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THE STUDY OF SOME AMINO ACIDS WITH A ROLE IN THE ADAPTATION OF THE BITTER CUCUMBER (*MOMORDICA CHARANTIA*) TO SALINITY STRESS

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Abstract

Bitter cucumber (Momordica charantia L.) is an annual tropical plant in the family Cucurbitaceae, cultivated worldwide for its bitter fruits that are used both for food and for its many medicinal properties. Salinity is a widespread problem globally and is constantly growing. This limits plant growth and biomass production, especially in arid, semi-arid and tropical areas. To adapt to new environmental conditions, plants can accumulate or consume different metabolites. In this work, proline and aromatic amino acids were determined by the spectrophotometric method. To carry out the determinations, two varieties of bitter cucumber and three experimental lines were used that were treated with saline solutions of different concentrations. Following the analyzes carried out, a tendency to increase the amount of proline was noticed in the variants treated with saline solutions compared to the control, and in the case of aromatic amino acids, a tendency to their decrease was observed proportional to the increase in the concentration of saline solutions. The determinations made highlight the degree of resistance of the studied genotypes to saline stress and the preferential accumulation of metabolites.

Key words*: Momordica charantia, saline stress, amino acids.*

INTRODUCTION

Momordica charantia L., or bitter cucumber, is an annual tropical plant in the Cucurbitaceae family that is cultivated in many places around the world for its particularly bitter fruits (Alisofi et al., 2020). Analysis have shown that this plant has the highest nutritional value of all Cucurbitaceae, as it is an excellent source of fiber, vitamins, minerals, carbohydrates and proteins. Vitamins C, A, P, thiamine, riboflavin, niacin and minerals are found in green leaves and fruits but also in young shoots (Bortolotti et al., 2019). The more than 60 medicinal properties of bitter cucumber fight almost 30 diseases such as diabetes, cancer and AIDS (Patel et al., 2020). Due to these properties, the plant is one of the most popular and valued fruit vegetables in the Cucurbitaceae family, cultivated both as food and for use in traditional medicine (Li et al., 2020).

The deterioration of food security is caused by the reduction of arable land due to abiotic stress. Food production must increase by 70% to meet the increase in global population, which is expected to reach 9.1 billion by 2050 (Iqbal et al., 2014). A major problem limiting plant growth and biomass production is salinity, found especially in arid, semi-arid and tropical regions where rainfall is scarce and irrigation systems are common (Mohsen et al., 1980; Gamalero et al., 2020).

Salinity manifests itself largely as osmotic stress and causes an ionic imbalance in the cell. The occurrence of this abiotic stress triggers cell signalling pathways and cellular responses, such as the accumulation of compatible solutes and the increase of antioxidants (Zhu, 2001).

Proline is a non-essential amino acid that plays a significant role in plant growth and development. There are two known pathways for proline synthesis: the glutamate pathway and the ornithine pathway. The most known and accepted pathway for the synthesis of this amino acid in plants subjected to abiotic stress is the glutamate pathway (Sharma & Verslues, 2010). Proline is synthesized from glutamate, as are γ-aminobutyric acid, glutamine and arginine. The main genes involved in the synthesis of this metabolite are: pyrroline-5-carboxylate

synthetase (P5CS), pyrroline-5-carboxylate reductase (P5CR), and ornithine-δ-aminotransferase (OAT), and those responsible for proline catabolism are: proline dehydrogenase (PDH) and pyrroline-5-carboxylate dehydrogenase (P5CDH) (Kishor et al., 2015).

In conditions of abiotic stress, proline fulfills an important role as a cellular osmoprotector. It regulates water absorption under conditions of water and salt stress. In addition to its role as a compatible solvent, proline is known to improve the stability of subcellular structures and cell membranes. It serves as a redox buffer, facilitates the activity of enzymes and is a powerful chelator of metals in the cytoplasm. Proline is also known as a cell signaling molecule (Wang et al., 2014).

In plants, aromatic amino acids (AAA) are synthesized from chorismate, the end product of the shikimate pathway, and are precursors to a wide range of secondary metabolites (Trovato et al., 2021). The enzymes 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DHS) catabolize the first reaction of the shikimate pathway (Keith et al., 1991). Phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) are aromatic amino acids characterized by the presence of an aromatic ring in their structures (Aydin et al., 2021). Aromatic amino acids are fundamental elements in plants, fulfilling multiple roles. These amino acids are important precursors in the biosynthesis of proteins, they play a role in the synthesis of some secondary compounds involved in the plants' fight against biotic and abiotic stress, and they also have the ability to function as antioxidants, ensuring the protection of plant cells against oxidative stress induced by environmental factors. adverse environment (Trovato et al., 2021).

MATERIALS AND METHODS

Materials

Spectrophotometer Specord 210 Plus, AnalitikJena, Vertex 70 FT-IR spectrometer, Bruker, Ninhydrin ($C_9H_6O_4$) - Merck, Sulfosalicylic Acid ($C_7H_6O_6S$) – Chemical Company, Glacial Acetic Acid ($C_2H_4O_2$) - Merck, Acid Phosphoric (H_3PO_4) - Merck, Toluene (C_7H_7) - Merck, L-Proline ($C_5H_9NO_2$) - Sigma Aldrich, Ammonium Hydroxide NH_4OH – Chemical Company, Nitric acid (HNO3) - Sigma Aldrich, *Momordica charantia* leaves.

Plant material

Five genotypes of *Momordica charantia* were used for the experiment, including two Romanian varieties (Rodeo variety and Brâncuși variety) and three experimental lines (Line 1, Line 3 and Line 4). The experience was located within the didactic resort "Ferma Vasile Adamachi" which belongs to the "Ion Ionescu de la Brad" University of Life Sciences in Iași.

Plants were treated with different concentrations of saline solutions: 100 mM and 200 mM. in total, three treatments were applied at intervals of 10 days between them. The leaves used for the determinations were harvested 7 days after the application of treatment 3. According to the BBCH scale, the plants were in phenophase 701 (corresponding to the appearance of the first fruit) at the time of application of the treatment and in phenophases 702-703 (corresponding to the appearance of the -second and third fruit) at the time of sampling. They were harvested from the main shoot of the plant and an average sample of 9 leaves was made. 3 leaves from 3 different plants belonging to the same variant (control, treatment V1 or treatment V2) were harvested and used.

Proline determination method

To perform the analysis, 1 g of plant material was used, which was mortared with 5 mL of sulfosalicylic acid 3%. The obtained mixture was centrifuged for 15 minutes at 5000 rpm. After centrifugation, the supernatant was used, from which 2 mL were extracted. 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were added to these. Acid ninhydrin was obtained by dissolving 1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6M phosphoric acid. For dissolution and homogenization, the substances were heated to 60 ºC.

The resulting mixture was placed on a water bath and subjected to heat treatment at 100 ºC for one hour. After the heat treatment, the resulting red solution was allowed to cool to room temperature. After cooling the mixture was extracted with 4 mL toluene.

The readings and calculation of the amount of proline were made using a calibration curve made in the range 1-18 mg/L. For proper fitting on the calibration curve, the samples obtained were diluted with another 2 mL of toluene.

Proline was read at 520 nm using a 1 cm quartz cuvette, after which the extract was dried for FTIR analysis.

Method for determining aromatic amino acids

AAAs were determined by means of the xanthoprotein reaction. An amount of 0.3 g of leaf was used, over which an amount of 10 mL of distilled water was added and which was then heated on a hot plate at a temperature of 60 ºC for 20 minutes. 2 mL of the obtained extract was used to which 0.5 mL of 65% HNO_3 was added and heated again at 60 ºC for 15 minutes. The solutions were cooled to room temperature, then 1 mL of 20% ammonia solution was added, after which the obtained solution acquired a yellow color. In order for the samples to be read by the spectrophotometer, they were centrifuged for 10 minutes at 2000 rpm.

An amount of 100 µL was used from the supernatant and diluted in 3 mL of distilled water. The resulting solution was read at 305 nm using a 1 cm quartz cuvette and then dried to perform FTIR analysis.

Statistical analysis

For the statistical analysis of the data, the Anova Two Factor test was used, following the influence of the concentrations of the saline solutions applied but also of the studied genotypes.

RESULTS AND DISCUSSIONS

Dramatic proline accumulation is a common physiological response in plants exposed to various types of abiotic stress. This increase may be due to de novo synthesis of proline, decreased degradation rate, or protein hydrolysis (Kaur & Asthir, 2015). Proline can preferentially accumulate in different plant organs. According to the literature, it can accumulate in roots, leaves, stems, flowers, fruits and seeds fulfilling different roles (Meenu Rani et al., 2022).

In leaves, proline accumulates with the aim of stabilizing cellular structures. Proline contributes to the stabilization of subcellular structures in chloroplasts. It helps maintain the integrity of membranes, proteins and enzymes. This stability is crucial for efficient photosynthesis even under stress conditions (Hayat et al., 2012)

Following the determinations made on the leaves of bitter cucumber after applying the last saline treatment (Figure 1), proline content values were recorded in the control plants between 1.5 mg/L in the case of the Rodeo variety and 2.18 mg/L registered in the Brâncuși variety. The differences between proline amounts in untreated plants were relatively low, with no statistically significant differences being recorded. Plants treated with 100 mM NaCl (V1) showed proline values between 1.94 mg/L in the case of the Rodeo variety and 5.59 mg/L in the case of Line 3. Following the V1 treatment, significant differences can be observed between the five various tested. The smallest differences compared to the control were also recorded in the Rodeo variety where the plants treated with V1 recorded 29.33% higher values, unlike Line 3 which showed a 225% increase compared to the control. In the case of plants treated with 200 mM NaCl (V2), very pronounced differences were observed between the amounts of proline in the five studied genotypes. Proline values fluctuated between 1.5 mg/L in the case of the Rodeo variety and 20.9 mg/L in the case of Line 3. Considerable amounts of proline were also recorded in the case of the Brâncusi variety (14 mg/L) and the Line 4 (13.9 mg/L). The smallest difference between the control and V2 was recorded in the Rodeo variety (150.66%) and the most pronounced difference was observed in the case of Line 3 (1115.11%). Significant and highly significant differences between controls and treatments are confirmed by two-way Anova test (Table 1). Among the genotypes studied, the Rodeo variety presented the lowest proline value both in the case of the control and the treated variants. This can be explained both by its poor resistance to salt stress and by the lack of the natural tendency of the genotype to accumulate proline. Compared to this, in the case of the Brâncusi variety and Line 4, the high amounts of proline may also be due to the general tendency of the genotype to accumulate proline under normal conditions. Following the analysis of the values, it can be concluded that the best adaptation to salt stress was recorded in the case of Line 3. Unlike the genotypes that present proline under normal conditions, Line 3 did not present this characteristic, which highlights this line as the most resistant to applied salt stress.

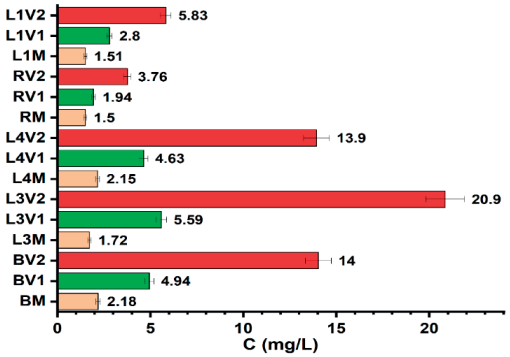

Figure 1. Free Proline content in bitter cucumber (*Momordica charantia*) leaves subjected to salt stress

After spectrophotometric analysis the toluene extract was dried. The residue formed was analyzed by infrared spectrometry in order to determine the type of functional groups present. This analysis was performed to highlight the presence of clusters characteristic of the analyzed compound. Analysis (403.11 m, 478.33 m, 621.05 m, 1018.37 w, 1220.89 vw, 1232.46 vw, 1244.03 vw, 1263.32 w, 1618.21 s (υC=O), 1637.50 m (υC=C), 1710.79 w (υC=O), 1753.22 vw, 1778.29 vw, 2925.89 vw (υC-H), 3236.42 w (υCH), 3413.86 vs, 3477.51 vs, 3552.73 s, revealed the formation of the compound of interest.

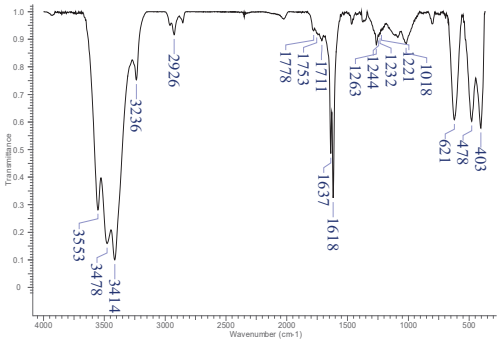

Figure 2. FTIR spectrum of the solid formed after drying the sample used for the determination of Proline

Table 1. Analysis of the variance of the amount of proline in bitter cucumber genotypes subjected to salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	*Significance*
Rows	99.392	4	24.848	1.933453	0.198253	3.837853	NS
Columns	268.829	2	134.4145	10.45895	0.005857	4.45897	**
Error	102.813	8	12.85162				
Total	471.0339	14					

Anova Two-Factor: NS non-significant statistical differences (p≥0.05); *significant statistical differences (p≤0.05); **distinctly significant statistical differences (p≤0.01); ***highly significant statistical differences (p≤0.001)

Following the performance of the Anova Two Factor test (Table 1), distinctly significant statistical differences ($p<0.01$) were revealed between the amounts of proline obtained following treatments with saline solutions, which demonstrates that the most important role is played by the concentration of NaCl in the amount of proline.

Aromatic amino acids

Studies on aromatic amino acids have shown that, under abiotic stress conditions, the expression levels of genes in the AAA biosynthesis pathway are up-regulated, leading to higher levels of them and their secondary metabolites in plants resistant to the abiotic stress encountered (Oliva et al., 2021).

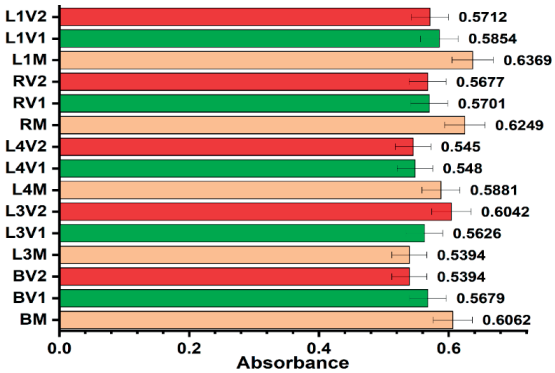

Figure 3. Aromatic amino acids from bitter cucumber leaves (*Momordica charantia*) subjected to salt stress

The analyzes of bitter cucumber leaves on the absorbance of aromatic amino acids, in the case of the five studied bitter cucumber genotypes, showed values between 0.5394 in Line 3 and 0.6369 in the case of Line 1, in the case of control plants. These values did not show statistically significant differences. In plants treated with V1, the absorbance values ranged from 0.548 in the case of Line 4 to 0.5854 in the case of Line 1. Following the comparison between the AAA absorbances of the control and V1-treated plants, a general downward trend was observed in the treated plants, except for Line 3 where a slight increase could be noted. The same phenomenon of decrease in absorbances was also noted in the case of the analyzes performed on the leaves that came from the plants treated with V2. The values fluctuated between 0.5394 in the Brâncusi variety and 0.6042 in the case of Line 3, which presented higher values than those of the untreated control and the V1 treatment. According to a study carried out on two varieties of chickpea (*Cicer arietinum* L.), by Kumar et al. (2021), it was highlighted that among the AAAs, only tryptophan was present in a greater amount compared to the control in the variety resistant to salt stress, while tyrosine decreased, compared to control plants, in both the salt stress-resistant and the sensitive variety (Kumar et al., 2021). Comparing the results obtained with this study, it can be concluded that Line 3 is a genotype resistant to abiotic stress conditions. The relatively low difference between the control and the treated variants in the case of this genotype can be explained by the increase in the plant only of the amino acid tryptophan.

According to specialized literature, AAAs not only play a role in protein synthesis, but also fulfill numerous other roles, including the adaptation of plants to abiotic stress (Trovato et al., 2021). Thus, the reduced amounts of AAA in the variants subjected to NaCl treatments could be explained by the plant's use of these amino acids in the synthesis of substances with a protective role.

After performing the spectrophotometric analysis, the yellow extract was dried in order to perform the infrared spectrometry analysis. This analysis was performed to determine the bands characteristic of the aromatic and aliphatic C-H vibrations, the C=O bands of carboxylic acids, and the NO vibration of nitroderivatives.

Analysis 387.68 vw, 586.34 vw, 626.84 w, 713.63 s, 827.43 vs, 1039.59 m, 1076.23 w, 1352.04 vs, 1400.26 vs, 1762.86 m, 2083.03 w, 2331.84 w, 2345. 34 w, 2354.99 w, 2395.49 m, 2505.43 vw, 2798.60 m, 3030.04 m, 3134.20 m revealed the presence of the compound of interest (Figure 4).

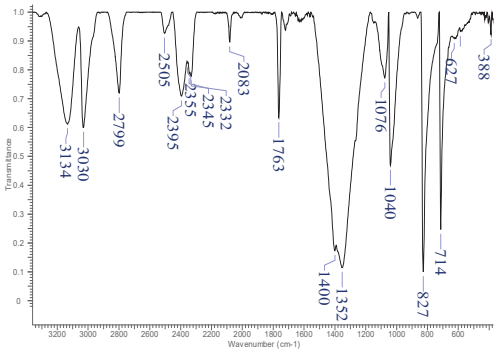

Figure 4. FTIR spectrum of the solid formed after drying the sample used to determine AAA

To carry out the statistical analysis of the AAA absorbance values in control and treated plants, the Anova Two Factor test was used (Table 2).
After performing the Anova Two Factor test (Table 2), insignificant differences were found in the AAA absorbances ($p \geq 0.05$) obtained after the treatments with saline solutions and those of the control plants. In this situation the null hypothesis is accepted.

The result obtained from the Two Factor Anova test can be explained by the different behavior of Line 3, which showed an increase in AAA in stressed plants compared to the general trend of decreasing absorbances in the case of treated variants in the other studied genotypes.

Table 2 - Analysis of the variance of the absorbance of aromatic amino acids in the leaves of bitter cucumber genotypes subjected to salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	*Significance*
Rows	0.002769	4	0.000692	0.86649	0.523528	3.837853	NS
Columns	0.003636	2	0.001818	2.275971	0.165012	4.45897	NS
Error	0.006391	8	0.000799				
Total	0.012796	14					

Anova Two-Factor: NS non-significant statistical differences ($p \geq 0.05$); *significant statistical differences ($p \leq 0.05$); **distinctly significant statistical differences ($p \leq 0.01$); ***highly significant statistical differences ($p \leq 0.001$)

CONCLUSIONS

Following the study of the proline content determined in the analyzed bitter cucumber (*Momordica charantia*) genotypes, an increase in the amount of this amino acid directly

proportional to the increase in the concentration of the applied saline solution was noted. The most pronounced difference between the control and the V1 and V2 treatments was recorded in the case of Line 3 where the amount of proline increased in V1 by 3.25 times compared to the control and in the V2 treatment by 12.53 times. This significant increase may demonstrate an adaptation of the genotype to the salinity conditions to which it was exposed.
Another evidence of the increased resistance to salt stress of this line is given by the lack of a large amount of proline in the control plants. Among the genotypes studied, Line 3 was highlighted by the lowest proline synthesis capacity under normal environmental conditions. This demonstrates that salt stress is the main factor that caused the increase in proline, a fact also observed by the result of the Two Factor Anova test.
The determinations regarding the analysis of aromatic amino acids in the leaves of the studied *Momordica charantia* genotypes revealed a tendency to decrease the absorbance of these substances proportional to the increase in NaCl concentration with which the plants were stressed. A distinct behavior was noted in Line 3 where AAA absorbance increased in saline-treated plants compared to the control. This phenomenon can be explained by the adaptation of this line to the abiotic stress conditions to which it was subjected, which allows us to recommend it to be cultivated on saline soils, in order to obtain a profitable harvest and some satisfactory nutritional properties.

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