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Effect of 2,4,5-Trichlorophenoxy Acetic Acid (2,4,5-T) on the Morphology of *Klebsiella* spp. from Environmental and Clinical Sources

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One of the herbicides that degrades very slowly in the soil is 2,4,5 trichlorophenoxy acetic acid (2,4,5-T). This degradation occurs through a co-oxidative metabolism by the natural soil microflora (Loos and Horvath 1970). In metabolizing 2,4,5 T and other recalcitrant compounds, no increases in microbial cell populations were observed nor was the carbon derived from the co-oxidative metabolism of these compounds incorporated into the cells (Alexander 1981).

The interaction of 2,4,5-T has received considerable attention with respect to degradation but relatively little is known concerning the potential effects on the free living heterotrophic bacteria. This study examines the effects of this compound on a major nitrogen-fixing genus, Klebsiella spp., found in the soil phyllosphere (Sengupta et al. 1981). Klebsiella pneumoniae has been reported (Pedersen et al. 1977) to be a major bacterial species displaying a strong nitrogenase activity and the energetics were studied (Anderson and Shannugan 1977). Since Klebsiella spp. occur widely in soil and contribute to the nitrogen economy through fixation, their potential sensitivity to herbicides such as 2,4,5-T was evaluated.

MATERIALS AND METHODS

Initial studies were conducted on a single strain of Klebsiella pneumoniae (ATCC 23356) obtained from American Type Culture Collection. It was cultured on Nutrient agar (Difco, Detroit, Mich.) and a modified mineral broth comprising (g/l): 0.7, K_2HPO_4; 0.3, KH_2PO_4; 0.2, $MnSO_4$; 0.2, $MgSO_4$; 0.005, $FeCl_2$; 0.005, MoO_3; 0.005, $CuSO_4$; 0.005, $ZnCl_2$; 1, KNO_3 and 5, glucose with the pH adjusted to 7.5. All of the chemicals were of analytical grade and obtained from Baker Chemical Co., Phillipsburg, N.J. All incubation for both growth rate and morphological studies were conducted at 37°C. Viable counts were performed in duplicate using lawn spread plates on Nutrient agar.

Cell dimensions were measured by a slide micrometer standardized to the stage micrometer. All cell dimensions are reported as the average measurement for 100 randomly selected cells.

Microrespirometric studies incorporated cultures grown on Nutrient agar. The cells were harvested using a sterile saline wash technique. Manometric procedures were performed using standard procedures (Umbreit, Burris and Stariffer 1959).

Standard capsule staining techniques (Cruickshank et al. 1973) were tried but found to inadequately differentiate the capsule for precise measurement. A new technique was developed in which a loop of the cell suspension was mixed with an equal volume of n-Alkyl dimethyl benzyl ammonium (formulated as the detergent-disinfectant A3, Airwick, Mississauga, Canada). The negative stain was provided by the addition of a loopful of Pelikan drawing ink (#50 special black, Pelikan AG, Hamburg, Germany). A smear was made after mixing in the ink under a cover slip. Under oil immersion microscopic examination, the capsules appeared to be clear with well defined outer edges separated from the Brownian movement of the ink particles. The grey colored cell could be easily differentiated within the capsule.

Upon completion of the initial studies, repeat studies were conducted using 565 strains of Klebsiella spp. isolated from various environmental and clinical sources.

RESULTS AND DISCUSSION

Table 1. Effect of Respiratory Activity in Klebsiella pneumoniae

TIME (mins)	2,4,5-T Concentration (mg/l)				
	0	2	20	200	400
30	95	71	65	33	39
60	83	143	141	76	13
90	73	143	147	72	0
120	59	40	82	61	0
150	59	19	48	33	0
180	59	0	0	0	0

Note: oxygen consumption reported as microml O_2/mg dried cell.

Initial experiments using 2, 20, 200 and 400mg/l of 2,4,5-T showed that the herbicide affected the oxygen uptake by Klebsiella pneumoniae with a termination of respiration occurring within 2 hours (Table 1).

At low concentrations of 2,4,5-T (2 and 20 mg/l), the respiratory activity increased over the controls by 70 and 101% at between 60

complete termination of respiration by 180 minutes. These low concentrations appear therefore to have stimulated respiratory activity prior to the onset of toxic effects. At 200 and 400 mg/l, toxic effects were registered by within 90 and 180 minutes of incubation with no apparent stimulatory effects.

Table 2. Influence of 2,4,5-T on the Growth (v.u./ml) of Klebsiella pneumoniae in a Modified Mineral Salts Broth

	2,4,5-T Concentration (mg/l)				
(hours)	0	2	20	200	400
0	1×10^2	1×10^2	1×10^2	1×10^2	1×0^2
3	5×10^3	2×10^2	2×10^2	2×10^2	2×10^2
6	3×10^4	5×10^1	4×10^1	6×10^1	6×10^1
9	3×10^5	6×10^2	4×10^2	8×10^2	1×10^3
12	3×10^6	5×10^3	7×10^3	3×10^3	6×10^4
15	2×10^7	6×10^4	2×10^5	1×10^5	7×10^6
18	4×10^8	8×10^5	2×10^6	2×10^6	5×10^7
21	5×10^9	5×10^7	2×10^7	1×10^8	3×10^8
24	4×10^9	7×10^9	4×10^9	4×10^9	4×10^9
27	3×10^9	2×10^8	4×10^8	5×10^8	3×10^8

Parallel studies were conducted upon the effect of the same concentrations of 2,4,5-T on viable cells of Klebsiella pneumoniae using the mineral salts broth. Enumeration was by the lawn spread plate technique (Table 2). At all concentrations of 2,4,5-T, one generation of cell reproduction occurred within 3 hours of incubation compared to almost six generations in the control. In the next three hours for conditions where respiration was inhibited, the viable count declined by 40 to 50% indicating a partial toxic effect. Reproduction was initiated again at these higher concentrations at between 6 to 9 hours. The growth rate appeared to be similar in the range between 2 and 200 mg 2,4,5-T/l. At 400 mg 2,4,5-T/l, the growth rate accelerated until a population maxima occurred at 21 to 24 hours.

This reproductive phase occurred without detectable oxygen uptake. Subsequent growth must therefore have been as a result of either nitrate respiration or anaerobic growth. Stationary phase occurred at 21 hours in the controls, followed by a slow

Table 3. The Influence of 2,4,5-T on the % of Capsulated Cells, Capsule Size and Cell Length of *Klebsiella pneumoniae*

	2,4,5-T Concentrations (mg/l)											
	0			20			200			400		
Time (h)	% cap	cap:cell ratio	cell length (microns)	% cap	cap:cell ratio	cell length (microns)	% cap	cap:cell ratio	cell length (microns)	% cap	cap:cell ratio	cell length (microns)
6	65	0.5:1	2	57	0.5:1	2	35	0.5:1	2.7	10	0.5:1	3
12	80	0.3:1	3	64	0.3:1	3.1	43	0.3:1	3.1	15	0.3:1	3.2
18	92	1:1	2.5	84	1:1	3.0	54	0.5:1	3.1	16	0.5:1	3.4
24	100	2:1	2	93	2:1	2.5	65	0.5:1	2.8	17	0.5:1	3.8

N.B. all the figures are based on the average of 100 cells.
% cap indicates percentage of cells capsulated.
cap:cell is the ratio of capsule thickness to cell radius on the lateral cell wall.

population decline. When compared to the controls, the cells had a shorter stationary growth phase (< 6 hours) in the presence of 2,4,5-T over the concentration range studied. The experiment was repeated three times with similar results.

In addition to the influence that 2,4,5-T had on the respiration, survival and growth of Klebsiella pneumoniae, routine confirmatory microscopic examination by negative staining revealed that the degree of capsulation was also affected (Table 3). When Klebsiella pneumoniae was cultured in the modified mineral salts broth with the addition of 20, 200 or 400 mg/l 2,4,5-T, the percentage of encapsulated cells declined. Mean values for the percentage of encapsulated cells over the four recorded intervals were (% ±1 standard deviation): 84±15 to 74±16, 49±13 and 14±3% respectively with increasing 2,4,5-T concentrations. This reduction in capsulated cells would indicate that the 2,4,5-T had directly interfered with the production of extracellular polymers (capsulation). At the same time, growth rates seemed to be unaffected (beyond the initial reduction) indicating that cell synthesis did not appear to be affected by 2,4,5-T (Table 2).

While observations were being undertaken on capsulation, it was noted that the average cell length (microns) increased with the concentration of 2,4,5-T (mean of four recorded intervals ± 1 standard deviation) 2.4±0.5 to 2.6±0.5, 2.9±0.2 and 3.4±0.3 microns respectively. Thus, it would appear that as capsulation diminished, the synthesized carbon compounds were diverted at least in part into cellular synthesis causing an elongation of the cell. No increases in cell diameter were observed.

2,4,5-T appears to influence the metabolic functioning of the Klebsiella pneumoniae strain ATCC 23356 in the following sequence of events. Firstly, exposure of the cells to 2,4,5-T caused only a single generation to be reproduced perhaps as a response to the adverse environmental stress (i.e. presence of 2,4,5-T). Secondly, a total loss in detectable respiratory function was observed with a concurrent decline in viable cell numbers. After this brief inhibitory phase, reproduction commenced with a progressive loss of capsulation and elongation of cell length. This would appear to be as a result of the diversion of organic compounds from capsule production to intracellular synthetic functions.

Upon the completion of this preliminary studies using the ATCC strain 23356, confirmatory studies were undertaken with 565 environmental and clinical isolates of Klebsiella pneumoniae. All were identified as K. pneumoniae or K. oxytoca and originally isolated using Wong's medium (Wong et al 1985). The sources were (number of isolates given in brackets): sewage (39), alfalfa sprouts (212), assorted vegetables (30), soils (20), freshwater (45), fecal (72), hospital labs (147), and ATCC strain 29665. All strains responded to the presence of 2,4,5-T at the same concentrations as used in the previous experiments in the similar manner to the original culture (i.e., elongated cells, loss of capsule thickness).

Klebsiella species in soil has already been shown to be capable of enhancing the available soil nitrogen by nitrogen fixation (Sengupta et al. 1981). Since 2,4,5-T has been demonstrated to influence the growth of Klebsiella spp. even at normal field application levels (i.e., 2 p.p.m.), these potential additional interactions are now under investigation in terms of their potential influence on the soil's nitrogen economy.

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