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The Effect of Auxins and Salicylic Acid on Chlorophyll and Carotenoid Contents in *Wolffia Arrhiza* (L.) Wimm. *(Lemnaceae)* Growing on Media of Various Trophicities

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Abstract

In *Wolffia arrhiza {Lemnaceae)* growing on municipal tap water (rich in mineral but poor in organic components) and thus being exclusively photoautotrophic, IAA (3-indolilacetic acid) and SA (salicylic acid) strongly stimulated action on the content of chlorophylls *a* and *b* and carotenoids (especially β-carotene and lutein + zeaxanthin). On the other hand, the chemical analogues of IAA, i.e. PAA (phenylacetic acid) and NAA (α-naphtylacetic acid), had a generally inhibitory effect on chlorophyll and carotenoid contents. In *Wolffia arrhiza* growing on raw waste water and a suspension of activated sludge from a sewage treatment plant (rich in organic substances) characterized by mixotrophism (that is photo- and heterotrophism) PAA had the highest stimulative action on the chlorophyll *a* and *b* content, SA negligible, whereas NAA had an inhibitory effect. IAA had a slight stimulative effect on raw sewage but inhibitory on activated sludge. Also, the greatest stimulative effect on carotenoids content was exerted by PAA; SA had a slight stimulative effect while IAA and, to a greater extent NAA had a clearly inhibitory influence.

Keywords: *Wolffia arrhiza,* nutrient availability, pigments, salicylic acid, auxins

Introduction

The rootless *Wolffia arrhiza* (L.) Wimm., of the duckweed family *{Lemnaceae),* is the smallest angiospermous plant which, depending on environmental conditions, may feed phototrophically and mixotrophically, that is simultaneously photo- and heterotrophically, and even entirely heterotrophically. *Wolffia arrhiza* is very resistant to the action of various stress and toxic factors, e.g. insufficient light, excess of ammonium salts, heavy metals, cyanides and other xenobiotics [1]. In the waters of Poland this plant is becoming more and more widespread, particularly in small and shallow eutrophic waters [2, 3].

It is known that depending on the chemical composition of the aquatic environment, mainly on its trophicity, the *Wolffia arrhiza* can be a phototrophic or mixotrophic organism, whereas when there is no light it becomes completely heterotrophic [2, 3, 4].

The quantitative and qualitative composition of chlorophylls and carotenoids, pigments directly connected with photosynthesis, in duckweeds, particularly the *Wolffia arrhiza* as a typical mixotrophic plant, has not as yet been analysed in detail. Furthermore, the effect of the chemical composition of the nutrient medium and its trophic character on the photosynthetic pigments has not been studied. For this reason, the effect of chemically

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differentiated auxins and salicylic acid as the main phenolic acid, on changes in the chemical composition of chlorophylls and carotenoids was studied.

Materials and Methods

The rootless *Wolffia arrhiza* was grown for 20 days under stable conditions (growth chamber), i.e. 22 (±1) °C with 12-hour fluorescent lighting giving a photosynthetically active radiation intensity of 50 μmol m^{-2} s^{-1}. The experiments were carried out in 2-litre crystallizers, 19 cm in diameter, containing 1 litre of fluid medium. The containers were covered with perforated plastic film of complete light transmittance.

The *Wolffia arrhiza* was grown on three fluid media of different trophicity: tap water (poor in organic components), raw municipal wastes (rich in soluble and colloidal organic compounds) and activated sludge (rich in suspended-colloidal organic substances). As a result the rootless *Wolffia* growing on the tap water was an entirely phototrophic organism, on the raw wastes it was mixotrophic (that is photo- and partly heterotrophic) whereas the *Wolffia arrhiza* on the activated sludge was mixotrophic with a domination of heterotrophism. Cultures were conducted in three replications.

In view of this, we undertook comparative biochemical studies of the effect of auxin compounds, differing in chemical structure, and salicylic acid (SA) as the main representative of phenolic acids on the changes in the content of chlorophylls and carotenoids in the *Wolffia arrhiza*, growing on three media differing in their trophicities. In the experiments the above compounds were applied in concentration ranges of 10^{-3} to 10^{-7} M. On the 15th day of the culture, when the *Wolffia arrhiza* had reached the maximum level of development and metabolism [2], the biomass was collected and after dehydration on filter paper, samples of approximately

Table 1. Effect of auxins (IAA, PAA, NAA)* and phenolic acid (SA)* on the total of photosynthetic pigment contents in *Wolffia arrhiza* (L.) Wimm. growing in various trophical conditions.

Culture medium	Names of chlorophylls and carotenoids	Content of pigments in μg g^{-1} biomass**				
		Control	IAA	PAA	NAA	SA
Raw sewage	chlorophyll *a*	52.92	54.26	60.61^{c}	49.23	52.81
	chlorophyll *b*	16.88	17.59	19.47^{c}	15.75	17.16
	epimer chlorophyll *a*	0.70	0.49^{c}	0.42^{c}	0.59	0.55
	pheophytin *a*	0.55	0.34^{c}	0.73^{b}	2.66^{d}	0.41
	β-carotene	7.95	7.76	8.95	7.54	8.20
	isomer β-carotene	1.64	1.77	1.54	1.45	1.56
	alloxanthin	0.31	0.82^{c}	1.09^{d}	0.87^{c}	0.98^{d}
	lutein + zeaxanthin	23.07	21.91	22.28	19.92	21.79
	neoxanthin	3.37	3.24	2.83	2.68^{c}	2.94
	violaxanthin	2.46	2.49	3.96^{c}	3.53	3.65^{c}
	19'-hexanoyloxyfucoxanthin	0.58	0.54	0.51	0.49	0.58
Suspension of activated sludge	chlorophyll *a*	54.91	46.74^{c}	74.25^{d}	42.43^{c}	53.50
	chlorophyll *b*	17.47	14.93^{c}	23.85^{c}	13.51^{d}	15.34
	epimer chlorophyll *a*	1.24	0.65^{c}	0.98	0.59^{c}	1.52
	pheophytin *a*	0.84	1.49^{c}	3.31^{d}	0.89	0.34^{c}
	β-carotene	8.80	8.22	15.47^{b}	6.23^{c}	8.86
	isomer β-carotene	1.81	1.84	3.55^{c}	1.36	1.45
	alloxanthin	1.17	0.84	2.18^{c}	0.94	0.75^{a}
	lutein + zeaxanthin	22.91	20.57	33.82^{c}	18.53^{b}	19.14
	neoxanthin	3.28	2.22^{b}	1.71^{c}	4.03^{a}	2.36^{a}
	violaxanthin	3.57	2.38^{b}	2.24^{c}	2.58	5.28^{c}
	19'-hexanoyloxyfucoxanthin	0.59	0.55	1.77^{d}	0.91^{d}	0.13^{d}
Tap water	chlorophyll *a*	71.84	111.79^{d}	66.02^{a}	65.78^{a}	104.77^{d}
	chlorophyll *b*	22.59	31.66^{c}	20.06^{a}	20.18	32.71^{d}
	epimer chlorophyll *a*	0.57	0.52	0.53	0.56	0.92^{a}
	pheophytin *a*	0.42	0.24^{a}	0.26^{a}	0.25^{a}	0.62
	β-carotene	11.23	18.26^{d}	9.13^{a}	9.97	16.35^{c}
	isomer β-carotene	2.12	4.02^{d}	1.91^{a}	2.04	3.18^{b}
	alloxanthin	0.67	0.29^{c}	0.96^{a}	0.63	0.98
	lutein + zeaxanthin	30.91	51.61^{d}	25.54^{a}	27.37	45.39^{c}
	neoxanthin	4.67	5.07	2.78c	2.57c	4.56
	violaxanthin	4.59	2.41c	4.70	3.71	6.43^{b}
	19'-hexanoyloxyfucoxanthin	1.08	0.78	0.70	0.43c	0.73

* optimal concentration 10^{-6} M

**SE lessthan5% (n=3)

Significantly different from the representative value in the control: ^{a} $p < 0.05$, ^{b} $p < 0.02$, ^{c} $p < 0.01$, ^{d} $p < 0.001$.

1 g were weighed immediately and covered with 2 ml of spectrally pure acetone. After homogenisation of the samples, extraction in a nitrogen atmosphere was complete after a period of 12 hours in darkness and a temperature of 20°C.

Pigments were determined by ion-pairing, reverse-phase HPLC. To 1000 µl of the clear extract, 300 µl of ion-pairing reagent (1.5 g tetrabuthylammonium acetate and 7.7 g ammonium acetate, made up to 100 ml with water) was added according to Mantoura, Llewellyn [5]. The HPLC equipment consisted of Shimadzu LC-6A double-system pump, driven by a gradient programmer Shimadzu SCL-6B and Rheodyne 7125 injetor equipped with a 20 µl loop. Detection was by a Shimadzu SPD-6AV UV-VIS spectrophotometric detector set on 440 nm and Shimadzu RF-535 fluorescence detector (λ_{ex} = 430 nm, λ_{em} = 670 nm).

Chromatographic separation was carried out with a Waters Spherisorb C_{18} ODS 2 column (250 x 4.6 mm, 5 µm particles). The solvents for the HPLC gradient (Baker) used were as follows: solvent A was ion-pairing reagent : water : methanol (10 : 10 : 80); solvent B was acetone : methanol (20 : 80) according to Mantoura, Llewellyn [5]. The flow rate was 1 ml min^{-1} and the gradient was linear. The program utilised was 41 min long. It started with 0% solvent B (100% solvent A), reached 100% solvent B in 11 min, stayed with 100% of solvent B for 25 min and returned to original conditions in 5 min. Chlorophyll *a,* chlorophyll *b* and β-carotene were obtained from Sigma Co. Derivatives were prepared by methods of Sartory [6]. Other pigment standards were obtained from the International Agency for ^{14}C Determinations, Denmark.

The results were evaluated using the Student's t-test for unpaired data.

Results and Discussion

In these studies the typical photosynthetic pigments, chlorophylls *a* and *b* were found in the rootless *Wolffia* irrespective of the trophic character of the nutrient medium. The ratio chlorophyll o/chlorophyll *b* was about 3.3 and was similar to presented earlier [7]. In addition, small amounts of chlorophyll *a* epimer and pheophytin *a* were found.

Of the carotenoids only β-carotene and a considerable amount of its isomer were identified, α-carotene commonly occurring in plant life was not found. The following xanthopylls were identified: alloxanthin, lutein + zeaxanthin, neoxanthin, violaxanthin and 19'-hexanoyloxyfucoxanthin (Table 1). Of these lutein + zeaxanthin, neoxanthin and violaxanthin, the latter in all probability actively participate in the xanthophyll cycle of the *Wolffia anhiza,* as they do in most vascular plants and green algae [8].

In the *Wolffia anhiza* growing on tap water, poor in organic substances but rich in mineral components, far more chlorophylls (particularly chlorophyll *a),* β-carotene and xanthophylls associated with the xanthophyll cycle were found than in the *Wolffia* growing on raw waste water and a suspension of activated sludge very rich in organic and mineral substances (in all cases $p < 0.05$). On the other hand, the *Wolffia anhiza* growing on a suspension of activated sludge contained rather more chlorophyll *a* and *b,* β-carotene and the xanthophylls: alloxanthin ($p < 0.01$) and violaxanthin ($p < 0.02$), than those growing on the raw waste waters (Table 1).

The results obtained in these studies show that in *Wolffia anhiza* growing on tap water photoautotrophism dominates and thus enhances the synthesis of chlorophylls and carotenoids essential to the light phase of photosynthesis. On the other hand, the *Wolffia* growing on raw waste water and a suspension of activated sludge containing large amounts of organic substances limits the process of photosynthesis to a minimum in favour of heterotrophism. That is why the chlorophylls and carotenoids content in these plants is much lower.

Earlier studies [2, 3] showed that *Wolffia anhiza* growing on raw waste water as compared with the *Wolffia* growing on tap water increases intensity of dark respiration by 100% while it reduces photosynthetic efficiency by approximately 70%. In these plants an increase in proteins of about 50%, and transketolase activity, related to the pentosophosphate cycle, by 80% was also observed.

Of the auxins applied to the *Wolffia anhiza* growing on tap water, the content of chlorophylls and carotenoids including β-carotene and xanthophylls, mainly lutein + zeaxanthin, were most intensively stimulated by IAA. Salicylic acid also acted stimulatively but somewhat weaker. On the other hand the chemical analogues of IAA, i.e. PAA and NAA had an inhibitory effect in comparison with the control culture (without exogenous auxins and SA). In this case the biochemical reaction of *Wolffia* to PAA and NAA is atypical in comparison with the numerous studies made of vascular plants [9, 10], mainly cultivated, and green algae, e.g. of the *Chlorella* genus and others [11-14]. In these plants NAA and PAA displayed a marked activity stimulating chlorophylls and carotenoids synthesis and the oxidation of carotenes into xanthophylls, mainly neoxanthin, violaxanthin and zeaxanthin analogically to the action of IAA.

On the other hand, in *Wolffia anhiza* growing on raw waste water characterized by mixotrophism, a marked stimulative action on chlorophyll and carotenoids content, including carotenes and xanthophylls; alloxanthin and violaxanthin was exerted only by PAA, whereas SA and IAA had only a slightly stimulative effect. NAA had an inhibitory effect on the chlorophyll and carotenoids content except for alloxanthin and violaxanthin, the content of which was stimulated.

In *Wolffia anhiza* growing on a suspension of activated sludge, the richest in organic components, where it becomes an organism with a domination of heterotrophism over photoautotrophism, PAA was also found to have the most potent stimulative effect on the chlorophyll *a* and *b,* β-carotene and xanthophylls contents with the exception of neoxanthin and violaxanthin. However, the other auxins IAA and NAA as well as SA had a slightly inhibitory effect on chlorophylls and carotenoids with the exception of violaxanthin, which was significantly stimulated under the influence of SA as compared with the controls. The strongest inhibitory effect on the chlorophylls, β-carotene and xanthophylls, except for neoxanthin and 19'-hexanoyloxyfucoxanthin was

Table 2. Effect auxins (IAA, PAA, NAA)* and phenolic acid (SA)* on total chlorophylls and principals groups of carotenoid contents in *Wolffia arrhiza* (L.) Wimm. growing in various trophical conditions.

Culture medium	Mains groups of pigments	Content of chlorophylls and mains genera of carotenoids (μg g^{-1} biomass)				
		Control	IAA	PAA	NAA	SA
Raw sewage	**chlorophylls**	71.05	72.68	81.23	68.23	70.93
	carotenoids:	45.79	44.80	48.46	43.18	46.87
	carotenes	9.59	9.53	10.49	8.99	9.76
	pool xanthophylls	29.79	29.00	30.67	27.49	29.94
	oxygen-rich xanthophylls	6.41	6.27	7.30	6.70	7.17
Suspension of activated sludge	**chlorophylls**	74.46	63.81	102.39	57.42	70.70
	carotenoids:	49.57	41.77	66.46	42.10	45.74
	carotenes	10.61	10.06	19.02	7.59	10.31
	pool xanthophylls	31.52	26.56	41.72	26.99	27.66
	oxygen-rich xanthophylls	7.44	5.15	5.72	7.52	7.77
Tap water	**chlorophylls**	95.42	144.21	86.87	86.77	139.02
	carotenoids:	65.61	90.70	53.90	53.43	89.34
	carotenes	13.35	22.28	11.04	12.01	19.53
	pool xanthophylls	41.92	60.16	34.68	34.71	58.09
	oxygen-rich xanthophylls	10.34	8.26	8.18	6.71	11.72

* optimal concentration 10^{-6} M
chlorophylls = chlorophyll (a + *b)* + epimer chlorophyll *a* + phaeophytin *a*
carotenes = β-carotene + isomer β-carotene
pool xanthophylls = alloxanthin + lutein + zeaxanthin + neoxanthin + violaxanthin + 19'-hexanoyloxyfucoxanthin
oxygen-rich xanthophylls = neoxanthin + violaxanthin + 19'-hexanoyloxyfucoxanthin

exerted by NAA, whereas IAA had only a slight inhibitory effect. The results of the studies clearly indicate that the chemical composition of the nutrient media applied and their trophic character exert in the rootless *Wolffia anhiza,* a typical mixotrophic plant, a significant effect on the intensity of the biosynthesis of the various photosynthetic pigments but do not change their qualitative composition (Table 1). Depending on the chemical structure of the auxins IAA, PAA and NAA and the phenolic acid SA, the content of the various types of chlorophylls and carotenoids also changes (Table 2). It was found that the qualitative composition of the chlorophylls and carotenoids in the rootless *Wolffia anhiza* is stable regardless of the type of medium used and differences in the chemical structure of the auxins studied and SA and that only the quantitative relations of the various pigments change.

The studies carried out on the *Wolffia anhiza* - a mixotrophic plant, are not completely in agreement with analogical studies of typical photosynthetic vascular plants [9, 10, 15-19] and green algae, e.g. of the green algae e.g. *Chlorella, Scenedesmus* and other [11, 13, 14, 20]. In these plants IAA and, more rarely, NAA usually exerted the strongest stimulative action on chlorophyll and carotenoid contents, PAA had a much weaker stimulative effect, whereas SA had the weakest. It is possible that the physiological-biochemical activity of the auxins studied in the *Wolffia anhiza* growing on raw waste water or a suspension of activated sludge, that is when it is mixotrophic, is analogical to their biological activity in the plumules of germinating seeds and alga spores [10, 11, 18] in their early stage of development, i.e. the hetero - and mixotrophic phase, before they completely convert to photosynthesis.

Abbreviations

IAA - 3 indolilacetic acid; NAA - α-naphtylacetic acid; PAA - phenylacetic acid; SA - salicylic acid.

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