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Differential Effects of Bentazon and Molinate on *Anabaena cylindrica*, an Autochthonous Cyanobacterium of Portuguese Rice Field Agro-ecosystems

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Abstract The effects of bentazon and molinate, two selective herbicides recommended for integrated weed management in rice, were studied in *Anabaena cylindrica*, an abundant cyanobacterium isolated from a Portuguese rice field agro-ecosystem. Comparative effects of both herbicides on *A. cylindrica* were estimated under laboratory conditions by measuring its dry weight yield, photopigments, and carbohydrate and protein contents in a time- and dose-dependent exposure throughout 72 h. Photosynthesis and respiration were also monitored. The results revealed that both herbicides exerted a pleiotropic effect on the cyanobacterium at the range of concentrations tested (0.75–2 mM). Growth, chlorophyll *a*, carotenoids and phycobiliproteins were more adversely affected by molinate than by bentazon. Cyanobacterial growth inhibitions of over 50% were observed after 48 h when 1.5–2 mM of molinate were applied. Bentazon concentrations ranging from 0.75 to 2 mM did not significantly modified chlorophyll *a* content with time, however, considerable reductions in chlorophyll *a*, carotenoids and specially phycobiliproteins were observed with molinate. Protein content increased with both herbicides although the effect was particularly noticeable with the highest concentration of molinate. Herbicide effects on carbohydrate content were contrasting: molinate increased this organic fraction whereas bentazon decrease it. Photosynthesis and respiration were inhibited by both herbicides and higher molinate concentrations (1.5–2 mM) completely ceased O_2 evolution after 48 h. Since *A. cylindrica* is abundant in Portuguese rice fields and could be used as an inoculum source in rice biofertilization programs, their protection from potential residual effects of herbicides is fundamental for a correct management of local soil fertility.

Keywords *Anabaena cylindrica* · Bentazon · Cyanobacteria · Growth · Molinate · Photosynthesis · Rice fields

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1 Introduction

Cyanobacteria are a large, diverse and widely widespread group of prokaryotes, comprising unicellular to multicellular microorganisms that carrying out oxygenic photosynthesis (Carr and Whitton 1982; Vermaas 2001). Their

main photosynthetic pigments are chlorophyll *a* (Chl*a*) and carotenoids together with phycobiliproteins. Due to these later pigments and mucilage, the color of cyanobacteria in nature may range from dirty yellow, through various shades of blue-green, to brown or black (Roger and Kulasooriya 1980; Whitton and Potts 2000). Some heterocystous genera are diazotrophic, *i.e.* they can use atmospheric N_2 as the sole nitrogen source, thus contributing to photodependent N_2-fixing in rice fields wordwide (*e.g.*, Asia, see Roger 1995; Europe, see Fernández-Valiente et al. 2000; South America, see Irisarri et al. 2001) and therefore play a vital role in the maintenance and building up of soil fertility. Native strains frequently found in rice field are the heterocystous genera *Anabena*, *Nostoc*, *Calothrix* and *Tolypothrix*, with a predominance of *Anabaena* and *Nostoc* (Whitton 2000; Silva and Silva 2007).

Rice is the most important food crop of the developing world and feeds more than two billion people worldwide as a staple food (Datta 2004). In Portugal, rice fields occupies an area of about 30,000 ha, distributed mainly by the river basins of Tejo and Sorraia (10,000 ha), Mondego (8,000 ha) and Sado and Caia (7,000 ha) (Calha et al. 1999). Portuguese paddy fields are unique artificial agro-ecosystems that are frequently disturbed by intensive agricultural practices such as flooding, drainage, ploughing and application of chemical fertilizers and pesticides (insecticides, fungicides and herbicides). Besides, nowadays it is necessary to apply great amounts of herbicides to control rice weeds whose growth is facilitated by present-day cultivation practices. Therefore, massive utilization of herbicides is an important factor for obtaining high crop productivities (Singh and Datta 2006). However, the subsequent dispersion of those agrochemical compounds and/or of their degradation products have negative effects to the environment (Castro et al. 2005; Park et al. 2005). The extreme sensitivity of cyanobacteria to herbicides is the main concern for a successful exploitation of these microorganisms as potential biofertilizers in rice culture agro-ecosystems. Furthermore, an ideal biofertilizer strain of cyanobacteria must have the ability to tolerate or even resist to toxic actions of herbicides (Singh et al. 2003).

Bentazon [3-(1-methylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide] is the active ingredient of the commercial herbicide Basagran™ and belongs to the chemical group of the benzothiadiazoles. It is a selective and contact herbicide used to control broad-leaved and grassy weeds in post-emergence rice. Bentazon inhibits photosynthetic electron transport chain and CO_2 fixation (Bethlenfalvay et al. 1979; Waxman 1998). Through joining to cellular membranes, interferes also with the transport of cations and cause alterations in cellular turgidity (Ashton and Crafts 1981; Böger and Sandmann 1998). However, its precise molecular mechanism of photosynthetic electron transport inhibition has not been yet characterized, but is assumed to act by binding to the exchangeable quinone site at the photosystem II (PS II) reaction centre (Bagchi et al. 2003).

Molinate (*S*-ethyl hexahydro-1*H*-azepine-1-carbothioate) is the active ingredient of the commercial herbicide Ordram™ and is a selective preemergence thiocarbamate herbicide applied once a year to flooded fields during rice seedling. Molinate caused effects on photosynthesis through inhibition of electron transport chain and it seems to interfere with the biosynthesis and composition of membrane lipids and fatty acids (Ashton and Crafts 1981; Yan et al. 1997; Waxman 1998; Böger and Sandmann 1998; Xia 2005). Chemical names, abstract numbers, structures and other properties of both herbicides are listed in Table 1.

The aim of this work was to establish the toxicity effects of the selected rice field herbicides bentazon and molinate on growth, photosynthetic pigmentation, protein and carbohydrate content of a rice field isolate of the diazotrophic cyanobacterium *Anabaena cylindrica*, which occurs abundantly in Portuguese paddy fields and contributes to the maintenance of local soil fertility. The present investigation was also aimed to elucidate the effect of both agrochemicals on photosynthetic and respiratory activities as important physiological parameters of herbicide toxicity evaluation. This investigation may thus further contribute to a more successful implementation of an effective integrated rice pest management and to a promising future utilization of cyanobacteria in rice biofertilization programs.

2 Materials and Methods

2.1 Microorganism and Culture Conditions

The filamentous and diazotrophic cyanobacterium *A. cylindrica* was isolated from a paddy field soil mud (pH

Table 1 Physico-chemical and partition coefficient properties of bentazon and molinate (Kidd and James, 1991; Kamrin and Montgomery, 2000; Tomlin, 2000)

Herbicide	Chemical class	Chemical name and number (Chemical Abstract Service)	Chemical structure	Molecular weight ($g.mol^{-1}$)	Melting point (°C)	Water solubility ($mg.l^{-1}$)	Vapour pressure (mPa)	Log K_{om}
Bentazon	Benzothia-diazole	3-(1-methylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide CAS no. 25057-89-0		240.28	137-139	500	< 0.46	0.46
Molinate	Thiocarba-mate	*S*-ethyl hexahydro-1*H*-azepine-1-carbothioate; CAS no. 2212-67-1		187.3	< 25	880	746	2.88

5.9) with an area of 2 ha, located at Montemor-o-Velho, eastern Portugal. The experimental work was undertaken in three sampling sites, located more than 10 m away from water inlet and outlet points, and randomly selected during the rice crop cycle of 2006. A sampling system was designed in order to sample either dry or submerged soil, according to Quesada et al. (1995). Isolation and purification of *A. cylindrica* were performed by the usual dilution and plating methods. The desired strain colonies were micro-isolated and subsequently cultured in N-free $BG11_0$ liquid medium (Rippka et al. 1979). Following the achievement of axenic methods, stock cultures for herbicide experiments were maintained in exponential growth by periodically transferring them to fresh medium and grew in 500 ml flasks containing 250 ml $BG11_0$. These stock cultures were incubated in a controlled growth chamber (Conviron mod. E7/2) at 28±2°C and grew photoautotrophic in a mechanical orbital shaker under a light/dark cycle of 16 h/8 h for a period of 3–4 weeks. A quantum flux density of 50 µmol m^{-2} s^{-1} (Quantum Radiometer Photometer, mod. Q102, Macam, Scotland) at the surface of cultures was achieved by a set of Osram Sylvania cool white fluorescent lamps. Cultures were gassed with dry sterilized air once a week and before herbicide experiments.

Basagran™ (liquid formulation, 480 g l^{-1} bentazon, sodic salt) and Ordram™ (granule formulation, 7.5% w/w molinate) were from BASF and Syngenta, respectively, and were purchased from commercial sources. Concentrated stock solutions of both herbicides were prepared by appropriate dilutions in pre-cooled sterilized $BG11_0$ medium and were sterilized through millipore membrane filters. For all experiments, exponentially growing cells were harvested by centrifugation (3,000×*g*, 5 min), washed thrice with sterilized double-distilled water and dispensed equally in assay flasks. An aliquot of the prepared stock herbicide was added aseptically to each culture medium to obtain final concentrations indicated for each treatment (0.75; 1.5; 2 mM). Each herbicide concentration given is for the respective active ingredient. This concentration range was selected for the present study in order to quickly stand out detectable and marked toxic effects of commercial formulations in a relatively short period of time (72 h). The effects of such higher concentrations will reflect the effects of small doses in long-term field experiments. All culture flasks received the same quantity of inocula (23.4 µg Chl*a* mg dw^{-1}) to a final volume of 85 ml and were incubated in 250 ml Erlenmeyer flasks under the prescribed growth conditions, with constant mechanical shaking at 100 rev min^{-1}. Untreated samples without herbicide served as controls. Various aliquots were removed at intervals of 24 h during 4 days (0, 24, 48 and 72 h) to measure the biochemical and physiological parameters.

2.2 Analytical Methods

Growth was estimated as biomass yield and was determined by the cell dry weight method. After being centrifuged and three-times washed thoroughly with distilled water through a cellulose nitrate filter, a definite volume of cyanobacterial suspension was oven-dried 24 h at 90°C. Growth was measured turbidimetrically at 750 nm (OD_{750}) and expressed by using the corresponding dilution curves.

For Chl*a* determination, aliquots of 1 ml washed cells were extracted with pure methanol for 24 h at 4°C, ultrasonicated (30 s using a Vibra-cell sonicator mod. VCX 130, Sonics Materials, equipped with a 3 mm diameter probe operating at 80% in 5 s pulses) and centrifuged at maximum acceleration for 5 min. Chlorophyll content was determined spectrophotometrically at 665 nm according to Marker (1972).

For determination of total carotenoids content, 1 ml of cell aliquots were homogenized with 4 ml of pure acetone at 4°C, ultrasonicated and dark incubated for 24 h. Cell extracts were then centrifuged at 20,000×*g* during 30 min for removal of cellular debris. The content of carotenoids was determined spectrophotometrically at 480, 663 and 645 nm. The obtained values were introduced in the formula given by Davies (1976).

Phycobiliproteins were extracted after osmotic shock according to Wyman and Fay (1986): 1 ml aliquots were centrifuged at maximum acceleration for 10 min and the pellets homogenized in 60–150 µl of glycerol, being thereafter dark incubated at 4°C for 2 h. Water was then added to osmotically lyse cells. Samples were centrifuged, ultrasonicated and the phycobiliproteins were estimated in the supernatant according to Bennett and Bogorad (1973). As a control, absorption spectra of supernatants were measured (400–700 nm) to confirm the absence of Chl*a* contamination because Chl*a* 665 nm peak can interfere with measurements of allophycocyanin at 650 nm. Chlorophyll and phycobiliprotein contents were then corrected for scattering by subtracting the OD_{750} from Chl*a* and phycobiliprotein peaks. All pigment extractions were subsequently repeated until no more pigment was extracted.

Total protein content was determined by Bradford method with minor modifications (Jones et al. 1989). Summarily, a sample of 1 ml was centrifuged at 20,000×*g* during 30 min and the pellet was resuspended in 0.1 N NaOH for 30 min. Afterwards, aliquots of 100 µl were mixed with 400 µl dilute Coomassie Brilliant Blue G-250 (1:5 in dH_2O) and 1,500 µl $BG11_0$. After incubation period, samples were read at 595 nm and converted to protein concentrations using bovine serum albumin as standard.

Total carbohydrates were extracted according to Myklestad and Haug (1972) and then determined by the phenol-sulphuric acid method using D(+) glucose as standard (Dubois et al. 1956). Briefly, aliquots of 1 ml were centrifuged at 20,000×*g* for 30 min and pellets discarded. A suitable volume of water was added and then samples were treated with 50 µl phenol 5% (v/v). After vigorously shaking, 1,600 µl of concentrated H_2SO_4 was added at once to a final volume of 2 ml and samples were kept in ice-water bath for 30 min. Final solution was then measuring at 490 nm. An UV/VIS Varian mod. Carry 50 spectrophotometer was used throughout all analytical determinations.

2.3 Photosynthetic Activity Measurements

Photosynthetic rate (P_m^{Chl}) and dark respiration rate (R_d^{Chl}), both normalized to Chl*a*, were determined as O_2 exchange in a Clark-type O_2 electrode, previously adjusted and calibrated in a Chlorolab 2 oxymeter (Hansatech Ltd., Norfolk, UK) coupled to a DW2/2 measuring cuvette (Hansatech Ltd., Norfolk, UK). Measurements were performed at culture growth temperature. Prior to start of measurements samples were dark adapted for 30 min. Thereafter, a 2 ml aliquot (dry weight, 0.30 mg ml^{-1}) of homogenized cyanobacterial suspension was transferred to measuring chamber, the medium saturated with CO_2 (2 mM $NaHCO_3$, pH 8.3) and then illuminated with a quantum flux density of 2,000 µE m^{-2} s^{-1} during 10 min. Light flux was provided by an high intensity probe light array with 11 red light-emitting diodes centered on 650 nm (ref. LH11/2R, Hansatech Ltd., Norfolk, UK). Dark respiration was immediately measured after net O_2 evolution in the same cuvette. Light was then turned off and after 10 min the rates were calculated by appropriate software (Oxylab ver. 1.15, Hansatech Ltd., Norfolk, UK).

All reagents and solvents used throughout were of analytical grade.

2.4 Statistics

Statistical differences were examined with ANOVA factorial analysis for all data, using the completely randomized experimental design and carried out using the Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA) and StatView for Windows ver. 4.53 (Abacus Concepts, Inc., Berkeley, USA) programs. Comparisons were made with Fischer's PLSD test with a significance level of 5%. Data were expressed as means and standard errors (SE) of at least three independent experiments.

Table 2 Effect of bentazon and molinate on dry weight, chlorophyll *a* (Chl*a*), carotenoids, phycobiliproteins (PBP), protein, carbohydrates, photosynthetic rate (P_m^{Chl}) and dark respiration (R_d^{Chl}) of *Anabaena cylindrica* after exposure for 24, 48, and 72 h

Parameters	Conc. (mM)	0 h		Basagran						Ordram					
				24 h		48 h		72 h		24 h		48 h		72 h	
Dry weight (mg $m1^{-1}$)	0	0.31±0.01	a	0.33±0.00	a	0.36±0.00	a	0.39±0.00	a	0.34±0.00	a	0.35±0.00	a	0.39±0.01	a
	0.75	0.32±0.00	a	0.33±0.00	a	0.35±0.00	a	0.39±0.00	a	0.31±0.00	b	0.30±0.00	b	0.30±0.00	b
	1.5	0.31±0.00	a	0.31±0.00	b	0.34±0.01	b	0.35±0.01	b	0.27±0.00	c	0.21±0.00	c	0.14±0.00	c
	2	0.30±0.00	a	0.30±0.00	b	0.32±0.01	c	0.32±0.01	c	0.18±0.00	d	0.13±0.00	d	0.06±0.00	d
Chl *a* (µg mg dw^{-1})	0	18.18±0.25	a	19.01±0.06	a	19.47±0.45	a	21.41±0.04	a	21.52±0.44	a	22.16±0.42	a	21.10±0.16	a
	0.75	18.47±0.22	a	18.59±0.21	a	19.54±0.24	a	20.10±0.16	a	18.72±0.99	b	19.66±0.28	b	18.91±0.84	b
	1.5	18.31±0.14	a	18.87±0.09	a	19.62±0.16	a	20.88±0.71	a	11.51±0.92	c	7.75±0.74	c	4.47±0.86	c
	2	18.63±0.31	a	19.74±0.18	b	20.04±0.18	a	20.15±0.79	a	10.76±0.58	c	7.41±0.13	c	4.99±1.10	c
Carotenoids (µg.mg dw^{-1})	0	5.07±0.06	a	5.03±0.04	a	5.71±0.04	a	5.71±0.04	a	4.19±0.00	a	4.67±0.26	a	5.50±0.12	a
	0.75	5.00±0.02	a	5.21±0.04	b	5.39±0.08	b	5.70±0.03	a	3.90±0.12	b	4.12±0.05	b	4.03±0.07	b
	1.5	5.10±0.02	a	5.57±0.08	c	5.80±0.11	a	5.93±0.04	b	3.57±0.07	c	2.48±0.11	c	1.36±0.09	c
	2	5.15±0.03	a	5.38±0.05	b	5.75±0.10	a	6.40±0.04	c	1.82±0.01	d	1.32±0.04	d	0.56±0.13	d
PBP (µg mg dw^{-1})	0	139.28±5.96	a	136.60±0.79	a	135.33±4.56	a	164.43±1.18	a	144.20±6.76	a	115.15±0.15	a	152.85±13.86	a
	0.75	135.28±2.13	a	147.39±1.41	b	135.06±3.82	a	179.50±3.91	b	125.03±3.95	b	90.25±0.65	b	110.37±5.20	b
	1.5	148.13±4.45	a	162.05±5.76	c	172.37±1.06	b	143.35±3.16	c	54.99±4.28	c	14.53±4.17	c	9.40±0.95	c
	2	136.36±4.19	a	166.70±1.80	c	175.39±3.57	b	154.82±2.46	d	1.02±0.76	d	4.48±1.29	d	10.83±0.68	c
Protein (µg mg dw^{-1})	0	287.61±3.89	a	276.97±2.39	a	277.67±5.58	a	325.21±6.69	a	261.12±2.53	a	250.37±4.11	a	264.24±10.07	ab
	0.75	291.43±1.12	a	280.77±11.70	a	314.13±4.82	b	299.13±0.27	b	275.45±0.94	ab	257.63±4.87	a	217.83±0.92	a
	1.5	284.68±13.62	a	363.66±3.46	b	343.13±6.61	c	314.74±5.24	ab	275.55±13.99	b	283.46±13.02	b	285.54±23.00	b
	2	294.27±2.41	a	368.25±5.20	b	351.66±2.28	c	361.88±6.33	c	399.50±8.32	c	581.09±9.77	c	1117.55±44.63	c
Carbohydrates (µg mg dw^{-1})	0	279.34±2.27	a	394.74±0.00	a	354.42±5.09	a	408.71±4.60	a	395.14±7.69	a	549.99±9.12	a	614.62±23.85	a
	0.75	225.88±6.51	a	387.71±3.77	a	330.96±7.42	b	339.63±15.50	b	328.54±21.35	a	431.19±17.12	a	609.40±47.26	a
	1.5	208.92±0.00	a	367.37±16.00	a	338.38±0.00	b	336.56±11.15	b	272.30±35.21	a	339.16±7.95	a	583.54±91.29	a
	2	222.44±3.49	a	326.16±6.97	b	323.98±2.58	b	340.01±3.90	b	540.23±12.25	b	773.59±40.68	b	1692.97±148.12	b
P_m^{Chl} (µmol O_2 mg Chl^{-1} h^{-1})	0	29.60±0.88	a	29.31±0.34	a	29.75±0.13	a	31.75±0.77	a	34.45±3.51	a	30.25±2.30	a	36.73±2.33	a
	0.75	26.38±0.55	a	15.02±0.16	b	14.25±0.12	b	9.77±0.52	b	25.88±1.50	b	22.75±0.91	b	29.00±0.37	b
	1.5	31.35±1.25	a	11.19±0.06	c	11.39±0.18	c	6.72±0.73	c	9.36±2.01	c	0	c	0	c
	2	30.32±0.76	a	9.84±0.45	d	5.78±0.13	d	5.49±0.07	c	0.97±0.07	d	0	c	0	c
R_d^{Chl} (µmol O_2 mg Chl^{-1} h^{-1})	0	18.31±0.52	a	18.32±0.33	a	21.73±0.42	a	19.88±0.11	a	17.76±0.79	a	15.39±1.42	a	19.32±0.02	a
	0.75	19.19±0.03	a	14.36±0.16	b	12.40±0.25	b	7.83±0.53	b	16.05±0.08	a	9.69±0.65	b	9.00±0.57	b
	1.5	19.43±0.32	a	10.97±0.32	c	8.46±0.18	c	7.43±0.11	bc	12.49±0.88	b	6.77±0.52	c	4.03±0.12	c
	2	18.41±0.68	a	10.33±0.33	c	5.91±0.00	d	1.54±0.32	d	11.10±0.86	b	5.46±0.78	c	5.83±0.19	d

Values are means ± SE of at least three independent experiments. Results of the one-way ANOVA factorial analysis. Values with a common letter are not significantly different according to Fisher PLSD test ($P<0.05$).

3 Results

Effects of bentazon and molinate on growth, photosynthetic pigments, protein and carbohydrate contents, photosynthesis and respiration of *A. cylindrica* colonies for 72 h were shown in Table 2. The results relatively to control (100%) are demonstrated by the so-called "carped plots" (Fig. 1). This kind of presentation was introduced (see, *e.g.* Vani et al. 1998 and Tsimilli-Michael and Strasser 2008) in order to facilitate comparison of the behaviour of biochemical and physiological parameters under different herbicide concentrations with time. The data presented in Table 2 show that, irrespective of some minor fluctuations, higher levels (1.5 and 2 mM) of bentazon induced a significant ($P<0.05$, LSD) decline on the biomass yield of *A. cylindrica* with time. Maximal decrease of biomass yield measured after bentazon exposure was 18.5% after 72 h, being significant ($P<0.05$, LSD) concerning to control values. Meanwhile, 1.5 mM of bentazon induced a percentage of inhibition to *A. cylindrica* which did not exceed 9.5% when compared to controls. The effect of molinate was, however, more pronounced, since all three concentrations of molinate markedly depressed the growth of the cyanobacterium in a time-dose dependent manner (Table 2; Fig. 1B). Prompt action of molinate on cell dry weight was observed soon after 24 h, inducing a significant ($P<0.05$, LSD) reduction of biomass yield (cell dry weight decreased from 0.31 mg.ml^{-1} at 0 h to 0.30, 0.14 and 0.06 mg ml^{-1} at 72 h for cells exposed respectively to 0.75 mM, 1.5 mM and 2 mM). Exposure of *A. cylindrica* cells to a concentration range of molinate between 0 and 2 mM showed that the concentration inducing 50% of growth rate inhibition after 48 h of exposure (48-h IC_{50}) was between 1.5 mM and 2 mM.

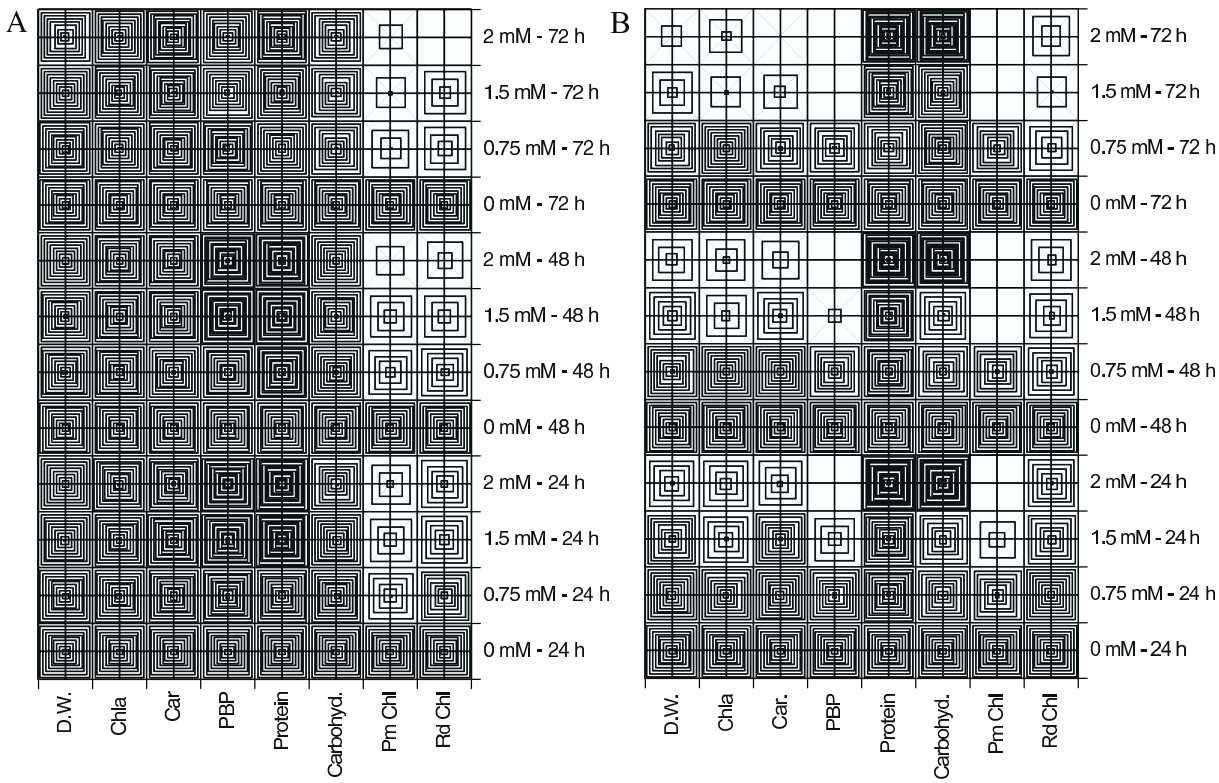

Fig. 1 Effect of bentazon (**A**) and molinate (**B**) on dry weight (D.W.), chlorophyll *a* (Chl*a*), carotenoids (Car), phycobiliproteins (PBP), protein, carbohydrates, photosynthetic rate (P_m^{Chl}) and dark respiration rate (R_d^{Chl}) of *Anabaena cylindrica* after exposure for 24, 48, and 72 h. Values are means ± SE of at least three independent experiments. The plotted values are visualised by the number of the contour lines, with successive lines corresponding to values differing by 0.05 (here above zero, *i.e.* bigger than the control). See Vani et al. (1998) and also Tsimilli-Michael and Strasser (2008) for additional details

Except for a minor negligible increase with 2 mM at 24 h, essentially Chl*a* contents of all bentazon-treated *A. cylindrica* samples were not significantly affected by this herbicide (Table 2; Fig. 1A). On the contrary, Chl *a* content in *A. cylindrica* cells was strongly affected, in a time-dose response manner, in cultures treated with molinate (Table 2; Fig. 1B). Immediately after 24 h of exposure to high molinate levels, Chl*a* contents showed a significant decrease relative to control ($P<0.05$, LSD). At the end of the experiment, after 72 h, Chl*a* contents were significantly ($P<0.05$, LSD) reduced relative to controls by 77.7% and 76,5% for 1.5 mM and 2 mM molinate, respectively.

The content of carotenoids was very slightly but significantly ($P<0.05$, LSD) stimulated in cyanobacterial cells after 72 h with high doses of bentazon (3.8% and 12.2% with 1.5 and 2 mM, respectively) (Table 2). Nevertheless, carotenoids in *A. cylindrica* revealed a different picture in molinate treated cultures, being significantly ($P<0.05$, LSD) depressed at all concentrations in a time-dose response manner (Table 2). At the end of the experiment, the values were reduced by 24.5%, 74.2% and 90.6% relative to control with 0.75 mM, 1.5 mM and 2 mM molinate, respectively.

After bentazon exposure, total phycobiliproteins (PBP) content exhibited an initial period of increased synthesis, from 0 to 48 h, that was succeeded by a period of depression, from 48 to 72 h, except for the lowest bentazon concentration (0.75 mM) (Table 2; Fig. 1A). After 48 h of incubation, the values significantly ($P<0.05$, LSD) increased 27.4% and 29.6% over control with 1.5 and 2 mM bentazon, respectively. These values dropped down to 12.8% (1.5 mM) and 5.8% (2 mM) below controls after 72 h. The effect of molinate on cellular PBP content was time-dose dependent (Table 2; Fig. 1B). The highest tested concentration (2 mM) of molinate almost completely suppressed the PBP contents at 24 h, although a negligible recovery was achieved at 72 h. At the end of experiment, the values were significantly ($P<0.05$, LSD) reduced relative to controls by 23.6%, 94.1% and 92.6%, with 0.75, 1.5, and 2 mM of molinate, respectively.

In cultures incubated with 0.75 mM of bentazon, protein content increased progressively up to 48 h, reaching 13.1% of increase relative to control at this time, but decreased by 8% below it after 72 h of exposure (Table 2). Protein content of cells exposed to 1.5 and 2 mM of bentazon significantly ($P<0.05$, LSD) increased to its maximum at 24 h (23.6% and 26.6% relative to control, respectively, in cultures exposed to 1.5 and 2 mM). These values tended to drop down with time, reaching 3.2% below control and 11.3% above it after 72 h, with 1.5 and 2 mM bentazon, respectively. A more pronounced effect of molinate than bentazon was observed on protein content of the studied cyanobacterium (Table 2). Soon after 24 h, protein content significantly ($P<0.05$, LSD) increased by 12% and 54.9% with 1.5 mM and 2 mM of molinate, respectively. After 72 h, the total protein content of *A. cylindrica* cells exposed to high molinate concentrations was significantly ($P<0.05$, LSD) increased by 13.6% and 327% for 1.5 mM and 2 mM molinate incubation, respectively.

The level of total carbohydrates decreased from 0 to 24 h in all bentazon treated cultures in a time-dose response manner (Table 2; Fig. 1A). After 72 h of exposure, carbohydrate content was significantly ($P<0.05$, LSD) reduced by 16.9%, 17.7% and 16.8% with 0.75 mM, 1.5 mM and 2 mM bentazon exposure, respectively. Contrary to bentazon, molinate contributed to a progressive increase of carbohydrate content that was only significant ($P<0.05$, LSD) for the highest concentration tested (2 mM) (Table 2; Fig. 1B). After 72 h, 2 mM of molinate greatly induced carbohydrate accumulation (344% comparatively to controls).

Photosynthetic oxygen evolution significantly decreased ($P<0.05$, LSD) after 24 h following bentazon exposure (Table 2; Fig. 1A). The IC_{50} for this parameter was achieved after 24 h between 0.75 and 1.5 mM of bentazon. After 72 h, photosynthetic oxygen evolution was considerably reduced by 69.2%, 78.9% and 82.7% when compared to controls, with 0.75 mM, 1.5 mM and 2 mM bentazon, respectively. Photosynthetic oxygen evolution of *A. cylindrica* cultures was significantly ($P<0.05$, LSD) inhibited at all molinate concentrations after 24 h (Table 2; Fig. 1B). At this time, photosynthetic rates were reduced by 20.9%, 75.8% and 97.6%, respectively with 0.75, 1.5 and 2 mM of molinate. Therefore, and as with bentazon, the IC_{50} for photosynthetic oxygen evolution was reached after 24 h with 0.75–1.5 mM molinate. Photosynthetic O_2 evolution was completely inhibited after 48 h of exposure to 1.5 and 2 mM molinate.

The effect of bentazon on dark respiratory activity (oxygen consumption) of *A. cylindrica* isolates were time- and concentration-dependent (Table 2; Fig. 1A). Bentazon treatment significantly ($P<0.05$, LSD) suppressed dark oxygen respiratory rates after 24 h. At the end of the experiment, the values significantly ($P<0.05$, LSD) decreased by 60.6%, 62.6% and 92.3% relative to controls with 0.75 mM, 1.5 mM and 2 mM bentazon, respectively. Oxygen consumption of cells exposed to molinate declined in a time-dose response manner from 24 to 48 h (Table 2; Fig. 1B) and, at the end of the experiment, after 72 h of exposure, dark respiration was significantly ($P<0.05$, LSD) inhibited by 51.2%, 80.4% and 71.1% with 0.75 mM, 1.5 mM and 2 mM molinate, respectively. For both herbicides, the IC_{50} of dark respiration inhibition was achieved after 48 h with a concentration between 0.75 and 1.5 mM.

4 Discussion

It is well established that the success of rice agriculture is increasingly dependent on use of herbicides in order to eliminate undesirable weeds. However, the extensive use of herbicides tends to raise the concentration of chemical residues to levels that might inhibit the growth of autochthonous microorganisms like cyanobacteria. Anything that affects these primary producers can be expected to affect the ecological balance of whole rice field agro-ecosystem. Cyanobacteria are quite sensitive to herbicides because they share many common characteristics with higher plants and green algae. This sensitivity of cyanobacteria towards herbicides varies, however, depending mainly on species and kind of herbicide (Leganés and Fernández-Valiente 1992). In Portugal, the usual field applied doses of Basagran™ and Ordram™ are 4 l ha^{-1} and 60 kg ha^{-1} of commercial formulations, respectively. Besides, in Portuguese real field situation, the average height of rice water column is 10 cm. Consequently, final concentrations of Basagran™ and Ordram™ in rice fields are equivalent to 6.1 μ M and 24 μ M of their active ingredients, bentazon and molinate, respectively. Herbicide concentrations used in the present investigation were therefore too high when compared to recommended levels for field application (RLFA). Nevertheless, it should be considered that is not easy to maintain a constant and uniform water level in paddy fields as deep as 10 cm following herbicide application, which consequently increases RLFA concentrations beyond the recommended ones, particularly at the moment of their application. Those levels, maybe higher than those found in this study to be toxic for *A. cylindrica*, will remain in the soil until effective dilution of remainder products were achieved in aquatic layer. It should also be noticed that cell density used in the present work was several orders of magnitude higher than those found in natural rice environment. Besides, the high herbicide concentrations used in this study were tested in a relatively short period of time (72 h) compared to the extended periods of exposure in real field situation. *In vivo* applied concentrations are lesser than those used here but the effect is more extended, *i.e.* cyanobacteria are exposed to toxicants during a longer period of time. Therefore, it can be expected that both bentazon and molinate, at their extreme toxic concentrations, may cause similar effects on the autochthonous population of cyanobacteria.

Data obtained in the present investigation revealed that *A. cylindrica* was more susceptible to molinate than to bentazon in most of the parameters studied. The reduction of the initial concentration of inocula dry weight with all tested concentrations of molinate (Fig. 1B) could be indicative of a progressive cell lysis mechanism, probably related with a possible effect on membrane lipids, since it is well known that thiocarbamate herbicides inhibit lipid biosynthesis (Moreland 1980; Böger and Sandmann 1998; Waxman 1998). Cell lysis mechanism after molinate exposure was further confirmed by microscopic examination of cultures treated with the toxicant. Our studies are in agreement with those of Yan et al. (1997) that verified a decreasing of *Anabaena sphaerica* growth rate with increasing concentrations of molinate. Eladel et al. (1999) and Battah et al. (2001) also reported that thiobencarb induced a decreasing of *Anabaena variabilis* specific growth rate and significantly reduced its biomass yield. The inhibition of biomass yield of *A. cylindrica* during bentazon treatment could be probably related to the inhibition of photosynthesis (Fig. 1A).

The constant levels of Chl*a* in *A. cylindrica* cultures exposed to bentazon (Fig. 1A) clearly demonstrated that this herbicide had no effect on this photopigment. However, Kobbia et al. (2001) verified

an inhibition of Chl*a* synthesis in *A. variabilis* and *Protosiphon botryoides* with all tested concentrations (0.2–0.8 mg l^{-1}) of simazine, a triazine and also a photosynthetic inhibitor at the reducing side of PS II. Our results are also contrary to those obtained by González-Tomé (1996). Working with *Nostoc* sp., this author found a progressive decrease of Chl*a* content using 100 and 200 times more bentazon than RLFA. However, in this study, bentazon was added *in vitro* together with MCPA, being difficult to establish the effects caused by an unique active ingredient. The decrease of Chl*a* in cells treated with molinate after 72 h (Fig. 1B) was probably a result of the degradation of lipid complexes associated mainly with pigments in thylakoid membranes of the cyanobacterium. However, Yan and collaborators (1997) verified an increase of Chl*a* content with molinate and a study done by Xia (2005) showed that thiobencarb had an insignificant effect on Chl*a* synthesis of *Nostoc sphaeroides* colonies. These contrasting results could be owed to different culture conditions and different herbicide concentrations that could significantly modify the composition of photosynthetic pigments of cyanobacterial cells.

Bentazon affected total carotenoids of *A. cylindrica* and the content of this accessory pigments were slightly increased after 72 h, mainly with the highest herbicide concentration (Fig. 1A). It is well established that carotenoids are involved in cyanobacterial photosynthesis in various ways: they act as light-harvesting pigments, contribute to the structure of thylakoid membranes and play an important role in photooxidative protection (Schagerl and Müller 2006). Therefore, herbicides that blocked photosynthetic electron transport chain, *e.g.* bentazon, permitted the interchange of excitation energy from Chl*a* to carotenoids and thus these latter accessory pigments will be progressively synthesized *de novo* as a consequence of the oxidative process caused by the herbicide. In fact, Buschmann et al. (1980) verified that bentazon enhanced the formation of lutein and carotenes in etiolated radish seedlings. These authors concluded that PS II inhibiting herbicides partially affect, but do not block, carotenoid and chlorophyll biosynthesis. The overall decrease of total carotenoids with molinate in a time-dose response manner (Fig. 1B) was probably related with two successive metabolic events. Firstly, it is well established that thiocarbamate herbicides like molinate increased membrane fluidity by inhibition of lipid biosynthesis, thus contributing to an increase of membrane permeability (Moreland 1980; Böger and Sandmann 1998; Waxman 1998). Secondly, since most of the enzymes which take part in cyanobacterial carotenogenesis are very labile, probably these membrane-associated proteins lose activity upon solubilization with molinate, thus contributing to a progressive decrease of carotenoid biosynthesis with time (Hirschberg and Chamovitz 1994).

The decrease of PBP content observed with both herbicides after 72 h of exposure (Fig. 1A, B) might be an adaptative mechanism of *A. cylindrica* under high concentrations of xenobiotics. The external localization of PBP on intracellular thylakoid membranes could be a possible reason for a more damaging effect of herbicides on PBPs than the other photopigments since they are more exposed to the action of both xenobiotics. However, the content of PBP increased from 0 to 48 h, with 1.5 and 2 mM of bentazon, and then decreased below controls after 72 h with the same concentrations of the herbicide (Fig. 1A). Based on these results and according to Hammouda (1999), two phases could be distinguished: (1) during the first 48 h of herbicide exposure, cells enter in an adaptative phase and the cyanobacterium has the ability of initially utilize the herbicide; (2) from 0 to 48 h, accumulation of higher bentazon concentrations and subsequent formation of breakdown products exerted toxic effects on PBP levels of the cyanobacterium. Again, the remarkable decreasing of total PBP content with time in cultures treated with molinate was probably due to a disturbance in lipid bilayer integrity at thylakoid membranes. Such modification in membrane lipid integrity may change membrane thickness, causing the detachment of phycobilisomes from the rest of the electron transport chain. A decline in PBP content due to adaptation to high levels of thiobencarb had also been reported in *N. sphaeroides* cells (Xia, 2005).

A bentazon doses of 1.5–2 mM increased cellular protein content of *A. cylindrica* after 24 h (Fig. 1A). The present data are in agreement with Kobbia et al. (2001), who reported that photosynthetic inhibitors enhanced cellular nitrogen metabolism thus leading to more amino acid and protein accumulation in *A. variabilis* cells. Of particular interest is the observation that higher molinate concentration (2 mM) caused a remarkably increase of protein content

(Fig. 1B). As Yan et al. (1997) stated, this happens not only because high concentrations of molinate can stimulate protein synthesis, but also because they can inhibit the hydrolysis of protein through inhibition of protease activity, thereby resulting in accumulation of total protein in cyanobacterial cells.

There has been relatively little research on the effect of pesticides, particularly of herbicides, on carbohydrate metabolism in cyanobacteria. Pesticides may generally decrease or increase the sugar content of treated organisms depending on type of the organism, age, treated part, duration of contact time and type of chemical used (Moreland 1980). The significant inhibition of carbohydrate synthesis with bentazon (Fig. 1A), which matched with the suppression of photosynthesis with all tested concentrations, may be attributed to the inhibition of this metabolic pathway. These observations are in agreement with the findings of Kobbia et al. (2001) for simazine, another photosynthetic inhibiting herbicide. However, the data herein obtained further reveal that total carbohydrate content of *A. cylindrica* increased significantly when cultures were exposed to the highest molinate concentration (Fig. 1B). Battah et al. (2001) reported that total carbohydrates increased in *A. variabilis* cells as a result of thiobencarb treatment. The increased production of carbohydrates in cells exposed to molinate could be probably attributed to extracellular rather than to intracelullar carbohydrates. Synthesis of extracellular polysaccharides (EPS) represents an adaptation strategy for survival and growth of cyanobacteria, especially under unfavorable environmental conditions (Ehling-Schulz and Scherer 1999; Nicolaus et al. 1999). Presumably, the stress caused by high molinate concentrations on cell membrane integrity triggered the synthesis and accumulation of EPS in *A. cylindrica* cells. These extracellular carbohydrates can thus bind molinate in order to decrease its toxicity. However, this strategy was not efficient since proteins and pigments were severely affected.

Photosynthetic O_2 evolution was one of the most sensitive parameters because of its early and pronounced inhibition, which was evident after 24 h of exposure to both herbicides (Fig. 1A, B). It is well know from literature that both herbicides cause inhibition of the thylakoid electron transport chain. They reversibly bind to the thylakoid membrane protein D1 located at the PS II heterodimeric centre (Böger and Sandmann 1998; Krieger-Liszkay and Rutherford 1998). In the presence of a PS II herbicide, the inhibition of linear electron transport to cytochrome b_6/f complex results in a shortage of reduced NADPH which is essential for CO_2 reduction (Moreland 1980; Campbell et al. 1998). Therefore, we can assume that the function of the oxygen evolving complex of PS II is affected by both herbicides. The effects of bentazon on photosynthesis are similar to those obtained with simazine, which inhibits *A. variabilis* photosynthesis mainly by preventing thylakoid electron flow through PS II. Xia (2005) also reported a marked decrease on *N. sphaeroides* photosynthetic rate at high thiobencarb concentrations. The inhibition of respiratory O_2 consumption by the two herbicides (Fig. 1A, B) could be related with the inhibition of photosynthesis since both processes share common intermediates in the electron transport chains in cyanobacteria (Scherer et al. 1988; Campbell et al. 1998).

5 Conclusions

The majority of herbicide toxicity studies in cyanobacteria were carried out with pure active substances. In this work, some of the effects caused by commercial products actually used in the field were studied. The results obtained gave valuable informations about residual inhibitory effects of bentazon and molinate herbicide formulations on growth, photopigments and photosynthesis of a filamentous cyanobacterium isolated from a common Portuguese rice agro-ecosystem. Pointing to safety environmental precautions, our findings suggest a constantly and regular use of bentazon in rice fields. On the contrary, we suggest the prohibition of molinate utilization in rice fields because of its strong inhibitory action on soil microflora organisms, specially on nitrogen-fixing cyanobacteria like *Anabaena cylindrica*.

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