ISOLATION AND CHARACTERIZATION OF ETHYLENETHIOUREA-DEGRADING BACTERIA FROM SOIL

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ABSTRACT - Isolation and characterization of ethylenethiourea-degrading bacteria from soil
Ethylenethiourea (ETU), the spontaneous degradation product of the widely used fungicide mancozeb is more stable in the environment than its parent molecule and has a carcinogenic effect. The accelerated degradation of ETU by soil microbes would be highly desirable. In our study, ETU-degrading bacterial communities from distinct soil and water samples were isolated using ETU as nitrogen source. The isolated strains from these communities were not able to use ETU as sole carbon source but were able to use ETU as sole source of nitrogen. A new colorimetric method was developed and optimized to measure the ETU consumption from the culture media. This method is based on the fact that 2,6-dichloroquinone-chloroimide give with ETU a pinkish-yellow product. The best ETU-degrading strains proved to be *Bacillus subtilis* and *Pseudomonas fluorescens* based on molecular level identification using sequences of their 16S ribosomal RNA genes. More than 40 *Bacillus* and more than 60 *Pseudomonas* strains deposited in our bacterium collection were screened for ETU transforming ability and about 10 % of the investigated strains were able to degrade 100 mg/l ETU within 10 days. However, the growth of all strains was totally inhibited at the ETU concentration of 1000 mg/l.

Keywords: ethylenethiourea, *Bacillus*, *Pseudomonas*, 2,6-dichloroquinone-chloroimid

INTRODUCTION

The fungicide mancozeb is very instable in the environment and its aqueous solution decomposes spontaneously within two weeks. The degradation product, ethylenethiourea (ETU) is carcinogenic and more stable than the parent molecule. The accelerated degradation of the fungicide and its derivative by soil microbes would be highly desirable (*Fig.1.*)
ETU degradation was found to be slower in autoclaved soils than in non-sterile soils, and only ethyleneurea (EU) was identified as degradation product. In biologically active soils, ETU was oxidized to carbon dioxide and to four other degradation products, two of which were identified as hydantoin and Jaffe's base (JACOBSEN AND BOSSI, 1997). Degradation of ETU to carbon dioxide in non-sterile soils was reported by LYMAN AND LACOSTE (1975). These results indicate that ETU is oxidized under both biological and non-biological conditions to EU, which considerably more stable than ETU, has less acute toxicity (*Table 1.*) not carcinogenic, and can be considered as the major breakdown product. EU, however, can be oxidized photochemically, using a catalyst, to

give glycine and carbon dioxide or could serve as nitrogen source for soil microbes (ROSS AND CROSBY, 1973),

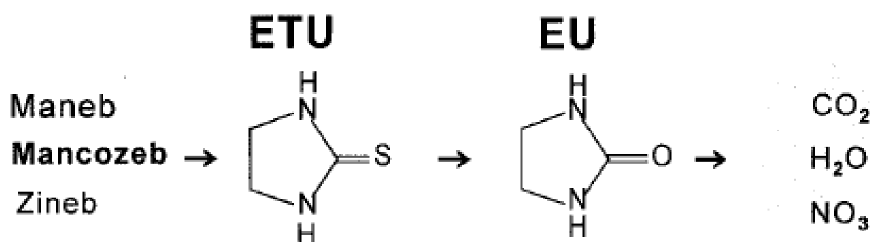

Figure 1. The main direction of microbial degradation of some dithiocarbamate fungicides. (from JACOBSEN AND BOSSI, 1997)

Table 1. Ecotoxicity of some dithiocarbamate fungicides, ETU and EU.

Compound	*Daphnia magna*	*Chlorella pyrenoidosa*	*Photobacterium phosphoreum*	*Nitrosomonas Nitrobacter*
	48-h LC_{50} (mg/litre)	96-h EC_{50} (mg/litre)	15-min EC_{50} (mg/litre)	3-h MIC (mg/litre)
Nabam	0.44	2.4	102	32
Maneb	1	3.2	1.2	56
Zineb	0.97	1.8	6.2	18
Mancozeb	1.3	1.1	0.08	32
Metiram	2.2	1.8	0.37	32
Na-DMDC	0.67	0.8	0.51	26
Ziram	0.14	1.2	0.15	100
Ferbam	0.09	2.4	0.20	10
Thiram	0.21	1	0.10	18
Na-DECD	0.91	1.4	1.22	43
Zn-DEDC	0.24	1.1	1.70	> 320
Disulfiram	0.12	1.8	1.21	> 320
ETU	26.4	6600	2100	1
EU	5600	16 000	3300	1000

[a] From: Van Leeuwen (1986).

MATERIAL AND METHOD

Soil samples were collected from agricultural fields in Hungary, where mancozeb was regularly used. Isolation of ETU-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): glucose 1.0, Na_2HPO_4 2.0, KH_2PO_4 1.0, $MgSO_4$ $7H_2O$ 1.0, NaCl 0.5, supplemented with ETU (100 mg/l). 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 spread 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 ETU degrading ability.

The taxonomical positions of the isolates with best degrading ability were determined by partial sequencing of 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'**.
ETU-degrading abilities of the isolates were measured in the enrichment medium supplemented with 100 mg/l ETU. After incubation, samples from the cultures were centrifuged at 10 000 g for 3 minutes and the remaining ETU in the supernatant was determined with the 2,6-dichloroquinone-chloroimide (DQC) reagent: to 1 ml sample 1 ml 1/15 M phosphate buffer (pH= 8) was added, then 0.1 ml 0.4% DQC (Sigma), dissolved in ethanol, was added to the mixture. After 60 min incubation at room temperature, the colour intensity was measured at 405 nm. The calibration curve regarding the colour reaction for ETU determination is shown on *Fig. 2*.

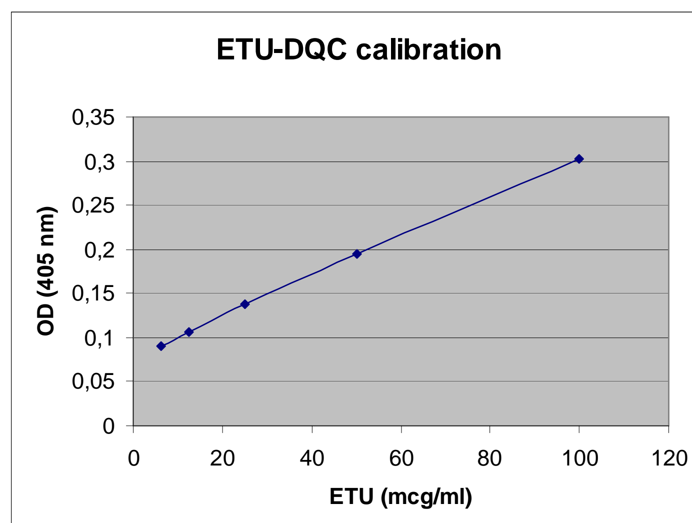

Figure 2. Calibration curve for determination of ETU with the DQC reagent.

RESULTS

We were only able to detect up growing bacterial communities if ETU was used as nitrogen source. The isolated strains from the enriched communities were not able to use ETU as sole carbon source but were able to use ETU as sole nitrogen source.
A new colorimetric method was developed and optimized to measure the ETU consumption from the culture media. This method is based on the fact that 2,6-dichloroquinone-chloroimide give with ETU a pinkish-yellow product. The best strains proved to be *Bacillus subtilis* and *Pseudomonas fluorescens* based on molecular level identification using sequences of their *16S RNA* genes.
The ETU degradation activities of the *Bacillus* and *Pseudomonas* strains were strongly repressed in the present of 0.1% ammonium chloride and were slightly repressed by 0,1% sodium nitrate in the medium (*Fig. 3*.).

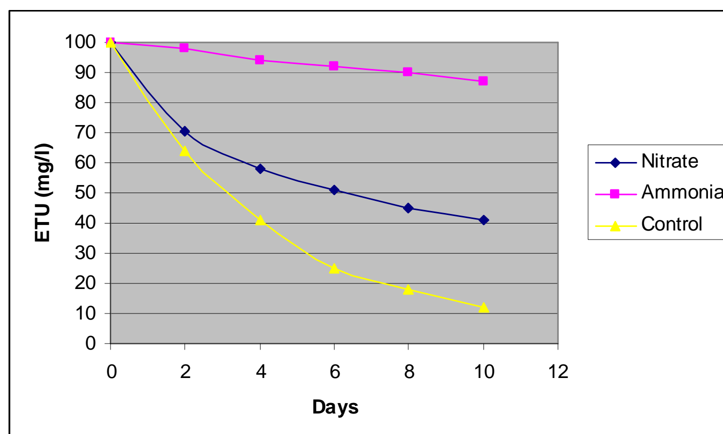

Figure 3. Effect of ammonia and nitrate on the ETU-degrading activity of the *Bacillus subtilis* B13 strain.

More than 40 *Bacillus* and more than 60 *Pseudomonas* strains deposited in our bacterium collection were screened for ETU transforming ability and about 10 % of the investigated strains were able to degrade 100 mg/l ETU within 10 days. However, the growth of all strains was totally inhibited at the ETU concentration of 1000 mg/l.

CONCLUSIONS

We proved that ethylenethiourea-degrading bacteria are frequent in the soil and belong mainly to the *Bacillus* and *Pseudomonas* genera. The best strains are able to degrade 100 mg/l ETU within 14 days of culturing, but 1000 mg/l ETU concentration is inhibitory for all strains. The quality of nitrogen source highly influenced the ETU degradation activities of the strains, ammonium ions strongly; nitrate ions slightly repressed the ETU degrading process.

ACKNOWLEDGEMENTS

The project was 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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