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Original Research

The Effect of Sodium Amidotrizoate on the Growth and Metabolism of *Wolffia arrhiza* (L.) Wimm.

A. Pietryczuk[1]*, R. Czerpak[1], M. Grabowska[1], T. Wolski[2]

[1]Institute of Biology, Department of Plant Biochemistry, University of Białystok, Świerkowa 20B, 15-950 Białystok, Poland
[2]Department of Pharmacology, Medical University of Lublin, Al. Racławickie 1, 20-059 Lublin, Poland

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Abstract

Sodium amidotrizoate is characterized by structural similarity with TIBA, and it could be speculated that it functions as a plant growth regulator. The aim of this work was to detect how it influences the growth and metabolism of the smallest vascular plant, *Wolffia arrhiza* Wimm., that is commonly used in biotechnological treatment of sewage, especially of human and agricultural origin. Sodium amidotrizoate strongly suppressed the growth of *W. arrhiza* (expressed as a fresh weight) by 21-30%, chlorophyll *a* by 9-30%, chlorophyll *b* by 26-30%, total carotenoids by 28-41%, monosaccharides by 6-10% and water-soluble proteins by 10-28% at the range of concentrations of 10^{-5}-10^{-4} M, in comparison to the control. On the other hand, sodium amidotrizoate at lower concentrations (10^{-7}-10^{-6} M) increased the content of analyzed biochemical compounds in *W. arrhiza*, except of the total pool of carotenoids. After seven days of treatment with 10^{-7}-10^{-6} M sodium amidotrizoate, cultures were analyzed by SDS-PAGE, which showed the presence of some new specific polypeptides. Moreover, 10^{-7}-10^{-6} M sodium amidotrizoate caused a two-fold increase in the activities of ascorbate peroxidase (APX) and NADH peroxidase. 10^{-8} M sodium amidotrizoate had no statistically significant effect on the *W. arrhiza*. Sodium amidotrizoate acts as a plant growth regulator and it could be speculated that it performs activities, similar to TIBA.

Keywords: 3,5-acetamide-2,4,6-triiodosodium benzoate (sodium amidotrizoate), chlorophyll *a* and *b*, total carotenoids, monosaccharides, water-soluble proteins, APX, NADH peroxidase

Introduction

Auxins are synthesized in the apical buds and translocated basipetally by polar transport. Earlier works have shown that the polar transport of auxins and its regulation played an important role in many aspects of plant growth and development, such as the control of cell elongation and division, tropisms, apical dominance, root formation, stem growth and differentiation of vascular tissues [1, 2]. The auxin transport at the cellular level involves two distinct processes associated with the plasma membrane, namely influx and efflux of auxin molecules, the latter being the rate-limiting component in the auxin transport system. The transport of auxin by the efflux carrier is non-competitively inhibited by a group of synthetic inhibitors such as naphthylphthalamic acid (NPA), 2,3,5-triiodobenzoic acid (TIBA),

*e-mail: annapiet@uwb.edu.pl

2,4,6-trichlorophenoxyacetic acid (2,4,6-T) and 9-hydroxyfluorene-9-carboxylic acid (HFCA). Two of the most frequently occurring inhibitors are TIBA and NPA [2, 3].

TIBA and NPA probably bind to the protein at the plasma membrane and, directly or indirectly, inhibit the auxin efflux carrier. It has been proposed that IAA efflux catalytic side and the NPA binding side be located on separate proteins linked by a third, transduction protein, having a rapid turnover rate [4]. In other studies made on pea seedlings, it was suggested that the polar auxin transport could be modulated by the protein phosphorylation and dephosphorylation and this modulation is correlated with change in binding affinity of the receptor of NPA [5-7]. Moreover, there is evidence that TIBA blocks the movement of calcium, which probably leads to the inhibition of auxin transport [8]. The accumulation of auxin inhibits tropic responses and cell elongation [9, 10]. Moreover, anti-auxins have been reported to promote or modify morphogenetic processes *in vitro* by negating the excess of exogenous or endogenous auxins in the cultures and activate the dormant buds by negating the apical dominance. Furthermore, it has been proposed that anti-auxins act as basipetal auxin inhibitors, thereby correcting the cytokinin-to-auxin ratio required for optimum axillary shoot proliferation [3].

3,5-acetamide-2,4,6-triiodosodium benzoate (sodium amidotrizoate, uropolinum) is a synthetic derivative of TIBA commonly used as a contrast in radiology. In contrast to TIBA, it has three iodine atoms, C2-, C4- and C6-substituted, and additional substituents – two acetamide groups C3- and C5-substituted (Fig. 1.). It can be supposed that structurally similar compounds may bind to the same receptor and share similar physiological activity. However, there is no information about the activity of sodium amidotrizoate in plants.

Wolffia arrhiza (L.) Wimm. (Lemanceae) is the smallest vascular greatly reduced plant that has no leaves, stems or roots. It may set flowers and seeds but in our environmental conditions it reproduces only vegetatively. Moreover, this plant is characterised by fast multiplication in a vegetative way of life, large resistance to various stresses and toxic conditions and the possibility of mixotrophic feeding. Thanks to these properties, *Wolffia* is used commonly in biotechnological treatment of sewage, especially of human and agricultural origin. In the waters of Poland this plant is becoming more and more widespread, particularly in small and shallow bodies of eutrophic waters [11, 12].

In the light of these facts, the fundamental aim of this study was to detect if 3,5-acetamide-2,4,6-triiodosodium can act as a plant growth regulator and determine its ability to influence the growth and development of *W. arrhiza* – one of the principal species in water environments. Our research focused on analyses of biochemical activity depending on different concentrations of sodium amidotrizoate.

Experimental Procedures

Plant Material and Growth Conditions

The cultures of the *Wolffia arrhiza* (L.) Wimm. (Lemnaceae) came from the collection of the Institute of Biology at the University of Białystok. The *Wolffia arrhiza* was cultivated for 7 days under controlled conditions at 25±0.5°C. Illumination was supplied during a 16-h photoperiod (8-h dark period) by a bank of fluorescent lights yielding a photon flux density of 50 µmol m^{-2} s^{-1} PAR (Photosynthetically Active Radiation) at the surface of the tubes. PAR was measured with the phytophotometre FF-01 (SOMOPAN, Poland). The culture mineral medium used was Hutner's medium in the following nutritive solution: 500 mg/l EDTA, 500 mg/l $MgSO_4 \times 7H_2O$, 400 mg/l KH_2PO_4, 354 mg/l $Ca(NO_3)_2 \times 4H_2O$, 200 mg/l KOH, 200 mg/l NH_4VO_3, 65.9 mg/l $ZnSO_4 \times 7H_2O$, 25.2 mg/l $Na_2MoO_4 \times 2H_2O$, 24.9 mg/l $FeSO_4 \times 7H_2O$, 17.9 mg/l $MnCl_2 \times 4H_2O$, 14.2 mg/l H_3BO_3, 3.95 mg/l $CuSO_4 \times 5H_2O$, 0.2 mg/l $Co(NO_3)_2 \times 6H_2O$. 1 / 20 dilution of the medium was used in this study. Analysis of growth and selected biochemical parameters was made after 7 days of cultivation. *W. Arrhiza* was cultured in sterile plastic boxes containing 100-ml medium. About 0.5 g of *W. arrhiza* cultures were treated with sodium amidotrizoate. Sodium amidotrizoate dissolved in distilled water was applied at five concentrations: 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M. Weaker solutions were prepared by serial dilution. An equal amount of distilled water was added to the control. Cultures were conducted in five replications.

Fresh Weight Determination

For fresh weight determination, the test plants were filtered and kept on filter paper for a few minutes to remove excess liquid, and weighed.

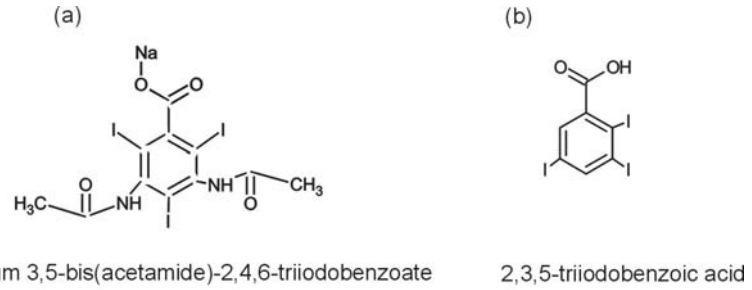

Fig. 1. Chemical structure of (a) 3,5-acetamide-2,4,6-triiodosodium benzoate (sodium amiditrizoate) and (b) 2,3,5-triiodobenzoic acid (TIBA).

Monosaccharide Determination

For monosaccharide concentrations, plant samples were first collected by filtration. Next, the monosaccharide content present in the fresh weight of *W. arrhiza* (0.1 g) was estimated after extraction in ethanol over 24h. The monosaccharide concentration was determined spectophotometrically, according to a method described by Samogyi and Nelson's using an arsenomolybdate reagent [13]. The absorbance was measured with a Shimadzu UV-Vis 1201 spectrophotometer.

Determination of Photosynthetic Pigments

The content of photosynthetic pigments followed filtration and homogenization of *W. arrhiza* fresh weight (0.1 g) in 99.9% methanol at 70°C for 30 min. [14]. The absorbance of the extract was measured with a Shimadzu UV-Vis 1201 spectrophotometer at 652.4 and 665.2 nm for chlorophylls *a* and *b* and at 470.0 nm for carotenoids. The amounts of photosynthetic pigments present in the methanol extract were calculated according to the equations of Wellburn [14].

Determination of Water-Soluble Proteins

The measurement of the content of protein soluble in water was done by the filtration and homogenization of *W. arrhiza* fresh weight (0.1 g), and extracting the fraction of water-soluble proteins overnight in 0.1 M NaOH at 4°C. Protein concentration was determined spectrophotometrically by the Lowry method [15] using Folin phenol reagent with a protein kit calibrated with bovine serum albumin as the standard. The absorbance was measured with a Shimadzu UV-Vis 1201 spectrophotometer.

SDS-PAGE

Soluble proteins for SDS-PAGE were extracted from seven-day-old cultures. The fresh weight of *W. arrhiza* was passed through filter paper under pressure and quickly homogenized using liquid nitrogen and subsequently a lysis buffer containing 0.5 M Tris-HCl (pH 6.8), 4% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.001% bromophenol blue. The extract was incubated at 100ºC for 5 min., then centrifuged at 800 × g for 10 min. The supernatant was removed and centrifuged at 10,000 × g for 5 min. 20 µl samples of SDS: protein, at a ratio of 4:1 (v/v) were loaded onto a 12% polyacrylamide gel containing 0.1% SDS and the buffer system as per Laemmli [16]. Gels were run at 20ºC at a constant current of 15 mA for approximately 4 h, and then stained with Coomassie brilliant blue [17].

Assay of Enzyme Activities

For extraction of APX and NADH peroxidase, the fresh weight of *W. arrhiza* was passed through filter paper under pressure and homogenized using liquid nitrogen and subsequently a lysis buffer containing 0.1 M sodium phosphate buffer (pH 6.0) containing 0.1 mM EDTA and 1% PVP. The homogenate was centrifuged at 12,000 g for 20 min and the resulting supernatant was used for the determination of APX activity. The entire extraction procedure was carried out at 4ºC.

APX was determined according to Nakano and Asada [18]. The decrease in ascorbate concentration declined in the optical density at 290 nm, and activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for ascorbate. One unit of APX was defined as the amount of enzymes that breaks down 1 mmol of ascorbate per min.

NADH peroxidase was determined according to Ishida et al. [19]. The decrease in NADH concentration was seen to decrease in the optical density at 340 nm, and activity was calculated using the extinction coefficient (6.3 mM^{-1} cm^{-1}) for NADH. One unit of NADH peroxidase was defined as the amount of enzymes that breaks down 1 mmol of ascorbate min^{-1}.

All enzyme activities were measured using a Shimadzu UV-Vis 1201 spectrophotometer.

Each treatment consisted of 5 replicates and each experiment was carried out at least twice at different times. Data was expressed as mean ± SE. A minitab statistical package was used to carry out a one-way ANOVA. The Student's t-test was used to estimate the difference between means. Differences at $p<0.05$ were considered statistically significant.

Results

The graphic presentation of the contents of fresh weight, chlorophyll *a* and *b*, carotenoids, monosaccharides, water-soluble proteins, SDS-PAGE of total proteins isolated from *W. arrhiza* and the activities of the antioxidant enzymes under the influence of the concentrations of sodium amidotrizoate from 10^{-8} to 10^{-4} M, is given in Figs. 2-9. The content percentage of the analyzed biochemical parameters was determined in relation to the control culture of *Wolffia arrhiza*.

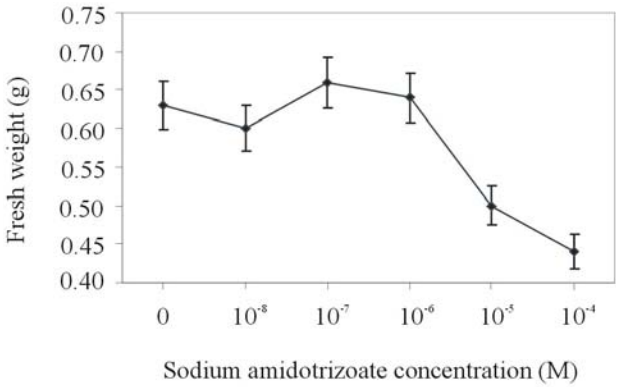

Fig. 2. The effect of the different concentrations of sodium amidotrizoate on the fresh weight of *W. arrhiza* after 7 days of cultivation. At day 0 fresh weight was 0.5 g. Mean values ± SE. (Significantly different from the control, $P \leq 0.05$, $n = 5$).

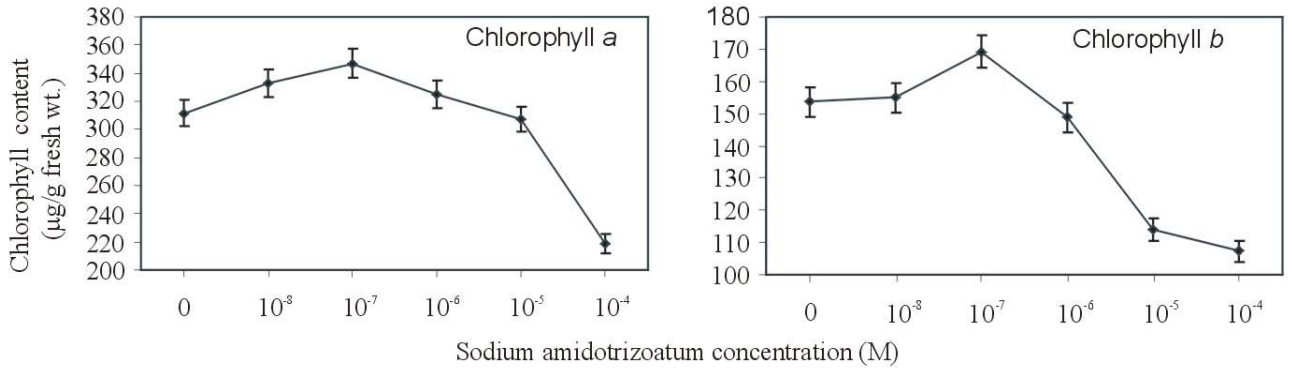

Fig. 3. The effect of the different concentrations of sodium amidotrizoate on contents of chlorophyll *a* and *b* in *W. arrhiza* after 7 days of cultivation. At day 0 content of chlorophyll *a* was 661.1 µg/g fresh wt. and the content of chlorophyll *b* was 228.7 µg/g fresh wt. Mean values ± SE. (Significantly different from the control, $P \leq 0.05$, n = 5).

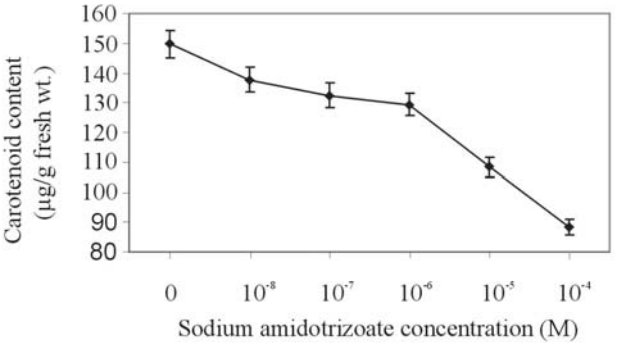

Fig. 4. The effect of the different concentrations of sodium amidotrizoate on the content of total carotenoids in *W. arrhiza* after 7 days of cultivation. At day 0 carotenoid content was 178 µg/g fresh wt. Mean values ± SE. (Significantly different from the control, $P \leq 0.05$, n = 5).

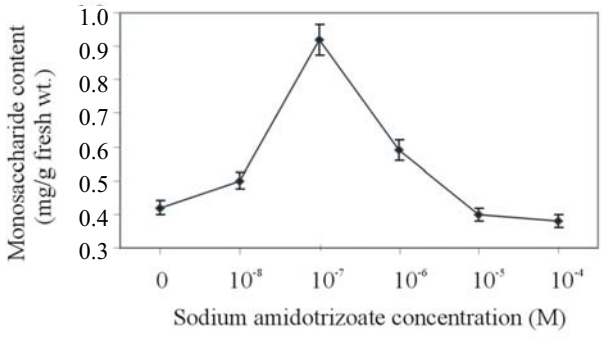

Fig. 5. The effect of the different concentrations of sodium amidotrizoate on the content of monosaccharides in *W. arrhiza* after 7 days of cultivation. At day 0 content of monosaccharides was 0.38 mg/g fresh wt. Mean values ± SE. (Significantly different from the control, $P \leq 0.05$, n = 5)

The most inhibiting influence on the content of fresh weight of *W. arrhiza* by sodium amidotrizoate was seen at the concentration of 10^{-4} M. It was noted that the fresh weight decreased by 30% in respect to control. The inhibitory action was observed also in the case of 10^{-5} M sodium amidotrizoate, which depressed fresh weight by 21% below the control. Other concentrations of sodium amidotrizoate had no statistically significant effect on the growth of *W. arrhiza* (Fig. 2).

The addition of sodium amidotrizoate at the concentration of 10^{-7} M resulted in a slight increase of chlorophylls. *W. arrhiza* treated with it contained more chlorophyll *a* and *b* (by 11%) than the control culture. At the concentrations in the range of 10^{-5}-10^{-4} M, this compound acted also as an inhibitor of chlorophyll accumulation and depressed chlorophyll *a* content by 9-30% and chlorophyll *b* by 26-30%, compared with the control culture. No statistically significant changes in chlorophyll content under the influence of 10^{-8} M sodium amidotrizoate was observed. (Fig. 3). Total carotenoid content decreased from 9 to 41% under the influence of all applied concentrations of sodium amidotrizoate. The greatest decrease was noted at 10^{-4} M concentration (Fig. 4).

Treatment with sodium amidotrizoate enhanced the content of monosaccharides in fresh weight of *W. arrhiza*. The two-fold increase in accumulation of reducing sugars, as compared with the control, occurred in the incubations with the addition of 10^{-7} M. Sodium amidotrizoate at a concentration of 10^{-6} M caused weaker increase of mosaccharide content by 40% over control. The analyzed compound at higher concentrations (10^{-5}-10^{-4} M) caused a slight decrease of the content of monosacharides only by 6-10% below the control (Fig. 5).

The addition of 10^{-7}-10^{-6} M sodium amidotrizoate caused a weak increase in the level of water-soluble proteins in *W. arrhiza*, in the range of 10-18% in comparison to the control culture. Moreover, the SDS-PAGE analysis of the total cellular proteins showed that under the influence of the most stimulating concentrations of sodium amidotrizoate (10^{-6} and 10^{-7} M), about 5 new proteins with molecular weights of 72, 94, 111.5, 120 and 138 kDa were detected. Furthermore, one polypeptide band with molecular mass of 70 kDa appeared to be in higher concentration than in the control (Fig. 7). The protein content in fresh weight in incubations with 10^{-5}-10^{-4} M addition fell from 10 to 28%, compared with the control. No statistically significant changes in protein content under the influence of 10^{-8} M sodium amidotrizoate was observed (Fig. 6).

The sodium amidotrizoate treatment had a significant effect on the activities of the antioxidative enzymes such as APX (ascorbate peroxidase) (Fig. 8) and NADH peroxidase (Fig. 9). It was discovered that the activity of both of the tested enzymes increased markedly (about twice in comparison to the control) in the presence of 10^{-7}-10^{-6} M sodium amidotrizoate.

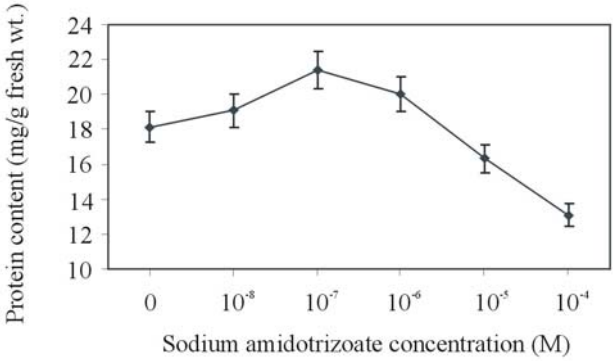

Fig.6. The effect of the different concentrations of sodium amidotrizoate on the content of water-soluble proteins in *W. arrhiza* after 7 days of cultivation. At day 0 content of water-soluble proteins was 17.31 mg/g fresh wt. Mean values ± SE. (Significantly different from the control, $P \leq 0.05$, n = 5)

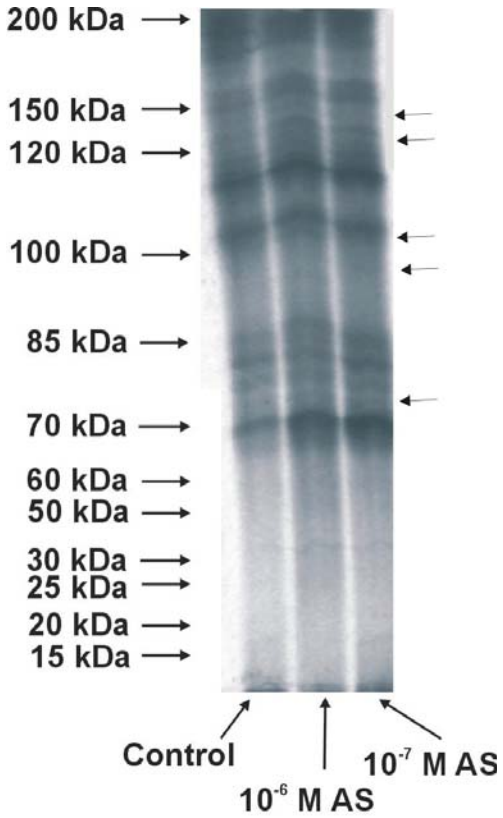

Fig. 7. The SDS-PAGE of the total water-soluble proteins isolated from *Wolffia arrhiza* cultivated in the presence of 10^{-7}-10^{-6} M sodium amidotrizoate (AS) compared to the control culture.

Discussion

The antihormones are a group of compounds that inhibit biosynthesis of natural phytohormones and block their receptors, transport or regulatory system. It is suggested that the affinity of analogues of bioregulators, each specific to their own receptors, is related to their physiological activity. The hormone-receptor interaction should initiate the physiological response typical for the type of cell. The antiauxin activity is manifested by TIBA, which inhibits polar auxin transport in plants [20]. The simplest chemical analogue of TIBA is sodium amidotrizoate.

Previous data indicated that TIBA at concentrations in the range of 0.02-4 × 10^{-5} M caused a significant reduction in the number of shoots and roots in *Brassica napus.* Moreover, TIBA was a more effective inhibitor of shoot formation [10]. Other studies revealed that the number of roots of tomato seedlings decreased with the increasing concentration of TIBA in the medium. Full inhibition of rooting was obtained with 0.8-0.75 μM [21]. It was also detected that 10^{-4} M TIBA was lethal to axillary buds, whereas shoots cultured on medium containing 10^{-6}-10^{-5} M TIBA were significantly shorter than control plants. Moreover, plants cultured on the medium containing TIBA were characterized by abnormal leaf and stem development. 10^{-7} M TIBA had no effect on the stem height and fresh weight of shoots. On the other hand,

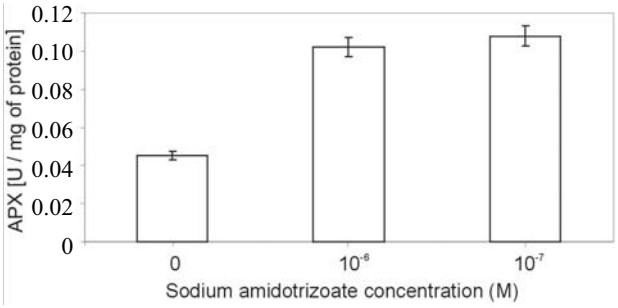

Fig. 8. The activity of the ascorbate peroxidase (APX) in *Wolffia arrhiza* after 7 days of cultivation. At day 0 the activity of APX was 3.0 U/mg protein. Mean values ± SE. (Significantly different from the control, $P \leq 0.05$, n = 5)

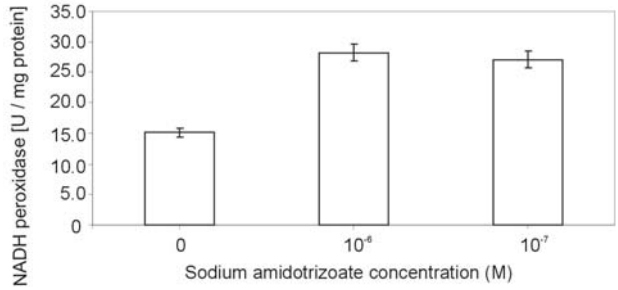

Fig. 9. The activity of the NADH peroxidase in *Wolffia arrhiza* after 7 days of cultivation. At day 0 the activity of enzyme was 13.9 U/mg protein. Mean values ± SE. (Significantly different from the control, $P \leq 0.05$, n = 5).

TIBA at the concentration of 10^{-8} M caused significant increase of stem growth of sweet potato (*Ipomoea batatas* L.) (by 21%) and fresh weight of the shoot (by 17%) [22]. This data corresponds with our results, where 10^{-4} M sodium amidotrizoaate acted as a strong inhibitor of the fresh weight of *W. arrhiza* and the levels of all analyzed metabolites. The inhibiting activity of the analyzed compound became weaker with decreasing concentrations. Sodium amidotrizoate at the concentrations of 10^{-8}-10^{-6} M had no statistically significant effect on *W. arrhiza* growth. It is speculated that TIBA and other plant growth retardants act by inhibiting cell division in the subapical zones of the shoot apex and furthermore reduce stem elongation [22]. In light of these facts and our findings, it could be supposed that sodium amidotrizoate could inhibit cell division in plants.

Other data showed that the application of TIBA to elongating inernoodes of plants (*Pea* sp.) markedly reduced the endogenous level of growth, promoting hormones – especially gibberelin (GA_1) and indole-3-acetic acid (IAA). There is evidence that inhibiting stem elongation by TIBA was at least partially connected with the reduction of IAA levels. Moreover, the movement of exogenous GA_1 may be inhibited by TIBA. This would explain why TIBA inhibited the growth of plants [23] and it could be speculated that sodium amidotrizoate may act in the same way.

Our examination showed that *W. arrhiza* grown under the influence of the 10^{-5}-10^{-4} M analyzed compound was characterized by lower levels of chlorophyll *a* and *b* in comparison to control plant, while the sodium amidotrizoate concentration of 10^{-7} M increased the chlorophyll *a* and *b* content over control. Moreover, our research revealed that sodium amidotrizoate at concentrations in the range of 10^{-7}-10^{-6} M strongly stimulated the content of monosaccharides in the fresh weight of *W. arrhiza* and caused a double increase of these metabolites. Previously, it was detected that TIBA treatments caused leaf abscission and decreased leaf area of the two sesame cultivars. Moreover, TIBA also suppressed the content of capsule wall chlorophyll [24]. On the other hand, it was reported that TIBA at lower concentrations enhanced chlorophyll synthesis in plant stems and leaves [22]. Research conducted on the *Rosa hybrida* L. indicated that the content of leaf chlorophyll *a*+*b* in 2-4 μM TIBA-treated plants was higher by 6-55% over control. Moreover, TIBA at low concentrations also increased the number of shoots per explants and the length of shoots of *R. hybrida* in comparison to the control plants. It is supposed that anti-auxins may negate the effect of high levels of endogenous auxins that get accumulated in the plant tissues cultivating in vitro. Furthermore, anti-auxins can correct the cytokinin to auxin ratio required for axillary shoot proliferation [3]. The increase of crucial metabolites as a response to the low concentrations of TIBA, observed also in our research concerning sodium amidotrizoate, is probably a result of the above.

Earlier works showed that TIBA enhanced glucose catabolism but had no effect on its anabolism [25]. Jacobson et al. [26] revealed that the accumulation of some of the Crebs cycle intermediates was reduced by TIBA. The concentration of malate decreased by 26% and fumarate by 21% in the presence of 2 μM TIBA. It was suggested that either entry into the Crebs cycle or some biochemical reaction within was blocked as a response to TIBA application. It was speculated that TIBA could inhibit metabolic entry into Crebs cycle by altering the physical and chemical properties of mitochondrial membrane [26]. Moreover, it is known that TIBA reduced phosphoenolopyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) activity [27]. It was also documented that TIBA in concentrations in the range of 10^{-8}-10^{-7} M caused an increase of accumulation of emergency sugars in the root system [22]. A marked increase of monosaccharide content was observed in *W. arrhiza* grown in a medium containing sodium amidotrizoate. It seems that, in contrast to TIBA, sodium amidotrizoate can probably act as an activator of glucose biosynthesis and the degradation of emergency sugars.

10^{-5}-10^{-4} M sodium amidotrizoate caused a reduction of water-soluble proteins up to 10-28% as compared with control. As in the case of the other analyzed parameters, concentrations of 10^{-7}-10^{-6} M increased the accumulation of proteins by 10-18% over the control. What is more, some new specific polypeptides with molecular masses in the range of 72-136 were synthesized in *W. arrhiza* under the influence of sodium amidotrizoate. Moreover, a polypeptide with a molecular weight of 70 kDa appeared to be in a higher concentration than in control as analyzed by SDS-PAGE. Numerous literary data revealed that TIBA influences the enzyme activity, especially anti-oxidant enzymes. It was reported that the GST (glutathione S-transferase) activity and the content of proteins increased markedly as the result of 100 mM TIBA application [28]. Our work focused on the activities of two enzymes: APX (EC 1.11.1.11) and NADH peroxidase (EC 1.11.1.1). These enzymes belong to the family of oxidoreductases and play an important role in detecting and protecting cells against the toxic effects of H_2O_2 in higher plants. Their activities are enhanced in plants by salinity and drought [29]. It was noted that the sodium amidotrizoate application markedly increased the activities of these enzymes in comparison to the control culture.

Conclusions

The above facts allow us to conclude that sodium amidotrizoate plays an important role in the growth and metabolism of *W. arrhiza*. First of all, sodium amidotrizoate acted in a dose-dependent manner. At the concentration of 10^{-5}-10^{-4} M, it depressed the content of fresh weight and the accumulation of analyzed metabolites. Lower concentrations of the examined compound had a stimulative effect on the content of primary metabolites in fresh weight of *W. arrhiza* (except of the content of total carotenoids) and on the activity of the antioxidant enzymes. In contrast to TIBA, 10^{-8} M sodium amdidotrizoate had no statistically significant influence on most analyzed parameters.

Sodium amidotrizoate is characterised by weaker activity than TIBA, which exhibits a stimulative effect also at the concentration of 10^{-8} M. It is probably connected with the presence of two additional acetamide groups in comparison to TIBA. It is commonly known that the additional substituents change the activity of phytohormones [30]. Sodium amidotrizoate is intensively involved in the metabolism and development of plants and in all likelihood its biological effects are similar to TIBA. However, more detailed studies are necessary to prove the correctness of this hypothesis.

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