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Research article

Osmotic adjustment in cowpea plants: Interference of methods for estimating osmotic potential at full turgor

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ABSTRACT

Osmotic adjustment is a persisting controversy in studies on the effect of salt and water stress in cowpea crops. Our hypothesis is that the osmotic potential determination method interferes with the osmotic adjustment calculation. The objective of this study was to consider the osmotic adjustment comparing results obtained by pressure-volume (P–V) curves and osmometry. The experiment was conducted in a randomized block design, with six salt water concentrations 0, 20, 40, 60, 80, and 100 mmol L^{-1} of NaCl in a Fluvisol. The osmotic adjustment found through osmometry were lower than those found through P–V curves. The apoplastic water fraction of the cowpea had a dilution effect, denoting overestimation of the osmotic potential by the methodology based on osmometry. This may be the source of the different interpretations of osmotic adjustment in cowpea plants. Thus, osmotic adjustment should be calculated preferably using the osmotic potential determined by method of pressure-volume curves.

1. Introduction

Soil salinity is one of the main abiotic stresses affecting plants and reduces yield in arid and semi-arid regions of the world due to osmotic effects, ionic toxicity and nutritional deficiency (Melo et al., 2018). Cell turgor and water content in the plant tissue are affected when the increase of solutes concentration in the soil solution exceeds the physiological capacity of the plants to adapt to the lower availability of water caused by the reduction in the soil osmotic potential (Truner Neil, 2017).

Some species are able to perform osmotic adjustment (Blum, 2017; Hessini et al., 2019) – as the water availability in soil is reduced (due to both the matric effect of the reduction in soil water content and the osmotic effect of accumulation of salts), thus maintaining a favorable gradient of water potential for water absorption by plants (Goufo et al., 2017).

The active osmotic adjustment occurs through both the synthesis and accumulation of organic solutes in the cytoplasm and the influx of inorganic solutes into the vacuole (Goufo et al., 2017). This mechanism results in the reduction of plant water potential, allowing a gradient of water potential favorable to water absorption and maintenance of cell turgor (Truner Neil, 2017).

This adaptive capacity is particularly important in crops better adapted to dry sub-humid, semi-arid and arid regions where water availability is affected by both a reduced amount of precipitation and greater susceptibility to salinization. Hence, cowpea is a prominent pulse widely cultivated in these environments.

Several adaptive responses of *Vigna unguiculata* (cowpea) to drought have been described (Goufo et al., 2017), such as reduction of stomatal conductance (prevention) affecting photosynthetic processes (Agbicodo et al., 2009), and osmotic adjustment that allows the absorption of water even in conditions of reduced water availability (tolerance) (Iwuagwu et al., 2017).

The importance of osmoregulation in response to water deficit in cowpea culture is still a controversial issue (Goufo et al., 2017). Some of the divergences on this adaptive response are explained by genetic variability between cultivars of the same species (Iwuagwu et al., 2017). However, gene variability does not explain divergent conclusions in studies on the same cultivar, as seen in the California blackeye cultivar in Augé et al. (1992) and Augé et al. (2001).

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Under these conditions in which the hypothesis of gene variability is disregarded, methodological aspects stand out when one seeks to explain the origin of the differences in these results, since the studies mentioned above, for example, have accessed the osmotic variable at full turgor by different techniques.

When the result is positive for performing osmotic adjustment in cowpea, Augé et al. (1992) access the osmotic variable by pressure-volume curves. When the result is negative, sap osmometry is quantified and the concentrations of solutes are used in the Van't Hoff equation to estimate the osmotic potential of the plant.

Moreover, the leaf osmotic potential reduces in environments with water deficit caused by high salt concentration in the soil solution due to passive and active accumulation of solutes in the plant tissue (Zhou and Yu, 2009; Blum, 2017; Hessini et al., 2019). Therefore, studies evaluating only responses of cowpea to water deficit caused by drought may not consider the common passive osmotic adjustment in these environments (Zeng et al., 2015).

Therefore, this study aimed to compare the results of osmotic potential at full turgor and total osmotic adjustment obtained by osmometry and pressure-volume curves to verify the effective performance of osmotic adjustment in cowpea, due to the specificities of each method.

2. Material and methods

2.1. Soil sampling and properties

Soil samples were collected in the 0–30 cm layer of a Neossolo Flúvico (Santos et al., 2013) Fluvic Neosol (Fluvisol), in the Nossa Senhora do Rosário farm, in Pesqueira, Pernambuco, Brazil (8°34′11″S; 37°48′54″W; and altitude of 630 m). The climate of this region is BSh, extremely hot and semiarid, according to the classification of Köppen, with total average annual precipitation of 730 mm, and average annual evapotranspiration of 1683 mm. The soil samples were air dried, homogenized, disaggregated, and sieved in a 4 mm mesh sieve to maintain soil microaggregates.

Disturbed soil samples were collected, air dried, sieved (2 mm), and subjected to particle-size distribution analysis using the pipette method (Gee and Bauder, 1986). Water-dispersed clay (WDC) was measured, and the dispersion index (DI) and flocculation index (FI) were calculated (Table 1). The bulk density was determined as the soil dry weight per unit volume of intact soil cores (Blake and Hartge, 1986). The particle density was determined using the volumetric flask method. The total soil porosity was estimated using the relationship between the bulk density and the particle density (Vomocil, 1965).

For chemical characterization of soil samples (Table 2), the following analyses were performed: pH and exchangeable Ca^{2+}, Mg^{2+}, Na^+, and K^+ (Thomas, 1982). In addition, the electrical conductivity, soluble bases, and chloride in the soil saturation extract (Richards, 1954) were measured. Flame emission photometry was used to determine the Na^+ and K^+ levels, and inductively coupled plasma optical emission spectrometry was used to measure the Ca and Mg levels. Cl^- was determined by silver nitrate titration.

Table 1
Physical properties of the soil used for cowpea (*Vigna unguiculata*) plants grown under different soil salinity levels. Pernambuco, Brazil.

Sand	Silt	Clay	WDC	Bd	Pd	DI	FI	TP	FC	PWP
g kg^{-1}			g cm^{-3}			%			U (%)	
433.09	466.04	100.87	50.4	1.37	2.63	50	50	48	19.1	8.1

WDC = Water-dispersed clay; Bd = Bulk soil density; Pd = Particle density; DI = dispersion index and FI = Flocculation index; TP = Total porosity; FC = Field capacity (0.01 MPa); PWP = Permanent wilting point; U (%) = Gravimetric moisture.

Table 2
Chemical properties of the saturation extract and soil sorption complex used for cowpea (*Vigna unguiculata*) plants grown under different soil salinity levels. Pernambuco, Brazil.

Saturation Extract*							
pH	EC	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SAR
	($dS\ m^{-1}$)	mmol L^{-1}					
7.3	3.36	13.51	2.13	9.12	8.63	25.47	4.54
Sorption Complex**							
pH	Na^+	K^+	Ca^{2+}	Mg^{2+}	H^+	SB	ESP
(1:2.5)	cmol$_c$ kg^{-1}						(%)
6.75	1.65	1.2	4.35	3.14	1.54	10.34	15.96

EC = Electrical conductivity; SB = Sum of bases; ESP = Exchangeable sodium percentage; SAR = Sodium adsorption ratio.

2.2. Experiment implementation

The experiment was carried out during 48 days in a greenhouse at the Federal Rural University of Pernambuco, in Recife, Pernambuco, Brazil (8°04′03″S; 34°55′00″W; and altitude of 4 m). Four seeds of the IPA206 cowpea cultivar were sown in 10-L pots. The seedlings were thinned, leaving two plants per pot, selected according to their sanity and vigor.

Temperature and relative humidity sensors (Instruterm, model HT70) were installed in the greenhouse during the experiment, and the recorded data are shown in Fig. 1.

2.3. Irrigation water preparation

The soil moisture content was maintained at 19% on a weight basis, correspondent to the matric potential of 0.01 Mpa at soil field capacity. The pots were weighed daily to evaluate the weight variation of each pot, and the water lost by evapotranspiration was replenished by

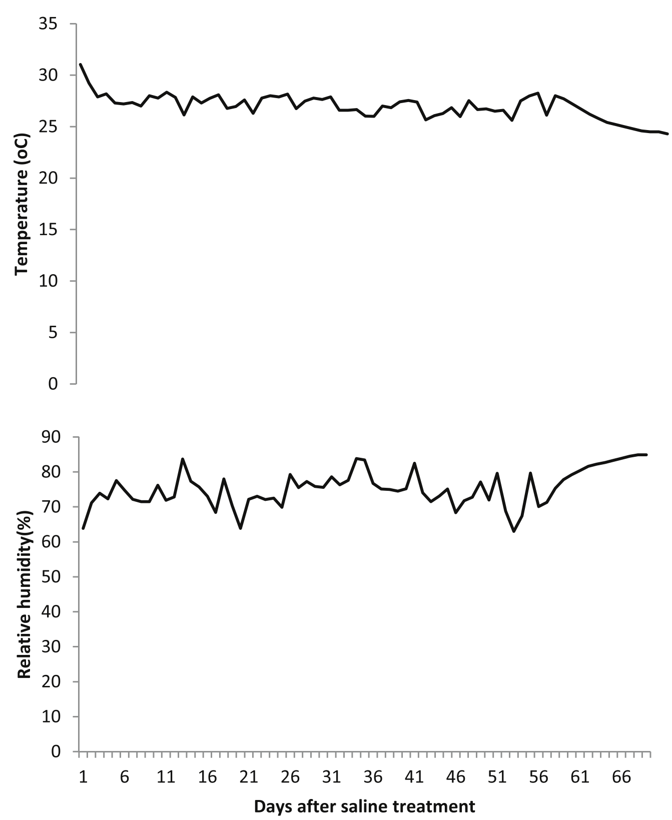

Fig. 1. Data of mean relative air humidity and mean daily temperature along the experiment in the greenhouse. Pernambuco, Brazil.

irrigation to maintain the pre-established soil moisture. The irrigation waters were prepared with NaCl concentrations of 0 (distilled water), 20, 40, 60, 80, and 100 mmol L^{-1}. The saline treatment started at 21 days after sowing, when plants were at the stage of vegetative development. To avoid osmotic shock, NaCl was progressively added every 2 day at concentration of 20 mmol L^{-1} until stabilizing at the highest concentration at 28 days after sowing.

2.4. Leaf sampling and evaluation

At the end of the experiment (48 days after irrigation with salt water), leaflets of completely expanded leaves were collected from the middle third of the plants to determine their water potential, osmotic potential, relative water content, and develop pressure-volume curves and determine the osmotic potential at full turgor. The same leaf was used to determine relative water content, water and osmotic potentials, and other two leaflets of the same leaves to construct pressure-volume curves and determine osmotic potential at full turgor by sap osmolality.

2.5. Relative water content and water, osmotic, and pressure potentials

The leaf water potential (Ψ_w) at predawn was determined using a Scholander pressure chamber (1515D, PMS Instrument Company, Albany, USA).

The total osmolality of the leaf tissue used to determine the osmotic potential (Ψ_o) was determined using sap samples of these leaves obtained through leaf maceration in liquid nitrogen. The sap samples were placed in Eppendorf tubes and centrifuged at 10,000 g for 15 min at 4 °C. A 10 μL aliquot of the supernatant was used to determine the total osmolality of the leaf tissue using a vapor pressure osmometer (Vapro 5600, Wescor, Logan, USA).

The Ψ_o was estimated using the Van't Hoff equation (Callister et al., 2006). The difference between the water potential and the osmotic potential was used to determine the pressure potential (Ψ_p), according to Equation (01):

$$\Psi_p = \Psi_w - \Psi_o \qquad 01$$

Seven leaf discs with diameter of 1 cm were used to determine the relative water content (RWC), using the fresh (FW), turgid (TW), and dry (DW) weights of this plant material, applying Equation (2):

$$RWC\ (\%) = \frac{(FW - DW)}{(TW - DW)} \times 100 \qquad 02$$

2.6. Osmotic potential of the apoplastic water, and apoplastic water fraction

Mild centrifugation was used to collect the apoplastic fluid of the leaves according to O'Leary et al. (2014) by the infiltration-centrifugation technique:

Leaves were detached from the plant with petiole using a shaving blade. The recently collected leaf was washed, immersed in distilled water to remove contaminants from the surface and carefully dried with paper towel. After this procedure, the leaf was placed in a 250-mL vacuum flask containing water, so that the liquid completely covered the leaf. Cycles of vacuum application and release (slowly to avoid damage to the tissue) were carried out until full water infiltration. After this procedure, the leaf was removed from the flask and carefully dried with paper towel.

The leaf was wrapped around a 5-mL pipette, covered with Parafilm and inserted in a 20-mL syringe. This syringe was inserted in a 50-mL plastic tube. Centrifugation was carried out for 10 min at 1000 g, at 4 °C (visualization of darkened areas indicated that additional centrifugation time was required to remove all the water infiltrated).

The apoplastic water collected by centrifugation was pipetted into Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4 °C. Then, the osmolality of the supernatant was measured using an osmometer. The osmotic potential of apoplastic water (${\Psi_o}^{AW}$) was estimated using the Van't Hoff equation.

The apoplastic water fraction was calculated using Equation (3) (Callister et al., 2006):

$$AWF = RWC\left(1 - \frac{\Psi_{o100}}{\Psi o^{CV}{}_{100}}\right) \qquad 03$$

wherein AWF is the apoplastic water fraction, RWC is the relative water content at full turgor, Ψ_{o100} is the osmotic potential at full turgor obtained through osmometry, and $\Psi^{CV}_{o\ 100}$ is the osmotic potential at full turgor obtained through P–V curves.

2.7. Osmotic potential at full turgor, and total osmotic adjustment

2.7.1. Pressure-volume curve development

The plant material collected to develop the pressure-volume curves were subjected to procedures of submersion in water—to avoid embolism, and loss of water by transpiration—and siphoning, maintaining the petiole submerged for overnight for saturation of the plant tissue, and then taken to the pressure chamber. The applied pressure was gradually increased until reaching the water potential of the tissue (sap exudation). This procedure was repeated and the successive sap exudations caused variation of the tissue water content and RWC, and a gradually more negative water potential of the leaf tissue. The weight of the exuded sap was measured and recorded according to the corresponding applied pressure to each potential equilibrium in the different RWC of the tissue. The curves were developed by plotting the inverse values of the water potential ($1/{-\Psi_w}$) as a function of the water deficit (1-RWC) of each time the exudation occurred. The adopted methodology followed the protocol used by Fanjul and Barradas (1987), and adaptations made by Lins et al. (2018).

2.7.2. Osmotic potential at full turgor obtained though P–V curves

The adjustment routine of these curves followed the model developed by Schulte and Hinckley (1985). The equation developed was used to determine the osmotic potential at full turgor considering the extrapolation of the line generated by the axes. This point represented the osmotic potential at full turgor obtained through the P–V curve (Ψ_{o100}^{CV}).

2.7.3. Osmotic potential at full turgor obtained through osmometry

Plant material was collected as described in item 2.4, packed in expanded polystyrene boxes, and sent to the laboratory where they were put into zipped plastic bags filled with water for saturation at 4 °C in the dark for overnight. Subsequently, the leaf osmotic potential was determined as described in item 2.5. The leaves were completely turgid after saturation, representing the full turgor to calculate the osmotic potential through osmometry (Ψ_{o100}).

2.7.4. Total osmotic adjustment

The difference between the osmotic potential at full turgor of control plants (Ψo_c^{100}) and the osmotic potential at full turgor of plants subjected to increasing salt stress levels (Ψo_s^{100}) was used to determine the total osmotic adjustment (OA_t), (Blum, 1989). This procedure was performed using the osmotic potential at full turgor data obtained through P–V curves, and osmometry.

2.8. Experimental design and treatments

The experiment was conducted in a randomized block design, with treatments consisting of six salt concentrations in the irrigation water (0, 20, 40, 60, 80, and 100 mmol L^{-1} of NaCl), with five replications, totaling 30 experimental units. The data was subjected to the Kolgomorov-Smirnov normality test and then subjected to ANOVA; the means were compared by the Tukey's test at 5% probability and fitted to regression equations.

Table 3
Relative water content (RWC) and pressure potential (Ψp) of leaves of cowpea (*Vigna unguiculata*) plants irrigated with increasing levels of NaCl, evaluated at 48 days after sowing. Pernambuco, Brazil.

NaCl (mmol L^{-1})	r	Ψp	RWC (%)
0	5	0.46 ± 0.14a	92.5 ± 1.00 a
20	5	0.37 ± 0.07a	91.3 ± 3.00a
40	5	0.48 ± 0.08 a	91.4 ± 2.00 a
60	5	0.37 ± 0.08 a	89.9 ± 3.00 a
80	5	0.38 ± 0.26a	82.6 ± 4.00 b
100	5	0.44 ± 0.17a	81.9 ± 4.00 b

Means followed by the same letter in the columns did not differ statistically ($p < 0.05$) by the Tukey's test at 5% probability.

3. Results

3.1. Relative water content and water, osmotic, and pressure potentials

Stress caused by increasing salt concentrations in irrigation water reduced relative water content (RWC) from the NaCl concentration of 80 mmol L^{-1}. The maximum RWC found was 92.5% in plants control's plant treatment (0 mmol L^{-1} of NaCl), and the minimum was 81.9% in plants subjected to the treatment with NaCl concentration of 100 mmol L^{-1} (Table 3). However, using high concentrations of NaCl in irrigation waters did not cause significant changes in leaf pressure potential (Ψ_p) (Table 3).

Water potential (Ψw) and osmotic potential (Ψo) of leaves had linear reductions with increasing NaCl concentrations in the soil solution (Fig. 2). The maximum water potential was −0.29 MPa, and the minimum was −1.3 MPa; and the osmotic potential ranged from −0.74 MPa to −1.70 MPa (Fig. 2) the highest values of Ψw and Ψo were observed in control and the most negative numbers in both analyses occurred under the condition of 100 mmol L^{-1}.

3.2. Osmotic potential of the apoplastic water

The osmotic potential of the apoplastic water (Ψo^{AW}) increased with increasing salinity levels (Fig. 3). The Ψo^{AW} found did not reached 0.1 MPa, therefore, it contributed little for the osmotic potential of the plants.

3.3. Apoplastic water fraction

Apoplastic water fractions (AWF) found were statistically similar in all NaCl concentrations, with mean value of 30.21% (Fig. 4).

3.4. Osmotic potential at full turgor and total osmotic adjustment

Increasing NaCl concentrations reduced significantly the osmotic potential at full turgor of the plants evaluated, regardless of the evaluation method—osmometry (Ψ_{o100}), and pressure-volume (P–V) curves (Ψ_{o100}^{PV}). The highest Ψ_{o100} and Ψ_{o100}^{PV} found were −0.53 MPa, and −0.72 MPa respectively, in control plants, and the lowest were −1.0 MPa, and −1.44 MPa, respectively, in plants of the treatment with NaCl concentration of 100 mmol L^{-1} (Table 4).

Ψ_{o100} was 44% higher than Ψ_{o100}^{CV} in all NaCl concentration evaluated (Table 4).

Cowpea plants accomplished osmotic adjustment with increasing salt concentration. The osmotic adjustment observed in the P–V curve's method (OA_t^{PV}) was 39% higher than those observed in osmometry (OA_t).

4. Discussion

Some species are able to perform osmotic adjustment (Blum, 2017) as the water availability in the soil is reduced (both through the matric effect of the reduction in soil water content and through the osmotic effect of accumulation of salts).

The adjustment can be made through the synthesis and accumulation of organic solutes in the cytoplasm, and/or by the influx of inorganic solutes into the vacuole (Goufo et al., 2017). This mechanism results in the reduction of Ψw, allowing a gradient of water potential favorable to water absorption and cell turgor maintenance (Truner Neil, 2017).

It is possible that Na^+ accumulation in cowpea leaves had a complementary role as an inorganic solute used in the osmotic adjustment performed by the species, since no more intense toxicity symptoms were identified with the increase of NaCl concentration in irrigation water. This response is similar to that observed by Mejri et al. (2016) in wild barley species subjected to drought.

The ability to use Na^+ in addition to K^+ in the osmotic adjustment promotes an advantage for the species, which may explain its tolerance to conditions of lower water availability, such as those found in environments similar to the site used to collect the soil of the present study, affected by Na^+ accumulation, but in which local farmers manage to maintain a tradition of cowpea cultivation.

Reduction of osmotic potential at full turgor (Goufo et al., 2017) and performance of osmotic adjustment (Iwuagwu et al., 2017) have been reported in some cowpea cultivars, which contests some studies that completely refuted the capacity of this species to perform osmotic adjustment (Shackel and Hall, 1983).

The indication of interspecific gene variability in cowpea may contribute for some cultivars to accumulate solutes in their tissue, adjusting their capacity to absorb water even under conditions of lower

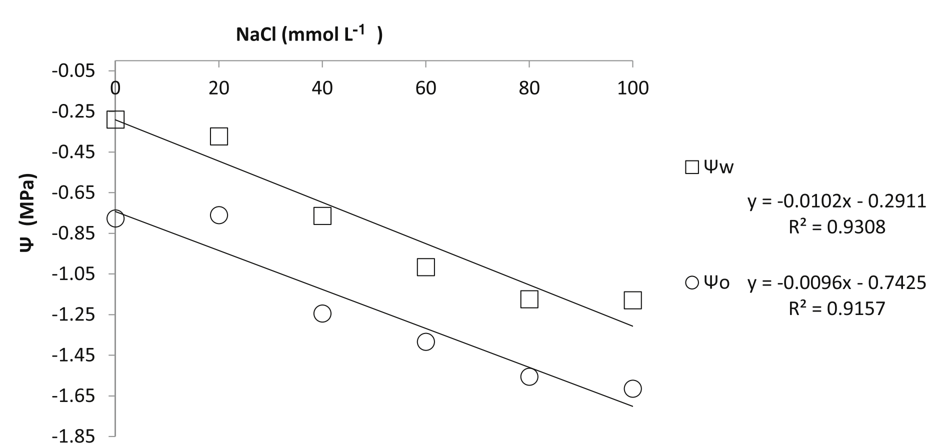

Fig. 2. Water potential (Ψw) and osmotic potential (Ψo) of leaves of cowpea (*Vigna unguiculata*) plants irrigated with increasing levels of NaCl. Pernambuco, Brazil.

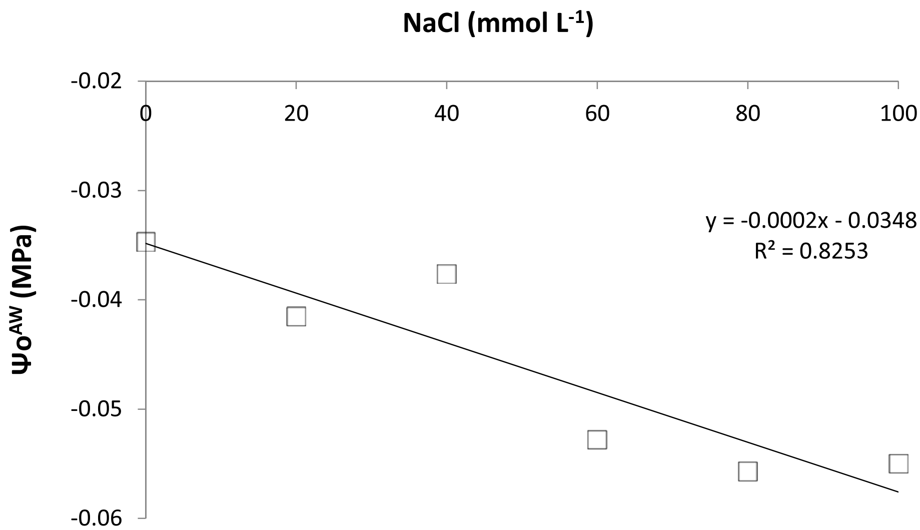

Fig. 3. Osmotic potential of the apoplastic water (Ψo^{AW}) collected by mild centrifugation from turgid leaves of cowpea (*Vigna unguiculata*) plants irrigated with increasing levels of NaCl. Pernambuco, Brazil.

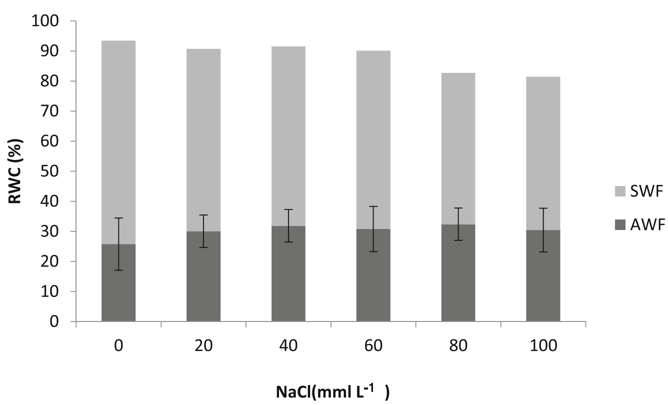

Fig. 4. Contribution of the symplastic water (SWF) and apoplastic water (AWF) fractions to the relative water content (RWC) of leaves of cowpea (*Vigna unguiculata*) plants, irrigated with increasing levels of NaCl. Pernambuco, Brazil.

availability, while others cannot (Iwuagwu et al., 2017).

In the present study, the cultivar IPA 206 showed reduction of osmotic potential at full turgor with increased salt stress, resulting in osmotic adjustment according to both methods evaluated (P–V curve or conventional method).

The differences found between the methodologies higher value of OA_t^{PV} compared to OA_t and lower value of Ψ_{o100}^{PV} compared to Ψ_{o100} (Table 4) prove that there is a methodological origin that goes beyond the genetic variability among cowpea cultivars to generate uncertainties regarding this species performing or not osmotic adjustment, because the species used was the same for the values obtained by both P–V curves and conventional method (considering sap osmolality).

In a situation analogous to that observed in here, in two different studies with the same cultivar (California blackeye), Augé et al. (1992) and Augé et al. (2001) drew different conclusions on the osmotic adjustment performed by cowpea. Augé et al. (1992) concluded that cowpea performed an osmotic adjustment of 0.19 MPa with osmotic potential data obtained by P–V curves, whereas Augé et al. (2001) understood that the value of 0.04 Mpa, obtained by sap osmolality and subsequently converted to osmotic potential by the Van't Hoff equation, was very low and considered that the species did not perform osmotic adjustment.

The processes for determining sap osmolality in osmometer involve destruction of the plant tissue by maceration or pressurization; thus, contents of symplastic and apoplastic waters are not considered separately (Callister et al., 2006; Arndt et al., 2015) as osmotic potential of a plant is in its symplastic solution the apoplastic solution may be an error cause.

A recent study reports that the error caused by the apoplastic fraction may be an underestimation of osmotic potential, especially in halophyte species (Lins et al., 2018); however, in general, solute content in apoplastic solution is lower than in symplastic solution (Arndt et al., 2015), as found for cowpea plants. Obtaining sap by maceration may result in dilution of symplastic solution by the apoplastic solution, generating an overestimation of osmotic potential (Wenkert, 1980; Wardlaw, 2005).

Cowpea plants had relatively high AWF (Fig. 2). Considering that the dilution effect is greater with a greater content of apoplastic water (Arndt et al., 2015), the apoplastic space was likely filled with distilled water in leaf saturation process for the osmotic adjustment methodology (Blum, 1989).

Table 4
Osmotic potential at full turgor and total osmotic adjustment of leaves of cowpea plants evaluated through the osmometry (Ψ_{o100}, OA_t), and pressure-volume curve (Ψ_{o100}^{PV}, OA_t^{PV}) methods. Pernambuco, Brazil.

NaCl (mmol L^{-1})	Ψ_{o100} (MPa)	Ψ_{o100}^{PV} (MPa)	OA_t (MPa)	OA_t^{PV} (MPa)
0	−0.53 ± 0.065 a A	−0.72 ± 0.133 a B	–	–
20	−0.68 ± 0.084 b A	−0.97 ± 0.086 abB	0.15 ± 0.04 B	0.25 ± 0.21 A
40	−0.80 ± 0.015 bcA	−1.17 ± 0.076 bcB	0.27 ± 0.105 B	0.46 ± 0.19 A
60	−0.83 ± 0.036 c A	−1.21 ± 0.101 bcB	0.30 ± 0.03 B	0.49 ± 0.23 A
80	−0.93 ± 0.042 cdA	−1.38 ± 0.061 c B	0.41 ± 0.08 B	0.67 ± 0.18 A
100	−1.00 ± 0.061 d A	−1.44 ± 0.131c B	0.47 ± 0.05 B	0.72 ± 0.26 A

Means followed by the same uppercase letter in the rows, and lowercase letter in the columns do not differ statistically by the Tukey' test at 5% probability. Uppercase letters compare osmotic potential and osmotic adjustment independently.

Ψ_{o100} was overestimated, as shown by the comparison with Ψ_{o100}^{PV} (Table 4). The P–V curves provide more realistic data for determination of osmotic potential because this methodology is not based on concentration of solutes in sap but on the equilibrium pressure that is applied by the pressure chamber in an equivalent leaf water potential at any given water content in plant tissue.

Therefore, differences in osmotic adjustment may be dependent not only on specificities of cowpea cultivars, but also due to the methodology used to access osmotic adjustment data.

5. Conclusions

Part of the existing controversy regarding the osmotic adjustment in cowpea is related to the diluting effect exerted by the apoplastic water fraction in methodologies that involve maceration or pressurization of the plant tissue to obtain the sap. Our results demonstrated that the P–V curve methodology is more suitable for studies on osmotic adjustment in this crop because this methodology is not affected by the dilution caused by the apoplastic fraction in the solutes concentration in the sap.

Although the results are more realistic, pressure-volume curves are not yet such an obvious choice. Many studies (if not most of them) still use sap osmometry for subsequent determination of osmotic potential by the Van't Hoff equation, because this method is fast. Pressure-volume curves are more difficult to be constructed, requiring a long time and a large number of operators, hence not being adequate for many samples.

Although it is not the scope of this study to validate the method suggested by Arndt et al. (2015), of generating a correction factor in a smaller number of samples based on pressure-volume curves and using it to correct a greater amount of data obtained by osmometer, this seems to be a good alternative.

This methodological aspect (diluting effect of the apoplastic water fraction) indeed has misled some researchers regarding the adaptive response of osmotic adjustment in cowpea under conditions of lower water availability. We reiterate the importance of evaluating whether the same error may be occurring in considerations about osmotic adjustment in other species, especially those whose fraction of apoplastic water is as high as that of cowpea.

Author contribution

Pablo R. M. D and Edivan R. de Souza planned and designed the research. Cíntia M. T. Lins, Hidelblandi F. Melo; Martha K. S. S. Paulino, Lucas C. Leal, Hugo R. B. Santos and Danilo R. Monteiro performed experiments, conducted fieldwork, analyzed data. Pablo R. M. D and Edivan R. de Souza wrote the manuscript.

Declarations of competing interest

None.

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References

Agbicodo, E.M., Fatokun, C.A., Muranaka, S., Visser, R.G.F., 2009. Breeding drought tolerant cowpea: constraints, accomplishments, and future prospects. Euphytica 167 (3), 353–370. https://doi.org/10.1007/s10681-009-9893-8.

Arndt, S.K., Irawan, A., Sanders, G.J., 2015. Apoplastic water fraction and rehydration techniques introduce significant errors in measurements of relative water content and osmotic potential in plant leaves. Physiol. Plant. 155 (4), 355–368. https://doi.org/10.1111/ppl.12380.

Augé, R.M., Stodola, A.J.W., Brown, M.S., Bethlenfalvay, G.J., 1992. Stomatal response of mycorrhizal cowpea and soybean to short-term osmotic stress. New Phytol. 120 (1), 117–125. https://doi.org/10.1111/j.1469-8137.1992.tb01064.x.

Augé, R.M., Kubikova, E., Moore, J.L., 2001. Jennifer L. Foliar dehydration tolerance of mycorrhizal cowpea, soybean and bush bean. New Phytol. 51 (2), 535–541. https://doi.org/10.1046/j.0028-646x.2001.00187.x.

Blake, G.R., Hartge, K.H., 1986. Bulk density. In: Klute, A. (Ed.), Methods of Soil Analysis: Physical and Mineralogical Methods. American Society of Agronomy, Madison (WI), pp. 363–376.

Blum, A., 1989. Osmotic adjustment and growth of barley genotypes under drought stress. Crop Sci. 29, 230–233. https://doi.org/10.2135/cropsci1989.0011183X002900010052x.

Blum, A., 2017. Osmotic adjustment is a prime drought stress adaptive engine in support of plant production. Plant Cell Environ. 40 (1), 4–10. https://doi.org/10.1111/pce.12800.

Callister, A.N., Arndt, S.K., Adams, M.A., 2006. Comparison of four methods for measuring osmotic potential of tree leaves. Physiol. Plant. 127 (3), 383–392. https://doi.org/10.1111/j.1399-3054.2006.00652.x.

Fanjul, L., Barradas, V.L., 1987. Diurnal and seasonal variation in the water relations of same deciduous and evergreen trees in a deciduous dry forest of the western Mexico. J. Appl. Ecol. 24, 289–303. https://doi.org/10.2307/2403805.

Gee, G.W., Bauder, J.W., 1986. Particle-size analysis. In: Klute, A. (Ed.), Methods of Soil Analysis. Part 1. Physical and Mineralogical Methods, second ed. American Society of Agronomy, Soil Science Society of America, Madison (WI).

Goufo, P., Moutinho-Pereira, J.M., Jorge, T.F., Correia, C.M., Oliveira, M.R., Rosa, E.A., António, C., Trindade, H., 2017. Cowpea (*Vigna unguiculata L. Walp.*) metabolomics: osmoprotection as a physiological strategy for drought stress resistance and improved yield. Front. Plant Sci. 8, 586. https://doi.org/10.3389/fpls.2017.00586.

Hessini, K., Issaoui, K., Ferchichi, S., Saif, T., Abdelly, C., Siddique, K.H.M., Cruz, C., 2019. Interactive effects of salinity and nitrogen forms on plant growth, photosynthesis and osmotic adjustment in maize. Plant Physiol. Biochem. 139, 171–179. https://doi.org/10.1016/j.plaphy.2019.03.005.

Iwuagwu, M.O., Ogbonnaya, C.I., Onyike, N.B., 2017. Physiological response of cowpea [*Vigna unguiculata*(L.) WALP.] to drought: the osmotic adjustment resistance strategy. Acad. J. Sci. 7 (2), 329–344 ISSN: 2165-6282.

Lins, C.M.T., de Souza, E.R., de Melo, H.F., Paulino, M.K.S.S., Magalhães, P.R.D., de Carvalho Leal, L.Y., Santos, H.R.B., 2018. Pressure-volume (PV) curves in Atriplex nummularia Lindl. for evaluation of osmotic adjustment and water status under saline conditions. Plant Physiol. Biochem. 124, 155–159. https://doi.org/10.1016/j.plaphy.2018.01.014.

Mejri, M., Siddique, K.H., Saif, T., Abdelly, C., Hessini, K., 2016. Comparative effect of drought duration on growth, photosynthesis, water relations, and solute accumulation in wild and cultivated barley species. J. Plant Nutr. Soil Sci. 179 (3), 327–335. https://doi.org/10.1002/jpln.201500547.

Melo, H.F., Souza, E.R., Almeida, B.G., Mulas, M., 2018. Water potential in soil and Atriplex nummularia (phytoremediator halophyte) under drought and salt stresses. Int. J. Phytoremediation 20 (3), 249–255.

O'Leary, B.M., Rico, A., McCraw, S., Fones, H.N., Preston, G.M., 2014. The infiltration-centrifugation technique for extraction of apoplastic fluid from plant leaves using *Phaseolus vulgaris*as an example. J. Vis. Exp. 94, 1–8. https://doi.org/10.3791/52113.

Richards, L.A., 1954. Diagnosis and Improvement of Saline and Alkali Soils. United States Salinity Laboratory, Washington (DC) (USDA: Agriculture Handbook, 60).

Santos, H.G., dos Jacomine, P.K.T., Anjos, L.H.C., dos Oliveira, V.A., de Oliveira, J.B., de Coelho, M.R., Lumbreras, J.F., Cunha, T.J.F., 2013. Sistema brasileiro de classificação de solos. Embrapa, Rio de Janeiro, BR.

Schulte, P.J., Hinckley, T.M., 1985. A comparison of pressure-volume curve data analysis techniques. J. Exp. Bot. 10, 1590–1602. https://doi.org/10.1093/jxb/36.10.159.

Shackel, K.A., Hall, A.E., 1983. Comparison of water relations and osmotic adjustment in sorghum and cowpea under field conditions. Funct. Plant Biol. 10 (5), 423–435. https://doi.org/10.1071/PP9830423.

Thomas, G.W., 1982. Exchangeable cations. In: Page, A.L. (Ed.), Methods of Soil Analysis. Part-2 Chemical Methods. American Society of Agronomy, Madison (WI), pp. 159–165.

Truner Neil, C., 2017. Turgor maintenance by osmotic adjustment, an adaptive mechanism for coping with plant water deficits. Plant Cell Environ. 40 (1), 1–3. https://doi.org/10.1111/pce.12839.

Vomocil, J.A., 1965. Porosity. In: Blake, C.A. (Ed.), Methods of Soil Analysis. American Society of Agronomy, Madison (WI), pp. 299–314.

Wardlaw, I.F., 2005. Consideration of apoplastic water in plant organs: a reminder. Funct. Plant Biol. 32 (6), 561–569. https://doi.org/10.1071/FP04127.

Wenkert, W., 1980. Measurement of tissue osmotic pressure. Plant Physiol. 65 (4), 614–617. https://doi.org/10.1104/pp.65.4.614.

Zeng, Y., Li, L., Yang, R., Yi, X., Zhang, B., 2015. Contribution and distribution of inorganic ions and organic compounds to the osmotic adjustment in Halostachys caspica response to salt stress. Sci. Rep. 5, 13639. https://doi.org/10.1038/srep13639.

Zhou, Q., Yu, B.J., 2009. Accumulation of inorganic and organic osmolytes and their role in osmotic adjustment in NaCl-stressed vetiver grass seedlings. Russ. J. Plant Physiol. 56 (5), 678–685. https://doi.org/10.1134/S1021443709050148.