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Biodegradation kinetics of the benzimidazole fungicide thiophanate-methyl by bacteria isolated from loamy sand soil

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Abstract Degradation of the fungicide thiophanate-methyl (TM) by *Enterobacter* sp. TDS-1 and *Bacillus* sp. TDS-2 isolated from sandy soil previously treated with TM was studied in mineral salt medium (MSM) and soil. Both strains were able to grow in MSM supplemented with TM (50 mg l^{-1}) as the sole carbon source. Over a 16 days incubation period, 60 and 77% of the initial dose of TM were degraded by strains TDS-1 and TDS-2, respectively, and disappearance of TM was described by first-order kinetics. Medium supplementation with glucose markedly stimulated bacterial growth; while the final rate of TM degradation was reduced by 21 and 27% for strains TDS-1 and TDS-2, respectively as compared to medium with TM only. Moreover, this additional carbon source changed the TM degradation kinetics, which proceeded according to a zero-order model. This effect was linked to substrate competition and/or a strong decrease of medium pH. Isolates degraded TM (100 mg kg^{-1}) in soil with rate constants of 0.186 and 0.210 day^{-1}, following first-order rate kinetics, and the time in which the initial TM concentration was reduced by 50% (DT50) in soils inoculated with strains TDS-1 and TDS-2 were 6.3 and 5.1 days, respectively. Analysis of TM degradation products in soil showed that the tested strains may have the potential to transform carbendazim (MBC) to 2-aminobenzimidazole (2-AB), and may be useful for a bioremediation of MBC-polluted soils.

Keywords Biodegradation kinetics · Thiophanate-methyl · *Enterobacter* sp. · *Bacillus* sp. · Soil

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Introduction

Continuous and extensive application of fungicides often has created environmental concerns due to their toxicity towards non-target organisms. One of the most widely used fungicide is thiophanate-methyl [dimethyl 4,4′-(*o*-phenylene)bis(3-thioallophanate); TM] (Fig. 1) belonging to benzimidazole group. TM is a systemic fungicide used to control a broad range of fungal diseases such as mould, spot, mildew,

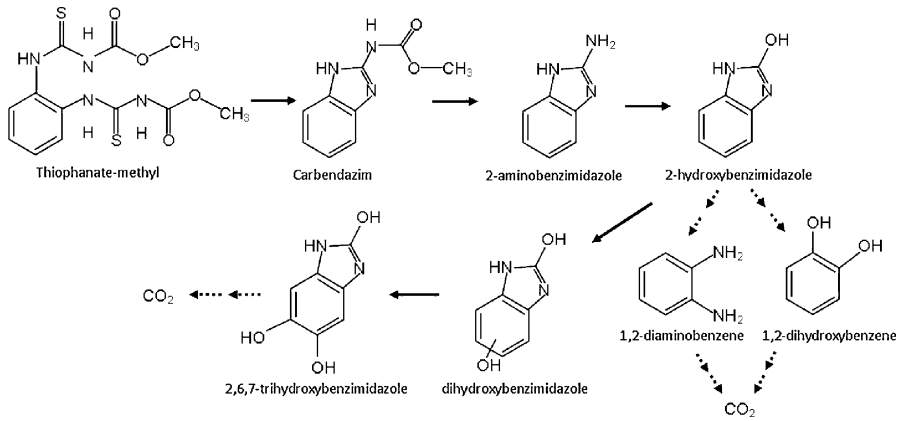

Fig. 1 Proposed biodegradation pathway of thiophanate-methyl

scorch, rot and blight in a variety of crops, i.e. cereals, fruits, and vegetables. It is also applied in post-harvest food storage, as a seed pre-planting treatment and as a timber treatment fungicide (Tomlin 2003). The antifungal activity of TM is based on the blockage of nuclear division during mitosis and destabilization of fungal cell structures. As a results of its action the development of fungal germ tube and formation of appressoria and mycelia growth are inhibited (Roberts et al. 1998).

Degradation of TM in water, soil, plants and animals follows a common pathway that involves the initial conversion into carbendazim [methyl benzimidazol-2-yl carbamate; MBC] (Fig. 1) that is also a systemic fungicide used to combat a wide range of diseases. MBC is degraded primarily via an oxidative mechanism in animals or through hydrolysis to 2-aminobenzimidazole (2-AB) (Fig. 1) (Roberts et al. 1998). In water TM degrades slowly and undergoes photolysis to MBC under UV light (254 nm) or natural sunlight exposure (Noguchi et al. 1971). A half-life of TM degradation in aerobic river sediment containing water is 15–20 days (Tomlin 2003). In turn, in soils via various processes TM is degraded by 90% during a 6–18 weeks depending on soil's texture, moisture and organic matter contents (Fleeker et al. 1974; Roberts et al. 1998).

Although fungicides may undergo adsorption, photolysis, volatilization and chemical transformation, microbial degradation is the major dissipation process. It's a reason that pesticide degradation by bacteria and fungi has received much attention (Cain and Mitchell 1996; Mercadier et al. 1998; Pai et al. 2001; Rama and Ligy 2009). A number of bacterial species are known to degrade benzimidazole structures and most of them were isolated from fungicide contaminated soils. Their potential for the degradation of various fungicides has been extensively studied both in liquid media and soils (Motonaga et al. 1996; Şahin and Tamer 2000; Nagase et al. 2006). Bacteria capable of degrading MBC have been isolated and characterized (Holtman and Kobayashi 1997; Zhang et al. 2005); however there is no information on identified species that participate in the transformation of TM to MBC. Despite the short-term persistence and rapid transformation of TM to the very stable compound MBC, it is interesting to recognize, which microorganisms participate in the degradation of this fungicide in soil environment. Therefore, the purpose of these studies was to isolate the bacterial strains capable of TM degradation and characterize their degradative potential. The ability of isolates to utilize TM as a sole carbon source in mineral salt medium (MSM) as well as the effect of glucose, as an easy degradable compound, on the TM disappearance were determined. The kinetics of TM degradation in both liquid media and sterile soil inoculated with a single bacterial strain were also studied.

Materials and methods

Chemicals

Certified standards of TM (99.8 ± 0.1% chemical purity), MBC (99.7 ± 0.3% chemical purity) and 2-AB (99.6 ± 0.2% chemical purity) were purchased from the Institute of Industrial Organic Chemistry,

Warsaw, Poland. All other chemicals and solvents were HPLC (high performance liquid chromatography) grade and obtained from Merck, Germany.

Soil contamination with TM

A composite soil sample not previously treated with TM and collected from the top layer (0–20 cm) at grass-covered field located in Upper Silesia, southern Poland, was used in this experiment. Based on the physico-chemical analyses, the soil was classified according to US/FAO System as loamy sand soil (Supplementary Table 1). In the laboratory, the soil was gently air-dried to the point of soil moisture suitable for sieving. After sieving to a maximum particle size of <2 mm, the soil was immediately used for the experiment. The aim of this step was to adapt the soil microflora to fungicide. For this purpose, the solution of TM in ethyl acetate (1 ml) was sprayed on the surface of 500 g of soil to obtain a final TM concentration of 500 mg kg^{-1} soil. After solvent evaporation, the water content of the soil was adjusted to 50% of the maximum water holding capacity. The soil was thoroughly mixed and incubated for 90 days in a darkened thermostatic chamber maintained at 30 ± 1°C. After 30 and 60 days of incubation, the soil was supplemented again with the same TM dose. Throughout the incubation period, the deionised water was added to the soil to compensate for any water losses exceeding 5% of the initial amount added.

Enrichment procedure, bacterial colony isolation and identification of isolates

At this step, MSM was used for the enrichment procedure. The medium contained 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.001 g of $FeSO_4 \cdot 7H_2O$, 1.5 g of $Na_2HPO_4 \cdot 12H_2O$, and 1.5 g of KH_2PO_4 per litre of deionised water. The final pH value was adjusted to 7.2. After autoclaving (121°C, 20 min) and cooling, the medium (100 ml) was supplemented with 100 µl of ethyl acetate containing a suitable amount of fungicide to give a final TM concentration of 50 mg l^{-1}. After solvent evaporation, the medium was kept in an ultrasonic bath to improve TM solubility. Next, 10 g of contaminated soil was added to 300 ml flasks with 100 ml of liquid medium supplemented with TM. Samples were incubated for 72 h on a rotary shaker (120 rpm) in a darkened thermostatic chamber maintained at 30 ± 1°C. After this time, 1 ml of soil suspension was transferred into flasks containing the same fresh medium supplemented with TM (100 mg l^{-1}) and incubated for an additional 72 h under the same conditions. After three subsequent transfers into the same medium, serial dilution of the flask samples were plated onto MSM agar plates supplemented with TM (100 mg l^{-1}) for isolation of individual colonies. Isolates exhibiting distinct colonial morphologies were isolated by repeated streaking on the same agar medium. For further analyses, two thiophanate-methyl degrading strains designated as TDS-1 and TDS-2 were used.

Identification of bacterial isolates was based on the profile of fatty acid methyl esters (FAMEs) analysed by gas chromatography (GC) using the Sherlock Microbial Identification System (MIDI Inc., Newark, USA). FAMEs were extracted from each isolate using the standard and recommended procedure, consisting of saponification, derivatization, extraction, and final base washing. Cellular FAME was separated by Hewlett Packard 6890 GC equipped with a capillary column Ultra 2-HP and flame ionization detector (FID) using hydrogen as a carrier gas. In addition, the biochemical properties of the isolates and the substrate utilization patterns using the API 20 E and API 50 CHB Systems (BioMérieux Inc., France) were determined. The biochemical identification was performed according to recommendation of the producer.

Studies on TM degradation in MSM

Degradation studies were performed in 500 ml Erlenmeyer flasks containing 200 ml of sterile MSM with TM as the sole carbon source. The medium was supplemented with 200 µl of ethyl acetate containing a suitable amount of fungicide to give a final TM concentration of 50 mg l^{-1}. After solvent evaporation, medium was kept in an ultrasonic bath to improve the TM solubility. MSM supplemented with glucose (MSM + TM + G) was used to study the effect of an extra carbon source on both bacterial growth and the TM degradation rate. A suitable amount of a sterile glucose solution was added to MSM after autoclaving to obtain a 1% (*w*/*v*) final concentration. The bacterial suspension was inoculated into MSM (in triplicate) to give a final bacterial

count of approximately 3×10^6 cells ml^{-1}. Triplicate samples of MSM + TM, MSM + TDS-1 or MSM + TDS-2 with or without glucose, were used as controls. Flasks were incubated on a rotary shaker (120 rpm) in a darkened thermostatic chamber maintained at 30 ± 1°C. Samples of MSM were periodically removed aseptically for bacterial growth rate and pH determinations, as well as for chemical analyses to determine the TM concentrations. The growth of isolates was recorded spectrophotometrically by measuring the OD at 660 nm using a UV–VIS spectrophotometer (Varian, USA), while the pH values of liquid medium were measured with a glass electrode using a Jenway pH-meter.

Studies on TM degradation in soil

The same soil (not contaminated with TM) from which bacteria were isolated was used for degradation studies. Before using, the soil was sterilized by autoclaving three times for 1 h at 121°C. To study the degradation dynamics, the solution of TM in ethyl acetate (200 µl) was sprayed on the surface of 200 g of soil by means of a micro-syringe that dispensed very small droplets and ensured thorough mixing. The applied amount of TM corresponded to a soil concentration of 100 mg kg^{-1}. After solvent evaporation, the bacterial suspension of each TM-degrading isolate was introduced into the soil (in triplicate) to give a final bacterial count of approximately 3×10^6 cells g^{-1} soil. In order to study the TM degradation rate under abiotic condition, the triplicate samples of sterile soil (SS), without bacteria were kept as controls. Additionally, the same TM dose was applied to the non-sterile soil samples (nSS), non-contaminated earlier with TM to study the degradative potential of the autochthonous microorganisms. The water content of the soil samples was adjusted to 50% of the maximum water holding capacity by the addition of sterile deionised water. All soil samples were incubated in a darkened thermostatic chamber maintained at 30 ± 1°C. Throughout the incubation period, the sterile deionised water was added to the soil treatments to compensate for any water losses exceeding 5% of the initial amount added. Samples of soil treatments (10 g) were periodically removed aseptically for chemical analyses to determine TM concentrations. In addition, the amounts of TM degradation products, i.e. MBC and 2-AB in each soil treatment at the end of the experiment were determined.

Chemical analyses

Concentrations of TM, MBC, and 2-AB were measured by high performance liquid chromatography (HPLC). For TM concentration determination in MSM, 5 ml samples were diluted tenfold with deionised water (45 ml) and extracted twice with 25 ml of dichloromethane on a rotary shaker (30 min). For TM concentration determination in soil, 10 g samples were taken for analyses and shaken twice with 30 ml of the same solvent. Next, the extracts were dehydrated with anhydrous Na_2SO_4, evaporated to dryness under a stream of N_2 at 40°C using a rotary evaporator (IKA, RV05 Basic, Janke and Kunkel-Ika Labortechnik, Germany), subsequently diluted to a final volume of 10 ml with the mobile phase, and reserved for chromatographic analysis. For MBC and 2-AB concentration determination, the same amount of soil was taken for analysis. The soil samples were extracted with 40 ml of ethyl acetate after adding 2.5 ml of 3 M NaOH on a rotary shaker (30 min). Next, the soil extracts were filtered through filter paper and shaken twice with 5 ml of 0.1 M H_2SO_4. The final extracts were adjusted to a final volume of 10 ml with the mobile phase, and reserved for chromatographic analysis. HPLC analyses were performed using a Varian ProStar System (Varian, Inc., USA) equipped with a UV–VIS detector (ProStar 325), solvent delivery module (ProStar 210), and reverse-phase column (Microsorb-MV 100-5 C18 (250 mm × 4.6 mm × 5 µm)). For TM detection, the mobile phase was acetonitrile–water (50:50, *v*/*v*) with a flow rate of 1.0 ml min^{-1} and a detection wavelength of 280 nm. For MBC and 2-AB detection, the mobile phase was acetonitrile-universal buffer, pH 2.36 (15:85, *v*/*v*) with a flow rate of 0.7 ml min^{-1} and a detection wavelength of 280 nm. The obtained data were analysed with Chromatography Workstation Software (Star, LC WS, Ver. 6.2). Retention times under these chromatographic conditions were 6.0, 8.2, and 6.6 min for TM, MBC, and 2-AB, respectively. To evaluate the usefulness of this chemical determination method, validation studies to determine the linear range of the calibration curves, recoveries, and limits of detection (LOD) and quantification (LOQ) were performed. The calibration curve proved to be linear within the range of

0.1–10.0 mg l^{-1} with $R^2 = 0.9986$, 0.9998, and 0.9995 for TM, MBC, and 2-AB, respectively. LOD, LOQ, and recovery of TM were 0.001, 0.005 mg l^{-1}, and 96.7 ± 3.2%; and 0.005, 0.01 mg kg^{-1}, and 95.5 ± 4.1% for MSM and soil, respectively. LOD, LOQ, and recovery for the concentration determination of MBC and 2-AB in soil were 0.02, 0.05 mg kg^{-1}, and 94.1 ± 3.1%; and 0.005, 0.01 mg kg^{-1}, and 97.0 ± 2.3%, respectively.

Kinetics and statistics analyses

Disappearance of TM in liquid medium or soil was fitted to zero- or first-order kinetic models. The rate constant (k) (day^{-1}) was determined using the algorithm $C_0 - C_t = kt$ for the zero-order model, and $C_t/C_0 = e^{-kt}$ for the first-order model, where C_0 is the amount of TM in MSM or soil at time zero, C_t is the amount of TM in MSM or soil at time t (days). Times in which the TM concentration in MSM or soil was reduced by 50% (DT50 values) were calculated from the linear equation obtained from the regression between $C_t - C_0$ (zero-order model) or $\ln(C_t/C_0)$ (first-order model) of the chemical data and time.

The results from three replicates of each treatment were also evaluated using analysis of variance and statistical analysis. The significance ($P < 0.05$) of differences were assessed by post hoc comparison of means using the least significant differences (LSD) test using the Statistica 6.0 PL software package. The data obtained for TM degradation kinetics and TM degradation products were treated statistically by one-way ANOVA, considering the effect of soil treatment, while the results concerning the disappearance of TM in MSM were analysed by two-way ANOVA, considering the effects of inoculum and medium type. For pH values, the data were treated statistically by three-way ANOVA, considering the effects of inoculum, medium type, and time.

Results and discussion

Identification of TM-degrading bacteria

Selection pressure is a common approach to isolate toxicant-degrading organisms. It was applied to screening of soil bacteria and fungi capable of degrading various types of pesticides (Rajagopal et al. 1984; Mohapatra and Awasthi 1999; Bhalerao and Puranik 2007). Using a soil enrichment procedure, two bacterial strains belonging to *Enterobacter* sp. (TDS-1) and *Bacillus* sp. (TDS-2) were screened and found to be capable of degrading the fungicide TM. The method based on the analysis of fatty acid methyl ester (FAME) profiles (Sherlock Microbial Identification System) designed by MIDI Inc., Newark, USA is suitable and reliable method for isolates identification. Fatty acid profiles of isolated strains are presented in Table 1. The similarity index (SIM) calculated by the MIDI system was determined to be 0.831 and 0.811 for strains TDS-1 and TDS-2, respectively, which were very good matches. SIM is an indication of the confidence with which the isolate is identified. Strains with a SIM level of >0.1, and especially those with a SIM of ≥0.3, are considered positively identified, whereas strains with a SIM of ≤0.1 are considered tentatively identified (Germida and Siciliano 2001). Analyses of biochemical patterns by the API 20 E and API 50 CHB test systems also supported reliable identification of the isolates to be *Enterobacter* and *Bacillus* species with 97.8 and 96.8% identity, respectively (Table 2). As far as we know, no previous studies have reported the isolation of TM-degrading bacterial strains. However, bacteria belonging to these genera have been isolated earlier from the soils contaminated with other pesticides, e.g. fungicide thiram (Şahin and Tamer 2000) and insecticide chlorpyrifos (Singh et al. 2006). In addition, it has been reported that bacteria from other genera such as *Acitenobacter*, *Arthrobacter*, *Burkholderia*, *Flavobacterium*, *Serratia*, *Pseudomonas*, and *Variovorax* are very metabolically active and capable of degrading many pesticides (Tixier et al. 2002; Dejonghe et al. 2003; Singh et al. 2006; Sørensen et al. 2008; Cycoń et al. 2009).

Dynamics of bacterial growth and TM disappearance in MSM

Culturing bacteria in medium containing TM as the only organic compound revealed that both strains were capable of using applied pesticide as a source of carbon and energy to growth (Fig. 2). The results showed the differences in growth kinetics of individual bacterial isolates in MSM + TM. The bacterial growth was most effective during the first 4 days of incubation. The growth curve for isolates attained a

Table 1 Fatty acid profiles of bacterial strains isolated from TM-treated soil

Enterobacter sp. TDS-1				*Bacillus* sp. TDS-2					
Fatty acid	%	Fatty acid	%	Fatty acid	%	Fatty acid	%	Fatty acid	%
14:0	4.34	17:0 *cyclo*	7.65	12:0 *iso*	0.65	15:0 *anteiso*	4.86	17:1 *iso* ω5c	0.86
14:0 2OH	1.48	16:1 2OH	0.94	12:0	0.93	16:1 *ω7c* alcohol	0.77	17:1 *anteiso A*	0.39
14:0 3OH/16:1 *iso* I	7.00	16:0 2OH	0.51	13:0 *iso*	7.58	16:1 *iso* I/14:0 3OH	0.82	17:0 *iso*	5.66
16:0 *iso*	0.49	18:1 *ω7c*	24.17	13:0 *anteiso*	1.01	16:0 *iso*	7.71	17:0 *anteiso*	1.69
16:1 *ω7c*/16:1 *ω6c*	22.73	18:0	0.59	14:0 *iso*	3.82	16:1 *ω6c*/16:1 *ω7c*	4.36	18:1 *ω9c*	0.59
16:1 *ω5c*	0.41	19:0 *cyclo ω8c*	0.41	14:0	5.08	16:0	8.58	18:1 *ω7c*	0.71
16:0	25.97	18:1 2OH	2.93	15:0 *iso*	40.67	17:1 *iso ω10c*	1.50	18:0	1.15

Table 2 Biochemical pattern of bacterial strains isolated from TM-treated soil

Strain/test	*Enterobacter* sp. TDS-1/API 20 E				*Bacillus* sp. TDS-2/API 50 CHB											
Active ingredients[a]	ONPG	+	GEL	−	Control	−	GAL	+	MDM	−	MEL	−	TUR	−		
	ADH	−	GLU	+	GLY	+	GLU	+	MDG	−	SAC	+	LYX	−		
	LDC	−	MAN	+	ERY	−	FRU	+	NAG	+	TRE	+	TAG	−		
	ODC	+	INO	−	DARA	−	MNE	−	AMY	+	INU	−	DFUC	−		
	CIT	+	SOR	+	LARA	−	SBE	−	ARB	+	MLZ	−	LFUC	−		
	H_2S	−	RHA	+	RIB	+	RHA	−	ESC	+	RAF	−	DARL	−		
	URE	−	SAC	+	DXYL	−	DUL	−	SAL	+	AMD	+	LARL	−		
	TDA	−	MEL	−	LXYL	−	INO	−	CEL	−	GLYG	+	GNT	−		
	IND	−	AMY	+	ADO	−	MAN	−	MAL	+	XLT	−	2KG	−		
	VP	+	ARA	+	MDX	−	SOR	−	LAC	−	GEN	−	5KG	−		

[a] *ADH* L-arginine, *ADO* D-adonitol, *AMD* amidon (starch), *AMY* amygdalin, *ARA* and *LARA* L-arabinose, *ARB* arbutin, *CEL* D-celobiose, *CIT* trisodium citrate, *DARA* D-arabinose, *DARL* D-arabitol, *DFUC* D-fucose, *DUL* dulcitol, *DXYL* D-xylose, *ERY* erythtritol, *ESC* esculin, *FRU* D-fructose, *GAL* D-galactose, *GEL* gelatin, *GEN* gentiobiose, *GLU* D-glucose, *GLY* glycerol, *GLYG* glycogen, *GNT* potassium gluconate, *H_2S* sodium thiosulfate, *IND* and *TDA* L-tryptophane, *INO* inositol, *INU* inulin, *2KG* potassium 2-ketogluconate, *5KG* potassium 5-ketogluconate, *LAC* D-lactose, *LARL* L-arabitol, *LDC* L-lysine, *LFUC* L-fucose, *LXYL* L-xylose, *LYX* D-lyxose, *MAL* D-maltose, *MAN* D-mannitol, *MDG* methyl-α-D-glucopyranoside, *MDM* methyl-α-D-xylopyranoside, *MDX* methyl-β-D-xylopyranoside, *MEL* D-melibiose, *MLZ* D-melezitose, *MNE* D-mannose, *NAG* *N*-acetyl-glucosamine, *ODC* L-ornithine, *ONPG* 2-nitrophenyl-β-D-galactopyranoside, *RAF* D-raffinose, *RHA* L-rhamnose, *RIB* D-ribose, *SAC* D-saccharose (sucrose), *SAL* salicin, *SBE* L-sorbose, *SOR* D-sorbitol, *TAG* D-tagatose, *TRE* D-trehalose, *TUR* D-turanose, *URE* urea, *VP* sodium pyruvate, *XLT* xylitol

maximum OD 660 nm after 12 days, while respective controls (MSM + TM, MSM + TDS-1 and MSM + TDS-2) showed no change in OD 660 nm for 16 days of incubation (Fig. 2A1). During the 16 days of the experiment, 60 and 75% of the initial dose of TM disappeared in MSM inoculated with strains TDS-1 and TDS-2, respectively. The TM disappearance curves showed that the most efficient degradation was within the first incubation period (0–4 days) and after this time approximately 49 and 58% of the initial TM doses were degraded by strains TDS-1 and TDS-2, respectively (Fig. 2B1). Kinetic data showed that the degradation process in MSM was characterized by rate constants of 0.057 and 0.093 day^{-1}, following first-order rate kinetics for strains TDS-1 and TDS-2, respectively (Table 3).

The TM disappearance in MSM supplemented with glucose (MSM + TM + G) was different compared to MSM + TM. The bacterial growth was significantly ($P < 0.05$) stimulated and approximately three times faster at the beginning of the incubation period, as compared to growth of isolates in MSM without an additional carbon source (Fig. 2A2). However, the growth dynamics were higher and the rates of TM degradation after 16 days were lower and reached 39 and 50% of the initial

concentration of TM for strains TDS-1 and TDS-2, respectively. The most effective degradation of TM was observed between 4 and 16 days. During the first incubation period (0–4 days) only 7.7 and 13.6% of TM were degraded by strains TDS-1 and TDS-2, respectively. Kinetic data showed that the degradation process in MSM + G was characterized by rate constants of 1.169 and 1.513 day^{-1} (Table 3). Moreover, in the presence of an extra carbon source, the TM degradation kinetics proceeded according to a zero-order model. The observed effect might result from substrate competition, and glucose as an easily degradable carbon source was utilized earlier than the fungicide. The same effect of glucose on the pesticide degradation rate was also observed by Singh et al. (2006), who studied the utilization of chlorpyrifos by *Enterobacter* sp. The observed initial inhibition of pesticide degradation in the presence of glucose may reflect the environmental adaptation of bacterial isolates in which easily available and rich carbon sources are preferentially metabolized. Once the readily available carbon source is depleted, the bacteria start to utilize the pesticides. This phenomenon gives pesticide-degrading microorganisms a competitive advantage in the environment, since they are able to utilize both readily available and less available carbon sources (Singh et al. 2006).

In addition, as a consequence of fast microbial glucose metabolism, a strong decrease of medium pH was found. For example, in MSM + G + TM inoculated with strain TDS-1, the pH of medium dropped from 7.20 to 5.41 during 16 days of incubation, but the highest pH decrease was observed during first 4 days of incubation (Supplementary Table 2). The higher pH increases the rate of TM transformation, whereas the acidic conditions may increase the TM stability and its resistance to chemical and microbial degradation (Fleeker et al. 1974; Roberts et al. 1998). It has been reported that pH has a marked influence on pesticide degradation by the bacterial isolates, and the increase stability under acidic conditions were observed for many pesticides (Karpouzas and Walker 2000; Vidali 2001). Moreover, lower pH may decrease the activity of bacteria and/or enzymes involved in pesticide degradation (Singh et al. 2006; Cycoń et al. 2009). The pH value measurements in MSM during the experimental period showed that this parameter was dependent on the bacterial inoculum ($P < 0.0001$), type of medium ($P < 0.0001$), time

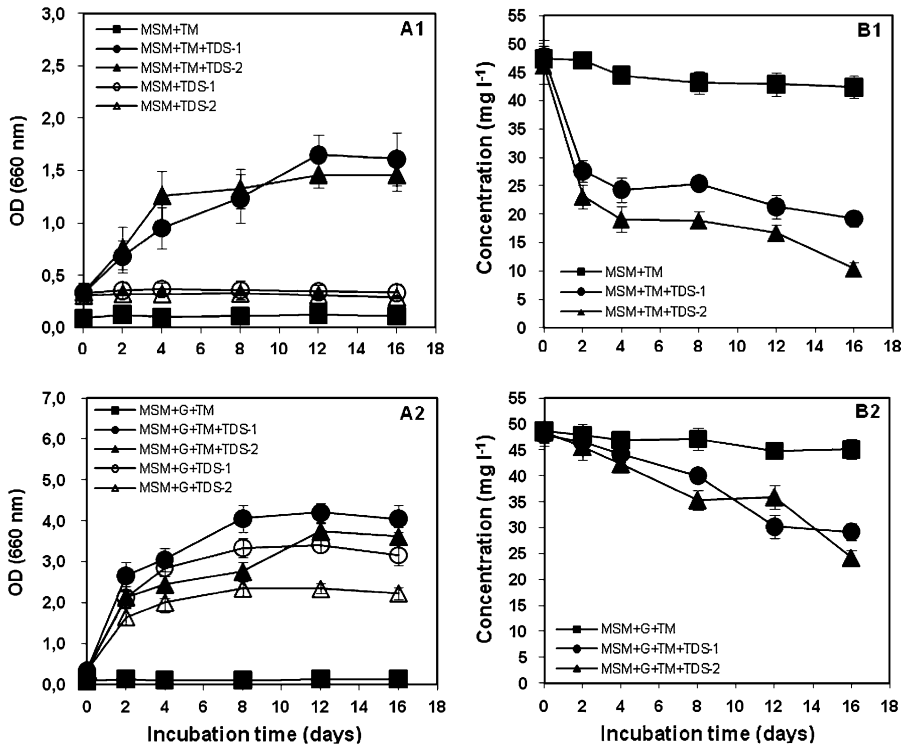

Fig. 2 Bacterial growth and TM disappearance dynamics in mineral salt medium (A1 and B1) and mineral salt medium with glucose as the additional carbon source (A2 and B2). *MSM* mineral salt medium, *TM* thiophanate-methyl, *G* glucose, *TDS-1* *Enterobacter* sp., *TDS-2* *Bacillus* sp. *Symbols* are the means of three replicates. *Error bars* represent the standard deviation which was within 5% of the mean

Table 3 Kinetic data of thiophanate-methyl disappearance

Treatments	Regression equation	R^2	k (day^{-1})	DT50 (days)
Medium*				
MSM + TM + TDS-1	$\ln(C_t/C_0) = -0.0127t - 0.2917$	0.6780	0.057 ± 0.008^a	9.7 ± 0.9^a
MSM + TM + TDS-2	$\ln(C_t/C_0) = -0.0700t + 0.3416$	0.7823	0.093 ± 0.014^b	5.0 ± 0.6^b
MSM + TM + G + TDS-1	$C_t - C_0 = -1.2979t + 0.8519$	0.9574	1.169 ± 0.018^c	19.1 ± 0.7^c
MSM + TM + G + TDS-2	$C_t - C_0 = -1.3742t - 0.1639$	0.9337	1.513 ± 0.126^d	17.4 ± 1.0^d
Soil**				
SS	$\ln(C_t/C_0) = -0.0254t - 0.0251$	0.9760	0.027 ± 0.005^a	26.3 ± 1.8^a
SS + TDS-1	$\ln(C_t/C_0) = -0.1686t + 0.3667$	0.9149	0.186 ± 0.016^b	6.3 ± 0.8^b
SS + TDS-2	$\ln(C_t/C_0) = -0.2036t + 0.3471$	0.9510	0.210 ± 0.017^b	5.1 ± 0.6^b
nSS	$\ln(C_t/C_0) = -0.3028t + 0.2116$	0.9914	0.300 ± 0.024^c	3.0 ± 0.4^c

MSM mineral salt medium, *TM* thiophanate-methyl, *G* glucose, *TDS-1 Enterobacter* sp., *TDS-2 Bacillus* sp., *SS* non-inoculated sterile soil, *SS + TDS-1* sterile soil inoculated with *Enterobacter* sp., *SS + TDS-2* sterile soil inoculated with *Bacillus* sp., *nSS* non-sterile soil without bacteria inoculum. The data presented are means of three replicates. * Different *letters* indicate significant differences, considering effects of medium and inoculum type (two-way ANOVA, $P < 0.05$, LSD test). ** Different *letters* indicate significant differences between soil treatments (one-way ANOVA, $P < 0.05$, LSD test)

($P < 0.0001$), and interaction between these factors ($P < 0.0001$) (Supplementary Table 2).

Dynamics of TM disappearance and amounts of MBC and 2-AB in soil

The TM disappearance dynamics in soil treatments are shown in Fig. 3. As indicated, the data obtained from the degradation kinetics studies showed that there were differences in the TM degradation potential between bacterial isolates. In sterile soil (SS) inoculated with *Enterobacter* sp. (SS + TDS-1) the complete degradation of TM occurred within 24 days, while in soil inoculated with *Bacillus* sp. (SS + TDS-2), 20 days. Kinetic data showed that the degradation process was characterized by rate constants of 0.186 and 0.210 day^{-1}, following first-order kinetics for SS + TDS-1 and SS + TDS-2, respectively (Table 3). The time within which the initial TM concentration was reduced by 50% (DT50) was 6.3 and 5.1 days for SS + TDS-1 and SS + TDS-2, respectively. Faster TM degradation was obtained in non-sterile soil (nSS), with a rate constant of 0.300 day^{-1}, following first-order kinetic and a DT50 of 3.0 days (Table 3). In these studies, soils were inoculated with 3×10^6 bacterial cells g^{-1} soil and this inoculum density appeared to be able to degrade TM. As indicated by other experiments, inoculum size is an important factor that determines whether or not target pollutants are efficiently degraded. It has been observed that when lower inoculum densities ($<10^6$ cells g^{-1} soil) are used, lower numbers of bacteria are able to survive the initial competition and population decline usually occurs following inoculation (Miethling and Karlson 1996; Rousseaux et al. 2003; Singh et al. 2006). This effect may be compensated for by using an inoculum with a higher bacterial density (Comeau et al. 1993; Duquenne et al. 1996).

Degradation kinetics of TM in SS and nSS indicated that apart from chemical processes microbial degradation is considered to be one of the main mechanisms of TM dissipation in soil. The chemical data showed that in non-inoculated sterile soil (SS) a significant amount of TM (60% of the initial dose) still persisted after 24 days (Fig. 3). The TM disappearance process in SS was characterized by a rate constant of 0.027 day^{-1}, following first-order kinetics, and the DT50 calculated from the linear equation was 26.3 days (Table 3). Results obtained for soils inoculated with individual isolates and nSS revealed that mixed bacterial strains participate in efficient degradation of TM, rather than a single microorganism. The observed fast disappearance of TM in nSS not previously treated with this fungicide suggested that TM applied even at a high concentration (100 mg kg^{-1}) did not affect the degradative potential of the autochthonous microorganisms. Generally, fungicides applied at recommended field rates are non-toxic to bacteria, as they do not have

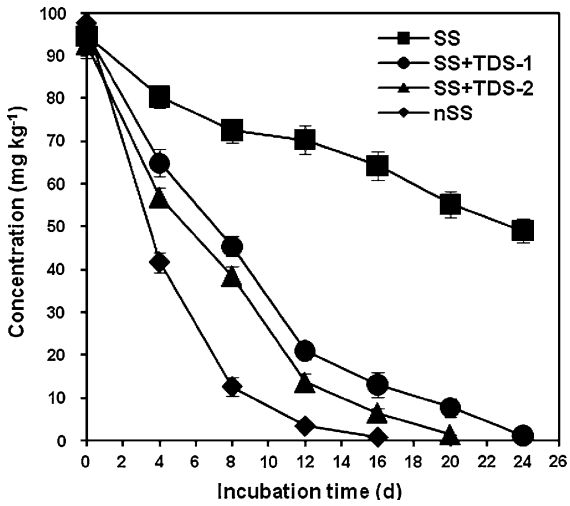

Fig. 3 The TM disappearance dynamics in soil. *SS* non-inoculated sterile soil, *SS + TDS-1* sterile soil inoculated with *Enterobacter* sp., *SS + TDS-2* sterile soil inoculated with *Bacillus* sp., *nSS* non-sterile soil without bacteria inoculum. *Symbols* are the means of three replicates. *Error bars* represent the standard deviation which was within 5% of the mean

sensitive targets (Strickland et al. 2004; Cycoń et al. 2006, 2010; Cycoń and Piotrowska-Seget 2009; Wang et al. 2009).

The DT50 of TM calculated for nSS was 3.0 days, which supported the previous study, showing that this fungicide breaks down rapidly (<4 days), and is transformed to the very stable compound MBC (Fleeker et al. 1974; Roberts et al. 1998). MBC is decomposed in the environment with a half-life of 6–12 months in bare soil, 3–6 months on turf, and 1–2 months in a water–sediment phase under aerobic conditions and 25 months under anaerobic conditions (Tomlin 2003). MBC is degraded to 2-AB and both chemicals are strongly bound or incorporated into soil organic matter and were found mainly in the topsoil. Residues of these compounds are probably due to the imidazole ring of the molecule, as the organic carbon sorption coefficient (K_{oc}) is relatively low (Gupta and Sharma 1989; Tomlin 2003; Zhang et al. 2005; Jing-Liang et al. 2006). Despite the high stability and long-term persistence of 2-AB in soil, this compound may also be degraded (Fig. 1). As indicated by data obtained from other experiments, some bacterial strains were isolated from soil and shown to be capable of degrading 2-AB in mineral salt medium. However, the complete degradation pathway of 2-AB is not known. For example, Fang et al. (2010) studying the degradation of MBC by *Pseudomonas* sp. CBW revealed that 2-hydroxybenzimidazole (2-HB) was detected as a metabolite of 2-AB. They also showed that 2-HB was degraded by strain CBW. Although no metabolite was identified from 2-HB degradation, they considered that it is reasonable to speculate 1,2-diaminobenzene and 1,2-dihydroxybenzene (catechol) as possible metabolites based on the structures of 2-HB and MBC (Fig. 1). The results obtained by Wang et al. (2010) revealed that strain *Rhodococcus jialingiae* djl-6-2 was able to degrade MBC to 2-HB via 2-AB. The authors also indicated to dihydroxybenzimidazole and 2,6,7-trihydroxybenzimidazole as the metabolites of 2-HB degradation (Fig. 1).

Chemical analysis of the TM degradation products revealed significant differences ($P < 0.05$) in the concentrations of MBC and 2-AB between the respective soil treatments (Fig. 4). After 24 days of incubation in soil inoculated with the isolate TDS-1, the concentrations of MBC and 2-AB were determined to be 26.5 and 16.3 mg kg^{-1} soil, respectively. On the other hand, in soil inoculated with the isolate TDS-2, a lower amount of MBC (17.5 mg kg^{-1} soil), but a higher level of 2-AB (22.3 mg kg^{-1} soil), was determined (Fig. 4). However, the MBC concentration in SS was at the same level as in SS + TDS-1, a small amount of 2-AB (1.5 mg kg^{-1} soil) was detected. The highest concentration of MBC was determined in nSS and this value was about two times higher than in the other soil treatments (Fig. 4). Analysis of TM degradation products in the respective soil treatments showed that transformation of MBC to 2-AB may also be done by the isolated bacteria. It is interesting to note that higher amounts of 2-AB were determined in SS inoculated with *Enterobacter* sp. and *Bacillus* sp. as compared to in nSS, non-contaminated earlier with TM. These results suggest that strains isolated from soils regularly treated with MBC have a considerable potential for biodegradation, since the organisms have adapted to the presence of pesticide. According to our knowledge, no information concerning the ability of bacteria belonging to *Enterobacter* and *Bacillus* species to utilize MBC is available. However, some studies have reported that several bacterial species such as *Pseudomonas* (Fuchs and de Vries 1978), *Ralstonia* (Zhang et al. 2005), and *Rhodococcus*

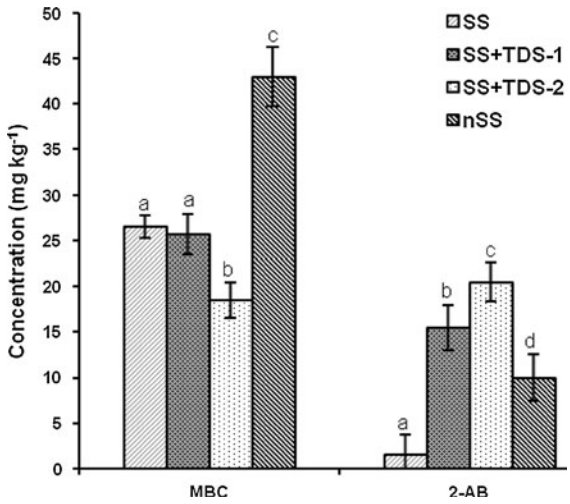

Fig. 4 The amounts of the TM degradation products in soil after 24 days of the experiment. *MBC* carbendazim, *2-AB* 2-aminobenzimidazole, *SS* sterile soil, *TDS-1 Enterobacter* sp., *TDS-2 Bacillus* sp., *nSS* non-sterile soil. The values are the means of three replicates. *Error bars* represent the standard deviation which was within 5% of the mean. Values followed by the *different letter* for a given chemicals differ significantly (one-way ANOVA, $P < 0.05$, LSD test)

(Holtman and Kobayashi 1997; Jing-Liang et al. 2006) are able to degrade MBC and use it as a source of carbon and energy.

Conclusions

The data obtained from TM degradation kinetics studies confirmed that apart from chemical processes microbial degradation is considered to be one of the main mechanisms of TM dissipation in soil. In these studies, bacteria belonging to *Enterobacter* sp. and *Bacillus* sp. were isolated and shown to be capable of degrading TM in soil. Moreover, they may also have the potential to metabolize MBC to 2-AB. Therefore, these isolated strains may be useful for a bioremediation of MBC-polluted soils. However, there is a need for further research on the biochemical and genetic aspects of TM and MBC degradation by the isolated bacteria. Additionally, the use of pesticide-degrading microbial systems for removal of MBC from contaminated sites also requires an understanding of the ecological requirements of the isolated strains.

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