

Evaluation of biochemical and free radical scavengers of *Digitaria exilis* L. under osmotic stress

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Abstract *Digitaria exilis* L. is an under-utilized crop with high nutritional and medicinal values. It thrives in and is well-adapted to arid areas with low soil nutrients. Using biochemical markers, this study investigates the mechanisms by which *D. exilis* responds to osmotic stress. Three accessions Dinat Iburua (DIN), Jakah Iburua (JAK) and Jiw Iburua (JIW) were collected from National Cereal Research Institute, Niger State. Two accessions, NG/11/JD/061 and NG/11/JD/062 were also collected from National Centre for Genetic Resources and Biotechnology, Ibadan. Murashige and Skoog medium of approximately 1.2 L was supplemented with polyethylene glycol 6000 to create osmotic pressures of -9.29, -13.93, -20.13, -26.32, -32.51, and 0 MPa (control). Sterilized seeds were inoculated in the medium and placed in the growth room for 4 weeks. Proline accumulation was significantly high in all JAK plants under osmotic stress. Proline and ascorbate peroxidase ($p < 0.05$) activities were directly correlated, thus reinforcing the survivability of JAK during stress. Catalase (CAT) activity was also significantly induced in JAK under osmotic stress, which synergistically improved its tolerability. As a result, >50% of OH^- , H_2O_2 , and NO radicals were scavenged. However, other accessions including DIN, NG061, NG062, and JIW showed variations in their responses to different levels of osmotic stress, although not significant. Therefore, JAK possesses a well-equipped free radical quenching system that is protected by the accumulation of the osmolyte proline; therefore, accession JAK is considered osmotolerant. CAT and superoxide dismutase activities were osmostabilized against oxidative

stress by proline.

Keywords free radicals, *D. exilis*, antioxidant enzymes, proline accumulation, lipid peroxidation

Introduction

Digitaria exilis possesses many nutritional, economic and phytochemical benefits to mankind. Gwete soup is an African delicacy that is locally prepared from *D. exilis* to treat diabetes¹. It yields and survives well in relatively poor climatic conditions such as arid areas. *D. exilis* is very important among other grains due to its high nutritional composition. *D. exilis* show generally mineral contents that are in the range of other cereals. However, it contains much more protein than other cereals like millets, maize, sorghum etc. and the protein is mainly concentrated in the grain and not in the husk¹. Methionine, which builds up sulphur, is accumulated in *D. exilis* twice the amount compared to corn or millet and three times compared to rice.

In vitro culture techniques minimize environmental variation due to defined nutrient controlled conditions and homogeneity of stress application. The simplicity of such manipulation enables to study large plant production and stress treatments in a limited space and short period of time. Simulation of drought stress under *in vitro* conditions during the regeneration process constitutes a convenient way to study the effect of drought stress on the plant. Application of osmotic stress to plants at juvenile stage is an effective method of selecting plants with drought tolerant traits. This will of course make the mature plants cope with the drought stress conditions during growth and reproductive periods. This will confer the potential in screening for drought tolerance. Poly-ethylene glycol 6000 had been used to simulate drought stress in plant as non-

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penetrating osmotic agent lowering the water potential in a way similar to soil drying. It is frequently assumed that plant water relations are similar whether the plants are growing in soil or in a PEG' solution having an equal water potential. Larger polyethylene glycol molecules such as poly-ethylene glycol 6000 are more useful for simulating soil drying⁵. The study therefore aimed at the biochemical mechanisms undertaken by *D. exilis* to survive osmotic stress.

Materials and Methods

Plant material

Five accessions of *D. exilis* were used in the studies, which were Dinat Iburua (DIN), Jakah Iburua (JAK), Jiw Iburua (JIW), NG/JD/06/11/062 (NG062) and NG/JD/06/11/061 (NG061). Three accessions DID, JAK and JIW were obtained from National Cereal Research Institute, Badeggi, Niger State, Nigeria, while the other two accessions NG061 and NG062 were obtained from National Centre for Genetic Resources and Biotechnology (NACRAB), Moor Plantation Ibadan, Nigeria.

Media preparation

To prepare 1.2 litre of MS (Murashige and Skoog, 1962) media, 60ml of macronutrients, 6 ml stock micro-nutrients, 36 g of sucrose, 0.12 g of inositol, 6 ml of vitamins, 0.04476 g of Sodium (Di) Ethylenediamine Tetraacetate Dihydrate (Na EDTA. 2H₂O) and 0.0278 g of Ferrous sulphate were added to 600 ml of deionized water. The mixture was divided equally into 6 sterilized jars. Polyethylene glycol PEG 6000 of 30 g/l, 45 g/l, 65 g/l, 85 g/l and 105 g/l and 0 g/l were added to create an osmotic conditions-of -9.29 MPa, -13.93 MPa, -20.13 MPa, -26.32 MPa, -32.51 MPa and 0 MPa (control) to represent A, B, C, D, E and F. Deionized water was added to make up to 200 ml in each jar. The hydrogen ion concentration i.e. pH of 5.7 ± 0.3 was taken using pH meter. About 0.46 g of phytagel (Agar) was added to each jar. All the media were solubilized for 15 minutes in an oven. Five millilitres (5 ml) were dispensed into an autoclaved test-tube. Five sterilized seeds were inoculated on the media inside the laminar airflow, sealed with paraffin and placed inside growth room.

Quantification of chlorophyll contents

Chlorophyll was extracted from the leaves. The extraction

of leaf pigments was performed with 75% ethanol, and the absorbance at 663 and 645 nm were measured with a spectrophotometer. The chlorophyll a, chlorophyll b, and total chlorophyll quantities were calculated according to the method of Arnon. The pigment concentrations were expressed as µg/ml. Chlorophyll contents were calculated using the formula stated below.

$$\text{Chl a} = 15.65A_{663} - 7.340A_{645}$$

$$\text{Chl b} = 27.05 A_{645} - 11.21 A_{663}$$

Determination of proline contents

All experiments were performed at 4°C. Leaf samples were homogenized in ice cold 50 mM sodium phosphate buffer (pH 7.8) for the proline extraction. The buffer contained 1 mM disodium EDTA and 2% (w/v) polyvinylipid peroxidationlpyrrolidone (PVPP). Supernatants collected after centrifugation at $13,000 \times g$ for 40 min were used to determine the proline contents. The free proline content was determined according to Bates.

Determination of radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl hydrate) Assay was determined using the stable radical DPPH (2, 2-diphenyl-1-picrylhydrazyl hydrate) as described by Brand-Williams. Nitric oxide radical activity of the extract was carried out according to the method of Green as described by Mar-cocci. The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the fractions for hydroxyl radicals generated from the Fe³⁺/ascorbate /EDTA/H₂O₂ system according to the method of Halliwell. The ability of plant extracts to scavenge hydrogen peroxide was determined according to the method of Ruch.

Determination of antioxidant enzyme

Superoxide dismutase (SOD) was described by Mccord and Fridovich. Catalase (CAT) activity was measured according to the method of Aebi. Ascobate Peroxidase (APX) activity was measured according to the methods of Nakano and Asada.

Determination of lipid peroxidation

Total amount of lipid peroxidation products present in the plant samples was estimated by the thiobarbituric acid

(TBA) method which measures the malondialdehyde (MDA) reactive products according to the method of Ohkawa.

Results

Chlorophyll contents of accessions JAK, NG062 and DIN were significantly high at osmotic stress D. Accessions DIN and NG062 had the highest chlorophyll content at osmotic level D. whereas, accessions NG061 and JIW had their highest total chlorophyll contents at osmotic level C. Furthermore, accession JAK had the overall highest Chl A and B at osmotic level D (Table 1). Proline contents in osmotic stressed JAK, DIN and JIW were significantly

higher than those without osmotic stress (control) except for accessions NG061 AND NG062 (Table 2). CAT activities of accessions NG061, NG062, JAK and DIN were significantly high at osmotic level A (Table 3). Accession JAK had high APX and CAT at all levels of osmotic when compared to control. Accession JIW had the lowest CAT activities. Though accession JAK had the highest SOD nevertheless, no significant different in the SOD activities was recorded in all levels of osmotic stress and Accessions. APX was significantly reduced in accessions NG061 and DIN but not significant in accession NG062. Highest APX was found in accession JAK with highest level osmotic stress E (Table 3). Percentage inhibition of OH, H₂O₂, NO and DPPH radicals during an osmotic stress in accession

Table 1 Chlorophyll content ($\mu\text{g/mL}$) of *Digitaria exilis* accessions under different osmotic potentials⁺

ACCESSION	Ψ_s	CHL a	CHL b	TOTAL CHL
NG061	A	14.93e	34.97cd	49.90d
	B	32.91ab	77.14b	110.04b
	C	42.12a	107.91a	150.04a
	D	19.17d	28.27e	47.43d
	E	19.13d	29.47de	48.6d
	F	24.24c	39.93c	64.17c
NG062	A	20.30b	46.06b	66.36bc
	B	20.42b	24.16d	44.58d
	C	7.24d	13.89e	21.13e
	D	32.82a	81.12a	113.94a
	E	16.97c	41.40bc	58.37c
	F	30.12a	48.35b	78.47b
JAK	A	25.72c	78.16b	103.88b
	B	23.84c	55.71bc	79.54c
	C	25.79c	41.35c	67.13d
	D	87.54a	211.07a	298.6a
	E	16.54d	17.87e	34.41e
	F	34.20b	38.39cd	72.6cd
DIN	A	18.86de	47.14c	65.99c
	B	29.21c	66.8b	96.01b
	C	37.93a	78.41ab	116.34ab
	D	33.65b	87.89a	121.54a
	E	19.77d	20.04d	39.8d
	F	33.44b	65.21b	98.65b
JIW	A	24.12ab	58.29ab	82.41b
	B	28.34ab	42.55b	70.89bc
	C	31.63a	78.30a	109.94a
	D	15.38c	37.65bc	53.03d
	E	18.13b	42.85b	60.99c
	F	34.00a	67.82a	101.82a

Values with the same letters in each column are not significantly different from each other at Duncan's multiple range test of $P < 0.05$. Ψ_s = osmotic potential, CHL = chlorophyll

Table 2 Proline content of *Digitaria exilis* under different osmotic potentials

ACCESSION	ψ_s	PROLINE (mg/mL)
NG061	A	0.044b
	B	0.038c
	C	0.043b
	D	0.034d
	E	0.038c
	F	0.050a
NG062	A	0.016e
	B	0.036a
	C	0.018e
	D	0.030c
	E	0.020d
	F	0.036b
JAK	A	0.052a
	B	0.019b
	C	0.013c
	D	0.014c
	E	0.015c
	F	0.009d
DIN	A	0.014d
	B	0.023ab
	C	0.025a
	D	0.015d
	E	0.020b
	F	0.017c
JIW	A	0.014a
	B	0.013a
	C	0.014a
	D	0.009d
	E	0.010cd
	F	0.010c
LSD (0.05)		0.009

Values with the same letters in each column are not significantly different from each other at Duncan's multiple range test of $P < 0.05$. ψ_s = osmotic potential, LSD = least significant difference

NG061 was significantly reduced as compared with to control (Table 4). On the contrary, osmotic stressed JAK scavenged above 50% OH, H_2O_2 and NO radicals significantly at different osmotic levels. It is important to state that osmotic stressed DIN and NG062 significantly scavenged OH. Lipid peroxidation of osmotic stressed accession NG061 was not significant with the control (Table 4). Osmotic stressed accessions NG062, DIN and JIW had their lipid peroxidation significantly higher when compared to the control. Accession JAK under all osmotic levels had their lipid peroxidation significantly low compared to control (Table 5). Nitric acid NO was positively correlated to SOD. Hydrogen peroxide was positively correlated to the activities of APX and Proline. Proline was positively correlated to CAT. MDA is negatively correlated to OH, H_2O_2 and NO (Table 6). Accession JAK

Table 3 Enzyme activities of *Digitaria exilis* under osmotic potentials

ACCESSION	ψ_s	APX (mmol/mL/ min)	SOD (units/mg protein)	CAT (units/mg protein)
NG061	A	0.010b	1.373a	2.155a
	B	0.003f	1.129b	0.451d
	C	0.007d	1.324a	1.937b
	D	0.008c	1.361a	0.682c
	E	0.005e	ND	ND
	F	0.020a	1.341a	1.608c
NG062	A	0.002c	1.412a	3.301a
	B	0.004a	1.406a	0.657f
	C	0.005a	1.119b	1.528d
	D	0.003b	0.054e	2.005c
	E	0.004b	0.589d	1.003e
	F	0.004b	0.765c	2.649b
JAK	A	0.010b	1.634a	3.628a
	B	0.005e	1.536b	1.477d
	C	0.008c	1.560b	2.330c
	D	0.006d	1.659a	2.344c
	E	0.016a	1.651a	2.710b
	F	0.003f	1.648a	1.146e
DIN	A	0.002e	0.874b	2.190a
	B	0.003d	0.723c	1.745b
	C	0.005c	0.810b	2.341a
	D	0.014a	0.852b	1.801b
	E	0.002e	1.615a	1.201c
	F	0.006b	1.476a	1.745b
JIW	A	0.002d	1.500ab	0.801d
	B	0.003c	1.563a	0.375e
	C	0.004b	1.556a	0.222f
	D	0.005a	1.395ab	1.246c
	E	0.005a	1.392b	1.948b
	F	0.002d	0.770c	2.328a

Values with the same letters in each column are not significantly different from each other at Duncan's multiple range test of $P < 0.05$. ψ_s = osmotic potential, ND = not determined, APX = ascorbate peroxidase, SOD = superoxide dismutase, CAT = catalase

had 85% osmotic tolerant level which was higher than the other accessions followed by NG061 (65%), DIN (55%), NG061 (48%) and JIW (47%). Tolerant level of *D. exilis* to osmotic stress ranged from 85% ~ 47% (Table 7).

Discussion

Accession JAK had a significant high level of proline during osmotic stress. It appeared that accumulation of proline protected plants against oxidative stress through stabilization of antioxidant enzymes. High levels of proline enabled the plant to maintain low water potentials. Due to

Table 4 Percentage (%) inhibition of radicals under different osmotic potentials

ACCESSION	ψ_s	OH ⁻	H ₂ O ₂	NO	DPPH
NG061	A	52.61b	35.67c	17.50e	21.99e
	B	55.02b	31.58d	27.13c	24.81d
	C	53.61b	45.61b	21.76d	21.75e
	D	46.59c	44.44b	25.37c	28.63c
	E	40.36d	50.88a	33.15b	40.07b
	F	62.27a	53.85a	57.64a	63.59a
NG062	A	49.60a	38.60d	38.24b	26.11b
	B	41.77b	45.61c	34.17c	15.99d
	C	50.20a	45.61c	42.13ab	18.21c
	D	51.00a	43.86c	41.20b	37.11a
	E	52.81a	50.88b	39.35b	16.91cd
	F	42.31b	60.07a	45.72a	27.06b
JAK	A	63.86b	ND	13.15e	19.33d
	B	65.26b	54.39a	20.00d	20.93d
	C	46.18d	59.65ab	12.04f	78.29a
	D	53.61c	19.30d	50.65a	49.13c
	E	80.22a	36.84c	29.54c	56.07b
	F	62.25b	51.28b	43.78b	10.17e
DIN	A	59.84a	45.61b	16.57cd	16.91d
	B	62.45a	28.65e	13.06e	26.89b
	C	60.04a	36.84c	17.59c	30.28a
	D	56.63a	32.16d	15.83d	31.15a
	E	60.24a	36.84c	26.39b	18.31d
	F	41.76b	62.27a	31.81a	24.15c
JIW	A	36.55e	31.58c	41.02b	23.69c
	B	43.98d	50.88a	38.61bc	14.34d
	C	56.83b	41.52b	31.02d	29.99a
	D	46.59c	40.35b	31.20d	26.55b
	E	40.36d	53.22a	49.72a	31.44a
	F	62.27a	52.38a	36.87c	26.58b

Values with the same letters in each column are not significantly different from each other at Duncan's multiple range test of $P < 0.05$. ψ_s = osmotic potential, ND = not determined, DPPH = 2, 2-diphenyl-1-picrylhydrazyl hydrate

low water potentials, accumulated compatible solutes osmoregulated the effect of the stress by allowing additional water to be taken up from the environment thus, buffering the immediate effect of water shortages within the organism. With the accumulation of solutes in JAK, the osmotic potential of the cell may have been lowered, which attracts water into the cell hence, provide and support turgor maintenance of the plant tissues. Osmotic adjustment helps to maintain the cell water balance with the active accumulation of solutes in the cytoplasm, thereby minimized the harmful effects of drought stress. The maintenance of turgor despite a decrease in leaf water may have permitted photosynthesis to go on unabated hence; high plant growth was recorded in osmotic-stressed JAK than their unstressed counterparts. Osmotic adjustment is an important trait in delaying dehydration damage in

water-limited environments by continued maintenance of cell turgor and physiological processes. The activity of SOD, CAT and APX varies with the level of drought/osmotic stress. Enzyme SOD was higher in osmotic stressed JAK which played a major role in quenching reactive oxygen. It works as a catalyzer which dismutated singlet O_2^- into H_2O_2 that are later eliminated by CAT and other antioxidant enzymes. Enzyme APX and CAT was high in osmotic stressed JAK than control. Accession JAK had good and consistent high value of APX when subjected to severe osmotic stress. Consequently, the singlet oxygen dismutated by SOD to hydrogen peroxide (H_2O_2) was later converted to water (H_2O) and oxygen (O_2) by CAT in accession JAK which actually made it drought tolerant.

Osmotic stressed accession JAK could scavenge above 50% of OH⁻, H_2O_2 , NO radicals. This could be as a result

Table 5 Lipid peroxidation in *Digitaria exilis* under different osmotic potentials

ACCESSION	ψ_S	MDA (Molarity M)
NG061	A	7.84E-07b
	B	8.99E-07a
	C	8.39E-07ab
	D	8.65E-07c
	E	8.74E-07a
	F	8.42E-07ab
NG062	A	5.96E-07b
	B	5.96E-07b
	C	5.47E-07c
	D	6.59E-07a
	E	6.54E-07a
	F	4.74E-07d
JAK	A	5.07E-07c
	B	4.58E-07e
	C	3.79E-07d
	D	6.18E-07b
	E	5.86E-07b
	F	7.80E-07a
DIN	A	1.58E-06a
	B	1.25E-06b
	C	7.76E-07cd
	D	7.52E-07d
	E	8.50E-07c
	F	6.66E-07e
JIW	A	1.41E-06c
	B	9.04E-07d
	C	2.03E-06a
	D	1.81E-06b
	E	6.94E-07e
	F	5.56E-07f
	LSD (0.05)	3.98×10^{-7}

Values with the same letters in each column are not significantly different from each other at Duncan's multiple range test of $P < 0.05$. ψ_S = osmotic potential, LSD = least significant difference, MDA = Malondialdehyde

Table 6 Correlation among the different assays

	APX	CAT	SOD	PROLINE	MDA	NO	OH ⁻	H ₂ O ₂
APX		0.15	0.16	0.49	-0.24	-0.05	0.25	0.02
CAT	0.15		-0.07	0.04	-0.37	-0.13	0.27	-0.03
SOD	0.16	-0.07		-0.12	0.04	0.01	-0.04	-0.02
PROLINE	0.48	0.04	-0.12		-0.26	-0.06	-0.03	0.05
MDA	-0.24	-0.38	0.04	-0.25		-0.09	-0.04	-0.29
NO	-0.05	-0.13	0.01	-0.06	-0.09		-0.31	0.18
OH ⁻	0.25	0.27	-0.04	-0.03	-0.04	-0.31		-0.24
H ₂ O ₂	0.02	-0.00	-0.02	0.05	-0.28	0.18	-0.24	

of positive correlation between APX and proline. Also, high SOD and CAT found in this accession must have directly caused the inhibition. Ascorbate peroxidase (APX), CAT and SOD were practically stabilized by osmo-regulator proline by removal of superoxide ions which was converted to OH⁻ and later to H₂O₂. Consequently, low lipid peroxidation was observed in osmotic stressed JAK. Lipid peroxidation, in both cellular and organelle membranes, takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also aggravating the oxidative stress through production of lipid-derived radicals. Osmotic stressed DIN, JIW and NG062 had a high lipid peroxidation. It has also been reported that water stress increased the lipid peroxidation, membrane injury index, H₂O₂ and OH⁻ production in leaves of stressed *Phaleolus vulgaris* plants.

Also, the positive correlation between APX and proline could have activated the activity of APX. The high accumulations of proline in JAK under osmotic stress could be responsible for high activities of antioxidant enzymes. These results suggested that accession JAK had higher capacity for osmotic adjustment in terms of accumulating proline, which could maintain water absorption under such harsh conditions. Proline stabilized the activities of CAT thus, low lipid peroxidation with high scavenging activities in accessions JAK were recorded. Osmotic tolerant scoring therefore explained that accession JAK is an osmotic tolerant accession, NG061 and DIN are might mild osmotic tolerant accessions and NG062 and JIW are susceptible to osmotic stress.

Conclusion

Osmotic tolerant ability of accession JAK was due to the accumulation of proline which helps to stabilize activities of enzymes CAT and APX consequently, approximately 50% of hydroxyl, hydrogen peroxide and nitric oxide

Table 7 Percentage osmotic tolerant scoring

ACCESSION	CHL a	CHL b	Total CHL	PROL	APX	SOD	CAT	MDA	OH	H ₂ O ₂	NO	DPPH	% DRGHT TOL
NG061	3	3	3	5	4	3	2	4	3	3	2	4	65
NG062	1	1	1	4	1	1	4	3	2	5	5	1	48
JAK	5	5	5	3	5	4	5	5	5	1	3	5	85
DID	4	4	4	2	3	2	3	2	4	2	1	2	55
JIW	2	2	2	1	2	5	1	1	1	4	4	3	47

PROL = Proline, APX = ascorbate peroxidase, SOD = superoxide dismutase, CAT = catalase, MDA = 2, 2-diphenyl-1-picrylhydrazyl hydrate, DPPH = Malondialdehyde, % DRGHT TOL = Percentage drought tolerant

radicals were successfully scavenged during osmotic stress. Hence, lipid peroxidation was drastically reduced during the stress.

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