

Isolation and identification of *Saprolegnia parasitica* from grass carp and screening of sensitive drugs

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Saprolegnia species are destructive pathogens in the freshwater aquatic environment. It is one of the most important causes of economic losses in the fish farming industry, affecting all developmental stages. Following the ban of malachite green in aquatic products, the new treatments of saprolegniasis are moving towards the selection of safer and environment-friendly products. In the present study, a strain of water mold was isolated from skin of grass carp *Ctenopharyngodon idella*. The isolated strain was initially suggestive of *Saprolegnia* sp. based on its morphological features. Genomic DNA of *Saprolegnia* sp. was extracted using proteinase K method and its partial sequencing of inter-transcribed spacer (ITS) sequence was amplified and aligned with other water molds. The sequence of ITS data showed 99% similarity with *Saprolegnia parasitica* (*S. parasitica*) C8 sequence. The constructed phylogenetic tree using neighbor joining method indicated that this isolated strain was closely related to *S. parasitica* C8 in one clade. Therefore, it was identified as *S. parasitica*. Further, *in vitro* activity of three commercial products against *S. parasitica* was tested using agar diluted method. The results showed that the order of inhibiting effect of three drugs was following: dithiocyano-methane > salicylic acid > diallyl trisulfide. By comparing the inhibition rate of three drugs at 24 h and 48 h, they showed a downward trend with the extension of time. But the inhibition rate of diallyl trisulfide decreased more slowly which indicated that its efficacy could maintain for a long time. Our obtained findings were highly suggestive for the efficacy of dithiocyano-methane as an antifungal disinfectant for fish. This study provides guidance for the rational selection and application of antifungal agents.

Keywords: *Saprolegnia parasitica*; antifungal drugs; inhibiting effect; treatment; *Ctenopharyngodon idella*.

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Introduction

Saprolegniasis is one of the common parasitic fungal diseases in freshwater aquaculture. Some genera of *Saprolegnia* and *Achlya* are very common. *Saprolegnia* infections have been reported to be the cause of mass kills among freshwater fish species from eggs to adult [1]. Saprolegniasis is more prevalent in lower water temperatures. Thereby, most of *Saprolegnia* associated mortalities are confined to late autumn, winter, and early spring seasons in

intensive culture ponds [2, 3]. Generally, *Saprolegnia* infections usually result from a wound on fish skin, while eggs are another main infection target, on which it may spread and cover more than 80% of body surface [4].

In the past two decades, routine application of disinfectants is a commonly used procedure during egg incubation at fish hatcheries worldwide, such as formalin, iodophor, malachite green (MG), and its reduced form leucomalachite green (LMG) [5-10]. Although

formalin was considered to control *Saprolegnia* infections in fish eggs effectively [5], there were growing concerns regarding the risks to human health and the environment. The potential use of iodophors for the treatment of fish is limited due to the high concentrations needed resulting in potential toxicity [6]. A lot of literatures have confirmed that MG and LMG generate public health issues related to their potential carcinogenicity, teratogenicity, and mutagenicity in humans [11, 12]. Hence, most of them have been strictly banned in aquaculture environment and in food chain. This ban has necessitated the search for acceptable, safe, and efficient alternatives. The low efficacy of prophylactic measures and the scarcity of effective treatments for the control of saprolegniasis also urge the screening of new products against *Saprolegnia* sp.

Currently, the anti-saprolegniasis drugs used in research or practice include antiseptics, disinfectants, antifungal agents, antibiotics, herbs, and pesticides; particularly, disinfectants and antiseptics are often used [13-17]. To seek the sensitive drugs against saprolegniasis, it is necessary to determine the minimal inhibitory concentrations (MICs). In previous studies, several drugs were identified as suitable compounds for the treatments or disinfection against *Saprolegnia* by measuring their MIC value, such as hydrogen peroxide [18], ozone [19], dithiocyano-methane [20], and boric acid [21]. In addition, natural plant agents are gradually receiving more attention as an alternative to chemotherapeutic agents [13, 15, 17].

In China, *Saprolegnia* infections are considered one of the most important causes of mortalities among grass carp *Ctenopharyngodon idella*. Thereby, the aim of this study was to identify aquatic fungi affecting the grass carp fry and measure the MICs of three commercial products (diallyl trisulfide, dithiocyano-methane, and salicylic acid). An ultimate aim was to screen promising drugs against *Saprolegnia* species, which provided guidance for the selection and

scientific applications of the anti-saprolegniasis drugs.

Materials and Methods

Isolation and culture of *Saprolegnia* species

The sampled infected grass carp fry (figure 1) was euthanized with Tricaine methanesulfonate (Sigma Aldrich, St. Louis, MO, USA) at 100 mg/L. The water molds were separated from fins using tweezers and disinfected for 2 to 3 s with 75% alcohol, then washed several times in phosphate buffered saline (PBS). A small amount of mycelium was inoculated into the Potato Dextrose Agar (PDA) (potato extract 12 g/L, dextrose 20 g/L, and agar 14 g/L) (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China) plate containing 100 mg/L of streptomycin and penicillin (Thermo Fisher, Carlsbad, CA, USA) and incubated at 20°C for 3-5 days with regular daily inspection for any expected fungal growth. A small amount of *Saprolegnia* sp. hyphae were transferred to the center of a new PDA plate, incubated at 20°C until the plate was covered with hyphae, and finally stored at 4°C until used.



Figure 1. The symptom of grass carp suffering from saprolegniasis. Arrows indicate the infected sites.

DNA Extraction

The protocol of DNA extraction was adopted from proteinase K method. The freshly harvested fungal mats were homogenized in 550 μ L of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), transferred to a 2 mL centrifuge tube containing 20% SDS, incubated with 20 μ L proteinase K (20 mg/mL) in a water bath at 55°C for 1 h, and added to a mixed solution (phenol:chloroform:isoamylol 25:24:1) of equal

volume, vortexed for multiple times, and centrifuged at 10,000 g for 10 min to collect the supernatant. The supernatant was added to an equal volume of pre-cooled isopropanol, mixed, and allowed to sit at -20°C for 2 h, then centrifuged at 10,000 g for 5 min to discard the supernatant. The precipitate was added to pre-cooled isopropanol of equal volume to mix, allowed to keep at -20°C for 2 h, and centrifuged at 10,000 g for 5 min to discard the supernatant. The precipitate was washed twice with 70% ethanol, dried at room temperature, dissolved with TE buffer, and stored at -20°C .

Amplification of the *Saprolegnia* sp. gene fragment and phylogenetic analysis

The 750 bp of the ribosomal internal transcribed spacer (ITS) gene was amplified by PCR using two universal ITS primers (ITS1: 5'-TCCGTAGGTGAA CCTGCGG-3'. ITS4: 5'-TCCTCCGCTTATTGATATGC -3') from Eissa *et al.* [1]. The extracted genomic DNA of *Saprolegnia* sp. was used as the template for PCR amplification. The PCR was carried out in a Mastercycler pro PCR (Eppendorf, Hamburg, Germany) using the Ex Taq Kit (TaKaRa Biotechnology, Dalian, Liaoning, China). The reaction system included 2.5 μL of 10 \times Ex Taq buffer, 2.0 μL of dNTPs, 1.0 μL of each primer, 1.0 μL of genomic DNA, 0.25 μL of Ex Taq enzyme, and volume to 25 μL with water, followed by mixing. The PCR reaction condition was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 40 s, 72°C for 1 min, and then a final elongation step at 72°C for 5 min. The resultant products were isolated by 1% agarose gel electrophoresis, purified using an E.Z.N.A gel extraction kit (Omega Bio-tek, Norcross, GA, USA), and then ligated into pMD19-T vector (TaKaRa Biotechnology, Dalian, Liaoning, China) by following the manufacturer's instruction. The constructs were transformed into *Escherichia coli* (*E. coli*) DH5 α by heat shock method. Putative clones were screened by PCR using the above primers, and selected transformants were sequenced using the dideoxynucleotides termination method on ABI 3730 automatic DNA Sequencer by Sangon Biotech Co., Ltd. (Shanghai, China).

The DNA sequence of the core gene of *Saprolegnia* sp. obtained was searched for homology against the other sequences in GenBank database by using BLASTn (www.ncbi.nlm.nih.gov/BLAST/). Sequences with high homology were used to perform multiple alignments using MEGA 6.0. The phylogenetic tree was established using the neighbor-joining (NJ) method to confirm the genetic relationship and classification of strains as described by Cooke *et al.* [22].

Preparation of test drugs

According to our preliminary results, the dilution ranges of the drugs were confirmed as salicylic acid 10^{-1} - 10^{-2} , diallyl trisulfide 10^{-2} - 10^{-3} , and dithiocyano-methane 10^{-2} - 10^{-3} . A new concentration gradient was set to dilute the drug solutions as follows:

(1) Salicylic acid solution: the 8% salicylic acid solution (Jincheng Animal Husbandry Co., Ltd., Henan, China) was diluted with sterile water by 8, 16, 32, 64, and 128-fold to prepare concentrations of 10,000, 5,000, 2,500, 1,250, and 625 mg/L, respectively.

(2) Diallyl trisulfide solution: the 80% diallyl trisulfide solution (Jincheng Animal Husbandry Co., Ltd., Henan, China) was diluted with sterile water by 100, 200, 300, 400, and 500-fold to prepare concentrations of 8,000, 4,000, 2,667, 2,000, and 1,600 mg/L, respectively.

(3) Dithiocyano-methane solution: the 45% dithiocyano-methane solution (Jincheng Animal Husbandry Co., Ltd., Henan, China) was diluted with sterile water by 100, 200, 300, 400, and 500-fold to prepare concentrations of 4,500, 2,250, 1,500, 1,125, and 900 mg/L, respectively.

***In vitro* antifungal experiments**

Before the antifungal experiment, the above prepared solutions were diluted 30-fold again. Experiments were performed following the method of Tedesco *et al.* [23]. Different concentrations of products were added to sterilized liquid PDA medium at a temperature of

about 50°C, 14.5 mL of the PDA culture medium was added to 0.5 ml of diluted drug solution or sterile water. Mixtures were then distributed in six plates (diameter of 90 mm) allowing to test five different concentrations and one negative control. Triplicates of each concentration were set. Following overnight solidification, a 6 mm diameter well was excised in the center of the agar by using a sterile stainless-steel punch. The well was then filled with a standard 6 mm inoculum of *S. parasitica*. Plates were incubated at 20°C and checked after 24 h and 48 h to determine the colony diameters of the growing mycelium as the average of two axes measured at 90 degree from each other. The growth inhibition rate of each drug on colony expansion was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{(\varnothing_c - \varnothing_m) - (\varnothing_p - \varnothing_m)}{\varnothing_c - \varnothing_m} \times 100\%$$

Where \varnothing_c is the diameter of control colony, \varnothing_m is the diameter of mycelium, and \varnothing_p is the diameter of processed colony.

The minimum inhibitory concentration (MIC) of each drug was obtained simultaneously. The MIC was defined as the lowest concentration inhibiting the growth of mycelium after 48 h of incubation.

Results

Morphological characterization

One water mold isolate was obtained from the grass carp fry saprolegniosis. Visual inspection of the cultured PDA plates has exposed the good growth of mold colonies within 48 h. The colonies can be morphologically described as cysts of whitish cottony long hooked hairs that shifted to yellow and contractive after 72 h (figure 2). By examining the symptoms of sick grass carp and the characteristic of fungal colonies, this strain was initially identified as a *Saprolegnia* isolate.



Figure 2. The morphology of *Saprolegnia* sp. cultured on PDA plate at 96 h.

Identification of *Saprolegnia* sp.

The 750 bp sequence of the isolated strain from grass carp fins was obtained by amplifying ITS sequence of *Saprolegnia* sp. (figure 3). We compared homology between the ITS sequence of the isolated strain and those of known *Saprolegnia* sequences in GenBank database, and the isolated strain was 99.4-99.7% homologous, which confirmed the initial identification. The constructed phylogenetic tree established by the neighbor joining method further demonstrated that the isolated strain was closely related to *Saprolegnia parasitica* C8 (GenBank accession No. JN400038) (figure 4). The molecular identification result from phylogenetic analysis was consistent with that found through morphological identification, so the strain was identified as *Saprolegnia parasitica* (*S. parasitica*).

Comparison of growth of *S. parasitica* hyphae in response to the three drugs

The agar plate dilution method was used to measure the MICs of diallyl trisulfide, dithiocyano-methane, and salicylic acid against the hyphae of *S. parasitica*, which was directly judged by measuring colony diameter. As shown in Table 1, all three drugs had inhibitory effects

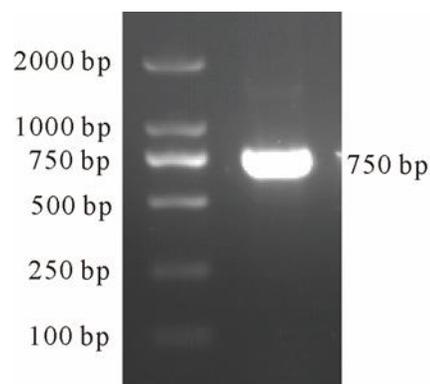
Table 1. Inhibition effect of three antifungal fishery drugs on the growth of *Saprolegnia* sp.

Fishery antibiotics	Concentration (mg/L)	24 h		48 h	
		Colony diameter (mm)	Inhibition rate (%)	Colony diameter (mm)	Inhibition rate (%)
Diallyl trisulfide	266.67	6	100.00	6	100.00
	133.33	7	95.00	9	92.31
	88.89	8	90.00	11	87.18
	66.67	10	80.00	14	79.49
	53.33	12	70.00	18	69.23
Dithiocyanomethane	150.00	6	100.00	6	100.00
	75.00	6	100.00	6	100.00
	50.00	6	100.00	6	100.00
	37.50	9	85.00	16	74.36
	30.00	11	75.00	19	66.67
Salicylic acid	333.33	6	100.00	6	100.00
	166.67	6	100.00	6	100.00
	83.33	7	95.00	11	87.18
	41.67	10	80.00	15	76.92
	20.83	14	60.00	22	58.97
control group	–	26	0	45	0

to different degrees. The effectiveness for killing the hyphae of *S. parasitica* was dithiocyanomethane > salicylic acid > diallyl trisulfide. The antifungal effect of each drug was lower at 48 h than that at 24 h. The effects of dithiocyanomethane and salicylic acid decreased significantly, whereas diallyl trisulfide maintained good efficacy after 48 h. The MICs of diallyl trisulfide, dithiocyanomethane, and salicylic acid were 266.67, 50.00, and 166.67 mg/L, respectively. As shown in figure 5D, hyphal growth in the control group is good, suggesting that the antifungal effect was attributed to the drug.

Influence of the three drugs on the morphology of *S. parasitica*

Figure 5 illustrated the effects of diallyl trisulfide, dithiocyanomethane, and salicylic acid on hyphal morphology of *S. parasitica*. The results showed that three drugs inhibited the growth and changed the hyphal morphology on PDA plates. As shown in figure 5, comparing to control group, the hypha of *S. parasitica* in experimental group was shorter and hyphal density was significantly sparser.

**Figure 3.** The amplified fragment of ITS gene fragment of grass carp *Saprolegnia* sp.

Discussion

In traditional taxonomy, the identification of *Saprolegnia* sp. often depends on morphological features including hyphal morphology, zoospore release pattern, and the generation of new sporangia. However, because the morphology of *Saprolegnia* sp. is unstable and the shape of the hyphae of each species is very similar, it is usually difficult or even impossible to classify these fungi by describing the hyphal morphology alone. Molecular identification based on ITS rDNA

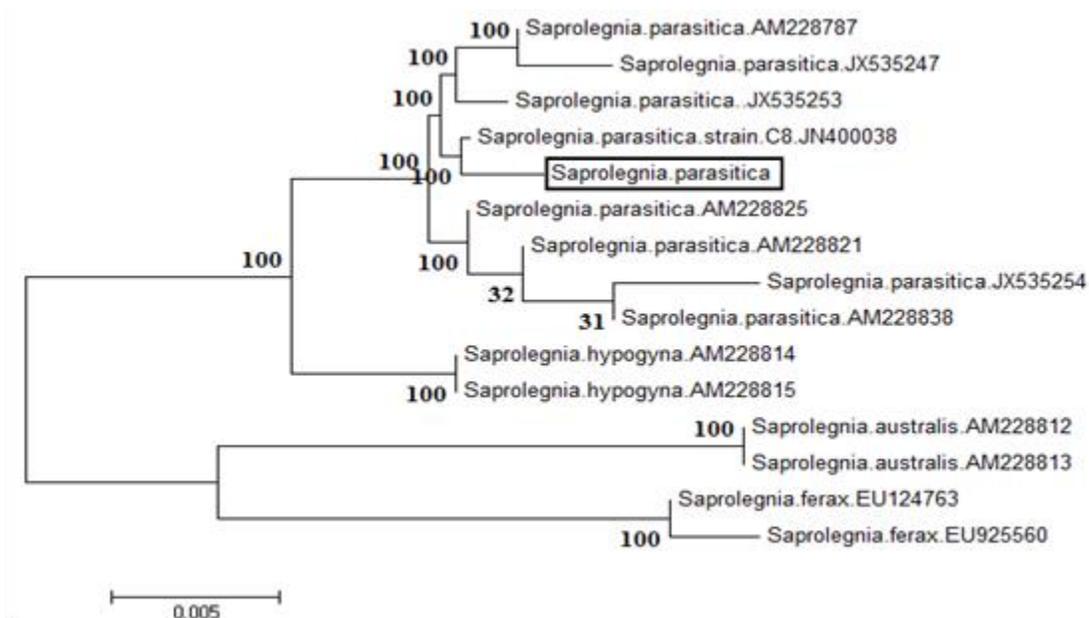


Figure 4. Phylogenetic tree constructed based on the ITS genes of *Saprolegnia parasitica*, *Saprolegnia hypogyna*, *Saprolegnia australis*, and *Saprolegnia ferax*. This phylogenetic tree was constructed with the neighbor joining method using Mega 6.0.

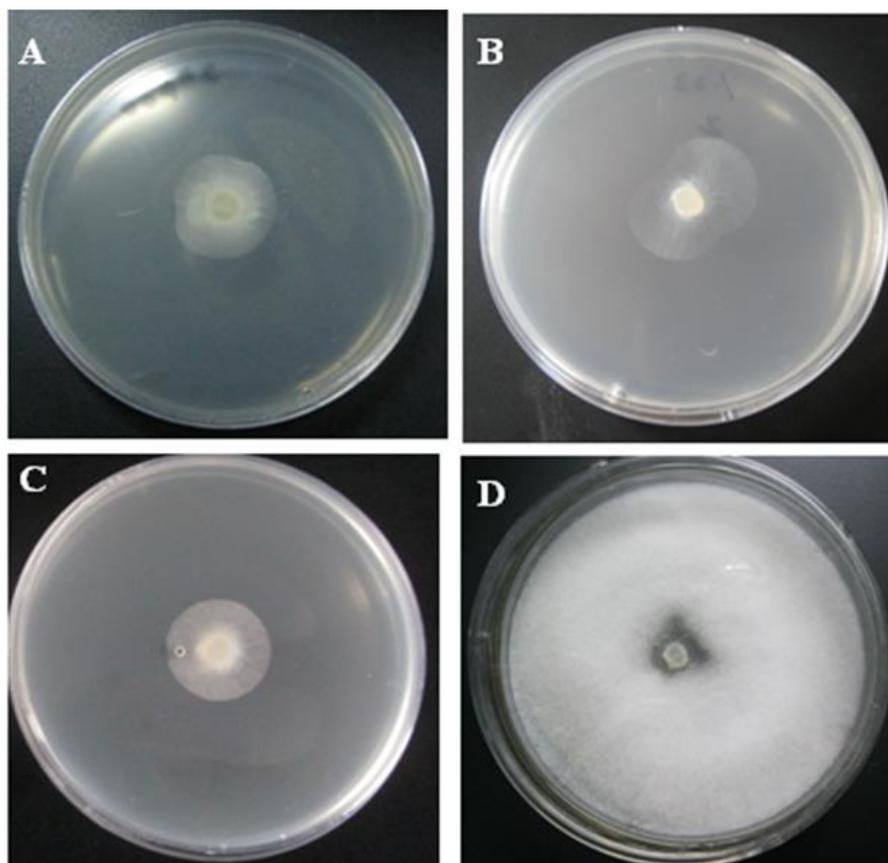


Figure 5. Changes of the growth of the *Saprolegnia* sp. by three antifungal drugs. A. 88.89 mg/L diallyl trisulfide. B. 37.50 mg/L disulfide-methane. C. 83.33 mg/L salicylic acid. D. control group.

sequence analysis has been widely adopted by mycologists [20, 23, 24]. In the present study, an isolate from grass carp water mold was initially identified *Saprolegnia* sp. based on its morphological characteristic, which was in line with the descriptions on *Saprolegnia* sp. by Eissa *et al.* [1]. Further, the phylogenetic analysis of ITS rDNA region clarified the taxonomic position of *Saprolegnia* sp. and confirmed its initial morphological identification.

Saprolegniasis is a common disease in freshwater fish causing severe losses in the farmed stock. To date, there were a few safe and effective chemicals used to control saprolegniasis in China such as formalin and dithiocyano-methane [20]. Diallyl trisulfide and salicylic acid are also the main antifungal drugs in current treatments. However, abuse of medicines approved for aquaculture increases drug resistance to microbes. The most significant reason is an incorrect dose of the drug, then leading to excessive dosing. Therefore, correct measurement of the MIC to an anti-saprolegniasis medicine can guide appropriate use.

Our antifungal effectiveness results indicated that the MIC of dithiocyano-methane against *S. parasitica* was around 50 mg/L, which was obviously lower than that of salicylic acid (166.67 mg/L) and diallyl trisulfide (266.67 mg/L). It follows that dithiocyano-methane possesses a strong inhibitory effect among three drugs. This result is consistent with other evidence that dithiocyano-methane can well inhibit the saprolegniasis of Yellow catfish (*Pelteobagrus fulvidraco*) eggs [20]. Dithiocyano-methane (structural formula: $\text{CNS-CH}_2\text{-SCN}$) can decompose into thiocyanate that blocks electron transfer in the microbial respiratory system and reacts with Fe^{3+} in cytochrome to generate $\text{Fe}(\text{SCN})_3$, and then generates red complex ion $\text{Fe}(\text{SCN})_6^{3+}$, in which the iron ions lose activity leading to death.

Diallyl trisulfide is an effective thioether constituent of allicin that possesses broad-

spectrum antifungal and antibacterial activities. It has been widely applied in medical and health industry. Liao *et al.* reported that multiple sulfocompounds are contained in a garlic extract liquid including diallyl monosulfide, diallyl disulfide, diallyl trisulfide, and dimethyl trisulfide. They have antimicrobial activities towards *Phytophthora capsici*, and that of diallyl trisulfide is the highest [25]. These sulfur compounds inhibit or kill multiple pathogens through three ways: (1) enzymes associated with microorganism growth and reproduction will be deactivated through sulfhydryl oxidizing; (2) they can competitively inhibit thioxy compounds (such as sarcosine, glutamic acid); (3) they can noncompetitively inhibit the activities of some enzymes [26]. In this study, although diallyl trisulfide has weaker microbicidal potential than dithiocyano-methane, the duration of efficacy is longer than the other two. Therefore, the advantage of using this drug when treating *Saprolegnia* sp. can reduce the frequency and avoid repeated use in the short time to prevent occurrence of drug resistance.

Salicylic acid, also named o-hydroxybenzoic acid, is a painkiller, keratolytic and fungicidal agent. When fish skin is infected with fungi, salicylic acid can soften the stratum corneum and cause the stratum corneum fallen off, hyphae detach from the skin. Rowe *et al.* demonstrated that benzoic acid and its derivatives have been used as antifungal agents in topical preparations for the treatment human infections [27] and show activity at different levels of fungal development [23, 28]. Salicylic acid helps other antifungal drugs penetrate and inhibit bacterial growth. Thus, salicylic acid can be cooperated with other anti-saprolegniasis drugs which are ideal to treat *Saprolegnia* sp. in practice.

In conclusion, the causative agent from grass carp fry is identified as *S. parasitica* based on morphological and phylogenetic analysis. The *in vitro* tests showed that diallyl trisulfide, dithiocyano-methane, and salicylic acid were effective against *S. parasitica*. Among these drugs, dithiocyano-methane showed the lowest

MIC while the effectiveness of diallyl trisulfide could maintain for a long time. In order to assess the possible application of these drugs, further tests will be necessary to evaluate the influence of different environmental factors on their efficacy.

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