https://doi.org/10.17221/136/2023-HORTSCI

The effect of rootstocks on morphological, physiological, and gene expression characters of citrus seedlings grown under drought conditions

Nirmala Friyanti Devy[1], *Siti Subandiyah*[2], *Sri Widyaningsih*[1], *Hardiyanto*[1]*, *Farida Yulianti*[1], *Dita Agisimanto*[1], *Agus Sugiyatno*[1], *Mutia Erti Dwiastuti*[1]

[1]*Research Center for Horticultural and Estate Crops, National Research and Innovation Agency of Indonesia, Cibinong Science Center Jalan Raya Bogor, Cibinong, Indonesia*
[2]*Department of Entomology and Plant Pathology, Universitas Gadjah Mada, Yogyakarta, Indonesia*

**Corresponding author: hardiyanto85@yahoo.com*

Citation: Devy N.F., Subandiyah S., Widyaningsih S., Hardiyanto Yulianti F., Agisimanto D., Sugiyatno A., Dwiastuti M.E. (2024): The effect of rootstocks on morphological, physiological, and gene expression characters of citrus seedlings grown under drought stress conditions. Hort. Sci. (Prague), 51: 255–269.

Abstract: To date, 'Japansche Citroen' (JC) has only been used commercially as a rootstock in Indonesia; however, the use of suitable rootstock remains challenging because of water limits in different places. The morphological, physiological, and gene expression changes of one-and-a-half-year-old mandarin cv. 'Keprok Batu 55' (KB) and sweet orange cv. 'Manis Pacitan' (MP) budded onto 'Japansche Citroen' (JC), 'Rough Lemon' (RL), 'Salam' (S), 'Volkameriana' (V), and 'Cleopatra Mandarin' (C) rootstocks, subjected to six-week drought-stress conditions, were examined under a shade house. The experiment was conducted in Junrejo Subdistrict (± 990 m a.s.l.), Batu City, East Java Province, Indonesia, from January 2022 to May 2023. The experimental design was a factorial randomized block design with two factors: rootstocks as factor I and two citrus species as factor II, with three replications. A low shoot/root (S/R) ratio indicates much more root development than shoots observed in KB/C (0.89) and MP/RL (0.87) plants under drought conditions; the MP/RL plant had the greatest stomatal density (16.0/0.015 mm^2) and percentage of stomatal closure (58.8%). In this condition, it had lower leaf relative water content (RWC) than the control, with comparatively high RWC ranging from 45–55% in KB/JC, KB/S, MP/C, and MP/RL. The drought did not affect cell membrane damage, although this did slightly increase the quantity of chlorophyll *a*, *b*, and carotenoids ($P < 0.05$). Aside from that, leaf proline and total soluble sugar levels were higher in the dryness than in the control, with the combination of KB/C (331.6 μ mol proline/g fresh weight), MP/JC, and MP/RL plants having the highest (347.9 and 337.1 μmol proline/g fresh weight). Meanwhile, the plants with the greatest quantities of soluble sugar were KB/C (35.9 mg/g dry weight) and MP/V (75.3 mg/g dry weight). Regardless of scion-rootstock interaction, KB enhanced the relative quantity of several genes such as 1-Aminocyclopropane-1-carboxylate (ACC) synthase, *Chlase*, ethylene receptors (*ETR1*), *osmotin*, plasma membrane instrinsic proteins (*PIP1*), and *PIP2* allowing it to respond more effectively to drought stress conditions. In contrast, MP only increased Chlase and *ETR1* ones. Under drought conditions, the KB/C and MP/RL combinations are assumed to be more adaptable than others.

Keywords: abiotic stress; water deficit; cellular osmolytes; scion species; tolerance; leaf pigments

---

Supported by Australian Centre for International Agricultural (ACIAR) Project No. HORT/2019/164.

Indonesia has commercially established citrus as one of its main fruit commodities. In 2022, the national production of this commodity (tangerines and mandarins is projected to reach approximately 2 551 999.00 tons, with corresponding to 57 000 hectares of harvested areas. This area is estimated to be 70% 'Siam' (tangerine) and 30% 'Keprok' (mandarin). In addition to them, the farmers also grow lemons, pummelo, and sweet orange s (BPS 2023). Mandarin citrus cv. 'Keprok Batu 55' (KB) and sweet orange cv. 'Manis Pacitan' (MP) are two common cultivars that customers like. Growers usually bud the scion onto a rootstock to cultivate them. 'Japansche Citroen' (CJ) (*Citrus* × *limonia* (L.) Osbeck), or JC, is a popular rootstock among Indonesian producers. Aside from that, a limited number of growers also employ 'Rough Lemon' (RL) (*Citrus* × *jambhiri* Lush.) and 'Salam' (S) (*Fortunella japonica* Thunb.) as rootstocks on their plants. Others, such as 'Volkameriana' (*Citrus 'Volkameriana'*), and 'Cleopatra Mandarin' (CM) (*Citrus reshni* hort. ex Tanaka), are less well-known.

The effect of rootstocks on scion performance has received a lot of attention. Rootstock may influence height, plant spread, canopy volume, scion and root diameters, leaf chlorophyll, and nutritional levels in citrus trees (Santana-Vieira et al. 2016; Hassanzadeh et al. 2019; Kumar et al. 2019; Zhu et al. 2020; Hayat et al. 2022).

The process of grafting on plants is a complicated one that might cause a change in the plants' essential characteristics (Greco et al. 2012; Sharma, Zheng 2019). The mobilization of genetic material between the two of them brings about this process of transformation (Stegemann et al. 2012; Thyssen et al. 2012). In addition to this, there is also the mobilization of endogenous hormones, such as gibberellis acis (GA), auxin, and cytokinin, as well as microelements (Asahina, Satoh 2015; Zhai et al. 2021). According to Hertle et al. (2021), the process of mobilization takes place via the formation of a new intercellular connection. Two different portions of the plant surface derived from rootstock and scion, come together and fit into one, creating this connection. It is suggested that gene flow from the rootstock occurs in citrus trees as well, resulting in genetic variations in the plant scions when different interstocks are used in mandarin Borneo Prima plants (Devy et al. 2023).

Furthermore, rootstock might affect the ability of a plant to adapt to its environment. According to Goncalves et al. (2019), 'Rangpur' lime rootstock produces better drought tolerance when budded with sweet orange scion by activating genes involved with cell wall formation, soluble glucose metabolism, and antioxidant metabolism. The activation of their signaling pathways increases in response to stress. Genes involved in starch metabolism, light responses, and ethylene signaling, on the other hand, are downregulated. Rootstock may transmit all stress responses to scion (Santana-Vieira et al. 2016; Balfagon et al. 2022).

Increasing the translation of ABA signaling genes might improve drought stress tolerance. Stomatal closure and transpiration decrease as ABA accumulates. In drought-tolerant rootstocks, changes in root zone moisture increase the movement of small amounts of ABA from roots to shoots, enhancing water consumption efficiency (Liu et al. 2016). Pine trees grafted with drought-tolerant rootstocks show higher photosynthesis, activation of genes involved in abiotic stress tolerance, and an increase in phytohormone transcript accumulation (Hinojosa et al. 2021). Under such conditions, drought stress frequently increases the expression of drought-related genes (Yulianti et al. 2021). In this research, two popular commercial citrus scions (mandarin cv. 'Keprok Batu 55' and sweet orange cv. 'Manis Pacitan') budded onto five rootstocks, namely JC, RL, 'Volkameriana', 'Cleopatra Mandarin', and 'Salam' were evaluated to determine their capacity to resist drought. Dry conditions were applied to plants for six weeks, and morphological, physiological, and gene-related drought expression features were examined.

MATERIAL AND METHODS

Research site. The research was conducted at a shade house in Junrejo Subdistrict (± 990 m a.s.l.), Batu City, East Java Province, Indonesia, from January 2022 to May 2023. The average temperature, relative humidity, and light intensity at the shade house were 30.6 ± 0.7 °C, 51.6 ± 1.4%, and 2936.5 ± 407.2 lux, respectively.

Materials. Rootstocks used in this study were 'Japansche Citroen' (JC) (*Citrus* × *limonia* (L.) Osbeck), 'Rough Lemon' (RL) (*Citrus* × *jambhiri* Lush.), citrus 'Volkameriana' (V) (*Citrus* × *limon*), 'Cleopatra Mandarin' (C) (*Citrus reshni* hort. ex Tanaka), and local rootstock 'Salam' (S) (*Fortunel-*

la japonica Thunb.). Meanwhile, two citrus scions used were mandarin (*Citrus reticulata* Blanco) cv. 'Keprok Batu 55' and sweet orange (*Citrus sinensis* L.) cv. 'Manis Pacitan' .

Rootstock seeds were sown in the seedbed containing mixed sand and soil (2 : 1), and it was maintained for one month under a shade house prior to this study (September 2021). Rootstock seedlings were then transplanted into polybags (10 × 30 cm) with a 2 : 1 : 1 mixture of fine sand, manure, and soil. Transplanted seedlings were kept in a shade house for three months (October to December 2021). Two citrus scions were then budded onto transplanted rootstocks, and they were kept for 13 months (January 2022 to February 2023) (Table 1).

One-and-a-half-year-old budded trees (from sowing the seeds) were transplanted in 2.4-liter plastic polybags filled with rice husk, manure, and soil (1 : 1 : 1) and watered twice a week for control and without water for six weeks for drought treatments. Plants undergo fertilization twice a month using a solution of 15 g of NPK (15 : 15 : 15) fertilizer and 10 g of ZA in 1 L of water, applied at a rate of 100 mL per plant. After the drought treatment had finished, all parameters were examined.

Table 1. Combination scion/rootstock plants

Treatment (scion/rootstock)	Code
'Keprok Batu 55'/'Cleopatra Mandarin'	KB/C
'Keprok Batu 55'/'Japansche citroen'	KB/JC
'Keprok Batu 55'/'Rough Lemon'	KB/RL
'Keprok Batu 55'/'Salam'	KB/S
'Keprok Batu 55'/'Volkameriana'	KB/V
'Manis Pacitan'/'Cleopatra Mandarin'	MP/C
'Manis Pacitan'/'Japansche citroen'	MP/JC
'Manis Pacitan'/'Rough Lemon'	MP/RL
'Manis Pacitan'/'Salam'	MP/S
'Manis Pacitan'/'Volkameriana'	MP/V

Methods

Shoot/root ratio. After six weeks of drying, plants were removed from their surroundings, cleaned, and divided into two sections: top (shoot) and bottom (root), and dried in an electric oven at 70 °C for two days. The S/R ratio was calculated by weighing the oven-dried shoots and roots.

Stomatal density (SD) and stomatal closure (SC). To generate an epidermal impression, the leaf surface was covered with nail polish. Peel off the dried layer of polish using sellotape and place it on a slide. SD was examined at a 400× magnification microscopeon an area of 0.015 mm^2.

Relative water content (RWC). The relative water content (%) in the leaves was calculated based on the equation:

Relative water content (%) = [(fresh weight – dry weight)/(fresh weight)] × 100

Membrane stability index (MSI). After washing with deionized water, 0.2 g of leaf tissue was cut into pieces and placed in 10 mL of deionized water. This material is heated for 30 minutes at 40 °C. A conductivity meter was used to measure its electrical conductivity (EC1). For EC2, the value is produced by re-boiling the content for 10 minutes at 100 °C. MSI was determined using the following equation: MSI (%) = [1 – (EC1/EC2)] × 100

Membrane injury (MI) was calculated based on the equation: MI (%) = $[1 - (MSI_d/MSI_c) \times 100]$

MSI_d = MSI drought; MSI_c = MSI control (Abid et al. 2018).

Chlorophylls and carotenoid content. Chlorophylls and carotenoid content were detected based on Sumanta et al. (2014). A total of 0.5 g of crushed leaf sample was added to 10 ml of 95% ethanol and centrifuged for 15 minutes at 10 000 rpm. The supernatant was separated and diluted ten times. The absorbance of the diluted mixture was measured spectrophotometrically at 664, 649, and 470 nm. The concentrations (µg/ml) were calculated by equation:

Chlorophyll $a = 13.36\ A_{664} - 5.19\ A_{649}$ (A = absorbance)

Chlorophyll $b = 27.43\ A_{664} - 8.12\ A_{649}$

Carotenoids = $(1\,000\ A_{664} - 2.13\ \text{Ch-}a - 97.63\ \text{Ch-}b)/209$

Determination of leaf proline and total soluble sugar content. The ninhydrin technique was utilized for the analysis of leaf proline (Bates et al. 1973); it included extracting 0.5 g of leaf sample from 10 mL of 3% aqueous sulfosalicylic acid. Proline concentrations are measured at 520 nm (µmoles of proline per gram of fresh-weight sample). The corresponding amounts of proline were determined using a standard curve.

The total soluble sugar was determined using the Anthrone method (Irigoyen et al. 1992). A 0.1 g crushed leaf sample was extracted with 80% (v/v) hot ethanol and centrifuged at 6 000 rpm for 15 minutes. The supernatant was extracted with 80% ethanol twice more. After mixing the three supernatants, 5 mL of 80% ethanol was added. The sugar content was measured at 620 nm. The corresponding amounts of soluble sugar were determined using a standard curve (mg/g DW).

Relative gene quantity analysis

Total RNA isolation. Young leaves from each sample plant unit were collected to serve as composite samples for each treatment. The composite samples provided total RNA. The isolation technique followed the guidelines of the PureLink® RNA Mini Kit. The quality and quantity of the isolated RNA were detected by using the Implen NanoPhotometer®.

Synthesis cDNA. The conversion of isolated RNA into cDNA was based on the guidelines of the iScript™ cDNA Synthesis Kit (BioRad).

Analysis of gene quantity. The quantity of genes was measured using the Mini Opticon Real-Time PCR System (BioRad). Each RT-qPCR reaction contained 10 µL of SYBR Green fluorophore (BioRad), 1 µL cDNA template, 0.5 µL (10 mM) of each primer (Table 2), and 8 µL of ddH_2O. The amplification program followed conditions: 10 minutes at 95 °C, 15 seconds at 95 °C for 40 cycles, and 1 minute at 60°C with the addition of a melting curve ranging from 65 °C to 95 °C. The Bio-Rad CFX Maestro software was used to estimate the relative gene quantity (relative to zero value). The primers used were based on Yulianti et al. (2021) (Table 2).

Experimental design. The research methodology used was a factorial randomized block design consisting of two factors: rootstocks as factor I and citrus varities as factor II, with three replications. Each treatment consisted of two plants, for a total of 120 polybags. The combination of five rootstocks and scions of 'Keprok Batu 55' and 'Manis Pacitan' were divided into two groups, then these groups were subjected to drought stress conditions for six weeks and normal conditions for control treatment, respectively.

Statistical analysis. A one-way analysis of variance (ANOVA) was used to analyze the data, subsequent to Duncan's multiple range tests ($P < 0.05$). The data was provided as mean ± standard deviation, using three replicates. R 4.3.2 (2023-10-31 ucrt) for Windows was used to create a heatmap (accessed on October 31, 2023). Trait mean values were normalized, and the library pheatmap was adapted to generate heatmap and hierarchical clusters.

RESULTS

Shoot-to-root ratio. Citrus budded plants were treated to water deficit to investigate the influence of rootstock on scion responses to drought. After stress treatments, the plant's oven-dry mass was measured, and the shoot-to-root ratio was established. The control treatment results in a significant ($P < 0.05$) difference in the S/R ratio. Plants of KB/S and MP/C exhibit a S/R ratio value greater than others, indicating that their shoot mass is greater than their root one. Other treatments, on the other hand, have a S/R value less than one (Table 3). Under drought conditions, all scion and rootstock combination plants had no significant ($P < 0.05$) effects on the S/R ratio. They vary from 0.76 to 1.18 under such conditions. On the 'Keprok Batu 55' and 'Manis Pacitan', the treatments with the lowest S/R values were KB/C (0.89) and MP/V (0.76), respectively. This may indicate that the root mass is greater than the shoot mass (Table 3).

Stomatal density (SD) and percentage of stomatal closure (SC). The interaction between the rootstock and the scion impacts the SD significantly ($P < 0.05$). At control conditions, MP/V has the highest SD (16.7) and KB/C has the least (11.2). The later treatment is not significantly different ($P < 0.05$) with KB/JC and KB/V. Meanwhile, under drought conditions, rootstock treatment in dry environments also affects the SD value of the plants. When compared to other plants, MP/C and MP/

Table 2. PCR primers forward and reverse sequences (Yulianti et al. 2021)

Primers	Forward sequences	Reverse sequences
Osmotin	CAACGACCTCTCCTCCTACG	ACAGCTCGTCCGTACCAAAC
PIP1	AGGATTACACGGAGCCACCT	TGCTTTTGGATTTGGACACG
PIP2	TGTGTTCATGGTTCACTTGG	TGAATGGTCCAACCCAGAAG
Chlase	ACCGCTTGTGCACCTGAAG	TGTCCATGTGACCATAATCTGTAGC
ACS2	CACAGTGTTCACCAAGGAGTC	CGAGTAAATGATACCGACCCTAA
ETR1	CAGGAGAGAAGCGGAAACAG	GCTCGGGTGTCAATTCAGTC

https://doi.org/10.17221/136/2023-HORTSCI

Table 3. The shoot-to-root ratio of a citrus budded plant

Treatments	S/R ratio	
	control	drought
KB/C	0.92 ± 0.1^{ab}	0.89 ± 0.1
KB/JC	0.85 ± 0.1^{ab}	1.13 ± 0.2
KB/RL	0.89 ± 0.1^{ab}	1.18 ± 0.3
KB/S	1.06 ± 0.1^{a}	1.07 ± 0.3
KB/V	0.95 ± 0.1^{ab}	0.94 ± 0.2
MP/C	1.02 ± 0.2^{ab}	1.03 ± 0.2
MP/JC	0.75 ± 0.1^{ab}	0.99 ± 0.3
MP/RL	0.74 ± 0.1^{ab}	0.87 ± 0.2
MP/S	0.98 ± 0.2^{ab}	1.06 ± 0.2
MP/V	0.62 ± 0.04^{b}	0.76 ± 0.1
P	*	ns
R^2 (%)	53.8	24.3

ns – letter in one column is not significantly different at $P < 0.05$; *different at $P < 0.05$ according to Duncan Multiple Range Test; C – 'Cleopatra Mandarin' ; JC – 'Japansche Citroen'; RL – 'Rough Lemon'; S – 'Salam'; V – 'Volkameriana'; KB – 'Keprok Batu 55' ; MP – 'Manis Pacitan' ; data are means ± standard deviation; $n = 6$

RL plants had the highest values (15.8 and 16.0, respectively) (Table 4). Drought treatment, in general, tends to increase SD in both 'Keprok Batu 55' and 'Manis Pacitan' .

In terms of the percentages of SC, all combination treatments were not significantly different both under control and drought conditions. The range of stomatal closure under control and drought conditions were 22.5 (KB/C) to 35.4% (KB/JC), and 35.3 (KB/RL) to 58.8% (MP/RL), respectively. In addition, the increment of stomatal closure ranged from 6.6 (KB/V) to 27.4 % (MP/RL) (Table 4).

Relative water content (RWC) and membrane injury (MI). The scion/rootstock combination showed significantly different leaf RWC under drought conditions ($P < 0.01$) (Table 5). The scion/rootstock combination with KB/S had the highest RWC (54.9%), followed by KB/JC (53.4%). In contrast, there was no significant difference among scion/rootstock combinations under control conditions. The RWC value varies from 64% to 74%. Nevertheless, drought stress reduced the RWC values of plants by 12.5 (KB/S) to 31.8% (MP/V).

When plants were exposed to drought treatment, the percentage of membrane damage in both 'Keprok Batu 55' and 'Manis Pacitan' plants was unaffected by the usage of rootstocks. Membrane injury (MI) ranged from 5.9 (MP/C) to 37.9% (MP/V) (Table 5).

Chlorophyll and carotenoids content. The leaf chlorophyll and carotenoids content were significantly affected by the interaction between the rootstock and the scion. The highest chlorophyll *a* content was observed in MP/C (8.0 µg/mL) under control conditions and KB/V and MP/JC (8.3 µg/mL) under drought stress. In addition, MP/C produced the highest chlorophyll *b* content under both control and drought conditions. The highest carotenoids content was detected in MP/V (1.7 µg/mL) and MP/JC (1.6 µg/mL) under control and drought conditions, respectively (Table 6).

Leaf proline (Pro) content. Plants subjected to drought stress will respond physiologically in or-

Table 4. Leaf stomatal density and stomatal closure percentage in citrus budded plants

Treatments	Stomatal density (SD/0.015 mm^2)		Stomatal closure (%)		
	control	drought	control	drought	increment
KB/C	11.2 ± 0.7^{d}	12.4 ± 0.5^{bc}	22.5 ± 6.4	39.0 ± 11.6	16.6 ± 10.6
KB/JC	11.9 ± 0.7^{d}	12.4 ± 0.3^{bc}	35.4 ± 2.0	47.9 ± 5.1	12.4 ± 4.7
KB/RL	12.0 ± 0.5^{cd}	11.6 ± 0.4^{c}	25.9 ± 2.4	35.3 ± 4.4	9.4 ± 5.9
KB/S	12.9 ± 1.2^{bcd}	12.4 ± 0.9^{c}	25.4 ± 8.2	37.1 ± 2.6	11.6 ± 10.7
KB/V	11.5 ± 0.8^{d}	12.2 ± 1.2^{c}	34.5 ± 4.4	41.2 ± 4.7	6.6 ± 5.5
MP/C	12.7 ± 0.7^{bcd}	15.8 ± 1.2^{a}	22.6 ± 4.6	37.0 ± 4.7	14.4 ± 9.0
MP/JC	15.2 ± 1.7^{abc}	14.6 ± 1.3^{abc}	34.5 ± 5.8	52.1 ± 2.7	17.6 ± 7.2
MP/RL	15.7 ± 1.4^{ab}	16.0 ± 0.7^{a}	31.4 ± 4.7	58.8 ± 5.8	27.4 ± 10
MP/S	15.1 ± 0.4^{abc}	14.6 ± 0.9^{abc}	28.3 ± 3.6	44.7 ± 2.0	16.4 ± 3.2
MP/V	16.7 ± 0.2^{a}	15.5 ± 0.3^{ab}	26.9 ± 5.3	43.2 ± 14.6	16.3 ± 9.8
P	*	*	ns	ns	ns
R^2 (%)	83.4	79.3	48.8	52.9	23.1

For abbreviations explanation see Table 1; *is significant different at $P < 0.05$ according to Duncan Multiple Range Test

Table 5. Levels of leaf relative water content (RWC) and membrane injury (MI)

Treatments	RWC (%)			MI (%)
	control	drought	reduction	
KB/C	65.9 ± 5.1	39.2 ± 3.0^{c}	26.7 ± 7.6	23.6 ± 11.5
KB/JC	74.0 ± 1.6	53.4 ± 1.6^{ab}	20.6 ± 1.4	17.0 ± 14.0
KB/RL	74.3 ± 1.6	43.1 ± 5.7^{bc}	31.2 ± 7.2	7.5 ± 2.1
KB/S	64.0 ± 5.4	54.9 ± 6.2^{a}	12.5 ± 6.7	7.9 ± 4.5
KB/V	69.5 ± 2.1	42.0 ± 9.3^{c}	27.6 ± 7.8	18.0 ± 10.3
MP/C	64.3 ± 3.01	46.3 ± 3.7^{abc}	18.0 ± 6.3	5.9 ± 6.7
MP/JC	68.9 ± 1.3	37.4 ± 1.2^{c}	31.5 ± 1.9	31.3 ± 8.3
MP/RL	72.9 ± 9.1	45.5 ± 3.9^{abc}	27.4 ± 13.0	18.2 ± 11.0
MP/S	67.3 ± 3.3	40.2 ± 4.4^{c}	27.1 ± 4.9	13.5 ± 4.5
MP/V	69.9 ± 2.3	38.1 ± 1.9^{c}	31.8 ± 4.1	37.9 ± 10.6
P	ns	**	ns	ns
R^2 (%)	52.2	85.3	44.3	60.5

For abbreviations explanation see Table 1; *is significant different at $P < 0.05$ according to Duncan Multiple Range Test

der to protect themselves from damage or death. The significant interaction was shown in leaf Proline content both under drought stress and control conditions. In general, Proline accumulation in leaves increased under dry circumstances compared to control ones (Table 7). Under drought conditions, leaf Proline content was highest in MP/JC (347.9 µmol Proline/g FW), followed by MP/RL (337.1 µmol Proline/g FW). Under the same circumstances, Proline concentration in KB was found to be high in plants budded on 'Cleopatra Mandarin' (KB/C, 331.6 µmol Proline/g FW) and 'Rough Lemon' rootstocks (KB/RL, 320.8 µmol Proline/g FW).

Soluble sugar content. A significant difference was observed in leaf soluble sugar concentrations both under control and drought conditions. MP/V and MP/C had the highest leaf soluble sugar concentration under drought (75.3 mg/g DW) and control conditions (37.9 mg/g DW), respectively (Table 8). It seems that the use of different rootstocks relatively did not affect the leaf-soluble sugar content in 'Keprok Batu 55', both under drought and control conditions.

Table 6. Levels of leaf chlorophyll and carotenoids content

Treatments	Levels of leaf chlorophyll (Chl) and cartenoids content					
	Chl-*a* (µg/mL)		Chl-*b* (µg/mL)		Carotenoids (µg/mL)	
	control	drought	control	drought	control	drought
KB/C	5.6 ± 1.3^{abc}	7.3 ± 0.1^{cd}	4.2 ± 0.1^{abc}	4.6 ± 0.1^{d}	1.4 ± 0.7^{ab}	1.0 ± 0.02^{de}
KB/JC	5.3 ± 0.1^{bc}	7.2 ± 0.1^{d}	3.2 ± 0.1^{d}	4.5 ± 0.2^{d}	0.8 ± 0.01^{abc}	1.0 ± 0.04^{de}
KB/RL	4.4 ± 0.5^{c}	7.9 ± 0.2^{abcd}	3.1 ± 0.2^{d}	4.7 ± 0.04^{cd}	0.4 ± 0.3^{c}	1.5 ± 0.05^{ab}
KB/S	4.9 ± 1.2^{bc}	8.1 ± 0.1^{abc}	3.9 ± 0.1^{bc}	4.8 ± 0.1^{bcd}	0.6 ± 0.3^{bc}	1.0 ± 0.01^{e}
KB/V	3.8 ± 1.1^{c}	8.3 ± 0.02^{a}	3.7 ± 0.5^{cd}	4.9 ± 0.03^{bcd}	1.0 ± 0.1^{abc}	1.3 ± 0.04^{bc}
MP/C	8.0 ± 0.2^{a}	7.6 ± 0.2^{abcd}	4.7 ± 0.2^{a}	5.7 ± 0.2^{a}	1.6 ± 0.01^{ab}	0.8 ± 0.01^{e}
MP/JC	6.1 ± 0.1^{abc}	8.3 ± 0.02^{a}	3.6 ± 0.3^{cd}	5.0 ± 0.1^{bc}	0.9 ± 0.1^{abc}	1.6 ± 0.03^{a}
MP/RL	6.0 ± 0.1^{abc}	7.4 ± 0.1^{bcd}	3.8 ± 0.1^{cd}	4.6 ± 0.04^{cd}	1.1 ± 0.01^{abc}	0.9 ± 0.03^{e}
MP/S	7.2 ± 0.1^{abc}	8.2 ± 0.3^{ab}	4.6 ± 0.1^{ab}	5.2 ± 0.2^{ab}	1.0 ± 0.04^{abc}	1.2 ± 0.05^{cd}
MP/V	4.8 ± 0.2^{bc}	5.1 ± 0.6^{e}	3.1 ± 0.2^{d}	3.6 ± 0.3^{e}	1.7 ± 0.1^{a}	0.8 ± 0.2^{e}
P	*	**	**	**	*	**
R^2 (%)	77.4	94.5	89.6	94.5	69.2	95.9

For abbreviations see Table 1; *, ** significatnly different at $P < 0.05$, and at $P < 0.01$ according to Duncan Multiple Range Test, respectively

Table 7. Level of leaf proline content

Treatments	Proline (μmol)/sample (g FW)	
	control	drought
KB/C	163.2 ± 3.3^{b}	331.6 ± 13.3^{ab}
KB/JC	31.8 ± 2.6^{f}	288.3 ± 8.7^{ab}
KB/RL	78.9 ± 1.3^{de}	320.8 ± 7.7^{ab}
KB/S	104.9 ± 10.4^{c}	273.3 ± 16.0^{bc}
KB/V	61.5 ± 4.2^{e}	319.8 ± 33.0^{ab}
MP/C	81.2 ± 8.4^{de}	206.4 ± 33.9^{c}
MP/JC	68.2 ± 10.3^{e}	347.9 ± 0.0^{a}
MP/RL	193.2 ± 6.3^{a}	337.1 ± 7.7^{ab}
MP/S	62.9 ± 4.6^{e}	330.9 ± 14.1^{ab}
MP/V	96.8 ± 2.9^{cd}	306.9 ± 35.0^{ab}
P	**	**
R^2 (%)	98.2	78.6

For abbreviations see Table 1; **significant different at $P < 0.01$ according to Duncan Multiple Range Test

Relative quantity of related-drought genes. Citrus plants exposed to stress enhance the relative expression of numerous genes involved in cell metabolism (Gonçalves et al. 2019). At control and drought conditions, patterns of quantity genes varied in 'Keprok Batu 55' and 'Manis Pacitan' (Table 9). In general, the relative quantity was higher under drought conditions than under control, with a bigger percentage rise in 'Keprok Batu 55' . Significantly more quantity genes were found in KB with *ACS* and *osmotin*, whereas *Chlase* and *ETR1* were found in greater numbers in MP. The KB/C and MP/RL combinations seem to have performed the best in gene quantity.

Table 8. Level of leaf soluble sugar content

Treatments	Total soluble sugar (mg/g DW)	
	control	drought
KB/C	15.9 ± 4.3^{c}	35.9 ± 3.6^{cd}
KB/JC	27.4 ± 4.8^{abc}	30.4 ± 0.9^{d}
KB/RL	24.1 ± 2. 8^{bc}	34.0 ± 6.5^{d}
KB/S	25.0 ± 3.1^{bc}	27.8 ± 2.6^{d}
KB/V	18.1 ± 2.0^{c}	23.8 ± 6.2^{d}
MP/C	44.6 ± 2.9 a	61.9 ± 1.0^{ab}
MP/JC	29.7 ± 9.8^{abc}	66.8 ± 5.4^{ab}
MP/RL	28.2 ± 3.2^{abc}	52.3 ± 2.8^{c}
MP/S	24.5 ± 8.3^{bc}	61.1 ± 4.8^{ab}
MP/V	37.9 ± 6.1^{ab}	75.3 ± 7.5^{a}
P	**	**
R^2 (%)	69.7	93.4

For abbreviations see Table 1; **significant different at $P < 0.01$ according to Duncan Multiple Range Test

Comparison of treatment results. A comparison heatmap analysis indicated two distinct groups among the parameters assessed in this research (Figure 1). Group 1 comprised all drought-related genes, soluble sugar content, proline, MI, and SC. The remainder is classified as the second one, which includes the S/R ratio, RWC, chlorophyll, and carotenoid concentration.

Drought-treated plants, on the other hand, were separated into two groups depending on their responses. The first group contains MP/V, KB/C, and MP/RL, with the main difference being a much higher concentration of all related-drought genes, as well as Proline and MI, than others.

The second group is divided into four sub-groups: 2a. MP/C, 2b. MP/JC and MP/S, 2c. KB/JC and KB/S, and 2d. KB/RL and KB/V. This group is distinguished by above-average amounts of chlorophyll *a, b*, and S/R ratios. There were differences in the amounts of *PIP1*, *ETR1*, *PIP2*, *Chlase,* SD, and soluble sugar in sub-groups 2a-b and 2c-d, which are 'Manis Pacitan' and 'Keprok Batu 55' , respectively. 'Manis Pacitan' had higher amounts of these substances than 'Keprok Batu 55' (Figure 1).

DISCUSSION

The lack of water and rootstock treatments on two citrus scion cultivars changed the morphology of the plants, as seen in the S/R ratio and stomatal characters. Drought also affects physiological and genetic characteristics such as leaf RWC and MI, chlorophyll, and the relative abundance of drought-related genes.

Drought stress affects shoot-to-root ratio. Genetic variations of rootstocks would impact scion biomass (Hayat et al. 2022). Under water stress, plants grafted onto drought-resistant rootstock will have a high drought-resistance coefficient. This is related to increases in root production and decreases in root dry weight (Li et al. 2021; Prinsi et al. 2021).

At control conditions, the rootstock had a significant ($P < 0.05$) effect on the growth of plant shoots and roots. 'Salam' rootstock stimulated more shoot development than roots in KB/S plants, while MP/V plants produced more root growth than

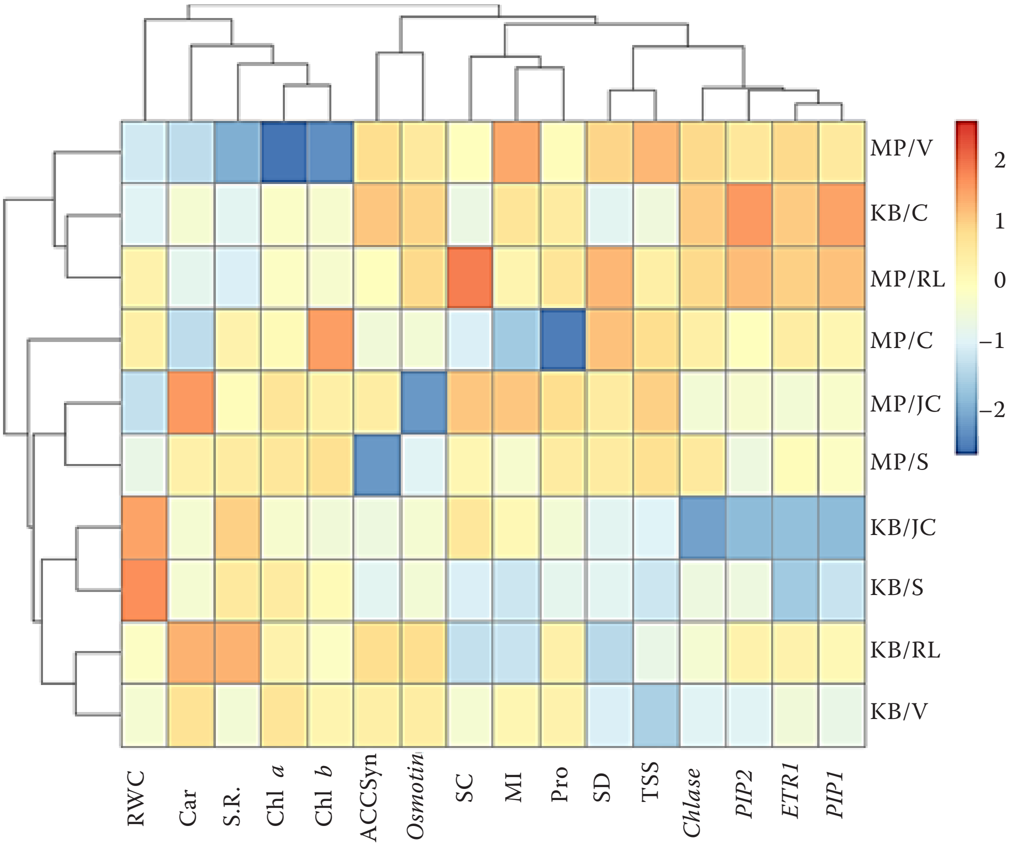

Figure 1. Heatmap and cluster analysis of the morphological, physiological, and relative quantity of related-drought genes in 'Keprok Batu 55' (KB) and 'Manis Pacitan' (MP) plants budded onto five rootstocks grown under drought stress treatment

C – 'Cleopatra Mandarin'; JC – 'Japansche Citroen'; RL – 'Rough Lemon'; S – 'Salam'; V – 'Volkameriana'; RWC – relative water content; SD – stomatal density; S.R. – shoot roor ratio; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; ACCSyn – 1-Aminocyclopropane-1-carboxylate synthase; Osmotin; SC – stomatal closure; Pro – proline; MI – membrane injury; TSS – total soluble sugar; *PIP2* – plasma membrane instrinsic proteins; *ETR1* – ethylene receptors; *PIP1* – plasma membrane instrinsic protein; Car – carotene

shoot growth. However, under drought treatments, rootstock treatment did not affect the value of the plant S/R ratio. Among all combinations of scion/rootstock, KB/C, MP/RL, and MP/V plants exhibit lower S/R ratios than the others, with values of 0.89, 0.87, and 0.76, respectively. This suggests that on the 'Keprok Batu 55', 'Cleopatra Mandarin' rootstocks favor root development more than shoot segments, as do the RL and 'Volkameriana' rootstocks on 'Manis Pacitan' (Table 3).

Dry stress causes plants to change their metabolism, enhancing water usage efficiency by decreasing water loss (Dahro et al. 2023). The initial phase in plant adaptation is to limit shoot development, decrease surface area, and promote root growth in order to maximize water absorption. However, according to Zhang et al. (2022), the gene suspected of encoding hyperosmolarity (*OSCA1*) does not function in this situation. This reduces calcium absorption in guard and root cells, resulting in decreased transpiration and plant root development (Yuan et al. 2014). In general, all of these activities result in a reduction in the fresh and dry weight of plant shoots and roots (Bashir et al. 2021). The rootstock's effect on the scion's performance is controlled by chemicals that move from the root to the shoot. These chemicals include peroxidase (POD), abscisic acid (ABA), gibberellins (GA_{24}), and many more (Jahromi et al. 2012; Sousa et al. 2022).

Researchers have classified 'Rangpur' Lime as a drought-tolerant citrus rootstock because it grows by consuming photoassimilates from the leaves and transporting them to the roots (Silva et al. 2021). Due to water scarcity, it altered

https://doi.org/10.17221/136/2023-HORTSCI

Table 9. Relative quantity gene of *ACS2*, *Chlase*, *ETR1*, *osmotin*, *PIP1*, and *PIP2* at the scion/rootstock combinations treatments

Treatments	*ACS2*	*Chlase*	*ETR1*	*osmotin*	*PIP1*	*PIP2*
Control						
KB/C	0.222 ± 0.037	0.121 ± 0.021	0.344 ± 0.069	0.413 ± 0.083	0.164 ± 0.024	0.083 ± 0.016
KB/JC	0.155 ± 0.060	0.053 ± 0.013	0.076 ± 0.027	0.069 ± 0.010	0.079 ± 0.043	0.044 ± 0.007
KB/RL	0.358 ± 0.023	0.142 ± 0.014	0.370 ± 0.036	0.416 ± 0.030	0.143 ± 0.010	0.100 ± 0.019
KB/S	0.627 ± 0.093	0.197 ± 0.030	0.686 ± 0.075	0.630 ± 0.107	0.295 ± 0.040	0.199 ± 0.011
KB/V	0.210 ± 0.018	0.095 ± 0.005	0.165 ± 0.015	0.216 ± 0.012	0.111 ± 0.003	0.084 ± 0.006
MP/C	0.269 ± 0.044	0.247 ± 0.059	0.268 ± 0.034	1.000 ± 0.254	0.613 ± 0.144	0.593 ± 0.110
MP/JC	0.447 ± 0.104	0.208 ± 0.107	0.289 ± 0.060	0.309 ± 0.018	0.221 ± 0.074	0.278 ± 0.052
MP/RL	0.253 ± 0.022	0.207 ± 0.062	0.211 ± 0.042	0.590 ± 0.095	0.297 ± 0.044	0.313 ± 0.125
MP/S	1.000 ± 0.158	1.000 ± 0.142	1.000 ± 0.195	0.929 ± 0.147	1.000 ± 0.135	1.000 ± 0.151
MP/V	0.808 ± 0.035	0.805 ± 0.049	0.684 ± 0.056	0.598 ± 0.050	0.447 ± 0.045	0.612 ± 0.041
Drought						
KB/C	1.000 ± 0.335	1.000 ± 0.189	1.000 ± 0.223	1.000 ± 0.324	1.000 ± 0.167	1.000 ± 0.152
KB/JC	0.076 ± 0.019	0.033 ± 0.008	0.068 ± 0.026	0.069 ± 0.012	0.095 ± 0.016	0.030 ± 0.006
KB/RL	0.649 ± 0.082	0.232 ± 0.017	0.504 ± 0.039	0.772 ± 0.025	0.385 ± 0.050	0.268 ± 0.019
KB/S	0.053 ± 0.019	0.189 ± 0.011	0.080 ± 0.024	0.062 ± 0.020	0.150 ± 0.010	0.111 ± 0.019
KB/V	0.341 ± 0.054	0.132 ± 0.021	0.246 ± 0.048	0.363 ± 0.085	0.217 ± 0.023	0.083 ± 0.013
MP/C	0.096 ± 0.000	0.527 ± 0.127	0.568 ± 0.172	0.062 ± 0.112	0.402 ± 0.054	0.190 ± 0.109
MP/JC	0.385 ± 0.045	0.219 ± 0.050	0.259 ± 0.051	0.001 ± 0.006	0.290 ± 0.041	0.147 ± 0.047
MP/RL	0.174 ± 0.230	0.856 ± 0.088	0.973 ± 0.134	0.848 ± 0.069	0.785 ± 0.061	0.693 ± 0.097
MP/S	0.006 ± 0.014	0.624 ± 0.077	0.383 ± 0.020	0.023 ± 0.015	0.315 ± 0.020	0.113 ± 0.031
MP/V	0.663 ± 0.033	0.867 ± 0.103	0.870 ± 0.107	0.427 ± 0.150	0.524 ± 0.066	0.373 ± 0.133

For treatments abbreviations see Table 3

plant development and shoot morphology sooner than the other rootstocks, with a considerable decrease in shoot and increase in root growth (Silva et al. 2023).

Stomatal density (SD) and percentage of stomatal closure (SC). Stomata are pores that allow water and CO_2 to enter and exit plants. Their size and density will influence their resistance, photosynthetic capabilities, and plant production (Chen et al. 2020). One of the most significant characteristics of drought-tolerant plants is their ability to adjust SD and SC.

In citrus plants, the utilization of the rootstock would influence their stomatal density (Oustric et al. 2021). Our results indicate that rootstocks had a substantial influence on plant SD in both control and drought conditions. This demonstrates that the rootstock affects the phenotype of the plant's stomata. This is consistent with a study on Macadamia sp. (Wakefield et al. 2022).

In general, SD was relatively similar to both control and drought treatments. The SD decreased by a relative slight under drought conditions in MP/V, MP/JC, KB/S, and KB/RL plants. These rootstocks are expected to adapt better to drought than others by reducing SD mechanisms in their scions to control transpiration, thereby limiting water loss and its adverse effects (Hughes et al. 2017).

Drought factors also promote a greater closure of stomata. This is consistent with the research of Zhao et al. (2015), who found that dryness would drastically enhance stomatal production in maize, resulting in an increase in SD but a decrease in stomatal size and a higher SC. ABA levels in leaves would increase under these conditions, resulting in reactive oxygen species (ROS). The higher ROS levels would induce the guard cells to create nitric oxide (NO) and Ca^{2+}. All of them contribute to the SC (Bharath et al. 2021; Jia et al. 2021). ABA-induced peptide 2; 1 (*PIP2; 1*) may also activate this pathway (Rodrigues et al. 2017). MP/JC, MP/RL, KB/C, and KB/JC plants likewise exhibited the highest percentage of stomatal closure. 'Cleopatra Mandarin', JC, and RL rootstocks are considered to alter water use efficiency in water-stressed conditions via changes in SD, stomatal size, and, finally, an increase in SC.

A six-week dry treatment affected the value of RWC, MSI, and MI. The impact is also found in plant chlorophyll and carotenoid levels. Each scion-rootstock combination plant exhibited distinct behavior. The scion/rootstock combination, according to Neves et al. (2017), would result in a different response to RWC as well as other physiological processes such as reduced MSI and increased in their MI level.

Relative water content (RWC) and membrane injury (MI). Drought stress reduced RWC in both species' leaves by 12–32% (Table 5). Reduced RWC has an influence on cell volumes inside the leaf, ABA accumulation, and SC (Sack et al. 2018). The outer layers of cells are the first to be stressed by many factors. This is thought to be a consequence of an adverse shift in membrane flexibility and protein organization. As a consequence, ensuring optimum membrane durability is crucial for plant adaptation to water scarcity (Yuan et al. 2014; Bashir et al. 2021). This situation is consistent with Mossad et al. (2018), who found that lowering irrigation circulation by 55% increases the leaf water deficit by 27% and reduces fruit growth by 15% without affecting shoot or trunk development.

Table 5 shows that RWC scion values did not differ under control. However, under drought conditions, scions responded with varying RWC levels (37–55%) depending on rootstock type. Both 'Keprok Batu 55' and 'Manis Pacitan' , which budded onto RL and 'Volkameriana' rootstocks, reacted to RWC stimuli similarly, with values of RWC of 43–45 and 38–42%, respectively. Plants budded onto the other three rootstocks behaved differently, with 'Keprok Batu 55' having a higher RWC than 'Manis Pacitan' on JC and 'Salam'. 'Keprok Batu 55' was less responsive to RWC stimuli on 'Cleopatra Mandarin' rootstock than on 'Manis Pacitan' .

The 'Cleopatra Mandarin' rootstock is thought to be drought-tolerant by driving the 'Manis Pacitan' scion to retain relatively high leaf RWC and low stomatal closure, allowing the photosynthesis process to operate more efficiently than plants with other rootstocks. It is hypothesized that this produces balanced development of the plant's shoot and root segments, which is represented in the S/R ratio value being approximately the same (1.0) under both control and dry situations. A similar phenomenon occurs with KB/S plants (Table 1).

MSI has a significant impact on membrane damage (MI). The lower the MSI score, the bigger the membrane damage. Low intake of carbon in drought environments creates reactive oxygen species (ROS), primarily superoxide ($O2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which cause membrane cell deterioration (Abid et al. 2018; Amoah, Seo 2021). Furthermore, drought-tolerant wheat plants had a greater MSI and a lower MI under these conditions.

When compared to other treatments, KB/RL, KB/S, and MP/C had a comparatively low MI. The low degree of membrane damage is assumed to be related to the rootstock's ability to stimulate the upper stem to maintain RWC in the plant, hence reducing ROS generation and the damage it causes. It suggested that 'Cleopatra Mandarin', RL, and 'Salam' are more efficient in utilizing water under drought stress, making them relatively resistant to drought.

Chlorophyll and carotenoid content. Plant chlorophyll levels have a significant impact on photosynthesis. The rootstock employed in this research affects the level of chlorophyll and carotenoids. These results are in line with prior citrus research (Gonzalez-Mas et al. 2009; Kumar et al. 2019). In control environments, 'Cleopatra Mandarin' rootstock induced the generation of chlorophyll *a* and *b* in 'Manis Pacitan' (MP/C) better than other rootstocks. However, KB/V and MP/JC generate this substance better than others under dry conditions. On the other hand, the highest carotenoid content was detected in MP/V (1.7 μg/mL) and MP/JC (1.6 μg/mL) under control and drought conditions, respectively (Table 6).

Variation in chlorophyll synthesis capability is thought to be a genetic trait of plants and a result of adverse environmental conditions (González-Mas et al. 2009; Kumar et al. 2019). This condition will impair rootstock's capacity to absorb nutrients from the soil (Bashir et al. 2021). 'Cleopatra Mandarin', 'Volkameriana', and JC rootstocks are regarded as relatively drought-resistant due to their tendency to generate more chlorophyll than others.

At Harumi Tangor citrus budded onto *Citrus junos* as the most drought-resistant rootstock, exhibited the highest chlorophyll levels in leaves under dry conditions (Dong et al. 2021). In addition to high levels of chlorophyll, dry situations promote the upregulation of five photosynthesis-related genes as well as greater Rubisco activity in Chrysanthemum plants grafted onto resistant rootstock (Chen et al. 2018). During drought, mobile mR-

NAs from the rootstock are transported to the scions of cucumber plants grafted onto pumpkin rootstock, which are identified as photosynthesis-related mRNAs. This chemical is thought to have increased chlorophyll concentration and photosystem II maximum (Davoudi et al. 2022).

Proline and soluble sugar levels generated from drought stress. Proline and soluble sugar are two compounds produced by stressed plants to protect themselves from injury or death (Goufo et al. 2017; Guo et al. 2018). Starch in the leaves causes proline and sugar synthesis during drought stress (Zanella et al. 2016; Dahro et al. 2023), which is then transported to the roots (Wang et al. 2022). Table 7. shows that, in general, proline accumulation in leaves increased under dry circumstances compared to control ones. The RWC is inversely related to the amount of Proline under drought conditions. In this research, a negative correlation was shown between RWC and proline content; the regression equations for proline level at 'Keprok Batu 55' and 'Manis Pacitan' are $Y_{Pro\text{-}KB}$ = 420.2 – 2.4 × (R^2 = 0.54**) and $Y_{Pro\text{-}MP}$ = 552.5 – 5.9 × (R^2 = 0.27*), respectively. Decreased levels of RWC would increase proline buildup.

Rootstocks JC and RL on 'Manis Pacitan' plants (MP/JC and MP/RL) produced more Proline than other rootstocks, as did 'Cleopatra Mandarin' on 'Keprok Batu 55' (KB/C). Meanwhile, as seen in Table 8, dry conditions encourage an increase in plant-soluble sugar concentration. When 'Manis Pacitan' and 'Keprok Batu 55' plants budded onto 'Volkameriana' (MP/V) and 'Cleopatra Mandarin' (KB/C), they had the highest sugar levels.

An increase in soluble sugar and sucrose content is followed by a reduction in starch content in soybean leaves (Du et al. 2020). Citrus plants' dehydration tolerance strategy may be controlled by glucose buildup as well as abscisic acid and salicylic acid accumulation in the roots and leaves, which protect plant cells from oxidative damage (Santana-Vieira et al. 2016).

According to the findings of a study by Silva et al. (2023), in drought conditions, citrus plants would induce the production of 58 major metabolites, the majority of which were amino acids, organic acids, and sugars. The rise in these compounds is a means for plants to adapt to dryness and to absorb water from their surroundings (Dahro et al. 2023). These organic solutes work as appropriate osmolytes to maintain turgor or as antioxidants to protect cell functioning (Goufo et al. 2017). These stress conditions will stimulate the synthesis of heat shock protein (HSP70) in scion cucumber grafted on drought-tolerant pumpkin, leading to an increase in Proline accumulation (Davoudi et al. 2022). Drought-tolerant plants absorb water and photosynthesize more efficiently than sensitive plants (Dien et al. 2019); Dahro et al. 2023.

Similar to Proline, the increase in soluble sugar in drought-stressed plants serves as an osmoprotectant as well as a source of plant carbon since the starch content in the leaves decreases under these situations (Silva et al. 2023). Research by Davoudi et al. (2022) showed a comparable large rise in sugar contents corresponding to activated genes encoding carbohydrate metabolism.

Relative gene quantity in response to drought. The variations in response to gene quantity in each combination plant suggested that physiological activation had occurred in the roots. According to Balfagón et al. (2022), a rootstock is more drought-resistant than others if the plant's antioxidant system is more activated.

Drought setting increased the relative amount level of *ACS* in KB/C, KB/RL, and KB/V plants by 62–350%; however, this situation also decreased the quantity of this gene at KB/JC and KB/S plants by about 51 and 91.5%, respectively. All rootstock treatments reduced the amount of this gene in 'Manis Pacitan' by 13–99%, with the MP/S plant having the lowest level (Table 9).

According to Jia et al. (2021) and Eun et al. (2019), *ACS* influences SD formation and ethylene synthesis. S-adenosyl-L-methionine (SAM) is converted by *ACS* activity into 1-aminocyclopropane-1-carboxylate (ACC) (Jia et al. 2021). Activating *ACC oxidase* (*ACO*) transforms ACC into ethylene. JC and 'Salam' may respond to drought by activating the *ACS* gene, which leads to a stomatal and ethylene production arrangement. Reduced *ACC* synthesis would reduce SD and ethylene production, allowing plants to save water. Once ethylene highly accumulates in this manner under stress, it causes plant mortality (Jha et al. 2021; Jia et al. 2021).

Chlase enzymes regulate chlorophyll degradation (Harpaz-Saadi et al. 2007). The presence of more ethylene in plants enhances the activity of this enzyme, resulting in more chlorophyll degradation (Jacob-Wilk et al. 1999). In contrast to most of the plants that were examined by Ben-Yaakov et al. (2006), during leaf senescence, the *Chlase* activity reduces but is not involved in chlorophyll breakdown (Hu et al. 2021).

Drought treatment, in general, promotes a rise in the amount of *Chlase* in plants, with the greatest increase in KB/C. However, there was a decline in its quantity at the KB/JC, KB/S, and MP/S plants. The reduction in *ACS* and *Chlase* activity in these three plants is thought to be a method for the plants to adjust to dry situations.

Plants under stress also induce changes in the ethylene receptor *ETR1*, allowing for relatively uninhibited plant growth and development (Cebrián et al. 2021; Wang et al. 2008). The lower the *ETR1* level, the more prone the plant is to ethylene, which limits growth (Zhao, Schaller 2004). In this research, a positive correlation was shown between *ACS* and *ETR1* levels, indicating that *ACS* activity determines the amount of ethylene produced. The regression equation for ETR1 quantity is Y_{ETR1} = 0.167 + 0.732 ACS** (R^2 = 49.8%; P = 0.001). Plants with a significant rise in *ETR1* were KB/C and MP/RL, whereas the decreased ones were KB/S and MP/S. It is expected that 'Salam' used as rootstocks at 'Keprok Batu 55' and 'Manis Pacitan' will respond to drought by reducing ethylene production at their scion.

The quantity of *osmotin* will increase to defend plants from abiotic stress. It has a role in the induction of osmo-tolerance in plants (Simsek 2018; Bashir et al. 2020). This protein activity is responsible for essential cell activity under controlled conditions (Bashir et al. 2020; Faillace et al. 2021). Soybean plants with osmotin had improved physiological responses and yield in dry conditions (Luis et al. 2014; Faillace et al. 2021). According to our findings, under dry conditions, KB/C, KB/RL, and MP/RL plants had a larger relative amount of osmotin than the others. These three plants may adjust to their surroundings by promoting the development of osmoprotectants such as proline, soluble sugar, and others.

PIPs are in charge of encoding aquaporins, which are water-restricting channels (Afzal et al. 2016). The expression pattern changes widely depending on the amount of disturbance and the plant type. During times of extreme drought, the plant lowers *PIP* intensity to minimize water loss via the roots into the surrounding soil and maintain suitable moisture levels (Afzal et al. 2016; Yıldırım et al. 2018; Opazo et al. 2020; Yang et al. 2022). Hou et al. (2019) and Shen et al. (2020) found that *PIP1* in *Arabidopsis* is a crucial driving factor that regulates the production of salicylic acid. It would promote the creation of the other *PIP1* family members responsible for SC. Under the same conditions, *PIP2* activation is also triggered by ABA (Rodrigues et al. 2017).

However, depending on the citrus cultivar, drought-induced stomatal conductance decreases are related to *PIP2.1* and *PIP2.5* downregulation (Miranda et al. 2022). It was predicted that lowering *PIP2* would result in a slight rise in SC. Under insufficient water conditions, Keprok Batu 55 budded onto JC rootstock (KB/JC) had the lowest relative amounts of *PIP1* and *PIP2* genes (0.095 and 0.030, respectively) (Table 9).

According to a comparative heatmap, in a dehydration situation, 'Cleopatra Mandarin' and 'Rough Lemon' rootstocks may stimulate 'Keprok Batu 55' and 'Manis Pacitan' scions, respectively to up-regulate transcription of all drought-related genes, synthesize Proline, soluble sugars, and chlorophyll *a* and *b* at average levels, allowing leaf RWC and photosynthesis to be comparatively maintained, and finally root development to be significantly higher than shoot development.The rise in the number of *ACS*, *ETR1*, *PIP1*, and *PIP2* genes in the two plants is regarded normal and has no negative effects on the plants.

CONCLUSION

Drought stress decreased leaf water content, membrane integrity, and stomatal aperture, resulting in higher Proline and soluble sugar concentrations. 'Keprok Batu 55' exhibited signs of stress by increasing all relative genes quantity, but 'Manis Pacitan' only elevated *Chlase* and *ETR1*. This indicates that cell membranes are being protected from stress damage and that water loss is being prevented, leading in lower MI values and improved rooting performance.

Based on the value of proline, MSI, shoot/root ratio, and all gene quantities, 'Keprok Batu 55' and 'Manis Pacitan' budded onto 'Cleopatra Mandarin' and 'Rough Lemon' rootstocks, respectively (KB/C and MP/RL), exhibited suitable plant growth and were thought to be more adaptable to dry conditions than the others.

REFERENCES

Abid M., Ali S., Qi L.K., Zahoor R., Tian Z., Jiang D., Snider J.L., Dai T. (2018): Physiological and biochemical changes during drought and recovery periods at tillering and jointing stages in wheat (*Triticum aestivum* L .). Scientific Reports, 8: 4615.

Afzal Z., Howton T.C., Sun Y., Mukhtar S. (2016): The roles of aquaporins in plant stress responses. Journal of Developmental Biology, 4: 9.

Amoah J.N., Seo Y.W. (2021): Effect of progressive drought stress on physio-biochemical responses and gene expression patterns in wheat. 3 Biotech, 11: 440.

Asahina M., Shinobu S.S. (2015): Molecular and physiological mechanisms regulating tissue reunion in incised plant tissues. Journal of Plant Research, 128: 381–388.

Balfagón D., Terán F., Oliveira R. De., Santa C. (2022): Citrus rootstocks modify scion antioxidant system under drought and heat stress combination. Plant Cell Reports, 41: 593–602.

Bashir M.A., Silvestri C., Ahmad T., Hafiz I.A., Abbasi N.A., Manzoor A., Cristofori V., Rugini E. (2020): Osmotin: A cationic protein leads to improve biotic and abiotic stress tolerance in plants. Plants, 9: 992.

Bashir S.S., Hussain A., Hussain S.J., Wani O.A., Zahid Nabi S., Dar N.A., Baloch F.S., Mansoor S. (2021): Plant drought stress tolerance: understanding its physiological, biochemical and molecular mechanisms. Biotechnology and Biotechnological Equipment, 35: 1912–1925.

Bates L.S., Waldren R.P., Teare I.D. (1973): Rapid determination of free proline for water-stress studies. Plant Soil, 39: 205–207.

Ben-Yaakov E., Harpaz-Saad S., Galili D., Eyal Y., Goldschmidt E. (2006): The relationship between chlorophyllase activity and chlorophyll degradation during the course of leaf senescence in various plant species. Israel Journal of Plant Sciences, 54: 129–135.

Bharath P., Gahir S., Raghavendra A.S. (2021): Abscisic acid-induced stomatal closure: An important component of Plant defense against abiotic and biotic stress. Front Plant Science, 12: 615114.

BPS (2023): Statistical Yearbook of Indonesia 2023. Badan Pusat Statistik Press. Available at www.bps.go.id.

Cebrián G., Iglesias-moya J., García A., Martínez J., Romero J., Regalado J.J., Martínez C., Valenzuela J.L., Jamilena M. (2021): Involvement of ethylene receptors in the salt tolerance response of *Cucurbita pepo*. Horticulture Research, 8: 73.

Chen H., Zhao X., Zhai L., Shao K., Jiang K., Shen C., Chen K., Wang S., Wang Y., Xu J. (2020): Genetic bases of the stomata-related traits revealed by a genome-wide association analysis in rice (*Oryza sativa* L.). Frontiers in Genetics, 11: 611.

Chen Y., Sun X., Zheng C., Zhang S., Yang J. (2018): Grafting onto artemisia annua improves drought tolerance in Chrysanthemum by enhancing photosynthetic capacity. Horticultural Plant Journal, 4: 117–125.

Dahro B., Li C., Liu J.H. (2023): Overlapping responses to multiple abiotic stresses in citrus: from mechanism understanding to genetic improvement. Horticulture Advances, 1: 4.

Davoudi M., Song M., Zhang M., Chen J., Lou, Q. (2022): Long-distance control of the scion by the rootstock under drought stress as revealed by transcriptome sequencing and mobile mRNA identification. Horticulture Research, 9: uhab033.

Devy N.F., Hardiyanto, Sugiyatno A. Dwiastuti M.E., Yulianti F. (2023): Leaf anatomy, photosynthetic characteristics, fruit quality, and genetic changes in ‘Borneo Prima’ mandarin (*Citrus Reticulata* Blanco) grafted onto different interstocks in dry highland conditions. Applied Ecology and Environmental Research, 21:1805–1822.

Dien D.C., Mochizuki T., Yamakawa T. (2019): Effect of various drought stresses and subsequent recovery on proline, total soluble sugar and starch metabolisms in Rice (*Oryza sativa* L.) varieties. Plant Production Science, 22: 530–545.

Dong T., Xi L., Xiong B., Qiu X., Huang S., Xu W., Wang J., Wang B., Yao Y., Duan C., Tang X., Sun G., Wang X., Deng H., Wang Z. (2021): Drought resistance in Harumi tangor seedlings grafted onto different rootstocks. Functional Plant Biology, 48: 529–541.

Du Y., Zhao Q., Chen L., Yao X., Zhang W., Zhang B., Xie F. (2020): Effect of drought stress on sugar metabolism in leaves and roots of soybean seedlings. Plant Physiology and Biochemistry, 146: 1–12.

Eun H.D., Ali S., Jung H., Kim K., Kim W.C. (2019): Profiling of ACC synthase gene (*ACS11*) expression in *Arabidopsis* induced by abiotic stresses. Applied Biological Chemistry, 62: 42.

Faillace G.R., Caruso P.B., Saraiva L.F., Timmers M., Favero D., Guzman F.L., Rechenmacher C., Oliveira-Busatto L.A., Souza O.N., Bredemeier C., Bodanese-Zanettini M.H. (2021): Molecular characterisation of soybean osmotins and their involvement in drought stress response. Frontiers in Genetics, 12: 632685.

Gonçalves L.P., Camargo R.L.B., Takita M.A., Machado M.A., Walter S., Filho S., Costa M.G.C. (2019): Rootstock-induced molecular responses associated with drought tolerance in sweet orange as revealed by RNA-Seq. BMC Genomics, 20: 110.

González-Mas M.C., Llosa M.J., Quijano A., Forner-Giner M.A. (2009): Rootstock effects on leaf photosynthesis in “navelina” trees grown in calcareous soil. HortScience, 44: 280–283.

Goufo P., Moutinho-Pereira J.M., Jorge T.F., Correia C.M., Oliveira M.R., Rosa E.A.S., António C., Trindade H. (2017): Cowpea (*Vigna unguiculata* L. Walp.) metabolomics: Osmoprotection as a physiological strategy for drought stress resistance and improved yield. Frontiers in Plant Science, 8: 586.

Greco M., Chiappetta A., Bruno L., Bitonti M.B. (2012): In *Posidonia oceanica* cadmium induces changes in DNA methylation and chromatin patterning. Journal of Experimental Botany, 63: 695–709.

Guo R., Shi L.X., Jiao Y., Li M.X., Zhong X.L., Gu F.X., Liu Q., Xia X., Li H.R. (2018): Metabolic responses to drought stress in the tissues of drought-tolerant and drought-sensitive wheat genotype seedlings. AoB PLANTS, 10: ply016 10.

Harpaz-Saad A.S., Azoulay T., Arazi T., Ben-yaakov E., Shiboleth Y.M., Hörtensteiner S., Gidoni D., Goldschmidt E.E., Eyal Y. (2007): Chlorohyllase is a rate-limiting enzyme in chlorophyll catabolism and is posttranslationally regulated. The Plant Cell, 19: 1007–1022.

Hassanzadeh H., Rastegar S., Golein B., Golmohammadi M., Aboutalebi A. (2019): Effect of rootstock on vegetative growth and mineral elements in scion of different Persian lime (*Citrus latifolia* Tanaka) genotypes. Scientia Horticulturae, 246: 136–145.

Hayat F., Li J., Liu W., Li C., Song W., Iqbal S., Khan U. (2022): Influence of citrus rootstocks on scion growth, hormone levels, and metabolites profile of 'Shatangju' mandarin (*Citrus reticulata* Blanco). Horticulturae, 8: 608.

Hertle A. P., Haberl B., Bock R. (2021): Horizontal genome transfer by cell-to-cell travel of whole organelles. Science Advances, 7: eabd8215.

Hinojosa M.L., María N., Guevara M.A., Vélez M.D., Cabezas J.A., Díaz L. M., Mancha J. A., Pizarro A., Manjarrez L.F., Collada C., Sala C. D., Goy M.T.C. (2021): Rootstock effects on scion gene expression in maritime pine. Scientific Reports, 11: 11582.

Hou S., Shen H., Shao H. (2019): PAMP-induced peptide 1 cooperates with salicylic acid to regulate stomatal immunity in *Arabidopsis thaliana*. Plant Signaling & Behaviour, 14: e1666657.

Hu X., Kha I., Jiao Q., Zada A., Jia T. (2021): Chlorophyllase, a common plant hydrolase enzyme with a long history, is still a puzzle. Genes, 12: 1871.

Hughes J., Hepworth C., Dutton C., Dunn J.A., Hunt L., Stephens J., Waugh R., Cameron D.D., Gray J.E. (2017): Reducing stomatal density in barley improves drought tolerance without impacting on yield. Plant Physiology, 174: 776–787.

Irigoyen J.J., Emerich D.W., Sanchez-Diaz M. (1992): Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum, 84: 55–60.

Jacob-Wilk D., Holland D., Goldschmidt E.E., Riov J., Eyal Y. (1999): Chlorophyll breakdown by chlorophyllase isolation and functional expression of the *Chlase1* gene from ethylene- treated Citrus fruit and its regulation during development. The Plant Journal, 20: 653–661.

Jahromi A.A., Hasanzada H., Farahi M.H. (2012): Effect of rootstock type and scion cultivar on citrus leaf total nitrogen. World Applied Sciences Journal, 19: 140–143.

Jha C.K., Sharma P., Shukla A., Parmar P., Patel R., Goswami D., Saraf M. (2021): Microbial enzyme, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase: An elixir for plant under stress. Physiological and Molecular Plant Pathology, 115: 101664.

Jia M., Liu L., Geng C., Jiang J. (2021): Activation of acid synthases sets stomatal density and clustered ratio on leaf epidermis of *Arabidopsis* in response to drought. Frontiers in Plant Science, 12: 758785.

Kumar S., Awasthi O.P., Dubey A., Sharma R.M. (2019): Effect of different rootstocks on growth, leaf sclerophylly and chlorophyll fractions of Kinnow mandarin . Indian Journal of Horticulture, 74: 505–509.

Li S., Cao Y., Wang C., Sun X., Wang W., Song S. (2021): Contribution of different genotypic roots to drought resistance in soybean by a grafting experiment. Plant Production Science, 24: 317–325.

Liu S., Li H., Lv X., Ahammed G.J., Xia X., Zhou J. (2016): Grafting cucumber onto luffa improves drought tolerance by increasing ABA biosynthesis and sensitivity. Scientific Reports, 6: 20212.

Luís R., Weber M., Wiebke-strohm B., Bredemeier C., Margis-pinheiro M., Brito G. G. De Rechenmacher C., Bertagnolli P.F., Eugênia M., Sá L. De., Campos M.D.A., Maria R., Amorim S.De., Beneventi M.A., Margis R., Grossi-de-sa, M.F., Bodanese-zanettini M.H. (2014): Expression of an osmotin-like protein from *Solanum nigrum* confers drought tolerancein transgenic soybean. BMC Plant Biology, 14: 343.

Miranda M.T., Espinoza-Nu´n˜ez E., Silva S.F., Ribeiro R.L., Boscariol-Camargo, Machado E.C., Ribeiro R.V. (2022): Leaf PIP2.1 and PIP2.5 are down-regulated in 'Mandarin' lime under drought. Theoretical and Experimental Plant Physiology, 34: 63–69.

Mossad A., Scalisi A., Bianco, R. (2018): Growth and water relations of field-grown 'Valencia' orange trees under long-term partial rootzone drying. Irrigation Science, 36: 9–24.

Neves D.M., Hora-Almeida L.A., Souza Santana-Vieira D.D., Freschi L., Ferreira C.F., Filho W.S.S., Gesteira A.S. (2017): Recurrent water deficit causes epigenetic and hormonal changes in citrus plants. Scientific Reports, 7: 13684.

Opazo I., Toro G., Salvatierra A., Pastenes C., Pimentel P. (2020): Rootstocks modulate the physiology and growth responses to water deficit and long-term recovery in grafted stone fruit trees. Agricultural Water Managament, 228: 105897.

Oustric J., Herbette S., Quilichini Y., Morillon R., Giannettini J., Berti L., Santini J. (2021): Tetraploid Citrumelo 4475 rootstocks improve diploid common clementine tolerance to long-term nutrient deficiency. Scientific Reports., 11: 8902.

Prinsi B., Simeoni F., Galbiati M., Meggio F., Tonelli C., Scienza A., Espen L. (2021): Grapevine rootstocks differently affect physiological and molecular responses of the scion under water deficit condition. Agronomy, 11: 289.

Rodrigues O., Reshetnyak G., Grondin A., Saijo Y., Leonhardt N., Maurel C., Verdoucq L. (2017): Aquaporins facilitate hydrogen peroxide entry into guard cells to mediate ABA- and pathogen-triggered stomatal closure. Proc. of the National Academy of Sciences of the United States of America, 114: 9200–9205.

Sack L., John G. P., Buckley T. N. (2018): ABA accumulation in dehydrating leaves is associated with decline in cell volume, not turgor pressure. Plant Physiology, 176: 489–493.

Santana-Vieira D.D.S., Freschi L., Aragão L., Henrique D., Moraes S., Neves D.M., Marques L., Bertolde F.Z., Soares S., Antonio M., Filho C. (2016): Survival strategies of citrus rootstocks subjected to drought. Scientific Reports, 6: 38775.

Sharma A., Zheng B. (2019): Molecular responses during plant grafting and its regulation by auxins, cytokinins, and gibberellins. Biomolecules, 9: 397.

Shen J., Diao W., Zhang L., Acharya B.R., Wang M., Zhao X., Chen D., Zhang W. (2020): Secreted peptide *PIP1* induces stomatal closure by activation of guard cell anion channels in *Arabidopsis*. Front Plant Science, 11: 1029.

Silva S.F., Miranda M.T., Costa V.E., Ribeiro R.V., Machado E.C. (2021): Sink strength of citrus rootstocks under water deficit. Tree Physiology, 41: 1372–1383.

Silva S.F., Miranda M.T., Cunha C.P., Domingues-Jr A.P., Aricetti J.A., Caldana C., Machado E.C., Ribeiro R.V. (2023): Metabolic profiling of drought tolerance: Revealing how citrus rootstocks modulate plant metabolism under varying water availability. Environmental and Experimental Botany, 206: 105169.

Simsek O. (2018): Effect of drought stress in *in vitro* and drought-related gene expression in Carrizo citrange. Fresenius Environmental Bulletin, 27: 9167–9171.

Sousa A.R. de O., Silva E.M. de A., Filho M.A.C., Costa M.G.C., Filho W. dos S.S., Micheli F., Maserti B., Gesteira, Da Silva Gesteira A. (2022): Metabolic responses to drought stress and rehydration in leaves and roots of three Citrus scion/rootstock combinations. Scientia Horticulturae, 292: 110490.

Stegemann S., Keuthe M., Greiner S., Bock R. (2012): Horizontal transfer of chloroplast genomes between plant species. Proceeding of the National Academy of Sciences of the United States of America, 109: 2434–2438.

Sumanta N., Haque C.I., Nishika J., Suprakash R. (2014): Spectrophotometric analysis of chlorophylls and carotenoids from commonly grown fern species by using various extracting solvents. Research Journal of Chemical Sciences, 4: 63–69.

Thyssen G., Svab Z., Maliga P. (2012): Cell-to-cell movement of plastids in plants. Proceedings of the National Academy of Sciences of the United States of America, 109: 2439–2443.

Wakefield S., Topp B., Alam M. (2022): Crown position and rootstock genotype influence leaf stomatal density in *Macadamia* sp. Biology and Life Sciences Forum, 11: 9.

Wang Z., Yang Y., Yadav V., Zhao W., He Y. (2022): Drought-induced proline is mainly synthesized in leaves and transported to roots in watermelon under water deficit. Horticultural Plant Journal, 8: 615–626.

Wang Y., Wang T., Li K., Li X. (2008): Genetic analysis of involvement of ETR1 in plant response to salt and osmotic stress. Plant Growth Regulation, 54: 261–269.

Yang L., Xia L., Zeng Y., Han Q. (2022): Grafting enhances plants drought resistance: Current understanding, mechanisms, and future perspectives. Frontiers Plant Science, 13: 1015317.

Yıldırım K., Yağcı A., Sucu S., Tunç S. (2018). Responses of grapevine rootstocks to drought through altered root system architecture and root transcriptomic regulations. Plant Physiology and Biochemistry, 127: 256–268.

Yuan F., Yang H., Xue Y., Kon D., Ye R., Li C., Zhang J., Theprungsirikul L., Shrift T., Krichilsk B., Johnson D.M., Swift G.B., He Y., Siedow J.N., Pei Z.M. (2014): OSCA1 mediates osmotic-stress-evoked Ca2+ increases vital for osmosensing in *Arabidopsis*. Nature, 514: 367–371.

Yulianti F., Adiredjo A.L., Soetopo L., Ashari S. (2021): Changes in physiological and relative genes expression response of mandarin citrus (*Citrus reticulata* Blanco) cv Rimau Gerga Lebong (RGL) grafted onto different citrus rootstocks. Indian Journal of Agricultural Research, 55: 549–555.

Zanella M., Borghi G. L., Pirone C., Thalmann M., Pazmino D., Costa A., Santelia D., Trost P., Sparla F. (2016): β-amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis during drought stress. Journal of Experimental Botany, 67: 1819–1826.

Zhai L., Wang X., Tang D., Qi Q., Yer H., Jiang X., Han Z., McAvoy R., Li., Li Y. (2021): Molecular and physiological characterization of the effects of auxin-enriched rootstock on grafting. Horticulture Research, 8: 74.

Zhang H., Zhu J., Gong Z., Zhu J.K. (2022): Abiotic stress responses in plants. Nature Review Genetics, 23: 104–119.

Zhao X.C., Schaller G.E. (2004): Effect of salt and osmotic stress upon expression of the ethylene receptor ETR1 in *Arabidopsis thaliana*. FEBS Letters, 562: 189–192.

Zhao W., Sun Y., Kjelgren R., Liu X. (2015): Response of stomatal density and bound gas exchange in leaves of maize to soil water deficit. Acta Physiologiae Plantarum, 37: 1704.

Zhu S.P., Huang T.J., Yu X., Hong Q.B., Xiang J.S., Zeng A.Z., Gong G.Z., Zhao X.C. (2020): The effects of rootstocks on performances of three late-ripening navel orange varieties. Journal of Integrative Agriculture, 19: 1802–1812.

Received: November 2, 2023
Accepted: March 3, 2024