Initial Studies on the Microbial Breakdown of Triallate

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Triallate (S-2,3,3-trichloroallyl N,N diisopropylthiolcarbamate) is used extensively on the Canadian Prairies as a pre-emergence herbicide for the control of wild oats in cereals and other crops. The chemical is rather volatile and must be mixed into the soil after application. When correctly incorporated herbicidal activity remains for several months (1), and residues can be carried over at the end of the growing season (2,3,4).

Studies by Banting (5) and Smith (3,6) have indicated that triallate is slowly degraded in moist non-sterile soils whereas little breakdown occurs in moist sterile soils. Thus biological degradation could be an important factor contributing to losses of the chemical under field conditions. Thiolcarbamate herbicides are known to undergo biological degradation and the subject has been reviewed by Kaufman(7). It has been noted (8) that triallate is degraded in moist soils much more slowly than other herbicidal thiolcarbamates.

Triallate is very strongly adsorbed to soil colloids and it has been reported (3) that the chemical is almost completely adsorbed to a variety of soil types from aqueous solution. This strong adsorption to soil colloids may account, in part, for the relatively slow biological degradation of triallate in soils.

Little is known concerning the rate at which triallate may be broken down by bacteria or molds in the soil. The object of this study was to establish whether breakdown could be attributed to microbial activity. The approach used was to isolate micro-organisms from soil containing triallate and attempt to establish if any of these organisms could degrade the herbicide.

Materials and Methods

The micro-organisms used in this study were isolated from three sources. Initial isolates were from stock solutions of triallate where fast degradation of the chemical had been noted. In all cases mycelial growth could be clearly distinguished. Four pure mold cultures were isolated and identified as belonging to the genera Penicillium (3 isolates), and Chladosporium (1 isolate). The second source of isolates was a soil sample of Regina heavy clay which had been treated with triallate. Of these, 39 cultures were purified consisting of 10 gram negative rods, 20 gram positive non-sporing rods, 7 gram positive cocci, 1 yeast (Rhodotorula) and 1 mold. Similar isolates were made

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from a Weybrun loam which had been trated with triallate when 14 garm negative rods, 4 gram positive non-sporing rods, 3 gram positive sporing rods, and 1 yeast (Rhodotorula) were isolated and purified.

In attempting to establish whether a specific organism would be capable of degrading triallate, a basal medium was developed containing: "DifCo" Proteose-Peptone (20 ppm); "DifCo" Yeast Extract (50 ppm), Ammonium nitrate (20 ppm), and Potassium dihydrogen phosphate (20 ppm). This medium was found to encourage the survival of pure cultures of Pseudomonas fluorescens, Aerobacter aerogenes, Aerobacter cloacae, Serratia sp, Chromobacterium viscosum, Proteus vulgaris, and Propionibacterium jensenii after 76 days of exposure at 20°C to 3 ppm triallate. Pseudomonas aerugenosa, Aerogenes hydrophilia, Xanthomonas campestris, Erwina amylovora, and Achromobacterium parvulus could not survive longer than 10 days under the same conditions. Reducing any component below the levels specified accelerated the death of these organisms. All bacterial cultures used in this survey came from the Department's culture collection. The molds isolated during the study were all found to survive and reproduce to a limited extent on the basal medium containing 3 ppm triallate over a 56 day incubation period at 20°C.

Some difficulties were experienced in incorporating triallate into the media because of volatility and adsorptive properties. It was found that triallate in a 3 ppm aqueous solution could not be sterilized by autoclaving, γ iradiation(2·4 M.Rads) or Seitz filtration. The chemical adsorbed onto polythene, polyurethane and onto some types of rubber liners. A 1% triallate solution in n-butanol was used as the source of triallate for cultural work. It was sterilized by membrane filtration (losses less than 5%) and aseptically added to the basal medium which had been sterilized at 15 p.s.i. for 20 minutes.

Two methods for evaluating the interaction of triallate and the pure cultures of micro-organisms were utilized. The first method involved the preparation of "basal" agar (1.6% agar) plates seeded with the culture under test. Four plates were prepared per culture. Sterile filter paper disks holding 0.1 $\pm$ 0.01 ml. of sterile n-butanol were placed in the center of two plates. The procedure was repeated on the other plates but using 1% triallate in the n-butanol. Incubation was at 20°C for 20 days and any diameter of inhibition around the disk due to n-butanol or triallate plus n-butanol was measured every other day (the difference between the two being attributed to the influence of the triallate). In the second method, the culture work was in a basal broth to which 3 ppm triallate had been added. The culture vessels selected were 2 oz. ground glass stoppered flasks and 300 ml. erelenmeyer flasks with ground glass stoppers. Work with the former vessels were discontinued when triallate losses of 0.7 $\pm$ 0.02

μ gm per day, due to volitility, were observed. Insignificant losses were found from the latter vessels. Sterilized basal medium (100 ml.) containing 3 ppm triallate were shaken vigorously for 10 to 20 hours to ensure even distribution of the triallate in solution. Molds B and C were then inoculated into 2 flasks each. These inocula were prepared by "washing off" spores from mycelial growth on a 5 ml. slope. In cubation was at $20^{\circ} \pm 1^{\circ}$C and the flasks were agitated using a wrist action shaker.

After 0, 7, 14, 21, and 45 days, 10 ml samples were taken and extracted by shaking with 10 ml benzene. The benzene layer was dried over sodium carbonate and the triallate content determined by injecting suitable aliquots into a Varian 204-20 gas chromatograph, equipped with a tritium electron-capture detector. The conditions have been reported previously (6). In later experiments, 10 ml samples of the media were extracted using 10 ml benzene and 5 ml iso-propanol. After shaking with a 2 x 15 ml portions of 2% aqueous sodium chloride to remove the iso-propanol, the benzene layer was dried over sodium carbonate and suitable aliquots analysed for triallate as described above.

Results and Discussion

The "Disk" method, after comparing the inhibitional effect of triallate dissolved in n-butanol showed a range of effects (Figure 1). In all cases the controls using n-butanol only gave inhibition zones of between 2 and 10 cm. diameter from the 2nd to the 17th day of incubation. After this period, the inhibition zone disappeared with 18 (63%) of the 30 strains. This was presumably due to two factors which were the volatilization of the solvent thus reducing its concentration in the agar, and the adaptation of the organisms to the n-butanol. With 9 strains (30%), triallate caused the inhibition zone to enlarge by at least 50% over the controls, indicating that the triallate had a significant and inhibiting effect on the growth of these microorganisms. Slight inhibitory effects were observed with a further 10 strains (33%). The remaining 11 strains (37%) showed no significant differences in the diameter of inhibition zones. Division of the organisms by gram character indicated that for the gram negative organisms 2 (17%) were highly inhibited by triallate, 6 (50%) were slightly inhibited, and the remaining 4 (33%) were not affected; for the gram positive organisms, the corresponding figures were 7 (41%), 4 (24%), and 6 (35%) respectively.

From the "Disk" experiment, it is apparent that triallate has an inhibitory effect on some bacteria over and above any effect caused by n-butanol. The gram positive bacteria appeared to contain a great percentage of sensitive organisms (41%) than the gram negatives (17%). Apart from this, no other characteristic studied showed a significant difference. Although this technique appeared to work well as a survey method for sensitivity to tri-

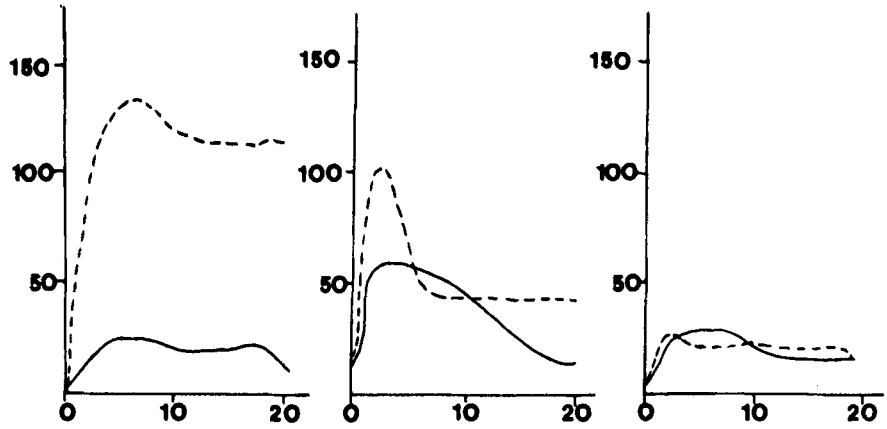

Figure 1. Graphical illustrations of the effect of the "disk" method for diffusing triallate through agar on the diameter of the inhibition zone (given in mm. as the vertical axis), over and incubation period of 20 days (horizontal axis). Inhibition is shown for n-butanol (continuous line), and for n-butanol plus triallate (discontinuous line). The three strains shown (S13, S9, and S10) exhibit continous inhibition by triallate, initial inhibition by triallate and no inhibition respectively.

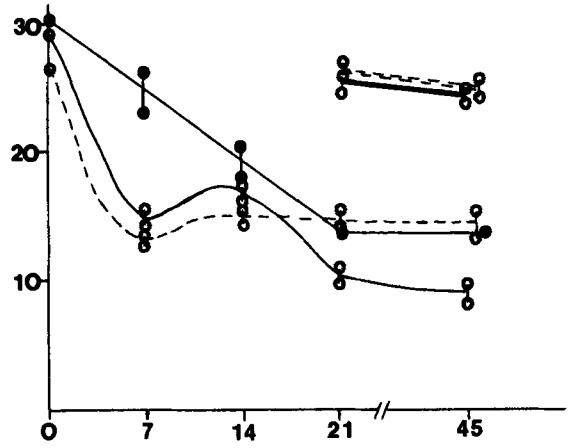

Figure 2. Graphical illustration of the effect of mold growth over an incubation period of 45 days at 20°C (horizontal axis) on the concentration of triallate in μgm/10 ml. (vertical axis). The control is shown as closed dots, continuous line and the molds B and C are shown as open dots with discontinuous and continuous lines. In the top left of the graph, the iso-propanol extractable triallate is shown as double lines for 21 and 45 days incubation only.

allate, the results were not confirmed by any alternative test system. This technique also failed to indicate wheter triallate was in fact degraded by any organisms. In all cases the inhibition for triallate in n-butanol was always at least the same width as the controls indicating that triallate could not, through its presence, increase the tolerance of organisms to n-butanol nor could triallate be degraded within this zone.

From the cultural experiment, using 2 molds both belonging to the genus Penicillium, the results are recorded as Figure 2. In this experiment, mold growth became visilbe after four days and appeared to reach a maximal level at seven days. After this time, the amount of mycelium in each flask remained constant. Loss of triallate again occured from the flasks during the first 24 days, after which time the concentration stabilized at 11.5 $\pm$ 1.0 μ gm per 10 ml media. After 7 days incubation both molds appeared to have degraded approximately 11μ gm of the 24.5μ gm still recorded as present in the controls. Strain B stabilized with approximately 13.0μ gm of benzene extractable triallate and strain C fluctuated between 9.0 and 11.0μ gms of benzene extractable triallate. The loss of triallate from the controls (5.5 gm. per 10 ml.) was attributed to volatilization by "creep" of the solution between the glass stopper and neck of the flask during the shaking. When 55% of the dissolved triallate had been lost from the inoculated media, the system appeared to stabilize. It was considered that this heavy loss of triallate may have been due to a failure of the benzene to extract all of the available triallate. Thus a 2:1 mixture of benzene and iso-propanol was used to extract aliquots taken after 21 and 45 days. This extraction resulted in the same values as previously for the controls but for the cultures higher concentrations of triallate were recorded:

Incubation period	21 days	45 days
Strain B	25.3, 26.2[a]	24.7, 26.3[a]
Strain C	24.7, 23.9	23.7, 22.8

[a]benzene-iso-propanol (2:1) extractable triallate as gm/10ml.

The level of triallate recorded by benzene-iso-propanol extraction was found to be much higher than by benzene extraction, indicating that half of the triallate had, in fact, become absorbed onto a component in the culture and was thus rendered poorly extractable by benzene. It would seem logical that this component could be the mycelial growth. Centrifugation of the culture, and removal of the medium revealed that most of the triallate was ab-

sorbed onto the mycelium and could be removed using the benzene and iso-propanol solution as extractant.

Absorption of triallate to mycelia was further confirmed in an experiment in which 8 flasks filled with basal medium containing 84^{+9}_{-7} gms. triallate per 100 ml. were inoculated with strain C mold and incubated as described. After 7 days incubation, when mycelial growth appeared to have reached a maximal level, the mold was removed by centrifugation, washed in distilled water, and the mold extracted with 15 ml of a benzene-iso-propanol (2:1) mixture. $16.5^{+4.9}_{-6.9}$ μ gms. triallate were thus extracted for the mycelium. This indicated a similar level of absorption as observed in the previous experiment. From these data it can be postulated that mold strains B and C did not degrade the triallate but that absorption occurred between the herbicide and the cell wall.

Since not all of the triallate was adsorbed, it would appear that the number of absortion sites was limited by some extraneous factor. One potential factor could be a requirement of the organism for sulphur containing compounds. In the basal medium, there was between 6 and 9 μ gms of sulphur containing amino acids (depending upon the quantity of yeast extract used). This gives a molecular relationship between triallate: sulphur containing amino acids of approximately 1.8:1 by weight. Such a parity could indicate some inter-relationship between the cell's uptake and sulphur containing amino acids and absorption of triallate. This absorption appeared to remain up to at least 8 weeks since incubated mycelia (still viable) retained the absorbed triallate.

From this study, it would therefore appear probable that after application to soil, some triallate becomes absorbed onto growing mold mycelia where it would remain during the life of the mold. Upon death, the chemical would presumably be released to be eiter readsorbed, degraded, or lost by volatilization.

Conclusions

1. Bacteria vary in their sensitivity to triallate when applied on a filter paper disk onto seeded agar plates.
2. Triallate is absorbed by strains of Penicillium during the phase of rapid mycelial growth and is retained whilst the mold remains viable.
3. The persistence of triallate in soil may be in part due to its absorbence onto microbial cells.

Summary

Two methods for studying the interaction of the herbicide triallate with micro-organisms isolated from soil and stock solutions is described. Some of the soil bacteria were found to be more sensitive to inhibition by triallate than the rest of the

micro-organisms. Two strains of the mold Penicillium were subjected to detailed cultural studies and were found to absorb triallate onto the mycelium in a way that made the herbicide poorly extractable by benzene but extractable by a mixture of benzene and iso-propanol.

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