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Cultural and Pathological Studies of *Pyricularia oryzae* Isolates at Abomey Calavi in Benin

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Abstract: A study was carried out to establish on one hand, the genetic diversity of the populations of *Pyricularia oryzea* at Abomey Calavi and the other, to find out the favorable culture media for mass production of the conidia and the mycelium of *P. oryzea* for the laboratory techniques. Thus, trapping virulence races present in the prospected ecosystem in Abomey-Calavi helped to highlight the fact that the resistance genes: Piz-t, Piks, Pik-p, Pi1, Pi5 (t), Pi7, Pia, Pish and Pik were overcome by a large section of the pathogen population. Virulence genes that are capable of overcoming resistant genes Pii, Pi9, Pi33, Pi5 (t) Pi7 and Pik^m are however absent or rare. The latter represented the genes that were effective against the pathogen population studied. On the other hand, this study showed that Pi5 and Pi7 gene association, which is individually ineffective, has conferred a durable blast resistance observed in Moroberekan. Four operational conditions were selected on the basis of the spores quantities produced and the fastness of the mycelium growth. The addition of starch in the medium does not influence the fungus mycelium growth. But this growth is clearly influenced by extract doses of yeast. The best sporulation is obtained for culture media consisting of 20 g of starch and 2 g of yeast extract, 10 g of starch and 2 g of yeast extract L^{-1}.

Key words: Pathological diversity, *Pyricularia oryzea*, resistance genes, rice, sporulation

INTRODUCTION

Rice blast is one of the most spread and the most damageable diseases of rice in the south Saharan Africa. It is caused by a pathogenic fungus which teleomorphe stage is called *Magnaporthe grisea* Herbert (Barr, 1977) and the stage anamorphe is called *Pyricularia oryzae* (Rossman *et al*., 1990). Upland rice represents approximately 44% of the total of West African rice production and covers 57% of the cultivated surfaces. More than 70% of the farmers of the region cultivate it for subsistence (Jones *et al*., 1997). The pathogen is most common on leaves, causing leaf blast during the vegetative stage of growth, or on neck nodes and panicle branches during the reproductive stage, causing neck blast (Bonman, 1992). Leaf blast lesions reduce the net photosynthetic rate of individual leaves to an extent far beyond the visible diseased leaf fraction (Bastiaans, 1991). Neck blast is considered the most destructive phase of the disease and can occur without being preceded by severe leaf blast (Zhu *et al*., 2005). Rice production is under a constant pressure of leaf, panicle, neck and node blast causing significant damage to upland rice grow is estimated at 21-64% in West Africa (Awoderu, 1970). In south Saharan Africa, the use of resistant varieties remains the only method of control accessible to the small rice producers among which more than half are women. Blast resistance is one of the major objectives in rice (*Oryza sativa* L.) breeding in both tropical and temperate countries. The causal organism, *P. oryzea*, is known for its genetic instability, allowing it to overcome the genetic resistance of host plants (Shull and Hamer, 1994). Because of their low income, the rice growers in south Saharan Africa are not able to get fungicides and the use of resistant varieties remains the only effective weapon at their support (Roumen, 1994; Wang *et al*., 1994; Correa-Victoria and Zeigler, 1995). This only method of fight available encounters numerous obstacles among which the narrowness of the genetic base of the selected material, the evaluation of genetic material under pressure of selection not very representative and the great variability of the pathogenic capacity of the agent responsible of *P. oryzae* disease. The principal objective of this work, is to establish on one hand, the genetic diversity of the populations of *P. oryzea* at Abomey Calavi and the other, to find out the favorable culture media for mass production of the conidia and the mycelium of *P. oryzea* for the laboratory techniques.

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MATERIALS AND METHODS

Experimental site and plant materials: The studies were carried out at the Station of the International Institute of Tropical Agriculture (IITA/AfricaRice) at Abomey-Calavi in the zone of southern Guinean savanna of West Africa. The plant materials used are consisted of the variety RAM 90 and 31 varieties and isogenic lines and differentials presented in Table 1.

Seedling tray: Differential varieties and lines were pre germinated in Petri dishes in the laboratory. The Petri dishes with seeds were placed in seed stray for 5 days at a temperature of 27°C. Five days after seed germination in seed tray, young s eedlings were transplanted into 3 plastic trays (30×30×15 cm) filled with 5 kg of soil. The pots were fertilized with 10 g of NPK 15-15-15 at planting and 5 g of ammonium sulphate 21 days after sowing. Seedlings were performed in rows and spaced of 3 cm at ten sprouted grains by variety. The experimental design is a randomized design. The three containers were placed in the field of URB sown with the variety Ram 90. The blast was observed 4 days after the filing of the trays and at interval of 7 days.

Culture media: Twelve different starch and yeast extract compositions were studied in this trial. In the laboratory, the components (starch, yeast extract and agar) were weighed, mixed and prepared for replication of strains of *P. oryzea*. Table 2 presents the twelve culture media and their respective doses per liter of solution. As it can be seen these compositions are the result of a combination of three different doses of starch and four different doses of yeast. Thus; S1 = 20 g of starch, S2 = 10 g of starch, S3 = 0 g of starch, Y1 = 0 g of yeast, Y2 = 0.5 g yeast Y3 = 1 g of yeast and Y4 = 2 g of yeast. The 12 media are from a combination of (S1, S2, S3) and (Y1, Y2, Y3, Y4). Twenty grams of agar were added to each solution to allow solidification of the culture media.

Table 1: Differential complete series for the characterization of the structure of the population of pathogenic *Pyricularia grisae*

Variety names	Resistance genes
IRBL1-CL/CO	Pi1
Nato	Pii
Co39	Pia
C104PKT	Pi-3
OB677	Temoin
IRBLta2-IR64/CO	Pita-2
IRBLta-Ya/CO	Pita
K1	Pi-ta
IRBLzt-IR56/CO	Piz-t
Shin2	Pi-k^{s}+Pish
IRBL5-M/CO	Pi5(t)
IRBL7-M/CO	Pi7
IRBLkh-K3/CO	Pik-h
Tetep	Pita
IRBLsh-S/CO	Pish
75-1-127	Pi9
Tsuyake	Pik^{m}
IRBLsh-Fu/CO	Piz
IR1529	Pi33
Moroberekan	Pi5, Pi7
IRBLkm-Ts/CO	Pik^{m}
IRBLz5-CA/CO	Piz-5
Toride 1	Piz^{t}
IRBLk-Ku/CO	Pik
K59	Pi-t
IRBLks-CO/CO	Piks
IRBLb-I T13/CO	Pib
IRBLkp-K60/CO	Pik-p
St1	Pif

Source: Ling and Ou (1969)

Evaluation scale of the of reaction of host x parasite: The first notation was performed four days after the onset of symptoms. The ratings were made at regular intervals of seven days. For each pot, the number of leaves per plant, number of tillers and number of lesions we recounted. Ratings are made by the method proposed by Kiyosawa (1967). This method distinguishes four types of blast lesion as follows.

Bs: Spots held, brown, not differentiated which size varies with the point head of a pin. It is a reaction of over-sensitiveness characteristic of a vertical resistance.

Bg: A center and a rather uniform brown edge are distinguished. The center is gray or yellow pale. A spot bg is round or almost round.

bG: This type of spot resembles the previous from the point of view of the colors. On the other hand the spot is larger, typically spindle-shaped or is much lengthened. On the susceptible varieties, the spots which edging can be irregular or discontinuous are often prolonged by a yellowish surface.

pG: This type of spot is very sensitive. It is spindle-shaped with a gray ashes center with glaucous and a more or less diffuse edge, crimson or green olive. It is only met on the very sensitive varieties or very young seedlings.

With the method proposed by Kiyosawa (1967) it was possible to study of the spectrum of virulence of *P. oryzea*.

Table 2: Composition of the media culture studied in the trial (for 1 L of solution)

	No. of culture media											
Products (g)	1	2	3	4	5	6	7	8	9	10	11	12
Starch	20	20	20.0	20	10	10	10.0	10	0	0	0.0	0
Yeast	2	1	0.5	0	2	1	0.5	0	2	1	0.5	0

Study of the spectrum of virulence of *P. oryzea*: It was calculated the average of the following parameters: The No. of leaves; the No. of lesions of bg type on the leaf; the No. of lesions of type bG on the leaves and the No. of lesions of type pG on the leaves of the variety. The average No. of lesions bg, bG and pG type of the variety is:

$$\frac{1}{f_i}\sum_{j=1}^{f_i}(nbg_{ij} + nbG_{ij} + npG_{ij})$$

For each cultivar, this number is compared to the average number of lesions in the control "Moroberekan".

Collection and purification of *P. oryzea*

Collection : Leave fragments of approximately 1-1.5 cm of blast lesions were collected and incubated immediately in Petri dishes containing Agar 20 g, distilled water 1000 mL cooled after pressure-sealing 1 g of Penicillin, 1 g of Streptomycin were added). At the laboratory, the collected leave samples were well laid out on the medium. The Petri dishes were then placed in an incubator at 28°C during 24 h.

Purification: In the laminar flow in the presence of a Bunsen burner flame, the white agar was taken using a sterile colony of spores observed in the stereoscopic binocular needle. The spores of *P. oryzea* collected were deposited on a medium composed of 20 g Agar, 10 g starch, 2 g yeast and distilled water 1000 mL, cooling after autoclaving 1 g of Penicillin, Streptomycin 1 g were added. The Petri dishes were again placed in an incubator at a temperature of 28°C. Seventy two hours after the incubation the mycelium of each isolate was left in five Petri dishes of 9 cm in diameter. These Petri dishes were left in an incubator at 28°C for 8 days.

Statistical analysis: The results were subjected to statistical analysis using GenStat and means were separated by Duncan's Multiple Range Test at 5% level of significance.

RESULTS AND DISCUSSION

Evaluation scale of the host and parasite interaction: The statistical analysis of the results obtained from the lesions sensitivity bg, bG and pG of the isogenic lines (Table 3) counted at the beginning and the end of the epidemic, revealed that at the beginning of the epidemic 10 days after the sowing, 31 lines and varieties were categorized into four groups of distinct varieties, while at the end of the epidemic they were categorized into three groupes.

Table 3: List of isogenic lines and varieties tested and the lesions sensitivity bg, bG and pG of the isogenic lines counted at the beginning and the end of the epidemic

		Rate sensitivity lesions (%)	
Variety	Code	Beginning of the epidemic	End of the epidemic
IRBL1-CL/CO	V1	10	6
Nato V2	4	0	
Co39	V3	15	5
C104 PKT	V4	8	2
OB 677	V5	1	1
IRBLta2-IR64/CO	V6	2	1
IRBLta-Ya/CO	V7	4	1
K1 V8	0	1	
IRBLzt-IR56/CO	V9	6	13
Shin2	V10	3	5
IRBL5-M/CO	V11	3	8
IRBL7-M/CO	V12	5	10
IRBLkh-K3/CO	V13	1	3
Tetep	V14	3	1
IRBLsh-S/CO	V15	4	5
75-1-127	V16	0	0
Tsuyake	V17	1	0
IRBLsh-Fu/CO	V18	5	3
IR1529	V19	0	0
Moroberekan	V20	0	0
IRBLkm-Ts/CO	V21	1	0
IRBLz5-CA/CO	V22	0	1
Toride 1	V23	2	5
IRBLk-Ku/CO	V24	1	3
K 59 V25	0	3	
IRBLks-CO/CO	V26	8	12
IRBLb-IT13/CO	V27	0	1
IRBLkp-K60/CO	V28	13	11
Sum		100	100

So, at the beginning of the epidemic, the distinguished groups were: First group includes the following lines: 75-1-127 (PI9), K1 (Pi-ta), IR1529 (Pi33) Moroberekan (Pi5 (t) Pi7), IRBLz5-CA/CO (Piz-5), K59 (Pi-t) and IRBLb-IT13/CO (Pib). These lines are highly resistant, that is to say, bear no blast lesion. Thus, it could be said the corresponding virulence genes capable of overcoming the resistance genes are absent from the population of the surveyed site. The second group includes lines IRBLk-Ku/CO (Pik), IRBLkm-Ts/CO (Pikm), Tsuyake ($Pi\text{-}k^m$), IRBLkh-K3/CO (Pik-h) and OB 677 whose gene is not yet identified. These lines are resistant to most of the components of the pathogen population. These genes of virulence are rare in the population of the surveyed site. The third group is the group of varieties was of the group of the resistance control. They are: Tetep (Pita), Toride ($Pi\text{-}z^t$) IRBLta2-IR64/CO (Pit-2) IRBL5-M/CO (Pi5 (t), Shin2 ($Pi\text{-}k^s$). The fourth group includes of susceptible and highly susceptible varieties. They are: CO39 (Pia), IRBLkp-K60/CO (Pik-p), IRBL1-CL/CO (Pi1), C104PKT (Pi3), IRBLks-CO/CO (Piks, IRBLzt-IR56/CO (Piz-t), IRBL7-M/CO (Pi7), IRBLsh-Fu/CO (Piz), Nato (Pii), IRBLta-Ya/CO (Pita), IRBLsh-S/CO (Pish). It is important notice that the virulence genes are very important in this surveyed site.

Contrary to all expectations, at the end of the epidemic, the studied lines fall into three distinct groups: The first group consists of highly resistant lines carrying no blast lesion until the end of the epidemic. This indicates that the virulence genes capable of overcoming them are absent in the surveyed site. These genes of resistance are those of the following varieties: Nato (Pii), 75-1-127 (Pi9), Tsuyake (Pi-k^{m}), IR1529 (Pi33), Moroberekan (Pi5 (t) Pi7) and IRBLkm-Ts/CO (Pik-m). The second group consists of resistant strains. The genes of virulence capable of overcoming are present but rare, these genes are of the following varieties: Tetep (Pita) (the resistance control), IRBLb-IT13/CO (Pib), IRBLz5-CA/CO (Piz-5) IRBLta-Ya/CO (Pita), IRBLta2-IR64/CO (Pit-2), K1 (Pi-ta), OB 677 whose resistance gene is not yet identified. The third group of varieties is varieties which resistance genes have been overcome by the virulence genes of the surveyed site. They are the followings: C104 PKT (Pi3), IRBLkh-K3/CO (Pik-h), IRBLsh-Fu/CO (Piz), IRBLk-Ku/CO (Pik), K 59 (Pi-t), CO39 (Pia), Shin2 (Pik^{s}), IRBLsh-S/CO (Pish), Toride1 (Pi-z^{t}) IRBL1-CL/CO (Pi1) IRBL5-M/CO (Pi5 (t), IRBL7-M/CO (Pi7), IRBLkp-K60/CO (Pik-p), IRBLks-CO/CO (Piks), IRBLzt-IR56/CO (Piz-t).

Observing the behavior of varieties can show that the genes (Pi5) in IRBL5-M/CO and (Pi7) in IRBL7-M/CO varieties are individually ineffective while their association within Moroberekan gives a high resistance. Such an effect of several genes in a variety explains the interest of pyramiding genes to confer a high level of resistance to blast. Such associations also provide the benefit of a durable type of resistance difficult to overcome in time and space by the virulence gene as it has been shown in the cases of (Pi5) and (Pi7) and it is established that they are difficult to circumvent by the pathogen (Conaway-Bormans *et al*., 2003). This work also showed that the resistance genes (Pi1) and (Pi2) would have additional spectra of resistance and the accumulation of these genes in a variety (pyrimidine) may confer a potentially broad spectrum of durable resistance.

In addition, two types of behavior were observed in lines and varieties with the same resistance gene. The isogenic IRBLkm-Ts/CO constructed by transferring the gene (Pik^{m}) of Tsuyuake within CO39 has the same behavior as Tsuyuake. Vertical resistance genes Pi-ta^{2}, effective in the surveyed site of Abomey Calavi is also effective with strains from Senegal and other African, Asian and Latin American countries (Notteghem, 1981). Bidaux (1978) reported that 6% of West African strains tested by artificial inoculation were able to infect the variety carrying the resistance gene Pi-ta^{2}. Resistance genes that have not been overcome by the virulence genes of pathogenic populations of surveyed site were: Pii, Pi-k^{m}, Pi33, Pi5 (t) Pi7 and Pik-m. Sehly and Balal (1993) reported that the resistance genes Pi-b, Pi-k, Pi-k, Pi-z^{t} were very effective in Egypt against the Egyptian strains artificial inoculation.

Study of mycelium growth and of the sporulation of isolates of isogenic lines and differential *P. oryzea*: Data analysis indicated that there is no significant difference between the starch doses. In other words, starch intake does not influence the growth of the fungus. But the mycelium growth is significantly influenced by the concentrations of yeast extract. The existence of a significant interaction between starch and yeast extract reveals that the influence of concentrations of yeast extract varies according to the amount of starch in the medium (Sere *et al*., 2004).

The analysis of the data showed that for all concentration of starch, there is no significant difference between the environments in which the yeast extract was brought, either at a concentration of 0.5 g L^{-1} or 1 or 2 g L^{-1}. In other words, for any amount of starch, just add 0.5 g of yeast extract for optimal growth (Table 4). However, the mycelium mass is only abundant at the dose of 1 g L^{-1} of yeast extract. Regarding the sporulation on other hand, there is a significant difference between the concentrations of starch; this reveals the influence of different concentrations of starch on sporulation. Similarly, the different concentrations of yeast extract affect differently on the sporulation. When no starch is provided, yeast extract contributions have no effect on the sporulation. With 10 or 20 g of starch per liter, the best sporulation was observed for 2 g L^{-1} of yeast extract followed by 1 g L^{-1} (Table 4). But no difference was observed between the concentrations of 0.5 g of yeast extract and 0 g.

Behavior in culture and on different culture media of *P. oryzea* isolates: The behavior of *P. oryzea* in culture and on different media of 10 isolates has been studied in order to highlight differences between the various strains. The analysis of variance at 5% of the mycelium radial growth observed 8 days after incubation revealed a

Table 4: Average spore after incubation of the pathogen on different media

Starch				
Yeast	S1 (20)	S2 (10)	S3 (0)	Mean
Y1 (0)	2.3^{b}	2.5^{c}	3.2^{b}	2.7
Y2 (0.5)	3.7^{a}	3.8^{a}	3.5^{a}	3.6
Y3 (1)	3.6^{a}	3.7^{b}	3.5^{a}	3.6
Y4 (2)	3.7^{a}	3.5^{a}	3.5^{a}	3.5
Mean	3.3	3.4	3.4	3.3

In a column, means followed by the same letter are not significantly different at 5% level according to Duncan's Multiple Range test

significant difference between the different culture media, but no significant difference between the isolates. The analysis revealed also no interaction media and isolates. The ANOVA at 5% indicate that the different doses of culture media influence the mycelium growth for most isolates. The culture media parameter is not significant; the composition of the culture media did not affect the sporulation of the fungus. This behavior of the different isolates could be linked to their genes (Silue *et al*., 1992; Singh *et al*., 1998). The parameters isolate and interaction "culture medium and isolate" are significant (Table 4).

In conclusion, the using of trapping design of virulence races of blast fungus in the ecosystem of Abomey-Calavi led to the study of the genetic diversity of populations of *Pyricularia oryzae*. It is clear from this work that the resistance genes were overcome by a significant proportion of the pathogen population in the experimental site. Thus, virulence genes capable of overcoming the resistance genes Pi9 of isogenic lines 75-1-127 , Pi33 of IR1529, Pi5 (t) of Pi7 Moroberekan, Pii Nato, Pikm of Tsuyake and of Pik- m of IRBLkm-Ts/CO are absent or rare. These are effective genes from this experiment. They could be ssociated two or three in the same variety to ensure the sustainability of rice resistance to blast.

Moreover, the study of culture media for the production of spores and mycelia of *Pyricularia oryzae* by laboratory techniques allowed evaluating the effect of doses of starch and yeast extract for mass spore production and better growth of mycelium of the fungus. The results obtained from the twelve evaluated culture media based on starch and yeast extract showed that starch intake did not affect the mycelium growth of the fungus. But, this growth is significantly influenced by the dose of yeast extract. Thus, for any amount of starch studied, just add 0.5 g of yeast extract for optimal mycelium growth . On the other hand, when no starch is brought, yeast extract contributions have no effect on sporulation. So starch and yeast have complementary actions on the optimal growth of the mycelium. The best sporulation is obtained for culture media consisting of 10 g of starch and 1 g of yeast extract, 20 g of starch and 2 g of yeast extract L^{-1}. This information is useful when you wish to produce mycelium for DNA extraction, or when it is desired to produce spores necessary for pathogenicity tests.

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