International Journal of Horticultural Science and Technology
Journal homepage: http://ijhst.ut.ac.ir

Screening Citrus Cultivars for Freezing Tolerance by Reliable Methods

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ARTICLE INFO

Article history:

Received: 26 September 2022,
Received in revised form: 4 April 2023,
Accepted: 11 April 2023

Article type:

Research paper

Keywords:

Plant breeding,
Principal component analysis,
Regression,
Low temperatures

ABSTRACT

Citrus species are comprised of susceptible plants that can barely tolerate freezing temperatures. To determine the relationship between cold stress tolerance (LT_{50}) and some important physiological traits, four commercial citrus cultivars of citrus species were studied. These were *Citrus unshiu*, *Citrus sinensis* var. 'Thomson navel', *Citrus paradisi* var. 'Star Ruby' and *Citrus limon* var. 'Lisbon'. Assessments of cold-stress tolerance were carried out at 4, -4 and -8 °C. Ultimately, the results showed that the Japanese mandarin and 'Thomson navel' had the highest stress tolerance (LT_{50}). *Citrus unshiu* tolerated cold stress up to -8.4 LT_{50}. The correlation coefficient demonstrated that significant, positive correlations were observed between several pairs of attributes, i.e. LT_{50} and total flavonoids, chlorophyll a and chlorophyll b, carotenoids and chlorophyll a, chlorophyll b and total chlorophyll, relative moisture content and malondialdehyde, as well as glycine-betaine and catalase. According to the regression coefficient, a change of one unit in lipid peroxidation caused a decrease of 1.9238 units and 5.9615 units in LT_{50} at +4 and -4 °C, respectively. While commercial citrus cultivars were selected for cold-tolerance and other traits, the efficiency of cold-tolerance correlated more with carotenoid content, chlorophyll content and lipid peroxidation, considering the assessments at particular temperatures.

Introduction

While the world population is growing rapidly and is predicted to reach 9.5 billion by 2050, food production is likely to decrease because of various abiotic stresses (Malhi et al., 2021). Therefore, ameliorating the adverse effects of abiotic stress on crop yield is increasingly becoming a focus in strategic planning for the supply of food security (Kajla et al., 2015). Crops are mainly affected by abiotic stress, such as cold temperature (Liu et al., 2019), salinity (Negrão et al., 2017), drought (Farooq et al., 2009), waterlogging (Ranger et al., 2013), oxidative stress (Shahid, 2021), pathogens (Fedele et al., 2020) and heavy metal toxicity (Pirhadi et al., 2020). Human activity has exacerbated the intensity of stress-inducing factors, thereby preventing plants from achieving their maximum genetic potential (Ghasemi-Soloklui and Ershadi, 2023). The irresponsible nature of anthropogenic activity has limited crop production all over the world (Rahemi et al., 2016; Shomali et al., 2021).

Citrus is among the three major subtropical fruits in the world (Wang, 2019). Citrus cultivation, especially orange, is a large industry in the United States, Brazil, Mexico, China, India, Iran and Mediterranean countries (Taghizadeh-Alisaraei et al., 2017). Citrus species are cultivated in more than 100 countries which are mainly situated in tropical and subtropical regions, where the soil and climate are suitable for their growth (Dahmoune et al., 2013). Citrus is valuable both in terms of fresh consumption and for the processing industry. So far, the citrus processing industry has been focused mostly on juice and

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value-added products. It is estimated that 33% of citrus harvested globally is used for juice production (Taghizadeh-Alisaraei et al., 2017). Iran ranks fifth in terms of global citrus production, given its climatic diversity and large citrus germplasm.

In Iran, citrus is produced in seven provinces. Mazandaran is the main center of citrus production in Iran and produces 1.5-1.7 million tons annually, followed by Golestan, Guilan, Fars, Hormozgan, and Kerman provinces (Salehi Sardoei and Fazeli-Nasab, 2021).

Weather fluctuations are a major obstacle to the production of crops (Salehi Sardoei et al., 2022b, 2022c), especially citrus fruits (Primo-Capella et al., 2021). Low temperatures and cold stress are one of the greatest challenges that citrus producers have faced since ancient times, primarily because citrus species are susceptible to low temperatures and are harvested in the winter (Mohammadian et al., 2012). Many citrus species are susceptible to -2.2 °C and lower temperatures. Nonetheless, some commercial species of citrus are cultivated in regions with a high risk of freezing (Lang et al., 2005). Winter frosts during citrus growth are a considerable threat to citrus orchards that exist adjacent to the Caspian shores, along the coasts of the Black Sea, coastal regions of France, the Adriatic Sea in Yugoslavia, some parts of Turkey, Spain, Palestine, and Greece. A sudden decline to subzero temperatures can make the trees susceptible to freezing. Citrus growers traditionally reduce the risk of winter frost by applying various methods such as sprinkler irrigation and the application of protective materials. However, introducing cold-tolerant citrus cultivars using genetic engineering methods can help to better protect against cold stress (Atta et al., 2020; Huang et al., 2011). Advances in biotechnology and the identification of relevant genes that regulate cold-stress tolerance can assist breeders in introducing cold-hardy citrus cultivars to the horticultural industry (Poles et al., 2020).

Multivariate statistical methods reportedly assisted in the evaluation of genotypes in terms of several features, used widely in genetic diversity assessments, regardless of the data type being attributed to morphological, physiological, or molecular aspects (Salehi Sardoei et al., 2022a). Among these methods, regression and principal component analyses are the most important (Mohammadi and Prasanna, 2003) as indicated in several reports on grapevine (Fattahi Moghadam et al., 2002), savory (Fathi et al., 2020), soybean (Majidian et al., 2020), vetch (Dehghani et al., 2020) and sideritis (Lotfi et al., 2020). Evaluations of physiological diversity can facilitate the selection of superior cultivars and contribute to cultivar management (Menzir, 2012).

There is a knowledge gap on the specifics of traits that affect cold stress tolerance in citrus species, especially through regression and principal component analysis. Therefore, the present study was conducted on several citrus cultivars to identify the physical and biochemical traits that significantly correlate with the efficiency of cold stress tolerance. The effects of these traits were evaluated on LT50 using multivariate statistics. The findings of the present study may be applied in the development of breeding programs for improving cold stress tolerance in citrus species.

Material and Methods

Plant materials

This experiment was conducted in a horticultural laboratory at Guilan University in 2021. To determine the tolerance of *Citrus unshiu*, *Citrus sinensis* var. 'Thomson navel', *Citrus paradisi* var. 'Star Ruby' and *Citrus limon* var. 'Lisbon', three levels of cold stress were applied, i.e. 4, -4, and -8 °C. For this purpose, two-year-old homogenous seedlings were selected and placed in 10 kg pots filled with regional field soil (silty-loam texture) (Tajvar et al., 2011).

Field experiment

Before applying the cold stress treatments and to allow plant adaptation to the decrease in temperature, the plants were moved to a temperate greenhouse (Pietrini et al., 2005) and then to a cool storage (+4 °C) with 65±5% relative humidity. The benchmark for this humidity is the percentage required for citrus growth in the north of Iran because the relative humidity in this region is typically 70%.

Cold stress treatment

The seedlings were placed in a programmable test chamber (Kato, Japan) for whole-plant freezing treatments. The test chamber started at +4 °C and decreased by 1.5 °C per hour. The chamber temperature was set to decrease stepwise by 1.5 °C/h, and, thereafter, by -4 °C/h until it reached -8 °C. Three replicates were considered for each treatment. A total of three treatments were applied for freezing (+4, -4, and -8 °C) and each took 10h. In addition, for the recovery treatment, after exposing the citrus plants to freezing temperatures for 8 h, the chamber temperature was increased stepwise to 4 °C and was held for 24 h to allow slow thawing. The relative humidity inside the chamber during the freezing and recovery treatments was maintained at 45±5% in the dark (Hashempour et al., 2019).

Measurement of physiological parameters

Due to the nature of symptoms such as ion leakage, relative water content (RWC), and LT50, these indices were measured immediately after the treatments. The RWC was studied using a relevant method, as outlined by Ritchie and Nguyen (1990). To measure traits such as leaf chlorophyll and carotenoids, total flavonoid, membrane lipid peroxidation, antioxidant enzymes of catalase, superoxide dismutase, and ascorbate peroxidase after stress, the samples were rapidly frozen in liquid nitrogen and stored at -80 °C in the freezer until the measurements were due.

Total flavonoids and membrane lipid peroxidation were studied using a method described by Campos et al. (2003), which is based on the content of the produced malondialdehyde (as a result of membrane injury) and its reaction with thiobarbituric acid which forms a colored compound of thiobarbituric acid-malondialdehyde. Antioxidant enzymes of catalase, superoxide dismutase, and ascorbate peroxidase were measured according to relevant methods described by Clarbone (1985), Shen et al. (2006), and Chance and Maehly (1995), respectively. Also, the LT50, chlorophyll, and carotenoid contents were measured using a method described by Barnes et al. (1992).

Electrolyte leakage

Before the first evaluation of electrical conductivity (EC1), each tube was filled with 20 ml of deionized water, spun at 23 °C (250 rpm) for one hour, and kept at room temperature for 24 hours. The samples were then autoclaved at 120 °C for 20 minutes to allow maximum ion leakage. They were then cooled at room temperature for 2 hours and the electrical conductivity (EC2) was measured again. The relative electrolyte leakage (REL) was calculated using the following formula: REL = (EC1/EC2) × 100. Cold tolerance was expressed as LT50, which is defined as the lethal temperature at which 50% of the total ion leakage occurs. The measurement involved fitting the response curves with the respective logistic sigmoid function (Sullivan and Ross, 1979).

Proline content

The concentration of free proline in stems was determined as described by Bates et al. (1973). Stem samples were crushed in liquid nitrogen and 0.5 g of the powdered tissue was homogenized in 10 ml of aqueous sulfosalicylic acid (3% (w/v)). The homogenate was then filtered using a No. 1 Whatman filter paper. Two milliliters of the filtered extract were obtained for analysis, along with 2 ml of ninhydrin and 2 ml of glacial acetic acid. The reaction mixture was incubated in a boiling water bath for one hour before termination in an ice bath. The organic phase was extracted with 4 ml of toluene added to the mixture. A UVvisible spectrophotometer (Bel Engineering Srl, Monza, Italy) was used for measuring the absorbance of the extract at 520 nm, while toluene served as a blank. Finally, using a calibration curve, the proline concentration was determined and expressed as μmg proline g-1 fresh weight (FW).

Statistical analysis

All data were analyzed using (ANOVA) (PROC GLM, SAS Institute). Duncan's multiple range test was used for differentiating the mean values ($p \leq 0.05$). Pearson's correlation coefficient (PROC CORR) was used in SAS to measure the correlation between pairs of attributes.

Results

LT_{50}

Citrus unshiu had the highest tolerance, which was associated with an LT50 of -7.4, whereas the lowest tolerance was observed in Citrus paradisi var. Star Ruby (LT50 = - 2.24) (Fig. 1).

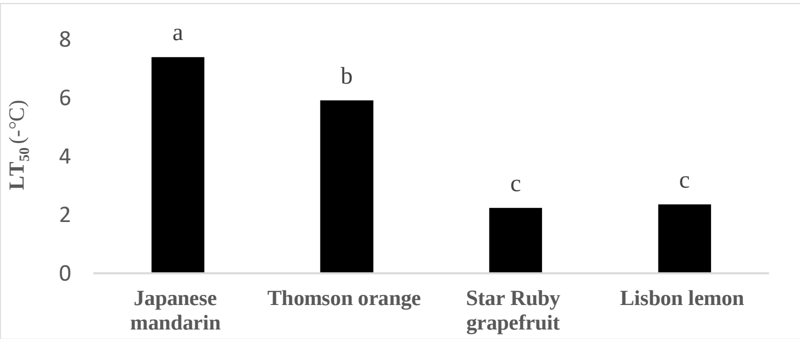

Fig. 1. Effect of cultivars and cold-temperature on LT_{50}.

Correlation Coefficient

The correlation coefficient showed significantly positive correlations between LT50 and total flavonoid (0.443**), chlorophyll b and chlorophyll a (0.613**), carotenoid and chlorophyll a (0.929**), chlorophyll b (0.573**) and total chlorophyll (0.849**), relative water content and malondialdehyde (0.559**), glycine-betaine and catalase (0.919**), hydrogen peroxide and total flavonoids (0.405**) and catalase (0.611**) (Fig. 2).

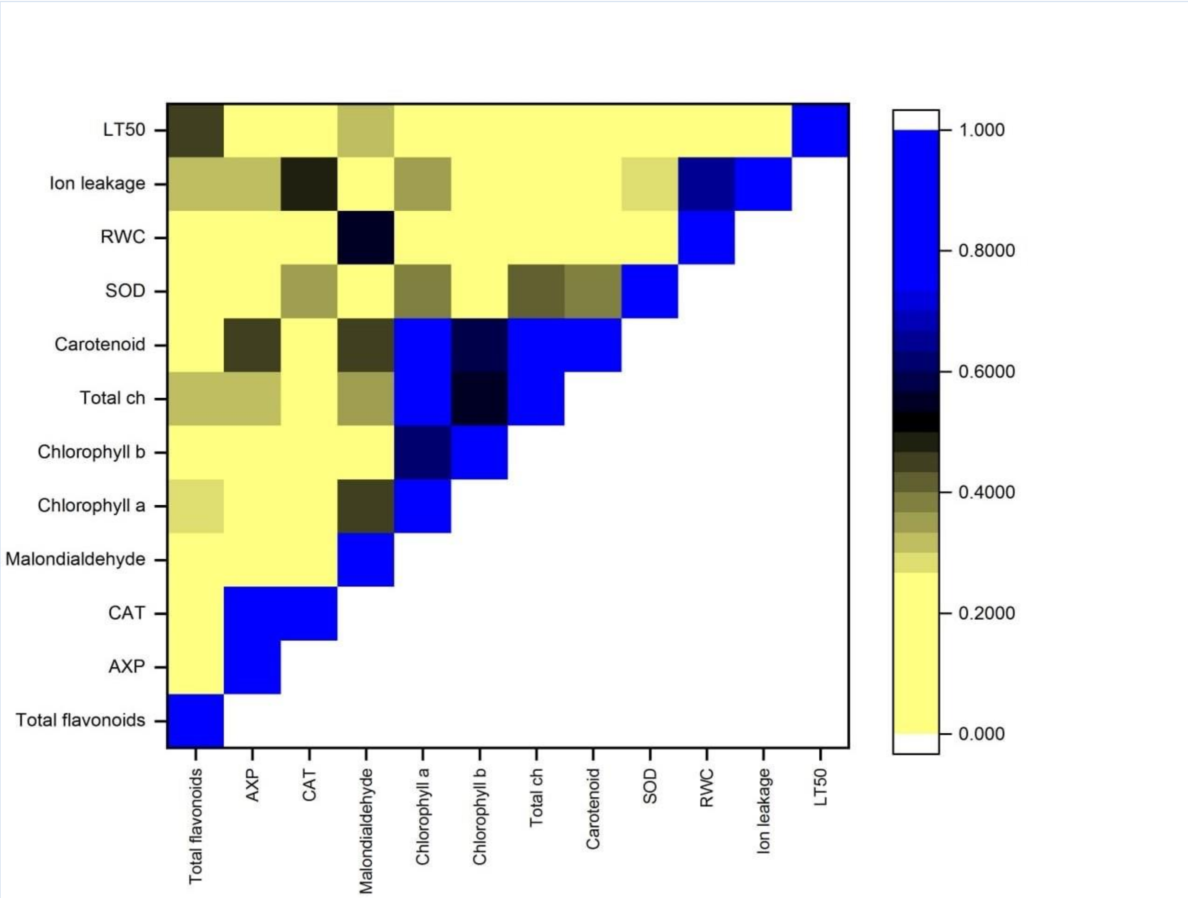

Fig. 2. Heat map of mutual relations of variables in the correlation coefficient

Principal component analysis

According to Table 1, three principal components were determined at +4 °C which explained 91.36% of the total variance. The first component included carotenoids and total chlorophyll with significantly positive coefficients, explaining 46.98% of the total variance alone and was regarded as the pigment component. The second component explained 24.85% of the variance and included flavonoids which had a positive coefficient. In contrast, RWC and ion leakage had negative coefficients and were considered influential in plant water potential. The third component was plant enzyme which included catalase and superoxide dismutase, with a negative coefficient, and lipid peroxidation with a positive coefficient, explaining 19.53% of the total variance (Table 1).

According to Table 2, three principal components were determined at -4 °C which explained 97.73% of the total variance. The first component was "pigment" which included carotenoids and total chlorophyll content, with positive coefficients, explaining 46.7% of the total variance (Table 2). The second component explained 36.48% of the total variance and included membrane peroxidation and RWC, with a positive coefficient, and ion leakage with a negative coefficient as a component influencing plant water potential. The third component was plant tolerance which included LT50 and superoxide dismutase, with negative coefficients, explaining 14.55% of the total variance.

Table 1. Eigenvectors, eigenvalues, relative and cumulative variances resulting from principal component analysis of citrus cultivars at +4 °C

Traits	First Component	Second Component	Third Component
Flavonoids	0.16	0.41	0.35
AXP	-0.37	0.08	-0.24
CAT	0.18	0.32	-0.40
Lipid peroxidation	-0.26	0.13	0.44
Ch. a	0.39	-0.10	0.16
Ch.b	-0.36	0.21	0.18
Carotenoid	0.40	-0.17	0.01
Ch. Total	0.40	0.16	-0.06
SOD	0.13	0.36	-0.44
Relative humidity content	0.23	-0.40	0.26
Ion leakage	-0.22	-0.42	-0.24
LT_{50}	-0.04	-0.34	-0.28
Eigenvalue	5.64	2.98	2.34
Relative Variance	46.98	24.85	19.53
Cumulative Variance	46.98	71.83	91.36

Table 2. Eigenvectors, eigenvalues, relative and cumulative variances resulting from principal component analysis of citrus cultivars at -4 °C

Traits	First Component	Second Component	Third Component
Flavonoids	0.28	0.32	0.19
AXP	-0.35	0.03	-0.37
CAT	-0.17	0.37	-0.33
Lipid peroxidation	-0.14	0.41	0.26
Ch. a	0.41	0.03	-0.10
Ch.b	0.41	0.07	0.09
Carotenoid	0.41	0.02	-0.02
Ch. Total	0.42	0.04	-0.03
SOD	0.111	0.28	-0.57
Relative humidity content	-0.06	0.47	0.066
Ion leakage	-0.05	-0.46	0.08
LT_{50}	0.19	-0.24	-0.52
Eigenvalue	5.60	4.38	1.74
Relative Variance	46.70	36.48	14.55
Cumulative Variance	46.70	83.18	97.73

According to Table 3, four principal components were determined at -8 °C which explained 93.28% of the total variance. The first component which explained 43.15% of the variance was the "pigment" component. It included carotenoids and chlorophyll a with a positive coefficient. The second component was plant enzyme which included catalase and superoxide dismutase with positive and negative coefficients, respectively, and explained 25.87% of the total variance (Table 3). The third component was "plant tolerance" and comprised ascorbate peroxidase, with a negative coefficient, as well as LT50, with a positive coefficient, explaining 15% of the total variance. The fourth component affected plant physiology and explained 69.02% of the total variance and included total flavonoids and chlorophyll b.

Regression analysis

Fig. 3 shows changes in the LT50 against ion leakage at +4 °C (A) and -4 °C (B). According to the regression coefficient, a change of one unit in ion leakage led to changes of 0.1661 and 0.3587 units in the LT50 at +4 and -4 °C, respectively (Fig. 3).

Table 3. Eigenvectors, eigenvalues, relative and cumulative variances resulting from principal component analysis of citrus cultivars at -8 °C

Traits	First Component	Second Component	Third Component	Four Component
Flavonoids	0.31	-0.06	-0.26	0.44
AXP	-0.07	0.34	-0.50	-0.20
CAT	-0.02	0.51	-0.24	-0.10
Lipid peroxidation	-0.34	-0.11	0.39	0.16
Ch. a	0.42	-0.06	-0.17	0.09
Ch.b	0.20	0.09	0.04	0.68
Carotenoid	0.41	-0.15	-0.04	-0.07
Ch. Total	0.34	-0.29	0.20	-0.24
SOD	0.21	-0.40	0.21	-0.32
Relative humidity content	-0.27	-0.36	-0.24	0.27
Ion leakage	-0.30	-0.30	-0.35	0.09
LT_{50}	0.25	0.35	0.40	0.06
Eigenvalue	5.18	3.10	1.80	1.11
Relative Variance	43.15	25.87	15.00	69.02
Cumulative Variance	43.15	69.02	84.02	93.28

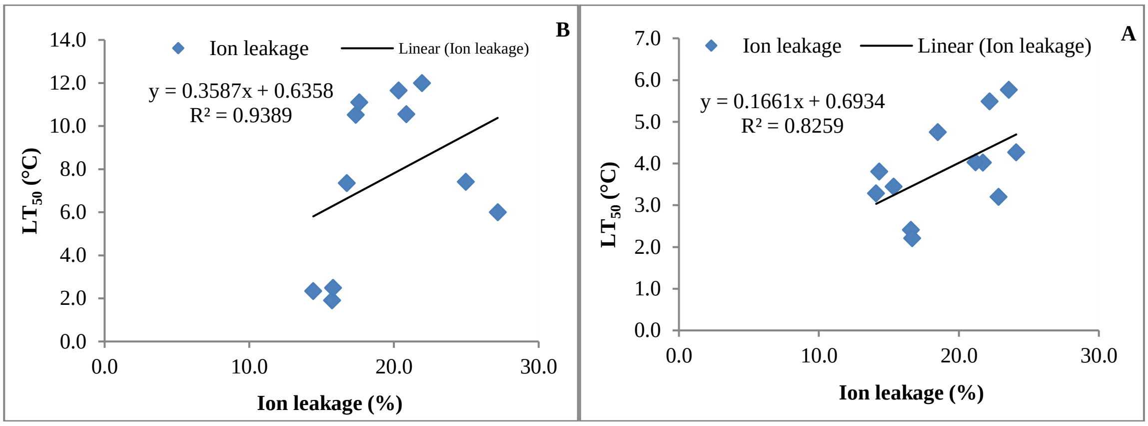

Fig. 3. Results of LT50 regression against Ion leakage at cold temperatures

Figure 4 illustrates the changes in LT50 as affected by changes in citrus lipid peroxidation at +4 °C (A) and -4 °C (B). According to the regression coefficient, a change of one unit in lipid peroxidation led to a decrease of 1.9238 and 5.9615 units in LT50 at +4 and -4 °C, respectively.

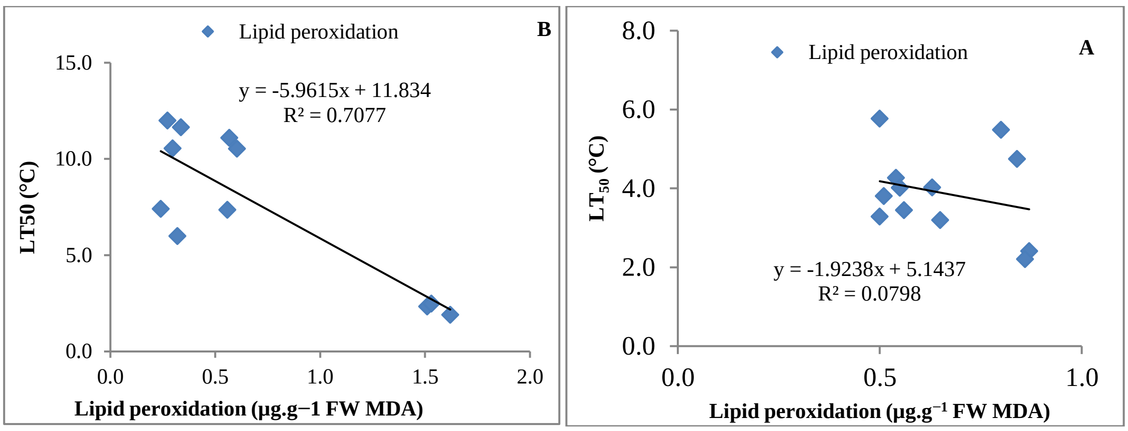

Fig. 4. Results of LT50 regression against lipid peroxidation at cold temperatures

Figure 5 shows the changes in LT50 against total flavonoid content at +4 °C (A) and -4 °C (B). According to the regression coefficient, a change of one unit in total flavonoid content led to a decrease of 0.0471 and 0.236 units in LT50 at +4 and -4 °C, respectively.

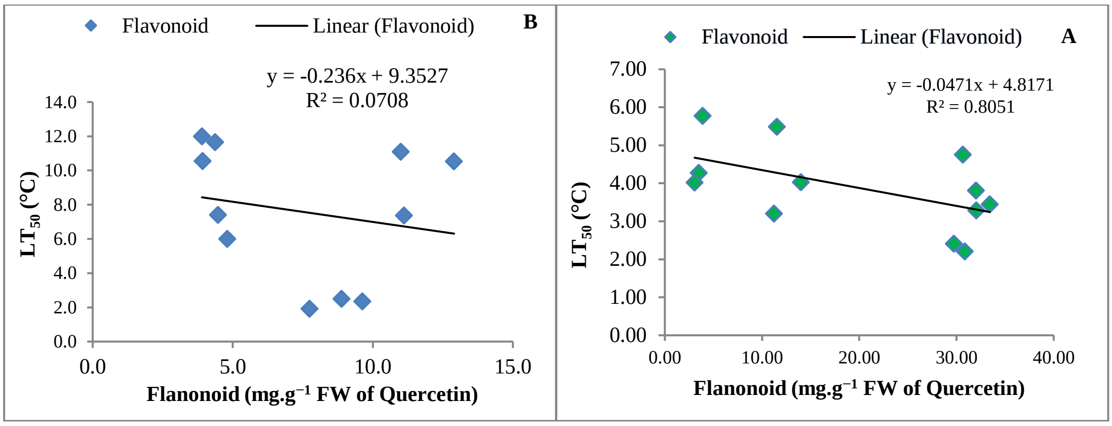

Fig. 5. Results of LT50 regression against flavonoid content at cold temperatures

Discussion

In this study, frost tolerance in some citrus cultivars was significant, which suggests that cold adaptation is not always associated with a halt in plant growth. Also, citrus can tolerate cold stress to some extent, such as in supercooling, similar to other woody perennials, thereby indicating a freezing-avoidance mechanism (Jiang et al., 2021). Although several subtropical fruit species can tolerate cold stress, the extent of frost tolerance varies among citrus cultivars which may be due to genetic variations (Arias et al., 2015). Previous studies on citrus revealed that freezing-tolerant cultivars usually have more stable cytoplasmic membranes and lower levels of electrolyte leakage, compared to susceptible cultivars (Moellering et al., 2020).

The correlation coefficient can help to determine the extent of a relationship between the linear changes of any two traits. This coefficient describes the degree of linear relationship and the direction of changes between any two traits. Quantification is highly important in breeding programs (Berry et al., 1985). Pearson's correlation coefficients on the traits (Fig. 2) showed significantly positive correlations between LT50 and total flavonoids (0.443**), chlorophyll a and chlorophyll b (0.613**), carotenoid and chlorophyll a (0.929**), chlorophyll b (0.573**) and total chlorophyll (0.849**), relative moisture content and malondialdehyde (0.559**), glycine-betaine and catalase (0.919**), hydrogen peroxide and total flavonoids (0.405**), as well as hydrogen peroxide and catalase (0.611**). These correlations indicated that an improvement in any of these traits may enhance plant tolerance to stress. Previous results (Abouzari et al., 2020) showed a positive, significant correlation between ion leakage and leaf water potential in citrus cultivars. A positive correlation between proline and carbohydrates was reported in page mandarin (Tajvar et al., 2011).

Correlations between traits may assist breeders in indirect selection for stress tolerance. This can be done by relying on traits that correlate with tolerance and are easier to measure (Zahedi et al., 2016). Although correlation coefficients between physiological traits can assist in determining correlations with stress tolerance, they fail to describe the relationships precisely and may require direct or indirect descriptions of the effects between these traits (Zahedi et al., 2016; Moosavi et al., 2016). In this study, since many traits correlated significantly and positively with the LT50, it is reasonable to further explore the traits via other statistical methods and determine key traits that affect citrus yield. Thus, an integrated pathway analysis was used for a further evaluation of the traits and their effects on citrus LT50.

The best genotypes may be selected according to different component plots when compared to each other, based on selected components. In most cases, two or three components best describe the variance and are used accordingly. In the present study, at +4 °C, the first component included carotenoids and total chlorophyll, with significantly positive coefficients, which described 46.98% of the total variance. The second component described 24.85% of the variance and the third component was an effective “plant enzyme” component, comprising catalase and superoxide dismutase enzymes, with significantly negative coefficients, whereas lipid peroxidation had a significantly positive coefficient. This component alone described 19.53% of the total variance. At -4 °C, three principal components were identified which described 97.73% of the total variance altogether. The first component included carotenoids and total chlorophyll, with significantly positive coefficients which described 46.7% of the variance. The second component described 36.48% of the variance. The third component was an effective “plant tolerance” component which included the LT50 and the superoxide dismutase enzyme, with significantly negative coefficients. This component alone described 14.55% of the total variance. At +8 °C, four principal components were identified which described 93.28% of the total variance. The first, second, third and fourth components described 43.15, 25.87, 15 and 69.02% of the total variance, respectively. Motaghi et al. (2009) and Asgar et al. (2010) used the principal component analysis and reported that the highest variation in data were described by the first two components. In the said research, it was concluded that traits such as stress tolerance, lipid peroxidation and antioxidant enzymes described 94% of the variance in stress tolerance. In another study, Zakizade et al. (2010) reported that the first three components described 96% of the total variance.

Conclusion

The four cultivars of citrus, i.e. Japanese mandarin (tolerant), Thomson orange (moderately-tolerant), Lisbon lemon and Ruby Star grapefruit (cold-susceptible) varied in their responses to freezing temperatures. Japanese mandarin was better suited to the drop in temperature, down to -4 °C, followed by Thomson orange, Lisbon lemon, and Ruby Star grapefruit. The current results facilitated the selection of commercial citrus cultivars for cold-tolerance in Citrus unshiu, Citrus sinensis ‘Thomson navel’, Citrus paradisi ‘Star Ruby’, and Citrus limon ‘Lisbon’. It may be indicated that carotenoids, chlorophyll, and lipid peroxidation perform better at specific temperatures. These traits can be used indirectly in selecting cultivars for cold-tolerance.

Declaration of Competing Interest

None.

Conflict of Interest

The authors indicate no conflict of interest for this work.

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