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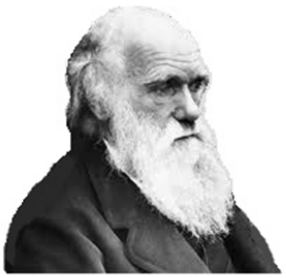

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Influence of *Rhizophagus irregularis* Inoculation on Salt Tolerance in *Cucurbita maxima* Duch.

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ABSTRACT

The influence of mycorrhizal fungi (*Rhizophagus irregularis*) on salt tolerance of *Cucurbita maxima* grown in saline soil was investigated in a pot experiment. The experiment was laid out in a completely randomized design, with treatments replicated thrice. Accordingly, soil salinity significantly (p=0.05) reduced total photosynthetic pigments from 58.06 to 38.06 mg/kg. Mineral contents, biomass yield, AMF colonization were also significantly (p=0.05) reduced. In contrast, mycorrhizal dependency was significantly (p=0.05) increased in saline soil plants (from 26.91% to 66.45%). Furthermore, inoculation with *R. irregularis* significantly (p=0.05) increased total photosynthetic pigments from 58.06 to 62.06 mg/kg; N from 4.88 to 5.47%; P 860.40 to 896.22 mg/kg; K 4430.00 to 4630.00 mg/kg; Mg 558.99 to 592.10 mg/kg and Ca 2810.00 to 3151.00 mg/kg; biomass yield; leaf dry weight from 0.06 to 0.14g; vine dry weight 3.68 to 5.09g; roots dry weight 0.32 to 0.74 and total dry weight 4.06 to 5.97g, *R. irregularis* colonization (from 33.77 to 58.44%) and mycorrhizal dependency in *C. maxima* was evident in both saline and non-saline soil treatments. The results of this work shows that *R. irregularis* can enhance the ability of *C. maxima* to resist salt stress - possibly through some morphological/ physiological changes, as well as improved vigour, probably via the extensive network of the mycorrhizal roots. This last is considered to be one of several mechanisms that magnify the salt tolerance of host plants through increased nutrient acquisition (N, P, K, Mg and Ca) and water uptake. Inoculation with appropriate AMF can, therefore, be used to increase the productivity of *C. maxima* in saline soils.

***Keywords*:** Arbuscular, *Cucurbita maxima*, Mycorrhizal, *Rhizophagus irregularis*, Salinity, Soil Salinity, Stress

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1. INTRODUCTION

Salinity is considered as one of the most important abiotic stresses that limits crop productivity, affecting several aspects of plant metabolism that generally results in the reduction of plant growth in non-halophytes plants [1, 2]. Generally, salinity effects are the combined result of the complex interaction among different morphological, physiological and biochemical processes. Under salt stress condition, plants are often stressed in three ways: (a) low water potential in the root medium which leads to water deficits in plants, (b) the toxic effects of ions, mainly Na and Cl, and (c) nutrient imbalance caused by depression in uptake and/or transport [2]. In addition, production of reactive oxygen species is also a major damaging factor in plants exposed to salinity stress. Osmotic stress as a result of salt stress leads to altered water potential, thereby reducing the water use efficiency of plant due to induction of physiological drought conditions. Whereas ionic stress causes disruption of ion homoeostasis at both cellular and whole-plant levels, oxidative stress elicits release of reactive oxygen species, which inhibit cell growth and plant metabolism.

Experiments carried out to understand arbuscular mycorrhizal fungi (AMF) salinity interaction revealed that mycorrhizal fungi reduce negative effects of these stresses and promote plant growth [3-6]. It has been widely accepted that AMF improve water use efficiency and nutrient uptake of plant under saline condition, thus helping in reducing negative impact of salt stress. AMF diminish detrimental effects of toxic ions on membrane permeability and cell organelle, maintain the level of compatible organic solutes and increase antioxidant production (both enzymatic and non-enzymatic), and positively control expression of salt-related genes. Researchers have presented several physiological, biochemical, and molecular approaches by which AM plants could alleviate salt stress [4, 7]. These are; increased accumulation of osmolytes; control over ion uptake by roots, compartmentation of ions, and their transport into plant tissues to maintain ion homeostasis; increased uptake of water and its distribution to plant tissues with the help of aquaporins.

C. maxima belong to the family Cucurbitaceae; its varieties are used for preparation of many dishes. In Nigeria and other Western African countries, seeds of *C. maxima* are widely used as a vegetable, roasted and salted, or ground into a thick paste that is mixed with vegetables in cooking. This study was undertaken to observe potential interaction between AM fungi and *C. maxima* in salinity conditions and further to observe influence of mycorrhizal inoculation (*R. irregularis*) on salt tolerance of *C. maxima*.

2. MATERIALS AND METHODS

2. 1. Study Area

Saline soil and salt water were collected from the saline ecosystem of Iwuochang, Ibeno Local Government Area (Latitude 4.56° N and Longitude 7.57° E), Akwa Ibom State, Nigeria, with an annual rainfall of about 4021 mm and mean temperature variation of 22–31 °C. The experiment was set up in a safe and secured environment at Mbioto 1, Etinan Local Government Area (Latitude 4.51° N and Longitude 7.50° E), Akwa Ibom State, Nigeria, with an annual rainfall of about 4000 mm and mean temperature variation of 26–36 °C. Non-saline soil for the control and non-saline treatments was obtained from a farmland in Mbioto 1,

Etinan Local Government Area; fresh water was used for watering the non-saline and control treatments.

2. 2. Experimental Materials

The experimental soils were sterilized in bits for two hours in the oven at 100^{o}C to kill weed seeds and soil microorganisms and sieved through a 2 mm mesh to remove pebbles. Matured seeds of *C. maxima* were collected from Akwa Ibom State Agricultural Development Project (AKADEP). AM Fungi *R. irregularis* (60–65 spores per 5 g) was purchased from International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria.

2. 3. Planting

About Five (5) seeds of *C. maxima* were sown in their respective earthen pots filled with about 10 kg of sterilized soils. The plants treated with species of AM fungi were inoculated with about 25 g of *R. irregularis* (60–65 spores per 5 g) was placed in the bucket at 15 cm depth, before planting, the plants inoculated were allowed to establish for up to 2 weeks before being treated with the first dose of salt (30 ml every 3 days). This is to ensure the establishment of AM colonization and avoid sudden plant death due to salinity shock.

Table 1. Experimental Design.

Treatments	Meaning
S- M-	- Salinity, - Mycorrhiza
S+ M-	+ Salinity, - Mycorrhiza
S+ M+ (*Gg*)	+ Salinity, + Mycorrhiza (*R. irregularis*)
S- M+ (*Gg*)	- Salinity, + Mycorrhiza (*R. irregularis*)

2. 4. Estimation of Photosynthetic Pigments

At Leaf chlorophyll meter was used for non-destructive estimation of the total photosynthetic pigments of *C. maxima*.

2. 5. Determination of Mineral Content

The plant samples were transferred to Ministry of Science and Technology, Akwa Ibom State for mineral analysis. Mineral contents: Nitrogen (N) was determined using the Macro-Kjeldahl method while calcium (Ca), magnesium (Mg), potassium (K) and phosphorus (P) of plant samples were determined by atomic absorption spectrophotometer (AAS), flame photometry and spectrophotometry according to the methods of [8, 9].

2. 6. Determination of Moisture Content

The moisture content of the plant samples was determined using the formula:

$$\text{Moisture Content} = \frac{\text{Difference Between Fresh and Dry Weight}}{\text{Fresh Weight}} \times 100$$

2. 7. Determination of Leaf, Shoot and Root Dry Weight

Leaf dry weight was determined by drying the plant leaves, shoots and roots to constant weight in oven at 70 °C.

2. 8. Determination of Root Length

The plate was prepared as follows: a transparent plastic plate/tray (296 × 210 × l mm) was placed on a paper where the appropriate size of the grids was drawn. The grids were masked with 1 mm wide adhesive tape. The grid size was determined depending on the sample size [10]. The number of intersections between grid lines and roots, which appeared as black dots or short lines were counted. To obtain the best estimation, we avoided counting the dots which did not reach the center of the grid width according to the counting rules proposed by Tennant [10].

2. 9. Determination of Root/Shoot Ratio

The plant sample was dried in an oven at 100 °C, the aerial part was cut from the root section and each section was weighed separately. Root/shoot ratio was calculated using the formula:

$$\text{Root/Shoot ratio} = \frac{\text{Dry Weight of Root}}{\text{Dry Weight of Shoot}}$$

2. 10. Quantification of Arbuscular Mycorrhizal Colonization in Plant Roots

Feeder roots of about 2–4 cm of *C. maxima* were separately collected, fixed in 50% ethanol and stored for colonization assessment. The fixed roots were rinsed in tap water before clearing in 10% KOH w/v and autoclaved for about 15 minutes at 121 °C autoclave-resistant glass containers that are less than one-third full to avoid overflow in the autoclave. Cleared roots were collected on a fine sieve and rinsed with water several times before being transferred into the staining solution. Staining of the plants roots was carried out using 5% ink diluted in vinegar (5% acetic acid). The roots segments were soaked in the ink and left in staining solution at room temperature for one day. Stained roots were later destained in 50% glycerol for 1 hour [11].

Stained roots were randomly dispersed in a 9 cm diameter Petri plate with grid lines. Vertical and horizontal gridlines were scanned at ×40 magnification with a dissecting microscope. The proportion of root length that is mycorrhizal and total root length can then be calculated from a conversion factor derived from the total length of grid lines and the area of the dish. A minimum of 100 intersections was used to assess the stained root samples; the samples were re-randomized and counted several times. Mycorrhizal root colonization was thus determined by estimation of percentage of root segments containing hyphae, arbuscules and vesicles [12].

$$\text{MC} = \frac{\text{Total number of roots infected intersecting gridlines}}{\text{Total number of roots intersecting gridlines}} \times 100$$

2. 11. Determination of Mycorrhizal Dependency (MD)

Mycorrhizal dependency (MD) was calculated according to the following formula:

$$\text{MD} = \frac{\text{DW inoculated Plants} - \text{DW non-inoculated Plants}}{\text{DW inoculated Plants}} \times 100$$

2. 12. Statistical Analysis

The study was conducted using complete randomized design with six (4) treatments with three (3) replicates. All data in the present study were subjected to analysis of variance (ANOVA) using Statistical package for Social Sciences and data are presented as standard error of mean (± S.E.M.) of triplicate experiments. The differences between the means were separated and compared using the Duncan's multiple range tests. However, a probability level of p=0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

Table 2. The Effect of *Rhizophagus irregularis* Inoculation on the Photosynthetic Pigments Content of *C. maxima* Grown in Saline Soil

Treatments	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Carotenoids (mg/g)	Total Photosynthetic Pigments (mg/g)
S- M-	*41.43 ± 3.41[a]	12.83 ± 1.67[a]	3.80 ± 0.34[a]	58.06 ± 4.13[a]
S+ M-	29.16 ± 1.24[b]	6.22 ± 0.12[b]	2.68 ± 0.21[b]	38.06 ± 4.05[b]
S+ M+ (*Ri*)	40.82 ± 2.39[a]	12.22 ± 1.89[a]	3.80 ± 0.63[a]	56.84 ± 2.66[a]
S- M+ (*Ri*)	43.98 ± 3.38[a]	13.66 ± 2.05[a]	4.42 ± 3.41[a]	62.06 ± 6.31[a]

*Mean of three replicates ± SEM. [a]Means within of each column followed by different letters are significantly different at p=0.05 according to Duncan's Multiple Range Test. S- (No salinity), M- (No mycorrhiza), S+ (Plus salinity), M+ (Plus mycorrhiza), (Ri) – *Rhizophagus irregularis*.

Results from this study revealed that total photosynthetic pigments (TPP) contents of *C. maxima* such as chlorophyll a, chlorophyll b and carotenoids grown in saline soil were significantly (p=0.05) reduced when compared to the control (Table 2). Inoculation with AMF significantly (p=0.05) increased these pigments in the test plant both in saline and non-saline soil treatments (Table 2). Lee *et al.* [13] in their study on *Paspalum vaginatum* L. and Siler *et al.* [14] in their study on *Centaurium erythraea* L. reported that chlorophyll 'a', 'b' and total chlorophyll decreased with the increase of salt concentrations. Supporting results also includes the works of Turan *et al.* [15] on bean plant *P. vulgaris* L., Cheruth *et al.* [16] on *Catharanthus roseus* L., Taffouo *et al.* [17] on cowpea (*V. unguiculata* L.) and Taffouo *et al.*

[18] on *Vigna subterranean* (L.). All demonstrated that salt stress of sodium chloride caused a decrease in total chlorophyll content. This could possibly have been as a result of the suppression of precursors of chlorophyll biosynthesis [19], and lowering of magnesium uptake [20]. Salinity-AMF interaction study on maize have shown that, improved photosynthetic capacity of maize plants by increasing the capacity of gaseous exchange and the efficiency of PS II as well as regulation of the energy flow between photochemical and non-photochemical reactions [20]. Wu *et al.* [5] observed increased photosynthetic rates and stomatal conductance in AMF over non-AMF plants under salinity stress.

From this study, salt stress significantly (p=0.05) reduced biomass yield of *C. maxima.* However, inoculation of the test plant with AMF significantly (p=0.05) increased the biomass yield of *C. maxima* in saline and non-saline soil treatments. The highest growth enhancement was observed when AMF inoculation was done (Table 3). Plant growth and biomass production is an integrative dimension of plant response to the stress conditions; therefore, the symbiotic effectiveness of AM fungi has been measured in terms of plant growth or biomass accumulation [7, 21]. Inoculation of *C. maxima* with arbuscular mycorrhizal fungi (AMF) (*R. irregularis*) in conjunction with saline soil significantly (p=0.05) increased their biomass accumulation above the control in both saline and non-saline soil treatments. Higher biomass yield in mycorrhizal plants grown in soil amended with organic matter has been reported by Okon *et al.* [22] who suggested a possible synergistic effect of AMF and organic matter.

Table 3. Effect of *Rhizophagus irregularis* Inoculation on the Biomass Yield of *C. maxima* Grown in Saline Soil

Treatments	Leaf Dwt (g)	Vine Dwt ($g \cdot plant^{-1}$)	Roots Dwt ($g \cdot plant^{-1}$)	Total Dry Weight ($g \cdot plant^{-1}$)	Root/Shoot ratio
S- M-	0.06^{b}	3.68^{b}	0.32^{b}	4.06^{b}	0.09^{c}
S+ M-	0.00	0.00	0.00	0.00	0.00
S+ M+ (*Ri*)	0.06^{b}	2.68^{b}	0.30^{b}	3.04^{b}	0.11^{b}
S- M+ (*Ri*)	0.14^{a}	5.09^{a}	0.74^{a}	5.97^{a}	0.15^{a}

*Mean of three replicates ± SEM. [a]Means within of each column followed by different letters are significantly different at p=0.05 according to Duncan's Multiple Range Test. S- (No salinity), M- (No mycorrhiza), S+ (Plus salinity), M+ (Plus mycorrhiza), (Ri) – *Rhizophagus irregularis*, 0.00 (Indicates that the plants were dead).

The mineral composition of *C. maxima* (N, P, K, Mg and Ca) were significantly (p=0.05) reduced in saline soil treatments in this study (Table 4). This is corroborated by the work of Evelin *et al.* [23] who reported that total N concentration in shoot and root of fenugreek plants was severely affected by NaCl-induced salinity in the soil. This negative effect of salt on N uptake of *C. maxima* is clearly seen in this study. Similarly, Cantrell and Linderman [24] on lettuce and onions plants, Silveira *et al.* [25] on cowpea plants and Colla *et al.* [26] in zucchini plants who all reported reduction of N, P, K, Mg and Ca composition with

increase in salinity. AMF application improved mineral uptake of *C. maxima* and this agrees with the findings of Evelin *et al.* [23] that inoculation with *G. intaradices* (*R. irregularis*) improved the N, P, K, Mg and Ca concentration in leaves, shoot and root of fenugreek plants over non-inoculated plants.

Table 4. Effect of *Rhizophagus irregularis* Inoculation on the Mineral Nutrient Contents of *C. maxima* Grown in Saline Soil

Treatments	N (%)	P (mg/kg)	K (mg/kg)	Mg (mg/kg)	Ca (mg/kg)
S- M-	*4.88[a]	860.40[b]	4430.00[c]	558.99[b]	2810.00[c]
S+ M-	0.00	0.00	0.00	0.00	0.00
S+ M+ (*Ri*)	4.87[a]	807.01[c]	4451.11[b]	562.77[c]	2870.01[b]
S- M+ (*Ri*)	5.47[a]	896.22[a]	4630.00[a]	592.10[a]	3151.00[a]

*Mean of three replicates ± SEM. [a]Means within of each column followed by different letters are significantly different at p=0.05 according to Duncan's Multiple Range Test. S- (No salinity), M- (No mycorrhiza), S+ (Plus salinity), M+ (Plus mycorrhiza), (Ri) – *Rhizophagus irregularis*, 0.00 (Indicates that the plants were dead).

AMF root colonization (MC) of *C. maxima* was significantly (p=0.05) reduced in saline soil treatment when compared to non-saline treatments (Table 5). This agrees with the work of Beltrano *et al.* [27] who reported roots of pepper plants were highly colonized by *R. irregularis* and were higher than other reports by Kaya *et al*. [28] with *G. clarum*, Cekic *et al*. [29] with *G. mosseae* and *R. irregularis*. The ability of *R. irregularis* to colonize the roots of pepper plants declined with increasing NaCl levels [31] as also observed in this study.

Table 5. *Rhizophagus irregularis* Root Colonization of *C. maxima* Grown in Saline Soil

Non-inoculated treatment	Root colonization (%)	Mycorrhizal Dependency (%)	Inoculated treatments	Root colonization (%)	Mycorrhizal Dependency (%)
S-M-	0.00	0.00	**S+M+ (*Ri*)**	*33.77[c]	66.45[a]
S+M-	0.00	0.00			
			S-M+ (*Ri*)	58.44[a]	26.91[b]

*Mean of three replicates. [a]Means within of each column followed by different letters are significantly different at p=0.05 according to Duncan's Multiple Range Test. S- (No salinity), M- (No mycorrhiza), S+ (Plus salinity), M+ (Plus mycorrhiza), (Ri) – *Rhizophagus irregularis*.

4. CONCLUSION

The influence of soil salinity and mycorrhizal fungi (*R. irregularis*) symbiotic association on salt tolerance of the *C. maxima* was analyzed in this study. Using different mechanisms, *C. maxima* by itself or in association with mycorrhizal fungi can tolerate or survive the stress. However, in the presence of *R. irregularis*, the test plant ability to resist the stress increases as a result of morphological and physiological changes. Production of different solutes, plant hormones, antioxidant products, extensive network of the mycorrhizal plant roots, and enhanced nutrient uptake are all among the processes that make the plant to survive under stress. Inoculation with appriopriate AMF can therefore be used to increase the productivity of *C. maxima* in saline soils.

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