Short Communication

STUDIES ON THE EFFECTS OF THE HERBICIDE SIMAZINE ON MICROFLORA OF FOUR AGRICULTURAL SOILS

MARIA VICTORIA MARTINEZ-TOLEDO, VICTORIANO SALMERON, BELEN RODELAS,
CLEMENTINA POZO and JESUS GONZALEZ-LOPEZ*

Group of Environmental Microbiology, Department of Microbiology, Faculty of Pharmacy and Institute of Water Research, University of Granada, E-18071 Granada, Spain

(Received 4 August 1995; Accepted 30 November 1995)

Abstract—The effects of 10, 50, 100, 200, and 300 µg simazine per gram of soil was studied under aerobic conditions. Compared with control soil, the herbicide simazine did not affect bacterial populations, fungi, aerobic dinitrogen-fixing bacteria, denitrifying bacteria, and nitrogenase activity. However, nitrifying bacteria were decreased at concentrations of 50 to 300 µg/g. The negative effects observed on nitrifying bacteria in soil amended with simazine were particularly evident after a second application of herbicide, showing that these microorganisms cannot tolerate certain concentrations of simazine.

Keywords—Herbicides  Simazine  Soil microflora  s-Triazines

INTRODUCTION

Many reports on the relation between pesticides and soil microorganisms are focused on the degradation of pesticides in unsterilized soils [1] or by microorganisms isolated from soils [2]. In many cases, studies of pesticides on soil microflora have been performed by measuring microbial activities such as CO2 evolution, ammonification, or denitrification [3]. Studies on the effects of pesticides on quantitative changes in microbial composition are limited [4,5]; studies on qualitative changes in broad microbial groups (bacteria, fungi, etc.) in response to applications of pesticides are even more limited [6].

The herbicidal properties of the s-triazines were discovered in the 1950s by J.R. GEIGY Ltd., Basel, Switzerland. Since then, many such compounds have been synthesized and evaluated for their herbicidal activity, and several major products have been established for agricultural usage (e.g., atrazine, simazine, cyanozine, ametryn, and prometryn). One such compound, simazine, is widely used in agriculture to control broad-leaved annual and perennial weeds and is applied at concentrations of 1.0 to 4.0 kg/ha. Duah-Yentumi and Johnson [7] reported that simazine caused no detectable effects on soil microflora, while De Felipe et al. [8] observed that the herbicide decreased the nitrogenase activity in nodulated roots of Lupinus albus L. In this context, the possibility that this herbicide may have an adverse effect on soil microbial activities could be of considerable importance.

We report the effects of simazine on fungal populations, total number of bacteria, aerobic N2-fixing bacteria, denitrifying bacteria, nitrifying bacteria, and nitrogenase activity in four agricultural soils under aerobic conditions in order to establish the interactions between the herbicide simazine and soil microflora.

MATERIALS AND METHODS

Soil samples were collected from the top 10 cm of four agricultural soils near Granada, Spain, that had never been treated with simazine. Soil samples were sieved through a 2-mm mesh screen and stored at 4°C before use. Various soil characteristics were measured by techniques described previously [9,10] and are presented in Table 1.

In the first experiment, each 50-g sample (five replicate 50-g samples were included in these experiments) was placed in a Petri dish, and 5 ml of distilled water was added (50 to 60% of field capacity). Each Petri dish was topped up with 5 ml of simazine (2-cloro-4,6-bis-ethylamino-1,3-triazine) at a concentration of 10, 50, 100, 200, and 300 µg/g of soil and then incubated at 20°C for 1, 2, and 4 weeks. To aid solubility and dispersion, 0.3% Tween 80 was incorporated into the solution of simazine. Control samples received equal amounts of Tween 80 for comparison. In the second experiment, 50-g samples of agricultural soils which 4 weeks earlier had received treatment with simazine at concentrations of 10 to 300 µg/g as previously described received a second herbicide application with a concentrated solution of simazine to a concentration of 10, 50, 100, 200, and 300 µg/g. The samples were then incubated at 20°C for 1, 2, and 4 weeks. Control samples without herbicide received equal amounts of Tween 80 as described above.

Total platable microorganisms were counted by a soil dilution spread-plating method. Total soil bacteria and fungi were counted using tryptic soy agar (TSA; Difco, Detroit, MI, USA) and Czapek-Dox® agar (Adsa-Micro, Barcelona, Spain) at pH 6.0. The inoculated agar plates (three replicates) were incubated at 28°C for 3 d for bacteria and 5 d for fungi before the colonies were counted. The populations of aerobic diazotrophs were estimated using a standard dilution series and plating on Burk’s N-free medium solidified with 1.5% agar as described by Martinez-Toledo et al. [11]. The inoculated agar plates were incubated at 28°C for 3 d, and the nitrogen-fixing capability of the colonies was measured in liquid shake cultures. Cells in the exponential phase were harvested by centrifugation at 3,000 g and dispersed in Burk’s medium (N-free) containing 0.5% glucose. Five milliliters of the suspensions were placed in 12-ml tubes, and the nitrogenase activity was measured as described below.

Numbers of denitrifying bacteria in soil were determined by

---

* To whom correspondence may be addressed.
### Table 1. Characteristics of agricultural soil samples

<table>
<thead>
<tr>
<th>Soil</th>
<th>Characteristics</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mollic xerovent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mollic xerovent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Typic xerorthent</td>
<td>50</td>
<td>50</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Calcixerolic xerochrept</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Texture</td>
<td>Silty loam</td>
<td>10</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>Clay loam</td>
<td>65</td>
<td>45</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>Loam</td>
<td>20</td>
<td>30</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>Clay loam</td>
<td>0.10</td>
<td>0.13</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.8</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>8.4</td>
<td>8.4</td>
<td>7.1</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means of three separate determinations.

the most probable number (MPN) technique according to Rodina [12]. The liquid enrichment medium contained the following per liter of distilled water: sucrose, 30.0 g; NaNO₃, 2.0 g; K₂HPO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.5 g; FeSO₄·7H₂O, 0.01 g; and pH, 7.0. Inoculated test tubes (three replicates) were incubated at 28°C for 7 d, and gas production in the test tubes served as an indicator of denitrifying activity.

The dilution tube technique (MPN) was employed for enumeration of nitrifying bacteria. An analysis was performed separately for nitrifying bacteria of phase I, which oxidize ammonium to nitrite, and for nitrifying bacteria of phase II, which oxidize nitrites to nitrates. Ammonium sulphate medium for *Nitrosomonas* spp. and *Nitrosolobus* spp. contained the following per liter of distilled water: Na₂HPO₄, 13.5 g; KH₂PO₄, 0.7 g; MgSO₄·7H₂O, 0.1 g; NaHCO₃, 0.5 g; (NH₄)₂SO₄, 2.5 g; FeCl₃·6H₂O, 14.4 mg; CaCl₂·2H₂O, 18.4 mg; and pH 8.0. Sodium nitrite medium for *Nitrobacter* spp. contained the following per liter of distilled water: NaNO₂, 0.3 g; MgSO₄·7H₂O, 0.18 g; CaCl₂·2H₂O, 0.01 g; KH₂PO₄, 0.5 g; K₂HPO₄, 0.5 g; FeSO₄·7H₂O, 0.01 g; NaHCO₃, 1.5 g; NaCl, 0.18 g; and pH 8.0. Inoculated flasks (three replicates) were incubated at 28°C, and the presence of nitrite-N and nitrate-N in these flasks was determined once a week for up to 8 weeks as described by Rodina [12].

The nitrogenase activity in the agricultural soils was detected by the acetylene reduction technique [13]. Two-gram samples of agricultural soil amended or unamended with simazine as described earlier was placed in 30-ml tubes, and 0.2% (w/w) glucose was added. The soil samples were sealed with rubber stoppers. After 10% of the atmosphere had been replaced by acetylene, the tubes were incubated at 28°C, and 0.5-ml gas samples were assayed for ethylene after 24 h by injection into a Perkin-Elmer model 8420 gas chromatograph with a Poropak T column and a hydrogen flame ionization detector. The acetylene was generated immediately before use from calcium carbonate and water. The degree of ethylene contamination of the acetylene was known (about 2 nmol/ml) and accounted for in the final calculations. Ethylene was measured by reading peak areas and comparing them with a standard prepared from a mixture of ethylene and nitrogen.

**Statistical analysis**

All data are means of five replicated samples and three sampling times (1, 2, and 4 weeks). Microbial counts were log-transformed before any analysis. Effects of simazine dose and treatment (first or second), with all interactions, were analyzed by multifactorial analysis of variance using the Statgraphics, version 5.0, statistical package. Least significant differences (LSDs) were calculated using 0.05% as the level of significance.

**RESULTS AND DISCUSSION**

The total platable count of bacteria in agricultural soils with silty loam, clay loam, and loam textures was not affected by the presence of simazine at a concentration of 10 to 300 μg/g (Fig. 1). Also, the plate count on Czapek-Dox agar indicated that fungal microflora was not significantly affected in the pres-
Effects of simazine on soil microflora

Fig. 3. Numbers of aerobic diazotrophs (log CFU·g⁻¹) in four agricultural soils in the presence of simazine. See soil characteristics in Table 1. Bars = least significant difference (p < 0.05). □ = first treatment; △ = second treatment.

Fig. 4. Numbers of denitrifying bacteria (log CFU·g⁻¹) in four agricultural soils in the presence of simazine. See soil characteristics in Table 1. Bars = least significant difference (p < 0.05). □ = first treatment; △ = second treatment.

The plate count data indicated that aerobic diazotrophs in four agricultural soils amended with 10 to 300 μg/g of simazine was not affected during 30 d of incubation at 20°C (Fig. 3). Also, the presence of this herbicide in agricultural soils did not affect the populations of denitrifying bacteria (Fig. 4). Therefore, the presence of 10 to 300 μg/g of simazine did not affect nitrogenase activity under the present experiment conditions (Fig. 5).

During the 30 d of incubation, the number of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in amended agricultural soils with simazine at concentrations of 50 to 300 μg/g was reduced compared to unamended soils (Fig. 6). This negative effect was particularly evident in samples of agricultural soils after a second treatment with the herbicide.
In the absence of clear data on simazine decomposition in agricultural soils, our results suggest that apparently heterotrophic bacteria, fungi, and denitrifying bacteria can tolerate high amounts (300 µg/g, concentrations up to 30 times greater than those normally used in agriculture) of the herbicide simazine without evident modification to their growth. Similar results have been reported by Duah-Yentumi and Johnson [7] in agricultural soil in response to repeated applications of simazine.

Our results show that simazine did not affect growth and dinitrogen fixation of heterotrophic nitrogen fixers. In this context, Martinez-Toledo et al. [14] suggest that frequent applications of this compound to the soil are not detrimental to the soil microflora comprised of species and is a dynamic population able to degrade a wide variety of chemically complex compounds, including herbicides [17]. However, the biodegradation of complex halogenated compounds such as simazine is generally considered to be a cometabolic process, and little information is available concerning the microbial catabolism of these herbicides [18].

In our study simazine considerably decreased the number of nitrifying bacteria, and this negative effect was more evident after a second soil treatment. These results suggest that repeated application of simazine in the range of 1.0 to 4.0 kg/ha (which is normally used in agriculture) may result in accumulation with a negative effect on nitrifying microflora. Similar results for other pesticides were reported by Martinez-Toledo et al. [19].

Among various microbial activities in soil, nitrification contributes significantly to the overall nitrogen economy of soils. Nitrifying bacteria are often used for testing the effects of pesticides on nitrogen cycling because of their sensitivity to environmental toxicants. In this context, the negative effects of simazine on nitrifying bacteria may disturb the nitrogen economy of an agricultural soil and thus the delicate balance established between nitrogen fixation, denitrification, and nitrification. Under that point of view, certain agricultural pesticides are used to inhibit the nitrification process and thus to limit the leaching of nitrate.

Our experimental results do support that the application of simazine to agricultural soils significantly affects the populations of nitrifying bacteria. It may be that numerous applications of this herbicide may result in their accumulation and affect the microbial equilibrium and thus disturb the fertility of the soil. In this context, it is clear that rotational and rational use of herbicides is important in preventing environmental hazard.

REFERENCES