ISOLATION AND CHARACTERIZATION OF CARBENDAZIM-DEGRADING BACTERIA FROM AGRICULTURAL SOIL SAMPLES.

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ABSTRACT - Isolation and characterization of carbendazim-degrading bacteria from agricultural soil samples.
The use of chemical pesticides in agriculture generates many ecological and human toxicological problems. One of the most frequently used fungicides is carbendazim, however, in spite of its importance, there are only a few reports dealing with its microbial degradation in the environment. It has high acute ecotoxicological effect, as well as a suspected endocrine disruptor potential, so its residues in food and feed are dangerous. Until now, single isolates of *Pseudomonas*, *Rhodococcus* and *Ralstonia* have been found to be able to degrade carbendazim. Among fungi, one isolate of *Alternaria alternata* and *Phanaerochete crysosporium* were described as good carbendazim degraders. Bacterial degradation pathways have been partially explored: the first step is the hydrolysis of the carbamate group, followed by a ring-fission in 2-aminobenzimidazole resulting 1,2-diaminobenzene. This compound is further metabolized via the beta-ketoadipic acid pathway. As part of our studies on pesticide biodegradation, new carbendazim-metabolizing bacteria were isolated from Hungarian agricultural soil samples. These degrader bacteria were isolated from soil samples by microbiological enrichment methods. The molecular analysis revealed that the best isolates belong to the *Variovorax paradoxus* species. The isolate 10/1 was able to use carbendazim as sole carbon and nitrogen source. The pH optimum and temperature optimum for growth were found to be pH 6.3 and 30 °C, respectively. This isolate seems to be an efficient tool for the bioremediation of carbendazim polluted agricultural soils.

Keywords: *Variovorax*, carbendazim, biodegradation

INTRODUCTION

The use of chemical pesticides in modern agriculture generates many ecological and human toxicological problems. One of the most frequently used agricultural fungicide both in Serbia and in Hungary is carbendazim (Fig. 1.). In spite of this, there are only few reports dealing with its microbial degradation. It has high acute ecotoxicological effect, as well as a suspected endocrine disruptor potential in human and animals, so its residues in food and feed are dangerous. Until now, single isolates of *Pseudomonas* (FUCHS and DE VRIES, 1978), *Rhodococcus* (HOLTMAN et al, 1997) and *Ralstonia* strain (GUI-SHAN ZHANG et al, 2005) proved to be able to degrade carbendazim. Taking into account fungi, one isolate of *Alternaria alternata* and *Phanaerochete crysosporium* described as good carbendazim degraders (SILVA et al, 1996: 1999). In case of bacterial degraders the degradation mechanism and pathway was also partially explored. In this process, at first the carbamate group is hydrolyzed to 2-amino-benzimidazole, which is

further degraded by ring fission leading to 1, 2-diamino-benzene. This intermedier is further metabolized via the beta-keto adipic acid pathway. As part of this, an investigation was made to isolate carbendazim-degrader bacteria from agricultural soil samples.

MATERIAL AND METHOD

Soil samples were collected from agricultural fields in Hungary, where carbendazim was regularly used. Isolation of carbendazim-degrading bacteria was carried out via the continuous enrichment culture method. Briefly: 5 g sample of soil was suspended in 50 ml sterilized NaCl solution 1.0 (g/l). From this suspension 0.1 ml was inoculated into the enrichment medium, (g/l): K_2HPO_4 1.0, $MgSO_4$ $7H_2O$ 1.0, NaCl 0.5, supplemented with carbendazim (200 mg/l). Carbendazim (purity: 94.6%) was firstly dissolved in 1 M hydrochloric acid at 20 mg/ml concentration. The pH of the medium was about 7.0 after the addition of carbendazim solution. The flasks were incubated in the dark at 20 °C on a rotary shaker at 200 rpm. After 14 days, dilution series were made from each culture and from these dilutions 50 µl aliquots were spreaded onto yeast extract glucose agar plates (YEG: (g/l) yeast extract 2.0, glucose 2.0, Bacto agar 18). Plates were desiccated and incubated for 3 days at 20 °C. The dominant colonies were picked up and tested for their carbendazim degrading ability.
The taxonomical positions of the isolates with best degrading ability were determined by partial sequencing the 16S ribosomal RNA genes. For PCR reaction standard conditions were applied with the following primers: Eub-341f 5'-CCTACGGGAGGC AGCAG-3' and UP-765r 5'-CTGTTTGCTCCCCACGCTTC-3'.
Carbendazim degrading abilities of the isolates were measured in enrichment medium supplemented with 50 mg/l carbendazim and 150 mg/l yeast extract. After incubation, samples from the cultures were diluted to twice fold with ethanol, centrifuged at 10 000 g for 3 minutes and the absorbance of the clear supernatants were determined in a spectrophotometer at 280 nm where both carbendazim and 2-amino-benzimidazole have strong absorbance. The degradation of carbendazim was correlated with the reduced absorbance values measured.

RESULTS

From the ten soil samples collected, eight different bacterium isolates were obtained after the enrichment step where carbendazim was the sole carbon and nitrogen source in the medium.
The carbendazim degrading abilities of these isolates are presented in *Table 1*.

Table 1. Carbendazim degrading abilities of different bacterial isolates obtained from Hungarian soil samples. Incubation time: 14 days.

Strain code	Species identity	Residual carbendazim (control 100%)
1/2	*Variovorax paradoxus*	73 %
6/2	*Acidovorax defluvii*	69 %
6/3	*A. delafieldii*	60 %
6/5	*Pseudomonas sp.*	64 %
6/8	*Microbacterium phyllosphaerae*	78 %
10/1	*V. paradoxus*	10 %
10/4	*Acidovorax sp.*	44 %
10/5	*Acidovorax sp.*	97 %

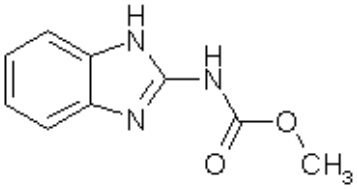

Figure 1. Structure of carbendazim

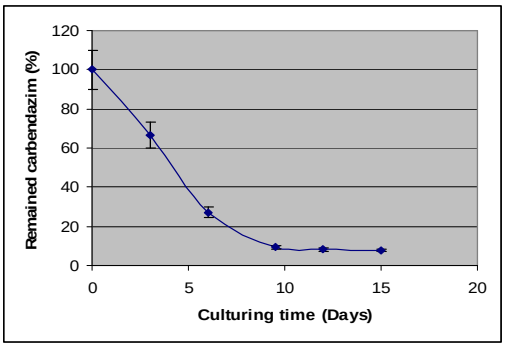

Figure 2. Degradation kinetics of carbendazim by *V. paradoxus* 10/1 isolate.

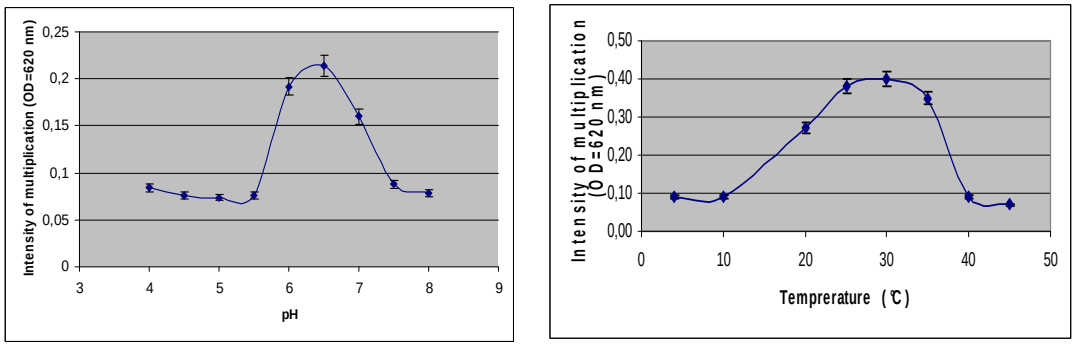

Figure 3. pH- and temperature-dependence of the growth of *V. paradoxus* 10/1 isolate.

After preliminary experiments, composition of the enrichment medium has been reformulated: it turned out that in the presence of NH_4Cl the bacterial isolates were

unable to degrade carbendazim. Further investigations were carried out with the isolate 10/1, which has the best degrading ability. A part of its 16S RNA gene was sequenced and analyzed: web-based similarity searches against the GenBank and Ribosomal Database Project databases revealed that 10/1 shared 100% identity with the 16S rDNA of strains of *V.* (formerly *Alcaligenes*) *paradoxus* (*Comamonadaceae*, WILLEMS et al, 1991). Our results show that *V. paradoxus* 10/1 was able to degrade 90% of carbendazim within ten days (*Fig. 2.*). The isolate was able to grow in ranges pH 5.5-7.5 and temperature 10-40 °C, respectively (*Fig. 3.*).

The carbon and nitrogen source utilization spectra of this bacterium were also investigated. From the tested 23 carbon sources, D-xylose, D-sorbitol, D-mannitol and some amino acids (L-leucine, L-izoleucine, L-proline, L-phenylalanine and L-tyrosine) supported its growth. In the presence of glucose, galactose and other common mono- and disaccharides, the growth of the strain was poor. From the compounds tested, urea, L-glutamine and L-asparagine (besides other L-amino acids) were the best nitrogen sources. *V. paradoxus* utilized NH_4Cl and $NaNO_3$ very poorly. In the carbendazim-degrading strains we detected highly active esterases in the periplasmic space or in the cytoplasm, but never in ferment broths. The same strains intensively used methylacetate and L-tyrosine methyl ester for growth (*Fig. 4.*). Probably these esterases are also able to hydrolyze the carbamyl-methyl ester group in carbendazim.

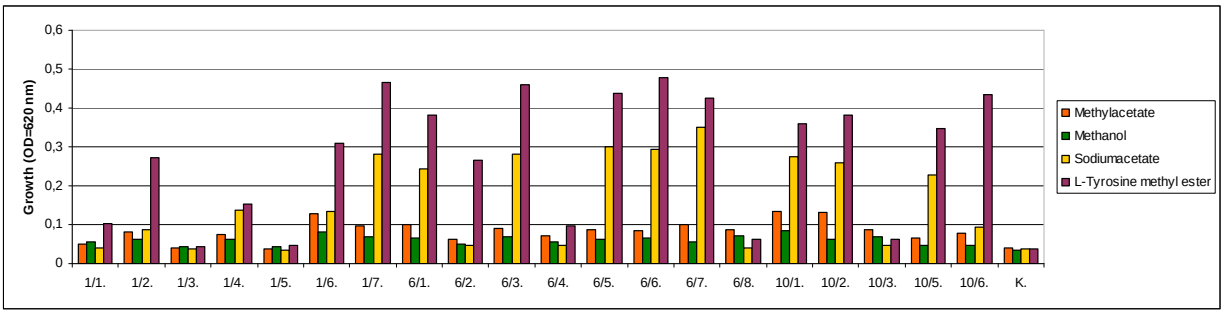

Figure 4. The use for growth of distinct esters by the bacterial strains isolated from carbendazim degrading communities.

CONCLUSIONS

Soil samples proved to be excellent sources of bacteria with carbendazim degrading ability. Key parameters of an efficient enrichment technique were optimized. It is proved that besides the ubiquitous soil bacteria *Ralstonia*, *Rhodococcus* and *Pseudomonas*, the *Variovorax* species also have great potential in the biodegradation of carbendazim. Until now only bacteria belonging to the genera *Pseudomonas*, *Rhodococcus* and *Ralstonia* were known as good carbendazim degraders (GUI-SHAN ZHANG et al, 2005). DEJONGHE et al. (2003) described *V. paradoxus* ability to degrade Linuron, a worldwide used herbicide. The molecular structure of Linuron has some common features with carbendazim: it contains an aliphatic carbamyl group and an aromatic ring. This suggests that some steps would be common in their degradation pathway in *V. paradoxus*.

ACKNOWLEDGEMENTS

The project is co-financed by the European Union through the Hungary-Serbia IPA Cross-border Co-operation Programme (BIOXEN, HU-SRB/0901/214/150).

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