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Leaf injury index: A quantitative approach for rapid screening of drought tolerance in citrus rootstocks

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ABSTRACT

The present study was carried out during 2019–2021 at ICAR-Indian Agricultural Research Institute, New Delhi to observe leaf injury symptoms and number of functional leaves in nine citrus rootstock genotypes subjected to drought stress. The experiment was laid out in a completely randomized block design (CRBD) with four replications. Qualitative leaf injury symptoms, namely yellow, rolled, scorched, defoliated leaves, and leaf wilting were used as weighted indicators after normalization. A composite weighted indicator-based index, the leaf injury index (LII) was then developed to evaluate the drought tolerance in citrus rootstocks. Citrus genotype RLC-2 exhibited the highest values for yellow (29.75), rolled (22.75), scorched (20.75) leaves, and leaf wilting score (4.50), while Grambhiri showed the highest defoliation count of 16.25. In contrast, X639 had the highest number of functional leaves (79.13) with lowest visible leaf injury in terms of yellow, rolled, scorched, and defoliated leaves, and leaf wilting score. The effectiveness of LII in measuring the drought sensitivity of citrus rootstock genotypes was tested using a heatmap and cluster analysis. Citrus genotype X639 was grouped into a single distinct cluster with the significantly lowest LII (0.250), indicating its drought tolerant nature. The other tested genotypes were classified into three sub-clusters: drought-sensitive (Cleopatra mandarin, RLC-2, RLC-1 and Grambhiri), intermediate drought response (RLC-7 and Troyer citrange), and some degree of drought tolerance (RLC-4 and RLC-5), with their higher, intermediate and lower LII respectively. This composite indicator ideally converts multiple qualitative indicators into a simple, rapid and cost-effective framework for screening citrus germplasm against drought stress.

Keywords: Composite indicator, Drought response, Leaf wilting, Scorching, Senescence

Citrus trees face multiple abiotic stresses throughout their life cycle due to their evergreen nature and longer fruiting period (Santana *et al.* 2016). Water and nutrient deficits impair vegetative growth, fruit yield-quality, and impose economic burden on citrus growers (Rodriguez-Gamir *et al.* 2010). Over the past century, the use of stress-resistant rootstocks has gained importance for their proven impact on growth, nutrient uptake, and fruit yield (Dubey and Sharma 2016). Citrus rootstocks show differential abilities to provide water and nutrients under stress, contributing to their overall adaptability (Rodriguez-Gamir *et al.* 2010). India possesses rich citrus genetic diversity, and promising rootstocks such as rough lemon and Karna Khatta were identified for Indian citriculture (Dubey *et al.* 2016). Soh Sarkar and RLC-5 have demonstrated moderate drought tolerance (Le *et al.* 2020) and rootstock hybrid CRH 21-13/14 performed well under polyethylene glycol-induced osmotic stress (Kadam *et al.* 2022). Therefore, breeding drought-tolerant rootstocks is crucial for sustainable citrus production in forecasted climate change and dwindling water resources.

Accurate assessment of drought tolerance remains challenging, requiring the monitoring of morpho-physiological, biochemical and molecular responses. Visual indicators such as leaf yellowing, wilting, and chlorosis provide a practical method for identifying stress responses (Salem-Fnayou *et al.* 2016). However, the severity of these symptoms varies by genotype with linear relationship between visual scores and drought tolerance (Fadel *et al.* 2018). Leaf area, leaf count and visual injury symptoms are reliable indicators of drought response, though inconsistencies in symptom presentation can lead to inaccurate assessments. A composite indicator is formed when individual indicators are compiled into a single index that measures multidimensional concepts. Earlier reports on quantitative and composite indices to screen drought tolerance include the stress susceptibility index

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(Mardeh *et al.* 2006), leaf wilting index (Pungulani *et al.* 2013), drought injury index (Yi-ling *et al.* 2015), drought resistance indices (Jie *et al.* 2020) and a combination of indices (Sabouri *et al.* 2022). We hypothesized that the development of a composite index based on quantifiable drought-induced visible leaf injury symptoms will enable the rapid and accurate identification of drought-tolerant citrus genotypes, complementing traditional physiological and biochemical assessments.

MATERIALS AND METHODS

The present study was carried out during 2019–2021 at ICAR-Indian Agricultural Research Institute, New Delhi. Nine citrus rootstock genotypes were subjected to drought stress to observe leaf injury symptoms and number of functional leaves (Table 1). Mature fruits of these genotypes from citrus field gene bank were harvested in December 2019. Subsequently, seeds were extracted and sown in nursery beds. In August 2020, six-month-old seedlings were transplanted in 12-inch plastic pots filled with native orchard soil and farmyard manure (2:1). The potted seedlings were regularly irrigated, and given single soil application of 20 g NPK (19:19:19). After establishment, one-year-old seedlings were subjected to drought stress (DS) by complete withholding of water for three weeks during March 2021. During sufficient moisture conditions (control), gravimetric soil moisture was 26%, and then it declined to 9% following DS. Visual observations of leaf injury traits, such as yellow, rolled, scorched and defoliated leaves, were recorded on individual seedlings at the end of the DS. Leaves with more than 50% functional leaf margins were also counted at this stage. Leaf wilting was assessed as described by Engelbrecht *et al*. (2007) using a six-point scale, where each category was defined as, 1, normal leaves; 2, slightly wilted leaves; 3, wilted leaves; 4, severely wilted leaves; 5, nearly dead leaves and 6, dead leaves.

For the computation of composite index for leaf injury, the observations of the number of functional leaves, yellow, rolled, scorched and defoliated leaves, and average leaf wilting scores were utilized as indicators to calculate the composite leaf injury index (LII). The values for these indicators were normalized using min-max method to render them comparable. Indicators with positive implications (yellow, rolled, scorched leaves and LWS) on the composite scores were normalized using the following formula:

$$X_{nv} = \frac{X_i - X_{min}}{X_{max} - X_{min}}$$

Two indicators i.e. the number of functional leaves and rolled leaves, which have a negative relationship with LII, were normalized using the following formula:

$$X_{nv} = \frac{X_{max} - X_i}{X_{max} - X_{min}}$$

In both equations, X_i represents actual value of the variable, X_{nv} is normalized value, X_{max} and X_{min} represent maximum and minimum values of the variable, respectively. Indicator normalization was followed by assigning weights and aggregations to derive the composite index. Expert opinion-based weights were assigned to the six indicators under consideration using Saaty's analytic hierarchy process (AHP) (Saaty 1980). The highest weightage of 25%, was assigned to 'number of functional leaves (FL)' followed by 'number of rolled leaves' at 20%, assuming their greater contribution to seedling performance under stress. Scorched leaves (SL), leaf wilting score (LWS), yellow (YL) and defoliation count (DL) were assigned weightages of 18%, 15%, 12% and 10%, respectively. Linear arithmetic aggregation was used, because most indicators had the same measurement units. The LII was then computed by the additive aggregation of the six indicators with their assigned weights, as mentioned previously. The mathematical expression of this composite index can be given as follows:

$$LII = \sum_{j=1}^{6}(y_{ij}w_j)$$

Where y_{ij}, Normalized value of indicator j and unit i and w_j, Weight of indicator j.

The value of the LII can range between 0 and 1, where a value of zero indicates no visible leaf injury, and a value of one represents the highest possible leaf injury. A more elaborate form of the above equation can also be expressed as:

$$LII = \sum (FL \times 0.25) + (YL \times 0.12) + (RL \times 0.20) + (SL \times 0.18) + (DL \times 0.10) + (LWS \times 0.15)$$

The experiment was conducted in completely randomized block design (CRBD) with four replications. Descriptive statistics and ANOVA with least significant difference (LSD) at $P<0.05$ was were calculated in MS Excel and RStudio, respectively. Pearson's bivariate correlation matrix examined relationships between LII and leaf injury traits using the 'corrplot' library. A heatmap based on Ward's minimum variance clustering (D^2) was visualized genotype clusters based on LII, using the 'heatmap 2' library in RStudio.

Table 1 Citrus rootstock genotypes used in investigation

Genotype	Botanical name	Accession number
Cleopatra mandarin	*Citrus reshni* Hort ex Tan	-
Grambhiri	*C. jambhiri* Lush.	-
RLC-1	*C. jambhiri* Lush.	IC273852
RLC-2	*C. jambhiri* Lush.	IC273847
RLC-4	*C. jambhiri* Lush.	IC274693
RLC-5	*C. jambhiri* Lush.	IC274698
RLC-7	*C. jambhiri* Lush.	IC255451
Troyer citrange	*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.	-
X639	*C. reshni* Hort ex Tan. × *P. trifoliata* L. Raf.	-

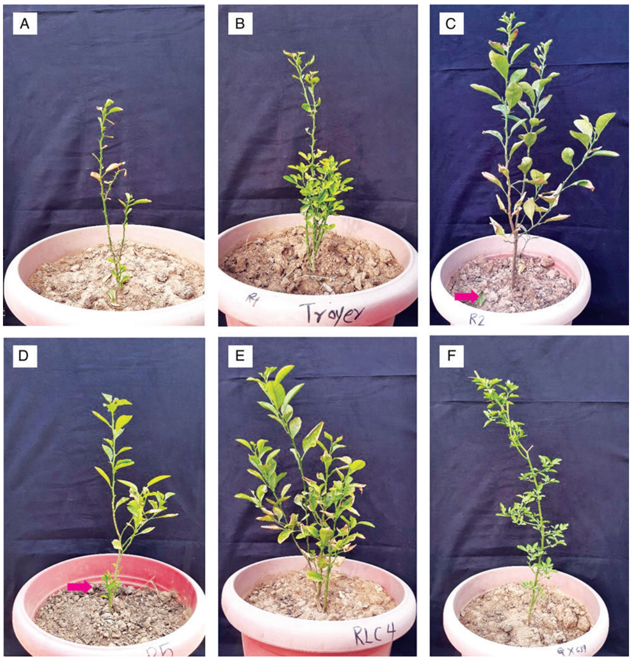

Fig. 1 Comparison of leaf injury symptoms in citrus genotypes under drought stress (A) Defoliation in Cleopatra mandarin; (B) Leaf yellowing in Troyer citrange; (C) Leaf rolling, wilting, scorching and defoliation in RLC-2; (D) New shoot emergence in RLC-5; (E) Number of functional leaves in RLC-4 and (F) X639 without any leaf injury symptoms.

RESULTS AND DISCUSSION

Drought stress induces osmotic imbalance, decreases turgor pressure, and reduces water content, all of which negatively affect plant growth and leaf count (Jaleel *et al*. 2009). We found significant variations in leaf injury symptoms in tested citrus rootstock genotypes, suggesting a differential drought response in citrus (Fig. 1).

Descriptive statistics of leaf injury traits under DS: Comprehensive overview of leaf injury parameters in studied genotypes are presented as descriptive statistics (Supplementary Table 1). The average number of functional leaves was 55.38, showing significant variability (σ = 19.49) and near-symmetry (skewness = 0.06). Similarly, yellow leaves had a mean of 15.31, with moderate variability (σ = 10.17) and near symmetry (skewness = -0.02). The average number of scorched leaves was 12.17, displaying moderate variability (σ = 6.57) and a slight left skew (skewness = -0.63). Similarly, defoliated leaves had a mean of 8.97, with moderate variability (σ = 5.50) and near symmetry (skewness = 0.05). The average number of rolled leaves was 10.75, exhibiting high variability (σ = 8.51) and a right skew (skewness = 0.59). Additionally, the LWS averaged 3.40, with low variability (σ = 1.12) and left skew (skewness = -0.94). Most parameters exhibited flattened normal distributions, except for LWS (kurtosis = 0.29). These statistics highlight the variability and distribution of leaf injury parameters in citrus rootstocks, which is crucial for understanding drought sensitivity.

Variations in leaf injury traits in citrus genotypes: Visual symptoms such as leaf yellowing, curling, rolling, wilting, and shoot tip burning serve as reliable indicators of water deficit in citrus (Salem-Fnayou *et al.* 2016, Santana *et al.* 2016). The significant variations in leaf injury traits were observed among the rootstock genotypes following drought stress (Table 2). Citrus genotype X639 registered the highest number of functional leaves (79.13), which was at par with RLC-5 (76.63) and RLC-4 (75.88). Cleopatra mandarin had the lowest leaf count of 35.75 (Fig. 1A), which was statistically similar with Troyer citrange. The higher defoliation counts of 16.25 and 15.25 were observed in *Grambhiri* and RLC-2, respectively, and X639 obsevred no leaf shedding. Furthermore, the RLC-2 (Fig. 1C) had the significantly highest number of yellow (29.75), rolled (22.25), and scorched (20.25) leaves which was closely followed by *Grambhiri*. In the other hand, X639 had zero counts for yellow, rolled, and scorched leaves (Fig. 1F). Following the trend of other leaf injury traits, the lowest LWS was observed in X639 (1), which was statistically at par with RLC-1 and RLC-4, while it was highest in RLC-2 (4.50), with no significant difference from those of Cleopatra mandarin (4.13) and *Grambhiri* (4.0).

Evaluation of leaf morphology through leaf movement and rolling has been adopted for drought screening in citrus (Santana *et al.* 2016). Leaf rolling might also play a role in osmotic adjustment to maintain internal plant

Table 2 Comparison of functional leaves and leaf injury traits following drought stress in citrus rootstocks

Genotype	No. of functional leaves	No. of defoliated leaves	No. of yellow leaves	No. of rolled leaf	No. of scorched leaves	Leaf wilting score
Cleopatra mandarin	35.75^{d}	7.50^{c}	4.25^{d}	3.75^{e}	12.75^{c}	4.00^{ab}
Grambhiri	45.25^{c}	16.25^{a}	22.25^{b}	17.75^{b}	16.00^{b}	4.13^{ab}
RLC-1	28.75^{de}	4.00^{d}	6.00^{d}	4.75^{de}	12.25^{c}	3.00^{de}
RLC-2	58.50^{b}	15.75^{a}	29.75^{a}	22.25^{a}	20.25^{a}	4.50^{a}
RLC-4	75.88^{a}	13.00^{b}	27.50^{a}	21.25^{ab}	17.50^{b}	3.00^{de}
RLC-5	76.63^{a}	11.50^{b}	17.25^{c}	12.25^{c}	10.75^{c}	3.50^{bcd}
RLC-7	60.25^{b}	7.75^{c}	14.50^{c}	7.75^{d}	16.75^{b}	3.75^{bc}
Troyer citrange	36.50^{d}	4.00^{d}	16.50^{c}	6.25^{de}	3.25^{d}	3.75^{bc}
X639	79.13^{a}	1.00^{g}	0.00^{e}	0.00^{f}	0.00^{e}	1.00^{h}

water status and conductance of water, heat, and gas exchange. It is hydronastic mechanism that inhibits light interception, transpiration, and dehydration. In the present study, the lowest leaf rolling in X639, Troyer citrange and RLC-1 suggested the least negative impact of drought. Previous studies have demonstrated that drought conditions inhibit leaf production in susceptible genotypes (Luvaha *et al.* 2008). Increased temperature accelerates leaf aging and senescence owing to elevated ABA levels and decreased cytokinin content in wilting leaves (Keller 2015). The drought conditions inhibit leaf production in susceptible genotypes (Al-Absi 2009, Le *et al.* 2020, Kadam *et al.* 2022) due to leaf senescence and abscission of mature leaves. The lowest leaf wilting in RLC-1 and X639 in present study is in agreement with the previous findings on apple (Wang *et al.* 2012) and citrus (Salem-Fnayou *et al.* 2016). Yellowing and scorching of leaves are linked to chlorophyll redistribution and degradation under water stress, which negatively affects photosynthetic activity (Osakabe *et al.* 2014). Turgidity of X639's green leaves was due to its ability to maintain chlorophyll levels and enhance antioxidant defense mechanisms during water deficit, which aligns with previous findings by Hussain *et al.* (2018) in Brazilian sour oranges. The absence of leaf injury symptoms in X639 and relatively lower injury in RLC-4 suggest their ability to maintain a higher leaf water status, attributable to their extensive root systems that sustained transpiration pull even under water deficit. Our results are in line with leaf wilting and rolling symptoms in citrus, which are negatively correlated with drought tolerance (Rodriguez-Gamir *et al.* 2010, Le *et al.* 2020, Kadam *et al.* 2022). Leaf movement traits have been reported to be useful for selecting drought-tolerant plants; however, their quantification is challenging. Leaf rolling and drying are more dependable traits for selecting crops under drought conditions (Salunkhe *et al.* 2011). The use of these scales requires experience in systematic and uniform leaf wilting scoring; otherwise, it may incur errors in drought tolerance assessment (Pungulani *et al.* 2013). Accurately estimating the actual severity of leaf injury by visible symptoms is difficult due to the variations in these symptoms (Hu *et al.* 2021).

LII correlation and heat map-clusters analysis: A heat map paired with the dendrogram based on leaf injury traits of citrus rootstock genotypes under DS is shown in Fig. 2. Citrus rootstock genotypes on the Y-axis with the same capital letter in parentheses are not significantly different in the LSD test (P<0.05). The highest LII (0.648) was observed in RLC-2, which was statistically similar to that of Cleopatra mandarin, Grambhiri and RLC-1. The first sub-cluster, comprising these four genotypes showed

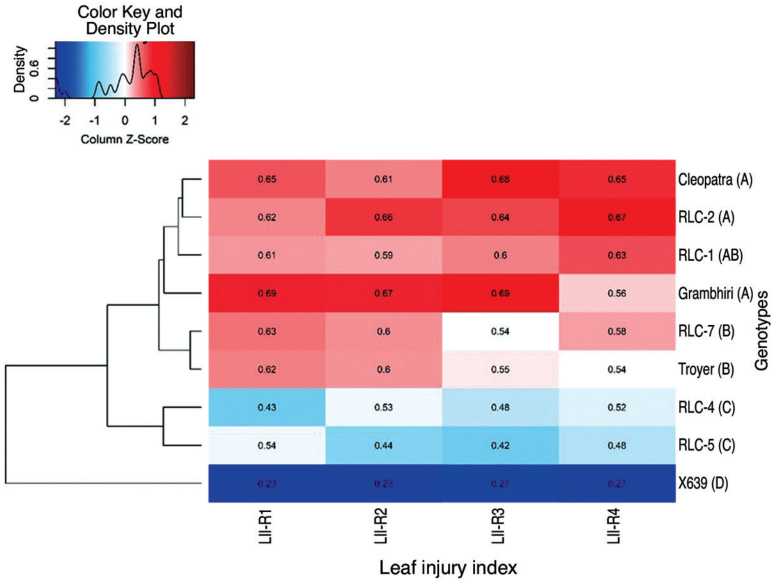

Fig. 2 Heat map representing LII in four replications under drought stress and hierarchical clustering of citrus rootstock genotypes.

Note: Values were scaled and normalized for each trait by Z-Fisher transformation. Red and blue indicates high and low LII values, respectively. Genotypes on Y-axis with the same capital letter in parenthesis were not significantly different in LSD test (P<0.05).

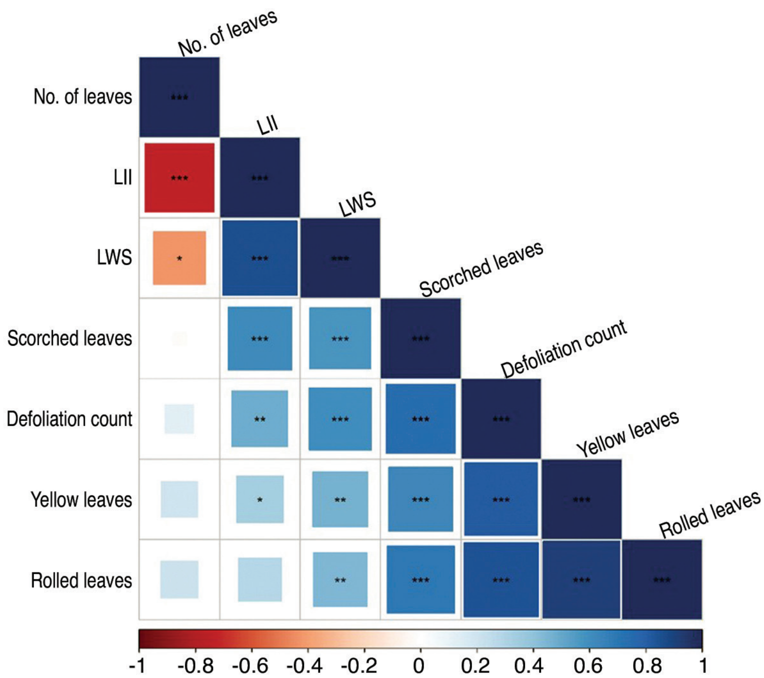

Fig. 3 Pearson's correlation matrix between LII and leaf injury symptoms during drought stress.

Note: Colour and size of square are in proportion to the strength of correlation. Non-significant correlations (P>0.05) are shown without any asterisk marks and single, double and triple asterisk marks corresponds to level of significance of 0.05, 0.01 and 0.001, respectively. LWS, Leaf wilting score; LII, Leaf injury index.

significantly higher LII values, indicating their drought-sensitive nature. Rootstock genotype X639 showed the lowest LII of 0.250 and hierarchical clustering distinctly separated this genotype, indicating to be most drought-tolerant genotype. Drought tolerance of the X639 rootstock was attributed to prioritizing growth, vigorous root system, elevated relative water content, membrane stability index and a robust antioxidant system (Chand *et al.* 2024). The remaining rootstocks were grouped into three sub-clusters, each representing different levels of drought sensitivity. The second sub-cluster, including RLC-7 and Troyer citrange, displayed moderate LII values of 0.589 and 0.579, respectively, suggesting an intermediate level of drought tolerance. The third sub-cluster, consisting of RLC-4 (0.489) and RLC-5 (0.472), exhibited lower LII values, indicating some degree of drought tolerance, although not as pronounced as X639.

The correlation matrix plot (Fig. 3) illustrates Pearson's correlation coefficients, revealing strong associations between the visual injury parameters and the LII developed in this study. The number of functional leaves showed a significant negative correlation with LII (r = -0.73***) and LWS (r = -0.42*). In contrast, the LII had the significant and strong positive correlations with the LWS (r = 0.85***), scorched leaves (r = 0.62***), defoliation count (r = 0.48**), and yellow leaves (r = 0.34). These correlations indicate that the higher LII values are associated with higher visual injury parameter scores. These findings underscore the interconnectedness of various leaf injury traits and their collective influence on the composite LII. Fadel *et al.* (2018) established a significant linear relationship between leaf water potential and visual scores in citrus species, which is consistent with previous drought screening studies in citrus and apple (Wang *et al.* 2012, Le *et al.* 2020, Kadam *et al.* 2022).

Our results confirmed the findings of Fadel *et al.* (2018), who reported the significant linear relationship between visual scores and drought tolerance in citrus rootstocks. They identified two drought tolerant 'Rangpur' lime clones for Valencia sweet orange. But abiotic stress generates gradients of symptoms and their specificity is determined by the degree of interaction between the stress factor and plant defense system (Vollenweider *et al.* 2005). The overlapping or absence of certain visual symptoms can lead to inaccuracies in screening protocols. In such cases, a composite index not only aggregates these variables, but also utilizes them more objectively. LII considered all visible injury symptoms and provided comprehensive understanding of drought response in our study.

The use of a composite index like LII for drought tolerance screening offers several advantages in citrus breeding programmes. By incorporating multiple leaf morphological traits such as leaf movement, rolling, weakening and shedding, the LII provides a comprehensive assessment of drought response, while minimizing biases and inaccuracy, associated with relying on a single trait. This approach, as noted by Pastenes *et al.* (2005), is particularly valuable in drought tolerance screening. The simplicity of the LII method, which does not require specialized equipment or extensive experience, enhances its applicability in other screening experiments. The heatmap visualization effectively illustrated the variations in drought tolerance among the citrus rootstocks, clearly identifying X639 as the most drought-tolerant genotype and RLC-4 and RLC-5 as moderately drought-tolerant. This information is crucial for breeding strategies aimed at developing drought-resistant citrus varieties, which is increasingly important in the face of changing climate conditions and water scarcity in many citrus-growing regions.

The construction of composite indicators relies more on the expertise of the model developer than on universally accepted scientific rules for encoding. This study has demonstrated the effectiveness of the LII as a composite indicator for assessing drought tolerance in citrus genotypes. The LII method offers several advantages, including simplicity, reliability, objectivity and versatility. It successfully identified Cleopatra mandarin, RLC-2 and *Grambhiri* as drought-susceptible, which exhibited the higher LII, while X639 proven drought tolerant with the lowest LII. Systematic observations of visual leaf injury variables during drought stress, followed by grouping of the composite index-LII-facilitated the informed decision that quantified drought response in citrus rootstock genotypes. This method is efficient, reliable and comprehensive, as it considers all six possible leaf injury parameters under drought stress and addresses the limitations associated with previous methods like leaf wilting scores. Such objective scoring generates a quantitative index that minimizes biases and enhances accuracy when assigning genotypes to distinct drought response groups, as compared to qualitative scales. The strong correlation between visible drought injury symptoms and LII-based clustering suggests its potential for efficient screening of large citrus germplasm collections. Furthermore, the LII framework showed promise for adaptation to other abiotic stresses and plant species. This research highlights the value of developing tailored composite indicators in agricultural research, particularly for quantifying complex traits like drought tolerance. LII may not be universally applicable, but serves as a model for creating specific tools to complement physiological and biochemical observations in citrus breeding programs. Future research should focus on validating the LII across diverse citrus varieties and exploring its applicability to other crops and environmental stresses.

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