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Effect of some pesticides on growth, nitrogen fixation and *nif*genes in *Azotobacter chroococcum* and *Azotobacter vinelandii* isolated from soil

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This study was designed to evaluate the effects of three pesticides (Imazetapir, Dimethoate and Bayleton 50) at the recommended concentration (in the field), on the growth of pure cultures of *Azotobacter chroococcum* and *Azotobacter vinelandii*, on the amount of fixed nitrogen and *nif* genes. Herbicide Imazetapir had no negative effect on nitrogen fixing bacteria, while Dimethoate and Bayleton 50 exhibited inhibitory effect on growth. Same effects were obtained on fixed nitrogen obtained when treated with studied pesticides. *nif*H1, *nif*H2, *nif*H3, *nif*U and *nif*V from *A. chroococcum*, and *nif*H, *nif*K, *nif*D, and *nif*M gene in *A. Vinelandii* were lost when pots were cultivated with wheat and treated with both Dimethoate and Bayleton 50, therefore, be deemed highly susceptible to them. While herbicide did not affect the *nif* genes, the bands on gel electrophoresis appeared as normal sample.

Key words: *Azotobacter chroococcum*, *Azotobacter vinelandii,* fungicide (Bayleton 50), herbicide (Imazetapir), insecticide (Dimethoate).

INTRODUCTION

Pesticides are used to control specific fungi, herbs, insects and other pests in crops (Johnsen et al., 2001). Pesticide application is still the most effective and accepted means for the protection of plants from pest (Bolognesi, 2003), but the extensive use of pesticide over the past four decades has resulted in tribulations caused by interaction with natural biological system (Ayansina and Oso, 2006).

In the last few decades, numerous soil microorganisms have been found to have a positive effect on plant development. Besides the well-known symbiotic nodular bacteria, free nitrogen fixers in the rhizosphere (*Azotobacter*) can also stimulate plant growth or reduce the damage caused by soil-borne plant pathogens (Kloepper et al., 1989) and has been used as a potential nitrogenous fertilizer to increase crop yield (Steinberga et al., 1996; Mrkovaaki et al., 2001). Some pesticides used in agriculture can be harmful to *Azotobacter,* not only to inhibit the nitrogen fixation process in *Azotobacter,* but also to reduce the bacterium's respiration rate and hence preclude its positive effects (San-Tos and Flores, 1995; Mrkovaaki et al., 2001).

The objective of the present study was to determine the effects of one herbicide (Imazetapir), one insecticide (Dimethoate) and one fungicide (Bayleton 50) on the growth, nitrogen fixation and *nif* gene of pure cultures of *Azotobacter chroococcum* and *Azotobacter vinelandii* isolated from Erbil City soil, Iraq.

MATERIALS AND METHODS

Pesticides

The pesticides were commonly used for wheat crop as recommended

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by Agriculture Research Centre in Erbil City, Iraq to the farmers where Imazetapir serves as herbicide (100 g/L), Dimethoate (Rogar) as insecticide (3 g/L) and Bayleton 50 as fungicide (2 g/L).

Wheat variety

Wheat variety *Triticum aestivum var. Aras* was used in the experiment, kindly provided by Agriculture Research Centre in Erbil City.

Bacteria used

The bacterial isolates used in the study were *A. chroococcum* and *A. vinelandii* isolated from the soil of Erbil City, Iraq, on the basis of cultural, morphological and biochemical characteristics as described by Forbes et al. (2002).

Plant-Microbial interactions

Pot experiment was carried out in green house; each pot was filled with 8 kg Ainkawa soil of 60% moisture content. Wheat seeds were planted at a rate of 10 seed/pot. After sowing, the pesticide was applied by spraying on the soil surface at known quantity/unit area of the pots. After 5, 15, 30 and 70 days, soil samples were taken under plant rhizosphere zone. The total number of *Azotobacter* was determined by MPN using nitrogen free Jensen's broth medium. The *Azotobacter* was isolated from the wheat rhizosphere of pots and identified depending on cultural, morphological and biochemical characteristics as described (Forbes et al., 2002), the *Azotobacter* were identified as *A. vinelandii* and *A. chroococcum*.

Total bacterial count

Soil samples were taken from each pot in duration of 5, 15, 30 and 70 days. The size of *Azotobacter* population of wheat rhizosphere was determined with MPN (Johnsen et al., 2001) using Jensen's broth medium.

Effect of pesticide on bacterial nitrogenase activity

The effect of pesticide on N_2-fixation of two *Azotobacter* species (*A. vinelandii* and *A. chroococcum*) that were used as inoculum in biofertilizer were examined by studying their nitrogenase activity in their respective growth inoculum with a 100 ml of individual bacterial culture containing 35×10^5 cells/ml of Jensen's broth medium. The flasks were prepared by adding stock solution of pesticides at the rate of 2 g/L for Bayleton 50 (fungicide), 100 g/L for Imazetapir (herbicide) and 3 g/L for Dimethoate (insecticide), with 3 replications for each treatment. The flasks were incubated at 28°C for 10 days. Then, the total nitrogen was calculated for each flask using micro-Kjeldhal method as described in Rowell (1996).

DNA extraction

Genomic DNA was extracted and purified from *A. vinelandii* and *A. chroococcum* cells isolated from rhizosphere of wheat plants in pot experiment using the QIA amp DNA Mini Kit (Jayashreet et al., 2007). Polymerase chain reaction (PCR) amplification of nitrogenase genes *nif*H1, *nif*H2, *nif*H3, *nif*U, *nif*V and FV genes for *A. chroococcum*, and *nif*K, *nif*D, *nif*M, *nif*H and FV genes for *A. vinelandii* was performed. Gene's sequences were obtained from NCBI site, and were designed in OPERON diagnostic Ltd, Germany. The primer length and melting temperature were designed with coordination between forward and reverse primers. The melting and annealing temperature were calculated following Womble (2000). Primers amplification was completed using the protocol and reagents followed by Rajeswari and Kasthuri (2009). The programmed temperature sequence was 96°C followed by 55°C for 1 min, and 72°C for 1 min, the temperature sequence was run for 30 cycles, the final product extension was conducted at 72°C for 6 min followed by 4°C temperature hold. The primers acquired from Operon Biotechnologies, Germany were used. The forwards and reverse primers are shown in Table 1.

Gel electrophoresis

DNA amplification was checked by electrophoresis of each PCR product in a 1.5% (w/v) agarose gel, in Tris-borate-EDTA (TBE) buffer for 1 h at 3.2 V/cm. Gels were stained in ethidium bromide for 15 min and thereafter washed for 5 min. DNA fragments were visualised at 312 nm with a UV-trans illuminator Image Master VDS (Amersham Biosciences) (Helmut et al., 2004).

RESULTS

The study has shown that the herbicide Imazetapir involved had no negative effect on the growth of nitrogen fixing bacteria in the soil as compared to the control, 3.1×106 cell/g of the soil from 34×106 cells/g survived. The insecticide (Dimethoate) and fungicide (Bayleton 50), however, did have inhibitory effect on the growth of nitrogen fixing bacteria (Table 2), 0.001×10^6 and 0.2×10 cells/g soil survived after 70 days after application with Dimethoate and Bayleton 50, respectively, as compared with the control 4.2×10^6 cells/g. While 3.1×10^6 cells/g survived when Imazetapir was applied.

The effect of pesticides on nitrogen fixation by nitrogen fixing bacteria in the nitrogen free Jensen's broth medium has been studied (Table 3), the results indicated that nitrogenase activity decreased in the presence of Dimethoate and Bayleton 50 and the amount of fixed nitrogen were 0.09 and 0.009 mg/ml when *A. chroococcum* was present, and 0.008 and 0.005 mg/ml when *A. vinelandii* was inoculated, while for control treatment 1.80 and 1.19 mg/ml for *A. chroococcum* and *A. Vinelandii*, respectively. Moreover, Imazetapir was found to have less inhibitory effect on nitrogenase when two nitrogen fixing bacteria were used as inoculum on the broth culture. The amount of fixed nitrogen was 1.09 and 0.82 mg/ml for *A. chroococcum* and *A. Vinelandii*, respectively (Table 3). In general, the obtained value of fixed nitrogen after 70 days of incubation was less than the control treatment, with negative significant differences of 0.55 and 0.54 for *A. chroococcum* and *A. Vinelandii*, respectively (Table 3).

To investigate the effect of the tested pesticides on nitrogenase activity through amplification of *nif*H1, *nif*H2, *nif*H3, *nif*U, *nif*V and FV genes for *A. chroococcum nif*H, *nif*D, *nif*K, *nif*M and FV genes for *A. vinelandii* by PCR technique, the PCR products are as shown in Figures 1 and 2. Band generation of PCR amplified nif genes fragments were used to evaluate the effect of tested

Table 1. The forwards and the reverse primers used.

Primer	Sequence (5'-3')	Nucleotide	Reference
	Azotobacter vinelandii		
*nif*H-F-	(taccgatacgcagttacgcggt)	22	Setubal et al. (2009)
*nif*H-R	(tcagacttcttcggcggttttg)	22	
*nif*D-F-	(taccgatacgcagttacgcggt)	22	Setubal et al. (2009)
*nif*D-R	(tcagacttcttcggcggttttg)	22	
*nif*K-F-	(tactcggtcgttcagctattttag)	24	Setubal et al. (2009)
*nif*K-R-	(ttagcgtaccaggtcgtggttgta)	24	
*nif*M-F-	(tcctattttgcctgtttgggac)	23	Setubal et al. (2009)
*nif*M-R-	(teagaggtcggccgacagcgcgg)	23	
V-F-	(tacagtagcggaaggttagggt)	22	Setubal et al. (2009)
FV-R-	(tcagccgccgaccttgatgccg)	22	
	Azotobacter chroococcum		
nifH1-F-	(cagacacgaagaagccgggc)	20	Setubal et al. (2009)
nifH1-R-	(gaccagcagcttgttgttga)	20	
nifH2-F-	(cgccggcgcagtgtttgcgg)	20	Setubal et al. (2009)
nifH2-R-	(cactcgttgcagctgtcggc)	20	
nifH3-F-	(cgatgactgaagactgaacgag)	22	Setubal et al. (2009)
nifH3-R-	(aaggtgcggtcaggagagaa)	20	
nifU-F-	(atgtgggattattcggaaaaa)	21	Setubal et al. (2009)
nifU-R-	(tcagcctccatctgccgtggg)	22	
nifV-F-	(gatggctagggtgatcatcgacga)	24	Setubal et al. (2009)
nifV-R-	(gccattcctcctgccgccagttcg)	24	
FV-F-	(tacagtagcggaaggttagggt)	22	Setubal et al. (2009)
FV-R-	(tcagccgccgaccttgatgccg)	22	

Table 2. Cell/g of nitrogen fixing bacteria applied with pesticides during 70 days incubation.

Days from infection	Control	Dimethoate	Bayleton 50	Imazetapir
5	36×10^6	5.6×10^6	0.1×10^6	34×10^6
15	23.5×10^6	2.4×10^6	0.06×10^5	21×10^6
30	14×10^6	0.05×10^6	0.04×10^3	12.5×10^6
70	4.2×10^6	0.001×10^6	0.2×10	3.1×10^6

pesticides on *A. chroococcum* and *A. vinelandii* isolated from soil cultivated with wheat plants and applied with pesticides. The number of bands present or absent is used to estimate the influence of pesticides on the bases of the number of shared amplification products. Figure 1, lane 5 shows the amplified nif genes in *A. chroococcum*, lanes 1, 2 and 3 are pesticide treatment, lane 1 applied with Dimethoate, lane 2 treated with Bayleton 50 and lane 3 treated with Imazetapir are as shown in Figure 1. Both insecticide and fungicide treated pots affected

Table 3. Total nitrogen of Jensen's broth cultures inoculated with *Azotobacter* and treated with pesticide after 15 days incubation.

Bacterial inoculum	Total nitrogen (mg/ml)			
	Control	Dimethoate	Bayleton 50	Lmazetapir
A. chroococcum	1.80	0.09	0.009	1.09
A. venilandii	1.19	0.008	0.005	0.82

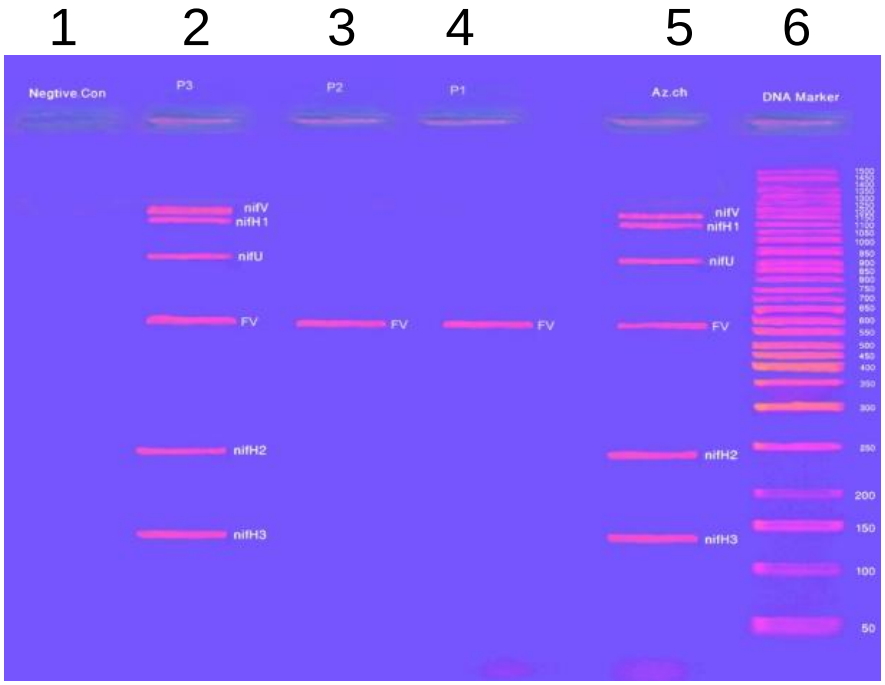

Figure 1. The PCR product of *Azotobacter chroococcum* isolated from the soil treated with pesticide as follows: Lane1, Negative control; Lane 2, Soil treated with lmazetapir; Lane 3, Soil treated with Bayleton 50; Lane 4, Soil treated with Dimethoate; Lane 5, Untreated *A. Chroococcum*; Lane 6, DNA marker.

negatively the nif genes, and *nif*H1, *nif*H2, *nif*H3, *nif*U and *nif*V were lost from the gel, while herbicide used in lane 3 did not have affected on the nif genes, and the bands appeared as normal sample of *A. chroococcum* in lane 4. Effect of tested pesticides on *nif* genes in *A. vinelandii* (Figure 2) was the same as *A. chroococcum* except *nif*M was not affected when Dimethoate was applied. FV gene was not affected when tested with pesticide applied in both isolated *Azotobacter* species.

DISCUSSION

This study has shown that the herbicide (lmazetapir) had no negative effect on the growth of nitrogen fixing bacterial population in the soil, that is, the percentage of survived bacteria after 70 days of incubation was 9.12%, and 11.66% for control. Fungicide and insecticide however did have a negative effect on bacterial population (Table 2); the percentage of the remaining survived nitrogen fixing bacteria was 0.012 and 0.0004% for Dimethoate and Bayleton 50, respectively. The number of nitrogen fixing bacteria reduced from 5.6×10^6 and 0.1×10^6 cell/g to 1×10^3 cell/ml when Dimethoate and Bayleton 50 were applied to the soil, respectively. When lmazetapir was applied, the number decreased from 34×10^6 to 3.1×10^6 cell/ml, while for control the population decreased from 36×10^6 to 4.2×10^6 cell/g. The results of this study are in agreement with previous studies, that is, the analysed strains of nitrogen fixing bacteria is resistant to herbicides and show no inhibition of growth observed when compared with the result of fungicide and insecticide application. Sudbakar et al. (2000) found that there were variable effects of pesticides on the growth of nitrogen fixing bacteria. *In vivo* study showed that amongst fungicides, Carbendazim reduced the bacterial

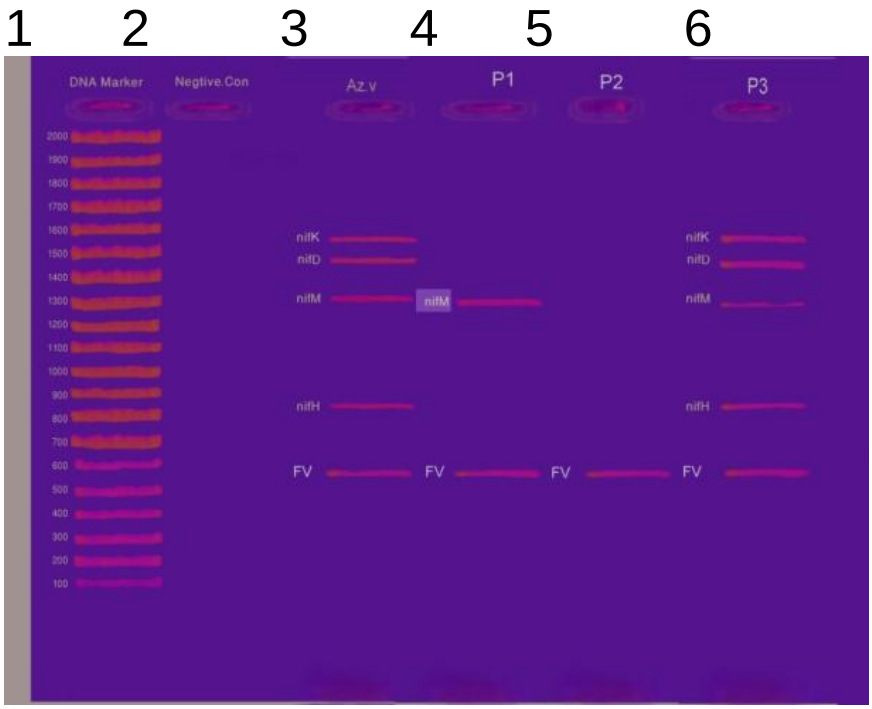

Figure 2. The PCR product of *Azotobacter vinelandii* isolated from the soil treated with pesticide as follows: Lane1, DNA marker; Lane 2, Negative control; Lane 3, Untreated *A. Vinelandii*; Lane 4, Treated with Dimethoate; Lane 5, Treated with Bayleton 50; Lane 6, Treated with Imazetapir.

population at all concentrations, but Dimethoate and wettable sulphur stimulated it and reduced it at higher concentration, and these results are supported by Gallori et al. (1991), Revellin et al. (1993), Taiwo and Oso (1997) and Dunfield et al. (2000) who reported that bacterial growth inhibition due to agrochemical, and they contain similar active ingredient that reduced the number of nitrogen fixing bacteria. Similar trend was reported by Martensson (1992) who observed that the fungicide treatment decreased the number of viable N2-fixing bacteria. Dimethoate decreased the growth of Rhizobium population (Castro et al., 1997).

The fixation of nitrogen was parallel to the population of both nitrogen fixing bacteria in soil treated with pesticide, Martinez et al. (1992) and Pozo et al. (1995) found that organo phosphorous insecticides profenofos and chloropyrifos reduced the number of aerobic nitrogen fixers and significantly decreased nitrogen fixation. Mubeen (2004) found negative effects of fungicides on nitrogen fixation.

Primer pair *nif*H1, *nif*H2, *nif*H3, *nif*V, *nif*U, FV genes of *A. chroococcum*, and *nif*H, *nif*K, *nif*D, *nif*M, and FV genes in *A. vinelandii* (Table 1) were used to amplify nif genes of *A. chroococcum* and *A. vinelandii* from three pots soil samples (Figures 1 and 2). The results show discrete bands of predicated size of approximately (140 to 1200 and 550 to 1550 bp) after being analyzed on conventional agaros gels. Lanes 3 and 4 are from Dimethoate and Bayleton 50 treatment which reduced n*if*H, *nif*K, *nif*D, *nif*M gene in *A. vinelandii* (Figure 1). Also, lanes 1 and 2 are from Dimethoate and Bayleton 50 (Figure 2) when applied on the soil lost *nif*H1, *nif*H2, *nif*H3, *nif*V, *nif*U genes from *A. chroococcum*, while soil without pesticide lanes 5 and 3 are from *A. chroococcum* and *A. vinelandii* (Figures 1 and 2), respectively. For negative control, no bands were observed in lanes 1 and 2 as shown in Figures 1 and 2. These results are supported by other researchers (Cernakova, 1993; Mubeen, 2004).

It is concluded from this study that the pesticides have differential effect on the growth of nitrogen fixing bacteria, and their action vary at different sites. Indication has been observed that the pesticides (Dimethoate and Bayleton 50) which are under field condition possibly due to its high toxic nature reduced the population of these bacteria under field condition.

Their direct effect on nitrogen fixing bacteria was a decrease in the number of viable bacterial population and a high indirect effect was a reduction of nitrogen fixing genes, that is, *nif*H, *nif*K, *nif*U, *nif*M, *nif*H1, *nif*H2, *nif*H3, *nif*V genes of studied *Azotobacter* species and finally on amount of fixed nitrogen. While the other pesticide (Imazetapir) did not show any effects (Figures 1 and 2).

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