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Physiological and biochemical responses of *Annona squamosa* genotypes to progressive salinity stress levels

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Abstract

Salinity stress is one of the major abiotic constraints limiting crop production worldwide, and its effect on fruit crops like *Annona squamosa* genotypes remains poorly understood. Using Factorial Randomized Block Design (FRBD), an experiment on the physiological and biochemical responses of *Annona squamosa* genotypes to progressive salinity stress levels was carried out at the North Farm Polyhouse, Karunya Institute of Technology and Sciences, Coimbatore, in 2025-2026 to determine the optimum salt-tolerant genotype. Five genotypes (Nattu Sita, Balanagar, NMK Golden, and Red Sita) were tested at different salt concentrations. The result revealed that out of 4 genotypes evaluated, NMK Golden was found to be superior, with the highest chlorophyll concentration (39.619), highest proline accumulation (0.143 $\mu\text{mol g}^{-1}$ FW), lowest electrolyte leakage (32.074%), lowest rate of decrease in chlorophyll concentration (3.31%), lowest rate of decrease in relative water content (3.11%), and highest rate of increase in proline accumulation (23.06%). Golden demonstrated the best performance under salinity stress. Hence, NMK Golden can be used as a salinity-tolerant genotype under Coimbatore.

Keywords: *Annona squamosa*, salinity, proline, electrolyte leakage, relative water content, chlorophyll concentration

Introduction

Salinity is a major setback in most of the fruit crops. Over 20 percent of the world's irrigated land is already compromised by salinity, and this number is rising at a rate of 10 percent annually (Shrivastava and Kumar, 2015) [30]. There are more underutilized fruit crops that possess a high level of health benefits. Among those, custard apple is one that belongs to the *Annona* genus, and it is widely distributed in the wild worldwide. Native to the Caribbean, Central America, and northern South America, this fruit has gained global recognition not only for its sweet, creamy pulp but also for its numerous health benefits and medicinal properties. (Martínez *et al.*, 2013; Gautam *et al.*, 2024) [22, 13].

It is also a powerhouse of essential nutrients, including vitamins, minerals, and antioxidants. It is particularly rich in vitamin C, a potent antioxidant that supports the immune system, helps combat free radicals, and promotes skin health. The fruit also contains significant amounts of dietary fibre, which aids in digestion and helps prevent constipation. The mineral content of custard apple is also noteworthy, with high levels of potassium, magnesium, and calcium, all of which are essential for maintaining heart health, bone density, and muscle function. Moreover, the fruit contains beneficial compounds such as polyphenols, flavonoids, and carotenoids, which contribute to its antioxidant and anti-inflammatory properties (Gautam *et al.*, 2024) [13]. Annonaceae is found in the tropics and subtropics of both the Old and New Worlds, well-adapted to altitudes below 1,000 msl and to annual rainfall of more than 1,000 mm, with a dry period during flowering. It is adapted to both tropical and subtropical areas. Fruits of all *Annona* spp. are climacteric with a rapid ripening after harvest. Besides their use in fruit production, *Annona* spp. are valued for their essential oils, insecticidal compounds, medicinal compounds, and wood (Leal and Paull, 2023) [17]. Salinity stress drastically reduces crop yields, affecting plant growth and restricting land use. As the world population is increasing at an alarming rate, agricultural land is shrinking due to industrialization and habitat use.

Hence, there is a need to utilize salt-affected land to meet food requirements. Excessive salt above what plants need limits plant growth and productivity and can lead to plant death (Safdar *et al.*, 2019) ^[27].

Increased chloride concentration and decreased nitrogen concentration near the root zone. The sodium response indicated that some form of exclusion or compartmentation occurred. Salinity reduced the osmotic potential of root tissue but did not influence foliar osmotic or predawn xylem potential. These results indicate that *A. squamosa* is sensitive to salinity stress and that its responses to salinity are consistent with those of other salt-sensitive woody perennial species (Marler *et al.*, 1996) ^[21].

Even though *Annona* is a popular dryland fruit crop, little attention has been given to the physiological response under abiotic stress conditions, particularly salinity. The present study aimed to evaluate the effects of salt stress on physiological and growth parameters of *Annona squamosa*, a locally available species in India. This may help breeders to develop abiotic stress tolerance in *Annona squamosa* varieties/hybrids.

Materials and Methods

Experimental Location

The research study was conducted on “Physiological and biochemical responses of *Annona squamosa* genotypes to progressive salinity stress levels” during 2025-2026 at the North Farm Polyhouse, Karunya Institute of Technology and Sciences, Coimbatore, Tamil Nadu, India, from December to February.

Collection of plants

A salinity tolerance screening experiment will be conducted using selected genotypes of *Annona*, obtained from diverse agro-climatic regions, to ensure genetic variability. Seedlings will be procured from certified nurseries located in and around Tamil Nadu to ensure genetic authenticity and quality of planting material. Healthy, uniform seedlings of each genotype will be transplanted into plastic bags filled with a well-drained potting mixture of soil, sand, and farmyard manure in a 2:1:1 ratio.

Application of Treatment

Five salinity levels were established using sodium chloride (NaCl) solutions at EC values of 3.5, 4.0, 4.5, 5.0, and 5.5 $dS\ m^{-1}$, with the 3.5 $dS\ m^{-1}$ treatment serving as the reference benchmark, representing a mildly saline condition. Salinity stress was imposed 60 days after transplanting by irrigating with NaCl-supplemented water to achieve these predetermined electrical conductivity (EC) levels, as measured using an EC meter, and maintained consistently throughout the experimental period. The physiological and biochemical responses of *Annona squamosa* genotypes were then evaluated against progressively elevated salinity stress conditions.

Plant height (cm)

Plant height was measured using a measuring scale from the soil level to the tip of the largest leaf. The average is taken from three replications and expressed in cm.

Chlorophyll Concentration

The amount of chlorophyll concentration in leaves after the 60th day of treatment imposition is measured using a digital

SPAD meter (SPAD-502 Plus).

Electrolyte Leakage (%)

A measure of membrane injury via ion leakage from tissue (Lutts *et al.*, 1996) ^[19]. Twenty discs (1 cm^2) of different *Annona* genotype leaves were put into flasks, each containing 25 mL of deionized water. The samples were shaken for 20h at ambient temperature. First, electrical conductivity (C_1) was recorded for each vial. Flasks were then submerged in a hot water bath at 80 °C for 1 hour. The vials were again shaken for 20 hours at 21 °C. Last, conductivity (C_2) was measured for each flask (Dionisio-Sese and Tobita, 1998) ^[10]. It was then calculated by using the formula.

$$EL\% = \frac{C_1}{C_2} \times 100$$

C_1 = conductivity after incubation

C_2 = conductivity after boiling

Relative Water Content (%)

Relative water content (RWC) is a critical physiological trait that indicates a plant's ability to maintain water balance under stress conditions. Different *Annona* genotypes of leaf discs (1 cm in diameter) were taken from the fully expanded leaves. The fresh weight (FW) was recorded, and then the discs were incubated in distilled water for 4 h. Turgid weight was recorded, and the discs were put in an oven at 70 °C for 24 h to calculate the dry weight (DW). Relative water content was measured by the methodology of (Sanchez *et al.*, 2004) ^[28]. Then it was calculated by using the formula.

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

FW-Fresh Weight

DW-Dry Weight

TW-Turgid Weight

Proline Accumulation ($\mu\text{mol g}^{-1}$ FW)

The measure of quantification of free proline as an osmoprotectant under stress. To measure proline accumulation, a solution of glacial acetic acid, proline, and ninhydrin acid (1:1:1) was incubated for 1 h at 90 °C. Then, the samples were cooled in an ice bath. The extraction was done using 2 mL of toluene, and the absorbance was determined at 520nm using a spectrophotometer as $\mu\text{g g}^{-1}$ FW (Bates *et al.*, 1973) ^[4]. It was then calculated by using the formula.

$$\text{Proline } (\mu\text{mol g}^{-1} \text{ FW}) = \frac{\text{Abs}_{520} \times \text{ml toluene}}{115.5 \times \text{g sample}}$$

Statistical Analysis

The experimental design followed was Factorial Randomized Block Design (FRBD) with genotypes and salinity levels as factors, replicated three times. Consisting of four genotypes (Balanagar, Golden Sita, Red Sita, Nattu Sita) and five salinity levels (3.5, 4.0, 4.5, 5.0 $dS\ m^{-1}$) respectively. All obtained data were subjected to an ANOVA analysis using OPSTAT, and significant differences between treatments were indicated by $p < 0.05$.

Results and Discussion

Chlorophyll Concentration

60 days after the imposition of salinity, the concentration of chlorophyll was measured. Table 1 shows that NMK Golden (V_3) outperformed all other *Annona squamosa* varieties, with the highest chlorophyll concentration (39.619) and the lowest (27.999) recorded in Nattu Sita. According to Table 2, the low EC concentration of 3.5 dS m^{-1} (T_1) had the highest chlorophyll concentration (78.698), while the high EC concentration of 5.5 dS m^{-1} (T_5) had the lowest (66.933). Table 3 depicts the combination effects of *Annona* varieties with different levels of EC concentrations, and the rate of reduction was calculated from the treatment combinations. Table 4 shows that a lower rate of reduction percentage of chlorophyll concentration (3.31%) is obtained in the treatment combinations of the NMK Golden variety compared to all other combinations. The above findings show that an increase in the EC concentrations led to a decrease in the chlorophyll concentration, thus proving that salinity affects the chlorophyll concentration. The presence of salts in the soil solution may favour increased chlorophyll content in salt-tolerant plants and decreased chlorophyll content in more susceptible plants, and the chlorophyll index reduction may be associated with reactive oxygen species formation and with the photoinhibition of photosynthesis (Heidari, 2012) [14]. These results are in line with Figueiredo *et al.* (2019) [12], who reported higher chlorophyll indexes at 0.5 dS m^{-1} and lowest values at 4.0 dS m^{-1} in sugar-apple plants, and Ferreira *et al.* (2021) [11], who observed the highest Chlorophyll total concentration ($2.74 \text{ mg g}^{-1} \text{ FM}$) at the lowest electrical conductivity (0.8 dS m^{-1}). The reduction in chloroplast pigment concentrations occurs due to degradation of pigment molecules and reduction in the chlorophyll synthesis process induced by high salinity conditions, which was further corroborated by Oliveira *et al.* (2018) [25], who reported reductions of up to 27.43% in total Chlorophyll concentrations in jackfruit plants irrigated with waters of 4.0 dS m^{-1} .

Electrolyte Leakage (%)

According to Table 1, NMK Golden (V_3) outperformed all other *Annona squamosa* types, with the lowest electrolyte leakage (32.074%) and the greatest (78.124%) recorded in Nattu Sita. Table 2 demonstrates that a rise in EC concentrations caused an increase in electrolyte leakage, with the lowest electrolyte leakage (56.040%) observed in low EC concentration of 3.5 dS m^{-1} (T_1) and the highest electrolyte leakage (66.943%) observed in high EC concentration of 5.5 dS m^{-1} (T_5). The combination effects of *Annona* types with varying EC concentrations are shown in Table 3, and the rate of reduction was determined from the treatment combinations. Table 4 demonstrates that, in comparison to all other combinations, the Red Sita variety treatment combinations yield a reduced rate of increase in the percentage of electrolyte leakage (5.28%). The above findings show that an increase in the EC concentrations led to an increase in the electrolyte leakage, thus proving that salinity results in increased membrane damage. Plasma membranes are the primary site of ion-specific salt injury (Mansour and Salama, 2004) [20], and electrolyte leakage is reported as one of the most important selection criteria for identification of salt-tolerant plants (Ashraf and Ali, 2008) [2], with a cell deemed damaged when electrolyte leakage

surpasses 50%. It was also observed that relative water content correlated positively with all variables, except electrolyte leakage (93%), further confirming the inverse relationship between water retention and membrane damage under salinity stress (Edmilson Júnio Medeiros Caetano *et al.*, 2024) [5]. These findings are in line with Santos *et al.* (2023) [29] and Lima *et al.* (2023) [9], who reported increased electrolyte leakage with increasing salinity levels in passion fruit plants, and similarly, Hnilíčková *et al.* (2019) [15] observed that electrolyte leakage progressively increased with increasing NaCl concentrations in lettuce and other plant species.

Relative Water Content (%)

Table 1 shows that Red Sita (V_4) outperformed all other *Annona squamosa* varieties, with the highest relative water content (83.456%) and the lowest (58.840%) recorded by Nattu Sita. According to Table 2, the low EC concentration of 3.5 dS m^{-1} (T_1) had the highest relative water content (78.698%), while the high EC concentration of 5.5 dS m^{-1} (T_5) had the lowest (66.933%). Table 4 demonstrates that treatment combinations of the NMK Golden variety had the highest rate of reduction percentage of relative water content (3.11%) when compared to all other combinations. This suggests that an increase in EC concentrations caused a decrease in relative water content, demonstrating that salinity has an impact on plants' relative water content. These findings are in line with Santos *et al.* (2023) [29], who reported that irrigation with water having an electrical conductivity of 0.6 dS m^{-1} induced a reduction in relative water content and gas exchange in sour passion fruit plants, and Lima *et al.* (2023) [9], who highlighted that irrigation with water EC of 0.3 dS m^{-1} or higher reduces the relative water content in passion fruit plants. It was also observed that relative water content significantly decreased (62 and 50%) at salinity levels of 1500 and 3000 ppm of NaCl compared to controls (82%) in sweet pepper plants (Abdelaal *et al.*, 2020) [1]. The decreased relative water content occurs as a result of the negative effect of salinity on water absorption capacity from the soil to the root system, reducing the internal water supply and negatively affecting plant organs (Liang *et al.*, 2002; Muneer *et al.*, 2014) [18, 23].

Proline Accumulation ($\mu\text{mol g}^{-1} \text{ FW}$)

Table 1 shows that NMK Golden (V_3) outperformed all other *Annona squamosa* varieties, with the highest proline accumulation ($0.143 \mu\text{mol g}^{-1} \text{ FW}$) and the lowest ($0.044 \mu\text{mol g}^{-1} \text{ FW}$) observed in Nattu Sita (V_1). According to Table 2, the low EC concentration of 3.5 dS m^{-1} (T_1) had the lowest proline accumulation ($0.075 \mu\text{mol g}^{-1} \text{ FW}$), whereas the high EC concentration of 5.5 dS m^{-1} (T_5) had the highest proline accumulation ($106 \mu\text{mol g}^{-1} \text{ FW}$). Table 4 shows that the highest rate of increase in the percentage of proline accumulation (23.06%) was obtained in treatment combinations of NMK Golden variety compared to all other combinations, indicating that an increase in EC concentrations led to an increase in proline accumulation, thus proving the positive effects of proline accumulation under salinity conditions. Proline plays multiple roles as an osmolyte, scavenging reactive oxygen species (ROS), controlling cell redox homeostasis, and supplying energy (Dawood *et al.*, 2014) [8], and this increment of proline is one of the typical responses to stress factors, particularly salinity stress, which plays a pivotal role in protecting

chlorophyll pigments from degradation (Yin *et al.*, 2013)^[33]. These findings are in line with Abdelaal *et al.* (2020)^[1], who reported that proline concentrations (18 and 24 $\mu\text{g g}^{-1}$

FW) significantly increased in sweet pepper plants under salinity conditions compared to controls (11 $\mu\text{g g}^{-1}$ FW), further corroborating the present findings.

Table 1: Influence of different varieties on the morphological and biochemical parameters of *Annona squamosa*

Variety	Chlorophyll concentration (SPAD value)	Electrolytic leakage (%)	Relative water content (%)	Proline ($\mu\text{mol g}^{-1}$ FW)
V ₁ : Nattu sita	27.999	78.124	58.840	0.044
V ₂ : Balanagar	31.086	49.252	77.946	0.100
V ₃ : NMK Golden	39.619	32.074	73.130	0.143
V ₄ : Red sita	34.233	88.266	83.456	0.074
C.D. (5%)	3.172	1.009	2.010	0.007
SE(d)	1.561	0.497	0.989	0.003
SE(m)	1.104	0.351	0.699	0.002

Table 2: Influence of varying EC concentrations on the biochemical parameters of *Annona squamosa* genotypes

Treatment	Chlorophyll concentration (SPAD value)	Electrolytic leakage (%)	Relative water content (%)	Proline ($\mu\text{mol g}^{-1}$ FW)
T ₁ : 3.5 dS m ⁻¹	78.698	56.040	78.698	0.075
T ₂ : 4 dS m ⁻¹	76.250	58.610	76.250	0.081
T ₃ : 4.5 dS m ⁻¹	74.955	63.088	74.955	0.090
T ₄ : 5 dS m ⁻¹	69.880	64.965	69.880	0.100
T ₅ : 5.5 dS m ⁻¹	66.933	66.943	66.933	0.106
C.D. (5%)	NS	1.129	2.247	0.008
SE(d)	1.745	0.555	1.106	0.004
SE(m)	1.234	0.393	0.782	0.003

Table 3: Interaction effect of different varieties and varying EC concentrations on the morphological and biochemical parameters of *Annona squamosa* genotypes

Variety + treatment	Chlorophyll concentration (SPAD value)	Electrolytic leakage (%)	Relative water content (%)	Proline ($\mu\text{mol g}^{-1}$ FW)
V ₁ T ₁	69.23	72.38	69.23	0.037
V ₁ T ₂	66.67	78.98	66.67	0.043
V ₁ T ₃	66.67	79.08	66.67	0.044
V ₁ T ₄	51.28	79.61	51.28	0.048
V ₁ T ₅	40.35	80.57	40.35	0.048
V ₂ T ₁	80.56	41.70	80.56	0.088
V ₂ T ₂	80.00	42.10	80.00	0.093
V ₂ T ₃	78.13	51.23	78.13	0.105
V ₂ T ₄	75.68	54.55	75.68	0.106
V ₂ T ₅	75.36	56.68	75.36	0.107
V ₃ T ₁	75.00	25.66	75.00	0.120
V ₃ T ₂	75.00	28.66	75.00	0.121
V ₃ T ₃	72.97	32.46	72.97	0.130
V ₃ T ₄	71.43	35.68	71.43	0.163
V ₃ T ₅	71.25	37.91	71.25	0.181
V ₄ T ₁	90.00	84.42	90.00	0.054
V ₄ T ₂	83.33	84.70	83.33	0.066
V ₄ T ₃	82.05	89.58	82.05	0.080
V ₄ T ₄	81.13	90.02	81.13	0.084
V ₄ T ₅	80.77	92.61	80.77	0.087
C.D. (5%)	4.494	2.257	4.494	0.016
SE(d)	2.211	1.111	2.211	0.008
SE(m)	1.564	0.785	1.564	0.005

Table 4: Rate of change in growth and physiological parameters of *Annona squamosa* genotypes under progressive salinity stress

Variety	Chlorophyll concentration	EL	RWC	Proline
V ₁ : Nattu sita	18.75%	9.00%	18.75%	1.55%
V ₂ : Balanagar	4.05%	17.00%	4.05%	17.43%
V ₃ : NMK Golden	3.31%	12.90%	3.11%	23.06%
V ₄ : Red sita	9.08%	5.28%	9.08%	9.03%

Conclusion

The study on physiological and biochemical responses of *Annona squamosa* genotypes to progressive salinity stress levels revealed that salinity stress significantly influenced all the biochemical and physiological parameters assessed. An increase in EC concentrations consistently led to a decrease in plant height, chlorophyll concentration, and relative water content, while proline accumulation increased

with increasing salinity levels, proving that proline plays a positive and protective role in plants against salinity stress conditions. Among all the genotypes evaluated, NMK Golden performed the best under salinity stress, exhibiting higher proline accumulation and better chlorophyll retention compared to Balanagar, Red Sita, and Nattu Sita, indicating a stronger osmotic adjustment mechanism and superior salinity tolerance. These findings confirm that genotypic variation exists among *Annona squamosa* varieties in their response to salinity stress. Therefore, future research can focus on utilizing NMK Golden as a potential parent in breeding programs aimed at developing salinity-tolerant *Annona squamosa* varieties, as well as exploring its use as a suitable rootstock in propagation studies to confer salinity tolerance to other genotypes. Such studies will help in utilizing salt-affected lands for *Annona* cultivation, ultimately contributing to enhanced fruit production and improved livelihood of farmers.

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