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Ullah, Abd; Tariq, Akash; Zeng, Fanjiang; [et al.]. «Application of GABA (g-aminobutyricacid) to improve saline stress tolerance of chufa (CyperusesculentusL.var.sativusBoeck) plants by regulating their antioxidant potential and nitrogen assimilation». South African Journal of Botany, Vol. 157 (June 2023), p. 540-552. DOI 10.1016/j.sajb.2023.04.027

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Application of GABA (γ-aminobutyric acid) to improve saline stress tolerance of chufa (*Cyperus esculentus* L. var. sativus Boeck) plants by regulating their antioxidant potential and nitrogen assimilation

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Abstract

The chufa is a perennial plant known for its almond-like underground tubers that have several health benefits for humans. GABA (γ-amino butyric acid) can regulate plants' tolerance to stress including tolerance to salinity. However, it is unknown how chufa plants respond to GABA application and salinity. Here, we analysed the impact of GABA (2 mM) on growth features and physio-biochemical changes in chufa plants under different saline stress levels (SS; 0, 100 and 200 mM NaCl and Na_2SO_4, 1:1 molar ratio). Our findings show that increasing SS levels negatively affected growth and metabolism. Increased SS reduced growth (shoot and root length, and their fresh and dry weights) and photosynthetic pigments, and significantly increased hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and osmolytes (i.e. soluble sugars, proline, soluble protein and superoxide dismutase (SOD). Peroxidase (POD) significantly declined at 50 mM and catalase (CAT) decreased under both stress levels. SS significantly enhanced salt ions (Na^+ and $Cl^–$) in roots and leaves but decreased root K^+ and increased Mg^{2+} at 100 mM, levels that then declined under 200 mM SS. However, foliar K^+ and Mg^{2+} remained unchanged. As well, $NO_3^–$ and NH_4^+ concentrations and nitrate reductase (NR) and glutamine oxoglutarate aminotransferase (GOGAT) declined significantly, while glutamine synthetase (GS) remained unaffected. However, exogenous GABA significantly reduced Na^+ and $Cl^–$ ions, H_2O_2 and MDA and improved chlorophyll pigments, osmolytes, Mg^{2+} and K^+, antioxidant enzymes and N metabolism compared to untreated GABA peers. GABA might thus be able to improve the salinity stress tolerance of chufa by improving its cellular metabolism and preventing the build-up of harmful substances. Hence, exogenous GABA could be an effective approach for increasing salinity tolerance in chufa plants in salt-affected regions. Future studies should aim to elucidate the molecular mechanisms that operate when GABA regulates saline stress tolerance in chufa plants.

Keywords: Abiotic stress, salinity, antioxidant mechanism, growth regulators, stress alleviation

1. Introduction

Worldwide agriculture is under threat from soil salinity, which today affects 3600 million ha (Mha) of arable land out of a global total of 5200 Mha. This results in an annual loss of USD 27.5 billion (Qadir et al., 2014; Zhang and Shi, 2013). Furthermore, it is estimated that the human population will rise to ≈ 10 billion by 2050, thereby requiring a 70% increase in agricultural food production, which will exacerbate the decline in available arable land (Calone et al., 2021). Climate change and inappropriate agricultural practices will further aggravate the problem of soil salinity, which renders soils infertile and unsuitable for agriculture (Wang et al., 2008). Salt-contaminated groundwater is still used for irrigation in many drought-prone regions worldwide (Minhas et al., 2019). Toxic levels of salt ions accumulate in plants growing in salt-affected soils, resulting in osmotic stress, impaired gas exchange, photosynthesis inhibition, cellular ionic imbalance, impaired nutrient acquisition, oxidative stress, membrane disorganization, metabolic toxicity and impaired respiration (Munns and Tester 2008; Zhao et al. 2020; Ullah et al., 2022a). Plants use several mechanisms to protect themselves from salinity-induced stress damages including (i) the protection of osmolytes, (ii) the exclusion of toxic salt ions (Na^+ and Cl^-) or their compartmentalisation in vacuoles and old tissues, and (iii) biosynthesis and the upregulation of antioxidant mechanisms (Mostofa et al., 2021; Rahman et al., 2019; Zaid and Wani, 2019).

Plants under stress accumulate salt-relieving solutes (proline, soluble sugars and glycine betaine, etc) known as osmolytes that help them maintain salinity homeostasis. Their functions can be summarized as maintaining water balance, protecting plants from salinity-induced damage, preventing ion toxicity and chlorophyll destruction, regulating cell division, stabilizing cellular structures, and scavenging ROS (Trovato et al., 2008; Singh et al., 2022). Soil salinity (SS) can also cause photoinhibition and, as a result, untapped energy accumulates, thereby producing reactive oxygen species (ROS) and diminishing photosynthesis (Gururani et al., 2015; Tokarz et al., 2021; Ullah et al., 2022b). Furthermore, SS can weaken antioxidant systems and damage membrane structure and permeability (Tanveer, 2020). In this regard, it has been demonstrated that plant salinity stress tolerance is linked to an increase in antioxidant defence mechanisms (Wang et al. 2009).

Plants need nitrogen (N) for optimum growth and development since N is a crucial constituent of their photosynthetic capacity. The activities of N-metabolizing enzymes have been reported to be impaired by salt ions and lead to a decline in NO_3^- and NH_4^+ concentrations in cells (Miura, 2012; Meng et al., 2016; Ashraf et al., 2018; Ullah et al., 2019; Ullah et al., 2022a). The impact of salinity in plants is highly affected by the regulation of N assimilation. As a result, there is an intricate relationship between salinity stress tolerance and N metabolism, which is highly dependent on the intensity and duration of soil salinity, and the form and quantity of soil N (Dai et al., 2015; Teh et al., 2016).

Chufa (*Cyperus esculentus* L. var. sativus Boeck; family Cyperaceae), also known as cultivated yellow nutsedge or tiger nut, is a perennial crop species that is often overlooked. The underground tubers of this plant are sweet, tasty and nutritious, and have several health benefits. The nutrients in its tubers include 5–10% protein, 15–20% sugar, 20–30% oil, 25–40% starch, 8–10% dietary fibres, and vitamins E and C (0.08–0.14 g/kg), as well as minerals such as Ca, K and P (Sanchez- Zapata et al., 2012; Bado et al., 2015; Maduka and Ire, 2018). *Horchata*, a milk-like non-alcoholic drink made from its tubers, has recently been the subject of scientific research (Sánchez- Zapata et al., 2012). Given the significant amount of nutrients and bioactive compounds they contain, tiger-nut tubers should be regarded as a significant species for both animals and humans (Maduka and Ire, 2018).

In light of the ever-increasing human population, the use of this underused and often neglected plant could contribute to alleviating poverty and enhancing food security (Calone et al., 2021). Chufa species prefer moist sandy soils (pH 5.0–7.5) and can withstand drought and flooding, as well as soil temperatures as low as -5 °C. Despite this, they are not tolerant of salinity (Tucson and Arizona, 2003). Tiger nuts have been investigated from various perspectives including plant characterisation, cultivar selection, cultivation methods, irrigation, fertilization and nutrition (Pascual-Seva et al., 2018), alkalinity stress and the application of exogenous naphthaleneacetic acid (NAA) (Ullah et al., 2023). However, it is unknown how chufa plants respond to exogenous GABA under saline stress (SS; NaCl and Na_2SO_4, 1:1 molar ratio). Alkaline salt stress results from alkaline salts (including $NaHCO_3$ and Na_2CO_3), while saline stress is caused by saline salts (such as NaCl or Na_2SO_4). Globally, both salinity and alkalinity stresses affect approximately 932 Mha of land area (Rao et al., 2008). Given this, further

research into improving saline stress resistance in chufa plants is urgently required to ensure production in salinized soils.

Extensive research is dedicated to improving the stress tolerance of crop species. According to recent research, exogenous growth factors including GABA are simple and effective ways of increasing salt tolerance and crop yields (Li et al., 2015; Li et al., 2016; Chen et al., 2018; Ullah et al., 2023). GABA is a 4-C, non-protein, amino acid that is highly water soluble. GABA rapidly increases in plants under environmental stress (Mekonnen et al., 2016; Shelp et al., 2017, 1999; Review; Li et al., 2021). A plethora of literature has highlighted the benefits of exogenous GABA that include improved growth features, photosynthesis, N metabolism and antioxidant responses (Priya et al., 2019; Salah et al., 2019; Vijayakumari et al., 2016; Ullah et al., 2023). Accordingly, there is a need to focus research on how exogenous GABA can benefit crop species in general and salt-sensitive chufa plants in particular.

We hypothesized that increasing saline stress (SS) would adversely affect chufa plants by impairing their growth attributes and physio-biochemical responses; however, exogenous GABA might plant growth and metabolism by mitigating the adverse impacts of SS. We used controlled and SS conditions with exogenous GABA application to test our hypothesis via a morphological and physio-biochemical investigation. Our aim was to gain deeper understanding of saline-stress tolerance mechanisms in chufa plants such as (1) growth and biomass, (2) salt ion accumulation, (3) osmolyte accumulation, (4) nitrogen metabolism and (5) antioxidant mechanisms. We found that GABA significantly decreased Na^+ and Cl^- levels, H_2O_2 and MDA levels. SS-treated chufa seedlings treated with GABA had enhanced chlorophyll photosynthetic pigments, K^+ and Mg^{2+}, osmolytes, antioxidant enzymes, NO_3^- reduction, and NH_4^+ assimilation compared to untreated seedlings.

2. Materials and Methods

2.1.1 Experiment design

The study was performed in August 2021 at the Cele National Station of Observation and Research (37◦00′ 56″ N; 80◦43′ 81″ E) located on the southern edge of the nutrient-deficient hyperarid Taklimakan desert. Typical mean annual precipitation and temperature are 35mm and

11.9°C, respectively. Four same-sized chufa tubers (cultivar, Fengchan No. 1) were sown in small plastic pots (1.5 L; basal diameter 11 cm; top diameter 15 cm; height 12 cm) with a small bottom hole (ca. 18%). The pots were filled with aeolian loamy sand (1 kg; organic C, total P, total N and total K values were 2.99, 0.60, 0.23, and 23.11 g/kg^{-1}, respectively; EC 177.7 $ms.cm^{-1}$ and pH 8.43). A complete randomised block design (RCBD) was used to arrange the pots and tap water was used to water them. In the study region, the groundwater table contains ≈ 40–50 mM Na^+ of salinity and is used for irrigation.

2.1.2 Saline stress and GABA treatments

Four weeks after sowing (fifth leaf stage), 48 pots with same-sized seedlings were divided into six sets (n=3) for the saline stress (SS; NaCl and Na_2SO_4, 1:1 molar ratio) with GABA treatments. Three groups of pots were subjected to three levels of SS, namely, 0 (CK; control), 100 and 200 mM. In the other three sets, SS levels were similar but exogenous GABA was administered. We applied 2 mM of GABA solution to each pot (150 ml after three weeks of sowing and 200 ml after five weeks). The optimal GABA level (2 mM) was selected based on seedling growth under 100 mM saline stress (supplementary data; Table S1). We harvested 45-day-old chufa plants, which were immediately dipped in liquid N and then stored in a refrigerator (-80 °C) for physio-biochemical analysis.

2.2. Determination of growth attributes

We estimated shoot and root lengths with a measuring tape, and their fresh and dry weights were weighed on an electric balance. Additionally, plant dry weight was determined by oven-drying at 75°C.

2.3. Estimation of chlorophyll pigments

An extraction of chlorophyll (0.1–0.3 g) was performed using 95% ethanol (vol/vol), and measurements were made at 665 nm (chlorophyll a) and 649 nm (chlorophyll b) in accordance with standard methods. Chlorophyll concentrations were calculated according to the formulae (mg g^{-1} FW) given below (Lichtenthaler and Buschmann, 2001).

Chlorophyl a = 13.98 A665 – 6.88 A649 (A)

Chlorophyl b = 24.96 A649 – 7.32 A665 (B)

Chlorophyl a/b = Chl a / Chl b (C)

2.4. Estimation of non-structural carbohydrates

We ground dried leaf samples into a fine powder. Following a standard procedure, glucose was used to estimate the concentration of soluble sugars (Yemm and Willis, 1954). A colorimetric measurement of starch was performed at 620 nm (Hansen and Møller, 1975). The NSC indicates the sum of the starch and soluble sugar concentrations.

2.5. Estimation of mineral elements

We used oven-dried root and leaf samples for Na^+, Mg^{2+} and K^+ estimates using an atomic absorption spectrophotometer (Mostofa et al., 2015). Ion chromatography was used to determine Cl^- ion (Ullah et al., 2019).

2.6. Estimation of H_2O_2 and cell membrane injury

For the estimates of the hydrogen peroxide (H_2O_2) content (Patterson et al., 1984), we homogenised leaf samples (0.2 g) in trichloroacetic acid (0.1%) before centrifugation for 10 min at 5000 × *g* (4°C). The supernatant was added to ammonia and 20% titanium tetrachloride and then centrifuged at 10,000 × *g* (10 min), and the absorbance was recorded at 410 nm.

For MDA concentrations, we homogenised 0.5 g of fresh leaves in trichloroacetic acid (5%) and then centrifuged them at 10,000 × *g* (10 min, 4°C). Next, the supernatant was mixed with 2 ml of 20% TCA. MDA concentrations were calculated by reading the absorbance at 600, 532 and 400 nm (Heath and Packer, 1965).

MDA (mol g^{-1} fresh matter) = 6.45 (A532− A600) – 0.56A450.

2.7. Antioxidant enzyme activities

A nitroblue tetrazolium (NBT) method was used to measure superoxide dismutase (SOD) (Fu and Huang, 2001). In brief, SOD was measured in terms of the quantity of enzyme needed to inhibit the NBT reduction by 50% at 560 nm. Following Fu and Huang (2001), peroxidase (POD) and catalase (CAT) were determined. To determine POD, the rise in absorbance at 470 nm for one minute was used to measure the degree of oxidation of guaiacol. POD was estimated by reading the absorbance (470 nm) after guaiacol was oxidised. To determine CAT, the reduction in spectrophotometric absorbance at 240 nm (1 min) was used to calculate the decomposition of H_2O_2. CAT activity was estimated by reading the absorption at 240 nm (1 minute) after H_2O_2 was decomposed.

2.8. Estimation of N-assimilative enzymes

We homogenised fresh samples in 25 mM PBS (pH 8.7) containing cysteine (10 mM) and EDTA (1 mM). We then centrifuged the mixture at 30,000 × *g* (20 min). Next, we collected the supernatant and treated it with sulphanilamide and naphthylamine reagents (2 ml each); the absorbance was noted at 540 nm (Ullah et al., 2019).

A fresh sample was homogenised in PBS [50 mM, pH 7.8; consisting of TritonX-100 (0.1%), EDTA (1 mM), glycerol (15%) and 2-mercaptoethanol (14 mM)] and centrifuged twice at 4°C (for 10 minutes) to determine glutamine synthetase (GS) activity. A 540-nm fluorescence measurement was made after the supernatant was complexed with acidified ferric chloride to determine the glutamine hydroxamate (Ullah et al., 2019).

GOGAT was estimated using the standard method (Rachim & Nicholas 1985). Glutamine (20 mM), ketoglutaric acid (100 mM), KCl (10 mM) and NADH (3 mM) were mixed with enzyme extract. Finally, the absorbance was checked at 340 nm to monitor the oxidation of NADH.

2.9. Determination of proline and soluble protein

The proline estimation (0.2 g) was performed using 2 ml of acetic acid (10%) and 5 ml of salicylic acid (3%). Based on a standard method (Liu et al., 2014), the supernatants were analysed for proline (Liu et al., 2014). The soluble proteins in 0.3 g samples were estimated using bovine serum albumin following a standard method (Bradford, 1976).

2.10. Statistical analysis

Microsoft Excel 2019 was used to sort the data values. ANOVA and descriptive statistics were performed with SPSS v. 16.0 (Chicago, IL, United States). The comparisons of mean values were performed using Duncan's multiple range test (p>0.05). The figure graphics were created using GraphPad Prism 8. Using OriginPro 2019 USA, Pearson correlation analyses were conducted for growth characteristics, chlorophyll content, osmolytes, nitrogen metabolism, mineral nutrition, H_2O_2 and MDA content, along with antioxidant enzymatic activities for interpretation purposes.

3. Results

3.1. Growth parameters

Saline stress (SS) had a negative impact on the growth performance of the chufa seedlings. The 100 mM and 200 mM SS resulted in 14 and 30.4, 38 and 55.6, 37.6 and 51.6, 12 and 22.9, 25.3 and 46.4 and 28.1 and 45.2% declines in shoot length (SHL), shoot fresh weight (SFW), shoot dry weight (SDW), root length (RTL), root fresh weight (RFW) and root dry weight (RDW), respectively (Fig. 1A-F). However, the exogenous application of GABA produced an increment (11.2, 16.6 and 4.2%) in SHL, (18.6, 20 and 14.3%) in SFW, (14.5, 28.6 and 4.3%) in SDW, (15.4, 9.7 and 3.5%) in RTL, (34.9, 11.9 and 1.4%) in RFW and (18.8, 33.6 and 8.3%) in RDW under 0 mM SS, 100 mM SS and 200 mM SS, respectively (Fig. 1A-F).

3.2. Response of photosynthetic chlorophyll pigments

The chlorophyll (chl) content of the chufa plants was influenced by the induction of saline stress (Fig. 2). A 4.9 and 6.2% inhibition was recorded in chl subjected to 100 and 200 mM SS, respectively (Fig. 3A). The only significant reduction (9.2%) was observed after exposure to 200 mM SS in chl b (Fig. 2B). The chl a/b ratio was reduced by 4.5% under 100 mM SS. Surprisingly, a 3.3% enhancement was recorded in the chl a/b ratio under 200 mM SS (Fig. 2C). The exogenous GABA produced an increment (11.5, 3.7 and 7.8%) in chl a and chl b (10, 9 and 15%) when it was applied with 0 mM SS, 100 mM SS and 200 mM SS mediums, respectively.

At the same time, adding GABA resulted in the inhibition of chl a/b at all saline stress levels (Fig. 2A-C).

3.3. Sodium (Na^+) and potassium (K^+) ions

Our results indicate that the nutritional profiles of the chufa seedlings were greatly modified by saline stress followed by GABA application (Fig. 3 & 4). Root Na^+ and Cl^- elements were substantially upregulated by the application of saline stress (Fig. 2A & B). The enhancement (82 and 119%) in Na^+ and (45 and 86.6%) in Cl^- concentrations occurred when plants were subjected to 100 mM SS and 200 mM SS, respectively. However, GABA application also significantly inhibited the concentrations of both these elements in roots. This inhibition was 18 and 32.5% for Na^+ and 15 and 13% for Cl^- after exposure to 100 mM SS and 200 mM SS, respectively. Unlike Na^+ and Cl^-, the saline stress led to the inhibition of K^+ concentration in chufa roots. Conversely, the GABA application improved it by 0 mM and 100 mM SS (Fig. 3C). Interestingly, the Mg^{2+} concentrations in roots were initially enhanced but then declined at 200 mM. At 0 mM stress, the GABA application did not result in any significant improvement. In all, 9.4 and 17% enhancement occurred when the plants were subjected to 100 mM SS and 200 mM SS, respectively (Fig. 3D).

More precisely, leaf Na^+ and Cl^- content rose as the saline stress increased (Figs. 4A & B). Na^+ and Cl^- were amplified by 136.6 and 34% in 100 mM SS and by 226.6 and 84.4% in 200 mM SS, respectively. Surprisingly, the GABA application enhanced Na^+ concentrations in plants without salt stress, while the addition of GABA under 100 mM SS and 200 mM mediums produced a decline of 17.6 and 15.2% in Na^+ concentration, respectively (Fig. 4A). Moreover, Cl^- was inhibited by 7.7, 22 and 32.8% in 0 mM SS, 100 mM SS and 200 mM SS after GABA application, respectively (Fig. 4B). Intriguingly, the saline stress had no significant effect on K^+ and Mg^{2+} concentrations in leaves, whereas GABA application did cause an amplification of these elements except at 100 mM SS in K^+ (Fig. 3C & D). Under 0 mM and 200 mM, an increment (43.5 and 10%) in K^+ concentrations occurred after the addition of GABA, respectively (Fig. 4C). As well, Mg^{2+} concentrations rose by 7.6, 2 and 5.2% after GABA application under 0 mM SS, 100 mM SS and 200 mM SS, respectively (Fig. 4 D).

3.4. ROS production and lipid peroxidation

Substantially elevated levels of MDA and H_2O_2 were recorded after both SS treatments (Fig. 5). MDA increased by 44.5 and 40% under 100 and 200 mM, respectively (Fig. 5A). Increments of 45.19 and 95.89% were observed in H_2O_2 levels under 100 and 200 mM SS, respectively (Fig. 5B). By contrast, the exogenously applied GABA reduced MDA and H_2O_2 levels by 7.7 and 1.9, 30.8 and 36.63, and 6.56- and 27.23% under 0 mM SS, 100 mM SS and 200 mM SS, respectively.

3.5. Antioxidant enzymes

SOD activity increased as the salinity level rose in the chufa seedlings (Fig. 5C). Compared to untreated plants, an enhancement of 25.9 and 64.2% was noted in SOD activity, respectively, after the application of 100 mM and 200 mM SS. In addition, the GABA application improved SOD activity by 32.8, 28 and 12.9% after 0 mM SS, 100 mM SS and 200 mM SS, respectively. Unlike the SOD activity, CAT activity was reduced by 7 and 38% under the 100 mM and 200 mM SS treatments, respectively, compared to control plants (Fig. 5D). The GABA application did not improve the CAT content when applied to 0 mM SS. Nevertheless, a 67.4 and 37.4% enhancement was recorded when applied under 100 mM SS and 200 mM SS, respectively. POD activity did not show any notable change after 200 mM SS but 100 mM SS did produce a 21% reduction in POD activity (Fig. 5E). Likewise, in other parameters GABA application also induced an improvement in POD activity, as indicated by 11.2, 60 and 9.6% increases under 0 mM SS, 100 mM SS and 200 mM SS, respectively.

3.6. NO_3^- reduction and NH_4^+ assimilation

Both the studied ions (NO_3^- and NH_4^+) declined as the saline stress in the chufa seedlings rose (Fig. 6). NO_3^- (56.4 and 89%) and NH_4^+ (19.6 and 35.7%) were inhibited by 100 mM SS and 200 mM SS, respectively. Although the exogenous application of GABA did not show any substantial modification under 0 mM SS and 200 mM SS in the case of NO_3^-, we did observe a 57% upregulation under GABA and the 100 mM SS medium (Fig. 6A). In case of NH_4^+, no significant change was observed after GABA application at 0 mM SS, although under 100 mM SS and 200 mM SS GABA application did lead to a significant improvement (Fig. 6B). NR

activity significantly decreased with increased saline stress levels; GABA application resulted in an upregulation of NR activity by 34.55, 117.36 and 69.05% under 0 mM SS, 100 mM SS and 200 mM SS, respectively (Fig. 6D).

Both salinity levels induced a 6% reduction in GS activity compared to untreated chufa seedlings (Fig. 6D). However, the application of GABA under 0 mM SS, 100 mM SS and 200 mM SS produced an improvement of 4.5, 9.6 and 1.8%, respectively. GOGAT activity decreased by 7.16 and 3.24% under 100 mM and 200 mM SS, respectively (Fig. 6C) but increased by 64.51, 27 and 5.29% at 0 mM, 100 mM and 200 mM after GABA supplementation.

3.7. Biochemical changes

Like the other studied parameters, the compounds that protect against oxidative stress were also influenced by salinity stress (Fig. 7A-F). The soluble sugar and non-structural carbohydrates initially increased (21.2 and 22.3%, respectively) at 100 mM SS but then decreased (17.2 and 15.2%, respectively) under 200 mM SS (Fig. 7A & D). However, a linear increment (4.4, 7.2 and 14.5%) in soluble sugar and (3.8, 6.7 and 15%) in non-structural carbohydrates were recorded under 0 mM SS, 100 mM SS and 200 mM SS, respectively. Starch concentrations progressively declined as the saline level was amplified (Fig. 7B). Starch content was reduced by 21.4 and 41.3% under 100 mM SS and 200 mM SS, respectively. By comparison, the GABA application improved the starch content (5.6, 9.8 and 1.7%) when applied with 0 mM SS, 100 mM SS and 200 mM SS, respectively. Starch and proline contents showed a similar trend since both progressively declined as the saline level increased (Fig. 7C & E). Starch content was reduced by 21.4 and 41.3% under 100 mM SS and 200 mM SS, respectively. However, the GABA application improved starch concentrations by 5.6, 9.8 and 1.7% at 0 mM SS, 100 mM SS and 200 mM SS, respectively.

Concentrations of the soluble sugar/starch ratio (55.2 and 60.6%), proline (19.82 and 240.68%) and soluble protein (16 and 58.9%) were increased by the addition of 100 mM SS and 200 mM SS solutions, respectively. In the case of GABA application, a different trend was observed for these compounds since the soluble sugar/starch ratio was not significantly improved by GABA application (Fig. 7C). However, the concentrations of proline were enhanced (76.75,

16.82 and 1.15%) at 0 mM, 100 mM, and 200 mM SS, respectively. As well, the soluble protein was improved by 16 and 58.9%, respectively, under 0 mM SS and 100 mM but was inhibited (14.8%) by GABA application when applied at 200 mM SS (Fig. 7EF).

3.8. Pearson's correlation analysis

The correlation analysis showed that GABA application significantly improved the studied parameters after salt stress and a revealed a positive correlation between N content and all the enzymatic activities. It is also indicated that plant fresh and dry weights are closely correlated with the N-mobilizing enzymes NH_4^+ and NO_3^-. A negative correlation between MDA and H_2O_2 was shown by plant dry and fresh matters. No correlation was observed between Cl^-, Na^+, Mg, NSCs and enzymatic activities for Chl a/b but Chl a and Chl b did exhibit a positive correlation with NR, GS, GOGAT, NH_4^+ and NO_3^- (Fig. 8).

4. Discussion

4.1. Exogenous GABA promoted growth and chlorophyll pigments in plants

In our study, saline stress (SS) caused significant reductions in shoot and root growth (length, fresh and dry weights) in chufa seedlings. Previous studies have shown that salinity stress disrupts plant growth due to excessive Na^+ levels, ionic imbalance, high H_2O_2 production and the inhibition of metabolic activities (Mittler et al., 2011; Ullah et al., 2022b). Chlorophyll pigments are necessary for photosynthesis. A decline in photosynthesis is the most common cause of a reduction in plant growth under saline conditions. Chlorophyll biosynthesis involves a chain of reactions and if any of these stepwise reactions are interrupted, biosynthesis is compromised (Min and Chengxiao, 2006; Tanaka and Tanaka, 2006). Several studies have demonstrated that salinity stress diminishes chlorophyll metabolism (Li et al., 2015; Xunling, 2002; Yuan et al., 2018), which corroborates our findings. However, exogenous GABA improved the growth and chlorophyll pigments in chufa plants compared to their untreated GABA counterparts. Exogenous GABA reduces chlorophyll degradation and enhances photosynthetic capacity and growth under salinity. For example, lettuce plants supplied with exogenous GABA were less susceptible to salinity toxicity than their untreated peers (Luo et al., 2011; Kalhora et al., 2018; Ullah et al., 2023). Likewise, a similar study has demonstrated that GABA positively regulates

chlorophyll biosynthesis, stomata conductance, transpiration and intercellular carbon dioxide levels, and reduces oxidative stress (Li et al., 2016). We thus believe that GABA can offset the detrimental impacts of salinity on growth features and photosynthetic chlorophyll pigments in chufa plants, which agrees with previous findings for other plant species (Jin et al., 2018; Kalhora et al., 2018; Wang et al., 2015; Wu et al., 2020).

4.2. Exogenous GABA regulated ion concentrations in chufa plants

Increasing salt ions can interfere with plant metabolism, development and growth. In our study, an investigation into chufa plants' response to saline stress demonstrated that the salinity-induced growth inhibition in chufa plants could be linked to ionic stress and cellular membrane damage, which is characterised by a marked accumulation of toxic salt ions (Na^+ and $Cl^–$) and a significant decline in K^+ and Mg^{2+} (Chartzoulakis, 2005; Ullah et al., 2019; Zhu, 2001). This salt-induced nutrient imbalance caused chufa seedlings to accumulate less dry matter and grow less, thereby substantiating previous research findings (Ashrafi et al., 2018; Sarker and Oba, 2020; Silva et al., 2015). It is believed that leaves are much more sensitive to salinity than roots due to the fact that Na^+ and Cl^- accumulate to a greater extent in leaves than in roots. Moreover, roots can regulate constant salt ions over time by exporting them to shoots or the soil. The xylem transports Na^+ to the shoots on the fast-moving transpiration stream (Tester and Davenport, 2003). It has been reported that roots have significantly lower levels of Na^+ and K^+ than shoots (Inal et al., 2009; Menezes et al., 2017), which likewise agrees with our findings. We suggest that chufa seedlings could benefit from the increase in Na^+, $Cl^–$, K^+ and Mg^{2+} ions in leaves as a survival strategy. This excess ion content could be used as osmolytes to increase water absorption by decreasing the water potential, thereby improving the plants' photosynthetic capability.

GABA application reduced Na^+ and $Cl^–$ levels but improved Mg^{2+} and K^+ levels in both roots and leaves, the exception being a decline in K^+ uptake in leaves under SS conditions. A recent study has shown that GABA can modulate SOS genes that arbitrate Na^+ efflux and Na^+/H^+ antiporters involved in storing surplus Na^+ in the vacuole (Li et al., 2020). Several other reports in the literature have indicated that increasing GABA under stress decreases Na^+ and ROS levels but also activates H^+ ATPase and inhibits K^+ deprivation (Shi et al., 2010; Li et al., 2021, 2019;

Su et al., 2019; Wu et al., 2021; Ullah et al., 2023), all of which are in line with our results. Furthermore, it has been shown that GABA binds to several transporters and channels, including the aluminium-activated malate transporter (ALMT) and the guard cell outward rectifying K^+ channel (GORK), which modulates ion homeostasis and improves stress tolerance (Xu et al., 2021). Hence, we suggest that GABA application to salt-stressed plants alleviates the negative impacts of salinity on chufa seedlings by regulating mineral nutrient uptake (Fig. 2), which is a clear indication that GABA can aid the optimisation of cellular metabolic processes.

4.3. Exogenous GABA upregulated the antioxidant mechanisms

Salinity increases reactive oxygen species (ROS) and can cause damage to membrane, thereby increasing malonaldehyde (MDA) levels (Mittler et al., 2004). Increasing MDA in plants indicates oxidative stress damage (Taulavuori et al., 2001). Antioxidant enzymes (POD, CAT and SOD) play a key function in the metabolism of ROS (Qiu et al. 2013). Our findings show that saline stress caused oxidative stress damage to chufa seedlings by upregulating the concentrations of H_2O_2 and MDA; however, GABA mitigates this damage by lowering these levels. The reduction in H_2O_2 under GABA application reveals the ability of GABA to decrease the levels of ROS oxidative stress damage (Jin et al., 2019; Kalhor et al., 2018; Y. Wang et al., 2017; Ullah et al., 2023). GABA can alleviate this effect by activating genes encoding ROS metabolizing enzymes (Shi et al., 2010). During abiotic stress, H_2O_2 interacts with calcium (Ca^{2+}) (Niu and Liao, 2016). There is evidence that Ca^{2+} contributes to root growth features (Han et al., 2015) and plant salinity tolerance (Hafize and Tulin, 2015). Stress increases Ca^{2+} levels and so upregulates the calmodulin (CAM) expression. Ca^{2+}/CAM complexes are also responsible for GABA synthesis by controlling glutamate decarboxylase (Allan et al., 2009). Thus, we believe that GABA/Ca^{2+} interactions modulate processes such as H_2O_2 signalling. Damage to the cell membrane is typically revealed as an increase in MDA levels under saline conditions, which is closely linked with high ROS accumulations. In our study, GABA-treated plants showed low MDA under control and saline conditions, which agrees with previous findings for poplars (Ji et al., 2018), rice (Sheteiwy et al., 2019), musk melons (Jin et al., 2019), wheat (Wang et al. 2017) and mung beans (Ullah et al., 2023).

Several antioxidant enzymes regulate ROS metabolism in plant cells by maintaining the equilibrium between ROS quenching and generation (Qiu et al. 2013). GABA reduces oxidative damage associated with ROS levels and hence improves tolerance to oxidative stress (AL-Quraan, 2015). For example, GABA application has been demonstrated to enhance the saline resistance of musk melons (*Cucumis melo*) due to greater SOD and ascorbic acid-glutathione activity (Xiang et al., 2015). GABA also profoundly affected the enzymatic antioxidant mechanism in corn plants subjected to salinity (Wang et al. 2017). Increased salt stress resistance in wheat has been observed following GABA application via improved enzymatic antioxidant enzyme mechanisms (Li et al., 2016). We observed significant improvements in SOD, POD and CAT enzymatic activities in controlled and SS-stressed plants supplied with GABA. These results endorse our findings regarding antioxidant enzymes under the co-application of saline stress and GABA, which indirectly suggests that GABA contributes to regulating lipid peroxidation and protecting cellular membranes by modulating the ROS scavenging mechanism. SOD enzyme dismutases and superoxide radicals into H_2O_2 (Hayyan et al., 2016). H_2O_2 removal is performed by enzymes such as POD and CAT. We found that CAT and APX activities decreased following salt exposure but that SOD activity increased compared to the control plants. Similar findings have been reported by Li et al. (2016) in wheat plants. Salinity may partially reduce the gene expression of POD and CAT, thereby decreasing enzyme activity; however, GABA either directly or indirectly offset the adverse effects of saline stress on antioxidant gene expression.

By contrast, in chufa seedlings under saline stress SOD activity increased as an indicator of the salinity concentration and the ability of SOD to diminish oxidative damage. Moreover, we found that GABA has a more notable effect on CAT under 100 and 200 mM SS stress and on POD under 100 mM stress than SOD. This concurs with our findings regarding the significant reduction in H_2O_2 and MDA levels after GABA application. Therefore, we suggest that the cellular injury to chufa plants caused by SS could be alleviated by GABA application, as demonstrated by reduced MDA and H_2O_2. Several studies have demonstrated that GABA can modulate antioxidant enzymes and ROS in plants under simulated salinity stress (Shi et al. 2010; Wang et al. 2017b; Jin et al. 2019; Khanna et al. 2021; Ullah et al., 2023), which is a further

confirmation of our results. Aside from oxidative stress, osmotic stress also contributes to salt toxicity in salinity-sensitive plants (Munns and Tester, 2008).

4.4. Exogenous GABA improved NO_3^- reduction and NH_4^+ assimilation

Nitrogen functions as a crucial component of several physiological processes and, for example, plays a role in salinity tolerance via a number of different pathways (Arghavani et al., 2017; Rais et al., 2013). We noticed that both NO_3^- and NH_4^+ decreased with increases in saline stress concentrations. It has been reported that Cl^- competes with NO_3^- during nutrient uptake and upward translocation (Abdelgadir et al., 2005). As a result of reduced NO_3^- concentrations, N assimilation and amino acid synthesis are inhibited, leading to lower dry weights (Queiroz et al., 2012). Salt ions impede NO_3^- reduction by inhibiting the activity of NR in general (Miura, 2012; Meng et al., 2016; Ullah et al., 2022b), which confirms our findings. A salt-induced reduction of NO_3^- prevents the supply of NH_4^+ by GS/GOGAT to the amino acid biosynthesis pathway (Wang et al., 2012). Although saline stress significantly reduced GOGAT activity, its effect on GS activity was insignificant. Several studies have demonstrated that saline stress decreases the amount of NH_4^+ incorporated into plant metabolism through the GS/GOGAT pathway (Sahu et al., 2001; Miura, 2012; Meng et al., 2016; Asharf et al., 2018; Wang et al., 2012; Ullah et al., 2023). Our results show that salinity-exposed chufa plants could not reduce NH_4^+ to replenish their glutamate pool, as demonstrated by the low GS activity. By contrast, exogenous GABA increased the concentrations of NO_3^- and NH_4^+ and enzymes of N assimilation (NR, GS and GOGAT) under saline stress. However, the effects on GS and GOGAT were insignificant. There is evidence to suggest that GABA plays a key role in N metabolism in plants (Salah et al., 2019) and, since it can alter nitrogen metabolism, GABA can lead promote growth and stress responses (Bouche and Fromm, 2004; Chen et al., 2020; Kinnersley and Turano, 2000). Recent studies have reported that GABA-treated plants have greater N-metabolism, as indicated by more activity by N-metabolizing enzymes and increased N concentrations (Khanna et al., 2021; Ullah et al., 2023), which supports our results. In some studies, GABA has also been noted to be a source of C and N (Reyes-García et al. 2012; Salah et al., 2019),two key elements for plants. Normal growth and yields are dependent upon the effective assimilation of both C and N. During the tricarboxylic acid cycle (TCA), C skeletons enter via the GABA shunt, where GABA can serve as N-storage biomolecules. Therefore, GABA plays a pivotal role in the biochemistry of C

and N assimilation in plants (Review Li et al., 2021). Hence, we believe that GABA application enhanced the reduction of NO_3^- and C and N assimilation by regulating their metabolizing enzymes.

4.5. Effects of exogenous GABA on biochemical changes in chufa plants

Plants accumulate stress-relieving solutes under stressful conditions (i.e. soluble sugar, glycine betaine, proline, etc., commonly known as osmolytes), which help maintain salinity homeostasis. Their main functions include maintaining water status and osmotic balance, preventing ion toxicity and chlorophyll destruction, regulating cell division, stabilizing cellular structures and scavenging excess ROS (Trovato et al., 2008; Singh et al., 2022).

In our study, soluble sugar and proline significantly increased in chufa seedlings subjected to saline stress, and exogenous GABA enhanced their concentrations. Sugar plays a crucial role as an osmolyte in plants by regulating cell division, controlling water loss, preventing chlorophyll degradation, scavenging free radicals, stabilizing membranes and proteins, and regulating gene expression (Rosa et al., 2009; Sami et al., 2016). In salt-stressed plants treated with GABA, the high proline content may help protect the photosynthetic machinery, eliminate excessive ROS and stabilize enzymes, proteins and membranes (Abdel Latif et al., 2018; Ahmad et al., 2016; Verdoy et al., 2006). GABA application improved proline levels under controlled and saline stress conditions, particularly at mid-range SS levels. Similar findings have been reported for black peppers (Vijayakumari et al., 2016), maize (Wang et al., 2017) and strawberries (Golnari et al., 2021) in which GABA application increased proline concentrations.

In addition, chufa plants subjected to saline stress and GABA application accumulated more soluble proteins than their untreated GABA counterparts; these proteins are considered to be necessary for osmotic adjustment and can serve as a source of N once the stress has abated (Ahmad et al., 2016; Abdel Latef and Tran2016).

5. Conclusion

Our study provides clear evidence that exogenous GABA positively modulates several physiological and biochemical mechanisms that can mitigate saline-stress-induced inhibitions in

growth and biomass. Specifically, it can (i) halt the decline in Na^+ and Cl^- contents, (ii) maintain beneficial K^+ and Mg^{2+} ions, (iii) improve chlorophyll content, (iv) mitigate oxidative stress via the suppression of H_2O_2 and MDA levels, (v) increase NO_3^- reduction and NH_4^+ assimilation and, finally, (vi) stimulate the production of compatible solutes. Na^+, Cl^-, K^+ and Mg^{2+} ion concentrations were higher in leaves than in roots, thereby illustrating their ability to increase cellular osmolality, which facilitates the upward transfer of water to leaves in hyperarid desert environments. According to our research, GABA can act as a powerful plant growth regulator that modulates the growth and physiological responses of chufa plants. We thus suggest that GABA application could be a viable strategy for reducing saline damage in chufa plants and help achieve sustainable food production in salinity-affected areas worldwide, particularly in hyperarid regions such as Taklamakan, where only saline groundwater is available for irrigation. In future studies, the molecular mechanisms underlying the regulation of salinity tolerance by GABA in tiger nut plants needs to be explored in greater depth.

6. Acknowledgements

We acknowledge the help provided by the concerned personnel on the field and in the laboratory.

7. Funding source

Natural Science Foundation of Xinjiang Uygur Autonomous Region [2021D01D02], National Natural Science Foundation of China [41977050], and Joint Funds of the National Natural Science Foundation of China and Xinjiang Uygur Autonomous Region of China [U1903102] supported this work. JS and JP acknowledge support from the Spanish Government Grant TED2021-132627B-I00 funded by MCIN, AEI/10.13039/501100011033 European Union NextGenerationEU/PRTR, the Catalan Government Grant SGR2021-1333, and the Fundación Ramón Areces Grant CIVP20A6621.

8. Declaration of competing interest

The authors report no competing interest.

9. Author contributions

Conceptualization: AU, ZF, Data curation; AU, Methodology; AU, Funding acquisition; ZF, Investigation; AU, AT; Project administration; ZF, Resources; ZF, Formal analysis; AU, AT, MAA, and JN, Software; AU, AT, JN, MAA, and ZZ, Visualization; JN, and MAA, Validation; ZF, AU, AT, and ZZ, Supervision; ZF, Writing - original draft; AU, Writing - review and editing; AU, ZF, AT, JS, JP, ZZ, JN

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Figures 1-8

Figure 1. Changes in **(A)** shoot length, **(B)** shoot fresh weight, **(C)** shoot dry weight, **(D)** root length, **(E)** root fresh weight, **(F)** root dry weight and **(G)** root-shoot ratios in chufa plants under saline stress (SS) and exogenous γ-Aminobutyric acid (GABA) application. Bars represent the SD of the mean; n=3. Letters indicate significantly different values at $P<0.05$ (Duncan's method).

Figure 2. Changes in concentrations of **(A)** Na^+, **(B)** Cl^- **(C)** K^+, and **(D)** Mg^{++} in leaves of chufa plants under saline stress (SS) and exogenous γ-Aminobutyric acid (GABA) application. Bars represent the SD of the mean; n=3. Letters indicate significantly different values at $P<0.05$ (Duncan's method).

Figure 3. Changes in concentrations of **(A)** Na^+, **(B)** Cl^- **(C)** K^+, and **(D)** Mg^{++} in roots of chufa plants under saline stress (SS) and exogenous γ-aminobutyric acid (GABA) application. Bars

represent the SD of the mean; n=3. Letters indicate significantly different values at P<0.05 (Duncan's method).

Figure 4. Changes in concentrations of **(A)** chlorophyll a **(B)** chlorophyll b and **(C)** chlorophyll a/b ratio in chufa plants under saline stress (SS) and exogenous γ-aminobutyric acid (GABA) application. Bars represent the SD of the mean; n=3. Letters indicate significantly different values at P<0.05 (Duncan's method).

Figure 5. Changes in concentrations of **(A)** MDA, **(B)** H_2O_2 and enzymatic activity of **(C)** SOD, **(D)** POD and **(E)** CAT in chufa plants under saline stress (SS) and exogenous γ-aminobutyric acid (GABA) application. Bars represent the SD of the mean; n=3. Letters indicate significantly different values at P<0.05 (Duncan's method).

Figure 6. Changes in concentrations of **(A)** NO_3^- and **(B)** NH_4^+ and enzymatic activity of **(C)** NR, **(D)** GS and **(E)** GOGAT in chufa plants under saline stress (SS) and exogenous γ-aminobutyric acid (GABA) application. Bars represent the SD of the mean; n=3. Letters indicate significantly different values at P<0.05 (Duncan's method).

Figure 7. Changes in concentrations of **(A)** soluble sugar, **(B)** starch, **(C)** soluble sugar/starch ratio, **(D)** NSCs, **(E)** proline and **(F)** soluble protein in chufa plants under saline stress (SS) and exogenous γ-aminobutyric acid (GABA) application. Bars represent the SD of the mean; n=3. Letters indicate significantly different values at P<0.05 (Duncan's method).

Figure 8. Correlation analysis between all the studied parameters. Red and blue colours represent positive and negative correlations. The size and intensity of colour indicates the significance of variables, while * represents the significance level at 0.05. SHL, shoot length; SFW, shoot fresh weight; SDW, shoot dry weight; RL, root length; RFW, root fresh weight; RDW, root dry weight; Chl a, chlorophyll a; Chl b, chlorophyll b; Chla/b, ratio; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; NH_4^+, ammonium; NO_3^-, nitrate; nitrogen; NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; SS, soluble sugar; ST, starch; SS/ST, soluble sugar/starch ratio; NSCs, non-structural carbohydrates; Pro, proline; SP, soluble protein; L-Na^+,

leaf Na^+; L-Cl^-, leaf Cl^-; L-K^+, leaf K^+; L-Mg^{++}, leaf Mg^{++}; R-Na^+, root Na^+; R-Cl^-, root Cl^-; R-K^+, root K^+; and R-Mg^{++}, root Mg^{++}.