Application of an Automated Quantitative Method to Determine Fungicide Resistance in *Botrytis cinerea*

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ABSTRACT

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An automated quantitative (AQ) assay used for measuring fungal growth with a microplate reader was compared with the linear growth method to determine fungicide resistance in *Botrytis cinerea*. The AQ assay uses absorbance in the range of 0.0–0.6 units as a measure of fungal biomass. This technique was successfully used to establish EC_{50} values (the concentration of fungicide that reduces absorbance by half) to iprodione and a mixture (1:5) of carbendazim and diethofencarb in an economical and rapid way. The AQ assay used 100 times less medium than did the conventional method of measuring radial growth (RG) of mycelium on fungicide-amended medium, and up to 96 samples (one microplate) could be processed at once. The assay was also performed with conidia stored at −20 C in a 20% glycerol solution for 4 mo, and EC_{50} values did not differ significantly from EC_{50} values using fresh conidia. Growth inhibition was measured most accurately when the spore concentration in a well of the microplate was 10 or 20 spores per microliter. EC_{50} values determined by the AQ assay were compared with EC_{50} values obtained by the RG method, and both were positively correlated. Regression lines to predict EC_{50} values by the RG method from AQ values were: $\log Y = -0.392 + 1.04 \log X$ for iprodione, and $\log Y = 0.742 + 1.38 \log X$ for the mixture of carbendazim and diethofencarb where $Y = EC_{50}$ value determined by the RG method and $X = EC_{50}$ value determined by the AQ assay.

One of the most widely used methods to measure fungicide sensitivity in a nonobligate pathogen population is to determine the dosage at which colony diameter on fungicide-amended agar medium is reduced to half (EC_{50}) that of the colony diameter on fungicide-free agar medium (RG method) (3,4,5). However, the RG method is cumbersome and time-consuming; its main disadvantage is that only linear growth is measured and density of the colony is not considered (3). A more accurate technique is to measure dry weight inhibition in liquid medium containing different concentrations of fungicide (5), but this method requires even more time. Monitoring fungicide resistance requires the testing of a large number of isolates, but usual methods (4) to determine it are time-, space-, and material-consuming.

An automated quantitative (AQ) assay was recently developed to measure fungal biomass increase (1,8). In this method, a fungus is grown in a microplate well and its growth is monitored spectrophotometrically. Absorbance measured in the range of 0.0–0.6 units is related directly to dry weight of microplate cultures (1). Therefore, the AQ assay offers the opportunity of measuring total fungal biomass inhibition in the presence of fungicide, overcoming the disadvantages discussed above. The AQ assay is fast, reproducible, and easy; these features make this method very suitable for rapid and accurate measuring of fungicide resistance in a fungal population.

Parameters of the AQ assay shown to influence the relationship between fungal biomass and absorbance include *(1)* total culture volume in the well, which should be between 75 and 200 µl; *(2)* initial spore concentration in the well, in the range of 10–100 spores per microliter; and *(3)* absorbance wavelength, which does not affect its relationship to dry weight.

The objectives of this study were to use the AQ assay to measure fungicide sensitivity in *Botrytis cinerea* Pers.:Fr. EC_{50} values for iprodione and a mixture of carbendazim and diethofencarb were calculated for seven isolates, and the results were compared with those obtained by the RG method. We also determined if there was any variation in EC_{50} values when conidia stored at −20 C in a glycerol solution were used, since this practice may simplify the AQ technique.

MATERIALS AND METHODS

Seven isolates of *B. cinerea* were collected from vegetables grown in commercial greenhouses in southern Spain during 15–17 December 1992. Isolates were stored on potato-dextrose agar (PDA) slants at 5 C and were grown on PDA at 25 ± 1 C in the dark for mycelial production.

Production, storage and viability of conidia. Conidia of *B. cinerea* were obtained by transferring a 5-mm-diameter plug of PDA from an actively growing colony to PDA (Oxoid, Unipath Ltd., England) in petri dishes. Parafilm-sealed cultures were incubated in a growth chamber for 7 days (16 hr photoperiod) at 25±1 C under fluorescent light (100 mE $m^{-2} s^{-1}$) and 21±1 C in the darkness. Conidia were harvested by flooding dishes with sterile water and rubbing them with a sterile transfer loop. The conidial suspensions were filtered through glass wool, and the concentration of conidia was determined using a hemacytometer. The final concentration was the mean of two replicates that did not differ by more than 10% from each other. Conidia were stored for 4 mo at −20 C in sterile 20% glycerol solution at a concentration of 60,000 conidia per milliliter.

Either fresh or stored conidia (20,000 conidia per milliliter) were germinated in tubes (15 × 65 mm) in 3 ml of double concentrated sterile potato-dextrose broth (2× PDB, 48 g/l; Difco laboratories, Detroit, MI) at 20 C in the darkness. They were germinated for 7 hr, because previous results showed that the percent germination at this time exceeded 65%. When stored conidia were incubated, the conidial suspension in PDB contained an additional 6% (v/v) glycerol. In a preliminary experiment, glycerol at a concentration of 6% (v/v) did not affect viability of fresh conidia. To determine viability of conidia, percent germination and germ tube growth of each isolate were determined from this spore suspension. Four 30-µl drops were mounted on slides and the number of germinated conidia out of 50 and the length of germ tubes of 25 conidia were measured in each drop. Estimated values were the average of these four counts. Each isolate was examined for viability two to five times.

To compare viability of fresh and stored conidia, percent germination (transformed to arc sine-square root values), and germ tube length were compared using a paired Student's *t* test for a total number of 22 observations.

Fungicide sensitivity. To determine fungicide sensitivity of each isolate by the AQ assay, conidia were germinated in 2× PDB as described for viability tests. The conidial suspension then was added to the wells of the microplate (Nunc-Immunoplate MaxiSorp, InterMed, Denmark) to obtain a final concentration of 1,000 conidia per well. Each well also

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had PDB medium (PDB, 24 g/l; Difco) and fungicide at different concentrations in a final volume of 100 μl. When stored conidia were used, wells also had 3 μl of glycerol in the final volume. After a preliminary screening for fungicide sensitivity, isolates were classified as sensitive or resistant. On this basis, isolates were tested on PDB amended with technical grade iprodione (Rhône-Poulenc Agro SA, Madrid, Spain) in the following ranges: 1, 2, 3, 4, 5, 6 μg a.i./ml for resistant isolates, and 0.2, 0.3, 0.4, 0.5, 0.6 μg a.i./ml for sensitive isolates. When a mixture (1:5) of technical grade carbendazim (Agrocros, SA, Madrid, Spain) and diethofencarb (Agrocros, SA) was tested, concentrations were 0.005:0.025, 0.01:0.05, 0.02:0.10, 0.03:0.15, 0.04:0.20 μg a.i./ml. Fungicides were diluted in acetone, and the final concentration of acetone in unamended or fungicide-amended medium was 1% (v/v) for iprodione and 0.1% (v/v) for carbendazim + diethofencarb. Each fungicide concentration was replicated five times in the microplate, and each isolate was tested at least four times in separate experiments.

Absorbance was measured with a microplate reader (Multiskan Plus P V. 2.01) at 492 nm wavelength. The first measurement was done just after filling the plate, and a second one after 46 hr of incubation at 20 C in the dark. Final absorbance values were calculated by subtracting the values of the first reading from those of the second reading.

Percent growth inhibition by iprodione was compared at initial concentrations of 1,000 and 2,000 conidia per well. One isolate of *B. cinerea* (ID number 12) was tested by the microplate assay as described above using iprodione in a range from 1 to 6 μg/ml. The experiment was repeated four times, each one with five wells per fungicide dosage.

To determine fungicide sensitivity of each isolate by the RG method, 5-mm-diameter plugs were cut from the margins of actively growing colonies with a sterile cork borer and transferred to petri dishes of PDA unamended or amended with technical grade fungicides that are described for the microplate assay. After the first test, isolates were classified as sensitive, or having low or high resistance, and then were tested, respectively, on PDA amended with iprodione in the following ranges: 0.1, 0.2, 0.3, 0.4; 0.5, 0.6, 0.75, 1.0, 1.2, 1.4; 0.75, 1.0, 1.2, 1.4, 1.6, 1.8 μg a.i./ml. For the mixture of carbendazim + diethofencarb (1:5) the ranges tested were: 0.005:0.025, 0.01:0.05, 0.015:0.075, 0.025:0.125, 0.033:0.165 μg a.i./ml for sensitive isolates; and 0.033:0.165, 0.045:0.225, 0.055:0.275, 0.066:0.33, 0.075:0.375 μg a.i./ml for resistant isolates. The final concentration of acetone in unamended or fungicide-amended PDA was 0.45% (v/v) for iprodione and 0.04% for the mixture. Each fungicide concentration was repeated four times. After 3 days of growth at 20 C in the dark, two perpendicular diameters of the colony were measured. The experiment was replicated four times for iprodione and three times for carbendazim + diethofencarb.

To calculate EC_{50} values for each isolate, the mean colony diameter (RG assay) and absorbance (AQ assay) were regressed on $\log_{10}$ of fungicide concentration. EC_{50} values were obtained by substituting half the mean colony diameter or absorbance values of the isolate on unamended medium into the regression equation. To establish the relationship between the EC_{50} values calculated by the two methods, EC_{50} values for each of the seven isolates determined by the RG method were regressed on EC_{50} values determined by the AQ method, using Minitab 7.1 for microcomputers (Minitab, Inc., 3081 Enterprise Drive, State College, PA). EC_{50} values of fresh and stored conidia were compared with a paired Student's *t* test to determine whether the use of conidia that were stored at −20 C affected the EC_{50} value calculated by the AQ method.

RESULTS AND DISCUSSION

The AQ assay was successfully used to determine sensitivity to iprodione and mixture of carbendazim and diethofencarb in seven isolates of *B. cinerea* collected from commercial greenhouses. EC_{50} values for inhibition of mycelial growth of sensitive isolates of *B. cinerea* determined by the RG method are in the range of 0.18–0.78 μg a.i. technical iprodione per milliliter (2,6) and less than 1 μg a.i./ml for the dicarboximide fungicide group (7,9). Based on these values, the AQ assay differentiated the same isolates as resistant and sensitive (Table 1). The AQ assay also distinguished between the two types of isolates for the mixture of carbendazim and diethofencarb.

EC_{50} values determined by the AQ method were about two times higher than those determined by the RG method for iprodione and aproximatedly two times lower for carbendazim + diethofencarb. A quantitative relationship between the EC_{50} values for each fungicide determined by both techniques was established by linear regression utilizing data from Table 1. The regression for iprodione was $\log Y = -0.392 + 1.04 \log X$ ($R^2 = 0.96$, $P = <0.01$), and for the mixture of carbendazim and diethofencarb was $\log Y = 0.742 + 1.38 \log X$ ($R^2 = 0.88$, $P = <0.01$), where X is the EC_{50} value determined by the AQ assay, and Y represents the EC_{50} values determined by the RG method. The equation can be used to predict the EC_{50} value determined by the RG method from that determined by the AQ assay. We do not know why the EC_{50} values determined with the AQ assay were higher than those obtained with the RG method with iprodione and lower with cabendazim + diethofencarb. The AQ assay uses a liquid nutrient medium, which may have affected the dose-response relationship between the fungus and each fungicide. The acetone concentration in the PDB medium ≤ 1% (v/v) should not have affected the fungal growth as determined in preliminary experiments (data not shown). Acetone is used to solubilize the fungicide and the differences in final acetone concentration in the AQ technique are only due to the different solubility of each fungicide.

Two conidial concentrations (10 and 20 conidia per microliter) were compared for iprodione inhibition (data not shown) and no significant differences ($P < 0.05$) in percentage of absorbance reduction were found. Although the relationship between fungal biomass and absorbance can be determined in the range of 10–100 conidia per microliter (1), a narrower range is necessary in the AQ assay since a binding fungicide-fungus reaction is involved.

Based on our results, the following procedure should be used with the AQ assay to determine fungal sensitivity to a fungicide. *(1)* Inoculum preparation.

Table 1. EC_{50} values for iprodione and mixture of carbendazim and diethofencarb (1:5) of seven isolates of *Botrytis cinerea* determined by measuring mycelial radial growth on petri dishes (RG) and absorbance in microplate culture (AQ)

Isolate ID[c]	Iprodione[a] RG (μg/ml)	Iprodione[a] AQ (μg/ml)	Carbendazim + diethofencarb[b] RG (μg/ml)	Carbendazim + diethofencarb[b] AQ (μg/ml)
12 (R, S)	1.25 ± 0.07[d]	3.40 ± 0.31	0.086 ± 0.0053	0.048 ± 0.0011
40 (R, S)	1.16 ± 0.06	3.21 ± 0.33	0.073 ± 0.0095	0.049 ± 0.0042
31 (R, R)	1.03 ± 0.10	2.40 ± 0.38	0.354 ± 0.018	0.1099 ± 0.0126
30 (R, R)	1.28 ± 0.09	2.10 ± 0.27	0.364 ± 0.008	0.1344 ± 0.0083
50 (S, S)	0.18 ± 0.02	0.50 ± 0.07	0.086 ± 0.0087	0.0525 ± 0.004
16 (S, R)	0.17 ± 0.01	0.47 ± 0.03	0.297 ± 0.0073	0.171 ± 0.026
22 (S, R)	0.20 ± 0.03	0.47 ± 0.03	0.438 ± 0.0069	0.128 ± 0.0176

[a] Data are means of a minimum of four replications.
[b] Data are means of three replications for RG assay and two to five replications for AQ assay.
[c] Letters in parenthesis following isolate ID indicate whether isolates are resistant (R) or sensitive (S) to iprodione and carbendazim + diethofencarb (1:5).
[d] Mean ± standard error.

Obtain conidia on PDA dishes inoculated with a 5-mm-diameter plug of PDA from an actively growing colony and incubated in a growth chamber for 7 days (16 hr photoperiod, 25 C daylight/21 C darkness). Prepare a conidial suspension of 20–40 conidia per microliter in 2× PDB and incubate for 7 hr at 20 C in the dark for spore germination. *(2)* Sensitivity assay. Add 50 μl of conidial suspension and 50 μl of graded series of fungicide solutions or suspensions to the microplate well. The final volume in the well should be 100 μl and the concentration of the medium, conidia, and fungicide are half of those initially prepared. Three replicate wells per fungicide concentration and isolate should be used. Absorbance at 492 nm is measured after incubation for 46 hr at 20 C in the dark. The mean absorbance is calculated for the three replicates per fungicide concentration and the values are used to calculate the EC_{50}.

There was no significant difference in the EC_{50} values determined with the AQ assay for conidia that had been stored for 4 mo at −20 C in 20% glycerol and freshly harvested conidia. The mean difference of EC_{50} values (fresh minus stored) was 0.084 units of absorbance (standard error of mean = 0.169) and its 95% confidence interval was of −0.27–0.44. The ability to use stored conidia makes the AQ assay much simpler, since time required for doing an experiment is reduced to that necessary to fill the microplate and read it automatically. After storage of conidia of *B. cinerea* at −20 C for 4 mo in a 20% glycerol suspension the percentage of conidial germination decreased significantly ($P = 0.002$) compared with fresh conidia (percent germination of fresh and stored conidia was 78±3.4 and 57±5.6 %, respectively). Germ tube length, however, was not significantly affected ($P = 0.4$). Since EC_{50} values for absorbance did not change significantly when using fresh or stored conidia, the result indicates that EC_{50} values were not affected by the number of conidia germinated, but only by the number of them in the well.

Culturing isolates in wells of a microplate and measuring absorbance with a microplate reader has several advantages over the RG method. With the AQ assay a large number of isolates can be processed simultaneously, since 96 samples can be cultured in one microplate. RG method in each experiment requires four 9-cm-diameter petri dishes per isolate and tested concentration, and 100 times more medium than is used in the AQ assay. Absorbance is measured automatically and determines the total fungal growth. On the other hand, linear measurement of a colony is operator-dependent and inaccurate when colony margins are not well delimited. In addition to this, RG method only accounts for the linear growth of the fungus. The AQ assay was performed with conidia that had germinated for 7 hr prior to their exposure to the fungicide. Percent germination for each isolate after this time varied from 62 to 89% with fresh spores. Pregermination of conidia separates the different effects that fungicides have on spore germination and on mycelial growth.

AQ technique also has some disadvantages. A considerable amount of time is necessary to make the conidial suspensions at the required concentration; however, this task is reduced if conidial suspensions are frozen until use in the microplate. For this reason, the AQ technique is more suitable for determining fungicide sensitivity by EC_{50} values and examining a given population in detail than for handling a very large number of isolates to monitor fungicide resistance of populations. This is more easily carried out by qualitative assessment using a critical fungicide concentration by which distinct sensitive and resistant isolates can be determined. Microbial contamination in the microplate well is possible and can be detected only by an abnormal increase of absorbance, which is more difficult than detecting contaminations with the RG method. This is why replications within the microplate are always recommended. Nevertheless, the fast growth rate of *B. cinerea* and the short incubation time make the effects of contaminants almost negligible. Volatility of fungicides may also need to be taken into account. High concentrations in some wells may inhibit fungal growth within the same plate in other wells unless adequate ventilation is ensured. If very high and very low concentrations of volatile fungicides are used, those concentrations should be kept in separate plates.

Results presented here show that the automated quantitative assay has many advantages in measuring fungicide resistance. A large number of samples can be processed in an economical and feasible way, and the technique can be used to quantify fungicide sensitivity in a fungal population.

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