African Journal of Biotechnology Vol. 6 (4), pp. 419-423, 19 February, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Biodegradation of the carbofuran by a fungus isolated from treated soil

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Accepted 27 November, 2006

Thirty microorganisms including two fungus and 28 bacteria were isolated from soil treated several times for many years by carbofuran. The carbofuran N-methylcarbamate insecticide-nematicide (2,3-dihydro-2,2-dimethyl-7-benzo-furanyl methylcarbamate), is extensively used as a soil-incorporated insecticide-nema-ticide to control a variety of insect pests that infest crops such as corn and rice. The carbofuran-degrading activities of the isolates were determined. A fungus capable of utilizing carbofuran as a sole carbon and energy source was characterized and identified as being a member of the genus *Gliocladium* (L_c); it exhibits the highest carbofuran degrading ability in the isolates. In addition, possibility of cross-enhancement between carbofuran and two organophosphate nematicides (fenamiphos and cadusafos) was investigated. The percentage of residual pesticide used was determined by chromatography. Metabolites formed from carbofuran by strain L_c were detected by thin layer chromatography. Strain L_c possesses a high level of degrading activity for only carbofuran but did not degrade the other organophosphorus nematicides tested at significant rates.

Key words: Carbofuran, biodegradation, methylcarbamate, organophosphorus, *Gliocladium*, soil.

Introduction

Pesticides are widely used to improve the quality and yield of food crops. They must persist long enough to control biological targets, but should not become a pollution problem. For some time now, some very effective pesticides used in treatment of soil have presented a significant reduction of their pesticidal effect (Kaufman, 1987; Racke and Coats, 1990). This problem is related to an increase in the biological capacity of the soil to degrade these products, due to the proliferation of microorganisms using the pesticide as source of carbon and/or energy. This phenomenon is called enhanced biodegradation of pesticides (Kaufman and Edwards, 1982), and occurs when microflora soil adapts to a chemical after its repeated application (Smelt et al., 1987). It is one of the major practical consequences of the structure and the evolution of the microbial populations responsible for the mineralization of the xenobiotic pesticides in the soil. It has often been observed in degradable pesticides such as organophos-phorus (Anderson et al., 1998) and carbamates nematicides (Suett et al., 1988).

The carbofuran (trade name Furadan) (2, 3-dihydro-2, 2 - dimethyl-7-benzo-furanyl méthylcarbamate) is extensively used as a soil-incorporated N-méthylcarbamate insecticide-nematicide to control a variety of insect pests (Kaufman, 1987). A number of isolates capable of carrying out some form of degradation of carbofuran have been isolated from soils and several bacterial taxa are represented; including *Pseudomonas sp.* (Felsot et al., 1981; Parekh et al., 1995; Slaoui et al., 2001), *Flavobacterium* (Chapalamadugu et al., 1991), *Achromobacter* (Chaudhry et al., 1988), *Arthrobacter* sp. (Ramanand et al., 1988) and *Sphingomonas* sp. (Feng et al., 1997).

The objective of this study was to isolate microorganisms with the ability to rapidly degrade carbofuran

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frequently used for the treatment of a soil of the area of Gharb (Morocco).

MATERIALS AND METHODS

Culture medium

A synthetic medium adopted at our laboratory containing per liter of medium: $((NH4)_2SO_4$, 2 g; KH_2PO_4, 3 g; $MgSO_4.7H_2O$, 0.5 g; glucose, 3 g, micro-elements mineral solution, 2 ml (Cooney and Levine, 1972) and distilled water, 1 liter. The solid synthetic medium is obtained by addition of bacto-agar, 15 g/l. The pH was adjusted to 7 and the media were sterilized at 120°C for 15 min.

Isolation and Identification of microorganisms

Soil samples used in this study were collected from greenhouses of banana, from the area of Sidi Allal Tazi Gharb, Morocco. These soils had a previous history of treatment with carbofuran. The samples were stored at 4°C for analysis. 1 g of a well-mixed soil sample was suspended in 25 ml of sterile physiological water at a rate, followed by dilution to an appropriate concentration. Various concentrations of carbofuran (analytical grade 99.5% purity) (50, 100, 200, 300 and 400 µg/ml) are prepared and added individually to plates containing synthetic solid medium. 0.1 ml of each suspension of soil was plated on synthetic solid medium supplemented by the carbofuran dissolved in methanol. The plates were incubated at 30°C for 3 days.

The isolation of the carbofuran degrading microorganisms was based on the appearance of cleared zones around the colonies. The putative positive strains were selectively isolated and were further purified by transferring them to synthetic agar medium plates supplemented by 200 µg/ml of carbofuran several times at 30°C for 48 h. The isolated strains were checked by a microscopic after five successive cycles in a liquid medium followed by an agar medium. The strain (L_c) exhibiting the highest activity in carbofuran degradation was selected for further analysis; it was identified in by API Auxacolor 20NE, whose principle relies on the physiological and biochemical characters of the strains.

Strain analysis

Growth of isolates:

Evolution of the biomass of the various isolates was carried out in presence and absence of the carbofuran. In 100 ml Erlenmeyer flask containing 30 ml of culture medium supplemented with 200 µg/ml of carbofuran (dissolved in methanol). The culture was agitated at 105 rpm for 48 h at room temperature (30°C). The growth was monitored at 600 nm

Mode of use of the carbofuran by the isolate L_C

In order to investigate if strain L_c could use carbofuran as the sole source of carbon or nitrogen, the culture was grown on semi-synthetic medium (synthetic medium added with the yeast extract, 3 g/l) in the absence of carbon or nitrogen but always in the presence of the alternative substrate (carbofuran 200 µg/ml) which was the only source of carbon and nitrogen. Incubation was carried out at 30°C on an orbital agitator (105 rpm) for 48 h. The cell multiplication was determined by measurement of turbidity at 600 nm

Evaluation of the residue

The degrading capacity of the isolates was determined by the evaluation of the residual carbofuran produced in the culture medium deprived of carbon or nitrogen sources but in the presence of the carbofuran. The cultures were incubated 0, 2, 4, 8, 12, 24 and 48 h and then were centrifuged at 12000 rpm for 15 min. Aliquots of supernatants were taken for high-performance liquid chromatography analysis using the following chromatographic conditions: Perkins Elmer Chromatograph equipped with a UV/Visible detector and a column, C18; 5 µm, ODS-MP 10 cm. The mobile phase was acetonitrile-water (1:1) with a flow of 1.2 ml/mn, λ = 280 nm and the volume injected was 20 µl of extract, dissolved in the acetonitrile. Carbofuran metabolites were detected by thin-layer chromatography (TLC), using silica gel plates (20 x 20 cm), which were developed with acetone-chloroform (1:10, v:v). 15 µL of each solution (cultures at various intervals of time (0, 4, 8, 12, 24 and 48 h) were spotted on the plate. Spots were detected by UV light. The ability of strain L_c to degrade a diversity of chemicals was also verified. We tested two nematicides of the organophosphates family: Fenamiphos (trade name, Nemacur) [ethyl 3-methyl-4-(methylthio) phenyl (1-methylethyl) phosphoramidate)] and Cadusafos (trade name Rugby) [O-ethyl S,S-bis(1-methylpropyl) phosphorodithioate)]. The later does not contain nitrogen in its molecule but has phosphorus. Both nematicides were pure and dissolved in methanol at 40 and 50 mg/l, respectively. The incubation conditions were the same as described previously.

The same experimental protocol for the evaluation of residual nematicides was followed, but the analysis was made by gas chromatography (CPG) according to the following chromatographic conditions: Shimadzu GC–14A gas chromatograph equipped with a flame photometric detector. The column contained 1% OV -1 (1.6 m x 3.0 mm i.d.) and the temperature was set at 260°C. The temperature of the detector and the injector was set at 280°C.

RESULTS AND DISCUSSION

Isolation and Identification of isolates

After several tests of culture on synthetic medium containing the carbofuran, 30 microorganisms are selected. These isolates are composed of 28 bacteria and two fungi (Table 1). The strains degrading carbofuran are diverse. The colonies which develop clear zones on solid synthetic medium are selected. These clear zones can be explained by the liberation of extracellular enzymes produced by the cells (Figure 1). Clark and Wright (1970) isolated *Arthrobacter* and *Achromobacter*, which utilizing a herbicide (isopropyl N –phenylcarba-mate) from soil and suggest that the process of clear zone formation was the gradual dissolution and diffusion of the herbicide in the culture medium. These processes of clear zone formation by these strains seem to be similar to that observed by Clark and Wright (1970). Only the strain L_c was retained for the rest of this work. It showed the highest potential of degradation. The analysis by the API20NE system (BioMerieux) establish L_c as a *Gliocladium* sp.

Growth of strain L_c

Figure 2 represents the evolution of the biomass of strain L_c examined in a semi-synthetic liquid medium containing 200 µg/ml carbofuran against a control culture without carbofuran. There were marked a difference in the growth of the strain L_c; it grew quite well in medium supplement-

Table 1. Cultural characterization of different strains isolated from banana green houses. Culturing was performed in Erlenmeyer flasks containing 30 ml of culture medium in the absence and presence of carbofuran (200 µg/ml) at 105 rpm and 30°C for 48 h.

Stain	OD_{600nm} in the absence of carbofuran	OD_{600nm} in the presence of carbofuran	Degradation (%)
F_s	0.6	0.8	44
F_3	0.9	1.2	52
F_J	0.6	0.9	36
F_{N1}	0.5	0.7	45
F_{N2}	0.7	1.0	50
F_{B3}	0.4	0.6	45
F_{CB}	0.3	0.5	30
L_c	0.5	1.43	81
L_s	0.28	0.42	24
L_n	0.3	0.5	41
B_n	0.25	0.4	28
B_M	0.1	0.36	24
B_K	0.18	0.3	22
B_J	0.46	0.65	47
N_1	0.18	0.2	16
N_2	0.71	1.0	49
N3	0.69	0.8	25
J_A	0.24	0.3	28
J_B	0.38	0.5	38
J_B	0.38	0.5	38
J_C	0.94	1.05	60
C_1	0.29	0.63	38
C_2	0.15	0.25	18
C_3	0.78	0.9	58
A_a	0.29	0.42	31
A_b	0.51	0.63	48
H_c	0.86	0.89	24
H_s	0.38	0.5	14
M1	0.32	0.22	11
M2	0.18	0.21	18

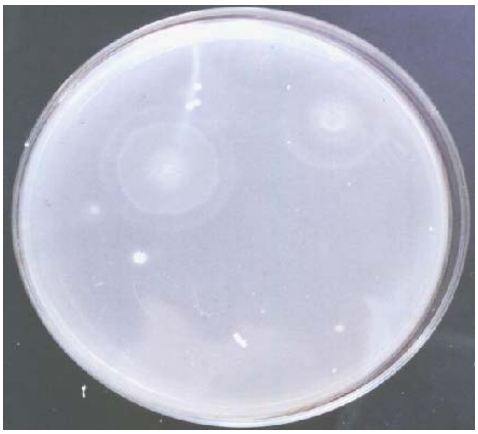

Figure 1. Clear zones formed by microbial colonies grown on a synthetic medium supplemented with carbofuran (200 µg/ml). The plates were incubated at 30°C for 3 days.

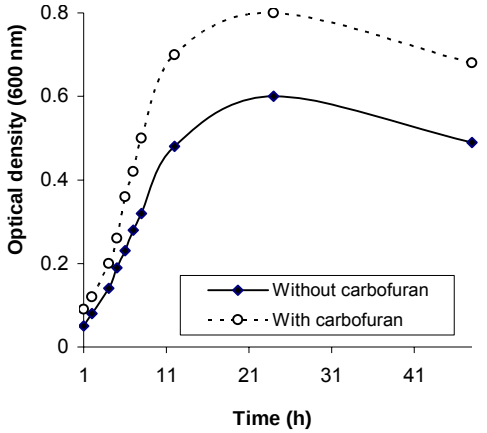

Figure 2. Growth of strain L_c. Culture was maintained in semi-synthetic medium in presence or absence of carbofuran on a rotary shaker 105 rpm at 30°C for 48 h.

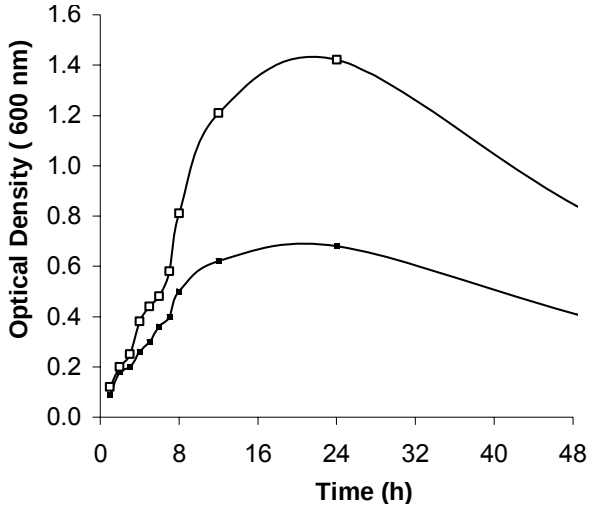

Figure 3. Growth of strain L_c on culture medium containing 200 µg/ml of carbofuran in the absence of carbon (■) or nitrogen (□) source on a rotary shaker 105 rpm at 30°C for 48 h.

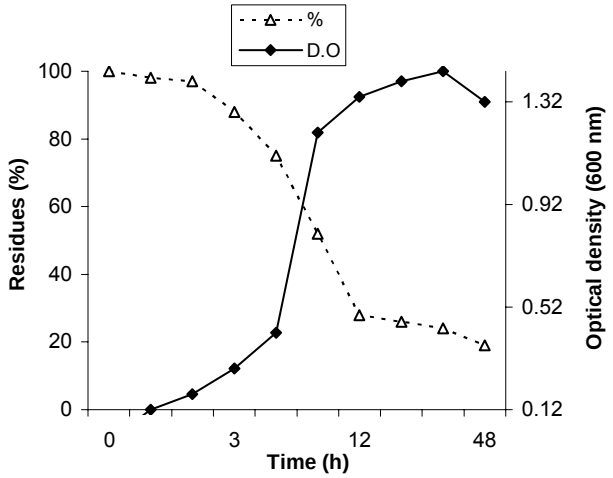

Figure 4. Growth and carbofuran-degrading activity of strain L_c (residual carbofuran). Strain L_c cells were incubated on a reciprocal shaker at 30°C for 48h in culture medium supplemented with 200 µg/ml carbofuran.

ted with carbofuran than in medium without carbofuran. These result indicate that strain L_c degrades carbofuran and uses it for its growth.

This increase in the biomass of the culture with carbofuran can be explained by the fact that this product constitutes, probably, an additional carbon and nitrogen contribution which allows the synthesis of new secondary metabolites favoring the production of microbial biomass and in consequence support a faster use of carbofuran.

The results presented in Figure 3 indicate that the biomass of the strain L_c on semi-synthetic liquid medium without source of nitrogen is more significant than that obtained on semi-synthetic liquid medium deprived of the source of carbon (glucose). We can conclude that the strain L_c uses the carbofuran preferentially as source of nitrogen than a source of carbon. The use of the carbofuran by certain micro-organisms as source of carbon and / or nitrogen can lead to degradation of this compound with loss of effectiveness against its target.

Evaluation of the residual compounds

The cultural characterization of the biomass and the hydrolytic characterization by determination of the residual carbofuran in high performance liquid chromatography of the different isolates are summarized in Table 1. According to these results, it appears that the various strains showed variable potentialities for degrading carbofuran. The residual carbofuran levels varied from 3 to 81%, with strain L_c exhibiting the highest degradation, 81% of the carbofuran within 48 h. Figure 4 shows the residual carbofuran of the strain L_c on semi-synthetic medium deprived of nitrogen or carbon.

The results indicate that L_c uses carbofuran as source of carbon and nitrogen, but more as source of nitrogen. This result suggested that the fungus possesses enzyme(s) which acts on amide and ester bond in carbofuran. We have previously isolated a more potent bacterium (*Pseudomonas*. sp) able to degrade at a very high rate the carbofuran by using it as source of nitrogen and carbon (Slaoui et al., 2001). These results join those of Rajagopal et al. (1984) who isolated *Bacillus* sp., *Micrococcu*c sp., *Arthrobacter* sp. and *Azospirillum* sp. capable of using carbofuran as source of carbon and nitrogen. Other works of Karns et al. (1986) showed that carbofuran was only used as source of nitrogen, whereas Venkateswarlu et al. (1984) isolated a bacterium able to use the carbofuran only like source of carbon.

The analysis in thin layer chromatography shows the presence of breakdown products. We noted the presence of a major metabolite, with retention times of 0.18 min and 0.72 min for carbofuran. The identities of the metabolites remain to be determined. They can indicate which type of enzymatic system is implied in the process of carbofuran degradation.

The results obtained on Figure 5 show residual fenamiphos and cadusafos by gas chromatography, indicating that the strain L_c is unable to degrade these two products at high rates as in the case of the carbofuran. This could be due to the absence of structural similarity between these two nematicides and the carbofuran; the enzyme(s) elaborated by strain L_c may be specific for carbofuran degradation. Racke et al. (1988) concluded that the phenomenon of enhanced degradation presented certain specificity. Moorman et al. (1994), Sharmila et al. (l989) and Smelt et al. (1987) showed that soils adapted to degrade carbamates at accelerated rates have not been found to attack organophosphates at high rates and vice versa. Read (1983) was the only author who observed a cross accelerated degradation between carbofuran and

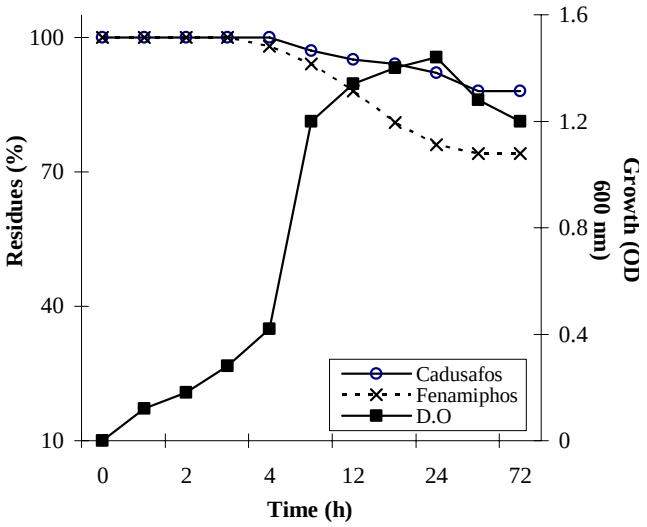

Figure 5. Fenamiphos and cadusafos degradation by strain L_c. Cultures were maintained in a semi-synthetic medium containing fenamiphos (40 mg/l) and cadusafos (50 mg/l) on a shaker at 30°C for 48 h.

an organophosphorus herbicide (fensulfothion). This effect is of great interest in the field. It makes it possible to restore the effectiveness of a pesticide by developing a rotation system of active matters belonging to different chemical families.

Conclusion

The strain L_c which is identified by API20NE system is a fungus of the *Gliocladium*. sp. It is able to apidly degrade carbofuran by using it as source of nitrogen and carbon. It is unable to degrade two organophosphates (fenamiphos and cadusafos) at high rates. Strain L_c will be very interesting in developing procedures of agricultural management which minimize the pollution of subsoil and surface water resources. L_c is also a fungus of industrial interest; it can be used in the depollution procedures of the environment. The description and the characterization of the enzymes responsible for the degradation of the carbofuran are under development.

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