Longevity and Pathogenic Stability of *Pyricularia oryzae*

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ABSTRACT

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Results are presented from several aspects of 30 years' studies on *Pyricularia oryzae*. These include techniques developed for production of dry spore inocula and evaluation of pathogenicity and host specificity toward the standard differential rice cultivars after long-term storage of the pathogen in various forms. Induced or natural mutations (or parasexual recombinations) occurred occasionally, usually resulting in broader host range and/or increased sporulating capacity. By far, the majority of >100 isolates surveyed retained their original pattern of specialization after 20–30 yr in culture with periodic transfer. With respect to the controversy regarding stability of pathogenic races of the rice blast pathogen, the results of our studies strongly support the concept that the species *P. oryzae* comprises a wide range of pathotypes (races) each characterized by its capacity to attack certain cultivars of rice; that these races are basically stable; and that mutations are the exception rather than the rule.

During studies initiated in 1952 on pathogenic specialization in *Pyricularia oryzae* Cavara, the pathogen of blast of rice (*Oryza sativa* L.) (6,7), we developed techniques for cultivation and long-term preservation of many races of the fungus in various forms on several substrates. More than 100 cultures have been maintained for 20–30 yr in one or more of these forms. We report here the results of recent tests of viability, pathogenicity, and host specificity of some of these cultures. Further, we describe methods for producing, testing, and storing dried spores (conidia) of *P. oryzae* that we have also found appropriate for other fungal pathogens such as species of *Bipolaris, Curvularia, Cercospora*, and *Colletotrichum*. These techniques are applicable for providing and maintaining standard inocula for use in field and greenhouse testing of crop cultivars for resistance to disease, as well as in biocontrol research for determining efficacy of selected facultative fungal pathogens as mycoherbicides.

We described our basic methods for production of dry spore inocula in 1971 (4). Here, we present a more detailed description of the procedures involved. We include information on dry spore longevity and on fungus survival in leaf and culm lesions collected from field and greenhouse plants.

MATERIALS AND METHODS

Specimen preservation. Specimens of leaf, node, and rachis lesions were obtained from field collections in many countries and from some 15,000 inoculations of greenhouse plants between 1952 and 1984. These specimens were cut into 3- to 5-cm pieces, placed in labeled coin envelopes, air-dried for several days, then the envelopes were closed with paper clips and stored in plastic bags at −18 C.

Isolation and growth of cultures. Pure cultures were isolated from sporulating lesions on fresh or stored specimens incubated in petri-dish moist chambers for 12–24 hr under light (20W cool-white fluorescent) at 25–28 C. Isolations were made onto slants of 2% rice polish agar (RPA) (11) by first slicing aseptically a tiny wedge-shaped sliver of agar from the slant with a dissecting needle flattened into a blade, touching the sliver to freshly formed spores on a lesion, and then returning it to the slant. By this method, a pure culture could be started after only 12 hr of specimen incubation. Depending upon their eventual use, cultures were grown primarily on one of three media. Rice polish agar in 125–300-ml Erlenmeyer flasks was used as the substrate for culture of colonies seeded by either the "plug" or "flood" method (5); colonies originating from plugs were grown to observe cultural variants and to isolate highly sporulating sectors, while flood-seeding (excess liquid poured off) was used to obtain fast coverage of the surface and rapid sporulation when making spore suspensions either for seeding liquid cultures or for inoculation of greenhouse plants. Yeast extract dextrose (YED) liquid medium (3 g of yeast extract and 15 g of dextrose in 1 L of H_2O) in 1-L Erlenmeyer flasks (400 ml per flask) was used to grow mycelium in rotary shake culture (130 rpm) for seeding inoculum in the spore production processes. Corn leaf piece (CLP) moist chambers (one piece of corn leaf from four- to six-leaf-stage plants autoclaved on two pieces of moistened filter paper in a 9-cm petri dish) were used (especially for poorly sporulating cultures) to produce spores for inoculating greenhouse plants and for culture preservation, described later.

Preservation of cultures. During our investigations on the extent of pathogenic variability in *P. oryzae*, and our development of a set of differential cultivars for race determination, we needed a simple method to preserve a large number of isolates (>2,000) in a form readily retrievable for retesting and evaluation of pathogenic specialization. Two such methods were used. In the first, rice nodes (1.5-cm sections) from rice straw collected at harvest time were cut and placed with 1–2 ml of distilled water in 8-cm culture tubes, plugged with 1-cm-diameter foam plugs, autoclaved, and seeded with mycelial or sporulating agar plugs from the cultures to be stored; node cultures were maintained at room temperature until most or all of the node surface was covered with mycelial and/or sporulating growth (about 1 wk). Tubes were then placed in a forced-air drying oven at 35 C for a period long enough to remove moisture droplets as determined under a dissecting microscope (usually 5–7 days). The dried cultures were then transferred to a freezer at −18 C. In the second method, CLP cultures were seeded either with mycelial plugs or with spore suspensions and incubated at 24–27 C until sporulation covered the leaf surface. Lids were then placed ajar until cultures became dry. The CLPs (with filter paper

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backing) (CLP/filter paper) were cut into strips (with aseptic technique used between successive cultures), which were placed inside coin envelopes and stored at −18 C.

Spore production processes. Two procedures were developed for producing dry spore inocula: the "corn grain" process and the "mycelial mat" process.

Cereal grains tested for support of sporulation included corn (*Zea mays* L.), oats (*Avena sativa* L.), sorghum (*Sorghum vulgare* Pers.), millet (*Setaria italica* (L.) Beauv.), rice, and hulls of oats and rice. Seed corn, with its low percentage of cracked and broken grain, was found to be best. The "corn grain" process is biphasic. During "phase 1," corn is steeped, rinsed, sterilized, aseptically seeded with fungus mycelium (grown in shake culture), and incubated in Fernbach flasks aerated (1 L/min) through sterile air samplers used as filters. For a 5- to 10-g yield of conidia, 20 flasks were utilized, each containing 200 g of steeped grain. In "phase 2," the fungus-covered grain is shaken from the flasks onto wire mesh trays and incubated under clean conditions in a growth chamber with 97–98% RH at 25–27 C under continuous light (30W cool-white fluorescent). Following 3–5 days of incubation, the sporulated grain is dried in a cabinet with circulating air at 40 C for 24 hr. After the corn is thoroughly dry (about 7% moisture), it is transferred by funnel to a Fernbach flask, agitated on a small shaker with Vythane (1,1,1-trichloroethane) in a chemical exhaust hood for 10 min. The resulting suspension of spores is filtered, the "cake" is dried at 40 C overnight, crumbled, and sieved; and this "spore product" is placed in appropriate containers for storage.

In the mycelial mat process, 1-L Erlenmeyer flasks (each containing 400 ml of YED liquid medium) are seeded with mycelial suspensions grown for 5–6 days in rotary shake culture (130 rpm) from spore suspensions seeded into 70 ml YED in 300-ml Erlenmeyer flasks. After 3–4 days in shake culture, or until shortly after pigmentation appears, the mycelial suspension is filtered onto moist filter paper in a 12-cm Büchner funnel, maintaining suction only until the liquid disappears. The mycelial mat is separated from the paper and incubated on a wire screen in a controlled environment chamber at 97–98% RH, 26 ± 1 C with continuous light (30W cool-white fluorescent) for 2–3 days, depending upon the sporulating capacity of the isolate. The mat is then dried overnight at 40 C, broken into pieces, and blended in a Waring Blendor with solvent until a slurry is formed (about 1 min). The suspension is filtered through a sieve to remove mycelial clumps, then filtered again onto filter paper in a Büchner funnel, leaving a cake of spores that is dried overnight at 40 C. The dry cake is pressed by spatula through a coarse screen to convert it to a powder, the "spore product."

Packaging and storage of spore products. Products are packaged and stored variously: in sealed ampoules under liquid nitrogen at −170 C, in glass baby food jars under partial vacuum with nitrogen gas, and in screw-cap vials at atmospheric pressure. The latter two types of containers are stored at 4 C and represent the majority of stored spore products.

Culture and spore product evaluation. Cultures and spore products were evaluated routinely as to pathogenic race, virulence (lesion size or toxic effect), sporulation, and spore concentration. Upon first isolation, >2,000 cultures (during 30 yr of testing) were inoculated onto differential cultivars and identified by lesion types (6) as to pathogenic race according to reactions shown in Table 1 (4,5,6). We characterized 51 races among >2,000 isolates from worldwide sources. One or more isolates of each race were retained and stored in frozen form as previously described. Frozen cultures stored on rice nodes or on CLP/filter paper were retrieved by transfer to agar media whenever needed for pathogenicity tests. Fresh cultures, or those reactivated from frozen storage, were grown for sporulation either on RPA in Erlenmeyer flasks or on CLP/filter paper in petri dishes. Spores for inoculation of plants were harvested from the former by agitation with distilled water and glass beads and from the latter by forceful spraying from a wash bottle with water (containing 2 drops of 5% Tween-20 in 10 ml) into a 100-ml beaker. Spore suspensions were filtered through a cone-shaped wire screen (500 μm), and sprayed onto sets of differential cultivars (three- to five-leaf stage). Plants were incubated in dew chambers (8) for 16 hr, transferred to greenhouse benches, and evaluated 5–7 days later as described previously (5,6).

Dry spore products were sampled for pathogenicity tests by suspending minute quantities (1–2 mg) in distilled water (containing two drops of 5% Tween-20 per 10 ml), adjusted to provide a suspension of about 3.5×10^5 spores per milliliter (5–10 spores per ×400 field). Plant incubations and evaluations were similar to those described above.

Germination tests of dry spore products were conducted by placing droplets of spore suspensions (about 3.5×10^5 per milliliter) on 3% water agar disks, two per microscope slide. The disks were cut from a thin layer (about 3–4 mm) of water agar with a sterilized 1.5-cm-diameter cork borer. Slides were placed on glass V-rods in petri-dish moist chambers and incubated in the dark for 24 hr. Percentage germination was determined by counting 100 spores per agar disk, 200 per slide. The numbers of spores per gram of product were determined with a haemacytometer.

Sporulation enhancement. Highly sporulating cultures were desirable for use in dry spore production processes and for fresh inoculum in greenhouse tests. Occasionally, isolates representing broadly pathogenic races (valuable in cultivar screening) sporulated poorly; e.g., isolate 825 (IB-1) from Costa Rica. We utilized several procedures to obtain variants with enhanced sporulation: UV irradiation of 24-hr germinating spores on RPA in quartz tubes (Hanovia lamp, wavelength 254 nm at 0.5 m for periods ranging from 2–24 hr); X-radiation of germinating spores at 500 to 10,000 roentgens, of dry spores at 10,000 to 500,000 roentgens; and selection of highly sporulating variants as they occurred naturally.

TABLE 1. Races[a] of *Pyricularia oryzae* of which dry spore inocula have been produced

Geographic origin of cultures / U.S. Type Culture No.	429 Louisiana, USA	802 El Salvador	499 India	748 Philippines	559 Malaysia	468 Cambodia	712 Colombia	476 Cambodia	740 Sierra Leone	600 Guinea	602 Nigeria	551 Taiwan	439 Okinawa	733 Hong Kong	520 Japan	438 Taiwan	603 Arkansas, USA
Int'l Race No.	IB 54	ID 6	ID 8	ID 14	ID 16	IG 2	II 1	IA 111	IB 35	IB 47	IB 63	IC 19	IC 23	ID 7	ID 15	IE 3	IH 1
Rice Cultivar																	
Raminad Str. 3	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Zenith	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
NP 125	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Usen	−	+	+	+	+	−	−	+	+	+	−	−	−	+	+	−	−
Dular	+	+	+	−	−	−	−	−	+	−	−	+	+	+	−	+	−
Kanto 51	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	+	−
Sha Tiao Tsao (S)	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
Caloro	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+

Geographic origin of cultures / U.S. Type Culture No.	923 Philippines	760 Guyana	479 Philippines	640 Nicaragua	455 Philippines	825 Costa Rica	710 Colombia	899 Louisiana, USA	453 El Salvador	793 Louisiana, USA	908 Peru	695 Indonesia	580 Japan	699 Indonesia	649 India	737 Sierra Leone	566 Indonesia	549 India	900 Louisiana, USA	820 Japan
Int'l Race No.	IA 109	IB 45	ID 13	IG 1	IA 65	IB 1	IB 5	IB 17	IB 33	IB 49	IC 1	IC 9	IC 17	IC 25	ID 1	ID 5	ID 9	IE 1	IE 5	IF 1
Rice Cultivar																				
Raminad Str. 3	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Zenith	−	+	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
NP 125	−	−	−	−	+	+	+	+	−	−	+	+	+	+	−	−	−	−	−	−
Usen	+	+	+	−	+	+	+	−	+	−	+	+	−	−	+	+	+	−	−	−
Dular	−	−	−	−	+	+	+	+	+	+	+	−	+	−	+	+	−	+	+	−
Kanto 51	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+	−	+	+	−	+
Sha Tiao Tsao (S)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Caloro	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]Symbols: + = susceptible and − = resistant.

RESULTS

Specimen preservation. The fungus remained viable in leaf, node, and rachis lesions from field and greenhouse collections stored at -18 C for as long as 20 yr. Without exception, the cultures isolated from 35 specimens stored 10-20 yr were vigorous in colony habit and showed pathogenic patterns on the differential cultivars identical to those of the original isolates from these specimens.

Preservation of cultures. Storage of cultures on sterile rice nodes at −18 C proved to be a highly satisfactory and simple method of preservation. Among a total of 100 node cultures sampled after various periods of storage, 90% survived 3-yr-storage, 50% survived 10 yr, and about 10% survived 20 yr. Upon rehydration of nodes in petri-dish moist chambers, cultures were readily reisolated from sporulating surfaces (Fig. 1). All reisolated cultures showed patterns of pathogenicity identical to those of the original cultures.

Storage of dried CLP/filter paper cultures in coin envelopes was also a simple and effective method of culture preservation. Twenty 10-yr-old cultures, the maximum period tested for this method of storage, were 100% viable upon incubation in petri dish moist chambers.

Spore production processes. Cooking time for corn in relation to volume was found to be critical: it must be steeped long enough for moisture to penetrate to the center of the grains, yet not so long that sterilization by pressure-cooking will make it too soft and cause excess bursting of grains, exposing too much starchy endosperm. Exposed endosperm causes stickiness and promotes mycelial growth rather than sporulation. Air flow should not exceed 1 L/min to avoid rapid drying-out of steeped corn. Aeration increases pigmentation and sporulation of the fungus in "phase 1" growth. Although these spores are not part of the final spore product, pigmented mycelium yields more conidiophores and spores than does the white vegetative mycelium typical of growth in unaerated flasks. The optimal growth period for "phase 2" incubation varies with the isolate or strain. Highly sporulating isolates can be incubated for as long as 4 or 5 days before drying, with increasing numbers of spores being produced throughout the period. Poorly sporulating isolates are likely to be overtaken by mycelial growth, or the spores that are formed tend to germinate in situ; such cultures are usually best dried after about 48 hr. It is important that the corn be thoroughly dry (about 7% moisture); otherwise, moisture reacts with the solvent used in harvesting these spores, giving the spore cake a rubbery consistency that does not pulverize uniformly. The solvents of choice are Vythane and Genetron 113 (Freon 113). We emphasize here that all extractions of spores by use of these chemicals must be performed in a chemical hood to avoid breathing the toxic fumes. The solvents can be redistilled following spore harvest and used repeatedly.

The mycelial mat process is less wasteful of substrate and more efficient, but is not amenable for use with all cultures of the fungus. It is especially good for highly sporulating isolates, and with such isolates this process has yielded our maximum purity of spore product, 4×10^9 spores per gram, which was achieved with isolate 455 of Race IA-65.

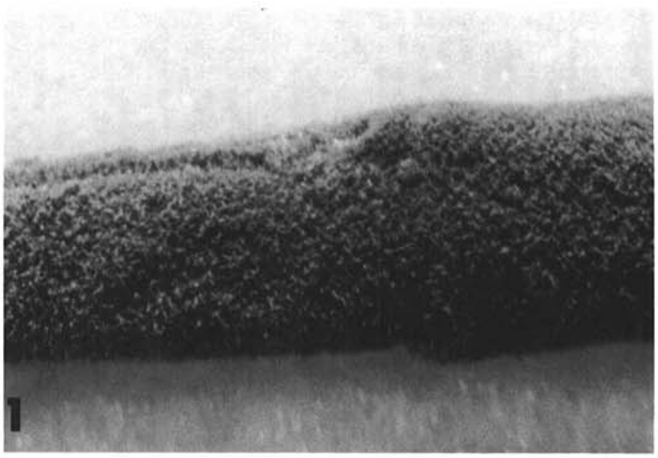

Fig. 1. Dense sporulation of *Pyricularia oryzae* from a rehydrated rice stem node after 10 yr of storage at −18 C.

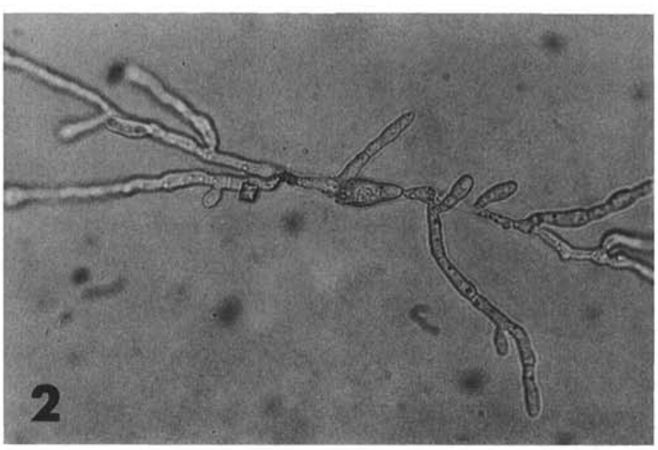

Fig. 2. Spore from 20-yr-old stored product of *Pyricularia oryzae* showing vigorous germination after 24 hr on moist membrane (26 C).

Fig. 3. Rice cultivar Frances. Plants on the left inoculated with race IA-111 (isolate 476) show susceptible type-4 lesions, but no "toxic" reaction; plants on the right show severe effect from inoculation with "toxic" strain 640 of race IG-1.

Spore products varied in virulence, just as the cultures from which they were derived. Different isolates of a single race often showed "quantitative" differences in virulence: i.e., they attacked the same cultivars in the differential series, but with more or less vigor; some caused larger, more definitive and uniform lesions, while others caused smaller lesions sometimes mixed with those typical of resistant reactions. In general, when choices were available, we selected the isolate that showed the strongest most definitive pathogenicity to susceptible cultivars for production of dry spore inoculum.

Between 1962 and 1970 we prepared 758 dry spore products representing 117 cultures and 50 races.

Spore product evaluation. Spore products of both processes stored since 1965 in sealed ampoules under liquid nitrogen retained high viability. Two samples that germinated in 1965 at 81 and 92% were 72 and 90% viable, respectively, after nearly 20 years' storage (Fig. 2). Tests of spores from 10 products so stored showed that they retained the vigor and pathogenicity to rice cultivars of the parent isolates. Other storage methods were quite satisfactory over a 20-yr period, even those in which screw-cap vials or jars were kept in an ordinary refrigerator at 4 C and opened frequently for sampling as inoculum. Five of these products (five races) stored since 1962 germinated poorly on agar and were overtaken by contaminants incurred during the spore production process, but were surprisingly pathogenic and always race-stable when inoculated onto differential rice cultivars. For example, cultures

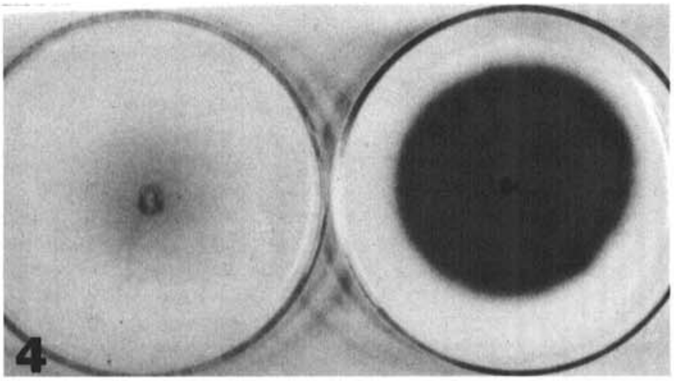

Fig. 4. *Pyricularia oryzae*. Left: poorly sporulating isolate (825) of race IB-1 that has not changed in cultural appearance or pathogenicity since its isolation in 1954. Right: densely sporulating X-ray mutant (825-D6) of culture on the left, selected in 1959, which has remained constant in growth habit and pathogenicity (race IB-1) since that time.

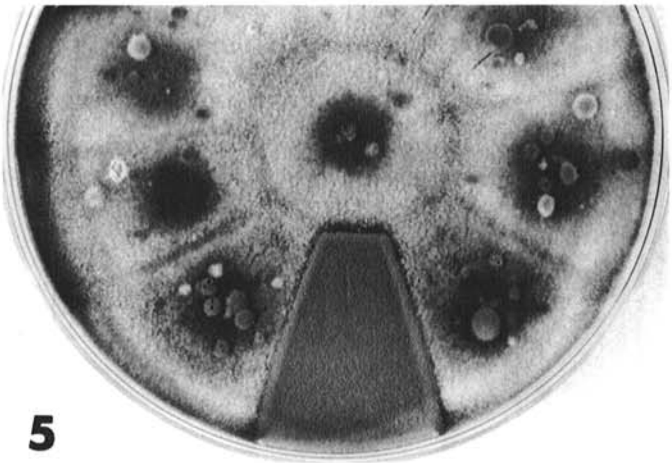

Fig. 5. Section of petri-dish culture (RPA) showing one densely sporulating variant among 10 single-spore isolates of *Pyricularia oryzae* from a previously single-spored culture. All 10 isolates showed identical race patterns.

reisolated from a 1962 product of 640 from Nicaragua, the first U.S. isolate of Race IG-1, infected susceptible cultivars in 1984 with the same intensity as had the original culture isolated in 1954. At that time, it was recognized as distinctive in causing susceptible cultivars to die without exposure to secondary dew periods beyond the initial night of incubation in a "dew chamber," as if a toxin were supplementing the infection process. Typically, susceptible cultivars in greenhouse tests "recover" if not exposed to repeated dew periods at night. After 22 years' storage in dry spore form, fresh isolates of this strain from a spore product caused plants of susceptible cultivars to die following standard inoculation procedures, just as had the original isolate in 1954. Comparison with a typical pathogenic strain is shown in Fig. 3.

Sporulation enhancement. Several isolates yielded improved sporulating capacity following UV irradiation of 24-hr germinating spores in quartz tubes, but no changes in pathogenic specialization occurred among 10 isolates exposed. Among 10 X-radiation experiments for each range and spore condition, several cultural changes were observed, but only one change in pathogenicity. This involved two irradiations of germinating spores from a poorly sporulating culture of Race IB-1; first at 500 roentgens, then a moderately sporulating single-spore isolate from this treatment was irradiated at 10,000 roentgens. Two densely sporulating mutants appeared among the 60 single-spored isolates obtained from the second treatment. One of these was of the same race as the parent culture, IB-1, and the other represented ID-8, a race with a much narrower host range on the differential cultivars. These cultures have maintained their growth habit and race type since they were selected from the X-radiated cultures in 1959, as has also the poorly sporulating but broadly pathogenic parent culture isolated in 1954 from a Costa Rican specimen. The parent isolate (825) and densely sporulating X-ray mutant (825-D6) of the same race (IB-1) are shown in Fig. 4.

Selection of natural cultural variants has yielded a number of highly sporulating isolates that have remained stable over many years of testing. Such variants for greater sporulation (Fig. 5) usually retained the pathogenicity of the parent isolate.

DISCUSSION

The various techniques described for culturing and harvesting spores for dry products and for preservation and inoculation of cultures in various forms have made possible the study of the pathogenic range of specific isolates over a period of years. As we had acquired isolates worldwide, we needed to find ways to maintain the many cultures in a state such that they would retain their original pathogenicity and degree of virulence in a form that could be used for testing at any time. We have developed techniques for producing dry spore inocula that, properly stored, remain viable and pathogenically stable for many years.

Two questions about the rice blast disease and its pathogen over which there is considerable controversy are: what is the true nature of the pathogen with respect to stability of its pathogenic races, and should breeding for resistance to specific races of *P. oryzae* be included in a breeding program for effective and stable resistance to blast? We have discussed previously (4) the effects of environment and nutrition on host reaction to blast and the discrepancies in evaluation of reaction that may result therefrom. Our experience has indicated that divergent results as to stability of races may be traced at least in part to seed impurity (genetic heterogeneity or heterozygosity) of differential cultivars, variation in reactions of differential cultivars under different growing conditions (especially nutrition), deficiencies in testing procedures and materials, and individual (subjective) differences in evaluation standards. The answer to this philosophical dichotomy must lie in materials, methods, interpretation, or all of these. We have described techniques for production and storage of dry spore inoculum suitable for quantitative dispersal (e.g., in a settling tower) for comparing the effects of different environmental conditions and nutrition on host susceptibility, as well as for qualitative determinations of cultivar reactions by spraying spores in water suspensions. Reproducibility of results and continuity among experiments is thus greatly enhanced.

That there is great pathogenic diversity within *P. oryzae* is not in question. In our own work we have characterized 50 races of the pathogen. However, phenotypic instability is not an inevitable consequence of the capacity for genotypic variability that may exist within populations of this pathogen. We have maintained isolates for as long as 30 yr in periodically transferred cultures with no apparent changes in pathogenic specialization. On the other hand, we have encountered occasional instances of striking pathogenic change during growth in culture. Our thesis is that, although mutation certainly occurs, and perhaps asexual, or even sexual recombination in light of recent findings (2,3,12), the rate of pathogenic change has been overestimated in some reports. We believe that the concept of "constant variability" (sensu Ou [9,10]) is misleading, and its acceptance would eliminate breeding for specific (vertical) resistance. We agree with Chien (1), who concluded regarding future research plans that, "It is important to select field resistance together with true resistance," and that "...a breeding program should be conducted under both controlled and natural conditions." There appears to be no obvious reason why the use of sources of general (horizontal) resistance should necessarily preclude the use of valuable sources of specific (vertical) resistance to virulent broad-range races when such are found. The two forms of resistance, for example, can be combined by using a horizontally resistant line as the recurrent parent in a program of backcrossing. We have proposed (4) that, through utilization of our spore production techniques, an international cooperative program could be set up such that cultivars selected for "field," "general," "horizontal," or "partial" resistance in any country of the world could be further screened by testing against dry inocula of specific races from all areas. These could be provided by any of several laboratories set up to receive blast specimens, isolate cultures, and produce dry inoculum of the different races. We can forsee, through the implementation of such a program, greatly increased communication and knowledge regarding sources of resistance and their effective use in rice breeding.

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