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Effects of jinggangmycin on peroxidase and esterase isozymes and phytotoxin from \textit{Rhizoctonia solani} AG-1 IA, the causal agent of rice sheath blight

Pirinç kabuğu yanığı nedeni \textit{Rhizoctonia solani} AG-1 IA tarafından üretilen peroksidaz, esteraz izozimleri ve fitotoksinler üzerinde jinggangmycin etkisi

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Abstract: Objective: To explore the effects of jinggangmycin on the pathogenicity of rice sheath blight.

Methods: The effects of jinggangmycin on peroxidase and esterase isozymes from \textit{Rhizoctonia solani} Kühn AG-1 IA were compared using non-denaturing polyacrylamide gel electrophoresis (PAGE). Rs-toxins were obtained from the improved Richard medium amended with jinggangmycin at different concentrations. Subsequently, bioassays of Rs-toxin, including virulence to rice tissues, seedling wilting and inhibition to the seed germination were conducted.

Results: The results of PAGE showed that when the jinggangmycin concentration increased to 50 μg/mL, two of peroxidase isozyme bands became apparently weaker compared with the controls, indicating that jinggangmycin could considerably weaken the activity of the peroxidase isozyme. In addition, three extra bands of esterase isozyme were induced by jinggangmycin when the concentration increased to 50 μg/mL, indicating that jinggangmycin could affect the metabolism of esterase isozyme. The bioassays of Rs-toxin showed that jinggangmycin could weaken the virulence of Rs-toxin in rice tissues.

Conclusion: It was concluded that the control role of jinggangmycin in rice sheath blight might be due to the effects of jinggangmycin on the peroxidase and esterase isozymes and Rs-toxin virulence.

Keywords: Rice sheath blight, \textit{Rhizoctonia solani} kühn AG-1 IA, Jinggangmycin, Peroxidase and esterase isozymes, Phytotoxin, Pathogenicity

Özet: Amaç: Jinggangmycinin pirinç kabuğu yanığı patojenüstesindeki etkilerinin araştırılması.

Metod: Jinggangmycinin \textit{Rhizoctonia solani} Kühn AG-1 IA tarafından üretilen peroksidaz ve estaraz izozimleri üzerinde jinggangmycin etkisi.
deki etkileri yapı-bozmayan poliaprilamid jel elektroforezi (PAGE) kullanılarak karşılaştırılmıştır. Rs-toxsinler, geliş-tirilmiş Richard besiyerinin değişik konsantrasyonlarda jinggangmycin eklenmesi ile elde edilmiştir. Bilahare, Rs-toxin biyoanjiz analizleri, pirinc dokularındaki viru-lans, ana çoğülenin solması ve tohumların çimlenmesin- en engellenmesi de dahil olmak üzere değerlendirilmiştir.

Buğular: PAGE sonuçlarına göre jinggangmycin konsan-trasyonu 50 μg/mLye yükseltildiğiinde, jinggangmycinin peroksidaz izomernin aktivitesini zayıflatması nedeni ile peroksidaz izozim bantlarında iki tanesi kontrolde gö-re daha zayıf elde edilmiştir. Buna ek olarak, jinggangmycin konsantrasyonun 50 μg/mLye yükseltilmesi ile esteraz izozim metabolizmasının etkilenmesi olması ile esteraz izozim bantlarında ekstra üç bant gözlenmiştir. Rs-tox-sin biyoanalyizleri ise jinggangmycinin pirinc dokusunda Rs-toxin virulansını zayıflatığı düşündürdükleridir.

Sonuç: Pirinc kabuğu yanığında, jinggangmycinin rolünün peroksidaz ve esteraz izozimleri ile Rs-toxin virulansı üzerinden olduğu sonucuna varılmıştır.

Anahtar Kelimeler: Pirinc kabuğu yanığı, Rhizoctonia solani Kühn AG-1 IA, Jinggangmycin, Peroksidaz ve esteraz izozimleri, Fitotoksin, Patotojensite

1 Introduction

Sheath blight is one of the most serious and economi-cally important diseases of rice (Oryza sativa L.) in most rice-growing countries [1]. It is caused by a soil-borne basidiomycete Rhizoctonia solani Kühn (teleomorph: Thanatephorus cucumeris [Frank] Donk), one of the most important fungal pathogens, which has a broad range of plant hosts and causes diverse necrotic symptoms such as sheath blight and root rot in mono- and dicoty-ledonous plants, respectively [2]. It is well-known that R. solani is recognized as a complex species consisting of at least 14 anastomosis groups (AGs) differing in morphol-ogy, pathogenicity, host range, molecular characteristics and distribution in nature [3,4]. In addition, 5 AGs of this fungus were further divided into intraspecific groups [4]. One important intraspecific group within the R. solani complex is AG-1 IA (R. solani AG-1 IA), the causal agent of rice sheath blight [1]. The taxonomy, biology, ecology and population genetics of R. solani have been well docu-mented by many researchers [5–9].

Phytotoxins and cell wall-degrading enzymes are con-sidered as the two important pathogenicity determinants of R. solani [10–14]. Previous studies revealed that Rs-toxin produced by R. solani are involved in lesion development and thus plays a significant role in pathogenesis [10–13]. Therefore, the understanding of Rs-toxin function in pathogenesis is of great significance.

Jinggangmycin, an antibiotic fungicide, is efficient, economic and of nice environmental compatibility in the control of rice sheath blight. Jinggangmycin from China and validamycin from Japan belong to the same kind of agricultural antibiotics produced by different variants of Streptomyces hygroscopicus, which have component A as their main efficient component [15,16]. Many researchers have studied the effects of jinggangmycin or validamycin on R. solani such as the inhibition of validamycin on R. solani mycelia growth [17–23], effect of jinggangmycin or validamycin on the production and activity of R. solani cell wall degrading enzymes [23,24], inflection of jinggangmycin or validamycin on R. solani cell wall [25] virulence and mechanism of jinggangmycin on R. solani [26]. They have equally studied the effects of jinggangmycin on cell wall degrading enzyme activity and soluble proteins of R. solani [27], genetic diversity of rice sheath blight fungus based on peroxidase and esterase isozymes and soluble protein analysis [7,28,29]. The peroxidase and esterase isozymes play important role on the immunity, which is raled with disease resistance, while Rs-toxin reflects the pathogenicity. Therefore the studies on the peroxidase and esterase isozymes and Rs-toxin have large value on exploring the relation between pathogenicity and disease resistance. However, there has been no report on the influ-ence of jinggangmycin on the changes of peroxidase and esterase isozymes, and the toxicity of Rs-toxin from R. solani so far. In this study, we aim to explore the effects of jinggangmycin on peroxidase and esterase isozymes, virulence to rice tissues, seedling wilting and inhibition to seed germination from Rs-toxin. We anticipate that the results will enrich and complement the control mecha-nism of jinggangmycin in rice sheath blight, and will be of practical significance in controlling rice sheath blight.

2 Materials and Methods

2.1 Fungal isolates and jinggangmycin origin

The virulent isolate GD-118 of R. solani AG-1 IA was main-tained by our laboratory and used in this study [6,13,14]. The isolate was incubated on potato dextrose agar (PDA) [30] in the dark at 26 ºC for the preparation of inocula. Jing-gangmycin with a purity quotient of 60% was bought from Wuhan Kernel Bio-tech Co., Ltd., China.
2.2 Effects of jinggangmycin on peroxidase and esterase isozymes from *R. solani*

The extraction of peroxidase and esterase isozymes was performed following the method described by Mohammadi *et al.* [7] with minor modifications. *R. solani* mycelial plugs, 5 mm in diameter, were removed from the margins of 36-h-old cultures on PDA plates and transferred to 250 mL Erlenmeyer flasks containing 100 mL potato dextrose broth (boiled exudate from 200g potato; dextrose, 20g; ultrapure water, 1000mL) with jinggangmycin at different concentrations of 0 μg/mL, 20 μg/mL, 50 μg/mL and 100 μg/mL separately. Flasks were shaken on a rotary shaker (150 r/m) for 5 days at 26˚C. The culture of each flask was filtered through four layers of Whatman filter paper No. 1 (Whatman International Ltd, Kent, UK) through a vacuum pump in order to obtain fungal biomass, which were washed three times with double distilled water. Mycelia of *R. solani* AG-1 IA isolate GD-118 were first frozen in liquid nitrogen, and then lyophilized for 8 h under vacuum condition and conserved at -80˚C before protein extraction.

Two grams of mycelia were ground to fine powder in a cold mortar using a pestle and then homogenized in 1.0 mL cold extraction buffer [0.05 M Tris–HCl (pH 6.9), 13% (v/v) glycerol and 0.5% (v/v) Tween 80]. The homogenate was transferred into 4-mL plastic tubes and centrifuged at 11, 000 r/m, 4˚C, for 20 min using Eppendorf 5804R centrifuge (Eppendorf, Germany). The supernatant was transferred to a new tube and conserved at -80˚C.

The analysis of peroxidase and esterase isozymes was conducted using non-denaturing polyacrylamide gel electrophoresis (PAGE). The preparation of gels followed the method described by Walker [31] with some minor modifications. Briefly, the gels for peroxidase isozymes were stained by benzidine and resolved in 8% separating gel and 4% (w/v) stacking gel, while the gels for esterase isozymes were stained by α-naphtyl acetate, β-naphtyl acetate and fast blue RR salt and resolved in 8% separating gel and 3% (w/v) stacking gel. After the electrophoresis of fungal proteins at 75V, 4˚C for 30min, and subsequently at 110 V, 4˚C for 4 h, the gel was stained accordingly. The experiment was repeated three times.

2.3 Effects of jinggangmycin on the seedling wilting from Rs-toxin

The Richard liquid medium, amended with jinggangmycin at different concentrations, was prepared following the method described previously [10,13]. The detailed culture and extraction of Rs-toxin were conducted according to the methods described previously by the author [13].

The rice cultivar 'yuexiangzhan' was taken as the experimental material. The roots of ten rice seedlings at 5-leaf-stage were washed and put into a test tube filled with 10 mL Rs-toxin solution. Seven treatments, such as sterile water, medium, medium + jinggangmycin complements, Rs-toxin + jinggangmycin at the concentrations of 0 μg/mL, 20 μg/mL, 50 μg/mL and 100 μg/mL respectively, were included in this experiment. All the rice seedlings in each treatment were cultured at 28˚C with 12 h-photoperiod and the wilting time of rice seedlings in each treatment was observed and recorded, followed by a comparison on wilting degrees of the rice seedlings incubated in Rs-toxin solution at different concentrations of jinggangmycin after three days. Ten replicates were maintained for each treatment, and all experiments were repeated twice.

2.4 Effects of jinggangmycin on the Rs-toxin toxicity

The leaves of rice cultivar ‘yuexiangzhan’ were cut into 4-cm-length fragments and transferred into 9-cm-diameter Petri dishes with 20 mL Rs-toxin solution. These Petri dishes were incubated at 28˚C for 48 h after sealing with Parafilm. Seven treatments, such as sterile water, medium, medium + jinggangmycin complements, Rs-toxin + jinggangmycin at the concentration of 0 μg/mL, 20 μg/mL, 50 μg/mL and 100 μg/mL respectively, were included in this experiment. Ten replicates were maintained for each treatment, and all experiments were repeated twice. The disease rate was calculated as follows.

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\text{Disease rate} (%) = \frac{\text{Diseased leaves}}{\text{Total leaves}} \times 100
\]

2.5 Effects of jinggangmycin on the Rs-toxin inhibition of seed germination

The rice cultivar ‘yuexiangzhan’ and the cucumber cultivar ‘jinmantian F1’ were included in this experiment. Seeds of rice and cucumber were disinfected in 3% H₂O₂ for 20 min and then were transferred onto the surface of wet, sterile two-layer filter paper placed inside the 9 cm diameter Petri dish. 100 seeds were placed in one dish, and the dish was sealed with Parafilm to maintain humidity, and the results were investigated after incubation at 30˚C for 48 h. Seven treatments, such as sterile water, medium, medium + jinggangmycin complements, Rs-toxin + jinggangmycin at the concentration of 0 μg/mL, 20 μg/mL, 50 μg/mL and 100 μg/mL respectively, were included in this experiment. The seed germination ratio, seed germ growth ratio, and radicle
growth ratio were evaluated separately according to the number of seed germination, seed germ growth or seed radicle growth in 100 seeds. Ten replicates were maintained for each treatment, and all experiments were repeated twice.

### 3 Results

#### 3.1 Effects of jinggangmycin on peroxidase and esterase isozymes from *R. solani*

When the jinggangmycin concentration increased to 50 μg/mL, two bands of peroxidase isozyme were obviously weaker than the controls, indicating that jinggangmycin could significantly reduce the activity of peroxidase isozyme of *R. solani* (Fig. 1a). When the jinggangmycin concentrations increased to 50 μg/mL, three extra bands of esterase isozyme marked as 'a', 'c' and 'd' were induced by jinggangmycin compared with the lower concentration of 20 μg/mL and the control (Fig. 1b), indicating that jinggangmycin could obviously enhance the metabolism of esterase isozyme.

#### 3.2 Effects of jinggangmycin on the seedling wilting induced by Rs-toxin

The wilting effects of Rs-toxin with jinggangmycin at different concentrations were obviously different. No seedling wilting was observed after 48-h incubation of these treatments groups, i.e. sterile distilled water, medium, and medium + jinggangmycin complements. The rice seedlings cultured in Rs-toxin solution without jinggangmycin showed wilting after 12 h, whereas those cultured in Rs-toxin solution plus jinggangmycin at 20 μg/mL, 50 μg/mL, and 100 μg/mL exhibited wilting at 14 h, 17 h and 18 h, respectively. After being treated three days, the different wilting degrees of the rice seedlings incubated in Rs-toxin solution at different concentrations of jinggangmycin could be obviously distinguished with the naked eye (Fig. 2). The results revealed that jinggangmycin could...
3.3 Effects of jinggangmycin on the Rs-toxin toxicity

RS-toxin could affect obvious symptoms (Fig. 3a), while rice leaves of sterile water, medium and medium + jinggangmycin complements treatments exhibited no symptoms, indicating that these treatments were of no significant effect on rice leaves and the medium or jinggangmycin complements had no effect on the toxicity of RS-toxin (Fig. 3b). Rice leaves at the treatments with 0 μg/mL, 20 μg/mL, 50 μg/mL and 100 μg/mL jinggangmycin showed obvious symptoms and the disease rates were negatively correlated with the concentrations of jinggangmycin. The disease rate was 98.70% when the rice leaves were treated with Rs-toxin and 85.35% when cultured in Rs-toxin with 20 μg/mL jinggangmycin. However, the disease rates decreased with the increasing concentrations of the jinggangmycin. The disease rate was 50.79% when cultured in Rs-toxin with 100 μg/mL jinggangmycin. The results indicated that Rs-toxin was of obvious toxicity to rice tissues, and jinggangmycin could actually reduce the damage to rice tissues caused by Rs-toxin (Fig. 4a).

3.4 Effects of jinggangmycin on the inhibition of the seed germination

The results revealed that Rs-toxin had high inhibition on the germination of rice and cucumber seeds, while the jinggangmycin could weaken this inhibition from Rs-toxin and improve the germination ratios. The seed germination ratios of rice and cucumber increased with the increasing concentration of jinggangmycin. When the rice and cucumber seeds were treated in sterile water, medium and medium + jinggangmycin complements, the germination ratios were both higher than 80.00%. However, when the rice and cucumber seeds were treated in Rs-toxin, the germination ratios were 0.00% and 27.22% respectively. When the rice and cucumber seeds were kept on the wet filter paper soaked in Rs-toxin solution with 20 μg/mL jinggangmycin, the germination ratios were 11.25% and 33.89% respectively. With the increase of the jinggangmycin concentrations, the germination rates increased. The germination ratios of rice and cucumber seeds were 20.62% and 53.89% when kept on the wet filter paper soaked in Rs-toxin solution with 100 μg/mL jinggangmycin. These results indicated that Rs-toxin was toxic to the seed germination and jinggangmycin could effectively reduce this damage on seeds caused by Rs-toxin (Fig. 4b).

The results revealed that Rs-toxin had high inhibition on the growth of seed germs, while jinggangmycin could weaken this inhibition induced by Rs-toxin and improve the growth ratios. As the jinggangmycin concentration increased, the growth ratios of rice and cucumber increased. When the rice and cucumber seeds were treated with sterile water, medium and medium + jinggangmycin complements, the growth ratios were both higher than 80.00%. When the rice and cucumber seeds were treated in Rs-toxin, the growth ratios were 13.34% and 0.00% respectively. When the rice and cucumber seeds were kept on the wet filter paper soaked in Rs-toxin solution with 20 μg/mL jinggangmycin, the growth ratios were 35.56% and 6.67% respectively. Increasing the concentration of jinggangmycin increased the growth ratios. When the rice and cucumber seeds were kept on the wet filter paper soaked in Rs-toxin solution with 100 μg/mL jinggangmycin, the growth ratios were 43.33% and 23.33% respectively. These results indicated that Rs-toxin was of obvious toxicity to the growth of seed germs, both for rice and cucumber, and jinggangmycin could effectively reduce the inhibition on the growth of seed germs caused by Rs-toxin (Fig. 4c).

The results revealed that Rs-toxin had high inhibition on the growth of seed radicles, both for rice and cucumber, while the jinggangmycin could weaken this inhibition and improve the growth ratios. When the rice and cucumber
seeds were kept on the wet filter paper soaked in sterile water, medium solution and medium solution + jinggangmycin complements, the growth ratios were both higher than 85.00%. When the rice and cucumber seeds were kept on the wet filter paper soaked in Rs-toxin, the growth ratios of seed radicles for both rice and cucumber were 0.00%. When the rice seeds were kept on the wet filter paper soaked in Rs-toxin solution with 20 μg/mL jinggangmycin, the growth ratios of rice and cucumber seeds were 4.51% and 7.50% respectively. With the increase of the jinggangmycin concentration, the growth ratios increased. When kept on the wet filter paper soaked in Rs-toxin solution with 100 μg/mL jinggangmycin, the growth ratios of rice and cucumber seeds were 18.51% and 21.67% respectively. These results indicated that Rs-toxin was of obvious toxicity to the growth of seed radicles both for rice and cucumber, and jinggangmycin could effectively reduced the inhibition of the growth of seed radicles caused by Rs-toxin (Fig. 4d).

4 Discussion

Jinggangmycin or validamycin had no killing effect on R. solani, but it could inhibit the hypha extension and stop the mycelia from branching, which would affect the pathogenicity of R. solani [17–23]. It was considered that jinggangmycin or validamycin had little effect on R. solani in vitro under general conditions. It cannot inhibit the production of mycelia, but it can inhibit the mycelium extension, causing frequent branching of the hyphal tip and further inhibiting its development. Jinggangmycin and validamycin had the same inhibition effect on the fungal penetration of plant tissues [32]. One disease-prevention mechanism of jinggangmycin was the dual role in both pathogen inhibition and plant defense responses [33]. The effects of jinggangmycin on peroxidase and esterase isozymes and the virulence of Rs-toxin from R. solani were investigated in this study, and this has not been previously reported. These findings will enrich and complement the control mechanism of jinggangmycin on rice sheath blight.

The analysis of peroxidase and esterase isozymes using PAGE could describe the biological characteristic of rice sheath blight fungus and is an important biochemistry technique, through which the differences between species could be reflected at the protein level [34]. The isozyme in vitro is the connection between the gene and the morphological characteristic and especially the esterase isozyme is also the reflection of biochemistry char-
acters in vitro [35]. The esterase isozymes from different R. solani isolates were analyzed and the results revealed that the main bands of R. solani isolates from different origins differed, which reflected the hereditary character of esterase isozymes [28]. The esterase isozymes of 20 isolates from R. solani AG-1 IA and AG-1 IB were analyzed, and results revealed that the esterase isozymes from the same subgroup were almost the same [7]. At the same time the esterase isozyme results of isolates with different pathogenicity revealed that the main bands of the isolate at the same pathogenic levels were almost consistent and the heterogeneity was reflected in the vice bands [29]. The previous reports did not refer to the effects of jinggangmycin on the peroxidase and esterase isozymes from R. solani [7,28,29]. The results of the present study revealed that jinggangmycin obviously affected the esterase isozymes. Three new bands of esterase isozyme were formed. These findings proved that jinggangmycin had obvious effect on rice sheath blight fungus by altering its metabolism in the esterase isozymes and weakening the virulence of Rs-toxin to rice.

The phytotoxin was considered to be one of the most important pathogenicity factors in the pathogenesis of R. solani [10–13]. The Rs-toxin can produce typical symptoms of rice sheath blight on rice tissues, inhibit the growth of the rice radicle, make the rice seedlings wilting and the effect is positive correlation with the concentrations of Rs-toxin [13,36]. The toxin-producing ability and the pathogenicity of different R. solani isolates were significantly correlated and the correlation coefficient was 0.8255, which revealed that the Rs-toxin played an important role in the pathogenesis of R. solani [36]. In this study, it was proved that jinggangmycin could obviously affect the inhibition of rice tissues and seed germination.

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Conflict of interest: None declared.

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