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PROLINE, SOLUBLE SUGAR, LEAF STARCH AND RELATIVE WATER CONTENTS OF FOUR MAIZE VARIETIES IN RESPONSE TO DIFFERENT WATERING REGIMES

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ABSTRACT. The purpose of this study was to evaluate the response of four maize varieties to different simulated watering regimes in term of proline, starch and soluble sugar contents as well as relative water content. Maize seeds were planted in 64 plastic pots of 20 litre capacity, arranged in a factorial fitted in completely randomized design (CRD), with four replications in the screen house of the Institute of Agricultural Research and Training (I.A.R&T), Moor Plantation Ibadan. The watering was done based on the designated field capacities (FC) of 25, 50, 75 and 100%. Fresh leaf samples were collected five weeks after planting and at the end of each stress period. The proline, soluble sugar, leaf starch and the relative water contents in the leaves were estimated. The results obtained showed that watering regime significantly influenced the leaf starch, soluble sugar, proline and relative water contents. The varieties also differ

significantly in the proline, soluble sugar content, leaf starch and the relative water contents. Watering regime and variety interaction was significant for soluble sugar, starch, proline and the relative water content. Highest soluble sugar of 1.28 mg/g and proline of 35.70 $\mu\text{mol/g}$ FW were obtained when FC was 25% and lowest when watering level was optimum. The starch and relative water contents were optimum under full watering (100% FC) and lowest when field capacity was 25%. Variations were observed with regards to different maize varieties. ART98SW6-OB accumulated the highest quantity of soluble sugar and proline under 25 and 50% field capacities alongside DTESYN, which is a drought tolerant maize variety. It could be concluded that water stress increased production of soluble sugar and proline, while water availability increases relative water content and favors starch accumulation. The consideration of these

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metabolites alongside other physiological features is a very fast and reliable method for drought tolerant plant selection even at the plant seedling growth stage.

Keywords: field capacity; proline; starch content; soluble sugar; relative water content.

INTRODUCTION

Water is an important component of life and is required for various physiological and biochemical processes involved in plant growth and development. It is a major abiotic factor which determines the distribution of natural vegetation in comparison with several other environmental factors (Kramer and Boyer, 1983). Several physiological and metabolic activities such as plant water content, turgor, total water potential, stomata closure, cell enlargement, cell growth and photosynthetic activities are affected or arrested under severe water stress (Mckersie- Leshem, 1994). Excessive amount of reactive oxygen species (ROS) are produced when drought stress becomes severe leading to peroxidation and degradation of membrane lipids, nucleic acids and various organelles, such as chloroplast, mitochondria and peroxisomes.

Naturally, plants employ several adaptive measures or mechanisms to cope with the threatening effects of the unfavorable weather conditions such as salinity and water deficit stress. Such adaptive measures bring about changes or adjustment in the

physiological and biochemical processes of plant. During such metabolic adjustment, compatible organic solutes like sugars, polyols, betaines and proline are usually accumulated (Yancey, 1981). These compatible solutes are of two types (i) nitrogen-containing compounds and the hydroxyl compounds. The nitrogen-containing compounds include proline, other amino acids, quaternary ammonium compounds and polyamines while the hydroxyl compounds consists of the sucrose, polyhydric alcohols and oligosaccharides (McCue & Hanson, 1990). Osmotic adjustment is a mechanism employed by plants to maintain plant- water relations under osmotic stresses. It involves the accumulation of a wide range of osmotically active molecules/ions including soluble sugars, sugar alcohols, proline, glycine betaine, organic acids, calcium, potassium, chloride ions, etc (Farooq *et al.*, 2009). These substances play antioxidant role, which help to minimize the damage caused by the reactive oxygen species during peroxidation in plants under water deficit stress (Doulis, 1994). Accumulation of proline in leaves at low water potential is caused by a combination of increased biosynthesis and slower oxidation in the mitochondria (Boggess *et al.*, 1976; Stewart *et al.*, 1977; Rhodes *et al.*, 1986; Samaras *et al.*, 1995). Accumulation of proline is a widespread plant response to environmental stresses, especially at

low water potential (Nayer and Reza, 2008), while increase in production of sugars in different parts of the plants has also been reported to occur in response and protection against various kinds of environmental stresses (Prado *et al.*, 2000). Hence accumulation of proline and sugars under severe water stress could be used as selection criteria for the selection of drought tolerant genotypes. Over the years, concerted efforts have been made by several investigators in the field of plant breeding and the allied disciplines to develop high yielding drought tolerant plant genotypes. The need for the development of drought tolerant plant genotypes is becoming increasingly necessary going by the prevailing climatic changes evident by the erratic rainfall pattern and unpredictable weather and climatic conditions, especially in the tropics. Drought tolerant is however a complex trait expression, which depend on action and interaction of different morphological traits, such as leaf rolling, efficient rooting system and biochemical parameters (accumulation of proline) (Nazarli and Faraji, 2011). The objective of this study was to determine the impact of varying watering regimes on the accumulation of free proline, soluble sugar, leaf starch content and the relative water contents of four maize varieties. In this experiment, only the total soluble sugar content would be considered without the identification of the specific sugar components.

MATERIALS AND METHODS

Four maize varieties, consisting of two high protein maize (ART98SW6-OB and ILE1-OB), derived from open pollinated populations developed by the Institute for Agricultural Research and Training, Ibadan (I.A.R.&T) and two other, consisting of one-non high protein maize (Tzpb-sr) and one drought tolerant variety (DTESTRSYN-w), were used for this study. The experiment was carried out in the screen house of I.A.R.&T., Ibadan.

Two seeds from each maize variety (which were later thinned down to one vigorous seedling at seven days after planting) were sown in each of the 64 plastic pots of 20 litre capacity filled with 15 kg of top soil whose textural classification and water holding capacity had been previously determined. The moisture content of the soil was determined by gravimetric/oven-dry method in order to determine the quantity of water needed to apply in order to give the required water equivalent to achieve the intended field capacity (FC). The soil was Loamy-sandy soil with 82% sand, pH of 6.63 and chemical properties of 5.3 g/kg Organic carbon, 0.1 g/kg Nitrogen, 7.0 mg/kg of P, 1.1 cmol/kg of Ca, 1.8 cmol/kg of Mg, 0.2 cmol/kg of K, 0.4 cmol of Na, 0.1 cmol/kg total acidity and 1.36 g/cm³ bulk density. The experimental design was a factorial arranged in a CRD with four replicates of four simulated water regimes equivalent to 25%, 50%, 75% and 100% field capacities. The water regimes constituted the main plot while the varieties were the sub plots. The amount of water lost during each measuring cycle was replaced bringing the pots back to their initial weight. A graduated measuring cylinder was placed at the centre of the screen

house, so as to determine the quantity of daily water evaporation. Measurements were taken every morning and the amount of watering requirement for each soil moisture treatment was added as at when required. Plants were watered regularly to the designated field capacity and the soil moisture potential was guided by quick and drawn tensiometer (Eijkelkamp.co).

Free proline content

Free proline content in the leaf tissue of the different maize genotypes was estimated by the method suggested by Nazarli and Faraji (2011) (a). An amount of 0.2 g of fresh leaf was homogenized in 5 ml of 95% ethanol. Above phase of filtrate was separated and its sediments were washed by 5 ml of 70% ethanol two times and its above phase added to the previous over compartment. The alcoholic extract was kept in refrigerator at 4°C (Paquin and Lechasseur, 1979). One ml of the alcoholic extract was diluted with 1 ml of distilled water and 2 ml of acid ninhydrin and 2 ml of glacial acetic acid was added, the mixture was placed in boiling water bath for 1h at 100°C. The reaction was stopped by placing the test tubes in cold water. The samples were rigorously mixed with 4 ml toluene. The light absorption of toluene phase was estimated at 520 nm using a UV Spectrophotometer. The proline concentration was determined using a standard curve. Free proline content was expressed as $\mu\text{mol g}^{-1}$ FW of leaves (Irigoyen *et al.*, 1992).

Soluble sugar estimation

A specific amount (2 ml) was taken from the extract preserved in refrigerator in ('a' above) and mixed with 3 ml anthrone (150 mg anthrone, 100 ml of 72% sulphuric acid, W/W). The samples were placed in boiling water bath for 10

minutes. The light absorption of each sample was estimated at 625 nm using a UV-Spectrophotometer. Contents of soluble sugar were determined using glucose standard and expressed as mg g^{-1} FW of leaves.

Leaf starch content

Estimation of starch content was obtained by the method of Thayumanavan and Sadasivam (1984). Plant residue from (a) above was washed thoroughly with 80% ethanol. The residue was dried over a water bath. To the residue 5.0 ml of water and 6.5 ml of 52% perchloric acid was added. The extraction process was repeated and the supernatant was made up to 100 ml. Out of this 0.2 ml of the supernatant was pipetted out and the volume made up to 1 ml with water. Working standards of 0.2, 0.4, 0.6, 0.8 and 1.0 ml were made up to 1ml in each tube with water. Anthrone reagent (4 ml) was added to each tube and the content of each tube was heated for 8 min in a boiling water bath. The tubes were cooled rapidly and the intensity of the green to dark green colour was read at 630 nm. The glucose content in the sample was determined using the standard graph. The value obtained was multiplied by a factor of 0.9 to arrive at the leaf starch content.

The relative water content (RWC)

The relative water content of the plant as affected by water stress during the period of the imposed stress were determined according to the method of Barr and Weatherly (1962). Large broad-leaves of maize leaf discs were cut from the leaves, to obtain about 5-10 cm^2 /sample. In the Lab, vials were weighed to obtain leaf sample weight (W), after which the samples were immediately hydrated to full turgidity for 4 hrs under normal room light and

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temperature. Leaf samples were then rehydrated by floating on distilled water in close Petri dishes. After 4 hrs the samples were then taken out of water and blotted dry for any surface moisture quickly and lightly with filter paper and immediately weighed to obtain fully turgid weight (TW). Samples were then oven dried at 80°C for 24 hrs and reweighed to determine the dry weight (DW). All weighing were done to the nearest mg.

Calculation: $RWC (\%) = [(DW-FW) / (TW-FW)] \times 100$, where DW = sample fresh weight and TW = sample turgid weight.

Data obtained from the various experiments were analyzed using SAS (2007) version 9, while the means were separated using Fisher's protected LSD test. This was performed only when the *F*-test indicated significant ($P < 0.05$) differences among the treatments

RESULTS

Starch content

The starch content was significantly ($P < 0.05$) influenced by varying watering regimes, variety (V) and interaction between variety and watering regimes (W x V) (Table 1).

Table 1 - Variance analysis for leaf starch content, soluble sugar, proline and relative water content of four maize varieties as affected by varying simulated watering regime in Ibadan

Source of variation	D.F.	STH mg/g FW	SS mg/g FW	PRO µmg/g	RWC %
Watering(W)	3	0.91*	0.07*	2412.9***	1764.1***
Error (W)	12	0.27	0.05	15.01	36.62
Variety (V)	3	0.08	6.22***	32.10	209.0**
W x V	9	0.66*	0.13***	62.32***	34.53
Error(V)	36				
Mean	63				

* ** *** Significant at $P < 0.05, 0.01, 0.001$, D.F. = Degree of freedom

STH= Starch content, SS=Soluble sugar content, Pro= Proline, and RWC= Relative water content

Main effect of watering regime effect showed that the highest starch content of 1.30 mg/g) was obtained when watering was 100% FC, starch content at 75% FC (0.91 mg/g), 50% FC (0.78 mg/g) and 25% (0.81mg/g) were low and not significantly different from each other. The varieties were not significantly different in content of starch (Table 2). Watering regime x variety effect showed that DTESYN had highest

starch content of 1.37 mg/g FW at 25% FC, followed by 0.92 mg/g FW and 0.78 mg/g FW of Tzpb-sr and ART98SW6-OB, which were not significantly different from each other, while Ile1-OB had the lowest leaf starch content of 0.34 mg/g FW. Tzpb-sr had the highest leaf starch content when FC was 50%, followed by ART98SW6-OB and Ile1-OB, which were not significantly different; the lowest leaf starch content of

0.43mg/g FW was obtained in DTESYN ARTSW98-OB had the highest leaf starch content of 1.55 mg/g FW when FC was 75%, Tzpb-sr and DTESYN were lowest in leaf

starch content and both were not significantly different. The four maize varieties were not significantly different in leaf starch contents when FC was 100 % (Table 3).

Table 2 - Main effects of watering regimes and variety on starch, soluble sugar, proline contents and results of F-tests for the effects of main factors and interactions of the leaf extract of four maize varieties in Ibadan

	STH mg/g FW	SS mg/g FW	PRO µmg/g	RWC (%)
Water regime				
25	0.81b	1.28a	35.70a	64.15d
50	0.78b	1.25ab	30.64b	73.29c
75	0.91b	1.15ab	18.73c	82.23b
100	1.30a	1.11b	4.35d	88.16a
Variety				
TZPB-sr	0.92a	0.94b	23.82ab	78.72a
Ile 1-OB	1.05a	0.78c	21.74b	79.57a
ART98SW6-OB	0.95a	0.92b	25.06a	77.93a
DTESYN	0.88a	2.12a	22.82ab	71.64b
F- test				
Water (W)	*	*	***	***
Variety (V)	ns	***	*	**
W x V	*	***	***	ns

* ** *** Significant at $P < 0.05, 0.01, 0.001$; D.F.= Degree of freedom; ns = not significant; STH= Starch content; SS=Soluble sugar content; Pro= Proline; RWC= Relative water content.

† Means followed by the same letter within a column are not significantly different Fisher's protected LSD test at $P=0.05$.

Soluble sugar

Soluble sugar was significantly influenced by watering regime ($P < 0.05$), the variety (V), variety and watering regime (V x W) interaction were significant at $P < 0.001$ (Table 1). Highest leaf soluble sugar of 1.28 mg/g FW was obtained when FC was 25%, the soluble sugar reduces as the watering regime increases. The soluble sugar content at 50% FC (1.25 mg/g) and 75% FC (1.15 mg/g) were not significantly different, lowest

soluble sugar of 1.11 gm/g FW was obtained when FC was 100%. Main effect of varying watering regime on the varieties showed that DTESYN had highest soluble sugar of 2.12gm/g FW, followed by Tzpb-sr and ART98SW6-OB, Ile1-OB was lowest with 0.18 gm/g FW (Table 2). DTESYN had the highest leaf soluble sugar of 2.26 gm/g FW, followed by ART98SW6-OB, which had 0.85 mg/g FW, while lowest values of soluble sugar content of 0.78 and

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0.71mg/g FW were obtained in Tzpb-sr and Ile1-OB when FC was 25%. DTESYN also exhibited highest leaf soluble sugar content of 2.26 mg/g FW, when FC was 50% ahead of ART98SW6-OB, while Ile1-OB and Tzpb-sr, which were lowest but not significantly different from one another. DTESYN also had the highest leaf soluble sugar of 2.38

gm/g FW, when FC was 75%, followed by ART98SW6-OB and Tzpb-sr, while Ile1-OB was lowest with 0.72gm/g FW. DTESYN, Tzpb-sr and ART98SW6-OB were highest in leaf sugar content at 100% watering field capacity and were not significantly different. Ile1-OB however had the lowest leaf soluble sugar content (Table 3).

Table 3 - Effect of water regime and variety interaction on starch content, soluble sugar, proline and relative water content of the leaves of four maize varieties in Ibadan

Source of variation	STCH mg/g FW	SS mg/g FW	PRO µmg/g	RWC (%)
25% FC x Tzpb-sr	0.92ab	1.07b	34.94a	69.24a
25% FC x Ile 1-OB	0.34b	0.79c	35.84a	68.20a
25% FC x ART98SW6-OB	0.62ab	1.15b	35.50a	63.83a
25% FC x DTESYN	1.37a	1.90a	36.53a	55.33a
<i>F</i> -test	*	***	ns	ns
50% FC x Tzpb-sr	1.04a	0.78b	26.82b	74.34ab
50% FC x Ile 1-OB	0.91ab	0.71b	25.89b	77.97a
50% FC x ART98SW6-OB	0.78ab	0.85b	34.66a	74.37ab
50% FC x DTESYN	0.43b	2.26a	35.81a	66.48b
<i>F</i> -test	*	***	***	*
75% FC x Tzpb-sr	0.53b	0.99b	20.21a	81.87ab
75% FC x Ile 1-OB	0.91ab	0.72c	18.90a	85.13a
75% FC x ART98SW6-OB	1.55a	0.93b	20.30a	84.72a
75% FC x DTESYN	0.63b	2.38a	15.51a	77.23b
<i>F</i> -test	*	***	ns	*
100% FC x Tzpb-sr	1.20a	0.90a	3.30a	89.42a
100% FC x Ile 1-OB	1.41a	0.70b	4.91a	88.78a
100% FC x ART98SW6-OB	1.50a	0.94a	4.76a	86.97a
100% FC x DTESYN	1.09a	1.93a	3.41a	87.50a
<i>F</i> -test	ns	***	ns	ns

* ** *** Significant at $P < 0.05, 0.01, 0.001$

† Means followed by the same letter within a column are not significantly different according Fisher's protected LSD test at $P=0.05$.

Free proline content

Mean square (MS) of proline as influenced by varying watering regime was significant at $P < 0.001$, variety (v) was significant at $P < 0.05$, variety and watering regime

interaction (V x W) significant at $P < 0.001$ (Table 1). Highest proline content of 35.70 µmol/g was obtained at 25% FC and reduces as the watering increases. Lowest amount of 4.35 µmol/g of proline was obtained

when watering was 100% FC (*Table 2*). Main effect of varying watering regime on the varieties showed that ART98SW6-OB produced the highest amounts of proline (25.04 $\mu\text{mol/g}$ FW), followed by Tzpb-sr and DTESYN, which were not significantly different, while lowest amount was obtained in Ile1-OB. The variety and water regime interactions showed that the varieties were not significantly different in the amount of proline produced at 25% FC. Highest amount of proline were produced by DTESYN and ART98SW6-OB when FC was at 50% FC, while lowest amount of proline was produced by Tzpb-sr and Ile1-OB. The varieties were not significantly different in the quantity of proline produced under 75% and 100% FC (*Table 3*).

Relative water content (RWC)

Relative water content was significantly influenced by varying watering regime at $P < 0.001$, the varieties were also significantly different at $P < 0.05$ (*Table 1*).

RWC was highest when FC was 100% (88.16%), followed by 75% (82.23%), and 50% (73.29%), while lowest RWC was obtained when FC was 25%. The main effects of the relative water contents were highest in Tzpb-sr, Ile1-OB and ART98SW6-OB, which were not significantly different from one another, while DTESYN had the lowest RWC value of 71.64 % (*Table 2*). The variety and watering regime interaction at 25% FC showed that the varieties were not

significantly different in RWC Ile1-OB had the highest RWC of 77.97% when FC was 50%, followed by ART98SW6-OB and Tzpb-sr, which were not significantly different from each other, while lowest value of 66.48% was obtained in DTESYN. Highest relative water content of 85.13% and 84.72% were obtained in Ile1-OB and ART98SW6-OB, when FC was 75%, which were not significantly different from each other, while lowest RWC of 77.23% was obtained in DTESYN. The varieties were not significantly different in relative water contents, when watering field capacity was 100% (*Table 3*).

DISCUSSION

Soluble sugar and proline increases as the watering regime reduces in this study, while starch accumulation and relative water content increases as the water levels increases. The increase in soluble sugar content as the water level decreases is well documented and agreed with the findings of Jones and Osmond (1981), Munns *et al.* (1979), Munns and Wier (1981), Irigoyen *et al.* (1992), Sanchez *et al.* (1998), Nayer and Reza (2008), Izanloo *et al.* (2008), while Lawlor and Fock (1977) and Thakur and Rai (1980) were of contrary view. Several possible explanations were provided by several investigators on the accumulation of soluble sugar by plants under water stress. Hsiao (1973) reported that drought induced conversion of hexoses and other carbohydrates, such

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as sucrose and starch, into sugar alcohols (polyols) and proline in a wide variety of plants and a remarkable increase in α -amylase enzyme activities was also observed. Watson and Wardlaw (1981) attributed the increase in soluble sugars to decreased translocation of assimilate from the leaves to other organs in plant under water stress. Sugars were reported to be responsible for protecting the cells during drought by mechanisms which involve substitution of the hydroxyl groups of sugar for water to maintain hydrophilic interactions in membranes and proteins during dehydration as sugars interact with proteins and membranes through hydrogen-bonding, thereby preventing protein denaturation (Leopold, 1994). Sugars also contribute majorly to vitrification, which is the formation of a biological glass in the cytoplasm of dehydrated cells (Leopold, 1994; Buitink *et al.*, 1998). Other view attributed increase in sugar concentration in plants under water deficit to degradation of starch (Fischer and Höll, 1991). Strong correlation exists between soluble sugars accumulation and drought tolerance in plants under water stress (Verslues and Sharp, 1999; Hoekstra *et al.*, 2001). Soluble sugar content was described to be a better marker for selecting improvement of drought tolerance in durum wheat (*Triticum durum* Desf.) than was proline content (Al Hakimi *et al.*, 1995).

The mechanisms of water stress tolerance may be associated with the

accumulation of solutes, such as sugars and proline. Nayer and Reza (2008) reported that proline itself can also be of principal importance in stress adaptation and can serve as a selection marker for tolerant cultivars, even in very young seedlings. Starch may play an important role in accumulation of soluble sugars in cells. Starch depletion in grapevine leaves was noted by Patakas and Noitsakis (2001) in response to drought stress. Decrease in the content of insoluble sugars (starch) observed in this study agreed with the findings of Hsiao (1973), which attributed the reduction in the starch content to reduced photosynthesis, due to stomatal closure in response to water stress and that sugar supply is necessary for free proline accumulation in plant under water deficit stress by supplying NADPH and ATP needed for proline synthesis. Sucrose is a principal component of soluble sugar, which enhances proline accumulation in plant under water stress (Larher *et al.*, 1993). The maize varieties under this study showed variation in the quantity of accumulated proline and soluble sugar in relation with plant relative water content.

The results obtained on the varietal responses to different watering regimes with the highest amount of proline and soluble sugar recorded in DTESYN, compared to other varieties could be attributed to the fact that this variety had original been bred for drought stress tolerance. Production of proline and soluble

sugar could probably be the major mechanisms being employed by this variety for tolerance.

CONCLUSION

In this study, the maize varieties showed variation in the contents of the various metabolites accumulated in relation to the relative water contents under the different watering regime. The use of soluble sugar and proline as criteria for selecting drought tolerant maize genotype under severe water shortage is a very fast and useful method of selection for drought tolerance in maize even at the seedling stage. Further work on the molecular identification of genes that code for increased accumulation of these metabolites under drought stressed condition would assist rapid development of drought tolerant maize genotypes.

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