Effect of creatine and creatine lysinate *on the in vitro* cultivation and antioxidant potential of *Stevia rebaudiana* Bertoni and *Leontopodium alpinum* Cass

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Abstract
Stevia rebaudina Bertoni and Leontopodium alpinum Cass. (Asteraceae) are valuable plants, largely applied in traditional and formal medicine. Six variants of Murashige and Skoog media containing creatine or creatine lysinate at different concentrations (1, 5 and 10 mg/l) were used. The application of creatine lysinate in the MS nutrient media influenced positively the apical growth and elongation of stevia and edelweiss in vitro plants. The higher activities of the antioxidant enzymes (SOD, APX and GPX), as well as the higher content of flavonoids and phenols, were detected in stevia compared to edelweiss. The activity of CAT was much more pronounced in edelweiss.

The activities of CAT, APX and GPX were found to decrease with the addition of creatine or creatine lysinate to the culture medium while the activity of SOD increased as this tendency is better expressed in L. alpinum. The selected concentrations of creatine and creatine lysinate did not show statistically significant differences in either of the treatments compared to control in both the species measured by free radical scavenging capacity (DPPH method).

Keywords: Medicinal plants, micropropgation, antioxidant enzymes, amino acids.

Introduction

Interest in medicinal plants has increased over the past three decades because of their health benefits in terms of safety and cost compared to synthetic drugs[4,64]. *Stevia rebaudiana* Bertoni and *Leontopodium alpinum* Cass. are valuable medicinal plants belonging to Asteraceae family. *S. rebaudiana* is a perennial herb, native to the northern Republic of Paraguay and South America, typical for regions with tropical and subtropical climates[23]. It is a natural sweetener plant known as Sweet Leaf, Sweet Herbs and Honey Leaf[2,15].

The leaves of stevia are a source of diterpene glycosides such as steviosides and rebaudiosides, which are 200 to 300 times sweeter than sucrose or cane sugar[25]. Stevioside is known as a valuable natural sweetening agent attributed to its relatively good taste and chemical stability[47]. It had been suggested for diabetic patients because it is a non-calorie sweetener; the powdered form of stevia leaves has hypoglycemic and body weight reducing potencies[10,61].

Edelweiss (*Leontopodium alpinum* Cass.) is a traditionally used medicinal plant for the treatment of gastrointestinal disorders such as diarrhea, dysentery and colic as well as bronchitis, angina and fever[19]. Various compounds such as terpenoids, phenylpropanoids (phenolic acids, flavonoids, coumarins, lignans), fatty acids and polyacetylenes isolated from different parts of edelweiss determine the pharmacological effects of the plant [20,24,56]. The species is native to the Pyrenees, the Alps, the Carpathians and the Balkan Peninsula and is enclosed within the Red Book of Bulgaria.

The high pharmacological potential of both species necessitates their cultivation. Conventional methods of stevia vegetative propagation are limited to a low seed germination percentage because of self-incompatibility and the low number of individuals that may be obtained from a single plant and successfully adapted to the soil[50,55]. Micropropagation, or *in vitro* culture appears to be the best method to overcome those problems and has the potential to provide giant numbers of plants in a brief time [50,53]. There are several studies of the micropropagation of *S. rebaudiana*[3,5,33,38,44,46,51]. Stevia can form multiple shoots from the nodal explants and appears to be appropriate for large-scale production[53]. In the literature, there are some reports on the *in vitro* cultivation of *L. alpinum* through direct or indirect regeneration using apical buds from mature, senescing plants or *in vitro* obtained seedlings[31,45].

The growth and morphogenesis of plant tissues under *in vitro* conditions are largely influenced by the composition of the culture media[35]. In the plant nutrient medium, nitrate ions, ammonium salt, amino acids and complex organic compounds supply nitrogen[8]. Amino acids provide plant cells with a source of nitrogen that is easily assimilated by tissues and cells faster than inorganic nitrogen source. They affect many physiological processes in plants by participating in the regulation of metabolic pathways, acting as intermediates of the final metabolites in certain metabolic pathways[71]. Amino acids used for enhancement of cell growth in culture media included glycine, glutamine, asparagine, L-arginine, cysteine and L-tyrosine[65]. Amino acids have been used as an organic nitrogen source in *in vitro* cultures of many species such as alfalfa, maize, sorghum, pineapple, rice, *Artemisia vulgaris* etc. to increase somatic embryogenesis and regeneration potential[16, 27, 28, 36, 52, 60].

Creatine is a natural substance and it is N-methyl-N-guanyl Glycine, (CAS Registry No. 57-00-1); (alpha-methyl guanido) acetic acid; N-(aminoiminomethyl)-N-glycine; Methylglycocyamine, Methylguanidoacetic acid or N-Methyl-N-guanylglycine. Creatine (methylguanidine-acetic acid) is endogenously formed from reactions involving the amino acids arginine, glycine and methionine in the kidneys and liver[70]. It probably occurs in soils, manures and green crops[59]. Creatine plays an important role in the metabolism of proteins in animal organisms and metabolizes to the waste product creatinine. In recent years, the recycling of animal waste for fertilizer production becomes more and more popular[11]. Given the rich content of creatine in animal tissues, its effect on plant growth and development is of great interest.

Skinner[59] reported the beneficial effect of creatine on the growth of wheat seedlings in a culture containing fertilizer combination without nitrate. In an aqueous solution, creatine is converted to creatinine which is a pH-dependent, non-enzymatic reaction (Figure 1). Aqueous and alkaline solutions contain an equilibrium mixture of creatine and creatinine. In acidic solutions, the conversion to creatinine is complete[37].

In order to create a stable aqueous creatine solution and to prevent the conversion of creatine into creatinine, salts of creatine and some natural acids like citric acid, orotic acid, pyroglutamic acid, ascorbic acid, taurine etc. have been prepared and published or patented [1, 12, 34, 39]. A successful approach for obtaining such salts is the complex of creatine and amino acids containing an amino group in the side strain[67]. They possess both simultaneously good water solubility and sufficient storage stability. L-Lysine is a proteinogenic amino acid containing a second amino group. It participates in the production of different proteins, enzymes, hormones, antibodies etc. That is why we found it intriguing to involve creatine lysinate as an additive to the nutrient medium and to compare its effect with creatine monohydrate.

Creatine lysinate (Figure 2) is a salt formed by creatine and L-Lysine and was synthesized following the method described in the patent[67]. Lysine is one of the most limiting essential amino acids in vegetative foods and cannot be synthesized by humans and animals [21, 71]. In plants, in addition to serving as a building block of proteins, lysine is also a precursor for glutamate and then to other stress-related metabolites in response to stress[21,22]. Lysine was found to be effective in supporting the growth of excised roots of *Senecio vulgaris* L. (groundsel) in the presence of nitrate as well as in the absence of another nitrogen source[58]. Harris[29] reported that L-lysine and L-arginine exhibit a joint restrictive impact on the growth of oat embryo cultures. Lysine and threonine antagonize each other however but do not inhibit the growth when present separately in the medium[14].

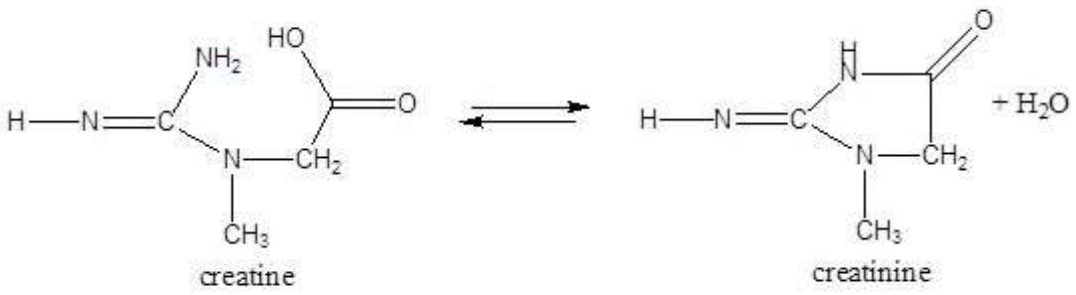

Figure 1: Creatine and creatinine in aqueous solutions exist in equilibrium depending on pH

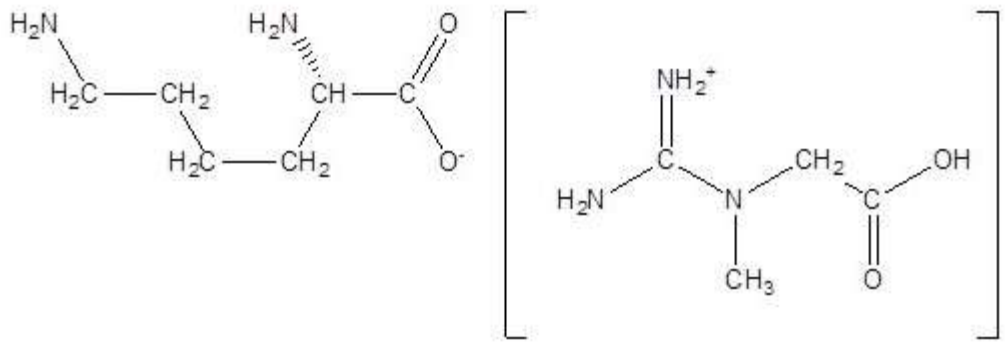

Figure 2: Structure of creatine lysinate

It was recently determined that this amino acid possesses antimicrobial activity against several microorganisms like *Escherichia coli*, *Staphylococcus aureus*, *Trichophyton rubrum* and *Candida albicans*[62]. The objective of the present study was to determine the effect of creatine and creatine lysinate added to MS nutrient medium on *Stevia rebaudiana* Bertoni and *Leontopodium alpinum* Cass micropropagation as well as their effect on antioxidant metabolites content and antioxidant enzyme activity of stevia and edelweiss extracts.

Material and Methods

Method of obtaining creatine-lysinate: Interaction of creatine and lysine in equimolar amounts takes place in an aqueous medium, lower alcohols or their lower esters, preferably ethyl acetate at a temperature of 20-40 ° C. The process leads to depletion of starting materials which is monitored via IR. The product obtained is filtered off and dried. The mother liquors can be returned to the process and used as a reaction medium. Yields are 85-100 % [67].

Initial plant materials: Seeds from *Stevia rebaudiana* Bert. were purchased from the commercial seed source company "Stevia-Paraguay", Paraguay, while these of *Leontopodium alpinum* Cass. were obtained from the garden centre of *Gotse Delchev* originated from Pirin Mountain, Bulgaria. The seeds of both species were sterilized after standard procedures using commercial bleach and germinated on MS based medium[40] containing 0.4 mg/l gibberellic acid. Multiple shoots of *S. rebaudiana* and *L. alpinum* were induced on MS medium supplemented with 1 mg/l BAP as previously described[73]. Stem segments excised from three months old *in vitro* shoots were used as initial explants for the current experiment.

Nutrient media and culture conditions: The MS based nutrient media supplemented with creatine (Cr1, Cr5, Cr10) or creatine lysinate (CrLys1, CrLys5, CrLis10) at a concentration of 1, 5 and 10 mg/l, were used to evaluate the growth and development of shoots. Control plants were grown on MS medium free of plant growth regulators and other additives. The media contained constant quality of agar-agar (0.7%) and sucrose (3%). The medium pH was adjusted to 5.8 before autoclaving at 121 ºC for 20 minutes at a pressure of 1.1 kg cm^{-2}.

Twenty stem explants were placed on each of the seven medium variants and each treatment was repeated twice. The mean number of shoots per explant, shoot height, root length and fresh weight of shoots and roots was assessed after 4 weeks of culture. The *in vitro* cultures were maintained under growth room conditions at a temperature of 22±2ºC, relative humidity of 70% and a 16 h photoperiod under 40 µmol $m^{-2}s^{-1}$ illuminations provided by white fluorescent lamps.

Antioxidant capacity assays: The extraction for the determination of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities was made according to Hristozkova et al[32]. Total SOD (EC 1.15.1.1) activity was determined according to Giannopolitis and Ries[26]. Total CAT (EC 1.11.1.6) activity was measured according to Beers and Sizer[7]. Total APX (EC 1.11.1.1) activity was assayed according to Nakano and Asada[42]. Total GPX (EC 1.11.1.7) activity was determined by Urbanek et al[66]. Soluble protein content was determined by Bradford[13] using bovine serum albumin as a standard. For the antioxidant testing dry samples (0.3 g) from four weeks *in vitro* plantlets were ground and extracted with 96% (v/v) methanol. Concentrations of total phenolic compounds were determined spectrophotometrically using the Folin–Ciocalteu reagent and calculated as caffeic acid equivalents[48].

Flavonoids in plant tissues were measured spectrophotometrically according to Zhishen et al[74] using the standard curve of catechin. Free radical-scavenging activity by using coloured, artificial stable free radicals DPPH• (1,1-diphenyl-2-picrylhydrazyl) was determined spectro-photometrically[63]. The percent inhibition of the DPPH• radical (I %) was calculated by the equation:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the extract) and A_{sample} is the absorbance of the extract. The ferric reducing antioxidant power (FRAP) was monitored by Benzie and Strain[9]. The level of water-soluble (WS-AOC) and lipid-soluble antioxidant capacity (LS-AOC) expressed as equivalents of ascorbate and α-tocopherol respectively were assayed spectrophotometrically by the method based on the absorption of phosphomolibdenum complex proposed by Prieto et al[49].

Statistical analysis: Data were subjected to one-way ANOVA analysis of variance for comparison of means and significant differences were calculated according to the Fisher LSD test at the 5% level using a statistical software package (Statigraphics Plus, version 5.1 for Windows). Data were reported as means ± standard deviation.

Results and Discussion

Effect of creatine and creatine lysinate on the growth of *in vitro* shoots of *S. rebaudiana* and *L. alpinum*: The effect of creatine applied to MS medium alone or as creatine lysinate on the shoot induction and growth of *S. rebaudiana* was evaluated (Table 1, figure 3). The higher number of shoots per explant and high shoot height and root length were induced on a nutrient medium containing creatine lysinate at all three studied concentrations compared with the values of these parameters in plantlets propagated on MS with creatine (1, 5 and 10 mg/l). The highest number of shoots (2.40) was recorded on MS medium containing 5 mg/l creatine lysinate CrLys5. The mean number of shoots per explant formed on the MS media supplemented with creatine varied from 1.55 to 1.65. The mean fresh weight results

followed almost the same trend as the mean number of shoots per explant.

The mean shoots FW was higher in creatine lysinate containing MS media and lower when creatine was applied alone in nutrient media at all three concentrations. The longest roots and higher root fresh weight were recorded on MS medium with the addition of 10 mg/l creatine lysinate CrLys10 medium.

Table 1
Effect of creatine and creatine lysinate on *in vitro* growth *Stevia rebaudiana* plantlets

MS nutrient media	Shoots			Roots	
	Number explant^{-1}	Height cm	FW mg	length cm	FW mg
Control	1.65±0.13ab	8.95±0.35cd	0.164±0.01c	1.34±0.10ab	0.04±0.008a
Cr1	1.55±0.15a	7.55±0.37b	0.158±0.01bc	1.22±0.16a	0.04±0.009a
Cr5	1.75±0.17b	7.50±0.38b	0.149±0.02b	1.44±0.19bc	0.05±0.009ab
Cr10	1.65±0.14ab	6.29±0.44a	0.128±0.01a	1.45±0.14bc	0.03±0.002a
CrLys1	1.80±0.18b	9.13±0.34e	0.166±0.01c	1.48±0.17c	0.05±0.009ab
CrLys5	2.40±0.18c	10.04±0.39e	0.185±0.02e	1.76±0.18d	0.06±0.004bc
CrLys10	2.00±0.16b	8.37±0.33c	0.172±0.01d	1.99±0.19e	0.07±0.006c
LSD	0.494	1.05	0.036	0.497	0.021

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test (5%) after performing ANOVA multifactor analysis.

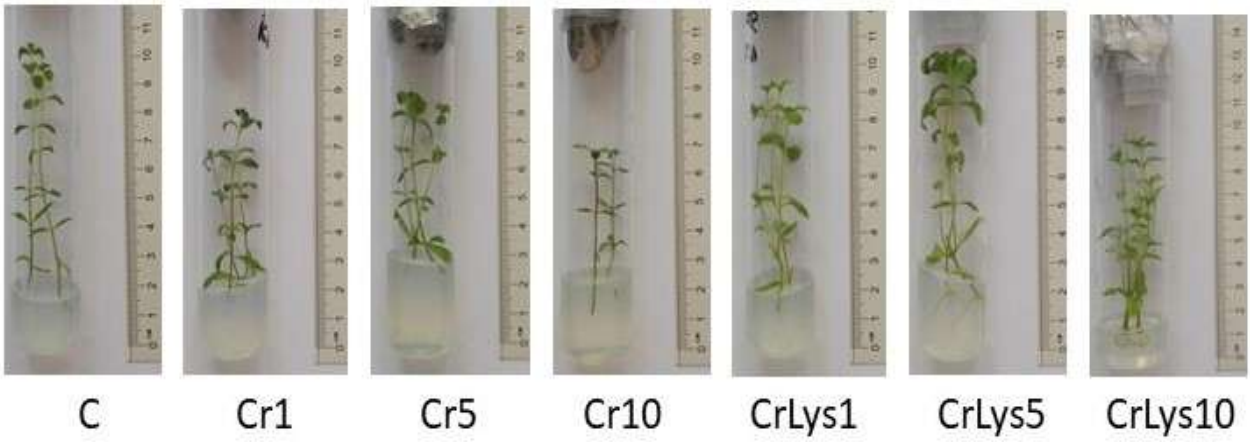

Figure 3: *In vitro* plantlets of *Stevia rebaudiana* cultured on MS media at different concentrations (1, 5 and 10 mg/l) of creatine and creatine lysinate

Table 2
Effect of creatine and creatine lysinate on *in vitro* grown *Leontopodium alpinum* plantlets

MS nutrient media	Shoots			Roots	
	Number explant^{-1}	Height cm	FW mg	length cm	FW mg
Control	24.50±1.05c	2.44±0.21a	0.686±0.09b	1.91±0.13c	0.040±0.04b
Cr1	23.00±1.29bc	2.31±0.21a	0.532±0.01a	1.24±0.17a	0.019±0.01a
Cr5	20.65±1.46ab	2.35±0.20a	0.588±0.05ab	1.62±0.11b	0.031±0.03b
Cr10	19.55±1.15ab	2.17±0.27a	0.512±0.06ab	1.64±0.04b	0.059±0.03c
CrLys1	19.05±1.22a	3.61±0.34bc	1.07±0.10d	1.94±0.17c	0.043±0.02b
CrLys5	18.20±1.28a	3.22±0.33b	0.943±0.09cd	2.62±0.17d	0.083±0.05d
CrLys10	17.05±1.32a	3.51±0.54bc	0.876±0.10c	2.77±0.31d	0.092±0.04e
LSD	3.530	1.330	0.439	0.506	0.059

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test (5%) after performing ANOVA multifactor analysis.

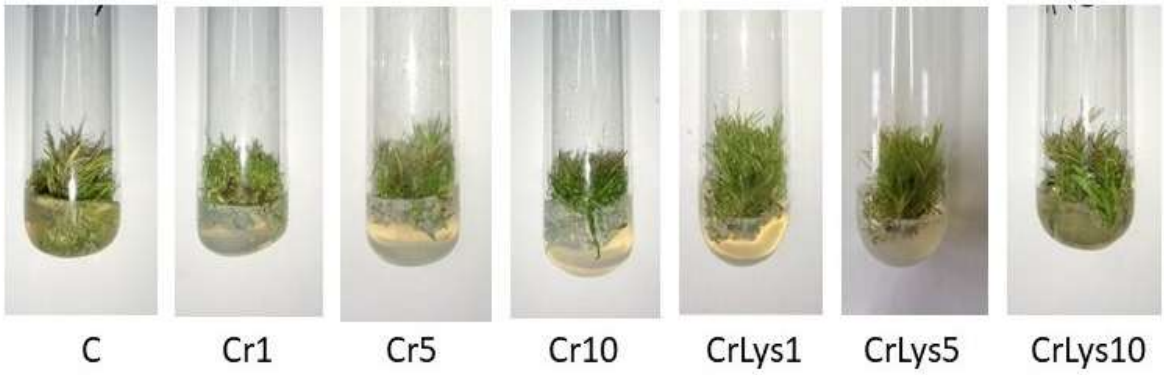

Figure 4: Multiplied shoots of *Leontopodium alpinum* developed on MS media at different concentrations (1, 5 and 10 mg/l) of creatine and creatine lysinate

Multiple shoots of *L. alpinum* were induced on all tested nutrient media. Besides the well-formed shoots, many small buds were noticed. The mean number of shoots per explant was the highest in plants grown on the control MS medium (24.50) (Table 2, figure 4). The explants cultured on the other tested media produced an average number of 17.05 to 23.00 shoots. The shoots grown on CrLys1 medium reached maximum height (3.61cm) and highest fresh weight (1.07g). The higher values of shoots height and FW were recorded in plantlets grown on MS medium by adding creatine lysinate (1, 5, 10 mg/l) than the values of these parameters of plants cultured on creatine containing media. The *L. alpinum* roots were the longest and had the highest FW on MS nutrient medium supplemented with 10 mg/l creatine lysinate CrLys10.

The present study demonstrates that creatine and lysine when applied together as creatine-lysinate salt in the MS nutrient media influenced positive apical growth and elongation of stevia and edelweiss *in vitro* plantlets. The shoots were vigorous and characterized by a large leaf area. Stimulation of axillary buds and development of adventitious shoots was observed when explants of stevia were grown on a medium supplemented with 5 mg/l creatine lysinate CrLys5. In edelweiss, the suppression of axillary buds induction was noticed in creatine and creatine lysinate containing culture media and the best number of shoots per explant was recorded on the control medium. But in plants cultivated at MS media with creatin lysinate salt, the plantlets were with higher height and fresh biomass accumulation.

The creatine had a stimulatory effect on the growth of wheat seedlings and plants were characterized by broader leaves and longer, better branched roots than those of the normal culture[59]. However, in our experiments, the creatine added to the nutrient medium separately did not affect growth and the *Leontopodium alpinum* plantlets were almost indistinguishable from the control. Even in the edelweiss plantlets, a decrease in the mean number of shoots per explants, height and FW was recorded. The favourable effect on the growth and development of *in vitro* plants (*S. rebaudiana* and *L. alpinum*) was established when using creatine lysinate salt on MS medium for micropropagation.

An increase in the formation and elongation of the cell wall and cell division has been observed due to the supplementation of amino acids[6]. It was found that amino acids (50 mg/l glutamine and 100 mg/l casein hydrolysate) produced calli of *S. rebaudiana* with more embryogenic and regeneration potential with the comparison with only PGR supplemented based culture medium[17]. The response to exogenous addition of amino acids during plants growth includes inhibition or stimulation of growth, morphological abnormalities and developmental alterations according to the concentration of the applied amino acids, their D- or L-configuration, the developmental stage of the plant and the source of nitrogen in the nutrient media[30]. It was found that an excessive increase of lysine content affects plant growth and development as these effects differ between different species due to the differences in metabolic flux and connections with lysine metabolism[71].

Endogenous biosynthesized lysine or exogenous lysine additions, plays an important role in reducing the stress of environmental conditions. Enhancing stress tolerance by an increase of the endogenous level of lysine content in potatoes and safflower plants grown under water-deficient conditions has been reported[41,72]. Furthermore, treating with lysine has been effective in reducing the adverse effects of heavy metal stress on wheat[54] and drought stress on radish[43]. These results follow the data presented in this study. The addition of creatine lysinate to the MS medium positively affects shoot proliferation and root induction.

Effect of creatine and creatine lysinate on the antioxidant potential of *S. rebaudiana* and *L. alpinum* plantlets: A significant difference in the enzyme's antioxidant activities in the two studied plants affected by creatine or creatine lysinate was observed (Figure 5, figure 6). The level of the activities of SOD, APX and GPX in stevia *in vitro* plantlets was significantly higher than in edelweiss while the activity of CAT is much more pronounced in edelweiss (Figure 5). According to Das et al[18], the activities of the antioxidant

enzymes SOD, CAT and GPX are species-dependent and the authors reported different values of the activities in ten studied plant species belonging to the family Combretaceae.

In stevia plantlets, the level of SOD activity increased slightly with increasing concentration of creatine and creatine lysinate added to the MS medium. In edelweiss plantlets, the activities of CAT, APX and GPX decrease with the addition of creatine or creatine lysinate to the culture medium at all three concentrations (1, 5 and 10 mg/l) compared with the control plants. In contrast, the activity of SOD increases significantly with increasing concentrations of creatine and creatine lysinate and this increase reaches 130 % compared to the control at the highest concentrations of the studied amino acids. Similar results of enhanced SOD activity, with a simultaneous decrease in the activity of GPO, CAT and ARX were obtained by Wójcik et al[69] working with the hyperaccumulator *T. caerulescens* under Zn stress.

Concerning catalase (CAT), there is a clear tendency to decrease the enzyme activity by increasing the concentration of creatine and creatine lysinate in both studied plant species (Figure 5B). A decrease in catalase activity was also observed in rice, wheat and cucumber seedlings exposed to NaCl and low temperature[57]. The authors suggested that the fall in catalase activity is a phenomenon occurring in many plant species under oxidative stress and is related to the accumulation of salicylic acid in oxidatively-stressed plants.

APX activity in edelweiss decreased in all variants compared to the control, especially in the treatment with creatine lysinate (Figure 5C). In stevia, there is also a drastic (over 50%) decrease in the activity of this enzyme in most variants, except for the variant with the lowest concentration of creatine (Cr1) and the average concentration of creatine lysinate (CrLys5). APX is the most abundant antioxidant enzyme in cells and is functionally more active than all other antioxidant enzymes for the scavenging of H_2O_2 under organic stress[68]. Our results suggest that adding creatine and creatine lysinate to the nutrient media has a favourable effect on physiological processes in the studied plant species.

In stevia plantlets, the addition of creatine to the nutrient medium led to a slight decrease in GPX activity while the addition of creatine lysinate at low (1 mg/l) and medium (5 mg/l) concentrations stimulated the enzyme activity (Figure 5D). A sharp decrease in the GPX activity of edelweiss plantlets was observed with the increasing concentrations of creatine and creatine lysinate added to the MS medium. In edelweiss plantlets, creatine and creatine lysinate reduce the GPX activity.

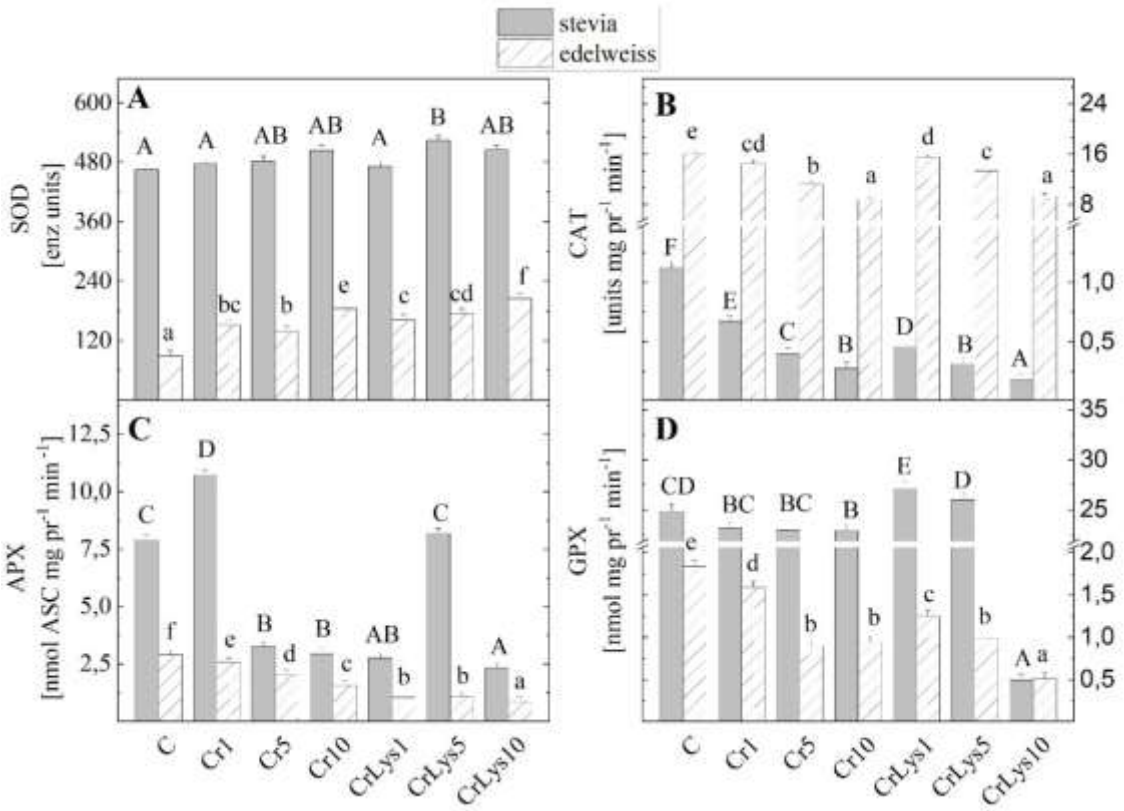

Figure 5: The activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) in the *in vitro* propagated *Stevia rebaudiana* and *Leontopodium alpinum* plantlets treated with creatine or creatine lysinate (at a concentration of 1, 5 and 10 mg/L). Values are means ± SE, n=20; different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multi-factor analysis. The statistical analysis of *Stevia rebaudiana* (uppercase) and *Leontopodium alpinum* (lowercase) was performed separately

Regarding the non-enzymatic antioxidant activity, our results showed that stevia plantlets are richer at secondary metabolites with antioxidant capacity (phenols, flavonoids water and lipid-soluble antioxidant metabolites) than edelweiss plantlets (Figure 6). Phenol content decreased in edelweiss treated with creatine, but increased after treatment with the lowest (CrLys1) and the highest (CrLys10) concentrations of creatine lysinate salt. In stevia, their amount is higher than the control in the variants with medium (CrLys5) and the highest (CrLys10) concentrations of the studied amino acids.

As the concentration of creatine at the MS medium increases, the content of flavonoids in the aboveground parts of edelweiss decreases (Figures 6B). The addition of creatine lysinate salt led to an increase in the amount of flavonoids, both compared to the values observed in the creatine treatments and compared to the control. There was no clear trend in the effect of creatine alone on flavonoid content in stevia plantlets. At a low concentration of creatine lysinate, the values of this indicator are lower than the control while higher concentrations led to a slight increase in flavonoid content.

The amount of water-soluble antioxidants was ambiguously affected by creatine and its combination with lysine as a salt (Figure 6C). An increase in their content is observed at the lowest (Cr1) and the highest (Cr10) concentration of creatine as well as at the highest concentration of creatine lysinate (CrLys10) in stevia plantlets. In the case of the edelweiss plantlets, the values of this parameter decreased or remain close to the control except in the case of the highest concentration of creatine lysinate (CrLys10).

Changes in the content of lipid-soluble antioxidant metabolites are analogous to those observed in catalase. In both the plant species, there is a gradual decrease in their amount with increasing concentration of creatine and creatine lysinate (Figure 6D).

Gradual increases in ferry reducing antioxidant power were recorded with increasing creatine and creatine lysinate content added to the nutrient medium for stevia propagation, which is more pronounced in the presence of creatine and less in creatine lysinate (Figure 6E). It can be assumed that in this case, lysine in higher concentration suppresses the effect of creatine. Henke et al[30] reported both synergistic and antagonistic effects of different amino acids added in different combinations in the nutrient media. In edelweiss, the values decrease to the same extent in the presence of creatine, regardless of its concentration and gradually reach the control values when combined with lysine.

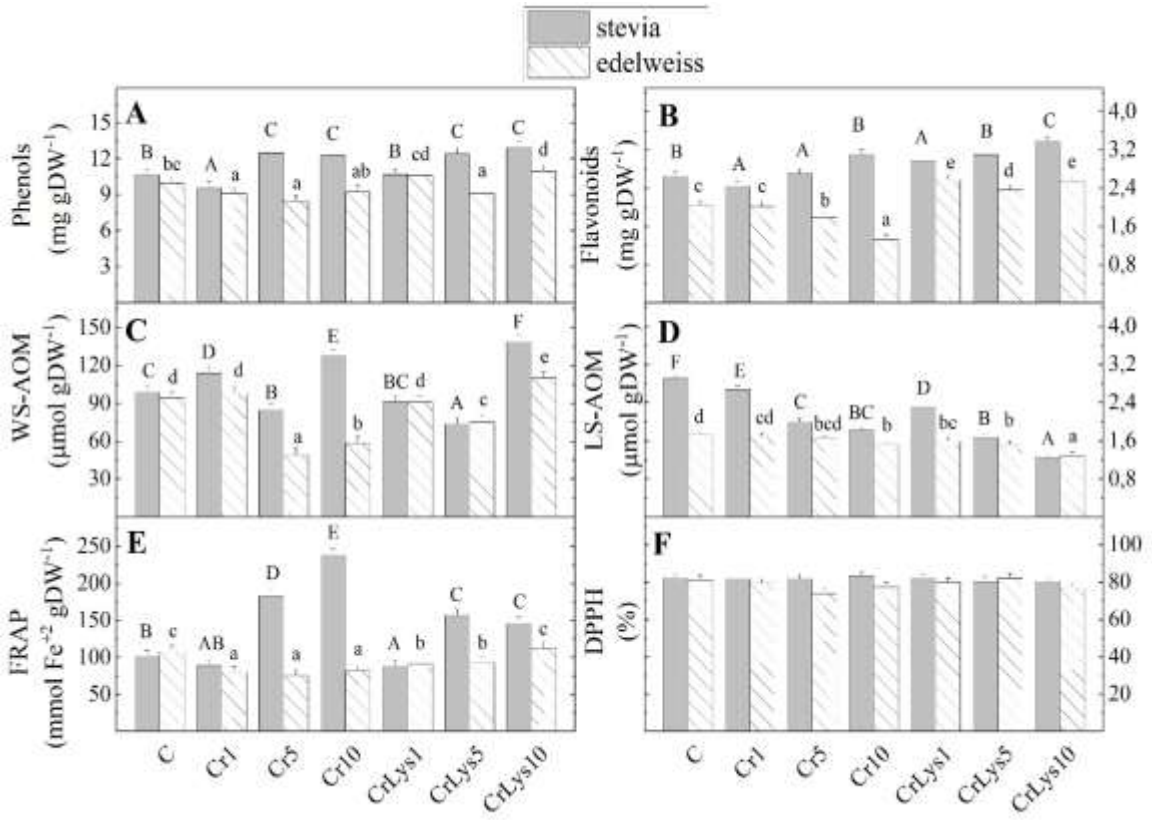

Figure 6: Antioxidant potential and content of metabolites with antioxidant power in the *in vitro* propagated *Stevia rebaudiana* and *Leontopodium alpinum* plantlets treated with creatine or creatine lysinate (at a concentration of 1, 5 and 10 mg/l). Values are means ± SE, n=20; different letters indicate significant differences assessed by the Fisher LSD test (P≤0.05) after performing ANOVA multi-factor analysis. The statistical analysis of *Stevia rebaudiana* (uppercase) and *Leontopodium alpinum* (lowercase) was performed separately

The results show that lysine normalizes the antioxidant activity measured by the FRAP method, which is altered by the addition of creatine. In the case of stevia, creatine increases it and lysine returns it closer to control and in edelweiss, the opposite is observed - creatine reduces it and lysine returns it to the control value. The antioxidant status of the two studied plant species measured by the radical scavenging assay (DPPH method) did not show statistically significant differences in either of the treatments compared to the control (Figure 6F). Most likely, creatine alone and in combination with lysine as a salt added at the selected concentrations did not cause changes in the overall antioxidant status of the two studied plant species.

Conclusion

The proper assessment of the nutritional and metabolic needs of the cells and tissues of the cultivated species is crucial for the positive impact on plant development. Furthermore, during *in vitro* propagation, plants are subjected to very harmful conditions - high relative humidity in the vessels, low ventilation rate, high concentrations of plant growth regulators and low light availability. Adding creatine lysinate to MS nutrient medium plays a role as an additional nitrogen source and increased the plant's tolerance to stressful environmental conditions. This is evidenced by an increase in shoot growth and root induction.

The results of this study indicated that the creatine and creatine-lysine in MS nutrient medium affected differently the growth of *in vitro* plantlets and the activity of some antioxidant enzymes of *Stevia rebaudiana* and *Leontopodium alpinum*. Adding the creatine lysinate to the MS medium influenced better *S. rebaudiana* and *L. alpinum* shoot and root growth more than creatine. The higher activities of the antioxidant enzymes (SOD, APX and GPO) as well as the higher content of flavonoids and phenols were measured in stevia compared to edelweiss. However, future research should be done in order to clarify the role of these amino acids.

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