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Bacterial Degradation of the Herbicide Pendimethalin and Activity Evaluation of Its Metabolites

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Pendimethalin (I, N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine), a preemergent selective herbicide of dinitroaniline group, has been extensively used for weed control in cotton, rice,soybean, tobacco, etc. In general, most dinitroaniline herbicides seem to be moderately persistent, i.e., they are degraded to non-phytotoxic levels within the growing season in warm, moist soils (Kearney *et al.* 1976) and undergo several transformation reactions including N-dealkylation, nitroreduction and cyclization when incorporated in the soil (Marquis *et al.* 1979). Although the microbial process of transformation is considered as the major or sometime the only means by which pesticides are eliminated from a variety of ecosystems (Alexander 1972) but little is known about the microbial transformation products of pendimethalin excepting the works of Barua *et al.* (1990) and Yoko *et al.* (1990). The present investigation has thus been intended to carry out the microbial transformation of pendimethalin by an important soil bacterium *Azotobacter chroococcum* Beijerinck in pure culture condition. Attempts have also been made to establish the structure-activity relationship among the different transformation products of pendimethalin for the safety evaluation of the environment.

MATERIALS AND METHODS

An unadopted strain of *Azotobacter chroococcum* Beijerinck (Saha *et al.* 1991) was grown in PO_4-buffered N-free mineral salts solution, pH 7.2 with 500 $\mu g\ mL^{-1}$ analytical grade pendimethalin dispersed in ethanol (5 mL/100 mL medium) at 31 ± 1°C in darkness in replicated erlenmeyer flasks of 150 mL capacity containing 20 mL of total volume as stationary culture. Cultures were extracted and analysed at 0, 2, 6, 10, 12, 15 and 20 d of growth in replicates to study the rate of pendimethalin degradation and to detect the presence of any other transformation products formed. Control sets contained ethanol equivalent to that of pendimethalin containing media. Each sample was

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centrifuged at 6000 rpm and the supernatant was extracted with ethylacetate (50 mL x 3) adding 100 mL of acidified water [0.5(N) HCl] and the bacterial mass was collected in an preweighed aluminium cup, dried at 80°C to constant wt for biomass estimation. The ethylacetate extract after passing through anhydrous sodium sulphate was concentrated on a rotary evaporator at 40°C. The concentrated material was diluted with acetone and analysed by gas chromatograph (HP Model 5890A) with the operating conditions as mentioned by Barua *et al.* (1990). Another pure culture set (2.0 L) was prepared containing 1.0 g of analytical grade pendimethalin. The total culture solution was centrifuged after 20 d of growth and extracted by ethylacetate as described earlier. The concentrated material thus obtained was subjected to column chromatography (2.0 cm x 60.0 cm) over silica gel and eluted successively with 2, 10, 25 and 50% of ethylacetate in *n*-hexane. Each fraction (500 mL) was concentrated separately on a rotary evaporator at 40°C and monitored by thin layer chromatography for the isolation of pure compounds. Melting points of the compounds (S_{1-6}) were determined on sulphuric acid bath and/or on an melting point apparatus and are uncorrected. IR spectra were recorded on Perkin Elmer 1310 spectrophotometer using KBr pellets. Mass spectral (EIMS, direct insertion) analyses were carried out on a JEOL JMS-DX 300 mass spectrometer at 70 eV. ^{1}H NMR spectra were recorded on JEOL JNM-FX 100 spectrometer (100 MHz) using TMS as an internal standard. The metabolites S_1 and S_2 were quantified by gc (Barua *et al.* 1990) and S_4 and S_5 were quantified by gc (HP 5890A) with flame ionisation detector using the glass column (6ft x 2 mm id) packed with 10% SE-30 on chromosorb W-HP. The oven temperature was programmed at 180-250°C with an increasing rate of 5°C per min and the detector and injector temperatures were maintained at 250°C each.

For the synthesis of 2-amino-6-nitro-N-(1-ethylpropyl)-3,4-xylidine (**III**) a solution of sodium sulphide nonahydrate (5 g, 0.02 M) and sodium hydrogen carbonate (1.7 g, 0.02 M) in 15 mL of water was added dropwise to a stirred methanolic solution of pendimethalin (1 g in 100 mL) at room temperature over a period of 10 min. The mixture was stirred for 3 hr and poured into water (500 mL). The suspension was filtered and the residue after washing with water was taken in hexane through anhydrous sodium sulphate, concentrated to about 50 mL and kept overnight under deep freezing condition. The separated solid (**III**) was filtered and further crystallised from chloroform-hexane as red needles, mp 78-80°C (Dureja and Walia 1989).

The isolated metabolites S_{1-5}, the synthesised product **III** along with pendimethalin were subjected to phytotoxic evaluation. A photometabolite, 2-methyl-4,6-dinitro-5-(1-ethylpropyl amino) benzaldehyde (**IX**), although not encountered in the present study, is a possible product formed by the aryl methyl group oxidation (Pal *et al.* 1991), was also subjected to toxicity evaluation. The experiment was set up in replicate pot culture

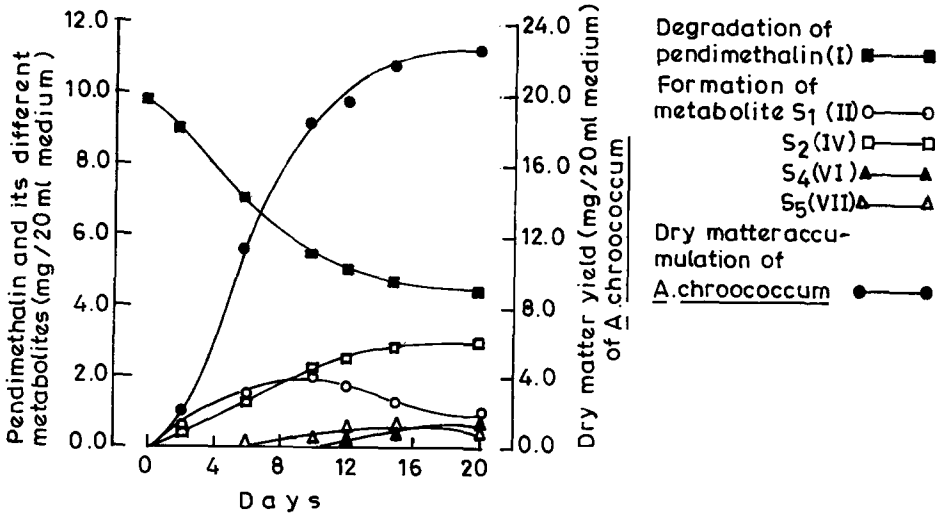

Figure 1. Kinetics of pendimethalin degradation and formation of its different metabolites in pure culture of *A. chroococcum*.

under growth chamber condition. Soil (air-dried, passed through 2 mm sieve was treated with each of the test compounds (dissolved in acetone) at 1.0 and 2.0 kg a.i./ha dose. The control soil sets were treated with acetone only. After 24 hr of soil treatment five wheat (cv. UP-262) seeds were sown in small plastic pots each containing 500 g treated soil maintained at 70% water holding capacity. Replicated pots were incubated at 25 ± 1°C for 14 hr in dark and 10 hr in light condition. Soon after germination only two seedlings were maintained per pot. Seedlings were harvested after 10 days of germination and gently washed under running tap water to remove root adhering soil particles. For evaluation of the phytotoxic effect of the test compounds the no. of seminal roots per seedling, length and dry wt of both shoot and root of each seedling were measured.

RESULTS AND DISCUSSION

Pendimethalin has been degraded effectively by *A. chroococcum* (45% in 10d and 55% in 20d) in pure culture with concomitant formation of metabolites S_1, S_2, S_4 and S_5 at different period of incubation (Figure 1). S_1 and S_2 were found to constitute 51% and 39% respectively of total conversion of I on the 2nd d and may be considered as the major metabolites. The concentration of S_1 was found to decrease after 10 d while that of S_2 was found to increase steadily during the period under study. The formation of S_5 was detected on the 6th d and that of S_4 on 10th d as the minor metabolites of I.

Column chromatography of the ethylacetate extract of *A.*

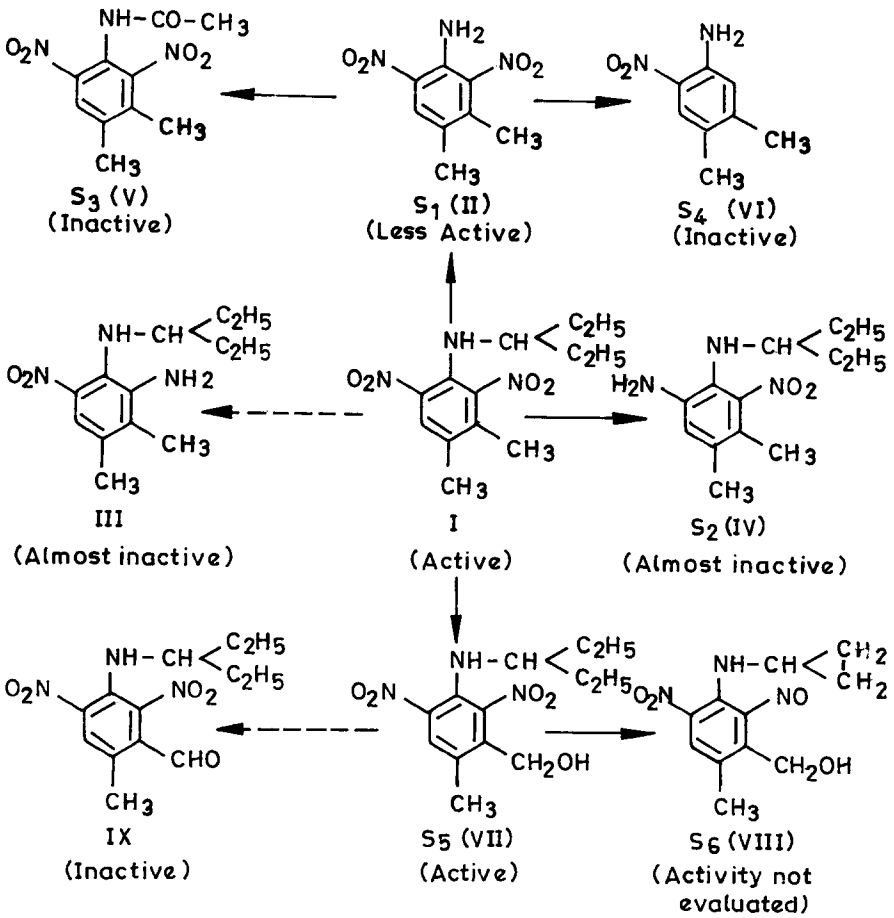

Figure 2. Proposed pathway of pendimethalin degradation by *A. chroococcum* (——→) in pure culture and structure activity relationship of its different metabolites (- - - - - → denotes the product has not been encountered in the present study).

chroococcum culture resulted in the isolation of six pure metabolites. S_1, S_2 and S_3 were obtained from the 10% ethylacetate S_4 and S_5 from 25% and S_6 from 50% ethylacetate in *n*-hexane respectively. The IR, MS and PMR spectral properties of S_1, a yellow orange crystal, mp 136-138°C; S_4, a light yellow solid, mp 138-140°C and S_5, a yellow orange solid, mp 49-52°C were found to be in excellent agreement with those reported for II, VI and VII (Pal *et al.* 1991) respectively and were confirmed by the direct comparison with the respective authentic samples. The spectral properties of S_2, a red needle, mp 80-81°C showed very close resemblence with those reported for III (Dureja and Walia 1989) but were not identical by co-tlc and co-glc. The discripancy was observed in the C_5 proton signal in the NMR spectrum which appeared at δ 6.64 in S_2 and at δ 8.0 in III. The significant upfield shift of C_5 proton in S_2

might be due to the reduction of the neighbouring NO_2 group to NH_2. Therefore, the structure of S_2 could be proposed as 2-nitro-6-amino-N(1-ethylpropyl)-3,4-xylidine (**IV**), a positional isomer of **III**. S_3, an orange needle, mp 235-238°C, showed the presence of imino (3320, 1260 cm^{-1}), amide (CO-NH at 1630 and 1610 cm^{-1} due to coupled interaction of C=O stretching and N-H deformation) and nitro (at 1520 and 1335 cm^{-1}) group absorptions in its IR spectrum. The PMR spectrum of S_3 disclosed the presence of one aromatic proton at δ 8.11 *s*, one imino proton at δ 6.68 b*s*. A sharp singlet appeared at δ 2.28 for 9 protons possibly due to the presence of two aromatic methylgroups and another methyl group in the amide side chain. Furthermore, the compound S_3 did not show the usual proton signals for N(1-ethylpropyl) group present in pendimethalin. Thus, the presence of an acetamide ($-NH-CO-CH_3$) side chain was expected in the molecule which was further corroborated by the appearance of mass fragments at m/z 210 (M^+-COCH_3) and 183 (M^+-COCH_3-HCN) beside the molecular ion peak (M^+) at m/z 253 along with the other important fragments at m/z 238, 236, 223, 209-207, 116, 99, 77. On the basis of these spectral evidence the structure of S_3 has been proposed as N (2,6-dinitro-3,4-dimethyl) phenyl acetamide (**V**). The IR spectrum of S_6, a light yellow crystals (mp 180°C), revealed the presence of hydroxyl group (3400 cm^{-1}), nitro group (1550, 1340 cm^{-1}), secondary amine (1250 cm^{-1}) in the molecule. Its PMR spectrum indicated an aromatic ring proton at δ 8.24 *s*, an aromatic methyl group at δ 2.0 *s*, an aromatic hydroxy methyl group at δ 4.72 *q* (2H) and at δ 1.56 *s* (1H, exchanged with D_2O). Instead of the usual ethylpropyl group proton signals of **I**, the S_6 PMR signals appeared at δ 2.96 *q* (1H) and at δ 1.28 *t* (4H) which could be fitted well in the N-alkyl side chain as N-cyclopropane. The molecular ion peak (M^+) of the compound appeared at m/z 251 and the other fragments at m/z 250, 233, 217, 206, 176, 162, 135, 91 and 77 are only explainable with the molecular formula $C_{11}H_{13}N_3O_4$. On the basis of the available spectral data S_6 has been formulated as 2-methyl-4-nitro-5-N-(1-cyclopropyl)-6-nitrosobenzyl alcohol (**VIII**).

Sequential N-dealkylation and reduction of one or both the nitro groups are very common pathways of photochemical and microbial transformation of substituted dinitroanilines (Helling 1976). S_1 was formed by oxidative complete N-dealkylation (Barua *et al.* 1990, Saha *et al.* 1991) which was subsequently undergone acetylation of the anilino nitrogen to form S_3 (Van Alfen and Kosuge 1974) and S_4 was formed from S_1 by direct elimination of nitro group at C-2 without any substitution (Pal *et al.* 1991). Contrary to earlier reports (Dureja and Walia 1989, Barua *et al.* 1990) S_2 was formed by the reduction of nitro group at C-6 which is being reported for the first time in pendimethalin degradation. The minor metabolite S_5 was found to be an arylmethyl group oxidation product at C-3 (Pal *et al.* 1991) which upon partial reduction of the 2-nitro group and N-dealkylation followed by oxidative cyclisation gave rise to product S_6 a novel product not evidenced earlier in the

degradation of dinitroaniline herbicides.

All substituted aromatic compounds are first transformed to dihydroxyphenolic compounds before ring cleavage can occur by the usual pathways (Wallnofer and Englehardt 1984). The bacterium utilized the C-dealkylation pathway, generally not observed for transformaton of substituted dinitroanilines to cause ring hydroxylation at C-3 and the products S_5 and S_6 may be considered as the intermediates of the process. Ring hydroxylation might also occur at C-1 (after N-dealkylation), at C-2 and C-6 after nitro reduction and oxidative deamination. Although, *A. chroococcum* was observed to prefer C-6 position than C-2 for this purpose. Thus the compounds S_1, S_2, S_4, S_5 and S_6 are the most obvious intermediates for cleavage of the aromatic ring of pendimethalin.

Results of the present study on phytotoxic effect of pendimethalin and some of its metabolites against the growth of wheat seedlings are presented in Table 1. Pendimethalin, like any other dinitroaniline herbicide showed the usual symptoms of growth inhibition (Olson *et al.* 1984) and the order of activity was found to be Pendimethalin > S_5 > S_1 > III ≃ S_2 and S_3, S_4 and IX were found to be completely devoid of any phytotoxicity. The varying degrees of the phytotoxic action may be explained in terms of structure-activity relationship (Figure 2). Reduction of any one of the nitro groups of parent molecule almost inactivated the herbicide as has been found in III and also in S_2 because 2,6-dinitroaniline is the basic moiety of the dinitroaniline group of herbicides (Probst *et al.* 1975). Oxidative N-dealkylation of pendimethalin reduced its herbicidal activity to a great extent and S_1 showed marked lower phytotoxic effect on the seedling growth than I. Unsubstituted dinitroanilines also exhibited some phytotoxicity and the alkyl substitution for amino hydrogens was reported to enhance the activity (Swanson 1972). Whereas, S_1 became completely inactive when one of the amino hydrogens was replaced by an acetyl group to form S_3 and also by the loss of one nitro group to form S_4. Substitutions at 3 and/or 4 positions were reported to modify the degree of herbicidal activity (Probst *et al.*1975) and when 3-arylmethyl group in I was transformed to an aldehyde group as in IX, the molecule completely lost its activity. However, this effect was not observed when the 3-arylmethyl group was oxidised to a primary alcohol as has been observed in S_5 which showed a comparable activity with the herbicide. Thus it is quite evident from the present study that all the transformation processes like nitro group reduction (III, S_2), oxidative N-dealkylation (S_1, S_3, S_4) and oxidative C-dealkylation (S_5, IX) were directed to detoxify the herbicidal molecule (I).

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Table 1. Effect of herbicide pendimethalin (I) and its different metabolites on the growth of wheat seedlings (10 d after germination)

Compounds tested	Dose	No. of seminal root	Length of root (mm)	Dry wt of root (mg)	Length of shoot (mm)	Dry wt. of shoot (mg)
Control	-	4.8^{a}	20.2^{a}	8.5^{a}	13.7^{a}	14.1^{a}
(I)	T_1	7.8^{c}	1.3^{d}	5.6^{b}	9.7^{e}	10.7^{c}
	T_2	7.2^{c}	0.7^{d}	6.6^{b}	4.0^{g}	6.3^{e}
S_1 (II)	T_1	5.4^{a}	15.9^{b}	9.3^{a}	12.1^{c}	10.1^{c}
	T_2	5.8^{b}	15.4^{b}	11.5^{c}	11.5^{c}	9.2^{d}
III	T_1	5.6^{a}	22.4^{a}	9.1^{a}	15.4^{d}	12.1^{a}
	T_2	5.4^{a}	16.8^{b}	8.2^{a}	13.2^{a}	10.3^{c}
S_2 (IV)	T_1	5.2^{a}	23.4^{a}	7.1^{a}	13.6^{a}	14.3^{a}
	T_2	5.6^{a}	16.4^{b}	7.3^{a}	14.3^{a}	11.4^{c}
S_3 (V)	T_1	5.6^{a}	21.5^{a}	9.5^{a}	14.1^{a}	13.6^{a}
	T_2	5.4^{a}	21.8^{a}	9.5^{a}	14.9^{a}	14.6^{a}
S_4 (VI)	T_1	5.6^{a}	21.6^{a}	10.8^{c}	17.5^{b}	16.9^{b}
	T_2	5.2^{a}	18.9^{a}	11.5^{c}	12.0^{c}	10.1^{c}
S_5 (VII)	T_1	4.2^{a}	9.2^{c}	5.0^{b}	9.4^{e}	7.4^{e}
	T_2	6.2^{b}	2.3^{d}	2.6^{d}	6.7^{f}	5.1^{f}
IX	T_1	4.4^{a}	20.4^{a}	11.6^{c}	13.2^{a}	12.2^{a}
	T_2	5.4^{a}	18.4^{a}	8.2^{a}	14.9^{a}	14.4^{a}

T_1 = 1.0 kg and T_2 = 2.0 kg a.i./ha. Each observation represents average of five replicates (seedlings). In a column means followed by same alphabet are not significantly different (P = 0.05) by DMRT.

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