Techniques

Radioimmunosorbent Assay for *Botrytis cinerea*

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ABSTRACT

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Radioimmunosorbent assay (RISA) for somatic antigens of the pathogen *Botrytis cinerea* can be used to detect the presence of Botrytis antigens in homogenized samples. As little as 100 ng of original fungal dry weight can be detected, and the sensitivity curve is log-log linear in response up to 10 mg. The assay is highly specific for *B. cinerea* although some reaction is obtained with other members of the Sclerotiniaceae. *B. allii* showed 48% reactivity relative to *B. cinerea,* and species of *Sclerotinia* and *Monilinia* showed 10–24% reactivity. All other fungi tested showed less than 0.1% reactivity. The usefulness of the assay for detection of the fungus within host tissue is demonstrated by the high correlation (r = .833) of the assay results with an estimation of rot weight from field-infected lots. Artificially produced infection levels representing 0.1% infected tissue mixed with sound tissue homogenates are easily distinguished from background by the assay. It was found that the use of microtiter plates made of polyvinyl chloride greatly increased the response of the assay so that its resolving power was improved.

Additional key words: Vitis vinifera, ELISA.

Recently a new type of serological assay, the immunosorbent assay, has been developed in which the basic antibody-binding reaction is combined with the adsorption of the γ-globulin reagent to a solid plastic matrix so as to facilitate selective retention of the antigen-antibody complex. The first procedure of this type to find use in the field of plant pathology was the enzyme-linked immunosorbent assay (ELISA) (2,15).

Other immunosorbent assays use a radioactively labeled antibody in place of the enzyme-linked reagent. Many variations of such "solid phase radio-immune assays" have been described (13). An assay of this latter type was recently developed by Ghabrial and Shepherd (5) for use in a plant virus system.

Immunological or serological assays have been used extensively for the detection of viral and bacterial plant pathogens and to a lesser extent for fungal agents. Immunofluorescent methods have been useful in many fungal systems (7,8,12) to qualitatively visualize pathogens within the host, soil, or other complex substrates. Such tests have even been used to distinguish between growth stages or structures of a given species (4). In another application, Adams and Butler (1) used agar immunodiffusion to distinguish between anastomosis groups of *Rhizoctonia solani.* Recently the use of an ELISA for the detection of a fungal product has been described in which antibodies were elicited to compounds in a flash-evaporated cultural filtrate of *Phoma tracheiphila* (10).

The study described here was undertaken to develop an assay for antigenic components of the mycelium of *Botrytis cinerea* Pers. ex Fries. *B. cinerea* is an extremely widespread plant pathogen; however, its epidemiology on various hosts is similar in certain respects. In many cases it has been hypothesized that the fungus colonizes senescing floral tissues and remains "latent" within that tissue or adjoining living tissue until the developing fruit ripens

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enough to become invaded. This general phenomenon has been reviewed by Verhoeff (14). Latent infections by *B. cinerea* have been proposed in strawberries (11), raspberries (6), and grapes (9).

In the case of *B. cinerea* on grapes, there has been substantial difficulty in reproducibly demonstrating the presence of "latent" infections in the pistil end of immature berries by sterile cultural procedures as suggested by McClellan and Hewitt (9). Sporulation in such latent infections by cultural manipulation has not been induced regularly and is hampered by inability to control surface sterilization procedures. Other theories, which account for the "latent" phenomenon and its efficacious control with fungicides applied to blossoms, postulate the colonization of loose floral debris that remains within the cluster. The confirmation of such colonization also encounters the same difficulties of surface sterilization. The development of a reliable, sensitive, and specific assay for the presence of *B. cinerea* in grape tissue would help to verify the actual occurrence of "latent infections." Further, an assay could be used to define the location and importance of such infections. It could also provide a valuable index of the threat from an epidemic of *B. cinerea* when the fruit matures. An immunosorbent assay would possess the aforementioned characteristics and thus would be extremely useful in the characterization of the epidemiology of *B. cinerea* on grapes.

MATERIALS AND METHODS

Antigen production. The isolate of *B. cinerea* used throughout this study was collected by mass transfer from a field-infected grape cluster (*Vitis vinifera* L. 'White Riesling') grown in the Napa Valley of California in 1976. The culture was maintained on potato-dextrose agar but occasionally was inoculated onto autoclaved grape shoots and reisolated, a process that tended to select for the prolific sporulation characteristic of the original culture.

Mycelium for antigen production was grown in a liquid Czapek's medium consisting of: $NaNO_3$(2 g), $FeSO_4$(.01 g), KCl(.5 g) K_2HPO_4(1 g), $MgSO_4$(.5 g) and dextrose (25 g) in 1 L of deionized water. Aliquots (10–15 ml) of medium were placed in petri dishes and autoclaved for 10 min at 121 C and 1.96 kgf/cm^2 (20 psi); the pH of the autoclaved medium was between 5.5 and 6. Cultures were grown at room temperature (23 C) and spore or agar block transfers filled the plates within 1 wk.

Four types of immunogen were prepared:

Type 1. Fresh mycelium was washed with distilled water, lyophylized, ground with a mortar and pestle at −5 C, and diluted in 0.02 M K-phosphate buffered saline, pH 7.4 (PBS). This preparation was centrifuged at 10,000 *g* for 15 min. Unless specified, all centrifugations were completed in a Sorvall centrifuge at 4 C. The supernatant solution from the preparation described above was called "soluble antigen."

Type 2. The pellet in the above process was suspended in PBS and labeled "solid antigen."

Type 3. Following two cycles of centrifugation in 30 ml of PBS to remove excess salts, mycelium was sonicated (Biosonic) at full power on ice in a series of short full-power bursts totaling 30 min. More than 90% of the cells appeared to be broken by the process. The broken cells were washed with PBS several times by centrifugations at 10,000 *g* for 10 min until the resulting supernatant buffer showed negligible absorption at 280 nm. This pellet was labeled "cell wall antigen."

Type 4. Cultures were initiated in Czapek's medium with large numbers of spores. After 24–36 hr of incubation at 20 C, the spores had germinated and formed thalli consisting of 10–30 cells. These cultures were harvested, washed by centrifugation as above, suspended in an equal volume of PBS, and labeled "whole antigen."

For tests of the specificity and sensitivity of the assay, *Botrytis cinerea* and mass-transfer isolates of fungi representing a wide range of other taxa were grown on Czapek's medium. Following growth at 23 C for 6 days, the fungal mat and remaining media were dialyzed extensively against distilled water to remove the nutrient salts. The cultures were subsequently frozen, lyophylized, weighed, and resuspended in a grinding buffer at a concentration of 10 mg dry wt per milliliter of buffer. The buffer was 0.1 M potassium phosphate, pH 7.4, with 2% polyvinyl pyrrolidone (Sigma PVP40, Sigma Chemical Co., St. Louis, MO 63178), 0.2% NaCl, 0.2% ovalbumin, and 0.02% sodium azide. This suspension was ground for 45 sec with a Polytron homogenizer (Brinkman Instruments, Westbury, NY 11590) on ice and then centrifuged for 20 min at 15,000 *g*. For assay, the supernatant liquid was diluted in PBS containing 2% PVP and 0.5% Tween-20.

In a separate study of the antigens present in the cultural filtrates from the various fungi, the medium was extracted by centrifugation from each culture and its volume was measured for comparison with the dry weight of fungus represented. The cultures had been grown at 23 C for 1 wk; however, the extent of growth varied between fungi. Dilutions of the cultural filtrates were used as test samples for RISA.

Antiserum preparation. Rabbits were given three biweekly subcutaneous injections of the antigens described above. Each injection consisted of 1 ml of antigen suspension emulsified with 1 ml of adjuvant. The first injection was in Freund's complete adjuvant and the remainder in incomplete adjuvant (Difco Laboratories, Detroit, MI 48232).

Serum was collected by cardiac puncture beginning 3 wk after the last injection, allowed to clot for 1–2 hr at 23 C, set over night at 4 C, ringed, and centrifuged at 10,000 *g* for 10 min at 4 C. The serum was stored in small bottles at −20 C. Purification of the globulin fraction was achieved as follows:

To 2 ml of serum stirring at room temperature was added 2 ml of saturated ammonium sulphate (SAS), adjusted to pH 7.8 with 3 M NaOH immediately prior to the precipitation. The SAS was added very slowly and stirring was continued for 2 hr. The precipitate was pelleted at 5,000 *g* for 10 min at 4 C. The supernatant solution was discarded and the pellet was resuspended in 1 ml of 0.85% saline. A second precipitation with newly adjusted SAS was followed as before. The second pellet was suspended in 1 ml of half-strength PBS and dialyzed at 4 C against three 600-ml changes of the same buffer to remove excess ions.

A column of DEAE-cellulose (DE-22, Whatman) was prepared according to the method described by the manufacturer and equilibrated to pH 7.4 with PBS. A short column 12 mm in diameter was used for each purification of 1.5 ml of SAS-precipitated globulin. Eluant (PBS) was run through the column at a rate of 40 drops per minute. Fractions of 15 drops were collected and scanned at 275 nm. The first peak to come through the column was diluted to give an extinction coefficient of 1.4 (approximately 1 mg/ml) and saved for future use.

Activity of the antibody at each stage of purification was determined by agar double diffusion with antigen in 0.6% Noble agar (Difco). Precipitin testing of the serum for titer determination was attempted, but the results were obscured by spontaneous aggregation of the antigen, even in the presence of dilute detergents. At some points, the antibody activity was checked by indirect immunofluorescence using fluorescein-labeled goat antirabbit serum (7), but these tests were subject to artifacts in certain instances.

Iodination of the purified γ-globulin was executed by the process described by Fraker and Speck (3) using the reducing agent IODOGEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycolurill, Pierce Chemical Company, Rockford, IL 61105). Optimal labeling was achieved when 1 ml of the DEAE-cellulose purified γ-globulin at 1 mg/ml was incubated on ice in tubes coated with 25 μg of IODOGEN. Ten microliters of 3×10^{-5}M Na ^{125}I with approximately 1 mCi activity (New England Nuclear, Boston, MA 02118) was added to each of these tubes and periodically mixed for 30–45 min. The excess NaI was removed by extensive dialysis against half-strength PBS, and the labeled γ-globulin was mixed with an equal volume of 10 mg/ml BSA for stabilization. The activity of the final preparation varied between 1.0×10^5 and 5.0×10^5 cpm/μl when counted in 2 ml of RIA-solve II (Research Products International Corporation, Kankakee, IL 60901) on the ^{14}C channel of a scintillation counter (Beckman Model LS 100C, Beckman Instruments, Fullerton, CA 92632).

The RISA procedure. The procedure used was a modification of that of Clark and Adams (2) and Ghabrial and Shepherd (5).

Coating. Plastic microtiter plates made either of polystyrene or polyvinyl chloride (PVC, Dynatech Laboratories Inc.) were incubated with 200 μl/well of DEAE-cellulose purified γ-globulin diluted to 5 μg/ml in 0.5 M carbonate buffer pH 9.6. Coating was generally completed during a 2–5 hr incubation at 37 C.

Washing. In this and other washing steps the plates were emptied and then flushed with PBS containing 0.5% Tween-20 in three washes of at least 3 min each. When emptying the plate, the most efficient method consisted of shaking followed by throwing the plate face-down onto several layers of paper toweling.

Antigen. Test samples of 200 μl in PBS-Tween with 2% PVP were incubated either for 2–5 hr at 37 C or overnight at room temperature.

Labeled antibody. Two hundred microliters of ^{125}I-labeled globulin at 6.25 μg/ml in PBS-Tween-PVP was added to all wells and incubated as before.

Final washing. In this step five to seven washes with PBS-Tween were used over a period of 30–60 min.

Release and counting. When polystyrene plates were used, the bound, labeled antibody was released from the emptied wells by the addition of 250 μl of 0.1 M glycine-HCl buffer, pH 2.3. Two hundred microliters of the buffer was then withdrawn and counted in 2 ml of scintillation cocktail for 5–10 min utilizing the ^{14}C channel of the counter.

When PVC plates were used, the emptied, washed wells were cut out of the plate with scissors and each well was placed directly into 2 ml of fluid for counting. A hot knife apparatus to facilitate this procedure is available commercially (D. Lee, Inc., Sunnyvale, CA 94086).

Procedures for tissue samples. Ripe grape berries (*V. vinifera* 'Emperor') were surface sterilized with 10% sodium hypochlorite, rinsed in sterile distilled water, injured with a scalpel, and inoculated with a suspension of conidia of *B. cinerea*. Some of the berries were not inoculated in order to serve as controls. All grapes were incubated in moist chambers until the inoculated berries were extensively colonized in a manner similar to that frequently seen in a field situation. Several grapes of each type, inoculated and uninoculated, were destemmed and weighed. Each gram of grape tissue was submerged in 1 ml of 0.1 M potassium phosphate buffer with 0.85% NaCl, 2% PVP, and 0.2% ovalbumin. The grapes were then coarsely chopped with a scalpel and homogenized on ice by using a Polytron homogenizer. Three full-power 10 sec bursts were sufficient to break all plant cells and a great number of fungal cells as verified by microscopic observation. The resulting slurry was degassed and centrifuged at 10,000 *g* for 20 min. The pH of the supernatant liquid was 6.5. Samples were held at 4 C until use in RISA; however, tests of the same samples 4 days later showed no change in reactivity. The supernatant solutions from the infected and noninfected tissues were mixed in different proportions to simulate samples representing different levels of colonization. Each mixture was diluted 1:1 with PBS-PVP-Tween for testing by RISA.

Individual lots of variously infected grape clusters (*V. vinifera* 'Sauvignon blanc') were harvested from field experiment sites near Yountville, California in September of 1979. Each lot, consisting of five clusters, was dissected into apparently sound and infected berries and each category was weighed. This procedure is similar to that used commercially to determine the level of rot in a load of grapes. All the grapes were then combined for each lot, destemmed, and pressed by using an electric screw-press device (Chisholm-Ryder Co., Inc., Niagara Falls, NY 14305) which applies a fairly constant pressure on each sample. The juice of each lot was collected and frozen.

Samples (1 ml) of juice from each lot were diluted in equal volumes of 0.1 M PBS with 2% PVP and 0.5% Tween-20. An additional 0.2 ml of 0.5 M PBS was added to each sample to raise the pH to 6. Duplicate aliquots of each sample were assayed by RISA.

The affinity of anti-Botrytis γ-globulin for mycelial cell surfaces was investigated. Ten μl of ^{125}I-labeled γ-globulin solution were incubated with mycelial suspensions of equal dry weight of *B. cinerea* and *Rhizopus stolonifer*. After several washings with PBS by centrifugation, the mycelium was incubated with the glycine-HCl buffer, pH 2.3, and centrifuged. Samples (100 μl) of the resulting supernatant buffer were counted by liquid scintillation.

RESULTS

The antiserum of highest quality was from rabbits injected with "whole antigen" which was composed essentially of unbroken *B. cinerea* thalli. This serum was used in all assays. The antiserum to the "solid antigen" preparation also formed precipitin bands in agar diffusion tests; however, the "soluble" and "cell wall" antigens failed to elicit an immune response sufficient to demonstrate such a reaction. ^{125}I-labeled γ-globulin was retained by the mycelium of *B. cinerea* at five times the level retained by the mycelium of *R. stolonifer*.

The sensitivity, specificity, and resolution of RISA for *B. cinerea* were investigated in a series of experiments as was the potential usefulness of the system for the detection of *B. cinerea* within several types of field tissue samples.

Sensitivity. The sensitivity of the assay was demonstrated by the relationship of the log of the counts per minute observed to the log of the dry weight of mycelium. The log-log plot is essentially linear ($r^2 = .982$) over five orders of magnitude representing mycelial dry weights from 100 ng to 10 mg (Fig. 1). A concentration of 100 ng/ml of original fungal dry weight elicited a reaction greater than background.

Specificity. The assay showed no appreciable reaction with any of the fungi tested that were not members of the Sclerotiniaceae (Table 1) while members of that family showed varying degrees of reactivity.

Culture filtrates of many fungi also were tested. In general, the pattern of reactivity followed that of the mycelial preparations except at a lower level. On the basis of mycelial dry weight the

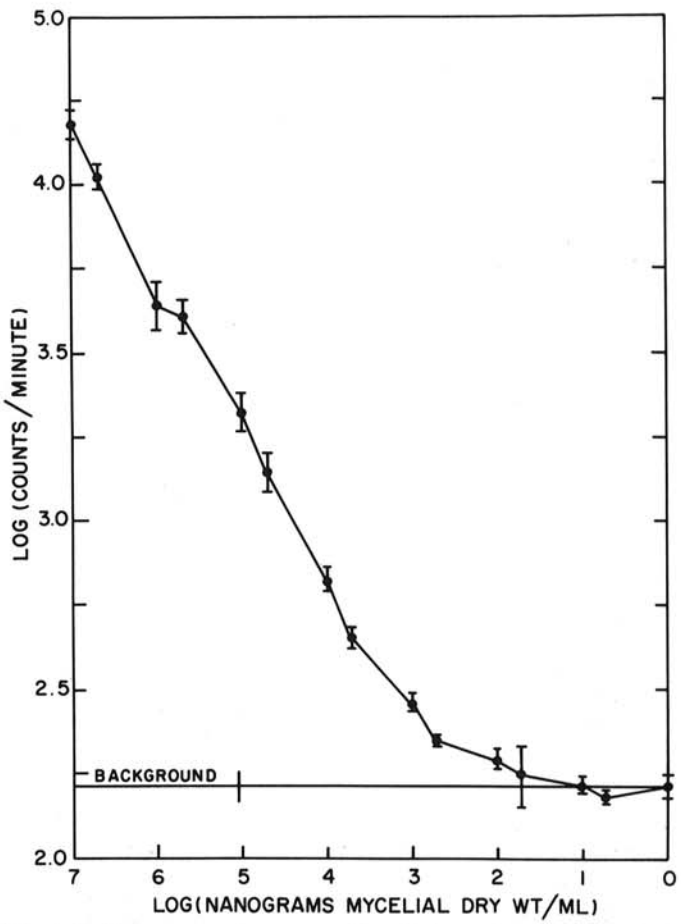

Fig. 1. Radioimmunosorbent assay of a dilution series of homogenized *Botrytis cinerea* mycelium obtained by using the polyvinyl chloride microtiter plate method. Each of 16 concentrations was replicated five times and 17 wells of buffer were included to define background levels.

filtrate of *B. cinerea* showed approximately 4% of the reaction of the corresponding mycelium. Similarly, the filtrate of *B. allii* reacted to 6% of the reaction level of its mycelium.

Performance of the assay for *B. cinerea* in grape tissue samples. In these assays, a mixture simulating 0.1% rotted tissue was easily distinguished from healthy grape backgrounds which were in turn only slightly above buffer controls (Fig. 2). The assay was most useful over the range of 0–5% rotted tissue.

In the assay of juice samples from field-infected clusters there was a high correlation ($r = .833$) between the estimate of percent rot by weight and the estimate by assay (Fig. 3).

DISCUSSION

The antigenicity of the "whole antigen" preparation along with immunofluorescent tests suggests that some portion of the active antigen in this system is associated with the mycelial surface, but the failure of the "cell wall" antigen to elicit a good serum suggests that the antigens are lost during extensive sonication. Other results demonstrated that soluble moieties within this antigen preparation and within the cultural filtrate reacted with the antiserum elicited by washed and essentially unbroken mycelium. Whether this suggests a connection between the "cell surface" antigens and the soluble components is unclear since the nature of mycelial breakdown within the rabbit is unknown.

Since the actual proportion of dry weight that is released by grinding and the proportion of that weight that represents antigenic moieties is unknown, it is not clear whether the 100-ng sensitivity of this RISA is comparable to the 1- to 10-ng sensitivities found in many ELISA and RISA procedures to detect viral antigens.

The high degree of specificity of RISA for members of the Sclerotiniaceae will make it very useful in the grape bunch rot system since the major saprophytic and parasitic competitors of *B. cinerea* (*Alternaria, Penicillium,* and *Aspergillus*) show almost no reaction with the antiserum. In a situation in which *B. cinerea* must be distinguished from other *Botrytis* species or from other members of the Sclerotiniaceae further selection of antigens would be required. The intermediate reaction of the related fungi suggests that some antigenic components are common throughout a given taxon and further suggests that the elicited antiserum contains antibodies specific for many separate components. The separation of the various antigenic determinants and selection of those unique to the desired species for immunization could potentially increase the specificity of RISA for fungi; however, it is also likely that the overall sensitivity of the assay would be reduced.

The importance of the slight reaction that occurs with cultural filtrates may be greater if the production of such antigens is influenced by substrate. This possibility prohibits the direct comparison of the reactivity of samples derived from mycelium grown in Czapek's medium and those from infected tissue.

The sensitivity of the assay allows detection of very low levels of infection in ripe fruit and the assay of juice samples from field-infected clusters demonstrates that *Botrytis* antigen may even be detected without extensive homogenization of the fruit. Sampling

TABLE 1. Radioimmunosorbent assay of the soluble preparation of various fungal cultures in polyvinyl chloride plates[a]

Species	Relative reactivity[b]
Botrytis cinerea	1.00
B. allii	.48
Sclerotinia sclerotiorum minor	.24
S. sclerotiorum major	.11
Monilinia fructicola	.10
Aureobasidium pullulans	<.001
Aspergillus niger	<.0005
Trichoderma viride	<.0005
Ulocladium chartarum	<.0005
Pythium aphanidermatum	<.0005
Stemphyllium vesicarium	<.0005
Penicillium sp.	<.0005
Alternaria alternata	<.0005
Epicoccum nigrum	<.0005
P. digitatum	<.0005
Phytophthora cinnamomi	<.0005
Rhizoctonia solani	<.0001

[a] Duplicate dilutions of homogenate representing .01, .1, 1, and 10 mg mycelium dry weight per milliliter were assayed for each species.

[b] The relative reactivity of each preparation was estimated by interpolation from a standard curve for *Botrytis cinerea*.

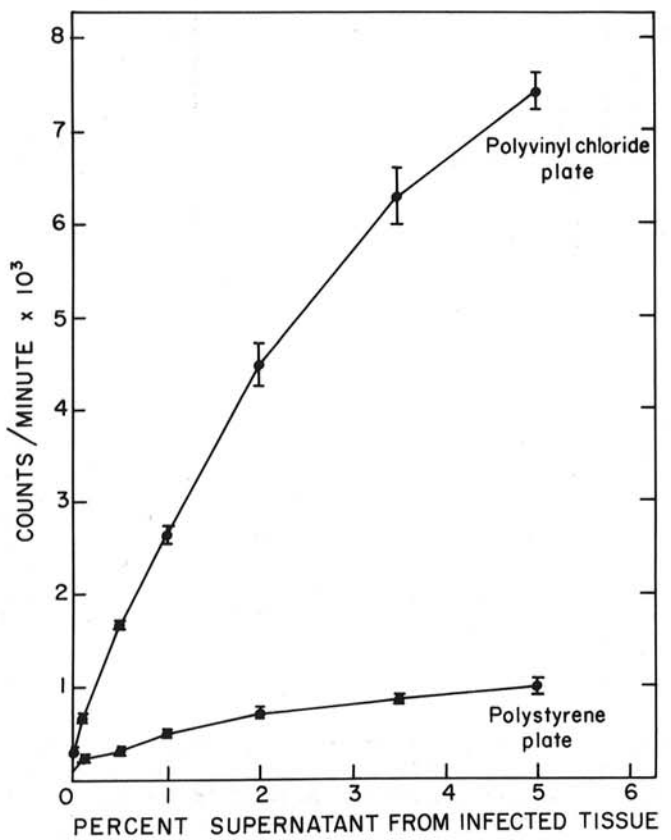

Fig. 2. Radioimmunosorbent assay for *Botrytis cinerea* in various mixtures of homogenized infected and noninfected ripe grape berries. Samples were replicated four times in either polyvinyl chloride or polystyrene plates.

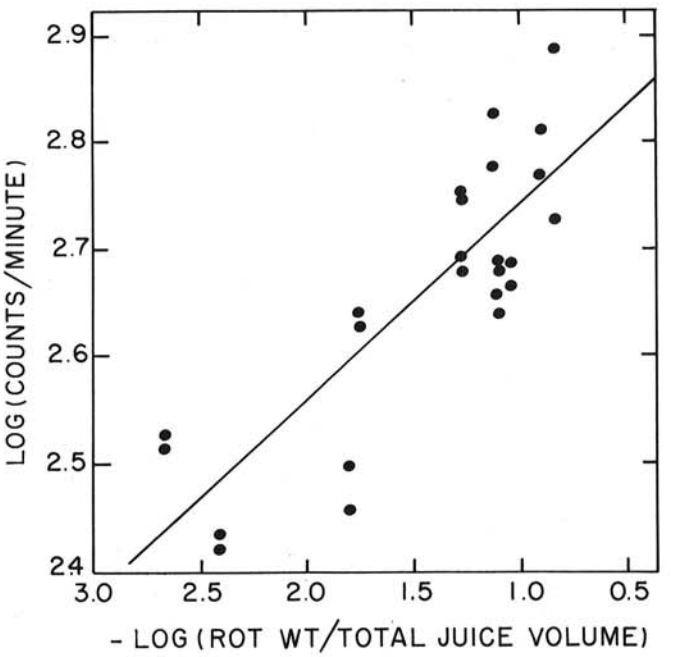

Fig. 3. Relationship of percent infection (based on the ratio of rot weight to juice volume) and the results of the radioimmunosorbent assay for *Botrytis cinerea* in duplicate samples of grape juice.

of large lots can be more conveniently done by using such juice samples rather than intact clusters.

It was found that the use of polyvinyl chloride microtiter plates conferred several advantages in this system. The elimination of the final low pH release step gives a considerable time savings as could the direct gamma counting of the separated wells. In addition, two characteristics of the PVC results combine to improve the resolving power of the assay. The uncertainty or error term associated with the use of a standard curve for estimation of a parameter by an assay is determined by two influences. The first is the variability of results for replicate samples and is estimated by the coefficient of variation (CV) which is the ratio of the standard deviation and the sample mean. The second influence is the slope of the standard curve in that uncertainty is reduced if the slope is steep. In the case of the assay of simulated rot levels (Fig. 2) the CV for the polystyrene plates averaged 7.3 over the useful 0–5% range while the CV for the polyvinyl chloride plate method averaged 3.9. In addition the PVC method resulted in counts per minute which were five to seven times those in the polystyrene plates. The combination of these influences results in an error term of 32% of the percent rot estimate in the case of the polystyrene system and 14% in the PVC system.

The specificity and sensitivity of the RISA in the detection of *B. cinerea* mycelium makes it a potentially powerful tool in the study of the epidemiology of Botrytis bunch rot. The assay system as described is now being implemented in investigations of the latency phenomenon described earlier.

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