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Quantifying water relations and evapotranspiration in pomegranate (*Punica granatum* L. var. *wonderful*) in response to salt stress

Parashuram Bhantana[1]* and Naftali Lazarovitch[2]

[1]Nepal Agricultural Research Council (NARC), National Soil Science Research center (NSSRC), Khumaltar, Lalitpur, Nepal.
[2]The French Associates Institute for Agriculture and Biotechnology of Drylands, The Ben Gurion University of the Negev, Midreshet Ben Gurion, Sdeboqer, Israel.
*Corresponding author's email: hailstormsea@gmail.com

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ABSTRACT
Water availability and salinity are major abiotic factors limiting plant growth and productivity worldwide. So, development of a tool that can estimate plant water demand is of prime importance. In the current study the effects of salt stress on water relations and plant stress were measured and different models were tested for evapotranspiration (ET) estimation. The measurements included (ET), leaf water potential (LWP), electrolytic leakage (ECL), stomatal conductance (g_s) and stomatal density (StoD) in lysimeter grown young pomegranate trees (*Punica granatum* L.) under salt stress. Trees were subjected to different irrigation treatments with electrical conductivities (EC_{iw}) of 0.8, 2.8, 5.0 and 8.5 dS m^{-1} for two years (2008-2009). Estimation of ET was made based on the measured value of ET, LWP, ECL, g_s and StoD. A significant reduction of ET, LWP, and g_s were recorded with increasing EC_{iw} from 0.8 to 8.5 dS m^{-1}. In contrary, a significant increment in ECL and StoD was observed at higher saline treatment. Among four parameters studied, StoD and ECL correlated best with ET (R^2 = 0.79 and 0.75). When a set of three factors was generated, the ET equation (ET = 14.77 – 0.015×StoD – 0.97× ECL + 0.005 × g_s) described about 93% (R^2 = 0.93) of the ET values and the model was significant at ($F_{(3, 16)}$ = 72.66, p<0.00001). The set of 3 factor model was stronger than the one to one factor correlation study. Since the measurement of ECL is relatively simple, an ECL generated equation for ET estimation is recommended for a practitioner. Moreover, a multiple factor model remains as an important tool for an agro ecosystem modeling and long term ecological planning.

Keywords: Pomegranate, evapotranspiration, salt stress, leaf water potential, electrolytic leakage, stomatal conductance and stomatal density

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INTRODUCTION

Water availability and salinity are major abiotic factors limiting plant growth and productivity. Plants that can make efficient use of water and maintain sufficient yields under salt stress will have great importance worldwide both environmentally and economically.

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits, among the seven kinds mentioned in the Bible (Blumenfeld et al 2000). Pomegrante is native to Iran and grown extensively in arid and semi-arid regions worldwide (Sarkhosh et al 2006). Pomegranate is one among the drought hardy crops (Kumar 1998) and can be grown successfully under salt stress (Bhantana and Lazarovitch 2010).

Sustainable crop production under stress environments requires efficient water management. An effective water management fetches broad consideration among soil-plant-atmosphere continuum. Numerous methods are being used for the water management issue since decades. Estimation of evapotranspiration (ET) is one among the various methods used. The measurement of ET is helpful to determine how much to irrigate. Mostly, the ET is being measured by weighing and non-weighing lysimeters, field experimental plots, soil mositure depletion studies, water balance methods (Michael 2006). Also, ET can be estimated by multiplying evaporation (E_o) and crop coefficient (K_C). Others approaches i.e. Blaney-Criddle method, Thornthwaite method, Penman method and Christiansen method are being used in estimating the ET by using climatological data and some other microclimatological methods are documented too. All of the methods in estimating ET have their own limitations. For example both weighing and non-weighing lysimeter study is very expensive and almost unaffordable by a farmer. And it is very hard to grow a tree crop in a lysimeter which can limit the root zone depth and can affect root to shoot ratio. Also the use of K_c approach in estimating ET requires prior speculation of the E_o data. Another limitation in this approach is lack of K_c data available for all varieties and during all stages of plant growth, as the K_c is affected by crop type, phenology and weather data (Allen et al 1998). Similarly, the use of mathematical formulae and equations in estimating ET by using climatological data is inaccessible to the general farmer. Thus alternative ways for estimating ET can be achievable by determining the plant physiological and morphological status grown under various soil water and stress environments.

Leaf water potential (LWP) is being used commonly for the indication of plant physiological status under stressed environments (Katerji et al 2003). LWP controls ET and root water uptake by the potential difference between the leaf and soil water (Campbell 1985). Both matric and osmotic potentials influenced reduction of root water uptake under water and salt stress environments respectively (Hopmans and Bristow 2002). Such a reduction in matric as well as osmotic potentials induces stomatal closure which prevents the transport of water vapor and decreases ET. A reduction in LWP, osmotic potential and turgor potential was observed in tomato (*Lycopersicon esculentum* L.) at increasing EC_{iw} levels (Maggio et al 2007). Likewise reduction of LWP was observed in olive at increasing salt concentrations (Ahmed et al 2008). The same authors showed stronger correlation between photosynthesis and LWP (R^2 = 0.63) than photosynthesis and Na^+ content in salt stressed plants (R^2 = 0.38). This showed LWP imparted stronger effect in photosynthesis than Na^+ content under salt stressed conditions. Moreover, a significant correlation of mid day photosynthesis and mid day stomatal conductance (g_s) with LWP was recorded in grapevine grown under deficit irrigation (de Souza et al 2005). Similarly, ET could be highly affected by LWP. Also, an increase in relative transpiration was observed with increasing LWP under salt stressed conditions in spring wheat (*Triticum aestivum* L. cv Haruyutaka) (Nishida et al 2009). And the same group of authors derived an empirical relationship of changes in transpiration ratio as a function of LWP. This is a quiet innovative idea and rarely documented. Thus derivation of such an empirical relationship of changes in ET can be done not only with the LWP, but also with the others ways of indicating plant physiological status.

Plant membranes are among the first line of defense against various environmental damages. Changes in membranes may constitute an initiation of plant response to stressful environments (Heckman et al 2002; Marcum et al 1998). An increase in membrane permeability up to 85% was observed in strawberry grown under 40 mM NaCl than without salt stress. But such damages to the membrane can be alleviated by foliar nutrient application of potassium, calcium and magnesium nitrate (Yildirim et al 2009). Thus measurement of the percentages of the membrane damage (MD) is taken into account for this study. Numerous methods are being used for the measurement of the MD of a plant grown under stress environments. Here an electrolytic leakage (ECL), a simple tool for assessing the effect of environmental stresses on plant membrane is used. As other method, ECL method has its own limitations (Whitlow et al 1992). The two limitations are it does not measure directly membrane stability and it does not take into account the effects of differences in anatomy between different species, which in turn can affect g_s. Still, the use of ECL as an indicator for assessing the effect of environmental stresses on membranes remains a strong and an important tool, especially when comparing samples from the same species (Rachmilevitch et al 2006).

Stomatal conductance (g_s) is a numerical expression of the rate of passage of water vapor or carbon dioxide through the stomata or small pores in the plant. Here an attempt has been made to measure the rate of passage of water vapor in terms of mmol of H_20 m^{-2} s^{-1}. Stomata consist of a pair of guard cells and the pores they enclosed, located in the plant epidermis. Regulation of stomatal opening is due to turgor changes driven by massive ion fluxes, which occur mainly through the guard cells, plasma membrane and tonoplast. Guard cells have an important role in a plant to regulate stomatal opening under environmental stresses. This is possible because guard cells are mechanistically separated from the surrounding cells and have sensitive reversible reactions to internal and external stimuli. It has been suggested that respond of guard cells for elucidation of environmental perception and ion transport mechanisms can take time scale of milliseconds to millions years (Assmann and Wang 2001). Moreover the stomatal aperture is determined by the capacity of mesophyll tissue to fix carbon and g_s correlated to the photosynthetic capacity of the plant (Wong et al 1979). Thus the measurement of g_s can be a key parameter in many ecological models (Chen et al 1999).

As the measurement of g_s is pretty easier, bunch of articles have already published for the response of plant grown under salt stress environments. A significant decrease in g_s was observed with increasing NaCl concentrations in spinach (*Spinacia oleracia*) (Downton et al 1985), *Chenopodium rubrum* (Warne et al 1990) and tomato (Maggio et al., 2007). Increasing levels of salt in irrigation strongly decreased g_s measured in *Cucumus sativus* leaves (Stepien and Klobus 2006) and also in *Populus alba* (Abbruzzese et al., 2009). A simultaneous decrease in g_s was observed along with decrease in transpiration and water use efficiency in tomato grown under increasing salinity (Romero-Aranda et al 2001) as well as a halophyte *Plantago coronopus* (L.), grown under varying sea water salinity, but not their regression study.

Stomatal characteristics i.e. length, width, area, number, index and distribution are affected by plant growth and environment (Bray and Reid 2002). A wide variation in stomatal frequency and distribution is recorded in increasing use of saline water for irrigation. Increased in the stomatal frequency but decreased in the stomatal size as well as pore size was reported while growing barley under saline soil compared to normal soil (Gill and Dutt 1982). At low to moderate salinities StoD was decreased but then increased again at high salinity in *C. rubrum* (Warne et al 1990). On an observation of the second trifoliate leaf of

Phaseolus vulgaris showed increased StoD on abaxial leaf surface and decreased StoD on adaxial leaf surface under salt treatment versus control, which remains true also for salt plus elevated CO_2 treatment versus elevated CO_2 treatment (Bray and Reid 2002). The same authors showed decreased in stomatal length, width and area under salt and salt plus elevated CO_2 treatment versus without salt and elevated CO_2 treatment. Also an increased in StoD was recorded in var. 2AS11 of *P. alba* tree subjected to salt stress than control along the days after the treatment, but the other two varieties i.e. 14P11 and 6K3 showed the reverse response (Abbruzzese et al 2009). Conversely, a proportional decrease in StoD was observed in two varieties of tomato Daniela and Moneymaker with increasing NaCl in nutrient solution (Romero-Aranda et al 2001). Beside the salt stress many studies have shown that drought stress also has variable effect on StoD. Wild type potato plant which is more tolerant to drought showed higher StoD than transgenic potato lines (Stiller et al 2008). Moreover, significant correlation (R^2 =0.558, $p<0.05$) between g_s and StoD has shown by Galmes et al (2007). Thus a study of StoD on ET could be more meaningful.

Pomegranate is considered to be moderately tolerant to salinity (Allen et al 1998). cuttings from pomegranate var. Malas Shirin showed tolerance to up to 40 mM NaCl in potted cultures of 1:1 sand-perlite medium irrigated with complete Hoagland's solution. However, the performance of other pomegranate varieties, such as Alak Torsh and Malas Torsh, in the presence of salinity showed a decline from 0 mM NaCl, as indicated by number of internodes, length of main stem, length of internodes and leaf surface area (Naeini et al 2006). Moreover, in a similar experiment with the same three pomegranate varieties, over the course of an 80-day experimental period, irrigation with concentrations of 0, 40, 80 and 120 mM NaCl resulted in increased Na, Cl and K concentrations and decreased Ca, Mg and N concentrations in the plant tissues (Naeini et al., 2004). A significant reduction of ET (mm day^{-1}) was observed in two varieties of pomegranate 'Wonderful' and 'SP-2', grown with saline water having an electrical conductivity of 8 dS m^{-1} than 0.8 dS m^{-1} (Bhantanna and Lazarovitch 2010). To date little has been studied about the response of pomegranate to the various physiological stress tolerance mechanisms grown under salt stress. A major objective of this study is to estimate ET based on different plant physiological and morphological stress indicators i.e. (a) Leaf water potential (LWP) (b) Electrolytic leakage (ECL) (c) Stomtal conductance (g_s) and (d) stomatal density (StoD). Thus an attempt has been made here in estimating the ET by using various stress indicators of the pomegranate (*P. granatum* L. var. Wonderful) trees grown under salt stress.

MATERIALS AND METHODS

Tree Establishment

Water relations study was conducted on var. Wonderful of pomegranate (*P. granatum* L.) with increasing levels of salt in the irrigation water. Plants were grown on lysimeters situated at the Jacob Blaustein Institutes for Desert Research, Sede Boqer Campus, the Ben-Gurion University of the Negev, Israel. Twenty 0.2-m^3 lysimeters were filled with Sede Boqer loess soil. One rooted cuttings, prepared from the variety Wonderful were transplanted into each lysimeter in February 2007. The total area surrounding each plant was 3 m × 2.25 m. Water application was automated and drainage water collected manually. Each lysimeter had a highly conductive drain filled with rock wool to control the matric potential at the lysimeter bottom. A detailed description of the lysimeter system is given in Ben-Gal and Shani (2002).

Treatments

The salinity of the irrigation water was brought to electrical conductivity (EC_{iw}) values of 0.8 ± 0.14, 2.8 ± 3.3, 5.0 ± 1.1 and 8.5 ± 1.5 dS m^{-1} by adding NaCl, $CaCl_2$ and fertilizers. The two salts (NaCl and $CaCl_2$) were added proportionally to have an equivalent effect on solution electrical conductivity. Hereafter, these treatments are denoted EC-0.8, EC-2.8, EC-5 and EC-8.5, respectively. Fertilizers (polyfeed water-soluble N:P:K fertilizers 14:7:28 + 1% MgO with micronutrient combination, manufactured by Haifa Chemicals, Israel) were applied with the irrigation water at a constant concentration of 0.5 kg m^{-3}. Calcium in the EC-0.8 treatment water was supplemented by the addition of 0.12 kg m^{-3} $CaNO_3$ with 0.4 kg m^{-3} fertilizer mix. Five-plant received each salinity treatment. Salinity treatments were begun in a 1-year-old orchard in March 2008. Volcanic tuff mulch was applied on the surface of the soil to minimize evaporative losses.

Measurements

Daily water balance, generating ET (mm) data for each lysimeter, was calculated based on:

$$ET = I - Dr + \Delta\theta D \qquad [1]$$

where *I* is the irrigation (mm), *Dr* is the drainage (mm) and $\Delta\theta$ is the change in stored root-zone water (-) to a root-zone depth of D (mm). Plants were irrigated with 120±5% of their back-day measured ET. Irrigation frequency varied from 1 to 5 times per day from the days after bud burst to peak season.

Measurement of LWP, ECL, g_s and StoD were made during the first week of August for both years. g_s was measured by steady state leaf porometer (SC-1, Decagon Devices, Pullman WA, USA). Nail police replica were used to determine the number of stomata under the inverted light microscope at 400× magnification. The stomata were counted by using the Soft Imaging System (Munster, Germany) software package. LWP was measured by a pressure chamber method (ARIMAD-3000, Israel). A finely cut stem was kept in an inverted position inside the pressure chamber and pressure was applied through a cylinder containing nitrogen gas. The amount of pressure requires for the appearance of water on the inverted stem tip was recorded as the LWP of that stem. For accuracy, the stem cut was viewed under 10× magnification with binocular. Measurement of the ECL was done by sampling five leaves from each tree. Leaves were sampled from the top fifth pair of leaves of a branch randomly. After sampling, leaves from each treatment were chopped into small pieces and were incubated in a 50 ml vial containing double distilled water. Electrical conductivity (EC) of the vials containing leaf tissues was recorded after shaking the solution (100 rpm) for 24 hours. Then all the leaf tissues were killed in autoclave 122°C for 90 minutes. Again the EC of the vials containing the dead leaf tissues was recorded after 24-hours of shaking. The ratio of EC before and after the autoclave gave the value of ECL. And ECL times 100 provided the value of membrane damage (MD).

Statistical analysis, correlation and regression study

One-way ANOVAs were used to determine the effect of salinity treatment on LWP, ECL, g_s and StoD by using the STATISTICA software (StatSoft, Inc., Tulsa, OK, USA). The significant differences in the means of all the physiological parameters recorded were separated using post-hoc Tukey HSD test (STATISTICA software). A correlation between LWP, ECL, g_s, StoD against ET was performed by multiple regression model with STATISTICA software. Instead of one to one factor correlation between each plant physiological stress indicators with the ET, a correlation of two factors i.e. LWP and ECL with the ET was performed by using multiple regression model with STATISTICA software in the year 2008 and 2009 respectively. Moreover a correlation of three factors i.e. LWP, ECL and g_s with the ET as well as a correlation of four factors i.e. LWP, ECL, g_s and StoD was performed by using multiple regression model with the STATISTICA software in the year 2009.

RESULTS

Daily ET measured data in the first week of August 2008 and 2009 have shown in Fig. 1. A significant decrease in the daily ET was recorded in the year 2008 ($F_{(3, 16)} = 118.41$, $p<0.00001$) and 2009 ($F_{(3, 15)} = 72.93$, $p<0.00001$) in increasing EC_{iw} levels (Table, 1). Furthermore, each salt treatment was significantly different from one another with respect to daily ET in the year 2008, which remained the same in the year 2009, except 5 and 8.5 dS m^{-1} saline treatment were statistically similar (data not shown). The daily ET changes from 4.64 to 1.14 mm day^{-1} and 6.62 to 1.05 mm day^{-1} during the year 2008 and 2009 respectively with increasing EC_{iw} from 0.8 dS m^{-1} to 8.5 dS m^{-1}. Comparing the result from the year 2008 to 2009, the daily ET demand of the plant increased when irrigated with 0.8 and 2.8 dS m^{-1} but decreased when irrigated with 5 and 8.5 dS m^{-1}.

Table 1. Sum of squares, numerator and denominator degrees of freedom, F-values and P-values computed with STATISTICA software under varying salinity treatments.

Parameters Recorded	Sum of Squares	Numerator Degrees of Freedom	Numerator Degrees of Freedom	F-value	p-value
Evapotranspiration (ET)-2008	37.6	3	16	118.41	<0.00001
Evapotranspiration (ET)-2009	85.85	3	15	72.93	<0.00001
Leaf Water Potential-2008	14.06	3	56	17.74	<0.00001
Leaf Water Potential-2009	14.02	3	37	23.23	<0.00001
Ratio of Electrolytic Leakage-2008	0.096	3	16	19.56	<0.00001
Ratio of Electrolytic Leakage-2009	0.36	3	40	20.45	<0.00001
Stomatal Conductance (g_s)-2009	87043.5	3	39	22.53	<0.00001
Stomatal Density (stomata mm^{-2})-2009	111329	3	39	40.65	<0.00001

Also the data of decreased in LWP by irrigating with increasing EC_{iw} levels has shown in Fig. 2. A significant decrease in the LWP in the year 2008 ($F_{(3, 56)} = 17.74$, $p<0.00001$) and 2009 ($F_{(3, 37)} = 23.23$, $p<0.00001$) was recorded (Table 1). There were not differences in LWP between the year 2008 and 2009 (Fig, 2). However within the year 2008, the first two salt treatment i.e. 0.8 dS m^{-1} and 2.8 dS m^{-1} were similar and differed from the last two salt treatment i.e. 5 and 8.5 dS m^{-1}, whereas the last two treatment were similar too (data not shown). But within the year 2009, the two intermediate salt treatments 2.8 and 5 dS m^{-1} were similar and different from 0.8 and 8.5 dS m^{-1}. Also the first and the last salt treatments were significantly different during the year 2009 (data not shown). The LWP changes from -1.6 to -2.9 Mpa and -1.7 to -3.14 Mpa during the year 2008 and 2009 respectively while increasing EC_{iw} from 0.8 dS m^{-1} to 8.5 dS m^{-1}.

Percentages of the membrane damage due to salt exposure have shown in Fig. 3 in terms of MD as the ratios of ECL before and after the autoclave times 100. A significant decrease of MD in the year 2008 ($F_{(3, 16)} = 19.56$, $p<0.00001$) and 2009 ($F_{(3, 40)} = 20.45$, $p<0.00001$) was recorded (Table 1). In agreement with the daily ET (Fig. 1), damages to the plant membrane was higher in the year 2009 than 2008 for all levels of salinity. Within the year 2008 and

2009 the first two saline treatments i.e. 0.8 and 2.8 dS m^{-1} was differed statistically from the last two saline treatments 5 and 8.5 dS m^{-1} (data not shown).

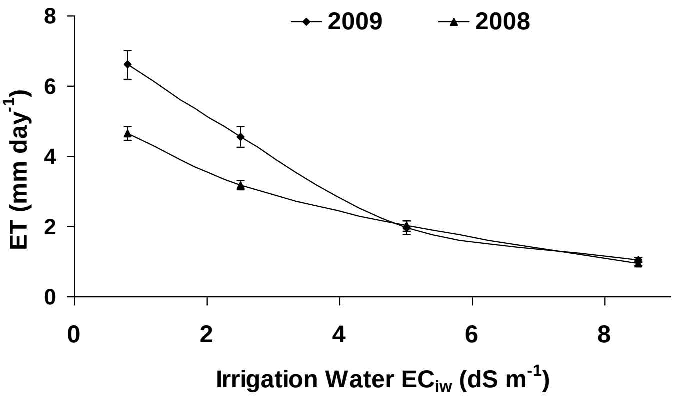

Figure 1. Means ± standard error of evapotranspiration (mm day^{-1}) under various irrigation-water electrical conductivities (EC_{iw}) recorded within the first week of August in 2008 and 2009.

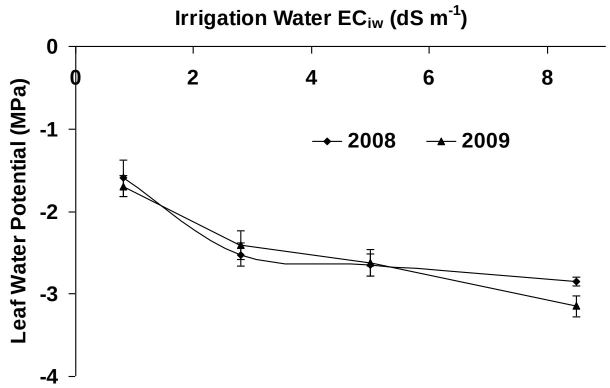

Figure 2. Means ± standard error of leaf water potential (-Mpa) under various irrigation-water electrical conductivities (EC_{iw}) recorded within the first week of August in 2008 and 2009.

In the Fig. 4, diurnal fluctuation of g_s has shown with increasing EC_{iw} levels. Plant grown under 0.8 dS m^{-1} had the highest value of g_s, whereas plant grown at 8.5 dS m^{-1} had the lowest value of g_s. Such difference in g_s with increasing salinity was highly significant ($F_{(3, 39)} = 22.53$, $p<0.00001$) (Table 1). Moreover comparing means among various saline treatments shown that last two treatments i.e. 5 and 8.5 dS m^{-1} were not different from each other but differed from the first two i.e. 0.8 and 2.8 dS m^{-1} saline treatment. Also 0.8 dS m^{-1} salinity treatment was significant from the 2.8 dS m^{-1} salinity treatment (data not shown). During the mid-day the g_s values of 187, 127, 81 and 79 mmol m^{-2} s^{-1} in increasing EC_{iw} levels of 0.8, 2.8, 5 and 8.5 dS m^{-1} respectively were recorded.

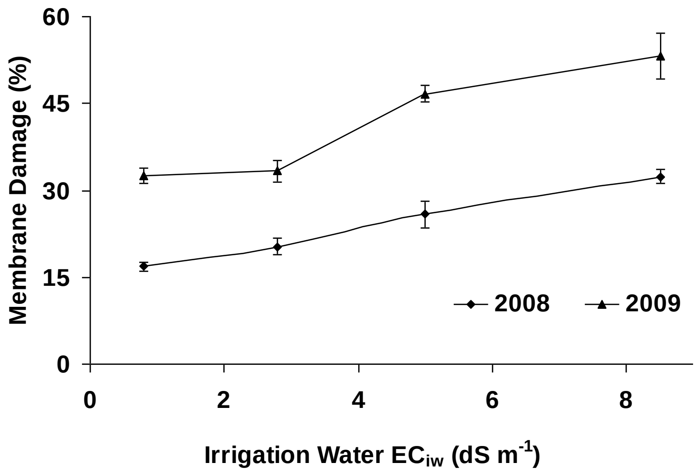

Figure 3. Means ± standard error of percentage of the membrane damage under various irrigation-water electrical conductivities (EC_{iw}) recorded within the first week of August in 2008 and 2009.

Similarly, the result from the measurement of StoD on abaxial leaf surface has shown in Fig. 5. It was observed that StoD increased from 317, 417, 437 and 466 with increasing EC_{iw} levels from 0.8, 2.8, 5 and 8.5 dS m^{-1} respectively. Furthermore, this result is statistically significant ($F_{(3, 39)} = 40.65$, $p<0.00001$) (Table 1). Comparing the means of the various saline treatments showed that 0.8 dS m^{-1} salt treatment was significantly different than the rest and not significant different were observed among 2.8, 5 and 8.5 dS m^{-1} saline treatment (Fig. 5).

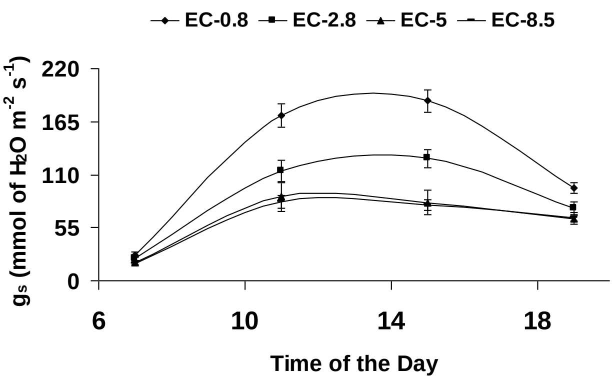

Figure 4. Means ± standard error of stomatal conductance (mmol m^{-2} s^{-1}) measured on abaxial leaf surface under various irrigation-water electrical conductivities (EC_{iw}) observed within the first week of August in 2009.

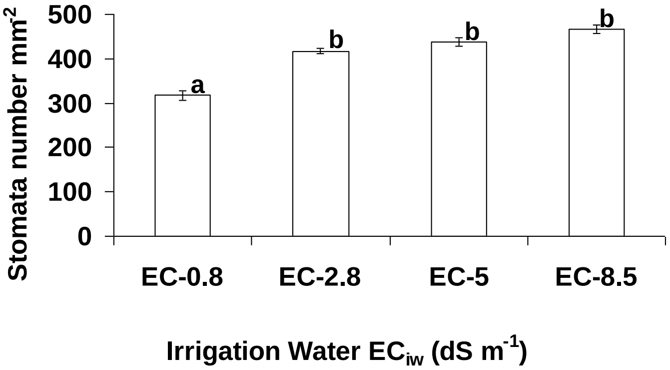

Figure 5. Means ± standard error of stomatal density (number of stomata mm^{-2}) under various irrigation-water electrical conductivities (EC_{iw}) recorded within the first week of August in 2009. Letters in the small cases represents the significant differences between the treatments

A correlation study between ET with StoD, ECL, LWP and g_s recorded within the first week of August 2009 has shown in Fig. 6. In the Fig. 6a an estimation of ET based on the StoD data has shown. About 79% (R^2=0.79, Fig. 6a & Table 2) of the ET value was described by the StoD equation (ET=18.01-0.04 × StoD) and their correlation was highly significant ($F_{(1, 18)}$ = 66.92, p<0.00001) (Table 2). Next to StoD generated equation, an ECL generated equation in 2009 (ET = 16.88 – 19.10 × ECL) described about 75% (R^2=0.75, Fig. 6b & Table 2) of the ET values and the correlation was highly significant ($F_{(1, 18)}$ = 54.73, p<0.00001) (Table 2). But in 2008, an ECL generated equation of (ET =10.22 –14.86 × ECL) described about 70% (R^2=0.69) of the ET values and the correlation was highly significant ($F_{(1, 18)}$ = 39.46, p<0.00001) (Table 2). Moreover a correlation study of ET with LWP and g_s has shown in the Fig. 6c and 6d respectively. LWP generated equation in 2009 (ET = 10.41 + 2.82 × LWP) described about 68% of the ET values and the correlation was highly significant ($F_{(1, 18)}$ = 37.83, p<0.00001) (Fig. 6c and Table 2). But in 2008 a LWP generated equation could describe about 42% of the ET values and the correlation was significant ($F_{(1, 18)}$ = 13.15, p<0.002) (Table 2). Instead g_s generated equation (ET =0.034 × g_s – 0.53) described 66% (R^2=0.66, Fig. 6d & Table 2) of the ET values and were highly significant at ($F_{(1, 18)}$ = 35.46, p<0.00001) (Table 2).

Table 2. Intercept, slope, R^2, F-value and P-value of simple linear regression models of various parameters with ET recorded within the first week of August in the year 2008 and 2009.

Simple Linear Regression with ET (mm day^{-1})	**Ratio of Electrolytic Leakage (ECL)**		**Leaf Water Potential (LWP) (Mpa)**		**Stomatal Conductance (g_s) (mmol H_2O m^{-2} s^{-1})**	**Stomatal Density (stomata mm^{-2})**
	2008	**2009**	**2008**	**2009**		
Intercept	10.22	16.88	5.74	10.42	-0.53	18.01
Slope	-14.86	-19.11	1.36	2.83	0.03	-0.04
R^2	0.69	0.75	0.42	0.68	0.66	0.79
F- value at (1, 18)	39.46	54.73	13.15	37.83	35.46	66.92
P- value	<0.00001	<0.00001	<0.002	<0.00001	<0.00001	<0.00001

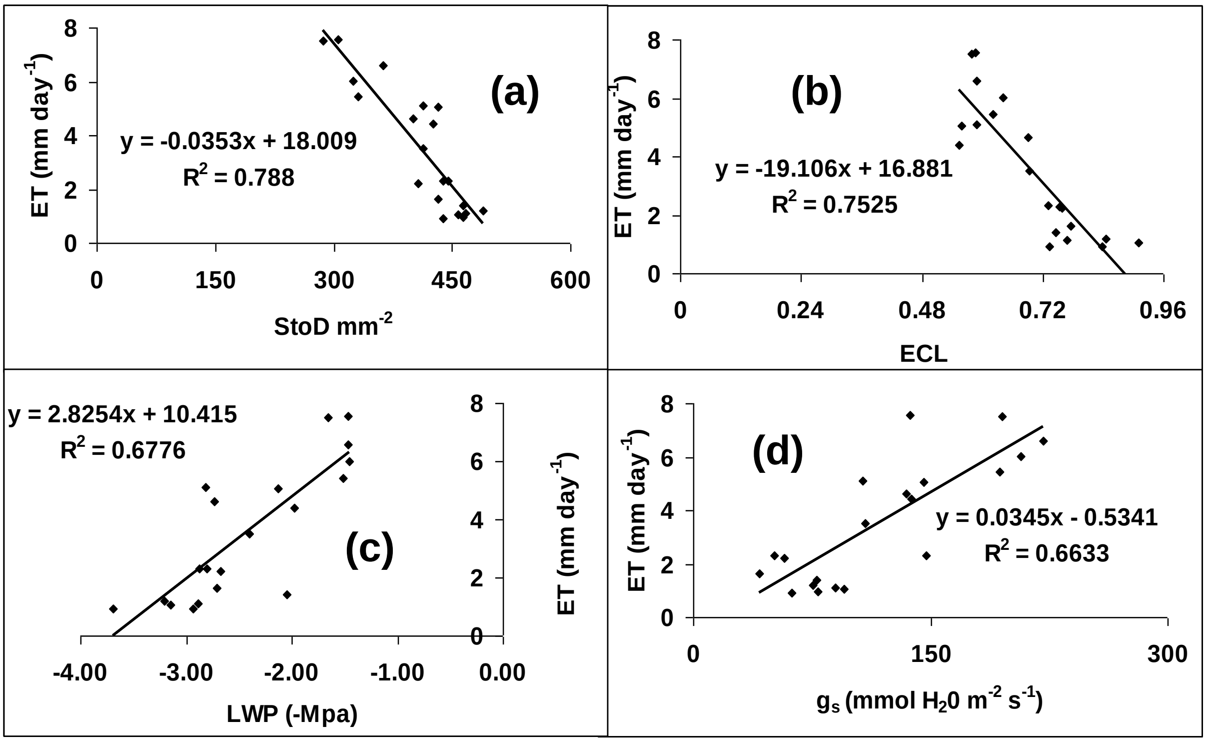

Figure 6. Simple linear regression between ET with (a) StoD (b) ECL (c) LWP and (d) g_s recorded within the first week of August in 2009.

In addition to one to one relationship of ET and different stress indicators, an effect of two or more stress indicators to the ET was studied in the year 2008 and 2009 (data not shown). The combination of two stress indicators LWP and ECL described 66% (R^2 = 0.66) of the ET in the year 2008 and proposed an equation of $ET_{(2008)} = (9.99 + 0.28 \times LWP_{(2008)} - 13.1 \times ECL_{(2008)})$. This function is highly significant ($F_{(2, 17)}$ = 19.43, p<0.00004) (data not shown). Similarly in the year 2009 the combination of two stress indicators LWP and ECL described about 82% (R^2 = 0.815) of the ET and proposed another equation of $ET_{(2009)} = (15.76 + 1.42 \times LWP_{(2009)} - 12.57 \times ECL_{(2009)})$. Also this function is highly significant ($F_{(2, 17)}$ = 42.94, p<0.00001) (data not shown). Moreover in the year 2009 a few multiple regression models were proposed by combining three and four parameters separately. While combining three parameters i.e. g_s, SWP and ECL resulted an equation of $ET_{(2009)} = (7.7 + 0.011 \times g_{s(2009)} + 0.26\times LWP_{(2009)} - 10.1 \times ECL_{(2009)})$ and described about 90% (R^2 = 0.9) and the model was highly significant at ($F_{(3, 16)}$ = 47.8, p<0.00001) (data not shown). Also combining another sets of three factors i.e. StoD, ECL and g_s proposed an equation of $ET_{(2009)} = (14.77 - 0.015\times StoD_{(2009)} - 0.97\times ECL_{(2009)} + 0.005 \times g_{s(2009)})$. This relationship described by 93% (R^2 = 0.93) and the model was highly significant ($F_{(3, 16)}$ = 72.66, p<0.00001) (data not shown). Last but not the least the combination of all four parameters described about 92% (R^2 = 0.919) of the ET and proposed an equation of $ET_{(2009)} = (16.72 + 0.007 \times g_{s(2009)} - 0.002 \times StoD_{(2009)} + 0.11 \times LWP_{(2009)} - 9.25 \times ECL_{(2009)})$. Also this model was highly significant ($F_{(4, 15)}$ = 53.23, p<0.00001) (data not shown).

DISCUSSION

The current study analyzed the daily ET as well as various physiological and morphological water status parameters in pomegranate trees. We found that ET, LWP and, g_s decreased in response to increasing EC_{iw} from 0.8 to 8.5 dS m^{-1}; however, ECL and StoD increased in response to increasing EC_{iw} from 0.8 to 8.5 dS m^{-1}. The decrease observed in ET, LWP and g_s could be due to reduction in osmotic potential (π) of soil solution in response to salinity. Ben-Gal et al 2009, presented a decline in relative yield of corn (*Zea mays* L.), melon (*Cucumis melo* L.), grapevine (*Vitis vinifera* L.) and bean (*Phaseolus vulgaris* l.) in response to decreasing π of the irrigation water. Furthermore, root water uptake is related to a decrease in ET, LWP and g_s. Root properties and growth environment such as root zone salinity root turgor pressure and root density might have effect on root water uptake. Root water uptake is guided by symplastic and apoplastic pathways. Salt built up in the root zone reduces π, which eventually reduces water uptake through symplastic pathways (Hopmans and Bristow 2002). The apoplastic pathways for root water uptake are less affected by salt stress as it is guided by matric potential. A five fold increase in the electrical conductivity of drainage water (EC_{dw}) was recorded in the same experiment with pomegranate trees at 8.5 dS m^{-1} than 0.8 dS m^{-1} during 2008 (Bhantana and Lazarovitch, 2010). Similarly, a 1.5 to 2 fold increase in the EC_{dw} was recorded at EC_{iw} of 9 dS m^{-1} (Ben-Gal et al., 2008) and a significantly higher EC_{dw} was recorded under varying EC_{iw} of 8, 18 and 28 dS m^{-1} versus 2.5 dS m^{-1} in the case of alfalfa (*Medicago sativa* L.) and tall wheat grass [(Agropyron elongatum (Host) P. Beauv] (Skaggs et al 2006). Other than the EC_{dw}, both root turgor pressure and root densities were observed to decline in tomato irrigated with 171 mM NaCl versus non-salinized treatment (Maggio et al., 2004). Not only root growth environment but also leaf area caused pronounced decreased in ET under salt stress. Assouline et al (2006) observed a reduction in leaf area at a salinity of 4.2 dS m^{-1} versus 1.8 dS m^{-1} in bell pepper (*Capsicum annum* L.), and an approximate 60% reduction in leaf area was observed upon irrigation with 171 mM NaCl versus non-salinized treatment in tomato (Maggio et al 2004). Likewise, an approximate 86% reduction in leaf area was recorded in both Serena and Seredo varieties of sorghum at 250 mM versus 0 mM NaCl (Netondo et al 2004). Skaggs et al (2006) concluded that the reduction in canopy cover under high saline treatment causes the decline in ET in alfalfa and tall wheat grass.

In increasing exposure to salt stress, decreases the values of LWP. Ahmed et al (2008) reported a decrease in LWP from -2.6 to -4.2 Mpa in one-year-old olive trees in response to increasing salt concentrations from 0 to 200 mM NaCl. Similarly a reduction of leaf osmotic potential from -0.59 to -0.75 Mpa and from -0.68 to -0.99 Mpa was reported in Moneymaker and Daniela varieties of tomato respectively in increasing salt concentrations from 0 to 70 mM NaCl (Romero-Aranda et al 2001). In another report, Maggio et al. (2007) reported a respective decrease in LWP and osmotic potential from -0.70 to -1.21 and -1.49 to -2.28 while increasing EC_{iw} from 2.5 to 15 dS m^{-1} in Licata, F1-COIS 94 variety of cherry tomato. Whereas in the case of pot grown spring wheat, a decrease in LWP observed from -1.5, -4 and -7 Mpa in response to increasing EC_{iw} levels from 0.2, 8 and 16 dS m^{-1} (Nishida et al., 2008). Moreover simultaneous decrease in LWP and leaf osmotic potential as well as root water potential and root osmotic potential was observed while increasing EC_{iw} from 0 to 1% salinity (Maggio et al 2004).

Salinity has been observed to damage plant membranes. In strawberry, membrane permeability increased to 85% with addition of 40 mM NaCl (Yildirim et al 2009). The same authors showed that foliar nutrient application of potassium, magnesium and calcium nitrate can improve such damage to the membrane under salt stress environment. Similarly increase in membrane permeability observed in strawberry with addition of 35 mM NaCl and pH from 5.5 to 8.5 (Kaya et al 2002). In the current experiment MD value changes from 16 to 32%

and 33 to 53% in the year 2008 and 2009 respectively while increasing EC_{iw} from 0.8 to 8.5 dS m^{-1}.

Regulation of stomatal opening is affected by numerous factors i.e. water, light, temperature and wind. Besides environmental factors some biotic factors i.e. type of plant, varieties and the leaf surface also affect much in g_s. The combined effects of salinity and time of the day have a significant impact on g_s. A decrease in g_s values of 164, 122 and 119 mmol m^{-2} s^{-1} was recorded in one year-old olive trees grown under salt concentrations of 0, 100 and 200 mM of NaCl respectively (Ahmed et al 2008). Also decrease in g_s values of 300, 150 and 100 mmol m^{-2} s^{-1} recorded in cucumber (*Cucumis sativus* L.) grown under salt concentration of 0, 50 and 100 mM of NaCl respectively (Stepien and Klobus, 2006). Moreover in two varieties of tomato i.e. Daniela and Moneymaker decrease in g_s values of 124, 74 and 40 and 78, 38 and 36 mmol m^{-2} s^{-1} were recorded with respect to increasing salt concentrations of 0, 35 and 70 mM NaCl (Romero-Aranda et al 2001). A significant reduction of g_s values was also observed in *P. alba* tree grown under saline treatment than non saline treatment (Abbruzzese et al., 2009). In the current experiment insignificant difference on adaxial leaf surface g_s was observed (data not shown) and concentrated on the abaxial leaf surface g_s with increasing EC_{iw} levels. Also the higher g_s in sunlit leaves than shaded leaves was recorded (data not shown). Abbruzzese et al (2009) presented a significant correlation between g_s and stomatal area in 14P1 and 6K3 varieties of *P. alba* with R^2 equals to 0.85 and 0.88 respectively. But the same authors showed no significant correlation between g_s and guard cell length in all the genotypes studied.

Stomata are gateway between the plant and atmosphere and play a vital role in alleviating plant from different environmental stresses. A significant correlation between StoD and LWP was observed 80 and 90 days after the treatment initiation in a grass *Leymus chinensis* (Xu and Zhou 2008). The same authors observed dramatic decreased in stomatal size with increasing water deficit and almost linear correlation was observed in stomatal size and water potential. Decreased in stomatal length and width was observed in *P. vulgaris* both in the adaxial as well as abaxial leaf surfaces under saline and saline plus elevated CO_2 treatment versus non-saline and elevated CO_2 treatment respectively. In the same experiment an increased in the StoD was observed on the abaxial leaf surface under saline and saline plus elevated CO_2 treatment versus non saline and elevated CO_2 treatment respectively but decreased StoD was observed on the adaxial leaf surface under saline and saline plus elevated CO_2 treatment versus non saline and elevated CO_2 treatment respectively (Bray and Reid 2002). Likewise in the current experiment a significant reduction in StoD on abaxial leaf surface of pomegranate was observed however the data of StoD from adaxial leaf surface was found similar. Furthermore, similarity in g_s on adaxial leaf surface (data not shown) possibly due to occurrence of same number of stomata. Though, nothing can be said critically without having detailed data, because stomatal characteristic is quiet diverse from environment to environment and species to species. Moreover a stronger correlation was observed between StoD and ET than g_s and ET. Likewise, a significant negative correlation was observed between g_s and specific leaf area (Xu and Zhou 2008). This finding could suggest us that decrease in leaf size could be a major fact of increasing StoD while irrigating with increasing EC_{iw} levels. Enlargement in leaf thickness could be a reason of having higher StoD under salinity and drought stress environment (Galmes et al 2007), because enlarged leaf thickness may produce more guard cells for a given leaf area. Another speculation of having higher StoD under stress environment can be for better stomatal regulation. Higher number of stomata per mm^{-2} enhanced plant's ability to regulate stomatal opening. As the value of g_s during morning and evening hours observed approximately similar in all salt treatments (Fig.

4), plants under higher salinity treatment can compensate their CO_2 demand by having more number of stomata during morning and evening. Conversely, a decrease in StoD mm^{-2} 163, 105, 87 and 157, 96, 91 were observed in Daniela and Moneymaker varieties of tomato respectively with increasing salt concentrations from 0, 35 and 70 mM NaCl (Romero-Aranda et al 2001). Thus the effect of salinity treatment on StoD could be species or environmental specific feature and fetch more investigation.

ET has been shown to be highly correlated to leaf area index (Graham et al 2009; Gong et al 2009) and vegetative cover (Tripler et al., 2007) and may reflect a scenario of the plants growing environment. Thus less daily ET at 5 and 8.5 dS m^{-1} in the year 2009 than 2008 is possibly due to high salt accumulation in the root zone (Dudley et al 2008) and more damage to the plant due to salinity (Munns 2002; Munns et al 2002; Grattan and Grieve 1999). In the current study, the correlations between the different physiological and morphological stress indicators and the ET were examined, in order to provide new improved tools for ET estimation. A negative correlation between photosynthesis and LWP (R^2 = 0.62) was recorded in one-year-old olive tree grown with increasing NaCl concentration varied from 0, 100 and 200 mM NaCl (Ahmed et al 2008) but relationship among ET with plant stress indicators were rarely reported. Moreover intercepts and slopes of the equation in both of the approaches described earlier increases in the year 2009 than 2008 (Table, 2). Such an increase in intercepts and slopes can be due to dynamicity of soil-plant-atmosphere continuum as plant acquires tolerance due to increasing exposure to salt. In the same experiment with pomegranate during 2008, monthly decrease in the slope parameters were recorded while fitting Mass and Hoffman salinity response function in increasing EC_{iw} (Bhantana and Lazarovitch 2010).

Summarizing all different approaches of ET estimation in the pomegranate grown under salt stress, the measurement of StoD best estimated the ET. However measurement of StoD is more difficult than ECL approach. No differences were observed in between two other approaches i.e. LWP and g_s in ET estimation. Comparing LWP and ECL approaches in the year 2008 and 2009, more accurate estimation to ET was observed in the year 2009 than 2008. Such discrepancies in ET estimation between years could be either due to increasing plant tolerance to salinity or salinity induced damage to the plant tissues (Grattan and Grieve 1999) with advancing time after the experiment. Furthermore, use of multiple factors regression models increased the accuracy of ET estimation than one to one factor correlation study. Obviously, in an open field numerous factors are affecting ET at variable rates. And more accurate result can be achieved taking into account for a number of factors. In the bottom line such multiple regression models can be useful for an agro climatic modeling or long term ecological planning of a crop grown under salt or the combined drought and salt stressed environments.

CONCLUSION

Exposure of pomegranate to salt stress decreased ET, LWP and g_s but increased ECL and StoD. Among the four different methods we tested to describe plant water status the StoD approach described 79% of the ET values. However, due to complexity in the measurement of the StoD, preference should be given to an ECL approach for a practitioner, whereas ECL described 75% of the ET values. Thus an ECL approach can be equivalent to water balance method used for ET estimation by weighing or non weighing lysimeter and strongly recommended for the future application. As the plant response to the environment is dynamic, frequent corrections to such model is inevitable. Moreover combination of two or more parameters in ET estimation improved fitness of the model and enhanced the accuracy of estimation. Such studies would serve to advance for an agro ecosystem modeling and long term agro ecological planning. For instance, a set of three parameters regression model including ECL, g_s and StoD described 93% of the ET and stronger than one to one factor correlation. Besides ET estimation increased in StoD with increasing EC_{iw} is fascinating point of this study.

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Authors' Contributions

Author Bhantana P being the main author has involved in article writing for publication, N Lazarovitch were engaged in data analysis and article final revision.

Conflicts of Interest

The authors have no relevant financial or non-financial interests to disclose.

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