

Water Relations of Well-watered Citrus Exposed to Cold-acclimating Temperatures

Smita Barkataky, Robert C. Ebel¹, and Kelly T. Morgan

University of Florida–Southwest Florida Research and Education Center, 2685 SR 29 N, Immokalee, FL 34142-9515

Keri Dansereau

Department of Horticulture, Auburn University, Auburn, AL 36849

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Abstract. This study was conducted on well-watered citrus to determine changes in water relations during cold acclimation independent of drought stress. Potted sweet orange and Satsuma mandarin trees were exposed to progressively lower, non-freezing temperatures down to 10/4 °C, light/dark temperatures, respectively, for 9 weeks in environmental growth chambers to promote cold acclimation. The trees were watered twice daily and three times on the day water relations data were collected to minimize drought stress. Although soil moisture was higher and non-limiting for plants in the cold than in the warm chamber, cold temperatures promoted stomatal closure, higher root resistance, lower stem water potential (Ψ_{stem}), lower transpiration, and lower leaf ψ_s . Leaf relative water content (RWC) was not different for cold-acclimated trees compared with the controls. Cold acclimation promoted stomatal closure at levels only observed in severely drought-stressed plants exposed to warm temperatures and where Ψ_{stem} and RWC are typically much lower than what was found in this study. Ψ_{stem} continued to decline the last 4 weeks of the experiment although air temperature, leaf ψ_s , RWC, stomatal conductance (g_s), and transpiration were constant. The results of this experiment indicate that water relations of citrus during cold acclimation vary from those known to occur as a result of drought stress, which have implications for using traditional measures of plant water status in irrigation scheduling during winter.

Citrus regions in many parts of the world receive very little rainfall during winter and thus irrigation is considered essential. California accounts for $\approx 32\%$ and Florida 65% of the total citrus production in the United States (Anonymous, 2012). Citrus grown in Florida receives very low rainfall during winter. Growers irrigate during winter based on evapotranspiration or soil moisture measurements with crop coefficients that range from 0.6 to 1.1; however, none of these methods incorporate changes in water requirements of citrus because they may change during cold acclimation independent of soil moisture content (Boman, 1994; Fares and Alva, 1999; Morgan et al., 2006). With water supplies in both regions limited, research is continuing to be conducted to optimize knowledge of water requirements.

Studies have typically concluded that citrus develop plant water deficits during winter (Ebel et al., 2004; Thomashow, 1999; Yelenosky, 1975, 1982, 1985). Most of these studies were conducted without adequate knowledge of soil moisture content or were conducted in dry soils that imposed drought

stress. Thus, it is not known how cold acclimation changes water relations of citrus independent of soil moisture content. Citrus cold-acclimate when air temperature is less than 10 °C, which typically occurs during winter in the citrus-growing regions of Florida and California (Attaway, 1997; Barkataky et al., 2012; Yelenosky, 1985; Yelenosky et al., 1984). There are several lines of evidence that would suggest that at least some changes in plant water deficits occur as a result of cold temperatures and not dry soil. Stomatal aperture and leaf water potential are typically reduced even on days of very low evaporative demand and after the trees have been fully irrigated (Ebel et al., 2004; Yelenosky, 1982).

Changes in citrus water relations during cold acclimation and independent of soil moisture content are not well understood. This study was conducted to characterize changes in plant relations of citrus plants with soil moisture carefully maintained at high levels to minimize drought stress. The results of this study are discussed with respect to implications for commercial irrigation of citrus during winter months as well as how the changes in plant water relations may be related more to freeze tolerance than plant water use.

Materials and Methods

Plant material and culture. Two experiments were conducted. The first experiment

was conducted at the University of Florida's Southwest Florida Research and Education Center, Immokalee, FL, using sweet orange cv. Hamlin (*Citrus sinensis* L.) budded on Carrizo citrange (*C. sinensis* L. Osbeck \times *Poncirus trifoliata* L. Raf.) rootstocks. The second experiment was conducted at Auburn University, Auburn, AL, using Satsuma mandarin [*Citrus unshiu* (Marc.) 'Owari'] budded on trifoliata orange rootstocks [*Poncirus trifoliata* (L.) Raf. 'Rubidoux']. The treatments and data-collecting procedures were very similar for both experiments.

In the first experiment, the plants were grown in 20 \times 40-cm (10.60-L) tree pots (manufactured by Steuwe and Sons Inc., Tangent, OR) filled with 11.79 kg of screened air-dried soil. The soil was Margate sand (siliceous, hyperthermic, mollic Psammaquents), which is typical of the Flatwoods region of southwestern Florida where citrus are commercially grown. Each pot was enclosed in fiberglass insulation to slow temperature declines of roots. The plants were fertilized once in a week with 500 mL of 33.6 g of a water-soluble fertilizer (Peters Professional, 18-8-17; Everris Int. B.V., Geldermalsen, The Netherlands) in 7.57 L of water. Keyplex 445, a combination of nitrate, iron manganese, and zinc (KeyPlex, Winter Park, FL), was applied as a foliar spray once every month. The pots were watered daily using a single 35-mL-min⁻¹ drip irrigation emitter. The irrigation emitters were programmed to operate two times daily for 10 min at each watering. On the day data were collected, plants were watered three times to minimize drought stress.

In the second experiment, the plants were grown in 3.8-L pots in a one pine bark:one sand substrate by volume amended with 4.45 kg-m⁻³ of dolomite lime, 8.9 kg-m⁻³ of 18N-6P₂O₅-12K₂O, (Polyon 12-14 month controlled release fertilizer; Pursell Technologies, Inc., Sylacauga, AL), and 0.89 kg-m⁻³ of a micronutrient package (Micromax; The Scotts Co., Marysville, OH). All plants were supplemented with water-soluble 20N-20P₂O₅-20K₂O with micronutrients, which were applied at 234 mg-L⁻¹ when watered. Each pot was enclosed in fiberglass insulation to slow temperature declines of roots. The plants were watered when soil moisture dropped below 85% of the water-holding capacity of the substrate except on days when data were collected. The temperature of the water used to water the plants was ≈ 20 °C.

Temperature treatments. Both experiments were provided with the same temperature treatments. Two temperature treatments were imposed on plants in two identical environmental growth chambers (Model PGR 15; Conviron Products of America, Pembina, ND, for the first experiment and Model E15; Conviron, Winnipeg, Canada, for the second experiment). The temperature treatment for cold-acclimated plants was 25 °C day/20 °C night for 7 d; 20 °C day/15 °C night for 7 d; 15 °C day/10 °C night for 7 d; and 10 °C day/5 °C night for 42 d. The irradiance was 450 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The temperatures were lowered after water relations data collection. In the control growth

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¹To whom reprint requests should be addressed; e-mail rcebel@ufl.edu.

chamber, air temperature was maintained at 25 °C day/20 °C night throughout the experiment. The day/night photoperiod for both treatments was 12 h/12 h, respectively.

Data collected. Data were collected every 7 d. On the days data were collected and 2 to 4 h after the start of the light period, the pots were thoroughly watered as demonstrated by water flowing from the bottom of the pots. Gravitational water was allowed to percolate out of the pots for 2 h before g_s and transpiration measurements were taken.

Stomatal conductance and transpiration. Stomatal conductance and transpiration data were obtained for both experiments on five recently matured leaves using a steady state porometer (Model LI 1600; LI-COR Inc., Lincoln NE) and a portable photosynthesis system (Model 6400; LI-COR Inc.).

Stem water potential. Ψ_{stem} was measured for both the first and the second experiments. Immediately after watering the pots and before g_s measurements were taken, one leaf per plant in the first experiment and two leaves per plant in the second experiment were wrapped in flexible plastic and aluminum foil (Garnier and Berger, 1985). The water potential of the leaf was allowed to equilibrate with the stem for 2 h before the leaves were excised and water potential measured using a pressure chamber (Model 3005 plant water status console; Soil Moisture Equipment Corporation, Santa Barbara, CA).

Osmotic potential. Ψ_{π} was measured in the first experiment. Immediately after measuring Ψ_{stem} , a 7-mm diameter disk was cut from each leaf using a cork borer, immediately dipped into liquid nitrogen, and held at -80 °C until measurement. Osmotic potential of each leaf disk was determined using a Dew Point Microvoltmeter (Model HR-33 T; Wescor, Inc., Logan, UT) that was calibrated with 0.550, 0.157, and 0.053 m solutions of NaCl of known ψ_s of -2.5, -0.75, and -0.25 MPa, respectively.

Relative water content. RWC was measured in the first experiment. After removing the leaf core used to determine Ψ_{π} , the leaves were immediately weighed to determine fresh weight (FW). The leaves were rehydrated by floating upright on water at 4 °C for 24 h, blotted dry with paper towels, and weighed to determine the turgid weight (TW). The leaves were dried at 54 °C for 72 h and weighed to determine dry weight (DW). RWC was calculated as $RWC = [(FW - DW)/(TW - DW)] \times 100$.

Estimation of root resistance. Root resistance (R_{root}) was estimated for both experiments using the same approach. Water movement in the soil-plant atmosphere continuum can be described using an analogy of Ohm's law (Landsberg and Jones, 1981; Van den Honert, 1948), which describes flow as being proportional to the driving force and inversely proportional to the resistance in the flow path:

$$T = \frac{(\Psi_{soil} - \Psi_{stem})}{(R_{root} + R_{soil})} \quad [1]$$

where T is whole plant transpiration, Ψ_{soil} is soil water potential, Ψ_{stem} is stem water

potential, R_{root} is root resistance, and R_{soil} is the resistance of water movement from the soil to the root surface. R_{root} of each plant was calculated by rearranging Eq. [1]:

$$R_{root} = \left[\frac{(\Psi_{soil} - \Psi_{stem})}{T} \right] - R_{soil} \quad [2]$$

It was assumed that Ψ_{soil} was zero, which is very close to values typical for soils at water-holding capacity (Mengel et al., 2001; Paul, 2007). R_{soil} was assumed to be zero, which is a reasonable assumption for well-watered soils (Malazian et al., 2011). These assumptions allowed Eq. [2] to be simplified to:

$$R_{root} = \frac{-\Psi_{stem}}{T} \quad [3]$$

Whole plant transpiration (T) was calculated by:

$$T = \frac{(PW_o - PW_t)/A_{leaf}}{t} \quad [4]$$

where PW_o was pot weight (g) measured 2 h after watering, PW_t was pot weight (g) measured ≈ 4 h later, A_{leaf} is the total leaf area of the plant (m^2), and t is the time elapsed between weight measurement (s). T was multiplied by $1 \times 10^{-6} m^3 \cdot g^{-1}$, the inverse of water density, to convert transpiration units from weight ($g \cdot m^{-2} \cdot s^{-1}$) to volume ($m^3 \cdot m^{-2} \cdot s^{-1}$) and thus give R_{root} units of $MPa \cdot s^{-1}$, units that are commonly used to describe root hydraulic resistance (Kriedemann and Barrs, 1981; Landsberg and Jones, 1981). The morning after all other measurements were taken, the potting media was refilled to water-holding capacity and after gravitational water was allowed to percolate from the base of the pots the pots were weighed to find PW_o and reweighed ≈ 4 h later to find PW_t . After measuring PW_t , the temperature in the cold-acclimated plants was lowered to impose the next temperature treatment.

Leaf area. Leaf area was measured at the beginning of each of the experiments using a portable leaf area meter (Model LI-3000A; LI-COR Inc.). Because the trees were growing when the experiment was initiated, each growing tip was labeled. At the end of the experiment, leaves of new growth were removed separately from leaves that were present before the experiment was initiated and total leaf area of each was measured separately. Total leaf area on the day water relations data were collected was estimated by adding the initial leaf area to an estimate of leaf area of new growth. Leaf area of new growth was estimated by assuming a linear growth rate for the entire treatment period for control plants and for the first 3 weeks for the acclimated plants after which growth had stopped. Growth of plants typically occurs in three phases: an early accelerating phase, a linear phase, and a growth termination phase (Erickson, 1976; Yin et al., 2003). The plants in the current study were in the middle of a typical growth flush and thus were assumed to be in the linear growth phase. Area of leaves used for Ψ_{stem} measurements were also measured after FW measurement and subtracted

from the total leaf area present when the experiment was initiated.

Soil moisture. Soil moisture was measured for the first experiment. To verify that soil moisture was not limiting for plants in the cold-acclimated chamber on the day data were collected, soil moisture was measured every 5 min on two sweet orange plants using dielectric aquameter sensors (ECHO; Decagon Devices, Inc., Pullman, WA) and a data logger (HOBO micro station Model H21 002; Decagon Devices, Inc., Pullman, WA).

Statistical analysis. There were eight plants used per treatment in each experiment. Means were separated using $2 \times$ the SEM.

Results

Soil moisture in the cold-acclimated chamber remained nearly constant during the 4 h that plant water relations measurements were made, whereas soil moisture in pots with control plants declined to $\approx 0.11 m^3 \cdot m^{-3}$ (Fig. 1). The amount of water removed in the control treatment would not have imposed significant drought stress (Morgan et al., 2006). Leaf RWC was similar for both cold-acclimated and unacclimated plants throughout the experiment (Fig. 2A).

Despite the well-watered conditions for cold-acclimated plants, changes in water relations typically found in drought stress plants occurred. Transpiration declined close to zero by the third week for *C. sinensis* (Fig. 3A) and the seventh week for *C. unshiu* (Fig. 3B). Transpiration remained low at the coldest temperatures in part because of stomatal closure (Fig. 3C–D), but evaporative demand was also less in the cold acclimation chamber as shown previously (Barkataky et al., 2013). Citrus stomates completely close only under extreme drought stress (Kriedemann and Barrs, 1981). Ψ_{stem} declined in a linear manner throughout the experiment for cold-acclimated plants to ≈ -0.9 MPa for *C. sinensis* (Fig. 3E) and -1.2 MPa for *C. unshiu* (Fig. 3F), which is typical for plants exposed to mild drought stress (Chone et al., 2001; Naor, 2000; Stagno et al., 2011). Ψ_{stem} of the unacclimated controls remained between -0.4 and -0.5 MPa, which is typical for citrus plants exposed to well-watered conditions and under very low evaporative demand (Chone et al., 2001; Naor,

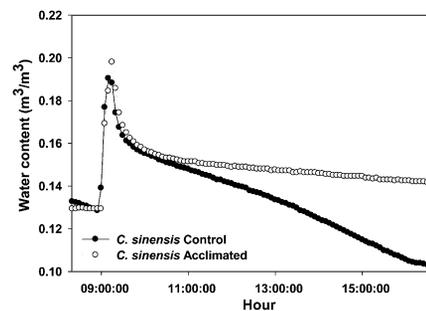


Fig. 1. Soil moisture content of potted *C. sinensis* trees on a day water relations measurements were collected. Data are means of two trees per treatment.

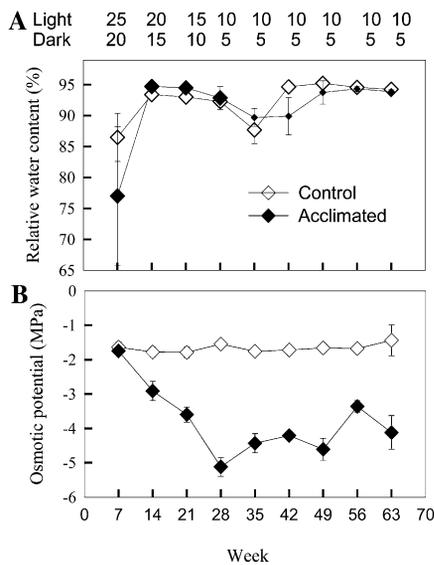


Fig. 2. Leaf relative water content (A) and osmotic potential (ψ_s) (B) of *C. sinensis* exposed to cold-acclimating temperatures and warm temperatures that minimize cold acclimation and promote growth (control). Temperatures of the cold acclimation treatment are shown at the top of the figure ($^{\circ}\text{C}$) during the light (12 h) and dark (12 h) cycles.

2000; Stagno et al., 2011). The Ψ_{π} of cold-acclimated *C. sinensis* trees decreased to a minimum of -5.3 MPa by the fourth week and remained between -5.3 and -3.8 MPa when exposed to $10/5$ $^{\circ}\text{C}$, whereas the controls remained between -1.0 and -2.0 MPa (Fig. 2B). Syvertsen et al. (1988) reported Ψ_{π} values of -2.16 and -2.27 MPa for trifoliolate orange and sweet orange, respectively, when exposed to drought stress for 60 d.

Cold acclimation increased R_{root} by six times in *C. sinensis* (Fig. 3G) and by 18% in *C. unshiu* (Fig. 3H) by the end of the experiment compared with control plants. Some error may have been introduced in the control trees by assuming R_{soil} was zero, but any error would have led to lower R_{root} estimates, which would have accentuated the difference in R_{root} between the cold-acclimated and unacclimated control trees. The R_{root} values are typical for citrus as reported previously (Elfving et al., 1972; Ramos and Kaufmann, 1979; Wilcox and Davies, 1981; Wilcox et al., 1983) and these resistances are similar to those of drought-stressed citrus (Kriedemann and Barrs, 1981).

Discussion

Our modern understanding of plant water relations has mainly evolved from studying growing plants at warm temperatures and in soils of varying moisture contents. It is understandable then that as studies have been conducted on cold-acclimated plants, the traditional relationships among water relation parameters of drought-stressed plants would be made. However, this study demonstrates that those relationships are not consistent for citrus trees exposed to cold-acclimating temperatures. By carefully maintaining high soil

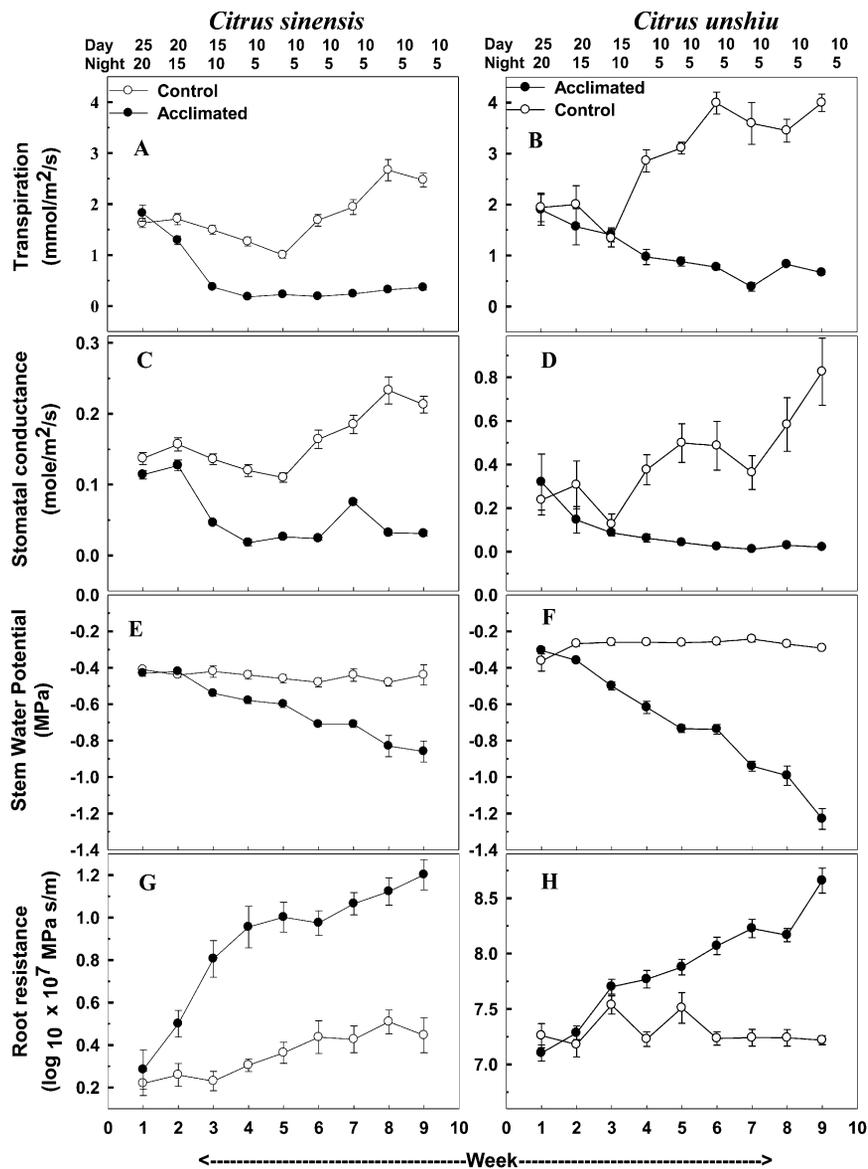


Fig. 3. Transpiration, stomatal conductance (g_s), stem water potential, and root resistance of the cold-acclimated and the unacclimated (control) *Citrus sinensis* (A, C, E, G) and *Citrus unshiu* (B, D, F, H) during cold acclimation. Temperatures of the cold acclimation treatment are shown at the top of the figure ($^{\circ}\text{C}$) during the light (12 h) and dark (12 h) cycles.

moisture contents throughout the experiments and especially on the day measurements were made, drought stress was minimized as evidenced by maintaining soil moisture near the water-holding capacity of the potting media and which was confirmed by the very high Ψ_{stem} of plants in the warm chamber. Thus, any changes in water relations of plants in the cold chamber would be mainly the result of temperature and not drought stress and should be considered with respect to the development of freeze tolerance.

Cold temperatures promoted stomatal closure, higher R_{root} , lower Ψ_{stem} , Ψ_{π} , and very low transpiration. Some of the changes we found in the current study have been shown in other studies and thus it is not surprising that others have concluded that cold acclimation promotes plant water deficits (Ebel et al., 2004; Kriedemann and Barrs, 1981; Huang et al., 2011; Yelenosky, 1978, 1982; Young, 1970; Young and Peynado, 1965) because

even under the well-watered conditions of this study, most of the water relation parameters changed in a manner consistent with the development of plant water deficits. Nevertheless, there are some striking inconsistencies in water relation parameters compared with those found in drought-stressed citrus plants. Stomatal closure, high R_{roots} , the low Ψ_{π} , and very low transpiration are at levels that occur for only extremely drought-stressed citrus trees (Kriedemann and Barrs, 1981), yet the Ψ_{stem} and RWC values are typical for actively growing, well-watered trees grown in commercial grove situations on warm, sunny days (Kriedemann and Barrs, 1981).

Because the changes in plant water relations are not related to soil moisture, we conclude that these changes are adaptive mechanisms that increase tolerance to freezes, although how these changes relate to freeze tolerance cannot be deduced from these data. Nevertheless, several interesting relationships should

be noted. First, Ψ_{stem} of Satsuma mandarin was lower than sweet orange on each measuring date although air temperature was the same. Satsuma mandarin is also substantially more cold-hardy than sweet orange (Yelenosky, 1985). Second, Ψ_{stem} continued to decline throughout the study for both genotypes even for the last 5 weeks when air temperature, soil moisture content, and Ψ_{π} were relatively constant. Although 3 weeks at low temperature have been suggested as being optimum for promoting maximum cold-hardiness of citrus (Yelenosky et al., 1984), it appears that Ψ_{stem} continues to change and may indicate a deeper level of acclimation beyond 3 weeks. Third, Ψ_{π} reached a minimum by the fourth week of cold acclimation and may indicate the limit to the accumulation of osmotically active substances (Cuilan et al., 2000; Kushad and Yelenosky, 1987; Ligeng et al., 2000; Lin and Liu, 1994; Lin et al., 1994; Yelenosky, 1985). Fourth, the decline in Ψ_{stem} beyond the fourth week of cold acclimation cannot be related to Ψ_{π} , which remained relatively constant during this same time period. Fifth, stomates closed reducing transpiration to close to zero although RWC remained high. We can conclude that the plant does not open stomates sufficiently to allow some drying that would reduce RWC.

The results from this study may have implications for commercial grove managers in managing irrigation during winter. However, it must be considered that the current study was conducted for potted plants where the roots were exposed to the same temperatures as the shoots, a phenomenon that rarely occurs in nature where soil temperatures are normally warmer during winter than air temperature as a result of heat storage of the soil and bedrock. In regions where freezes occur and irrigation is required, deficit irrigation is recommended to impose mild drought stress to promote cold acclimation (Besigner, 2004), but how overlaying drought stress on the changes that are associated with air temperatures that promote cold acclimation are not known. Current irrigation scheduling methods generally incorporate only evaporative demand and soil moisture measurements (Morgan et al., 2009). Changes that occur in citrus that affect water use during cold acclimation are ignored and as such lead to excessive irrigation (Barkataky et al., 2013). Some have proposed using Ψ_{leaf} or Ψ_{stem} measurements for irrigation scheduling (Ortuno et al., 2006; Shackel et al., 1997), but their modification, independent of soil moisture status during cold acclimation, would have to be considered during winter for citrus.

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