# Acquired Tolerance to Blasticidin-S in Pyricularia oryzae

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ABSTRACT

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Thirteen naturally-occurring isolates of *Pyricularia oryzae* varied in tolerance to Blasticidin-S [1-(1′-cytosinyl)-4-[L-3′-amino-5-(1″-N-methylguanidino) valeryl amino]-1,2,3,4-tetradeoxy-β-D-erythro-hex-enuronic acid benzylamino-benzene sulfonate]. Isolates tolerant to 500, 700, 900, and 1,300 μg/ml of Blasticidin-S were obtained using ultraviolet (UV) irradiation and a high concentration of Blasticidin-S. Tolerance to Blasticidin-S was unstable in all isolates except Bu7, which was obtained from UV-irradiated conidia; that isolate retained a high level of tolerance even after 20 transfers on fungicide-free media. Successive monoconidial cultures of Bu7 were genetically homogeneous for tolerance. The tolerant isolate Bu7 sporulated more profusely and showed a higher percentage of germination of conidia on media with or without fungicide than did the parental isolate. The Blasticidin-S-tolerant isolate also was tolerant to the antibiotic, Kasugamin, but not to the organophosphorus fungicides, Hinosan and Kitazin. Isolate Bu7 was more virulent on seedlings of some rice cultivars than was the parental isolate.

*Additional key words:* rice blast, antibiotics.

Rice blast, which is caused by *Pyricularia oryzae* Cavara, is one of the most important limiting factors in rice production in rice-growing countries. Recently, the agricultural antibiotics and organophosphorus fungicides were found to be very effective against rice blast. The antifungal antibiotic, Blasticidin-S [1-( 1′-cytosinyl)-4- [L-3′-amino-5- (1″-N-methylguanidino) valeryl amino]-1, 2, 3, 4-tetradeoxy-β-D-erythro-hex-enuronic acid benzylaminobenzene sulfonate], has been widely used for the control of rice blast in Asia. As Blasticidin-S becomes more extensively used, the development of tolerance to it in *P. oryzae* may pose a serious practical problem.

Mutants of *Ustilago hordei,* tolerant to benomyl and carboxin, were induced by ultraviolet (UV) irradiation and were used to study the genetic and physiologic aspects of tolerance in the fungus (1). Yamasaki et al. (10) obtained copper-tolerant isolates of *P. oryzae* by repeated exposure of cultures to copper, although some cultures failed to adapt to certain copper concentrations. Similarly, tolerance to Blasticidin-S has been observed in *P. oryzae,* whereas development of tolerance to phenylmercury acetate was inconclusive (5, 8).

The lack of a sexual stage in *P. oryzae* limits the testing of the genetic aspects of fungicide tolerance by sexual crosses. Dutta and Garber (2) claimed a genetic origin of tolerance to acriflavine and filipin in *Colletotrichum lagenarium* by observing that the tolerance was maintained even after serial transfers were made on fungicide-free media, and that the heterokaryon was able to grow on a medium containing both fungicides.

Investigations on the genetic and physiologic aspects of tolerance to Blasticidin-S, which might have occurred in nature, could lead to a better understanding of the mechanisms of tolerance development. This paper reports on the tolerance of field isolates of *P. oryzae* to Blasticidin-S and on the genetic and physiologic characteristics of tolerant isolates originating from both UV-irradiated and nonirradiated conidia.

## MATERIALS AND METHODS

Isolates of *P. oryzae* were collected from the rice blast nursery at the College of Agriculture, Seoul National University, where no fungicides have been applied since in 1972. In preliminary experiments, a large number of pathogenic races were identified on the basis of the international set of differential host cultivars (9). Thirteen pathogenic races were selected for this study. Monoconidial cultures of each isolate were used throughout. The isolates used (race designation in parentheses) were: 2A2 (ID-15), 2A10 (IC-31), 2A15 (IA-65), 3A1 (IH-1), 3A5 (IA-79), 3A12 (IA-95), 3A13 (IA-109), 3A14 (IA-127), 3A17 (IE-3), 3A19 (IG-1), 3A20 (IA-125), 3A25 (IC-19), and 3A29 (IF-3).

The principal antibiotic used was Blasticidin-S, formulated as an emulsifiable concentrate with 2% active ingredient (2% EC). Fungicides tested for cross-resistance included the antibiotic Kasugamin (kasugamycin) at 2% EC; and two organophosphorus fungicides, namely Hinosan (O-ethyl-S-S-diphenyldithiophosphate) at 30% EC and Kitazin (O,O-diisopropyl-S-benzylthiophosphate) at 48% EC. The fungicides were added to

molten agar (50-55 C) at the required concentration before it was poured into petri dishes. The media containing fungicides were used 20 hr after preparation.

All cultures were grown on potato-sucrose agar (PSA) at 28 ± 1 C, unless indicated otherwise. Petri plates received agar disks 5 mm in diameter cut from the periphery of 10-day-old cultures, or were seeded with conidial suspensions ($10^5$ conidia/ml).

The experiments for production of tolerant isolates were carried out on PSA containing 200 μg Blasticidin-S/ml, about 15 times the concentration required to inhibit conidial germination of the wild-type isolates. Twenty ml of conidial suspension ($10^5$ conidia/ml) were irradiated by UV light at a wavelength of 254 nm from a distance of 45 cm and at about 130 erg/mm$^2$/second. During irradiation, the suspension was mixed continuously with a stirrer. Irradiation time (5-7 min) was regulated according to the UV-sensitivity of each isolate, to obtain a 4% conidial survival rate. The survival level was determined on the basis of a plate count of conidia germination on fungicide-free PSA. The irradiated conidia in suspension (0.5 ml/plate) were distributed on the surface of the medium containing Blasticidin-S. Twenty plates were seeded for each of the 13 isolates. The nonirradiated conidial suspension was applied in the same way to 20 plates for each isolate. After incubation for 5 days, colonies that developed were individually transferred to fungicide-free PSA and then tested for mycelial growth at various concentrations of Blasticidin-S.

Data obtained from the experiments for germination and sporulation were analyzed by analysis of variance and differences between means were evaluated using Duncan's multiple range test, $P = 0.01$.

TABLE 1. Minimum inhibitory concentrations of Blasticidin-S in potato-sucrose agar seeded with mycelial disks of 13 isolates of *Pyricularia oryzae*

| Isolate no. | Concentration (μg/ml) |
|---|---|
| 3A19 | 50 |
| 3A12 | 150 |
| 2A10, 3A1, 3A5, 3A29 | 200 |
| 3A20, 3A25 | 250 |
| 2A15 | 300 |
| 2A2, 3A13, 3A14, 3A17 | 350 |

TABLE 2. Numbers of tolerant isolates[a] of *Pyricularia oryzae* on potato-sucrose agar (PSA) containing various concentrations of Blasticidin-S

| Parental isolate | Blasticidin-S concentration (μg/ml) | | | | |
|---|---|---|---|---|---|
| | 500 | 700 | 900 | 1,100 | 1,300 |
| 2A2 | 2(1) | ... | ... | ... | ... |
| 2A15 | ... | 1 | 2 | ... | ... |
| 3A14 | (1) | ... | ... | ... | ... |
| 3A17 | (2) | 1 | ... | ... | 1 |
| 3A20 | ... | (2) | (2) | ... | ... |

[a]Obtained from colonies grown on 200 μg/ml Blasticidin-S-PSA seeded with $10^6$ conidia of each isolate. Numbers in parentheses are from nonirradiated isolates and the others from ultraviolet-irradiated ones.

## RESULTS

**Tolerance of field isolates.**—The concentration of Blasticidin-S necessary for complete inhibition of vegetative growth of each of 13 isolates was determined by measuring mycelial growth after incubation for 7 days on a series of media containing gradually-increasing concentrations of Blasticidin-S. The minimum inhibitory concentrations of the 13 isolates ranged from 50 to 350 μg/ml (Table 1). There was no apparent correlation between pathogenicity and tolerance to Blasticidin-S. The isolates, 3A5 and 3A25, highly virulent on many of the international differential cultivars, were moderately tolerant to Blasticidin-S (growth inhibited at 200 to 250 μg/ml); whereas the isolates 3A14 and 2A2 (less virulent than the other isolates) were the most tolerant (growth inhibited at 350 μg/ml).

**Acquisition of tolerance.**—Tolerant isolates of *P. oryzae* were obtained from colonies grown on PSA with 200 μg Blasticidin-S/ml, after $10^6$ conidia were seeded to 20 plates for each of the 13 isolates (Table 2). An equal number of plates were seeded with UV-irradiated or nonirradiated conidia, respectively. Three of the 13 irradiated isolates produced seven colonies tolerant to Blasticidin-S. Likewise, among the nonirradiated isolates four yielded eight tolerant colonies. The eight isolates not listed in Table 2 did not produce any tolerant isolates on media with 200 μg Blasticidin-S/ml. The isolates, 2A2, 2A15, 3A14, 3A17, and 3A20 (which in the first test were inhibited at relatively higher concentrations; i.e., 250-350 μg/ml, than the other isolates) developed tolerant isolates. Tolerant isolates varied in the level of tolerance to various concentrations of Blasticidin-S. Where UV-irradiation was carried out, of seven tolerant isolates, two were tolerant to 500 μg/ml, two to 700 μg/ml, two to 900 μg/ml, and one (designated Bu7) to 1,300 μg Blasticidin-S/ml. This latter isolate was derived from parental isolate 3A17 whose growth was completely suppressed at 350 μg Blasticidin-S/ml. Where no irradiation was carried out, four of eight tolerant isolates were tolerant to 500 μg/ml, two to 700 μg/ml, and two to 900 μg/ml. In general, tolerant isolates from the irradiated conidia exhibited greater tolerance than those from the nonirradiated conidia. With both methods, the frequency of tolerance development was 0.5-0.6 × $10^{-6}$ with some variation among isolates. These results also indicate that only five of 13 sensitive parent isolates could produce the isolates tolerant to Blasticidin-S, regardless of treatments.

TABLE 3. Tolerance of isolate Bu7[a] of *Pyricularia oryzae* on potato-sucrose agar containing Blasticidin-S

| Generation[b] of Bu7 | Monoconidial isolates | No. of tolerant isolates at Blasticidin-S concentrations of: | | |
|---|---|---|---|---|
| | | 900 μg/ml | 1,100 μg/ml | 1,300 μg/ml |
| First | 20 | 3 | 2 | 15 |
| Second | 20 | ... | ... | 20 |

[a]Tolerance induced by ultraviolet-irradiation of isolate 3A17.
[b]The first generation was obtained from conidia of Bu7 and the second one was from Bu7-3, one of the 15 first-generation isolates tolerant to 1,300 μg/ml of Blasticidin-S.

**Stability of tolerance.**—Mycelial disks of the tolerant isolates were transferred at intervals of 7 days to fungicide-free PSA. At each transfer, mycelial growth on PSA containing Blasticidin-S was tested to check the stability of tolerance of the isolates. After four to nine transfers on PSA without fungicide, all the tolerant isolates except Bu7 gradually reverted to a sensitive condition. Tolerance was not maintained by these isolates. In contrast, the tolerance of Bu7 was stable after 20 transfers or more on fungicide-free PSA.

Twenty monoconidial lines were isolated from Bu7. In the first generation, 15 of these showed the same level of tolerance as Bu7; the other five survived on 900 to 1,100 μg/ml. In the second generation from Bu7-3 (tolerant to 1,300 μg Blasticidin-S/ml) 20 of 20 isolates showed the same tolerance that the original monoconidial isolate showed (Table 3). Thus, Bu7-3 appeared to be genetically homogeneous for tolerance and the original Bu7, which was not a single-spored isolate, apparently was heterokaryotic as a culture.

**Physiologic traits of tolerant isolates.**—The tolerance of conidia to fungicide was tested by adding 0.3 ml of conidial suspension ($10^5$ conidia/ml) to 2% water agar containing Blasticidin-S, and then assessing percentage germination after 24 hr. One hundred conidia were examined from each of three plates per each isolate. The tolerant isolate Bu7 on water agar alone was similar in percentage germination to its parent isolate 3A17. In the presence of Blasticidin-S, however, there were marked differences in percentage germination between 3A17 and Bu7; 3A17 was highly sensitive and Bu7 was relatively insensitive (Table 4). Germination of 3A17 and Bu7 was inhibited completely at 11 and 60 μg/ml, respectively.

Sporulation of 3A17 (sensitive parent) and Bu7 (tolerant subculture from 3A17) was determined by crushing four 2-$cm^2$ squares from each culture in 10 ml of sterile water, and uniformly flooding 0.5 ml of this conidia and mycelial suspension onto the surface of PSA containing Blasticidin-S in a 9-cm diameter petri dish. After incubation for 7 days under continuous fluorescent light, the conidia were harvested by flooding the plate with 20 ml of water, and the resultant production of conidia was estimated with a haemocytometer. In the absence of Blasticidin-S, Bu7 produced many more conidia than 3A17 (Table 5). Sporulation of 3A17 was greatly reduced in the presence of Blasticidin-S, whereas sporulation of Bu7 decreased gradually as the concentration of Blasticidin-S increased (Table 5).

The isolate Bu7 grew better than 3A17 on 300, 500, 700, and 900 μg/ml of Kasugamin, but showed the same reaction as 3A17 on 20 and 40 μg/ml of Hinosan and on 90 and 110 μg/ml Kitazin. Thus, isolates tolerant to Blasticidin-S were also tolerant to Kasugamin, but not to Hinosan and Kitazin.

Tests were made to determine whether 3A17 and Bu7 differed in pathogenicity to rice seedlings in the greenhouse. The eight international differential cultivars were individually inoculated at the four- to five-leaf stage with conidial suspensions ($10^5$ conidia/ml) of each isolate. Disease ratings were made after 7 days according to the system proposed by the American and Japanese Cooperative Study (9). The two isolates were equally virulent on five of the cultivars, but 3A17 was less virulent than Bu7 on Zenith and Dular, and it showed no virulence on NP-125 in contrast to Bu7 which was moderately virulent on NP-125.

TABLE 4. A comparison of the percentage germination of conidia between parental (3A17) and ultraviolet-induced tolerant (Bu7) isolates of *Pyricularia oryzae* on water agar containing various concentrations of Blasticidin-S

| Blasticidin-S (μg/ml) | Germination of conidia of isolates: 3A17 (%) | Bu7 (%) |
|---|---|---|
| 0 | 96[a] | 93 |
| 5 | 51 | 91 |
| 10 | 5 | 84 |
| 11 | 0 | . . . |
| 15 | . . . | 77 |
| 20 | . . . | 72 |
| 40 | . . . | 24 |
| 60 | . . . | 0 |

[a]Percentages were determined by examining 100 conidia from each of three plates after 24 hr.

TABLE 5. Sporulation by parental (3A17) and ultraviolet-induced tolerant (Bu7) isolates of *Pyricularia oryzae* on potato-sucrose agar containing Blasticidin-S

| Blasticidin-S concentration (μg/ml) | No. (× $10^5$) of conidia/plate[a] 3A17 | Bu7 |
|---|---|---|
| 0 | 11.2 w | 62.2 w |
| 20 | 3.2 x | 26.7 x |
| 40 | 1.0 x | 16.7 y |
| 60 | 0.0 x | 0.9 z |

[a]Mean of six replicates in each of two experiments. Numbers followed by different letters are significantly different from each other according to Duncan's multiple range test ($P = 0.01$).

## DISCUSSION

Thirteen naturally-occurring isolates of *P. oryzae* varied in tolerance to Blasticidin-S. A similar observation has been reported (8). Since these isolates were selected randomly from the blast nursery where fungicides were not used, it is presumed that variation in tolerance may occur in natural populations of *P. oryzae*. This may reflect the variability of the fungus itself and/or mechanisms that operate in nature, regardless of the presence or absence of the chemical. No correlations were found between pathogenicity of isolates of *P. oryzae* and tolerance to Blasticidin-S, as has been observed in *Rhizoctonia solani* tolerant to chemicals (4). However, Shatla et al. (7) reported some correlation between the pathogenicity of *R. solani* isolates and tolerance to pentachloronitrobenzenes.

Attempts were made in this study to induce tolerance to Blasticidin-S in *P. oryzae* with UV-irradiation and with a high concentration of Blasticidin-S in the growth medium. In other studies (5, 8), other approaches were used to induce tolerance to Blasticidin-S. Our data indicate that the frequency of development of tolerance is low and varies among the parental isolates that are capable of growing on higher concentrations of

Blasticidin-S. This suggests that high levels of tolerance may develop in isolates inhibited at higher concentrations under field conditions rather than in those inhibited at lower concentrations. There could be several reasons why tolerant isolates were not obtained with more frequency from irradiated than from nonirradiated conidia, but the most likely reason is that the UV-irradation doses and Blasticidin-S concentrations tested in the present study may not have been suitable. Additional studies based on varied UV-irradiation doses and Blasticidin-S concentrations will be necessary to determine which factors are associated with tolerance development.

The tolerant isolates tended to revert to their original sensitive state when transferred on fungicide-free PSA. Isolate Bu7 was an exception. It is likely that Bu7 is a mutant which not only maintains tolerance, but also possesses physiologic traits that differ from those of the parental isolate. The variation in tolerance exhibited by the monoconidial isolates from Bu7 probably was due to the fact that the original Bu7 was a mass transfer and thus probably carried several nuclei through each subculture. It is probable that the monoconidial cultures of Bu7 were homokaryons and thus genetically homogeneous for tolerance.

Mutation to tolerance in *Hypomyces solani* f. sp. *cucurbitae* occurred only after some exposure to the fungicides (3). Our study suggests that *P. oryzae* may adapt physiologically to Blasticidin-S with no genetic change. It should be possible to obtain mutations toward tolerance using UV light as a mutagenic agent. The only isolate with permanent tolerance was obtained from irradiated conidia, but further work is required to establish a causal relationship.

Blasticidin-S and Kasugamin are related antibiotics with a similar mode of antifungal action. Thus, it is not surprising that isolates tolerant to Blasticidin-S also were tolerant to Kasugamin. There was no cross-resistance between Blasticidin-S and the organophosphorus fungicides such as Hinosan and Kitazin. This compares with the findings of Uesugi et al. (8), who showed that isolates tolerant to EBP (S-benzyl O,O-diethyl phosphorothioate) did not tolerate Blasticidin-S.

Mycelium of isolate Bu7 grew on 1,300 $\mu$g Blasticidin-S/ml, but germination of conidia of this isolate was completely inhibited at 60 $\mu$g Blasticidin-S/ml. This suggests a differential sensitivity between mycelium and conidia of Bu7. Parry and Wood (6) reported that the difference in tolerance between conidia and mycelium of fungi may be due to differential permeability of the cell walls.

Isolate Bu7 and the parental isolate 3A17 differed greatly in percentage germination in the presence of Blasticidin-S, whereas their germination was the same without the antibiotic. Sporulation of Bu7 was also greater than that of the parental isolate. The potential of the tolerant isolate to sporulate profusely is probably due to an independent mutation induced by UV and it should not be overlooked that the development of tolerance to Blasticidin-S appeared to increase rather than reduce pathogenicity. Indeed, the development of tolerance to Blasticidin-S would seem to be of epidemiological significance if tolerance can be accompanied by these other important traits in a natural population of *P. oryzae,* in spite of the fact that tolerance rarely was induced by UV or Blasticidin-S in the present study.

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