



Response of citrus (*Citrus* spp.) rootstock hybrids to PEG induced drought under hydroponic system

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ABSTRACT

Present study was carried out at the glasshouse facility of the ICAR-Indian Agricultural Research Institute, New Delhi during 2020–21 using a simple hydroponic platform to assess the polyethylene glycol (PEG) induced drought impact on shoot-root morphology and physio-biochemical traits of 4 newly developed citrus (*Citrus* spp.) rootstock hybrids. Hybrid CRH 21-13/14 displayed superior shoot morphological performance in drought, articulated in terms of increment in plant height (4.5%), leaf numbers (9.5%), and dry weight of shoot (7.0%) with the lowest leaf wilting index (LWI). A contrasting pattern was noticed in CRH 23-5/15, CRH 23-9/17 akin to sensitive check Jatti khatti. Hybrid CRH 21-13/14 exhibited extensively, ramified and improved root system, and was able to sustain root growth under drought. It also showed an increment in root tips (116.1%) and forks (12.6%) during drought, whereas the rest hybrids along with sensitive check (Jatti khatti) exhibited a declining trend. The strong positive association of most root traits with relative water content (RWC) and membrane stability index (MSI) underscored the contribution of root traits towards drought avoidance by optimizing water resources. Although all tested hybrids declined total chlorophyll, chlorophyll a/b ratio and CSI (chlorophyll stability index) to varying degrees during drought, CRH 21-13/14 exhibited minimum decline. Study postulate superior drought tolerance of CRH 21-13/14 conferred by the drought avoidance mechanism and moderate performance of CRH 21-14/14 (Pummelo × Troyer) as against CRH 23-5/15 and CRH 23-9/17 (Pummelo × Morton hybrids).

Keywords: Drought physiology, Hydroponic, Root architecture, Rootstock, Root traits

Drought dominates abiotic stresses due to its huge impact on agriculture production *vis-a-vis* food security worldwide. Global warming prompted an increment in average temperature and altered rainfall patterns. Consequently, inconsistent rainfall and a rise in drought frequency are major issues for agriculture systems. Globally, citrus (*Citrus* spp.) rank first in the international fruit trade with cultivation spanning over 140 countries and production of 146.6 million tonnes (FAOSTAT 2019). On account of their evergreen nature and extended fruiting period, citrus trees are subjected to multiple abiotic stresses throughout their life-cycle (Santana-Vieira *et al.* 2016). Inadequate water and nutrients impair plant development and lower fruit yield and quality, leading to the perpetual decline of citrus orchards (Rodriguez-Gamir *et al.* 2010). Therefore, sustainable citrus farming necessitates assured irrigation during critical growth periods.

Rootstocks have been valued for more than a century in the global citriculture sector. Their impact on plant growth, nutrient acquisition, fruit yield, and quality as well

as abiotic stress tolerance is well documented (Dubey and Sharma 2016). Furthermore, differential abilities of citrus rootstocks to provide water and nutrient to shoots have been established (Rodriguez-Gamir *et al.* 2010). Citrus rootstock response to water deficit is determined by root architecture, biomass, distribution, and water and nutrient use efficiency (Rodriguez-Gamir *et al.* 2010). Therefore, breeding citrus rootstock with superior root systems could boost citrus production in light of recurring drought and dwindling water resources.

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 *in vitro* testing (Rodriguez-Gamir *et al.* 2010, Zaher-Ara *et al.* 2016). Alternatively, hydroponic platforms have been increasingly employed for rapid and effective drought screening of agronomic crops. However, relatively few studies have been conducted on fruit crops using hydroponic systems. Hence, present study aimed to investigate morphological and physio-biochemical reactions of citrus rootstock hybrids to PEG induced drought with

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special emphasis on root attributes under hydroponic conditions.

MATERIALS AND METHODS

The present study was carried out during year 2020–21 using a simple hydroponic platform in glasshouse facility available at the Division of Plant Physiology, ICAR-Indian Agricultural Research Institute, New Delhi. Four newly developed citrus rootstock hybrids generated from 2 different cross combinations i.e. CRH 21-13/14 and CRH 21-14/14 from Pummelo × Troyer and CRH 23-5/15 and CRH 23-9/17 from Pummelo × Morton were selected to screen against PEG-6000 induced drought along with the known drought-susceptible check Jatti khatti (*Citrus jambhiri*). Mature fruits were harvested from the citrus hybrid block and field gene bank maintained at the main orchard of ICAR-Indian Agricultural Research Institute, New Delhi. Seeds were sown in nursery beds in polyhouse just after extraction from mature fruit. Fifteen-day-old uniform nucellar seedlings were selected for the current study. A hydroponic setup was established in plastic trays, each with a dimension of 0.56 m × 0.37 m × 0.12 m (L × W × H), covered with thick a thermocole sheet having 18 holes evenly spaced at 8 cm in 3 rows (each row had six holes). The complete hydroponic setup was comprised of 4 trays (1A, 1B, 2A, and 2B). Trays 1A and 1B were exact replicas of each other, so were 2A and 2B (Fig 1). Each tray holds 2 citrus rootstock hybrids and sensitive check (Jatti khatti), with 6 seedlings of each in separate rows. Seedling roots were submerged in the half-strength Hoagland solution (replaced every four-days), continually maintained to pH of 4.7, and aerated using an aquarium air-pump. The temperature of the glasshouse was maintained at 35±2.5°C for 16 h photoperiods provided by 60 W LED bulbs (600 µmol/m²/s) and the relative humidity was 65%. Post-transplant seedlings were established for 15 days until new shoot and root growth was observed. Subsequently, drought stress was imposed via PEG-6000 (80 g/l) to achieve osmotic stress of -0.10 MPa in trays 1B and 2B while, trays 1A and 2A were maintained under control (without PEG). After the imposition of drought treatment for one-week, all seedlings were harvested for evaluation of shoot-root morphology and physio-biochemical attributes.

The plant height and total number of leaves were recorded (n=6) under both treatments, at the end of the experiment while, the number of rolled leaves were recorded only for drought stress seedlings. The LWI was scored from 0 to 6 (n=6) as per leaf wilting categories, viz. 1, normal (not wilted); 2, slightly wilted; 3, wilted; 4, severely wilted; 5, nearly dead; and 6, dead (Engelbrecht *et al.* 2007). The shoots and roots were then separated carefully and their fresh weight was recorded, subsequently, dry weights were determined following oven-drying for 48 h at 70°C. Furthermore, the shoot to root dry weight ratio was calculated. The root study (n=6) was performed using WinRhizo™ system (Regent Instruments Inc., Québec, QC, Canada, 1996). A set of 7 root traits was recorded: total

length of the root system (cm), projected and surface root area (cm²), root volume (cm³), and number of root tips, forks, and crossings. While newly emerged white roots counted by visual observation.

Leaf RWC was determined using 8 mm leaf discs from 4 samples according to Barrs and Weatherley (1962). Leaf MSI was measured following method of Bajji *et al.* (2002). Proline content in matured leaves was assessed by rapid colorimetric method as suggested by Bates *et al.* (1973). The leaf chlorophyll ‘a’ and ‘b’ were extracted using DMSO solvent and chlorophyll a to b ratio and total chlorophyll was estimated as suggested by Barnes *et al.* (1992). The chlorophyll stability index (CSI) was estimated according to Murphy (1962).

Statistical analysis was performed using Proc GLM procedure in SAS 9.3 version software (SAS Institute Inc. Cary, NC, USA), followed by a t-test (LSD). P value <0.05 was considered significant. Association between studied traits was estimated using Pearson’s simple correlation at P≤0.05, P≤0.01 and P≤0.001, represented in a heatmap generated using R software (v3.4.3, <https://www.r-project.org>).

RESULTS AND DISCUSSION

We found significant differences among tested citrus rootstock hybrids to drought stress as revealed by most of their shoot-root morphological and physio-biochemical attributes, suggesting differential drought tolerance capability. Treatment-wise means comparisons revealed that drought treatment caused a significant decline in the majority of studied traits, excluding shoot to root ratio which showed no alteration, alternatively mean proline and chlorophyll ‘b’ content increased under drought compared to control conditions.

Shoot morphology: Genotype × drought interaction effect revealed that under drought CRH 21-13/14 (10.03 cm) and CRH 23-5/15 (9.91 cm) displayed superiority over sensitive check Jatti khatti (7.93 cm) in relation to plant height (Table 1). Regarding the change in plant height under drought, CRH 21-13/14 displayed an increment of 4.5%, while the rest hybrids diminished. Similarly, during drought, the highest number of leaves per seedling was sustained by CRH 21-13/14 (13.50) significantly, followed by CRH 21-14/14 (9.50 nos.) (Table 1). Alternatively, CRH 23-5/15 and CRH 23-9/17 reported a decline in leaf numbers compared to sensitive check (Jatti khatti). Leaf shedding in drought could be the response to increased synthesis and sensitivity of ABA hormone in plants. Genotype × drought interactions revealed that, during drought, CRH 21-13/14 exhibited 7% and 11.1% boost in shoot and root dry weight, respectively (Table 1). Root biomass and shoot to root biomass partitioning are vital for optimizing water resources and plant productivity in drought. The ability of CRH 21-13/14 to sustain growth in above and below-ground portions could be attributed to an efficient physiological and biochemical mechanism. The minimal decline in shoot and root weight in CRH 21-14/14 suggests the lesser impact of

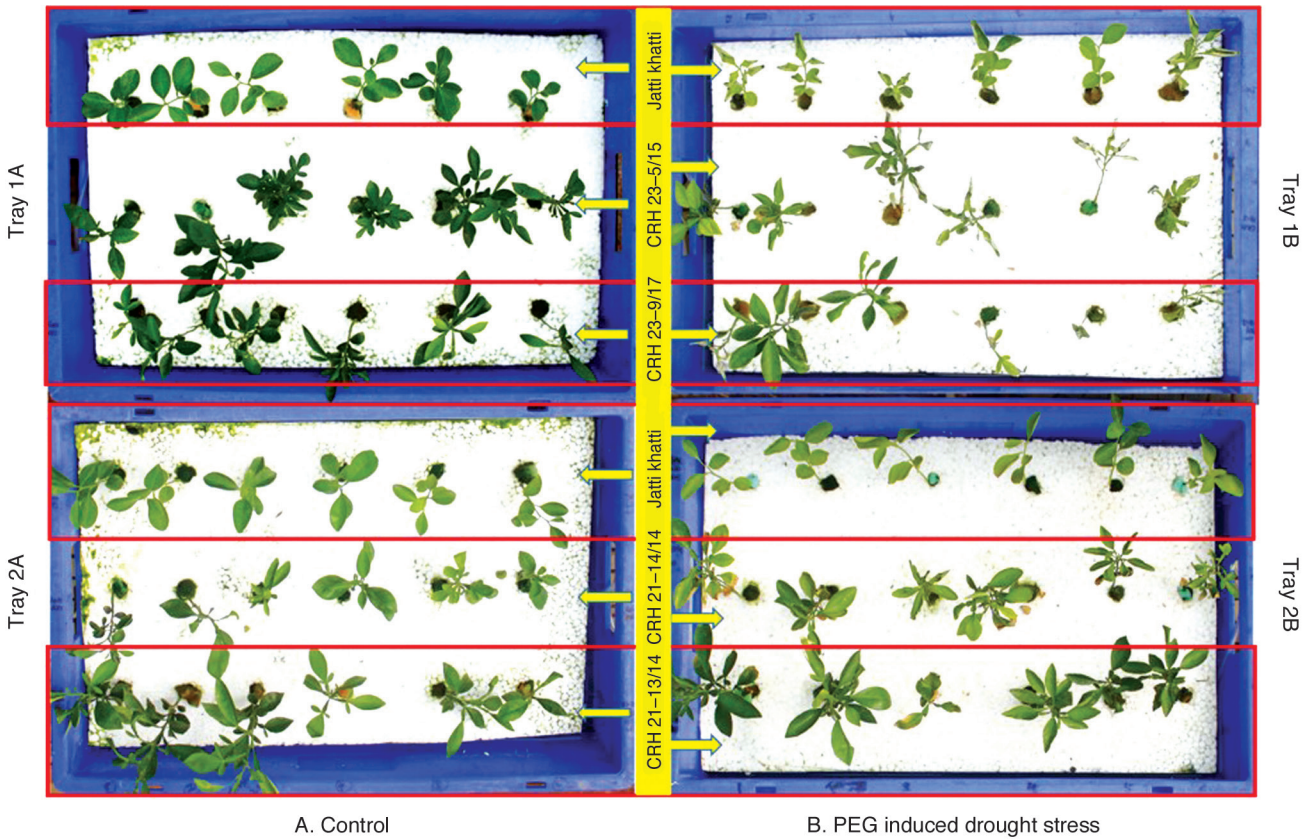


Fig 1 Hydroponic setup of experiment depicting comparative performance of 4 citrus rootstock hybrids and sensitive check (Jatti khatti) under control and drought conditions.

drought (Pedroso *et al.* 2014). Under drought, the highest shoot to root dry weight ratio was reported for CRH 21-13/14 (2.02) (Table 1) followed by statistically similar CRH 23-9/17 (1.95), while CRH 23-5/15 (0.55) displayed the lowest ratio, statistically analogous to sensitive check (Jatti khatti) (0.61). Our result corroborates the findings of Zaher-Ara

et al. (2016). Alteration in leaf morphology traits, viz. leaf movement and rolling, is adopted for drought screening in citrus (Santana-Vieira *et al.* 2016). In the present study, CRH 21-13/14 had the lowest per cent leaf roll (22.17%) and LWI score (0.67), suggesting the least drought induced negative impact (Fig 2).

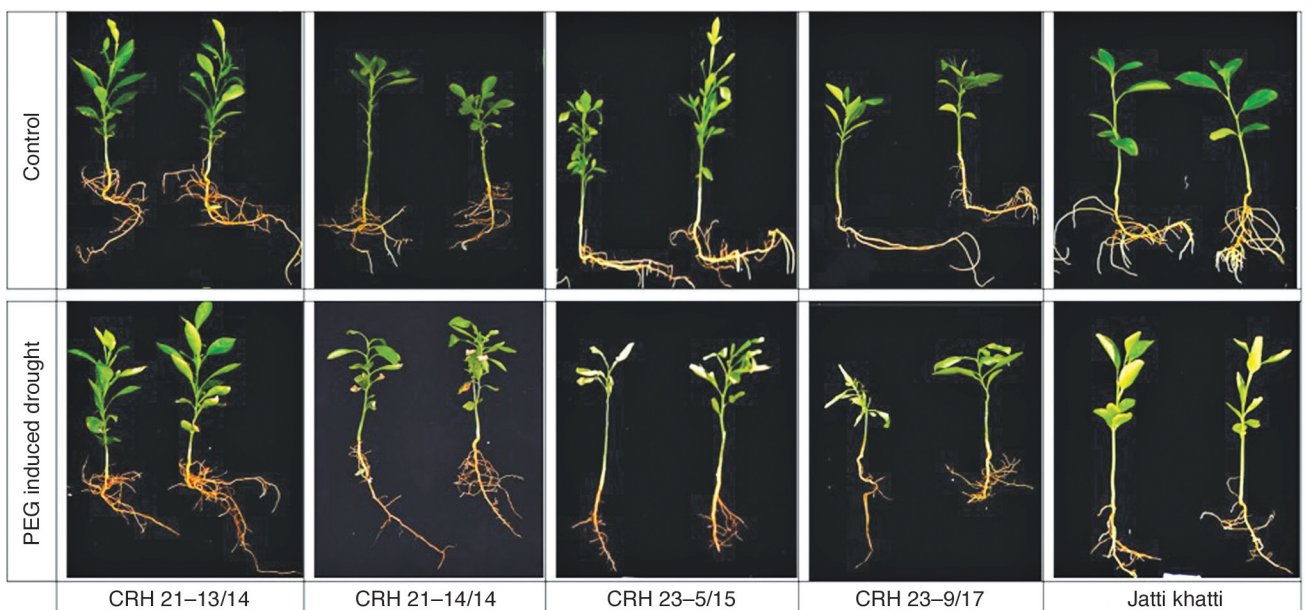


Fig 2 Leaf scorching, leaf rolling and root growth of citrus rootstock hybrids and sensitive check (Jatti khatti) under control and drought conditions.

Table 1 Comparative morphological performance of citrus rootstock hybrids subjected to drought under hydroponic condition

Genotype	Plant height (cm)		Number of leaves		Shoot dry weight (mg)		Root dry weight (mg)		Shoot to root dry weight ratio		Total length of root system (cm)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
CRH 21-13/14	9.60 ^{bc}	10.03 ^{ab}	12.33 ^a	13.50 ^a	148.9 ^a	159.3 ^a	76.65 ^e	85.17 ^b	1.94 ^{ab}	2.02 ^a	85.43 ^b	106.40 ^a
CRH 21-14/14	8.77 ^{cd}	8.40 ^{de}	9.33 ^{cd}	9.50 ^{bc}	94.4 ^b	89.3 ^b	59.63 ^d	52.18 ^f	1.60 ^c	1.71 ^{bc}	67.80 ^c	57.67 ^d
CRH 23-9/17	8.37 ^{de}	7.78 ^e	10.83 ^b	8.00 ^{ed}	91.3 ^b	79.3 ^{bc}	54.15 ^{ef}	40.61 ^g	1.70 ^{bc}	1.95 ^{ab}	52.67 ^d	35.22 ^e
CRH 23-5/15	10.71 ^a	9.91 ^{ab}	12.83 ^a	7.83 ^e	81.1 ^{bc}	43.5 ^d	117.90 ^a	79.53 ^c	0.69 ^e	0.55 ^e	81.38 ^b	41.80 ^e
Jatti khatti	8.51 ^{de}	7.93 ^{de}	7.00 ^e	6.83 ^e	67.7 ^c	25.9 ^e	58.95 ^{ed}	42.71 ^g	1.15 ^d	0.61 ^e	100.90 ^a	35.32 ^e
LSD (P≤0.05)												
Genotype (G)	0.68		0.98		11.73		3.80		0.20		5.14	
Drought (D)	0.43		0.62		7.42		2.40		0.12		3.25	
G × D	0.96		1.39		16.59		5.38		0.30		7.28	

Each data represents the mean value of six samples. Values representing different small letters are significant at P≤0.05.

Root morphology: With respect to the total length of root system, CRH 21-13/14 exhibited a 24.6% increment under drought (Table 1). While rest hybrids along with sensitive check (Jatti khatti) declined to the tune of 14.9% (CRH 21-14/14) to 65.0%. Previously, role of deep root development in tomato and brinjal was stated to be associated with the ability to extract water from deeper soil layers (Kulkarni and Phalke 2009). In contrast to the rest hybrids, CRH 21-13/14 demonstrated an increment in root volume (8.8%), projected root area (14.6%), and surface root area (15.5%) during drought (Table 2). While sensitive check (Jatti khatti) displayed >60% decline in these traits. Therefore, signifying better drought avoidance capability of CRH 21-13/14 was owing to higher access to water and nutrients in terms of area. Our findings are consistent with Wijewardana *et al.* (2019). Hybrids CRH 23-5/15 and CRH 23-9/17 produced negligible new roots in drought (Table 2), while hybrids CRH 21-14/14 and CRH 21-13/14 displayed 13.8% and 48.0% increments in new roots, respectively (Table 2). It demonstrated the ability to optimize growth and maintain efficient physiological functions during drought (Wijewardana *et al.* 2019). In relation to root architecture traits, CRH 21-13/14 excelled in terms of >150%, >50% and >60% increments in root tips, forks and crossings (Table 2), respectively under drought. Similarly, CRH 21-14/14 also displayed >100% and >10% growth in root tips and forks, respectively. Nevertheless, a reverse trend was reported in the rest hybrids. Root architecture reflects the spatial arrangement and distribution of roots, which regulates water and nutrient absorption of plants (Wijewardana *et al.* 2019). Inclusively, citrus rootstock hybrids CRH 21-13/14 and CRH 21-14/14 with Pummelo × Troyer parental lineage displayed improved, ramified root system under drought (Fig 2) than hybrids CRH 23-5/15 and CRH 23-9/17 (Pummelo × Morton).

Physio-biochemical traits: The leaf RWC reveals plant water status and is regularly used to estimate physiological consequences of cellular water deficit (Barrs and Weatherly 1962). In this study, high RWC values (>80%) indicate that sufficient plant water status was maintained in control

(Table 3). During drought only CRH 21-13/14 sustained >70% RWC, indicating superior drought tolerance capability over rest hybrids. Reduction in RWC was more pronounced in hybrids CRH 23-9/17 and CRH 23-5/15. Our results are consistent with Khoe *et al.* (2020). Cell membranes are one of the first components to be affected by moisture stress and the integrity and stability of cell membranes is a key indicators of drought tolerance (Bajji *et al.* 2002). Among G × D interactions, during drought, the highest MSI was sustained for CRH 21-13/14 (69.84%) followed by statistically different CRH 21-14/14 (61.10%) (Table 3). While lowest MSI was noted for sensitive check (Jatti khatti) (37.01%). Similar findings of lower electrolytic leakage were reported in drought tolerant citrus hybrids (Khoe *et al.* 2020). Proline plays a key role in osmoregulation and act as a ROS scavenger and molecular chaperon to protect protein structures. We observed >100% augmentation in leaf proline level of CRH 21-13/14 as against rest hybrids and sensitive check (<50% increment) during drought (Table 3), demonstrating a superior osmoregulation mechanism. These findings are in agreement with Zaher-Ara *et al.* (2016).

All tested hybrids and sensitive check noted a substantial impact of drought-induced oxidative stress on photosynthetic apparatus, manifested in terms of decline in total chlorophyll to varying degrees. With regard to total chlorophyll, under drought, CRH 21-13/14 (1.84 mg/g FW), CRH 21-14/14 (1.76 mg/g FW) and CRH 23-9/17 (1.64 mg/g FW) excelled over sensitive check (Jatti khatti) (1.53 mg/g FW) (Table 3). Interestingly, although under control conditions all hybrids exhibited a chlorophyll a/b ratio of >1, it was CRH 21-13/14 which retained >1 chlorophyll a/b ratio during drought (Table 3). The decline in chlorophyll a/b ratio during drought suggests impairment of chiral macro aggregates of chlorophyll 'a' or 'b' pigment-protein complexes which guard photosynthetic machinery. With respect to CSI, among G × D interactions, in drought, the superior value was noted for CRH 21-13/14 (77.9) significantly followed by CRH 21-14/14 (56.72) and CRH 23-9/17 (53.84) than sensitive chick (Jatti khatti) (44.6) (Table 3). CSI is the measure of

Table 2 Comparative root system of citrus rootstock hybrids subjected to drought under hydroponic conditions

Genotype	Projected root area (cm ²)		Root surface area (cm ²)		Root volume (cm ³)		Number of newly emerged root		Number of root tip		Number of root fork		Number of root crossing	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
CRH 21-13/14	7.90 ^b	9.05 ^a	24.75 ^{bc}	28.58 ^a	0.57 ^{ab}	0.62 ^a	4.83 ^{de}	5.50 ^{cd}	116.0 ^e	290.2 ^b	95.80 ^{cd}	145.2 ^b	6.83 ^d	11.17 ^b
CRH 21-14/14	6.15 ^c	4.60 ^{de}	19.28 ^d	16.08 ^e	0.44 ^{bc}	0.36 ^c	4.16 ^{ef}	6.17 ^c	85.0 ^f	183.7 ^d	83.50 ^d	94.0 ^{cd}	8.50 ^c	7.00 ^{cd}
CRH 23-9/17	5.47 ^{cd}	4.00 ^e	16.67 ^e	12.47 ^f	0.42 ^{bc}	0.33 ^c	8.33 ^b	0.50 ^g	95.5 ^f	81.7 ^{fg}	64.67 ^e	62.50 ^e	6.50 ^d	4.00 ^e
CRH 23-5/15	8.47 ^{ab}	4.17 ^e	26.65 ^{ab}	13.12 ^f	0.70 ^a	0.33 ^c	5.50 ^{cd}	0.00 ^g	208.2 ^c	66.0 ^{gh}	100.5 ^c	51.67 ^e	6.00 ^d	4.00 ^e
Jatti khatti	7.48 ^b	2.78 ^f	23.48 ^c	8.75 ^g	0.44 ^{bc}	0.16 ^d	21.00 ^a	3.50 ^f	322.2 ^a	57.7 ^h	182.3 ^a	61.17 ^e	16.00 ^a	7.50 ^{cd}
LSD (P≤ 0.05)														
Genotype (G)	0.76		1.79		0.11		0.70		13.03		9.55		1.07	
Drought (D)	0.48		1.13		0.07		0.44		8.24		6.04		0.67	
G × D	1.08		2.53		0.15		0.99		18.42		13.51		1.51	

Each data represents the mean value of six samples. Values representing different small letters are significant at P≤ 0.05.

Table 3 Comparative physio-biochemical performance of citrus rootstock hybrids subjected to drought under hydroponic condition

Genotype	Relative water content (%)		Membrane stability index (%)		Chlorophyll 'a' (mg/g FW)		Chlorophyll 'b' (mg/g FW)		Leaf total chlorophyll (mg/g FW)		Chlorophyll stability index		Proline (mg/g FW)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
CRH 21-13/14	84.98 ^a	72.15 ^d	79.76 ^a	69.84 ^d	1.25 ^b	0.97 ^e	0.68 ^{ef}	0.82 ^c	2.09 ^b	1.84 ^d	88.1 ^a	77.91 ^b	3.28 ^e	6.94 ^a
CRH 21-14/14	81.32 ^c	63.35 ^e	76.13 ^b	61.10 ^e	1.42 ^a	0.80 ^f	0.69 ^e	0.85 ^{ab}	2.35 ^a	1.76 ^e	74.9 ^{cd}	56.72 ^f	4.39 ^d	6.06 ^b
CRH 23-9/17	83.13 ^b	56.66 ^f	73.00 ^c	45.33 ^f	1.03 ^d	0.73 ^g	0.74 ^d	0.84 ^{bc}	1.84 ^d	1.64 ^f	73.2 ^d	53.84 ^g	4.35 ^d	5.32 ^c
CRH 23-5/15	82.70 ^{bc}	58.32 ^f	71.59 ^{cd}	45.08 ^f	0.92 ^e	0.65 ^h	0.66 ^f	0.83 ^{bc}	1.85 ^d	1.56 ^g	76.2 ^{bc}	45.21 ^h	3.53 ^e	4.45 ^d
Jatti khatti	82.41 ^{bc}	53.80 ^g	70.02 ^d	37.01 ^g	1.21 ^c	0.61 ⁱ	0.67 ^{ef}	0.87 ^a	1.97 ^c	1.53 ^g	66.3 ^e	44.57 ^h	3.28 ^e	4.50 ^d
LSD (P≤ 0.05)														
Genotype (G)	1.26		1.36		0.03		0.02		0.03		1.4		0.29	
Drought (D)	0.79		0.86		0.02		0.01		0.02		0.9		0.18	
G × D	1.78		1.93		0.04		0.03		0.05		1.9		0.41	

Each data represents the mean value of four samples. Values representing different small letters are significant at P≤ 0.05.

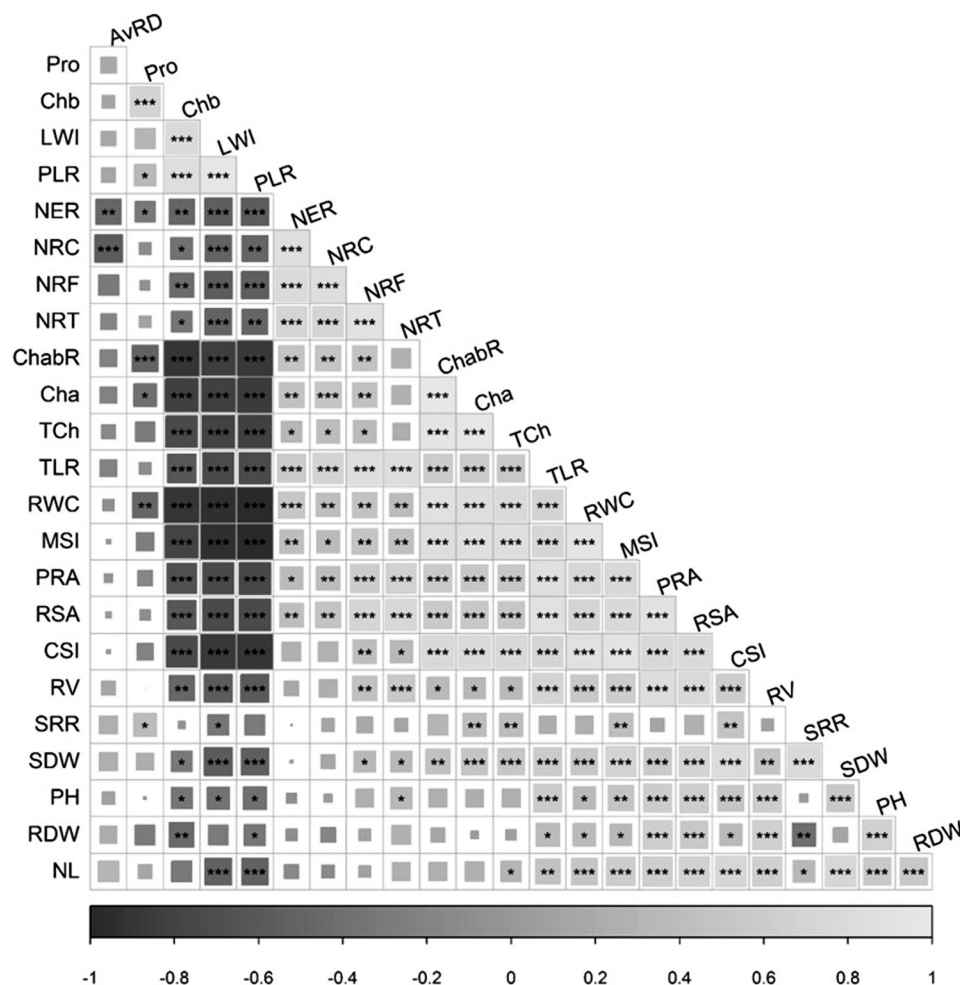


Fig 3 Heatmap represent Pearson Correlation Coefficients among all studied traits under control (without-PEG) and PEG induced drought conditions.

integrity and stability of chlorophyll and the key indicator of the stress tolerance capability of plants.

The correlation heatmap (Fig 3) depicts positive and negative correlations in light grey and dark grey colour, respectively. The intensity of colour and square size are proportional to correlation coefficients. ‘*’, ‘**’, and ‘***’ indicate significant correlations at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. A strong significant positive relationship was noted amongst root architecture traits i.e. number of root tips (NRT), number of root forks (NRF) and number of root crossings (NRC) and with other root and physiological traits such as total length of root system (TLR), projected root area (PRA), root surface area (RSA), RWC and MSI (Fig 3). This relationship indicates the significance of root traits in securing water resources and consequently sustaining turgor pressure in leaves. Improved root biomass under drought stress is linked with the preservation of leaf water status (Becker *et al.* 2016). Significant positive association of TLR with key parameters i.e. RWC, MSI, CSI, chlorophyll a/b ratio (ChabR), chlorophyll ‘a’ (Cha), total chlorophyll (TCh), PRA, RSA, root volume (RV), newly emerged roots (NER), shoot and root dry weight (SDW and RDW), plant height (PH) and the number of leaves (NL)

indicate root length is a key regulatory factor to optimize water uptake in drought. TLR had a strong negative correlation with chlorophyll ‘b’ (Chb), LWI and per cent leaf roll (PLR), while it had no association with shoot to root ratio (SRR) and proline (Pro).

Overall, the result indicates better resilience of CRH 21-13/14 (Pummelo × Troyer) against drought, articulated in terms of least morphological injury symptoms. Superior membrane integrity and chlorophyll stability might have played a crucial role in sustaining photosynthetic efficiency of CRH 21-13/14 during drought. Consequently, photo-assimilate accumulation and its partitioning towards roots may have improved root traits, as evidenced by the increment in root traits in CRH 21-13/14. The physiological plasticity induced by an efficient root system could be a key mechanism supporting the presence of drought avoidance phenomena in CRH 21-13/14. While, the intermediate performance of CRH 21-14/14 (Pummelo × Troyer) could be attributed to its ability to produce new roots and better root architecture. However, both Pummelo × Morton hybrid seedlings CRH 23-5/15 and CRH 23-9/17 performed poorly during drought. We advocate further field evaluation of CRH 21-13/14 as rootstock for yield, quality, and abiotic stress tolerance studies. It could also be argued that citrus species too could be efficiently screened against drought using a hydroponic platform like other field crops.

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