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Inhibitory activity of aloesin and aloe gel against *Curvularia lunata*

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Abstract: In order to find natural active substance alternative to the chemical fungicides currently used against the devastating *Curvularia* leaf spot of rice, the bioactivity of aloesin and aloe gel against *Curvularia* lunata was determined. The filter paper method showed that 300 mg/mL aloesin led to an obvious inhibitory band (3.37 mm) at 28 h, and a inhibitory zone (Φ = 51.27 mm) against spores (3 × 10^4 cfu/mL), and these inhibitory effects remained until 6 d. The mycelial inhibition rate by aloe gel at 1, 2, 4, 8, 16, 32 and 64 mg/mL ranged from 9.25% to 56.21% at 5 d, and it increased with increased concentration. Additionally, the inhibition rate of 64 mg/mL aloe gel increased by 21.87% from 2 to 5 d. The mycelial growth of *C. lunata* treated with 160 mg/mL aloesin was weaker than control, and no appressorium produced. The germ tube of *C. lunata* treated with aloe gel was much slower than control, and the inhibition rate was 75.39%-96.58%. This study was first reported that aloesin effectively inhibited mycelia growth and conidia germination of *C. lunata*, and aloe gel effectively inhibited mycelia growth of *C. lunata*.

Keywords: aloesin; aloe gel; *Curvularia lunata*; antifungal activity

芦荟苦素和芦荟凝胶对新月弯孢的抑菌活性

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摘 要：通过研究芦荟苦素和芦荟凝胶对新月弯孢 *Curvularia lunata* 的抑菌效果，以期寻找天然活性物质用于水稻新月弯孢叶斑病的防治。滤纸法显示：300 mg/mL 的芦荟苦素处理 28 h 后对新月弯孢可产生明显的抑制带 (3.37 mm)；在孢子液 (3 × 10^4 cfu/mL) 平板上可产生直径为 51.27 mm 的抑菌圈，且抑制效应可达 6 d。用 1、2、4、8、16、32、64 mg/mL 系列质量浓度的芦荟凝胶处理新月弯孢 5 d 时的菌丝抑制率范围为 9.25% ~ 56.21%，抑菌率随芦荟凝胶质量浓度的增加而升高。此外，从处理后 2 d 到 5 d，64 mg/mL 芦荟凝胶的抑制率增加了 21.87%。显微镜检表明，160 mg/mL 芦荟苦素处理的 *C. lunata* 菌丝生长弱于对照，且无附着胞产生。经芦荟凝胶处理后，分生孢子芽管的生长速度显著低于对照，其生长抑制率可达 75.39%-

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96.58%。本研究首次报道芦荟苦素能有效抑制 *C. lunata* 的菌丝生长和分生孢子萌发，而芦荟凝胶能有效抑制 *C. lunata* 的菌丝和芽管生长。

关键词: 芦荟苦素；芦荟凝胶；新月弯孢 (*Curvularia lunata*)；抑菌活性

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Rice (*Oryza sativa* L.) is an important food crop grown under worldwide，one of the major classes of organisms that cause plant disease are phytopathogenic fungi. Over the past decades, the actions of these organisms worldwide have caused millions of dollars[1-2]. The diseases are spread worldwide, but its occurrence and severity vary by year, location and environmental conditions[3-5]. *Curvularia* leaf spot, caused mainly by *Curvularia lunata*, is a widespread plant disease in China. *C. lunata* could cause the seeds of the rice to lose the germination capacity and cause the seedling blight and leaf spot of the rice. In recent years, directional host selection by the pathogen, which likely results in the virulence differentiation in pathogen population, is widely reported[6-7]. This need further enough study establish a foundation for the effective managment of *C. lunata*.

Management of leaf spot is difficult because the pathogen is seed-borne. To date, management approaches have mainly focused on the use of synthetic chemicals and resistant rice varieties[8-10]. However, their efficacy can be limited by the rapid development of resistance to these chemicals, which applied as either seed dressing or spray, such as benomyl, benomyl + copper sulfate, probenazole, thiabendazole and pyroquilon fungicides, have been used in different rice growing countries to manage rice disease[11].

Many plant diseases caused by fungi have been controlled by plant extracts[12-16]. Plants produce a high diversity of natural products with a prominent function against pathogens on the basis of their toxic effect on growth and reproduction of microbes[17]. Thus, the exploration of plant resources for their antifungal potential against pathogens is important for the sustainable and ecofriendly management of pathogens. Moreover, Prisana *et al.* reported that all the *Streptomyces* species inhibited the growth of *C. lunata*[18]. The application of the *Streptomyces angustmyceticus* NR8-2 spore suspension and cell-free culture filtrate reduced the disease severity index of leaf spots[18].

Aloe is rich in a variety of active ingredients, including anthraquinones, polysaccharides, active enzymes, amino acids and organic acids, antibiotics, and saponins[19-20]. Among these, anthraquinone compounds and anthraquinone derivatives have strong inhibitory action toward many fungi and bacteria[21]. Aloesin is an anthraquinone compound with a chemical structure of 2-acetyl-8-*D*-pyrrolidone-7-hydroxy-5-methylparaphthone that has anti-hepatitis, neutralizing toxin, anti-tumor, and leukemia effects[22]. Aloe gel is the most widely recognized herbal medicine in the world today because it contains a wide range of enzymes and provides preservative, anti-inflammatory, and anti-fungal benefits[23]. The main focus of this study was to evaluate the bioactivity of aloesin and aloe gel against *C. lunata*.

1 Materials and methods

1.1 Plant material and fungal material

Aloesin (95%) was obtained from Xi'an Yunyue Biotechnology Co., Ltd. Aloe gel (100%) was acquired from Yunnan Wan Green Biological Co., Ltd. *C. lunata* was obtained from the Agricultural Products Quality Safety Laboratory of the College of Agriculture, Guizhou University. *C. lunata* was cultured on the Potato Sucrose Agar (PSA) at 28 ℃ for 2-3 d; filter paper (6 mm in diameter) was sterilized at 121.3 ℃ for 20 min.

1.2 Inhibition effect of aloesin against *C. lunata* by filter paper method

The anti-fungal activity against *C. lunata* was tested by the disc diffusion method, and clear zones appeared around the treatment discs were recorded[24-25].

But we were slightly modified. First, *C. lunata* discs were placed in the center of PSA plates. Next, filter paper containing three different plant extracts at three different concentrations (300, 200, or 100 mg/mL) was also placed around the fungal discs. In addition , all experiments were repeated in triplicate and all plates were incubated at 28 ℃ for 2-3 d. Meanwhile, inhibition effect was assessed using conidial suspensions. A hemocytometer was used to adjust the conidial concentration to 3×10^4 conidia/mL. After the medium solidified, conidial suspensions (0.1 mL) were then placed in treated PSA plates, filter papers of various concentrations were added to each dish. All experiments were repeated in triplicate and all plates were then incubated at 28 ℃ for 2-3 d.The width and the diameter of the inhibition zone were then measured, and the average value of the three treatments was taken.

1.3 Inhibition rate of mycelium growth against *C. lunata* by aloe gel

A series of sterile water containing different concentrations of aloe gel (10, 20, 40, 80, 160, 320 and 640 mg/mL) were prepared. Next, PSA(40℃) was melted in an electric furnace, after which 1 mL aliquots of test solution were mixed with 9 mL PSA agar and added to 90 mm culture dishes. The test compounds were present at concentrations of 1, 2, 4, 8, 16, 32 and 64 mg/mL. In addition, untreated media was used as a CK. A piece of fungus disc was placed in each plate with five treatments (1, 2, 4, 8, 16, 32 and 64 mg/mL) and CK. Three piece of fungus disc was placed in each plate, and each treatment was performed in quadruplicate *C. lunata* was incubated at 28 ℃ for 5 d, and the colony diameters of the tested fungus were recorded[26]. SPSS 19.0 was used for analysis. The percent inhibition of fungal growth was estimated according to the Ogbeborand Adekunle[26] methods as formula (1).

$$I/\% = \frac{D_c - D_t}{D_c - D_d} \times 100 \quad (1)$$

In formula (1): I means mycelial inhibition, %; D_c means mycelial growth diameter in control, mm; D_t means mycelial growth diameter in treatment, mm; D_d means mycelia discs diameter, mm.

1.4 Microscopic examination

The spore morphology and germination of *C. lunata* in water were observed using a US3 multifunction digital microscope (400 ×). *C. lunata* was also observed in mixtures with aloesin and aloe gel. For clear observation, 0.05% aniline blue staining was conducted. In addition, the spore morphology and germination of *C. lunata* in aloesin and aloe gel were observed and recorded. The spore morphology and germination of *C. lunata* in water were observed using a US3 multifunction digital microscope (400 ×). *C. lunata* was also observed in 200 mg/mL aloesin and aloe gel. In addition, the spore morphology and germination of *C. lunata* in 200 mg/mL aloesin and aloe gel were observed and recorded. A piece of fungus disc was placed in each plate with three treatments.

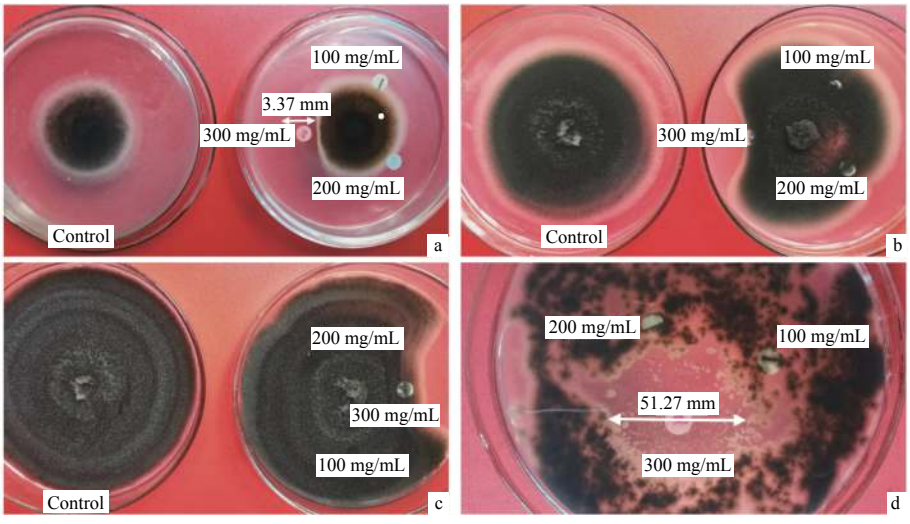
2 Results and Analysis

2.1 Inhibition effect of aloesin against *C. lunata* by filter paper method

After 24 h, the filter paper with 300 mg/mL aloesin showed the best antimicrobial activity and could produce an obvious 3.37 mm inhibition zone (Fig.1a) that could persist for 40 h (Fig.1b). However, the filter paper with 100 and 200 mg/mL aloesin had low antimicrobial activity and did not produce obvious inhibition zones, and instead, only made colony color fade, and these inhibitory effects remained after 6 days (Fig.1c). The filter paper method showed that 300 mg/mL aloesin led to an obvious inhibitory band (Φ = 51.27 mm) against 3×10^4 cfu/mL spores (Fig.1d).

2.2 Inhibitory effect of aloe gel on *C. lunata* mycelium growth

At the same concentration, the inhibitory effect was enhanced with time (2-5 d). As the aloe gel concentration increased, the rate of inhibition increased, too. Specifically, 64 mg/mL aloe gel showed the lowest inhibition rate of 9.25% at 2 d and the highest of 56.21% at 5 d, representing an increase in inhibition of 46.96% (Fig. 2).



Inhibitory zone of aloesin against *C. lunata* at 24 h (a), 40 h (b) and 6 d (c), inhibitory zone of aloesin against *C. lunata* spores at 28 h (d).

Fig. 1 Inhibition effect of 100, 200, and 300 mg/mL aloesin against *C. lunata*

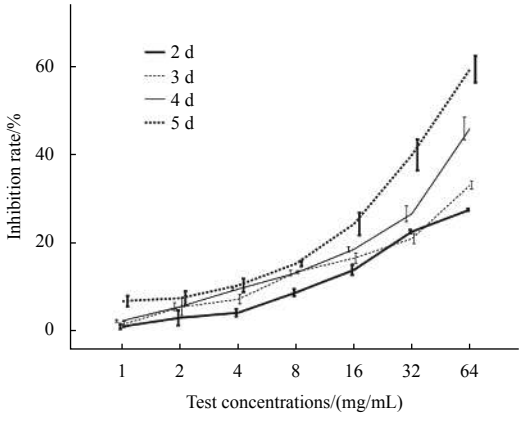
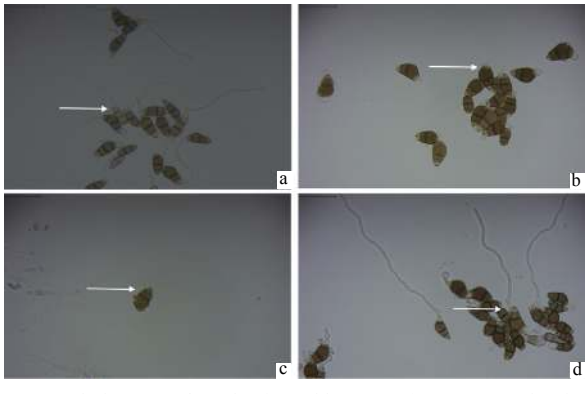


Fig. 2 Inhibition rate against mycelium growth of *C. lunata* at the test concentrations of aloe gel (2-5 d)



(a) Control; (b) 200 mg/mL aloesin could cause *C. lunat* spores to break at one or both ends; (c) 200 mg/mL aloesin could cause *C. lunata* spores to rupture; (d) The unbroken end of *C. lunata* spores could germinate in the presence of 200 mg/mL aloesin (10 × 40; Bar = 10 μm).

Fig. 3 Inhibitory effect of aloesin against *C. lunata* germination

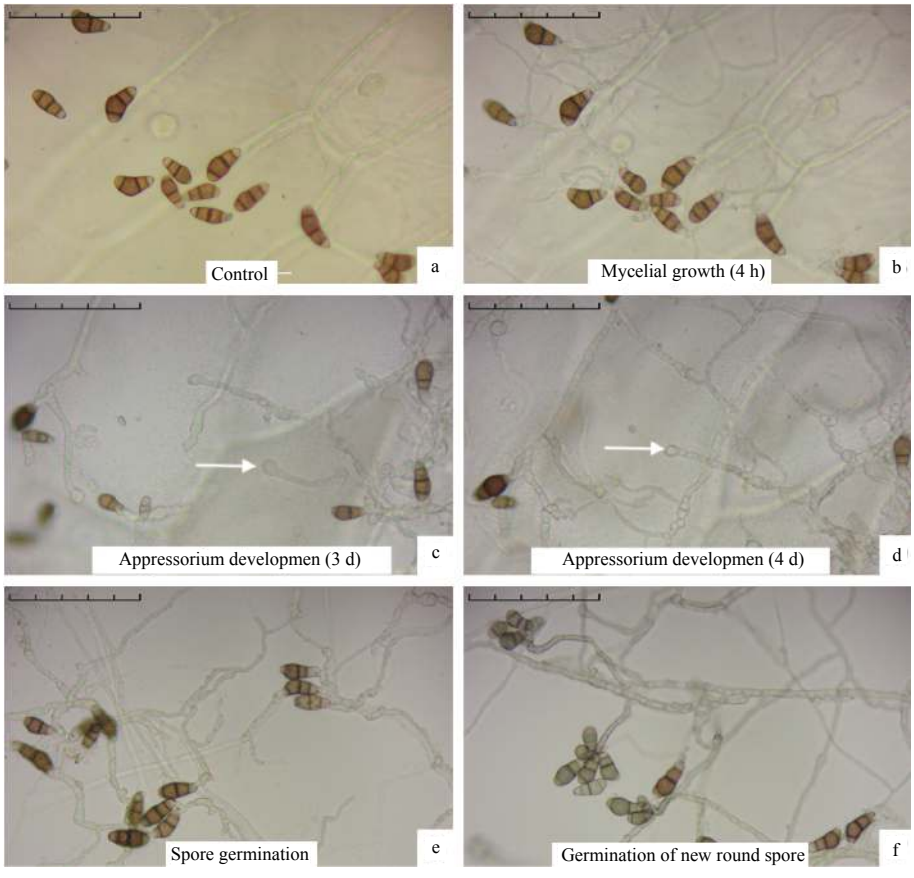
2.3 Microscopic examination

2.3.1 Inhibition effect of aloesin against conidial germination The *in-vitro* antimicrobial activity of aloesin against *C. lunata* spores revealed that 200 mg/mL aloesin could cause both or one end of the *C. lunata* spores to break or rupture and 200 mg/mL aloesin also cause *C. lunata* spores to rupture, the broken ends could not germinate to produce germ tubes, and the unbroken ends could still germinate (Fig.3).

C. lunata spores could germinate at 4 h on onion skin. Appressoria development of *C. lunata* after 3 d with onion skin. After 4 d, a large number of appressoria were produced. *C. lunata* spores could germinate in water without appressorium produced. After 6 d, a new round of *C. lunata* spores were produced (Fig. 4).

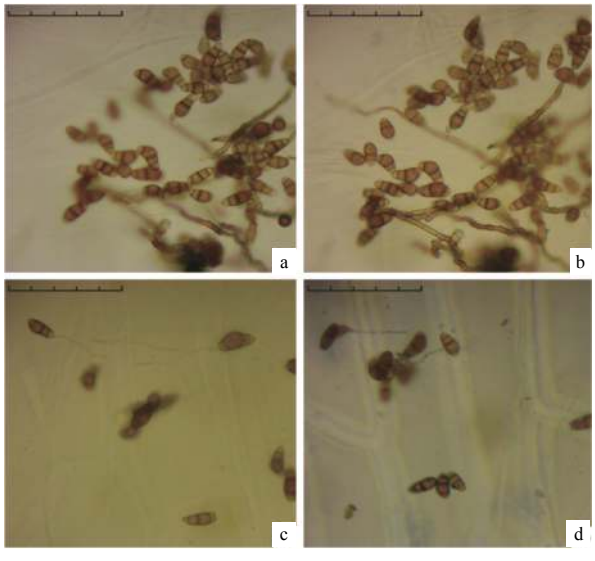
After 22 d, the *C. lunata* spores on onion skin were essentially unchanged in response to 160 mg/mL aloesin. In addition, there were no appressoria produced, only a small amount of spores germinated, mycelial growth was weak, and the average diameter of hypanthium was 1.25 μm (Fig.5), which was weaker than control (3.75 μm).

2.3.2 Inhibition effect of aloe gel against *C. lunata* conidial germination Most *C. lunata* spores in CK could germinate in 6 h, the germinating of *C. lunata* were colorless and tubular, and the sporoduct length was 96.64-106.78 μm (16.11-17.80 μm/h). However, in aloe gel, the germinating *C. lunata* were colorless and sporoduct growth was very slow (Fig.6). The sporoduct length was 3.31-26.28 μm with in 6 h



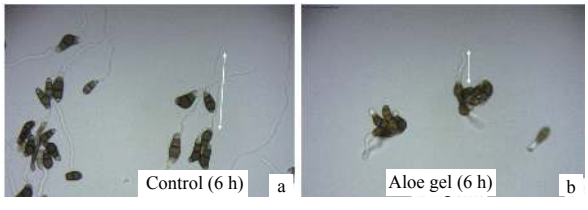
(a) Control. (b) Germination of *C. lunata* spores with onion skin (4 h). (c) Appressorium development of *C. lunata* with onion skin (3 d). (d) Appressorium development of *C. lunata* with onion skin (4 d). (e) Germination of *C. lunata* spores without appressorium in water (6 d). (f) A new round of *C. lunata* spores was produced in 6 d (10 × 40; bar = 20 μm).

Fig. 4 Mycelial growth, appressorium development and spore germination of *C. lunata* with onion skin



(a) *C. lunata* spores at 160 mg/mL aloesin at 1 d. (b) *C. lunata* spores at 160 mg/mL aloesin at 22 d. (c) Mycelial growth of *C. lunata* spores at 160 mg/mL aloesin was weak without appressorium (22 d). (d) Mycelial growth of *C. lunata* spores at 160 mg/mL aloesin was weak without appressorium based on aniline blue stain (22 d) (10 × 40, Bar = 20 μm).

Fig. 5 Mycelial growth and appressorium development of aloesin against *C. lunata* with onion skin



(a) Germinating of *C. lunata* spores in control. (b) Germinating *C. lunata* spores in aloe gel. (Bar = 10 um)

Fig. 6 Morphology of germinating *C. lunata* spores in control and aloe gel (6 h)

(0.55-4.38 μm/h) and the inhibitory rate was 75.39-96.58%. Because the sporoduct growth was slow, the total mycelium quantity was less than that of the CK.

3 Conclusions and Discussion

The pathogenic fungus *C. lunata* causes rice production serious loss, biological control technology to reduce the use of chemical fungicides has received a great deal of attention. Studies were conducted to determine the effects of aloesin and aloe gel on the

control of *C. lunata*. Microscopic examination showed that there was an obvious inhibitory effect of aloesin against *C. lunata* spores. The spores of *C. lunata* were damaged and reduced the number of germinating spores and hyphae, only a small amount of spores could germinate in aloesin. Aloesin can inhibit appressorium, while the presence of melanin is a necessary condition for germination of *C. lunata*[27]. Aloesin had long-term and stable inhibitory effects. In the aloesin and aloe gel treatments, spores were ruptured at one or both ends, leading to direct death of the spores. Aloesin and aloe gel have the potential for use as biocontrol materials, indicating that it is necessary to continue screening and add other components to improve the inhibitory effect as a biocontrol material. The inhibition effect of aloe was similar to that of antibiotics, but its function was superior[25].

Lynch *et al.* reported that aloesin may be useful as a functional food ingredient[28]. There were no toxicologically or statistically significant changes in body weight gain or in feed and water consumption and the no-observed-adverse-effect level was considered to be 1 000 mg/kg body weight/day, the highest dose tested, supporting the potential use of aloesin as a functional food ingredient[28]. Based on these advantages, further studies on the use of aloe to induce bacteriostasis of plant pathogens and the preservation of agricultural products are necessary. In this study, aloesin and aloe gel in aloe were studied, and their inhibitory effects on plant pathogenic microorganisms were evaluated. However, this study investigated the antimicrobial effects of specific components of *A. vera* against *C. lunata*. Chemical preservatives as traditional methods were often used for preservation, many of which are harmful to health. Therefore, it is important to identify new and highly efficient natural active substance to improve food safety. Moreover, consumers are increasingly choosing products with natural preservatives[29-30]. Aloe active ingredients are nontoxic to humans and have been shown to have good efficacy. Therefore, they have the potential for use in the research and development of non-toxic and harmless food preservatives.

This study was reported that aloesin effectively inhibited mycelia growth, conidia germination, and that aloe gel effectively inhibited mycelia growth of the rice pathogen (*C. lunata*). The results presented herein supported that aloesin and aloe gel mostly control spore germination and inhibit spore formation, thereby effectively preventing spore germination and reducing the production of blast spots. The current findings suggest that the extracts from most of the tested plants possess antifungal properties and can be used as seed and foliar treatments against the pathogen *C. lunata*. These plant extracts are safe and eco-friendly when compared to synthetic chemicals. However, additional studies are required to identify and characterize the active antifungal compounds in the extracts of these plants, as well as their roles in *Curvularia* leaf spot control.

Overall, this is the first report of antifungal activity of aloesin and aloe gel against *C. lunata*. The results presented herein provide a basis for the investigation of biological control and food preservation of rice fungus. Fungicide application and planting resistant varieties are considered the two main methods to control disease[31]. Recently, the increasing environmental and economic concerns caused by synthetic fungicides have led to increased efforts to produce safer agricultural products and develop new and safer antifungal agents such as plant based essential oils and botanical extracts to combat fungal plant diseases[32]. The tendency towards application of natural products and botanical extracts as safer antimicrobial agents against plant pathogens has recently increased[33].

Conflicts of Interest: The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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