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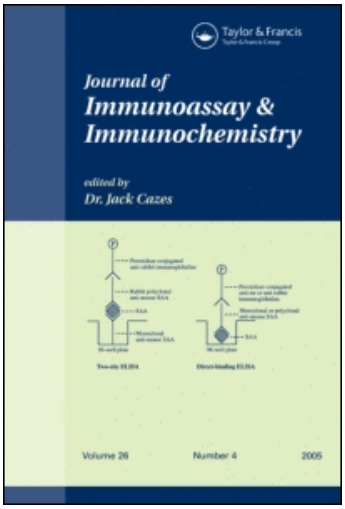

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J. L. Montillet[a]; M. Rossignol[a]; P. Auriol[a]
[a] Centre de Physiologie Végétale de l'Université Paul Sabatier Unité Associée au C.N.R.S., Toulouse cédex, France

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RADIOIMMUNOASSAY OF THE PHYTOTOXIC COMPOUND ZINNIOL

J.L. Montillet, M. Rossignol and P. Auriol
Centre de Physiologie Végétale de l'Université Paul Sabatier
Unité Associée au C.N.R.S. n° 241, 118, route de Narbonne
31062 Toulouse cédex, France

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ABSTRACT

The phytotoxic compound zinniol, produced by phytopathogenic fungi such as Alternaria spp. and Phoma macdonaldii was conjugated to bovine serum albumine by the mixed anhydride method. Antiserum against zinniol was obtained by injection at multiple intradermal sites of rabbits. Sensitivity of the R.I.A. was 0.14 ng/tube, the within and between-assay coefficient of variation were less than 10 and 14 % respectively. Negligible binding occurred when analogs of zinniol were tested for cross reactivity. The excellent accuracy of this R.I.A., applied to ethanolic extracts of plants, might allow to determine the production of toxin by the parasite during the infection process.

INTRODUCTION

Zinniol, (3-methoxy-4-methyl-5 (3-methyl-2-butenyloxy) 1,2 benzenedimethanol (fig. 5a), is a phytotoxin isolated from in vitro culture of phytopathogenic fungi such as Alternaria spp. (1-7) and Phoma macdonaldii (8) ; it induces necrosis in all dicotyledonous plants tested (1-8) and chlorophyll retention in three cereal species (9).

Zinniol has been suggested to be involved in the pathogenesis process of Alternaria species. Thus, symptoms caused either by zinniol, or the fungus were identical (3). In addition a non pathogenic isolate

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of Alternaria zinniae did not produce detectable amounts of zinniol (6). Barash et al. have pointed out that, infecting forms (conidia) of Alternaria dauci during germination phase, rapidly secrete the toxin (3).

However, the intervention of zinniol in infections by Alternaria spp. is discussed yet. Thus, its toxicity on plants is relatively low (3) and while, recently found in excised hypocotyls of sunflower artificially infected by Phoma macdonaldii (8), it has never been characterized from naturally infected plants (1-7) . The failure to detect zinniol in infected tissues could be explained in two ways : zinniol is rapidly metabolized by plant (3), or it is present in minute amounts in some localized sites where it could, by some manners be responsible for dramatic tissues destruction (8). The previously used techniques (TLC ; GLC ; HPLC) did not allow to give clear-cut answer to this problem (1-7). In these conditions, the only way likely to go through was to set up a far more sensitive assay. Thus, we have developed a radioimmunoassay for zinniol in order to estimate its production by the parasite during the development of the disease. Such a technique is a new tool in the field of phytopathology, since up to date there are only a few examples of radioimmunoassays of phytotoxins (10, 11, 12).

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MATERIALS AND METHODS

All chemicals and solvents used, were of analytical grade. Zinniol synthesized by martin and Vogel (13) was purchased from Roche products Ltd.

Preparation of immunogen monosuccinyl-zinniol-BSA conjugate

Zinniol (10 mg) and succinic anhydride (30.7 mg) were dissolved in 2 ml dry pyridine. The reaction was carried out at 28.5° C in a capped vial during 18 hours, under nitrogen. Then, 2 ml distilled water were added to hydrolyse the excess of succinic anhydride and the reaction mixture was taken up in 1 ml methanol and chromatographied in preparative T.L.C. using chloroforme : methanol (50:50) as solvent. Three short wavelenght UV-quenching bands could be detected at Rf 0.73, 0.9 and 1. Two of them, Rf 0.73 and 0.9, were positive to 0.1 % bromophenol blue in ethanol spray, suggesting the presence of a carboxylic acid group. The last one Rf 1 corresponding to zinniol does not react. Acid compounds were eluted with methanol, taken to dryness under reduced pressure and the residue was taken up in 2 ml of a solution of 10 % ethanol in water. This solution was loaded onto a 27 x 1 cm column of Q.A.E. Sephadex (Pharmacia) equilibrated in water and eluted with a 0.02-0.4 M NaCl gradient. The elution was monitored at 272 nm and two absorbing fractions were detected. The fraction corresponding to monosuccinylzinniol (MSZ) eluted at the lowest ionic strength (0.075 M) was desalted by another Q.A.E. Sephadex chromatography in distilled water.

Fractions, eluted with 0.02 M HCl were pooled and lyophilized to yield 4 mg MSZ.

MSZ was conjugated to bovine serum albumine (BSA, Nutritional Biochemical Corporation) by the mixed anhydride method. MSZ (3.73 mg) dissolved in 300 µl of dimethylformamide (DMF) was reacted with 20 µl triethylamine (TEA) mixture (1/11 in DMF)

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and 20 µl ethylchloroformiate (ECF) mixture (1/16 in DMF). All reagents were freshly redistilled ; TEA and ECF were equilibrated at 0°C before use. The reaction leading to mixed anhydride was allowed to proceed for 12 min at 0°C. Then the MSZ activation mixture (340 µl) was added to a solution of BSA (6 mg of BSA in 2 ml of borate buffer 5 mM, pH 9) and allowed to stand at room temperature for 30 min. The conjugate was purified on a Sephadex G 25 medium column equilibrated in 0.01 M phosphate buffer, pH 6.7 containing 0.015 M NaCl. The column was eluted by the same buffer and elution was followed at 280 nm. Fractions corresponding to the void volume were pooled and dialysed during 72 hours against distilled water. When [1,4-^{14}C] succinic anhydride (4.29 GBq/mMole, Amersham) was used as a marker in the immunogen preparation, it was estimated that 14 molecules of monosuccinylzinniol were conjugated to each BSA molecule.

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Immunization

Monosuccinyl-zinniol-BSA conjugate, suspended in a 0.01 M phosphate buffer containing 0.8 % NaCl, was emulsified with an equal volume of Freund's complete adjuvant to give a final concentration of 250 µg/ml. Rabbit antisera were obtained by injection at multiple intradermal sites with 1 ml of the immunogen emulsion. Booster injections were given at 3 weeks intervals using 1 ml of emulsion in Freund's incomplete adjuvant containing immunogen at 200 µg/ml.

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Preparation of (^{3}H) monoacetyl-zinniol

Zinniol (0.33 mg) in 1 ml dry pyridine was added to (^{3}H) acetic anhydride (1.275 mg ; 296 GBq/mMole, Amersham) under nitrogen and the resulting solution protected from moisture, was incubated at 32.5°C for 4 h. The reaction was stopped by 1 ml distilled water and the solution evaporated to dryness under nitrogen. The residue was taken up in 1 ml methanol and subjected to HPLC on a semi-preparative Lichrosorb RP 18 (5 µ) column (150 x 7 mm), using methanol : water (65/35) at a flow rate of 3 ml.min^{-1}. The elution was monitored at 233 nm and the radioactivity of each fraction measured by liquid counting scintillation in ready solv-MP (Beckman) using a Packard 460 C counter. The (^{3}H) monoacetyl-zinniol was collected and stored at -20°C in the chromatography solvent.

Radioimmunoassay

Incubation was carried out in a final volume of 240 µl of a medium containing : 100 µl 0.04 M phosphate buffer, pH 7.4 (PBS) with 0.2 % BSA, 2 % triton X 100 and 0.02 % NaN_3 ; 40 µl of a dilution tracer (10,000 dpm) in (PBS) with 10 % ethanol ; 50 µl of 1/36 dilution in (PBS) 0.2 % BSA of the antiserum or of a non-immune rabbit serum (control) and 50 µl of appropriate amounts (0-50 ng) of zinniol standards in (PBS) 10 % ethanol. The assays were performed in 4 ml polyethylene tubes. The binding reaction was initiated by adding antiserum and the mixture was incubated for 1 hour at room temperature. Then the tubes were placed

on ice and supplemented with 0.5 ml of a chilled solution of 25 % PEG in (PBS). The reaction mixture was left on ice for 1 hour, afterwards, the tubes were centrifuged at 3500 g for 1 hour at 4°C. Aliquots (0.5 ml) of the supernatant were counted for radioactivity. Bound radioactivity was calculated after substracting the background count obtained with control serum. The standard curve was obtained by plotting concentration versus percentage binding on log-logit paper.

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RESULTS

Antibody production

Two rabbits immunized with MSZ-BSA for 4.5 months produced antibodies against zinniol. The titer of the sera after the fourth boosting immunization was followed as a function of time (Fig. 1). It could be noticed that both rabbits produced very similar antibody responses. Another run of immunization failed to substantially increase the level of anti-zinniol activity. The sera of both rabbits were collected 13 days after the fourth boosting immunization and serum of rabbit number 1 was used for all further studies reported here.

Purification of the tracer

Zinniol (RT = 7 min), monoacetyl-zinniol (MAZ, RT = 13 min) and diacetyl-zinniol (RT = 26 min) were separated by HPLC (fig. 2). It is noteworthy that the esterification of hydroxy group by

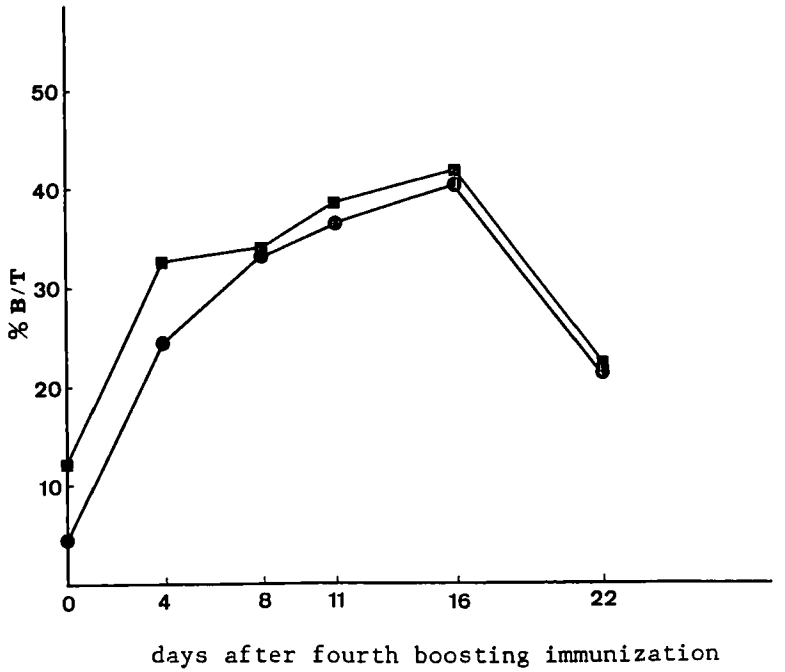

FIGURE 1 : Binding of (^{3}H) monoacetylzinniol by two rabbit antisera at a 1/36 dilution as a function of time after the fourth boosting immunization with monosuccinyl-zinniol-BSA . ■ Rabbit 1 , ● Rabbit 2 .

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acetic anhydride greatly decrease the polarity of the molecule and allows a good separation of compounds on a reverse phase C_{18} column. The yield of MAZ, used as tracer, was 0.17 mCi with a specific radioactivity of 4 Ci/mMole. Nervertheless in our conditions of chromatography, a (^{3}H) acetic acid contamination estimated to 25 % by radioactive profile (fig. 2) led to a maximum B/T ratio of 75 %. Thus a 1/36 antiserum dilution (B/T = 37 %) was used for the standard curve.

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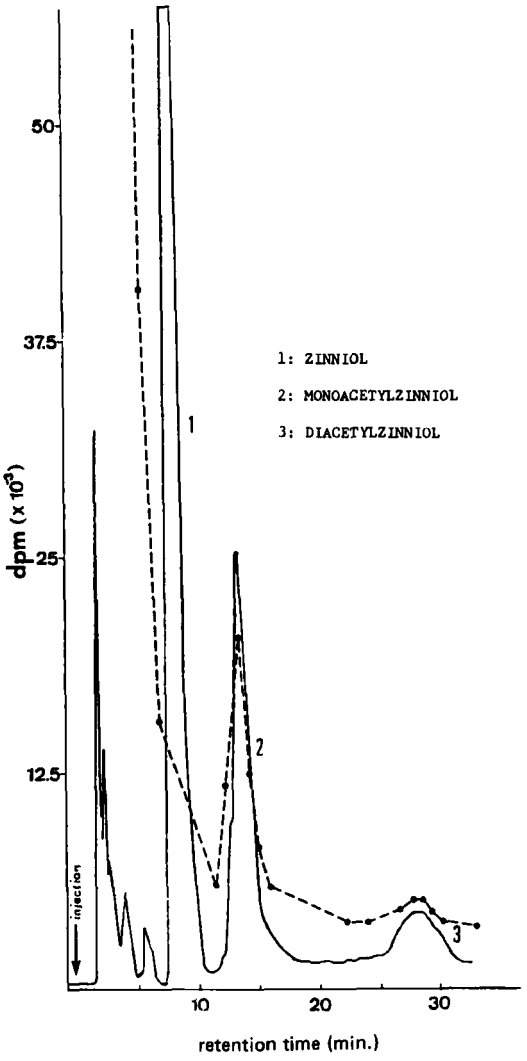

FIGURE 2 : Chromatogram of a 100 µl tritiation mixture
column : Lichrosorb RP 18 (5 µ),(7 x 150 mm); solvent: methanol/water (65 : 35); flow rate: 3 ml/min; room temperature.
•---• radioactivity of 5µl of each fraction; —— UV profile at 233 nm .

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Radioimmunoassay

An erratic non-specific binding of the tracer often reached 30 % of the total count but was dramatically reduced to 0-2 % by adding triton X 100 at a final concentration of 0.83 % in the incubation mixture. In these conditions the non ionic detergent had no effect on the specific binding of the tracer to antibodies.

The time dependency of the binding reaction (fig. 3) clearly indicates that an equilibrium is reached within 30 min and is maintained over a period of 24 hours.

Moreover the binding of (^{3}H) MAZ to antibody was not significantly changed by pH values ranging from 6.4 to 8.4.

Standard curve

The ability of different amounts of zinniol to compete for binding of (^{3}H) MAZ to the antiserum is shown in figure 4. A linear curve was obtained by logit/log plot for concentrations of zinniol ranging from 0.19 to 25 ng/assay tube (i.e. 0.71-94 pmoles/tubes).

Limit of detection

The minimum amount of zinniol that significantly displaces (^{3}H) MAZ was 0.14 ng/tube (i.e. 0.52 pmole/tube), (n = 10).

Precision

All samples have been performed in duplicates and the whitin-assay variation has been estimated by means of the coefficient of variation for the central part of the curve (0.195-6.25 ng/tube).

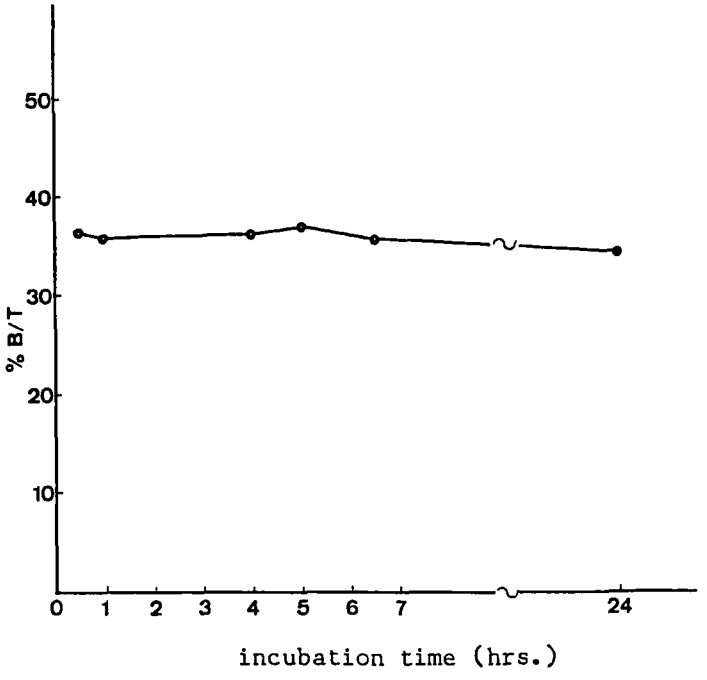

FIGURE 3 : Effect of time on binding of (^{3}H) monoacetylzinniol to antiserum at a 1/36 dilution .

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In our conditions of assay the relative error was less than 10 % ; in another experiment, with a fixed quantity of zinniol (1 ng/tube) the error was to 9 % (n = 10 duplicates).

Reproductibility

The between assay coefficient of variation calculated on the midrange point of the standard curve, gave a value of 14 % for this related amount (i.e. 1.069 $\pm$ 0.149 ng ; n = 8)

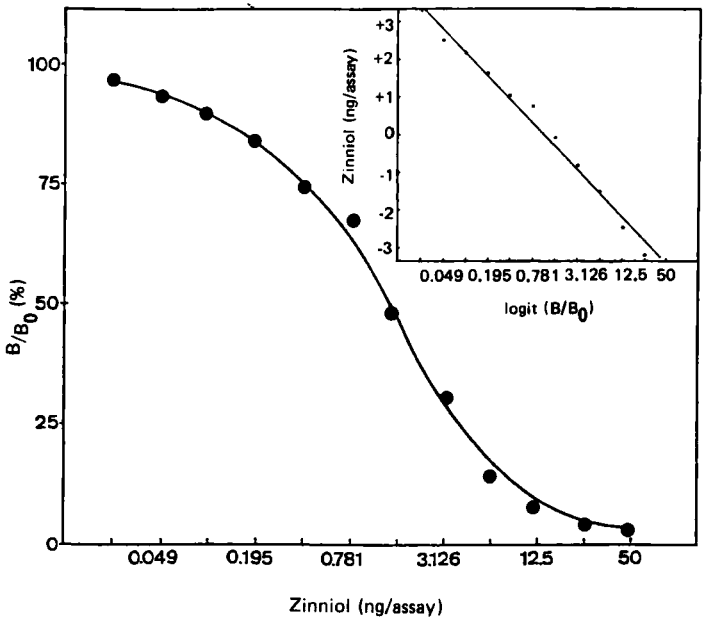

FIGURE 4 : Typical standard curve of zinniol radioimmunoassay shown in two plots;
B = binding of tracer in the presence of zinniol standard;
B_o= binding in the absence of zinniol;
Logit (% B/B_o) = Ln ((% B/B_o) /100 - (% B/B_o));
antiserum dilution factor was 1/36 .

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Specificity

The antibody specificity in the R.I.A. was checked by challenging zinniol with various compounds including phenolics or substitued benzene rings in addition with 3 methyl-2-butene-1-ol (fig. 5). Among the compounds tested the most cross reacting ones were compounds -C- and -B- (fig. 5) with respectively 0.08 % and less than 0.04 % cross reactivity. All other molecules did not react at all at concentrations higher than 1 mM (tab. 1).

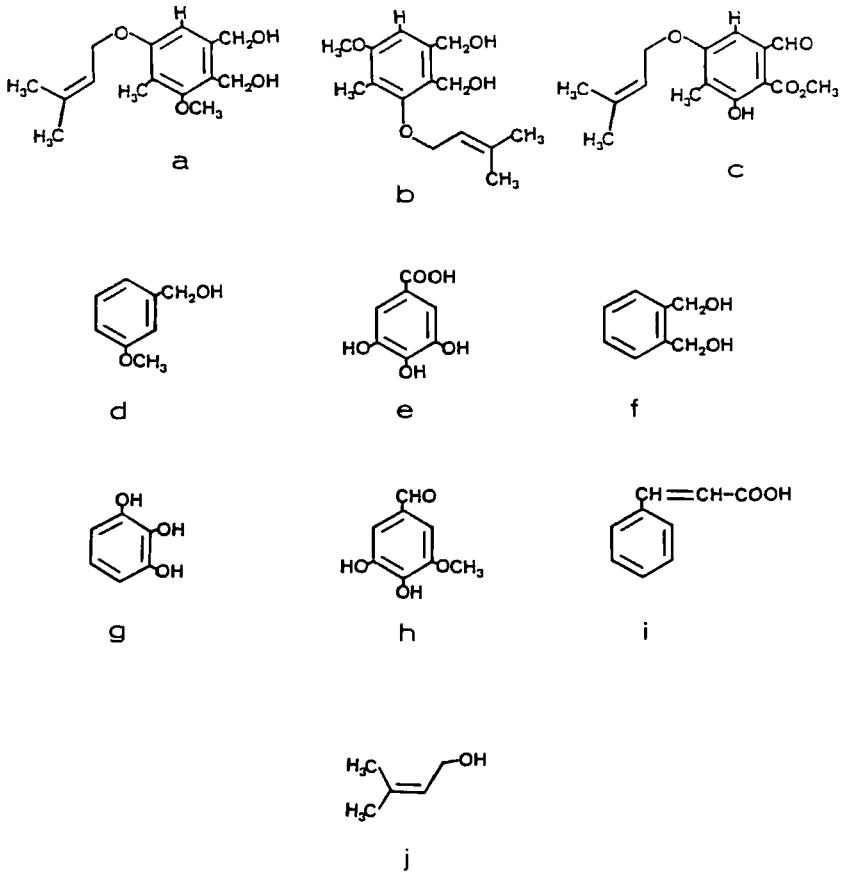

FIGURE 5 : Strutures of compounds assayed for cross-reactivity .

Accuracy

Accuracy was defined as the recovery of known amounts of zinniol (X) added to ethanolic extracts of carrot leaves and the amount of zinniol measured in these media by R.I.A. (Y). As shown in figure 6, there is a good correlation between X and Y. This is indicated by the regression line : $Y = 0.93\ X + 0.665$ ($r = 0.9998$) .

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TABLE 1

Cross-reactivities

Compound	Per cent Cross-reactivity*
a : Zinniol (3-methoxy-4 methyl-5 (3-methyl-2 butenyloxy) 1,2 benzene dimethanol	100
b : Isozinniol (5-methoxy 4 methyl-3 (3-methyl-2 butenyloxy) 1,2 benzene dimethanol	< 0.04
c : Methyl 2-formyl-3-hydroxy-4-methyl-5-(3-methyl-2-butenyloxy) benzoate	0.08
d : 3-methoxybenzylalcool	$\ll 0.01$
e : Gallic acid	$\ll 0.01$
f : 1.2 benzenedimethanol	$\ll 0.01$
g : Pyrogallol	$\ll 0.01$
h : Vanillin	$\ll 0.01$
i : Trans cinnamic acid	$\ll 0.01$
j : 3-methyl-2-buten-1-ol	$\ll 0.006$

* $\%\ CR = \frac{\text{mol of zinniol necessary to displace 50 \% of tracer}}{\text{mol of compound necessary to displace 50 \% of tracer}} \times 100$

DISCUSSION

Up to date there was still no specific, and very sensitive method for determination of the phytotoxic compound zinniol, in several plants of economical importance infected by Alternaria species. Techniques such as TLC, GLC, HPLC, did not allow to

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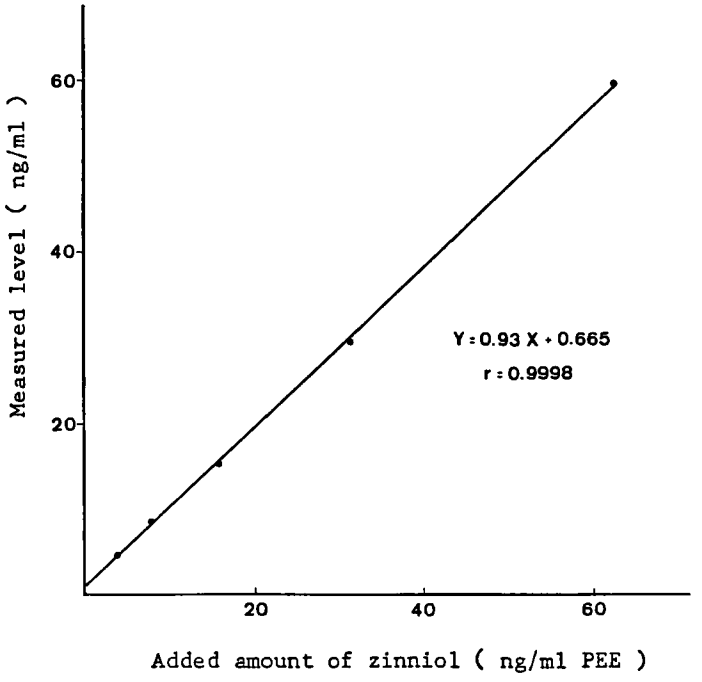

FIGURE 6 : Recovery test in plant ethanolic extract (PEE); to one ml of PEE (ethanol/water, 50:50) increasing amounts of zinniol were added: 3.9; 7.8; 15.6; 31.2; and 62.5 ng ; the zinniol assay was performed with 50 µl of PEE .

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demonstrate the occurence of the toxin in diseased plants. In this paper, a R.I.A. for zinniol is described and has been shown to be sensitive, reproducible, specific and accurate.

In addition another example of the utilization of (^{3}H) acetic anhydride to label haptens is described. As suggested by Fong et al. (14), this method could be of use for molecules where hydroxy or amino groups are available. In this way, the introduction of an acetyl group into bruceantin (14) and zinniol, only slightly

alter the structure of the parent molecule, consequently affinity to the antibody of the resulting tracer remains high enough, while, specific activity of the tracer (4 Ci/mmole) is sufficiently high to be used in a R.I.A. procedure.

We obtained a good sensitivity with 0.14 ng/tube, i.e. 0.52 pmole/tube approximatively. This sensitivity could allow to detect 10 nM toxin that is 2 or 3 orders of magnitude more sensitive than for other methods such as UV measurement or GLC. Precision and reproductibility were satisfactory since the relative error concerning the estimation of zinniol levels was less than 10 % in the range of 0.195-6.25 ng/tube and the between-assay variation coefficient was approximatively 14 % at a concentration of 1 ng/tube. Furthermore, the antiserum showed a narrow specificity since compounds closely related to zinniol showed a slight reactivity ; several phenolics and substitued benzene rings had no interaction with antibodies. This was of most importance because plants generally synthesize aromatic compounds in reaction to infection, leading to localized accumulation of these molecules. Finally, accuracy is excellent since the amounts of zinniol added to biological fluid and measured level by the R.I.A. gave a correlation coefficient equal to 0.9998, the slope of the curve being close to 1.

All these characteristics are consistent with the fact that the radioimmunoassay of zinniol is very sensitive and specific. Such a method opens the way for the study and estimation of zinniol in several diseases where it could be implicated. After fusicoccin (10-11) and bredfeldin A (12), zinniol is the third phytotoxin of low molecular weight that can be determinated by R.I.A. procedure.

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Send reprint request to J.L. Montillet, Centre de Physiologie Végétale de l'Université Paul Sabatier, Unité Associée au C.N.R.S. n° 241, 118, route de Narbonne, 31062 Toulouse cédex, France.

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REFERENCES

1) White, G.A. and Starratt, A.N. The production of a phytotoxic substance by Alternaria zinniae. Can. J. Bot., 1967 ; 45 : 2087-2090.

2) Stoessl, A. and Unwin, C.H. Metabolites of Alternaria solani part 5. Biosynthesis of altersolanol A and incorporation of altersolanol A ^{13}C into altersolanol B and mecrosporin. Tetrahedron Letters, 1979 ; 27 : 2481-2484.

3) Barash, I., Mor, H., Netzer, D. and Kashman, Y. Production of zinniol by Alternaria dauci and its phytotoxic effect on carrot. Physiological Plant Pathology, 1981 ; 19 : 7-16.

4) Tietjen, K.G., Schaller, E. and Matern, U. Phytotoxins from Alternaria carthami (chowdhury) : structural identification and physiological significance. Physiological Plant Pathology, 1983 ; 23 : 387-400;

5) Cotty, P.J., Misaghi, I.J. and Hine, R.B. Production of zinniol by Alternaria tagetica and its phytotoxic effect on tagetes erecta. Phytopathology, 1983 ; 73 : 1326-1328.

6) Cotty, P.J. and Misaghi, I.J. Zinniol production by Alternaria species. Phytopathology, 1984 ; 74 : 785-788.

7) Cotty, P.J. and Misaghi, I.J. Effect of light on the behaviour of Alternaria tagetica in vitro and in vivo. Phytopathology, 1985 ; 75 : 366-370.

8) Sugawara, F. and Strobel, G.A. Zinniol, a phytotoxin, is produced by Phoma macdonaldii. Plant Science, 1986 ; 43 : 19-23.

9) Robeson, D.J. and Strobel, G.A. Zinniol induces chlorophyll retention in barley leaves : the selective action of a non host-specific phytotoxin. Phytochemistry, 1984 ; 23 : 1597-1599.

10) Pini, C. Vicari, G., Ballio, A., Federico, R., Evidente, A. and Randazzo, G. Antibodies specific for fusicoccins. Plant Science Letters, 1979 ; 16 : 343-353.

11) Federico, R., Aducci, P., Marra, M. and Pini, C. A radioimmunoassay for fusicoccin. Zeitschrift Pflanzenphysiologie, 1981 ; 104 : 275-279.

12) Tietjen, K.G., Hammer, D. and Matern, U. Determination of toxin distribution in Alternaria leaf spot diseased tissue by radioimmunoassay. Physiological Plant Pathology, 1985 ; 26 : 241-257.

13) Martin, J.A. and Vogel, E. The synthesis of zinniol. Tetrahedron, 1979 ; 36 : 791-794.

14) Fong, K.L.L., Ho, D.H.W., Carter, C.J.K., Brown, N.S., Benjamin, R.S., Freireich, E.J. and Bodey, G.P. Radioimmunoassay for the detection and quantitation of bruceantin. Analytical Biochemistry, 1980 ; 105 : 281-286.

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