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# The puzzle of bakanae disease through interactions between *Fusarium fujikuroi* and rice

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## 1. ABSTRACT

Bakanae disease, one of the most noteworthy seedborne rice diseases, is caused by *Fusarium fujikuroi*, a member of the *Gibberella fujikuroi* species complex. The decreasing availability of chemical seed-dressing products over the last few years has raised the concerns of rice seed companies regarding bakanae disease. Therefore, new research trends require a deeper investigation into the main aspects of bakanae disease through interactions between rice and *F. fujikuroi*, in order to find new resistant or tolerant cultivars and alternative bakanae disease control strategies, as well as to develop more sensitive molecular diagnostic techniques. Here, some new aspects of *F. fujikuroi* epidemiology and pathogenicity, as well as its interactions with rice, are reported, and recent approaches applied to control bakanae disease are summarized.

## 2. INTRODUCTION

Bakanae disease is one of the most important diseases affecting rice (*Oryza sativa* L.). It was first identified in 1828 in Japan (1), and is present in the all of the major rice cultivation areas throughout the world. Over the last few decades, disease incidence and yield losses have increased in several Asian countries: Pakistan (2), Malaysia and Indonesia (3), Bangladesh (4), India (5), South Korea (6) and Taiwan (7). Yield losses ranging from 5 to 15%, with an increasing trend, have also been found in Italy, the main European rice producer (219,500 ha and 1,386,100 t in 2014). Bakanae disease continues to spread throughout the world on rice, with reports in California (8), FYR Macedonia (9), Russia, the Philippines and Thailand (10). *F. fujikuroi* has also been isolated from other hosts, such as grapevine (*Vitis vinifera*), little bluestem (*Andropogon scoparius* Michx.) and water grass (*Echinochloa* spp.), but Koch's postulates to prove *F. fujikuroi* pathogenicity of these hosts are still missing (8, 11, 12, 13). *F. fujikuroi* has recently been reported to cause pre- and post-emergence dumping off on soybean, and it fulfilled Koch's postulates (14).

*Fusarium fujikuroi* Nirenberg (teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura), the causal agent of bakanae disease, belongs to the *G. fujikuroi* polyphyletic taxon species complex (GFSC; 15). The most striking disease symptoms are yellowing and anomalous elongation of infected plants, due to the production of plant/fungal gibberellic acid, which has resulted in the disease being called with the Japanese word 'bakanae' which means the

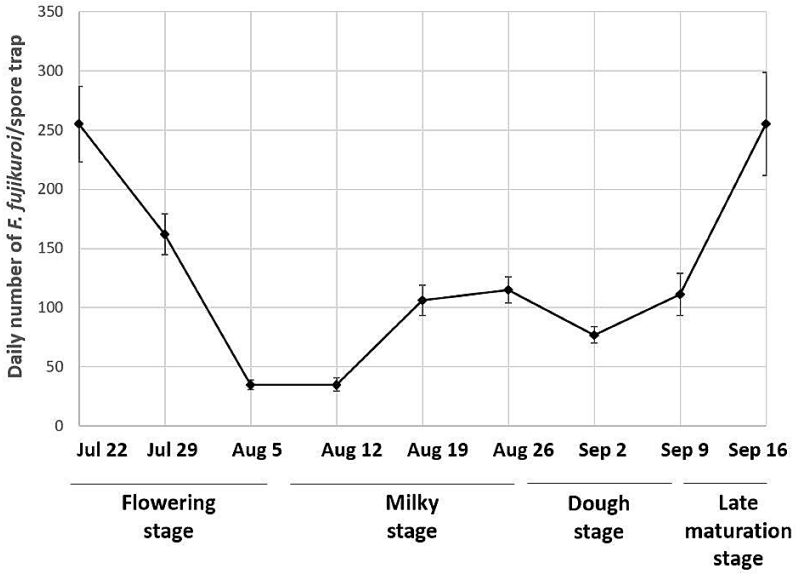


**Figure 1.** Trend of aerial dispersion of conidia of *F. fujikuroi* during the rice cropping season. Monitoring carried out during July-September 2010. Error bars indicate standard deviations from the measurement of three samples.

'foolish seedling'. Gibberellins are also involved in spore germination and the elongation of young fungal hyphae (16). Apart from gibberellins, the fungus is also capable of synthesizing other secondary metabolites, such as beauvericin, fusaproliferin, moniliformin, bikaverin, fusaric acid, fusarin and fumonisins (17, 18, 19, 20, 21, 22, 23).

Since bakanae disease is seedborne, the best preventive strategy for disease control is the use of healthy seeds. Furthermore, the application of curative methods can mainly be applied to seeds in the form of chemical, physical, or biological seed dressings (24). This review describes some recent achievements in the epidemiology, pathogenesis and control methods of *F. fujikuroi* on rice.

## 3. EPIDEMIOLOGY

Bakanae is a monocyclic disease. The fungus produces conidia on diseased plants, which are easily spread by wind and water (5). The high production of conidia on infected or dead culms in the field coincides with flowering and ripening of rice, when the conidia are able to infect or contaminate the seeds (25). Infected kernels develop a reddish color due to the presence of conidia, and the whole seed becomes discolored when severely infected. The fungus can also be isolated from asymptomatic seeds, if they are collected from a highly infected rice field.

Airborne ascospores have also been reported, as an infection source, at the flowering stage of the crop (26). Moreover, the fungus can infect seedlings at an early stage of development, when it becomes systemic in the plant, but without any colonization of the floral organs. The first 72 hours after seed germination are critical for the development of the disease, which is favored by high amounts of exudates (sugars and amino acids) from germinating seeds. *F. fujikuroi* growth is also stimulated by temperatures from 27°C to 30°C, and by higher levels of nitrogen in the soil. The assumed form of the disease is further affected by humidity; high humidity leads to elongation of the culms, while low humidity causes rice plant stunting. The microconidia and mycelia of the pathogen develop in vascular bundles, particularly in larger vessels and in the xylem gaps, while the phloem and parenchyma do not seem to be infected. The fungus overwinters in infected seeds, and these represent the main source of inoculum for the following season.

A study has been performed to confirm the influence of the wind on the conidial dispersion and diffusion of bakanae within the rice field (personal communication). A seven-day spore trap (Burkard Scientific) was positioned to trap air-borne particles in Northern Italy (Trino, VC) in the center of a rice field sown with local rice lines (SAPISE Soc. Coop., Vercelli, Italy). The rice field was surrounded by a susceptible rice cultivar, 'Galileo', which originated from a seed lot with a high infection rate of *F. fujikuroi*. Spore monitoring was carried out daily, from flowering until harvest. Microscopic observations of the tape indicated that there was no uniform trend of diffusion of *F. fujikuroi* conidia over the monitored period, but there was an increase during the flowering (July 22-30, 2011) and late maturation stage (September 9-16, 2011; Figure 1). A slight increase in conidial transmission was also observed at the milky stage of grain maturation

(August 19-26, 2011). A greater presence of winds and rains was also registered at flowering and at the end of maturation in the experimental area (http://www.ilmeteo.it/portale/archivio-meteo/Vercelli), than for the rest of the monitoring period and the previous growing seasons, which indicates that wind and rain might contribute to the transmission of *F. fujikuroi* conidia.

Briefly, the present results show that aerial conidial diffusion of *F. fujikuroi* occurs, as a result of the transmission of conidia from the highly infected cultivar 'Galileo'.

## 4. PATHOGENICITY AND MONITORING OF *FUSARIUM FUJIKUROI*

A total of 146 strains of Fusarium *spp.* were isolated from infected rice plants and seeds from several growing areas in Northern Italy (Piedmont) in 2008, 2009 and 2010. The strains were molecularly characterized by sequencing a portion of the translation elongation factor 1-α (*TEF 1-α*) gene (27). The molecular characterization revealed that 85% of the strains belonged to GFSC, and 79% of the strains within GFSC were *F. fujikuroi*, thus confirming that this species is the principal *Fusarium* species on bakanae-infected rice. The other *Fusarium* spp. (*F. proliferatum*, *F. verticillioides*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. avenaceum, F. brevicatenulatum*, *F. napiforme*, and *F. sporotrichioides*) were isolated from seeds that had a lower level of incidence. Of the 146 isolates, 121 were tested for their pathogenicity on 'Galileo' rice. The typical bakanae symptoms were visible 14 days post-germination (dpg), but only on the plants inoculated with *F. fujikuroi* strains. The heavily infected plants died within 28 dpg. Eighty-eight percent of *F. fujikuroi* strains resulted pathogenic; 5% were weakly virulent, while 7% were non-pathogenic. Several *F. fujikuroi* strains did not cuase the typical bakanae symptoms, but did cause plant yellowing, in agreement with the observations of Ou (25) and Zainudin *et al*. (3). All the *F. fujikuroi*, *F. proliferatum and F. verticillioides* strains were re-isolated from inoculated rice seedlings, while the other seven tested *Fusarium* spp. were not re-isolated, thus suggesting their possible presence in the form of saprophytic or epiphytic fungi. Similar study has been carried out on basmati rice in India, where *F. fujikuroi* was found as the most dominant species, and all the 41 isolates of *F. fujikuroi* resulted pathogenic (29% highly virulent, 34% medium virulent, and 37% moderately virulent (28).

Different degrees of virulence were observed among the *F. fujikuroi* strains from the same cultivation area and from the same cultivars, thus indicating the possible influence of genetic features of the strains, rather than the involvement of environmental factors. *Fusarium fujikuroi* genetic structure is mainly clonal in Italy, but the 1:1 ratio of mating type alleles found in 6 out of 8 Italian fungal populations indicates that sexual-reproduction of *F. fujikuroi* may occur in the field (29). Similar coexistence of both mating types was also observed within *F. fujikuroi* populations in Taiwan (7).

## 5. MOLECULAR DIAGNOSIS

Although *F. fujikuroi* is the causal agent of bakanae disease, several other *Fusarium* spp. could also be present on rice, but at a lower incidence. In particular, two species of GFSC, *F. verticillioides* and *F. proliferatum*, have been isolated in different rice areas in the world (10, 30, 31), and these species have also been considered as thebakanae-disease causative agents for decades. Since some species belonging to GFSC are morphologically indistinguishable (*e.g. F. fujikuroi* and *F. proliferatum*), the support of more sensitive molecular techniques is necessary. To this end, molecular techniques were initially developed using random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) techniques, and electrophoretic karyotype analysis to separate *Fusarium* isolates with different degrees of virulence, and to distinguish among the different species of GFSC (32, 33). Recently, a set of various polymorphic simple sequence repeat (SSR) marker have been developed as a sensitive molecular technique for characterization of the genetic variation among different *F. fujikuroi* populations (7, 29).

Internal transcribed spacer region (ITS), which is commonly used in the identification of fungal species, was found to not be reliable in the case of *Fusarium* species belonging to GFSC, as it leads to incorrect phylogenetic inferences. A portion of the *TEF 1-α* gene, because of its elevated sequence polymorphism among the highly related *Fusarium* spp., was found to be well suited for the development of the polymerase chain reaction (PCR) to molecularly characterize *Fusarium* at a species level (15, 34). The multiple alignment of the *TEF 1-α* sequences of different *Fusarium* spp. has evidenced a six-nucleotide deletion in the *F. fujikuroi* sequences, and a two-nucleotide variability in *F. proliferatum* in the same region. Two species-specific primer pairs were designed by amplifying a 179-bp product for *F. fujikuroi* and a 188-bp product for *F. proliferatum*, and their specificity was confirmed by analyzing 298 strains of *Fusarium* spp., isolated from Italian rice plants and seeds. The variability of the same *TEF 1-α* region was used to develop a real-time PCR for the screening of a high number of bakanae-infected plants. A Sybr-green real-time PCR assay was developed and was found to be a sensitive and specific tool for *F. fujikuroi* detection from infected rice plants, but not sensitive enough for a reliable detection from rice seeds (35). PCR assays, in which the primers from other gene sequences, such as those involved in the biosynthesis of mycotoxins have also been described as an additional tool for *F. fujikuroi* detection (23, 36,

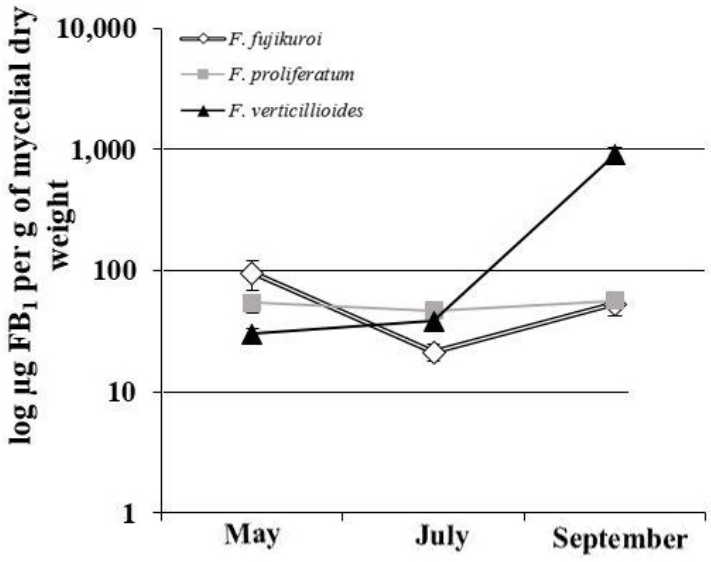


**Figure 2.** Effect of different temperatures and day lengths simulating the average May (23°C and 15 h of light / 12°C and 9 h of dark), July (32°C and 15 h: 30 min of light / 19°C and 8 h: 30 min dark) and September (25°C and 12 h: 30 min of light / 11°C and 11 h: 30 min of dark) conditions in northern Italy on $FB_1$ production in *F. fujikuroi* (strain Augusto2), *F. proliferatum* (strain 11-471) and *F. verticillioides* (strain 19-115). Strains were grown in PDB for 10 days by shaking (100 rpm) at 20 °C. Initial concentration was 2 x $10^3$ spores/ml. Error bars indicate standard deviations for three assays.

37). A primer-introduced restriction analysis PCR (PIRA-PCR) has recently been developed to determine *F. fujikuroi* genotypes resistant to methyl benzimidazole carbamate-group fungicides (38).

## 6. MYCOTOXIN POTENTIAL OF *FUSARIUM FUJIKUROI*

*Fusarium* spp. produce a broad spectrum of biologically active secondary metabolites, such as mycotoxins, which pose health risks to animals and humans (39). One of the most important classes of *Fusarium* mycotoxins is fumonisins. The role of fumonisins in the fungal life cycle and pathogenicity is still unclear. The main fumonisin ($FB_1$) inhibits the enzyme ceramide synthase, which is included in sphingolipid biosynthesis in plants and animals. This affects the cell function and may result in cell autolysis and inhibition of the plasma membrane ATPase (40).

Three members of GFSC (*F. fujikuroi*, *F. proliferatum*, and *F. verticillioides*), originating from rice, were screened to establish their fumonisin-production ability ($FB_1$ and $FB_2$) (23). Various light conditions (red, yellow, green, blue, white, and light/dark alternations) and different temperatures were applied during the experiments. Most of the strains of the three *Fusarium* spp. stimulated a significantly higher fumonisin production under light than under dark ($P$<0.0.5). A more abundant fumonisin production was observed in the *F. fujikuroi* and *F. proliferatum* strains under white and blue light, and light/dark alternations, and in the *F. verticillioides* strains under yellow and green light. The similar light-regulated fumonisin biosynthetic pattern of *F. fujikuroi* and *F. proliferatum* is probably related to their close phylogenetic relationship, compared to the other members of GFSC. In spite of the high conservation of the fumonisin gene cluster for both *F. fujikuroi* and *F. verticillioides*, the production of fumonisins was found to be different under the same growing conditions (23, 41). It was concluded that *F. fujikuroi* might have a good and similar fumonisin production capacity to the other two species (*F. verticillioides* and *F. proliferatum*), a conclusion that differs from the previous reports (31, 37, 42). Furthermore, the overexpression of the fumonisin-specific transcription factor (*fum21*) can lead to an increased fumonisin production of up to 1000-fold (41). Interestingly, in a recent report by Bolton *et al*. (13), the potential of *F. fujikuroi* to produce fumonisins has also been observed on wine grapes, thus emphasizing the need for further investigations on the ability of this fungus to produce mycotoxins.

The fumonisin-producing potential of *F. fujikuroi, F. proliferatum,* and *F. verticillioides* has also been tested at different temperatures. One strain of each *Fusarium* species was grown under average temperatures and day lengths of May, July and September in northern Italy, which represents the main rice production area in Europe. For *F. fujikuroi* strain Augusto2 (personal communication) $FB_1$ production was the highest for the May conditions, followed by the September conditions, whereas $FB_1$ synthesis in July was significantly reduced (Figure 2). *F. proliferatum*

(the strain 11-471) showed a similar production of $FB_1$ for the three conditions, with a slight decrease during July. *F. verticillioides* (the strain19-115) produced less $FB_1$ during May and July, but its production significantly increased during September (24-30 fold).

## 7. GENOMES OF *FUSARIUM FUJIKUROI* STRAINS AND THEIR GENE CLUSTERS

During 2013, two complete *F. fujikuroi* genomes were sequenced (43, 44) with a size of 43.8-43.9 Mb and an arrangement of 12 scaffolds, referring to the 12 fungal chromosomes. When the *F. fujikuroi* genome was compared with those of six other *Fusarium* spp., it was observed that some *F. fujikuroi* related species contained GA-biosynthetic genes, but GA production was restricted to *F. fujikuroi* on rice (31, 44). Furthermore, comparisons with the draft genomes of three geographically distant strains of *F. fujikuroi* (12) indicate diverse secondary metabolite profiles among different strains. *F. fujikuroi* genetic diversity based on species and lineage-specific genes were found concentrated in subtelomeric regions, which might be associated with fungal adaptation to environmental changes and host colonization (12).

Moreover, three Italian strains of *F. fujikuroi* (Augusto2, CSV1 and I1.3), which had previously been characterized for a different pathogenicity and mycotoxin profile (23), were sequenced, and the genome was assembled through a reference-guided approach, in order to identify the allelic variants in the genes responsible for the production of secondary metabolites, such as fumonisin, fusaric acid, and gibberellins (45). About 44.3 Mb of draft genomes, containing the 12 chromosomes were generated. Overall numbers of 14080, 14064, and 14194 proteins were predicted for the three genomes, respectively. The gene clusters involved in gibberellin, fumonisin, and fusaric acid production were determined; the gibberellin and fusaric acid clusters did not show any relevant amino acid differences. On the other hand, the fumonisin cluster of strain I1.3 showed amino acidic differences within *FUM1, FUM21,* and a possible deletion in the transcript *FUM13* (45). All these changes in the I1.3 strain might be involved in the fumonisin biosynthetic pattern, and this could result in its lower fumonisin production ability compared to the other two sequenced *F. fujikuroi* strains (23). Furthermore, identical amino acid sequence of the gibberellin synthesis cluster, observed for the three fungal strains with different pathogenicity patterns, might be explained with an influence of epigenetics in the GA gene expression. Thus, the COMPASS Component Ccl1 deletion mutants of *F. fujikuroi* with reduced GA biosynthesis did not show reduced virulence, suggesting that other unknown plant signals might compensate the role of gibberellins during pathogenesis (46).

## 8. RESISTANT GENOTYPES

Since the first report of bakanae disease was drawn up, great efforts have been made to find resistant rice cultivars. Several resistant genotypes have been identified, but they only represent a minor portion of the rice germplasm. Ito and Kimura (1) were the first to identify three bakanae-disease resistant Japanese genotypes. However, recent studies have reported thirteen genotypes with moderate or high resistance, five genotypes with medium resistance, and one genotype with moderate resistance, respectively (47, 48, 49). Three resistant accessions have been found among the rice genotypes that contained various dwarf or semi-dwarf genes (50). Moreover, using inclusive composite interval mapping, three quantitative trait loci regulating resistance of rice basmati to *F. fujikuroi* were reported: *qBK1.1*, *qBK1.2*, and *qBK1.3* (51).

No resistant varieties have been reported in rice cultivated in temperate climatic areas, until now. Twelve commercial rice varieties have been evaluated in greenhouse conditions to establish their resistance to bakanae disease (52). *F. fujikuroi* pathogenic strain I1.3 was used for inoculation of rice seeds at final spore concentration of $10^6$/ml by seed soaking and shaking in the spore suspension. Plants were maintained at temperatures of 25 °C (day) and 17 °C (night) and they were watered 3 times per day. Disease symptoms were evaluated at 21 dpg according to a bakanae disease index (d.i.; 27): symptomless plants (0%), plants with chlorotic leaves and delayed growth (25%), plants with thin and elongated internodes (50%), plants with crown necrosis (75%), and dead plants (100%). Among all the genotypes tested against *F. fujikuroi*, the 'Selenio' genotype (d.i.: 17%) showed the highest level of resistance, while the 'Dorella' genotype presented the highest susceptibility (d.i.: 83%; 23, 52). A field trial performed during summer 2016 (Castello d'Agogna, PV, Italy) confirmed these data under a high pressure of *F. fujikuroi* infection, where 'Selenio' exhibited no typical bakanae symptoms, whereas 'Dorella' showed a higher disease index than 3.5 (personal communication). These recent results suggest that rice resistant accessions might be found among the cultivars and lines under development (52). The choice of the cultivar, based on its resistance to bakanae, could be advantageously combined with preventive control methods, such as the use of healthy rice seeds.

## 9. PLANT-*FUSARIUM FUJIKUROI* INTERACTIONS: MOLECULAR PATTERNS

The molecular regulatory mechanisms of rice defense responses against *F. fujikuroi* have not yet been fully clarified. In order to elucidate the factors involved in rice resistance against bakanae disease, an RNA-seq transcriptome study has been carried

out. The molecular events that take place during the response of the resistant 'Selenio' cultivar and susceptible 'Dorella' cultivar were identified at 7 and 21 dpg (52). More meaningful transcriptional changes were found at 21 dpg, thus emphasizing the importance of this infection stage in the defense strategy of rice. The number of differentially expressed genes (DEGs) was 3,119 in 'Selenio', and 5,095 in 'Dorella'. The basic rice resistance machinery against *F. fujikuroi* involved PR genes, glucanases and peroxidases, since they were upregulated in both the resistant and susceptible cultivars. The specialized and evolved resistance mechanisms, which were only found in 'Selenio', included WRKY transcriptional factors, MAPK cascades, some cytochrome P450 genes, and the following gene ontology (GO) categories: 'response to chitin', 'jasmonic acid biosynthetic process', and 'plant-type hypersensitive response', which might constitute the rice resistance platform against bakanae disease. These mechanisms were further confirmed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) identification (53) of the $Ca^{2+}$-dependent protein kinase gene, JASMONATE ZIM-DOMAIN-like genes, *CEBiP*, *CERK1*, and *MYC2* genes that were found only in 'Selenio'. These genes participate in one of the molecular patterns: response to chitin, jasmonic acid biosynthesis, and plant hypersensitive response, as described above. 'Dorella' instead specifically activated chitinases, gibberellin-metabolic genes, and the GO categories: 'response to salicylic acid stimulus' and 'gibberellin metabolic process', which could be the key factors of the response of susceptible rice to *F. fujikuroi*. Overall, the transcriptome profiling has suggested a different mode of action of *F. fujikuroi* between the resistant and sensitive genotypes. Thus, the fungus in 'Selenio' acts locally, at lower concentrations, and causes a rice hypersensitive response without any further damage to the plants, while it behaves like a necrotroph in "Dorella" through a systemic infection that provokes a gibberellin imbalance, irregular growth and eventually plant death.

At the same time, an analogous transcriptomic study has been recently published (54), studying the response of two rice cultivars (moderately resistant cultivar '93-11' of *O. sativa indica*, and susceptible cultivar 'Nipponbare' of *O. sativa japonica*) to *F. fujikuroi*. Similarly, defense-related DEGs, such as WRKYs and MAPK cascades, were up-regulated in '93-11', and the GO categories correlated to chitin including 'chitin metabolic process', and 'chitin catabolic process' were found only in resistant cultivar, confirming their association in rice resistance machinery to bakanae disease.

Finally, the transcriptomic study permitted the molecular defense responses of the bakanae-induced rice to be revealed. Molecular patterns may be further compared with the chemical responses included in the *F. fujikuroi*-rice interaction puzzle, described in the paragraph below.

## 10. PLANT-*FUSARIUM FUJIKUROI* INTERACTIONS: CHEMICAL PATTERNS

The accumulation of phytohormones, whose genes were found to be involved in the response of rice against *F. fujikuroi,* was further investigated by means of high performance liquid chromatography (HPLC). The upregulation of the gibberellin metabolism genes in 'Dorella' that exhibited specific bakanae symptoms and systemic fungus infection, matches the gibberellin production data, which showed 12 times higher levels than 'Selenio' (52). The results confirmed the importance of this group of secondary metabolites in the response of rice to bakanae disease. When the gibberellin production was controlled, the 'Selenio' plants activated the jasmonic acid metabolic pathway, which was previously found in plants stressed under different conditions (55), and confirmed by our enriched JA biosynthetic data. In this way, 'Selenio' maintained its bakanae-resistance level. A similar situation was also observed for other phytohormones. Jasmonic acid was produced uniformly in 'Selenio' during the observation period, while it was highly decreased in 'Dorella' at 14 dpg. The salicylic acid value was high in 'Dorella', particularly at the end of disease development, and its concentration was always higher than in the healthy control, whereas 'Selenio' produced more salicylic acid in the healthy control than the infected plants (56).

Moreover, the production of rice phytoalexins, which are in general included in the rice response against various adversities, was examined since some of the phytoalexin-associated genes were found to be activated in 'Selenio' (52). Four rice phytoalexins were measured (naringenin, sakuranetin, and momilactones A and B), and increased concentrations of all of them were only found in 'Selenio'. The greatest increase in phytoalexin biosynthesis was observed for sakuranetin and momilactone A. The obtained chemical responses suggest that the secondary metabolites of the rice are involved in the development of bakanae disease, and that their production is different between resistant and susceptible genotypes. Finally, the chemical data are in agreement with the molecular data, and the activated genes lead to biosynthesis of the signaling components and phytoalexins two to three weeks after rice germination (52, 56).

## 11. SEED DRESSING AND LEGISLATION

Until recently, chemical seed dressings have been the most common way of controlling rice bakanae disease. In some Asian countries, such as China and Japan, the seeds are dressed by dipping them in an aqueous suspension containing fungicides

(prochloraz, carbendazim, trifumezol, thiram, or others; 57). In the last few years, due to the decreasing availability of authorized chemicals for rice seed dressings, particularly in the European Union (EU) and the United States, the importance of the disease has grown considerably.

Seed companies, in both temperate and tropical countries, have to commercialize certified seeds free from *F. fujikuroi,* but reduced number of registered fungicides is urging the companies to find solutions to overcome *F. fujikuroi* incidence. During the previous decade, prochloraz was the most frequently used active ingredient for the control of bakanae disease, but, since 2015, its application has been banned in the EU. Moreover, the technical issue of increasing occurrence of prochloraz-resistant strains of *F. fujikuroi* is posing a new risk (58).

A chemical alternative, fludioxonil (the first lower risk seed fungicide) is allowed, and it has shown an 80% reduction in bakanae disease incidence in Italy (59). A survey conducted in rice fields destined for the production of certificated seeds has indicated that around 70-100% of seeds had previously been dressed and used to establish those fields. Our monitoring in rice certification fields in northern Italy over the last eight years has shown an average presence of 15% bakanae affected plants, after manual removal of symptomatic plants at the early disease stage during routine agricultural operations (59).

## 12. ALTERNATIVE SEED TREATMENTS

Because of the low number of active ingredients authorized for seed dressings, alternative methods for bakanae control have been proposed, including physical (hot water, hot steam and dry heat) and biological seed treatments (60, 61). Thermotherapy, which is conducted on rice seeds either by applying hot water (60° C for 15 minutes) or steam (90% relative humidity, at 73-75° C for 2 minutes) has provided a comparable efficacy to that of a chemical control (prochloraz, carbendazim, and mancozeb). Thermotherapy treatments have been shown to be capable of reducing bakanae disease to 90 %, both in laboratory tests and in field tests, with similar results to those obtained through the use of chemical products (24, 62).

A biological control, based on the use of antagonistic microorganisms, can be an effective tool to control seedborne diseases (63, 64). Several antagonistic microorganisms isolated from different sources have been tested against *F. fujikuroi*. Gram-positive and gram-negative bacteria (*Paenibacillus polymyxa* from tomato leaves and *Bacillus subtilis, B. megaterium, B. oryzicola* and *Pseudomonas fluorescens* from rice) were tested to evaluate their biocontrol potential (65, 66, 67, 68). The antagonistic fungus *Trichoderma asperellum* was able to penetrate the hyphae of *F. fujikuroi* and to degrade its cell wall, thus suggesting its biocontrol ability (69). The authors have recently tested 62 yeasts, isolated from rice seeds, as potential biocontrol agents (70). Four yeast isolates were selected because of their efficacy against *F. fujikuroi*, based on dual culture assays (with reference to mycelial growth inhibition), and seed tests (with reference to decreases in the infection rate). Three selected yeasts reduced the incidence of bakanae disease on rice in greenhouse assays. Rice seeds treated with *Pichia guilliermondii* R9, and *Metschnikowia pulcherrima* R23 and R26 showed a more significant reduction in *F. fujikuroi* infection than a few tested commercial biofungicides (*Bacillus subtilis* QST 713-Serenade, *Streptomyces griseoviridis* K61-Mycostop mix, and microorganism mixture-Ekoseed Pro). Biological seed treatments with *P. guilliermondii* R9 decreased the disease index to 20.0%, while *M. pulcherrima* R23 reduced it to 28.5% (compared to 93.0% in an untreated control). The selected yeasts were also tested in combination with thermotherapy/hot water (60° C for 10 min), which improved their effectiveness in the control of *F. fujikuroi,* compared to each treatment on its own (thermotherapy or antagonist). The highest disease reduction was obtained when *P. guilliermondii* R9 or *M. pulcherrima* R23 were applied together with thermotherapy, after which the bakanae index dropped to 5.0%, and seed germination was improved as it showed the seed priming efficiency; germination rate was increased to 96.0% with *M. pulcherrima* R23, and to 100% with *P. guilliermondii* R9 when compared to the 87.0% in untreated control.

Although the pathogen was first reported more than 100 years ago, only old and rarely detailed phytopathological studies can be found on *F. fujikuroi*. However, molecular and chemical studies were carried out for industrial purposes in the past in order to improve the biotechnological application of *F. fujikuroi* as a gibberellin producer. When several fungicides were available for seed dressing, bakanae disease was rarely taken into consideration by rice seed producers. In the last few years, due to the reduced availability of chemicals, the importance of the disease has steadily increased. Seed companies have to sell seeds that are certified free of *F. fujikuroi.* The new lines of research include the characterization of the molecular processes involved in fungal pathogenesis and the pathogen interactions with rice, the elucidation of the epidemiology, the evaluation of new sustainable control strategies, and the search for tolerant or resistant rice genotypes. The recent sequencing of the *F. fujikuroi* genome has opened up new and interesting fields of research, including the study of the regions of the genome involved in the pathogenicity and differentiation processes (including horizontal gene transfer) that are necessary to clarify this specific

pathosystem. In the coming years, this knowledge will allow innovative defense strategies to be developed to control bakanae rice disease.

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