of the most critical factors that affect citrus development and sustainability in many regions around the world. Therefore, screening for drought-tolerant rootstocks among citrus genotypes is an important step in breeding programs. In this investigation, *in vitro* evaluation of drought stress on three important rootstocks, i.e., volcameriana, sour orange, and lime by using polyethylene glycol (PEG-6000) at 5% was conducted based on morphological and chlorophyll analysis. Morphological characteristics involving proliferation rate, shoot/root length ratio, shoot/root biomass ratio, number of leaves per explant, rooting percentage, and number of roots per explant were considerably reduced under PEG stress compared to the control treatment. Volcameriana rootstock showed the greatest stability under drought stress compared to sour orange and lime rootstocks. Furthermore, total chlorophyll was highly declined under drought stress in the three rootstocks over their control. Data revealed also that volcameriana rootstock reflected the maximum chlorophyll (Chla/Chlb) ratio followed by sour orange rootstock, then lime rootstock. The results suggest that volcameriana was the most tolerant rootstock to drought stress, while sour orange rootstock was moderate to drought and lime rootstock was the most sensitive rootstock. This work offers an efficient protocol for drought screening in citrus that could help in enhancing breeding programs.

***Keywords:*** *Citrus rootstocks, Drought, polyethylene glycol, In vitro, Morphological traits.*

## Introduction

Citrus is one of the most important fruit crops worldwide, belonging to the family Rutaceae. Citrus is one of the major fruit crops in tropical and subtropical regions of the world according to its area and productivity. It is believed that citrus is native to the Himalayan area of Southern Asia and Northeastern India. It is currently cultivated in over 130 countries around the world with a substantial annual production that recorded 161.8 million tons in 2019 in a planting area of 9.89 million hectares (Liu *et al*., 2022). China, United States, Brazil, Mexico, India, and Mediterranean countries like Spain and Turkey are among the leading citrus producing countries in the world (Singh *et al.*, 2020). In Egypt, citrus fruits represent around 37.49% of the total fruit production (FAOSTAT, 2021). As for statistical data of total area was 469.912 (fed.) and the

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production was 4,344,458 (ton) of citrus in Egypt according to Annual Reports of Statistical Inst. and Agri. Ec on. Res. Egypt (2020). Citrus fruits are greatly favored for their nutritional value and human health benefits. They are a good source of phytochemicals and nutritionally active compounds like multivitamins, polysaccharides, carotenoids, flavonoids, fatty acids, fibers, and especially polyphenols (Lu *et al.,* 2023). They are widely consumed because of their outstanding flavor, pleasing taste, reasonable economic reach, and consumer awareness owing to the increasingly recognized potential health benefits. In addition to their edible fruits, they are also grown for essential oils derived from their leaves, flowers, and fruit rind (Hamid *et al*., 2024). Therefore, citrus cultivation and industry are increasing every day. In Egypt, citrus is a very valued fruit crop due to the increased demand of these fruits for local and export markets. However, due to the adeptness of citrus in tropical, subtropical, and temperate regions, drought stress is considered one of the greatest problems that threatens plant growth and productivity in many regions of the world, including Egypt. Global warming has worsened the adverse effects of drought stress (Bolan *et al*., 2024). By 2050, drought stress is predicted to trigger severe plant growth complications in dry lands and influence two-thirds of the population of the globe (Biswas *et al*., 2025).

Drought stress was reported in citrus to cause inhibition of shoot and root growth, leaf chlorosis and decreased chlorophyll content, imbalance of nutrient uptake, suppression of carbohydrate and protein biosynthesis, and negative disorders of physiological processes such as sugar metabolism, photosynthesis, and respiration (Savé *et al*., 1995; Zaher-Ara *et al*., 2016; Shafqat *et al*., 2021). Furthermore, severe water shortage induces secondary osmotic stress, which increases the formation of malondialdehyde (MDA) and reactive oxygen species (ROS), causing oxidative damage and eventually leading to plant death (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Unfortunately, plants in their natural habitats are frequently subjected to drought stress as a threatening ecological factor that cannot be avoided.

Hence, to reduce the effects of drought stress and maximize the use of water resources, the screening and proper choice of drought-tolerant cultivars is very critical. Citrus is composed of rootstock and scion varieties with edible fruits. Several investigations have demonstrated that some citrus rootstocks have high tolerance to abiotic stresses, including drought (Zaher-Ara *et al*., 2016; Hussain *et al*., 2018). Rootstocks have been valued over the years in the global agriculture sector. Their impact on plant growth, productivity, and quality as well as abiotic stress resistance have been well-established (Dubey and Sharma, 2016). Therefore, breeding citrus rootstock with superior root systems could boost citrus production in light of recurring drought. The accurate determination of drought resistance in plants is a challenging task and monitoring the morphological, physio-biochemical and molecular responses to the drought could be crucial for the identification of tolerant genotypes. Previous efforts in drought tolerance screening of citrus species were restricted to in vivo and due to variable and environmental conditions and long-term breeding cycles. Alternately, *in vitro* screening of the elite rootstocks through plant tissue culture technique could provide a promising alternative for the selection of stress tolerant rootstocks to drought stress over traditional screening tools (El-Mahdy *et al*., 2022). Thereby, the present study was conducted under *in vitro* conditions to examine the impact of polyethylene glycol (PEG) drought stress-inducing agent on the growth performance of three important citrus rootstocks widely grown in Egypt.

## Materials and Methods

### 1. Plant material

In this experimental work, fruits were collected from the adult growing citrus trees of three rootstocks, i.e., key lime (*Citrus aurantifolia*), Volkameriana lemon (*Citrus volkameriana*), and sour orange (*Citrus aurantium*) within the period of December till March. Trees were grown in the Farm of the Pomology Department, Faculty of Agriculture, Assiut University, Egypt. After washing and cleaning the fruits, seeds were carefully extracted and rinsed under running tap water for 1 h. Seeds of three citrus rootstocks were used as explant for further *in vitro* experiment. Seeds were then brought to the Tissue Culture Laboratory at the Pomology Department, Faculty of Agriculture, Assiut University, Egypt, for further sterilization.

### 2. Preparation and sterilization of plant material

Well-developed seeds were first cleaned with liquid soap under tap water, then soaked in distilled water for 12 h (4°C) a day before culture. For surface sterilization, under aseptic conditions in a laminar flow cabinet, seeds were treated with 30% Clorox (sodium hypochlorite $<5.2\%$ w/v) with 0.1 µmol drops of 0.1% (v/v) Tween-20 (to optimize surface cleanliness) per 100 mL solution continuously shaken on a rotary shaker for 25 min. Following this treatment, seeds were cleansed in double-distilled autoclaved water several times until the foam disappeared.

### 3. Culture establishment and growth conditions

After surface sterilization, the disinfected seeds were inoculated in glass vessels containing MS medium (half strength) amended with vitamins (Murashige and Skoog, 1962) and fortified with 3% sucrose and solidified with 2.5% gelrite. This media was separated into two groups: the first one for drought stress (contains 5% PEG-6000), and the second one served as a control (free of PEG). No hormones were added to either medium. Next, the pH of all media was regulated at 5.7–5.8 by using drops of 1 N HCl or 1 N KOH prior to autoclaving (1.06 kg/cm$^2$ pressure at 121°C) for 20 min. The medium was equally dispensed into culture vessels (250 ml) and the cultures were preserved in a growth room at $24 \pm 2$°C in darkness for germination induction for 10 days and then transferred to a 16-h photoperiod under white, fluorescent light lamps with a photosynthetic photon flux of 100 µmol m$^{-2}$ s$^{-1}$.

### 4. Morphological assessment

Observations of each plantlet were reported with 40-day-old cultures at the end of the experiment. Plantlets were gently removed from the jars. Plantlets were separated into shoots and roots. Roots were washed with running tap water carefully to remove adhered media without damaging the root. Several growth parameters were recorded to determine the effect of drought stress on plants of three rootstocks.

## 5. The following parameters were measured

Proliferation rate (number of shoots/explant), and the shoot and root lengths (cm) were assessed to calculate shoot-to-root length ratio (S/R L ratio) through dividing shoot length by root length.

To consider the shoot-to-root of biomass (S/R biomass ratio), dry tissues were measured by collecting fresh tissues of shoots and roots and drying tissues in an oven for 48 hours at 72◦C. Subsequently, the shoot-to-root biomass ratio was computed using the given equation as follows:

$$\textbf{Shoot:Root} = \frac{\textbf{Shoot dry weight}}{\textbf{Root dry weight}} \times \mathbf{100}$$

Moreover, the number of leaves per explant (LN), rooting percentage (RP), and number of roots per explant (RN) were calculated and counted manually.

## 6. Chlorophyll content analysis

The photosynthetic pigments of total chlorophyll (Chl a + Chl b) content, and Chlorophyll a/Chlorophyll b (Chla/Chlb) ratio were evaluated in citrus leaves following the improved method previously explained by Lichtenthaler (1987). In brief, fully expanded fresh leaves (100 mg) from stressed and unstressed plants were submerged in test tubes filled with 10 mL ethyl alcohol (95%, v/v). The samples were heated in a water bath to 60–70°C until the complete removal of chlorophyll pigments and the leave's color totally turned into white. The concentrations of Chl-a and Chl-b were measured by UV spectrophotometer at 663 and 644 nm, respectively. Ethanol (95%, v/v) was employed as a blank. The final concentrations of total chlorophyll content were considered following the equations in the Lichtenthaler (1987) protocol:

Chlorophyll a (mg/g FW) = (13.36 A663) – (5.19 A644).

Chlorophyll b (mg/g FW) = (27.49 A664) – (8.12 A663).

Total chlorophyll (chlorophyll a + chlorophyll b).

## 7. Statistical analysis

The current trial was set up in a completely randomized design (CRD) and repeated twice. For each treatment, three replicates with five repetitions were exploited. The analysis of variance (ANOVA) was applied using Proc Mixed of the SAS package version 9.2, and means were compared by using Duncan Multiple Test at $P \leq 0.05$.

# Results

## 1. Effect of PEG on proliferation rate and morphological parameters of citrus rootstocks

Table 1 demonstrates the effect of drought stress by using PEG on the proliferation rate and morphological growth parameters of the three selected citrus rootstocks grown in MS media. Data indicated that PEG stress significantly decreased the morphological traits like proliferation rate, S/R L ratio, S/R biomass ratio, LN, RP, and RN in the three different rootstocks. Sour orange rootstock showed moderate decrease in the above parameters by 50%, 27.9%, 27.8%, 54.7%, 31.2%, and 63%, respectively, compared to the control. Volcameriana rootstock showed the lowest decline by 55.9%, 47.5%, 39.8%, 28.7%, and 46.5%, respectively, except S/R biomass ratio under drought stress. Lime rootstock had the highest drop by 32%, 36.1%, 46.6%, 25.6%, 41.2%, and 42.3%, respectively, showing that volcamerina rootstock was the most tolerant cultivar, while lime rootstock is the most sensitive one to drought stress.

**Table 1. Effect of drought stress (PEG 5%) on some morphological traits of three citrus rootstocks**

| Rootstock | Treatment | Proliferation rate | S/R L ratio | S/R biomass ratio | LN | RP | RN |
|---|---|---|---|---|---|---|---|
| **Sour orange** | **Control** | $2.20^{a}$ | $62.70^{bc}$ | $320.56^{b}$ | $5.30^{a}$ | $85.33^{b}$ | $2.07^{a}$ |
| | **PEG** | $1.1^{bc}$ | $45.2^{c}$ | $231.6^{b}$ | $2.4^{d}$ | $58.7^{c}$ | $1.0^{b}$ |
| **Volcameriana** | **Control** | $2.27^{a}$ | $56.13^{bc}$ | $341.46^{b}$ | $4.93^{a}$ | $81.33^{b}$ | $2.00^{a}$ |
| | **PEG** | $1.00^{c}$ | $66.79^{bc}$ | $293.95^{b}$ | $2.97^{c}$ | $58.00^{c}$ | $1.07^{b}$ |
| **Lime** | **Control** | $1.47^{b}$ | $142.95^{a}$ | $779.89^{a}$ | $4.97^{a}$ | $94.67^{a}$ | $2.20^{a}$ |
| | **PEG** | $1.00^{c}$ | $91.31^{b}$ | $416.08^{b}$ | $3.70^{b}$ | $55.67^{c}$ | $1.27^{b}$ |

Means followed by the same letter are not significant at $P \leq 0.05$, according to Duncan Multiple Test. S/R L ratio: shoot to root length ratio; S/R biomass ratio: shoot to root biomass ratio; LN: number of leaves per plant; RP: rooting percentage; RN: number of roots per plant.

## 2. Effect of PEG on chlorophyll content of citrus rootstocks

As shown in Table 2, drought stress had negative effects on chlorophyll analysis. PEG remarkably declined the total chlorophyll content in sour orange, volcameriana, and lime rootstocks by 40.2%, 40.4%, and 38.6%, respectively, over the unstressed plants. On the other hand, the rootstocks showed variable responses of Chla/Chlb ratio under drought stress. Volcameriana rootstock showed the highest Chla/Chlb ratio (10.80) followed by sour orange rootstock (2.93) then lime rootstock (2.22).

**Table 2. Effect of drought stress (PEG 5%) on some morphological traits on three citrus rootstocks**

| Rootstock | Treatment | Total chlorophyl (mg/g FW) | Chlorophyll a/Chlorophyl |
|---|---|---|---|
| **Sour orange** | **Control** | $2.19^{a}$ | $1.69^{b}$ |
| | **PEG** | $1.31^{b}$ | $2.93^{b}$ |
| **Volcameriana** | **Control** | $2.08^{a}$ | $2.72^{b}$ |
| | **PEG** | $1.24^{b}$ | $10.80^{a}$ |
| **Lime** | **Control** | $2.15^{a}$ | $1.83^{b}$ |
| | **PEG** | $1.32^{b}$ | $2.22^{b}$ |

Means followed by the same letter are not significant at $P \leq 0.05$, according to Duncan Multiple Test.

## Discussion

Plants are continuously subjected to various abiotic and biotic stresses in their communities. Drought stress is considered one of the most menacing factors to plant growth and productivity (El-Agamy *et al*., 2010). In temperate, tropical, and subtropical regions, especially during spring and summer seasons, the evaporative requirement for the atmosphere leads to remarkable drought stress in crop plants, causing one of the most adverse environmental stresses. In these situations, water stress instigates a strong decline in growth, quantity, and quality, influencing almost all plant functions and metabolisms. It was reported earlier that drought stress negatively affects morphological, physiological, and molecular responses in several plant species, particularly fruit crops (Wang *et al*., 2012; Min *et al.,* 2019; El-Mahdy *et al*., 2021). Therefore, the selection of rootstock is among the most important priorities to develop new varieties tolerant to drought stress. Numerous investigations have illustrated the importance of citrus rootstock cultivar in response to drought stress and water limitations (García-Sánchez *et al.,* 2007; Pedroso *et al*., 2014; Miranda *et al*., 2021).

The morphology of various cultivars can determine their tolerance to drought stress. In the current study, drought stress induced by PEG negatively declined growth parameters of citrus rootstocks. PEG inhibited several parameters such as proliferation rate, shoot/root length ratio, shoot/root biomass ratio, number of leaves per explant, rooting percentage, and number of roots per explant. Volcameriana cultivar showed the highest tolerance to drought stress, while lime cultivar showed the lowest. These results well match with the study of Incesu *et al*. (2025) that showed higher growth measurements in volcameriana rootstock compared to sour orange rootstock under different irrigation deficiency levels. Moreover, similar observations were stated earlier by (Zaher-Ara *et al*., 2016), who documented a strong decrease in vegetative parameters in ten citrus cultivars; however, the extent of drought stress varied depending on the cultivar. The authors stated that growth traits like the root dry weight and shoot fresh weight could be employed as significant markers of tolerance to osmotic stress in citrus. Recently, Modica *et al*. (2025) observed significant differences in eight rootstock responses to drought stress according to morphological analysis. This growth decline could be attributed to the reduction in the water potential and the decrease in relative water content of roots under water shortage conditions, which could affect the whole growth. Thus, drought stress initiated by PEG induces turgor defeat, which disturbs mitosis, negatively inhibiting cell expansion and differentiation, and suppressing overall development.

Chlorophyll analysis is a vital indicator of photosynthetic performance, which could be used to determine the negative effect of abiotic stresses on plants (El-Mahdy *et al*., 2018; Elazab *et al*., 2021). The harmful impact of drought stress on chlorophyll biogenesis could be due to the photo-oxidation of the chlorophyll pigment and the suppression of enzymes involved in the pigment biosynthesis (Monteoliva *et al*., 2021). Moreover, Mafakheri *et al*. (2010) explained that owing to the weakening in chlorophyll synthesis, the capacity of light harvesting is lessened, which eventually raises the formation of ROS. These changes lead to the degradation of chlorophyll and damage the internal configurations of the chloroplast, altering the photosynthetic process. In agreement with our results, Jafari and Shahsavar (2022) reported similar declines in chlorophyll content in Mexican lime under *in vitro* conditions. In addition, Hussain *et al.* (2018) observed a significant drop in chlorophylls content in six citrus rootstocks, and this drop was more severe in sensitive rootstocks. In our study, volcameriana rootstock exhibited the highest Chla/Chb ratio, confirming the morphological results.

## Conclusion

Our study revealed that *in vitro* screening technique could be used as a reliable tool to identify the impact of PEG-induced drought stress on three different citrus rootstocks. The results demonstrated that drought stress negatively affects the growth parameters including, proliferation rate, shoot/root length ratio, shoot/root biomass ratio, number of leaves per explant, rooting percentage, and number of roots per explant. However, this effect varies among different rootstocks. Moreover, PEG stress showed an adverse impact on total chlorophyll. According to morphological and chlorophyll analysis, volcameraina rootstock reflected the highest tolerance to drought stress, followed by sour orange rootstock, while lime rootstock was the most sensitive to drought stress.

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**الاستجابة المعملية لثلاث اصول موالح لإجهاد الجفاف الناتج عن البولى ايثلين جليكول**

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قسم الفاكهة، كلية الزراعة، جامعة أسيوط، اسيوط، مصر.

**الملخص**

الموالح هي واحدة من أكثر محاصـيل الفاكهة شـيوعًا في جميع أنحاء العالم. ومع ذلك، يتأثر نمو الموالح وإنتاجيتها على نطاق واسـع بالإجهادات الحيوية وغير الحيوية غير المحدودة. ومن بين الإجهادات، يعد الجفاف أحد أهم العوامل التي تؤثر على نمو الموالح واسـتدامتها في العديد من المناطق حول العالم. لذلك، فإن فحص الأصـول المقاومة للجفاف بين الأنماط الجينية للموالح يعد خطوة مهمة في برامج التربية. في هذا البحث، تم إجراء تقييم معملي في المختبر لإجهاد الجفاف (باسـتخدام مادة البولي ايثلين جليكول) على ثلاثة أصـول مهمة وهي الفولكاماريانا والبرتقال المر والليمون بناءً على التحليل المورفولوجي وتحليل الكلوروفيل. انخفضت الخصائص المورفولوجية التي تتضمن معدل التفريع ونسبة طول السيقان إلى الجذور ونسبة وزن السيقان إلى الجذور وعدد الأوراق لكل نبات ونسبة التجذير وعدد الجذور لكل نبات بشـكل كبير تحت تأثير الجفاف، مقارنةً بمعاملة الكنترول. أظهر الفولكاماريانا أكبر قدر من الثبات تحت اجهاد الجفاف مقارنةً بالبرتقال المر والليمون. علاوة على ذلك، انخفض الكلوروفيل الكلي بشـكل كبير تحت اجهاد الجفاف في الأصـول الثلاثة، مقارنةً بمعاملة الكنترول. أظهرت النتائج أيضًا أن فولكاماريانا عكست أعلى نسبة كلوروفيل أ/كلوروفيل ب، يليه البرتقال المر، ثم الليمون. تشير هذه النتائج مجتمعةً إلى أن فولكاماريا كان الأصـل الأكثر تحملًا لإجهاد الجفاف، بينما كان البرتقال المر معتدل التحمل، وكان الليمون هو الأصـل الأكثر حسـاسـية. يقدم هذا العمل بروتوكولًا فعالًا لفحص الأصول المقاومة للجفاف في الموالح، مما قد يُسهم في تحسين برامج التربية.

***الكلمات المفتاحية:*** *اصول الموالح، معمليا، الجفاف، بولي ايثيلين جليكول، صفات مورفولوجية.*