Effect of organic matter and chemical fertilizers on 2, 4-D degradation: Changes in microbiological status of 2, 4-D-perfused soil

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SUMMARY

Various groups of microbial populations and 2,4-D-degrading microbes in 2,4-D-enriched soils amended with rice straw, compost and N-P-K fertilizers combined or separately applied were enumerated by the most probable number (MPN) and plate count methods, respectively. No recognizable differences in the numbers of yeast and total bacteria were found between the different amendments. The population of 2,4-D-degrading bacteria, however, was greater in N-P-K and compost amendments than in rice straw and control (non-amendment). 2,4-D-degrading bacteria were isolated from the perfused soil and all the strains degraded 2,4-D rapidly with increasing phosphorus concentration. Moreover, in a medium with glucose or sucrose as organic source, the isolates utilized these materials for growth without attacking 2,4-D to any extent and *vice versa* in medium containing cellulose or starch.

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RÉSUMÉ

DUAH-YENTUMI, S. & KUWATSUKA, S.: *Effet de la matière organique et l'engrais sur la dégradation de 2, 4-D: Changements du niveau microbiologique du sol aspergé avec 2,4-D.* Les groupes différents de la population microbienne et des microbes qui dégradent 2, 4-D dans des sols aspergé avec 2,4-D et amendé avec de la paille du riz, du compost et de l'engrais (N-P-K), appliqué en combiné ou *séparément, ont été énumérés* par des méthodes du nombre le plus probable et compte sur plaque de verre, respectivement. Il n'y a pas de différence significatif dans le nombre des levures et des bactérie totales entre les amendements différents. La population des bactérie qui dégradent 2,4-D, cependant, etait plus grand dans des sols traité avec de l'engrais et du compost que des sols traité avec de la paille du riz et le temoin (sans amendement). Les bactérie qui dégradent 2,4-D ont été isolé du sol aspergé avec 2,4-D et toutes les bactérie ont dégradé 2,4-D très rapidement avec une teneur en phosphore plus élevé. En plus, dans un milieu avec du glucose ou de la saccharose comme une source organique, les bactérie isolés ont utilisés du sucre pour pousser sans dégradant 2,4-D pour autant, et vice versa d'un milieu avec du cellulose ou de la fécule.

Introduction

The phenoxyacetate herbicides such as 2,4 dichlorophenoxyacetic acid (2,4-D) and 2-methyl 4-chlorophenoxyacetic acid (MCPA) are degraded readily by microorganisms in the soil. Soil perfusion experiments of these herbicides have shown that the lag period and subsequent rapid degradation of the two herbicides are due to proliferation of the degrading bacteria and/or induction of degrading enzymes (Audus, 1950; Audus, 1952). Different soil managements influence the level of microbial population of soil and the types of microorganisms that make up the population (Martyniuk & Wagner, 1977). Duah-Yentumi & Kuwatsuka (1980) found that organic matter and chemical fertilizers promoted the degradation of benthiocarb and MCPA herbicides in soil and also investigated the factors by which organic matter and chemical fertilizers affected the degradation of these herbicides and 2,4-D using the perfusion technique (Duah-Yentumi

& Kuwatsuka, 1982). It was suggested that differences in the size and composition of the microbial population of the amended soils may play a role in the observed effects in promotion activities.

In this report, 2,4-D was selected as one of the herbicides to assess quantitatively and qualitatively the levels of 2,4-D- degrading microbial population with a view to establishing the characteristics of the main population in the amended soils.

Materials and methods

Soil

Anjo soil (Anthrotroaquic Dystrochrept, *p*H 5.3 (H_2O), total-C 1.9 per cent, CEC 10 meq/100 g, kaolinite, dominant clay mineral) was collected from 0-15 cm depth of an unsubmerged paddy field at Aichiken Agricultural Research Center in Anjo City, Japan. The soil (sieved moist) in the perfusion column (Duah-Yentumi & Kuwatsuka, 1980), was amended with 0.5 g of rice straw and 1.0 g of compost (prepared from rice straw) per 50 g soil. The N-P-K chemical fertilizers, combined or applied separately, were dissolved in the perfusion solution as urea, Na_2HP0_4, and KCl respectively, at rates corresponding to 22.5 mg for each of N, P and K. The soil was circulated with 350 ml of 50 ppm aqueous solution of 2,4-D at 30°C in the dark at a speed of 5 ml/min. Unamended soil served as control.

2,4-D degradation in a medium inoculated with perfusate of amended soil

In a previous report (Duah-Yentumi & Kuwatsuka, 1980), the authors found that the addition of rice straw, compost and chemical fertilizers all promoted 2,4-D degradation from the initial periods but the N-P-K combined amendment resulted in faster degradation of the herbicide than the others. Furthermore, the promotive effect of P was more evident than N or K.

To confirm the pattern of microbial enrichment in the different soil amendments, samples of the perfusate were taken after 2, 5 and 7 days of perfusion for preliminary examination of the changes in microflora during the period of perfusion. The selection of these time intervals were based on the changes in 2,4-D concentration with time as reported previously (Duah-Yentumi & Kuwatsuka, 1982). Two millilitre samples of the perfusate were inoculated into 18 ml of 2,4-D nutrient broth (meat extract, 0.3 g; peptone, 1.0 g; and NaCl, 0.5 g/*l* of water) medium containing 50 mg/*l* of the herbicide at *p*H 7.0 and incubated at 30 °C on a reciprocal shaker. Samples of the culture media, centrifuged if necessary, were taken at appropriate intervals and the amount of 2,4-D remaining was analysed by gas chromatography (Duah-Yentumi & Kuwatsuka, 1980).

Most probable number (MPN) count of 2,4-D-degrading microbes in perfusate

Samples of the perfusate were used for counting the microbial populations because the amendments resulted in rapid fermentation, especially in the soil treated with rice straw and compost and, therefore, direct sampling of the wet soil during perfusion created ponding of water on the soil surface. This did not, however, occur in the undisturbed soil. In this regard, Audus (1951) isolated 2,4-D-degrading microbes using drops of perfusate from soil. The MPN method was used for counting the 2,4-D-degrading microbes. Samples of the perfusate were taken into a 20 ml test tube and distributed via a ten-fold dilution to give inoculation of 10^{-1} to 10^{-7}. Further dilutions were prepared from these dilution series by inoculating 1ml of the corresponding dilution ratio above into test tubes containing 9 ml of 2,4-D (50 mg/*l*)/nutrient broth medium at *p*H 7.0 to give inoculations of 10^{-1} to 10^{-7}. Five test tables were inoculated for each dilution. A sample of the perfusate from unamended soil was inoculated, after 1 h perfusion with water, to serve as the value for the initial population. Periodically, 2.5 ml samples of the cultures, centrifuged if necessary, were examined with a UV Spectrophotometer (Hitachi, Model 124) for the presence or absence of 2,4-D in the wavelength range 250-320 nm (Loos, Schlosser & Mapham, 1979). MPN values for the populations of organisms degrading 2,4-D were estimated (Cochran, 1950).

Enumeration of total bacteria, fungi and yeast in perfusate by plate count method

One ml each of the 10^{-4} and 10^{-7} dilutions with the sterile distilled water prepared for the MPN experiment was inoculated into plates at five plates per dilution with the following medium (meat extract, 0.3g; peptone, 10g; NaCl, 0.5g; and agar, 15g per *l* of water) for counting the total bacteria. The numbers of fungal propagule colony forming units (fungi and yeast) were counted using rose-bengal-streptomycin agar (Martin, 1950).

Enumeration of microbial population and 2,4-D-degrading microbes in the perfused soil

At the end of perfusion, 2 g of the wet soil was transferred into 18 ml of sterilized distilled water, and the soil was dispersed thoroughly using a micro-thermo mixture. The soil suspension was distributed via ten-fold dilutions to give inoculations of 10^{-1} to 10^{-8}. One-ml portions of the 10^{-4} - 10^{-8} dilutions were inoculated into plates at five plates per dilution. Meat extract-peptone-NaCl-agar medium of the same concentration as above was used for counting the total bacteria while fungi and yeast were counted in rose-bengal-streptomycin agar (Loos, Schlosser & Mapham, 1979). The 2,4-D-degrading microbes in the soil were counted by the MPN method by inoculating 1-ml samples of 10^{-5} - 10^{-8} dilutions into test tubes containing 9 ml of 2,4-D-nutrient broth medium as already described. Five tubes were inoculated per dilution. The tubes were incubated by horizontal shaking at 30 °C in the dark.

Isolation and characterization of 2,4-D-degrading bacteria from soil

About 20 bacterial colonies were removed from the plates of the dilution plate method above after 10 days incubation and purified by further streaking on slant cultures containing 50 mg 2,4-D, 1.8 g nutrient broth and 15 g agar in 1 litre of water. After incubating stationary for 7 days at 30 °C, one loop-ful from each slant was scraped into 2,4-D-inorganic salt medium (Loos, Roberts & Alexander, 1967) buffered at *p*H 7.0. The 2,4-D-degrading ability of each bacteria was examined by gas chromatography. Further examinations on the shape, motility, oxidase and catalase activity, formation of spores, gelatin liquefaction, reduction of nitrate to nitrite (Komagata, 1975), and acid formation from glucose under aerobic and anaerobic conditions (Hugh & Leifson, 1953) were carried out to determine the morphological and physiological characteristics of the strains.

Organic materials and phosphorus utilization by isolated bacteria

To investigate the effect of organic materials other than rice straw and compost on the degradation of 2,4-D, glucose, sucrose, cellulose and starch were used as energy sources for the isolated bacteria. The isolates were cultured in a medium containing 2,4-D (50 mg/*l*), inorganic salt (KH_2PO_4, 0.4 g; K_2HPO_4, 1.6 g; NH_4NO_3, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.025 g; $FeSO_4.7H_2O$, 8 mg) and 0.1 per cent of the above organic materials.

Since phosphorus promoted the degradation of 2,4-D as reported in our previous paper (Duah-Yentumi & Kuwatsuka, 1982), a loopful of each isolate was inoculated into 10 ml of 2,4-D-inorganic salt medium as described above with varying concentrations of the phosphate buffer. All the cultures were buffered at *p*H 7.0 and incubated at 30 °C by horizontal shaking. An aliquot of the cultural medium was taken periodically for analysis of 2,4-D persistence by gas chromatography as described above and for bacterial growth by measuring the optical density (OD) of the medium at 660 nm.

Results and discussion

The changes in amount of 2,4-D in a medium inoculated with 2 ml perfusate of soil amended with rice straw, compost and N-P-K after designated periods of perfusion are shown in Fig.1. In the medium inoculated with perfusate from compost and N-P-K amendments after 2 days of perfusion (2-days perfusate), degradation of 2,4-D was noted after a period of 6 and 7 days, respectively. The 20-

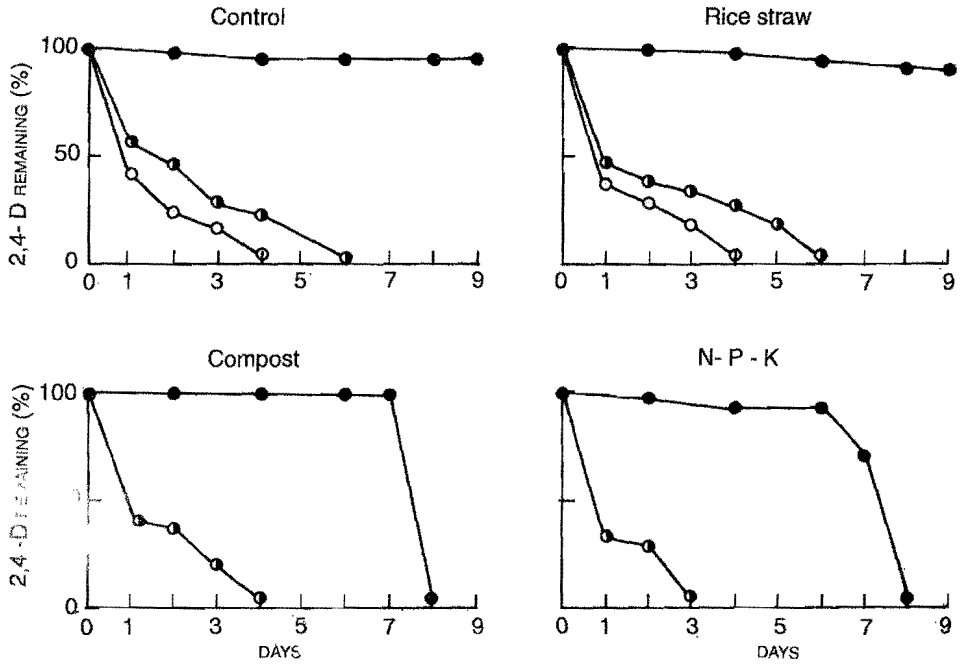

Fig. 1. 2, 4-D degradation in a medium inoculated with 2 days (●), 5 days (O) and 7 days (O) perfusate of rice straw, compost and N-P-K fertilizer amended soils.

days perfusate of control (non-amendment) and rice straw amendments were not sufficiently enriched with microbes to degrade the herbicide even after 9 days which suggested that after 2 days of perfusion, the compost and N-P-K amendments contained more numbers of 2,4-D degrading microbes than that of rice straw and compost. By inoculating with sample of the perfusate taken after 5 days of perfusion, rapid degradation of 2,4-D was noted especially in N-P-K and compost amendments.

Compared with the control, 2-days perfusate of N, P and K applied separately were active in degrading 2,4-D, and 5-days perfusate of P showed faster degradation rate than the others (Fig. 2). No recognizable differences were observed in 2,4-D degradation when inoculated with perfusate taken at the 7th day of perfusion (7 days-perfusate) in N, K and control treatments though the degradation was faster than 5-days perfusate of these treatments. Duah-Yentumi & Kuwatsuka (1982) found that 2,4-D was degraded after a period of 7 days in control and rice straw amendments and 4 days in compost and N-P-K combined amendments.

Furthermore, the initial lag periods observed in control and rice straw amendments were absent in the compost and N-P-K combined amendments. This may be partly explained by the high activity of 2,4-D degrading microbes in the compost and N-P-K treatments compared with those of rice straw and control within 4 days after perfusion as shown by the results of this experiment. In a similar manner, the promotion action of P compared to N, K and control previously reported by Duah-Yentumi & Kuwatsuka (1982) may be related to the high activity of the herbicide-degrading microbes.

The changes in the microbial population in perfusate of the amended soils are shown in Fig. 3 and Fig. 4. The initial numbers of yeast and total bacteria (including actinomycetes) counted by the plate method were about 10^3/ml and increased rapidly attaining a peak value of 10^7/ml in 5 days. The population of total bacteria did not show much variations between the different amendments. The

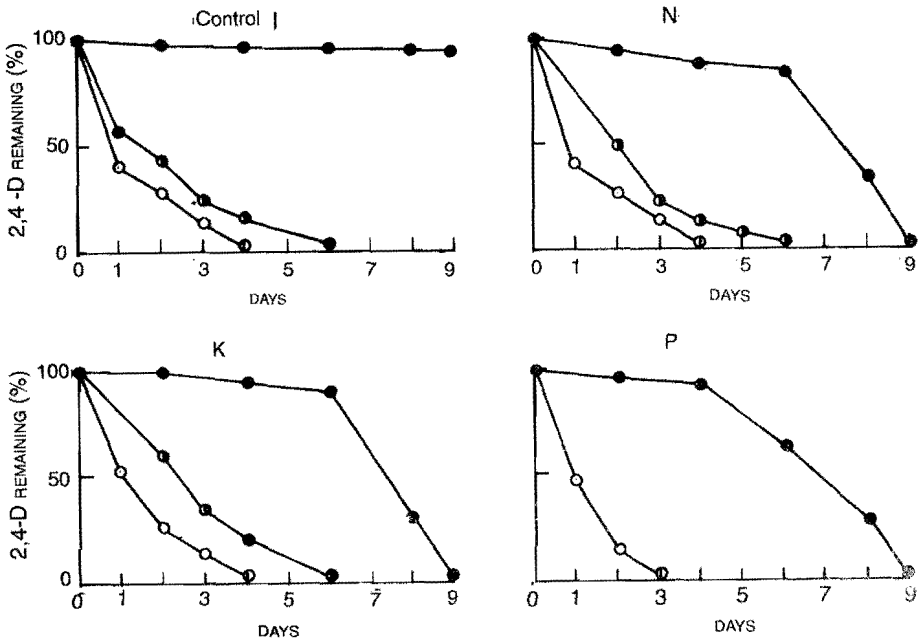

Fig. 2. 2, 4-D degradation in a medium inoculated with 2 days (●), 5 days (O) and 7 days (O) perfusate of N, P and K amended soils.

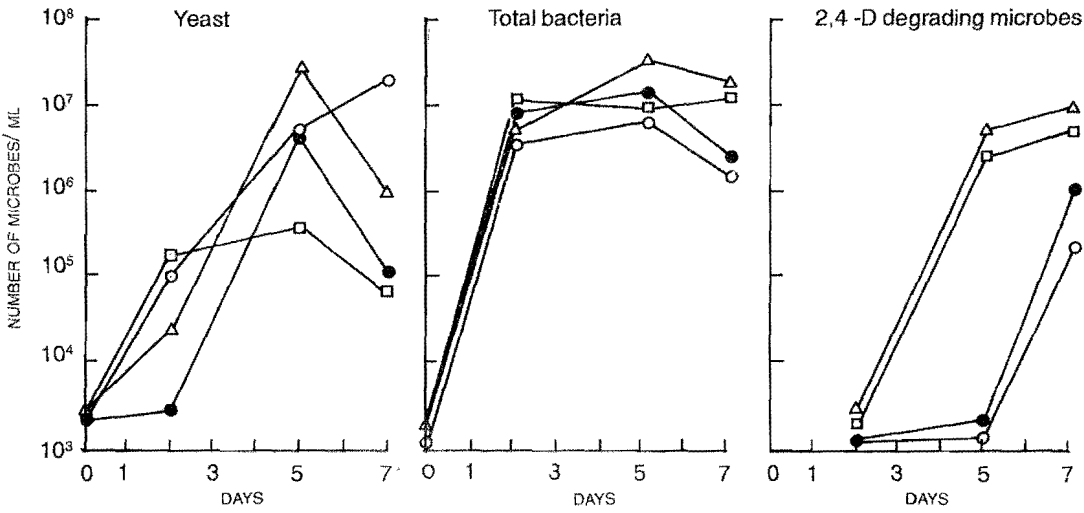

Fig. 3. Changes in microbial population in perfusate of soils amended with rice straw (●), compost (□) and N-P-K fertilizer (Δ) and control (O).

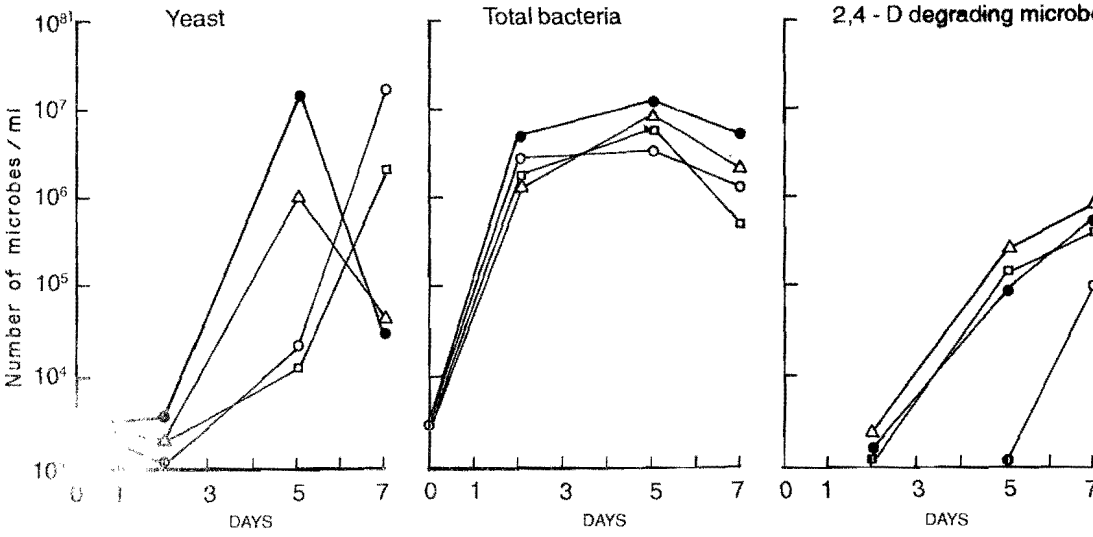

Fig. 4. Changes in microbial population in perfusate of soils amended with N (●), P (Δ) and K (□), and control (○).

numbers of 2,4-D degrading microbes counted by the MPN method were less than 10^3/ml even after the 3rd day of perfusion and increased after 3 days in compost or N-P-K amended soil and after 5 days in straw or control amendment (Fig. 3). This result is in agreement with the rapid degradation of 2,4-D in compost and N-P-K amendments. Phosphorus amendment also promoted proliferation of 2,4-D degrading microbes (Fig. 4). Comparing the results of Fig. 3 and 4 to those of Fig. 1 and 2, it may be inferred that the microbial activity in the 2, 5 and 7 days perfusate described above corresponds with the numbers of 2,4-D degrading microbes in the perfusate. Moreover, the absence of any lag and the rapid degradation of 2,4-D in the compost, N-P-K and P amendments reported previously (Duah-Yentumi & Kuwatsuka, 1982) may be associated with high numbers of 2,4-D degrading microbes.

The populations (colony forming units) of the various microflora in the soil after 7 days perfusion are presented in Table 1. The numbers of 2,4-D degrading microbes were in the same order of magnitude for the different amendments which indicate that once enriched, the population of 2,4-D-decomposers attained the same level irrespective of the soil amendment.

TABLE 1

Microbial Populations (colony forming units) in Soil of Various Amendments after 2,4-D Perfusion for Seven Days*

Microbial flora	*Soil amendment*						
	Control	*Rice straw*	*Compost*	*N-P-K*	*N*	*P*	*K*
Fungi (× 10^3)	9.2	29.1	15.4	12.7	6.2	5.1	10.3
Yeasts (× 10^5)	11.3	17.5	24.6	20.4	10.3	12.3	20.4
Total bacteria (× 10^7)	16.1	23.0	22.1	28.2	17.2	11.2	15.5
2, 4-D degrading microbes (× 10^7)	6.1	6.7	10.9	11.1	5.6	8.4	6.3

* Replicates of five plates.

Loos (1969) reported that 2,4-D is degraded by a variety of soil bacteria. The fungus, *Aspergillus niger* is the only species of fungi reported to degrade the herbicide (Faulker & Woodcock, 1964) and little is known of degradation by yeast. Thus, the detoxification of 2,4-D in this experiment may be related mainly to the action of soil bacteria. Three of the 20 bacterial isolates (Strain Nos. 2, 5 and 18) could utilize the 2,4-D as sole carbon source. On nutrient agar plate, Strain No. 2 produced yellow colonies with smooth surface, Strain No. 5 light-orange flat colonies and Strain No. 18 light-yellow translucent colonies. All the strains were rod shape, gram-negative, non-spore forming and catalase-and oxidase-positive. Neither of the strains formed indole nor liquefied gelatin. Strain No. 18 formed nitrite from nitrate but Strain No. 2 and 5 did not. Both Strain Nos. 2 and 5 produced acid from glucose under oxidative conditions while Strain No. 18 had no action on glucose under aerobic or anaerobic conditions.

Strain Nos. 2 and 5 were tentatively identified as *Flavobacterium* sp. and Strain No. 18 as *Moraxella* sp. using Bergy's Manual, 8th ed. (Buchanan & Gibbon, 1974). The growth of the strains was very fast on 2,4-D nutrient broth agar medium and 2,4-D-degrading ability was maintained after 3 months culture. Fig. 5 shows the degradation of 2,4-D and the growth of the isolates in inorganic salts medium containing various amounts of phosphorus. Although the growth of bacteria was limited due to the absence of any carbon source except a small amount of 2,4-D, rapid degradation of 2,4-D was observed when phosphate was added. In the medium without phosphorus, the degradation of 2,4-D by the isolates, especially Strain No. 18, stopped after about 1 week of inoculation. All the strains, especially Strains No. 5 and 18, showed increased rate of 2,4-D degradation with increasing phosphate concentration in the culture medium.

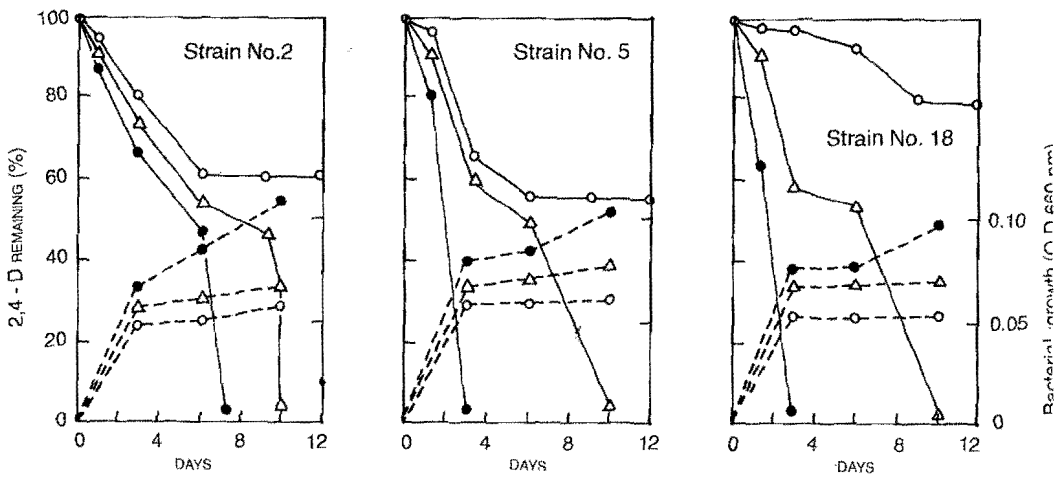

Fig. 5. 2. 4-D degradation in a medium containing 0 g (o), 0.3 g (Δ) and 1.1 g (●) phosphorus. 2, 4-D remaining (—), Bacterial growth (OD 660 nm) (- - - -).

As shown in Fig. 6, the growth of all strains on glucose and sucrose were very rapid as indicated by measurement of the optical density (OD) at 660 nm. However, the rates of metabolism of 2,4-D in these media were very slow. On the contrary, in cellulose, starch and control (non-amendment)

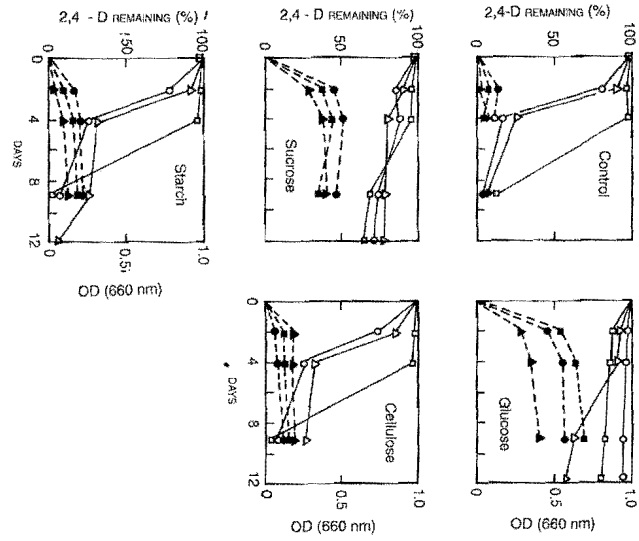

Fig. 6. 2, 4-D degradation by Strain No. 2 (Δ), Strain No.5 (□) and Strain No. 18 (O) of isolated bacteria in a medium containing various organic materials. 2, 4-D remaining (——), Bacterial growth (OD 660 nm) (- - -).

treatments, bacterial growth was very slow but 2,4-D degradation was rapid. Kaufman (1964) reported that the presence of an additional carbon source resulted in an increased utilization of dalapon. Organic materials such as glucose and sucrose, may serve as energy sources for bacterial growth and therefore the microorganism may be adapted to attack these materials in preference to 2,4-D, resulting in slow degradation of the herbicide. Furthermore, larger amounts of the supplemental organic materials (0.1%) in cultural medium compared to 2,4-D (0.005 %) may have resulted in the greater ease with which the organisms were able to utilize the glucose and sucrose. On the other hand, cellulose and starch were hardly employed, thus allowing these organisms to utilize 2,4-D in similar manner as in control (non-amendment).

From the results of the present study, it may be inferred that different factors are involved in the promotion activities of organic matter and chemical fertilizers in pesticide degradation. Day *et al.* (1961, 1963) correlated the rates of herbicide decomposition among various soils to differences in population of effective soil microorganisms. In this study, MPN count revealed that population differences in 2,4-D degrading organisms existed among the different soil amendments which correlated with the promotion activities of these amendments. Since different microorganisms are involved in the degradation of the herbicide, the promotion activities may not be attributed to differences in numbers of effective organisms alone but also differences in the kinds of species that may be important. Moreover, the effect of phosphorus and other organic materials indicate that the nature and kind of substrate play an important role in microbial degradation of 2,4-D.

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