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Note

Estimation of the systemic fungicide Ridomil by thin-layer chromatography

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Ridomil or Acylon [methyl DL-N-(2,6-dimethylphenyl)-N-methoxyacetyl)-alaninate](Fig. 1) is a systemic fungicide recently developed by Ciba-Geigy. It is probably the first systemic fungicide effective against phycomycetous plant pathogens including *Phytophthora* spp and peronosporales that cause downy mildew diseases of economically important crop plants[1,2]. These pathogens could not be controlled by any of the several systemic fungicides available for plant chemotherapy before the development of Ridomil. As such Ridomil holds great promise in the control of many devastating plant diseases caused by phycomycetous fungi, especially the downy mildews. One of the pertinent questions asked in the use of the systemic fungicides is whether the chemical residues are left in the edible plant parts, especially because of the uptake of these fungicides and their distribution through the plant system. So far, published methods for thin-layer chromatographic (TLC) analysis of Ridomil are, to the best of our knowledge, not available. We report here a simple TLC system and chromogenic reagents capable of detecting very low levels of Ridomil. Simple regression equations for estimating the quantity of Ridomil are also presented. The method has been successfully used to analyse Ridomil from treated maize and pearl millet plants.

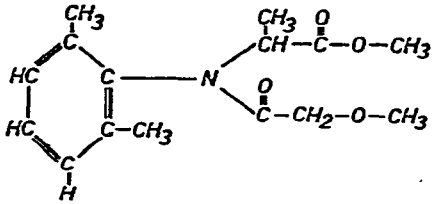

Fig. 1. Molecular structure of Ridomil.

EXPERIMENTAL

Chemicals

Ridomil (Ciba-Geigy CGA 48988), formulated as a wettable powder, containing 25% active ingredient, was used. Solutions of it were prepared in 40% acetone. Most chemicals and solvents were of analytical grade.

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Preparation of thin-layer plates

Thin-layer plates were prepared by coating glass plates (20 × 20 cm) with a 0.25-mm layer of a slurry made by uniformly suspending 30 g silica gel G containing 13% calcium sulphate (BDH, Bombay, India) with 64 ml of glass-distilled water[3,4]. The addition of a few drops of methanol to the slurry greatly aided in the uniform coating of the gel. The coated plates were first dried at room temperature and then activated in an oven at 110°C for 30 min. In some experiments precoated plates (Eastman-Kodak, Rochester, NY, U.S.A.), without fluorescence indicator, were used.

Chromogenic detection reagents

A large number of general chromatographic detection reagents and chemical compounds capable of reacting with Ridomil to produce coloured spots was tested. These included compounds capable of causing substitutions in the aromatic ring of Ridomil or of reacting with the nitrogen atom, the keto group of the methoxyacetyl moiety or the carboxylic group of the esterified alaninate moiety (see Fig. 1). Attempts were also made to hydrolyze the fungicide and cause the product(s) to react with reagents on the TLC plates. Of the reagents tested, the following were satisfactory for the detection of Ridomil:

(a) Iodine–azide [1.27% iodine in 95% ethanol and 3.25% sodium azide in aqueous ethanol (1:3), mixed in equal volumes just before spraying].

(b) Iodine–potassium iodide [5% iodine in 10% potassium iodide made up in 2 *N* acetic acid and distilled water (2:5:3, v/v)]. This reagent can be stored for several weeks but we recommend the use of fresh solutions.

(c) Reagent a followed by b.

Chromatography

Some of the effective solvent systems are listed in Table I.

The plates were spotted with 5 μl of Ridomil solutions of different concentrations or 10–20 μl of the plant extract in as small and uniform spots as possible. After development, the plates were air dried and sprayed with the reagents. The spots were demarcated with a needle and their diameters, as the average of two directions at right angles to each other, and areas (using graph paper) were measured. The correlation and regression were then calculated by taking fungicide quantity or concentration as independent variable and spot area or average diameter as dependent variable.

Treatment of plant material

Although we have separated and analysed Ridomil from both maize and pearl millet plant tissues we give here data only for maize. The treatment was applied to both seeds and foliage. Thirty grams of seeds were soaked for 24 h at room temperature in a suspension of 150 mg commercial (37.5 mg active ingredient) fungicide in 5 ml water. These seeds were germinated for 7 days on water-soaked blotting-paper in glass petri dishes. The fungicide analysis was made on seeds, roots and shoots. For foliage treatment, two month old maize plants grown in a glass-house were sprayed with 500 ppm (active ingredient) of an aqueous suspension of Ridomil. Control plants were sprayed with water.

Extraction of Ridomil from plant tissues

(a) From seedlings. Seven-day old maize seedlings grown from Ridomil-treated seeds were washed with tap-water to remove any surface-contaminating fungicide. Different parts of the seedlings were ground in a mortar and the homogenate was extracted for 2 h with 125 ml of dichloromethane in a Soxhlet apparatus. The extract was reduced in volume to 2 ml at 40°C.

(b) From leaves. Thirty grams of leaves removed 24 h after spraying with fungicide were washed thoroughly to remove Ridomil from the surface. The leaves were then chopped into small pieces, ground and extracted in a Soxhlet apparatus for 3 h in 300 ml of acetone. The extract was reduced to 12 ml by flash evaporation and 18 ml water were added to make the final acetone concentration 40%. After 10 h the extract was filtered through bacteria-proof filter. Following evaporation of acetone, Ridomil was extracted from the remaining aqueous suspension four times each with 25 ml dichloromethane. The extract was concentrated to 2 ml at 40°C. In a separate experiment, 30 g untreated leaves were ground in a mortar along with 10 mg (active ingredient) of Ridomil. Further extractions were done as above.

RESULTS AND DISCUSSION

Iodine–azide (reagent a) produced brown spots on a light yellow background while iodine–potassium iodide (reagent b) produced dark brown spots on a yellowish-brown background. The colour of the spots was, however, not very stable and quickly faded. When reagent a was first sprayed followed by reagent b, more stable spots developed on a light brown background. The detection limit for all reagents was 2.5 μg.

Table I lists the R_F values of Ridomil in different solvent systems. The fungicide did not move in non-polar solvents, *e.g.*, benzene and carbon tetrachloride (dielectric constants 2.284 and 2.238, respectively), but relatively polar solvents such as methanol and ethyl acetate (dielectric constants 33.62 and 6.02) caused good movement of Ridomil with R_F of 0.86 and 0.83, respectively. Mixtures of polar and non-polar solvents in different ratios gave good solvent systems out of which three, benzene–methanol (150:5, v/v), benzene–ethyl acetate (9:8, v/v) and carbon tetrachloride–ethyl acetate (10:9, v/v), have been routinely used in our laboratory with good separation of Ridomil. The criteria for selecting these solvents were circular, concentrated and non-diffuse spots and a high degree of correlation between spot area or average diameter and fungicide concentration.

TABLE I

R_F VALUES OF RIDOMIL IN TLC WITH DIFFERENT SOLVENT SYSTEMS

Solvent system	R_F
Benzene	0.00
Carbon tetrachloride	0.00
Methanol	0.86
Ethyl acetate	0.83
Benzene–methanol (150:5, v/v)	0.44
Carbon tetrachloride–ethyl acetate (10:9, v/v)	0.55
Benzene–ethyl acetate (9:8, v/v)	0.62

The areas and average diameters of the spots increased with increasing fungicide concentration in each of the solvent systems (*e.g.*, Fig. 2 for benzene–methanol) with correlation coefficients as high as 0.99. The regression coefficient was also very highly significant ($P = 0.01$) for all the solvent systems. The high correlation coefficient (0.99) indiates that the variation in spot areas and diameters is due to variations in the concentration of Ridomil. When linear regression curves were drawn between average spot diameters or areas and the amount of fungicide spotted, the observed concentrations of the fungicide were found to lie very close to the expected points, *i.e.*, on the regression line (Fig. 3). In Table II we give regression equations for each of the solvents, from which the Ridomil concentrations can be calculated. These

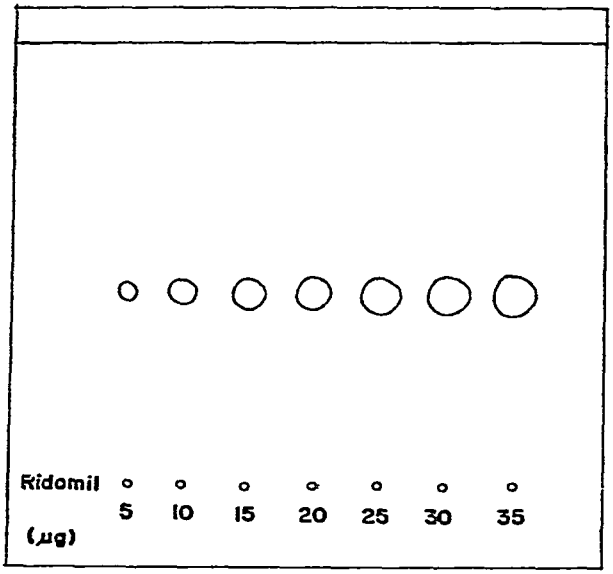

Fig. 2. Fascimile of a TLC plate showing increase in spot area and diameter with increasing amounts of Ridomil in benzene–methanol (150:5, v/v) as the solvent system.

TABLE II

CORRELATION COEFFICIENTS (r), REGRESSION COEFFICIENTS (b) AND COEFFICIENTS OF DETERMINATION (r^2) AND REGRESSION EQUATIONS

Solvent systems: A = benzene–methanol (150:5, v/v); B = carbon tetrachloride–ethyl acetate (10:9, v/v); and C = benzene–ethyl acetate (9:8, v/v). All values of r and b are significant at $P = 0.01$. The independent variable (x) in each case was the fungicide concentration.

Solvent system	*Dependent variable (y)*	r	b	r^2	*Linear regression equation* $y = a + bx$
A	Average spot diameter	0.951	0.229	0.906	$y = 5.571 + 0.229x$
	Spot area	0.993	3.486	0.986	$y = 12.714 + 3.486x$
B	Average spot diameter	0.995	0.252	0.989	$y = 5.107 + 0.252x$
	Spot area	0.998	3.707	0.997	$y = 8.857 + 3.707x$
C	Average spot diameter	0.971	0.191	0.942	$y = 6.679 + 0.191x$
	Spot area	0.992	2.914	0.984	$y = 30.571 + 2.914x$

equations are derived under TLC conditions of a solvent front of 14.5 ± 1.0 cm and chromatography at 22 ± 3°C.

These linear regression relationships are applicable up to 50 μg fungicide per spot. Therefore the spotting solutions should be adjusted so as to give an amount of $\leqslant 50$ μg Ridomil per spot.

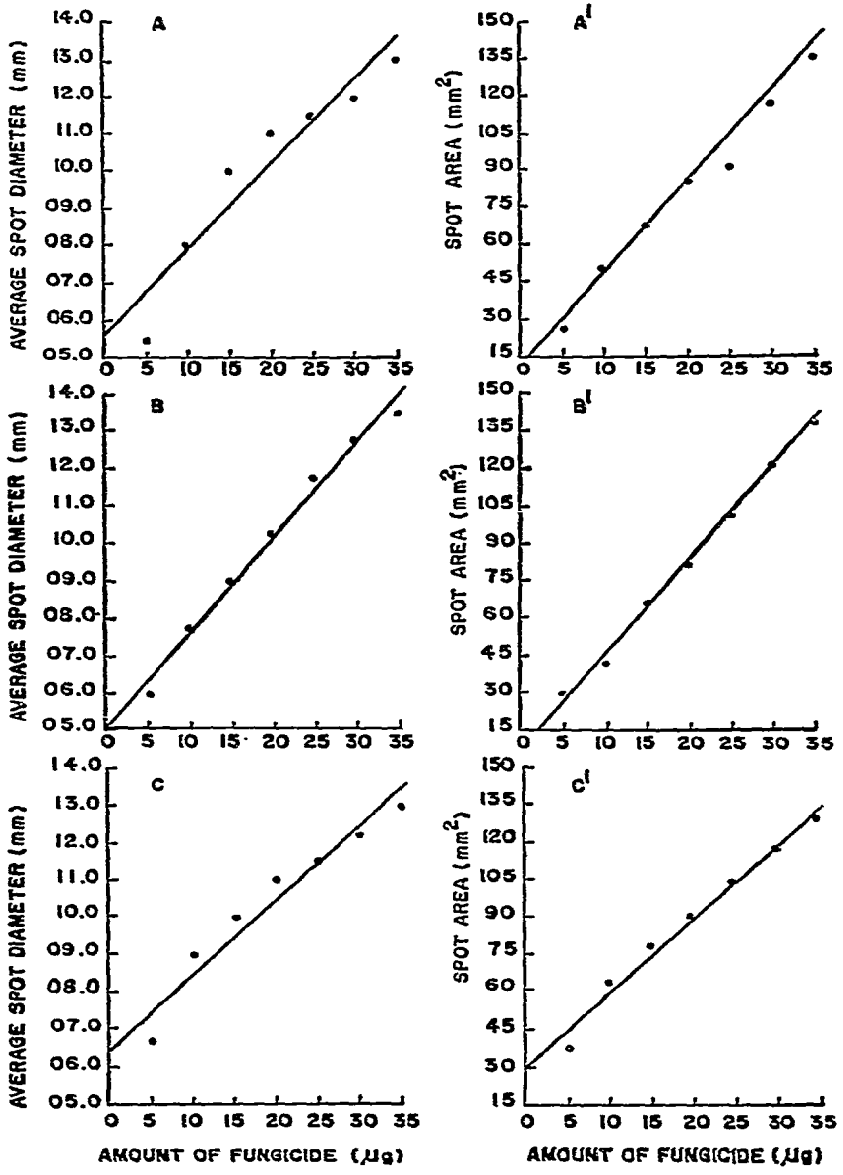

Fig. 3. Regression curves of the quantity of Rodomil against spot area (A′, B′ and C′) and spot diameter (A, B and C). Solvents: A and A′, benzene–methanol; B and B′, carbon tetrachloride–ethyl acetate; and C and C′, benzene–ethyl acetate.

In analysis of Ridomil from plants a fairly good separation of the fungicide was obtained from different parts of the seedlings and sprayed foliage in each of the three solvent systems. The plant constituents did not interfere with the fungicide separation (Fig. 4). The extracts from roots, shoots and seeds produced nearly circular spots in

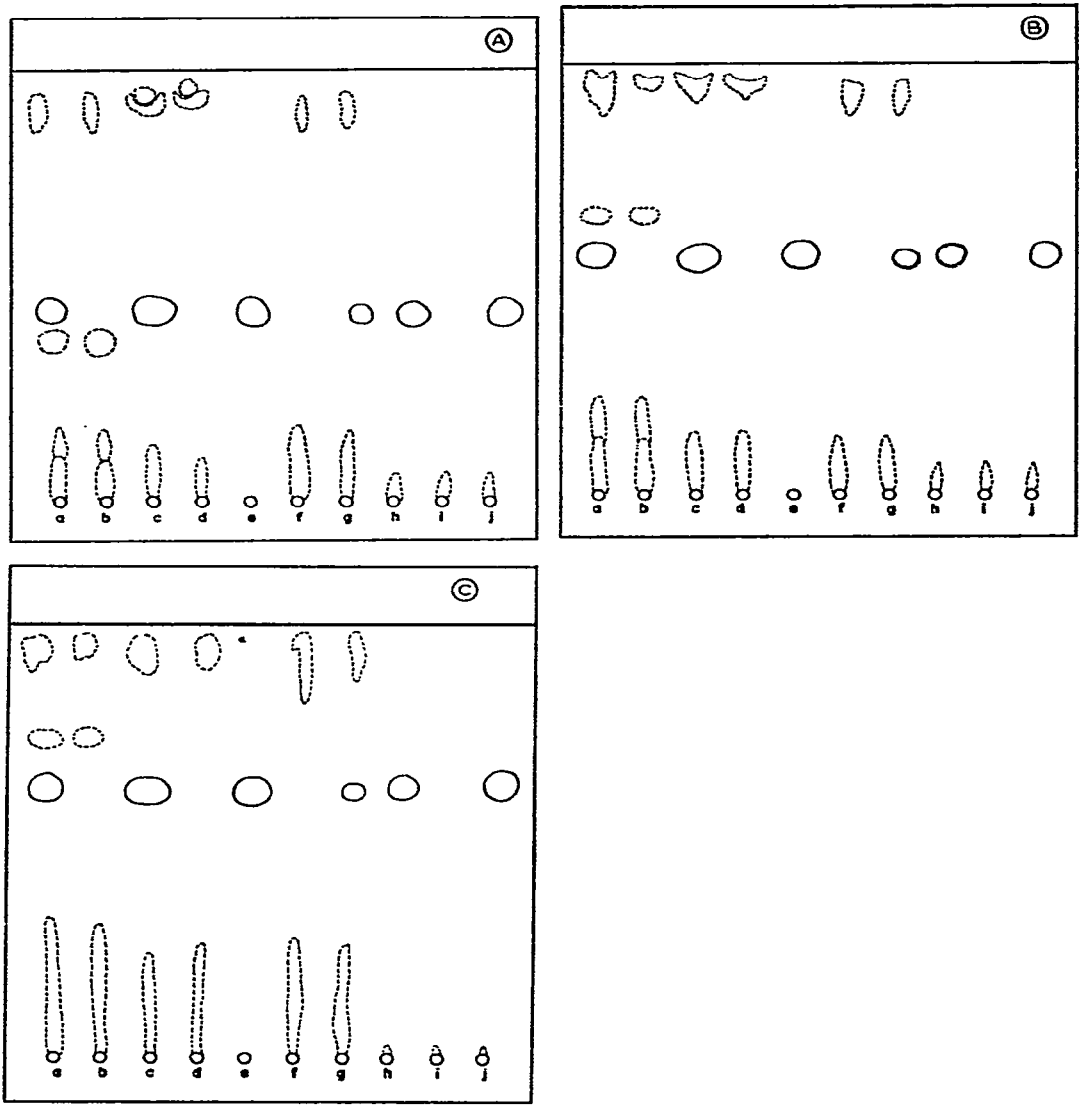

Fig. 4. Fascimiles of TLC plates showing separations of Ridomil from different parts of maize plants: a, c and g, extracts of shoot, seed and root of 7-day old seedlings grown from fungicide-treated seeds; b, d and f, controls from untreated seedlings; e, h, i and j, 20 μg of standard Ridomil, extract of treated maize foliage, extract of untreated leaves and extract of leaves with Ridomil added only during homogenization, respectively. Twenty microlitres of each extract, except j (10 μl), were spotted. Dotted spots represent plant components while solid, circular spots are those of Ridomil. Solvent systems: benzene–methanol (150:5) (A); carbon tetrachloride–ethyl acetate (10:9) (B); and benzene–ethyl acetate (98:8) (C). Detection reagent: iodine–azide followed by iodine–potassium iodide.

all solvents. Ridomil can, therefore, be estimated either from average spot diameter or area by using the regression equations of Table II.

One of the main problems in the separation of any chemical compound from plants, especially foliage, is interference from the plant pigments, chlorophyll, xanthophyll, etc. These pigments must be completely separated from the fungicide spots in order to use the chromogenic detection method, and, if possible, should be removed from the extract applied to the plates. In our extraction method Ridomil was fairly soluble in 40% acetone in which most of the plant pigments and some lipids remained insoluble, thus giving an almost colourless extract. When such an extract was applied to the TLC system the spot area or average diameter increased with increasing volumes of the extract (Fig. 4).

To find the percentage recovery of Ridomil from leaf homogenates, untreated leaves were ground with Ridomil added during homogenization. Based on the calculation of the fungicide concentration from spot j in Fig. 4, a recovery of about 95% was obtained. However, it is not certain from this experiment whether some fungicide is bound in a non-extractable form in plants exposed to the fungicide for a longer period. Some preliminary data indeed show that such binding comprises about 15% of the total Ridomil applied.

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