Residues of Triforine and Its Metabolites in Barley Grain and Straw

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The systemic fungicide triforine (N,N'-bis(1-formamido-2,2,2-trichloro-ethyl)-piperazine) is widely used against powdery mildew, rust and other leaf diseases on cereals. The residues of triforine have been analysed in barley grain and straw (SCHICKE et al., 1971; EICHLER, 1972). In barley shoots grown under laboratory conditions and treated with ^{3}H triforine, the following compounds were observed: N-(1-formamido-2,2,2-trichloro-ethyl)-piperazine (TF/2); piperazine; unidentified chloroform soluble and basic metabolites which gave piperazine after acid hydrolysis; unidentified metabolites bound to the solid residue left after the first chloroform extraction of the plant, and which generated piperazine after acid hydrolysis (ROUCHAUD et al., 1976). In the present work, we analysed the residues of triforine and its metabolites in barley (grain and straw) grown in the field and treated with triforine at the rates of the agronomic practice. Combined preparative thin-layer-chromatography (PTLC) and gas liquid chromatography (GLC) were used following a described procedure (ROUCHAUD, 1976).

EXPERIMENTAL

Chemicals. Saprol (commercial formulation which contains about 190 g triforine/liter),triforine, and TF/2 were from Cela Merck (Germany). PTLC were performed with activated plates (Kieselgel 60F 254, 20x20 cm/2 mm, Merck). Porapak P was from Waters Assoc., polyethyleneimine (PEI) from Varian, and Silyl-8 from Pierce Chem. The adjuvant powder was provided by Protex & Co (Deurne, Belgium); 100 g of adjuvant powder contained 6 g of Vanicell E (non-ionic dispersing agent), 6 g of Supragil INB (anionic wettering agent), and 88 g of Argirec B24 (non-ionic diluent). The other chemicals were of analytical grade from Merck.

Barley culture and treatment. Different cultural and treatment experiments were conducted with barley (Hebe), untreated plants (control) being always grown and analysed simultaneously.

Experiment 1. Barley plants were grown in a glasshouse at about 25°C and 10,000 lux during a day length of 16 hr, and 20°C at night, the relative humidity being 50-60%. Plants were sown (17.03.76) and grown in plastic pots (50 plants/pot) of 20 cm diameter, the soil being a mixture of sand from the Rhine and of expanded Perlite (1/1, liter/liter). The soil was dren-

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ched with the nutritive Shive and Robbin' s solution I (0.2 g/ liter of ferric monosodium salt of ethylenediaminetetraacetic acid was used in place of iron sulfate)(JOHNSON et al., 1959). When the plants had 3 leaves (growth stage I or 6) and a height of 35 cm, the first treatment was applied to the plants (26.04. 76). Triforine (15 mg/pot) and an equal weight of adjuvant powder was emulsified in water (100 ml/pot), the emulsion being used for soil drenching. The second treatment, identical with the first, was applied (25.05.76) when the last leaf was developed (M or 10). At harvest (23.06.76), the ears and straw were analysed separately. The ears were underdeveloped, and the kernels could not be separated, so that the ears were analysed as a whole.

Experiment 2. Barley test plots were located in a field at the University of Louvain, Louvain- la- Neuve, Belgium. The plots were treated by spraying first (3.06.76) at stage I (or 6), and second (1.07.76) at stage L (or 9). During each treatment, the rate was 1.25 liter Saprol/ha in 300 liters of water. Barley was harvested on 22.08.76 (stage W or 11.4), and the grains were extracted from the ears. Grains and straw were analysed separately.

Experiment 3. This experiment was performed simultaneously with experiment 2, and under the same conditions, except that the rate for each treatment was 2.50 liters Saprol/ha in 300 liters of water.

Experiment 4. Barley test plots were located in a field near Veurne, Belgium. The plots were treated by spraying first (19.05.76) at stage K (or 8), and second (1.06.76) at stage M (or 10). At each treatment, the rate was 1.25 liter Saprol/ha in 300 liters of water. Barley was harvested (16.07.76), and only the grains were analysed.

Analysis. The previously described analytical procedure has been used (ROUCHAUD, 1976) with the following modifications. For GLC of piperazine (retention time of 1.7 min), the column (1 m, 140°C, 15 g% PEI on Porapak P 80-100 mesh) was not silylated.

For the analysis of grains, these were first milled with external ice cooling (Sorvall omnimixer, 8,000 rpm, 5 min), and then extracted with chloroform as described. This primary chloroform extract was concentrated to 150 ml (rotatory vacuum evaporator, 15°C), and an equal volume of absolute ethanol was added. The mixture was centrifuged (4,500 rpm, 15 min), and the precipitated starch was added to the solid residue left after the primary chloroform extraction. The solution was concentrated to 5 ml (rotatory vacuum evaporator, 15°C), 200 ml chloroform was added, and the mixture was extracted 3 times with aqueous 0.1 N HCl (3x30 ml), giving the secondary chloroform extract and, after combination, the 0.1 N HCl extract. Both these extracts were processed as described (ROUCHAUD, 1976).

For the analysis of piperazine (free in the plant, or generated by the acidic hydrolysis of concentrate B or of the solid residue) the chloroform phase (5 ml) of the final described GLC sample (ROUCHAUD, 1976) was further purified by PTLC (ammonia- ethanol, 4:6; 16 cm development; R_f of piperazine = 0.57). The zone in the region 0.59 and 0.47 (R_f units) was scraped off; the powder was eluted through a column (30 cm, 2.5 cm diam.) with 150 ml of a mixture (chloroform-methanol-ammonia, 6:6:3). The organic solvents were distilled from the eluate (rotatory vacuum evaporator, 40°C). To the aqueous residue (about 25 ml) an equal volume of saturated aqueous NaOH was added. The mixture was extracted 3 times with 3x50 ml chloroform. The combined chloroform phases were made slightly acidic (indicator paper) by addition of a few drops of concentrated aqueous HCl, concentrated to 5 ml in a rotatory vacuum evaporator (40°C) and, in a small flask (15 ml), to dryness under a flow of nitrogen (40°C). 0.5 ml Aqueous 6 N NaOH and 1 ml chloroform were added to the residue. The sample was agitated on a Vortex mixer, and the chloroform phase was analysed for piperazine by GLC. These modifications gave, for the analysed compounds, "recoveries" similar to those obtained previously (ROUCHAUD, 1976); however, as shown by analysis of control samples, they suppressed the interferences which occured sometimes at the GLC, and the performances of the GLC column for piperazine analysis were more durable. The results were the mean values relative to 3 analyses.

RESULTS AND DISCUSSION

In barley grown in the field (experiments 2, 3, and 4;Tables 1+2) and treated by doses of triforine corresponding to those of the agronomic practice, the same metabolites of triforine were observed as in barley grown under laboratory conditions (ROUCHAUD et al., 1976). In the latter case, these metabolites were thus not abnormal, i.e. produced by the overloading of metabolic routes by high rates of triforine.

In the present work, and for barley grown in the field, analysis showed that triforine corresponded to more than 90% of the foreign compounds in the secondary chloroform extract. The whole analytical procedure gave the total amount of triforine, and of its identified and unidentified metabolites containing the piperazine ring, which were present in barley (ROUCHAUD, 1976).

In barley grown in the field, the percentages (usually more than 75%, in piperazine equivalents, harvest having been made 80 days after the first treatment in experiments 2 and 3, and 58 days in experiment 4) of unidentified metabolites bound to the solid residue were higher, in both grain and straw, than those observed under laboratory conditions (23 and 38% when harvest was made after respectively 15 and 30 days; ROUCHAUD

TABLE 1

Residues of triforine and its metabolites in barley grain and straw (ppm relatively to the fresh weight of tissue).

Experiment/ Analysed part of barley	Free compounds (soluble in chloroform)				Unidentified of the solid residue[b/]
	Triforine	TF/2	Piperazine	Unidentified of the 0.1N HCl extract[a/]	
Experiment 1					
Ear	0.90	0.31	0.11	0.05[c]	1.32[c]
Straw	3.21	0.92	0.42	0.17[c]	5.85[c]
Experiment 2					
Grain	0.04	0.01	0	0[c]	0.06[c]
Straw	0.07	0.03	0	0[c]	0.13[c]
Experiment 3					
Grain	0.06	0.02	0	0[c]	0.06[c]
Straw	0.13	0.04	0	0[c]	0.21[c]
Experiment 4					
Grain	0.05	0.02	0	0[c]	0.08[c]

[a/] Unidentified metabolites soluble in the 0.1N HCl extract obtained by partition of the first chloroform extract with 0.1N HCl.

[b/] Unidentified metabolites bound to the solid residue left after the first chloroform extraction.

[c/] Piperazine equivalents.

TABLE 2

Distribution of triforine and its metabolites in barley grain and straw (% in piperazine equivalents).

Experiment/Analysed part of barley	Free compounds (soluble in chloroform)				Unidentified of the solid residue[b/]
	Triforine	TF/2	Piperazine	Unidentified of the 0.1N HCl extract[a/]	
Experiment 1					
Ear	10	6	6	3	75
Straw	9	4	6	2	79
Experiment 2					
Grain	11	4	0	0	85
Straw	10	6	0	0	84
Experiment 3					
Grain	15	9	0	0	76
Straw	10	5	0	0	85
Experiment 4					
Grain	11	7	0	0	82

[a/] Unidentified metabolites soluble in the 0.1N HCl extract obtained by partition of the first chloroform extract with 0.1N HCl.

[b/] Unidentified metabolites bound to the solid residue left after the first chloroform extraction.

[c/] Piperazine equivalents.

et al., 1976). This was understandable since, in the first case, the time elapsed between harvest and the first treatment was longer. These were conjugated metabolites insoluble in chloroform and containing the piperazine ring, and which afforded piperazine after hot acid hydrolysis.

In barley grown in the field, neither free piperazine nor unidentified metabolites soluble in the 0.1 N HCl extract were observed, on account probably of the analytical limit of sensitivity (0.03 ppm) for piperazine, and of the low percentages of these metabolites in barley straw and grain (ROUCHAUD et al., 1976). The concentrations of triforine and its metabolites were usually more than twice as high in straw than in grain. Treatment with twice the dose usually gave higher residues of metabolites,but not by twice the amount (experiments 2 and 4 compared to experiment 3). In every experiment, two treatments were performed, and at about the same stage of growth. Similar residue values were obtained for both experiments 2 and 4, notwithstanding that they were carried out at different places and dates, which corroborated the significance of the results.

The triforine contents we observed here in grain and straw of barley, grown in the field, were similar to the ones observed occasionally by SCHICKE et al. (1971; one treatment at the rate of 1 liter Saprol/ha, at the growth stage H or 5), and EICHLER (1972; one or two treatments at the rate of 2 liters Saprol/ha/treatment, and harvest after between 67 and 125 days after the last treatment); their analytical procedure would have given approximately the sum of both triforine and TF/2 residues. Our results show that, besides the triforine residue, there were low but significant concentrations of metabolites which corresponded usually to more than 85% (in piperazine equivalents) of the total residue of triforine and its metabolites in both grain and straw of barley.

Experiment 1 was performed in order to perfect the analytical procedures when using "cold" triforine. The distributions of triforine and its metabolites were similar as with experiments (2, 3, and 4) in the field. However, low percentages of free piperazine and of unidentified metabolites soluble in the 0.1 N HCl extract were observed in experiment 1. This also suggested that the apparent absence of piperazine in barley grown in the field was due to the analytical limit of sensitivity for this compound.

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