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Enantiomeric Resolution and Growth-Retardant Activity in Rice Seedlings of Uniconazole

Jianqiang Sun,[†] Anping Zhang,[†] Jing Zhang,[§] Xuemei Xie,[‡] and Weiping Liu*,[§]

[†]Research Center of Environmental Science, College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou 310032, China

[‡]Lishui Environmental Monitoring Centre, Lishui 323000, China

[§]MOE Key Laboratory of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China

ABSTRACT: The increasing application of chiral pesticides has enhanced interest in their enantioselectivity. However, little relevant information is currently available for enantioselective activity of chiral plant growth regulators. In an attempt to screen active enantiomers of uniconazole, this work investigated enantiomeric separation and the enantioselective effect of uniconazole on the growth of rice seedlings and cyanobacteria. Baseline resolution of uniconazole enantiomers was achieved on a Chiralpak AD column by chiral high-performance liquid chromatography (HPLC). The relationship among circular dichroism (CD), optical rotation (OR), and absolute configuration was successfully established by coupling of CD and OR detection. The *t* test at the 95% level of confidence indicated significant differences between the enantiomers in their retardant activity toward growth of rice seedlings and stimulation effect on growth of cyanobacteria, the natural biofertilizers in rice paddy fields. The *S*-(+)-enantiomer was more active than the *R*-(−)-enantiomer in retarding growth of rice seedlings and stimulating growth of *Microcystis aeruginosa*. This special enantiomeric selectivity was further elucidated by probing the binding mode of enantiomers to gibberellin (GA) 20-oxidase by molecular docking. The *S*-(+)-enantiomer was found to bind tightly with GA 20-oxidase. The results suggested that the *S*-(+)-enantiomer instead of a racemate of uniconazole should be used to improve rice seedling quality.

KEYWORDS: *enantioselectivity, retardant activity, Xiushui 63, cyanobacteria, molecular docking*

INTRODUCTION

The enantioselectivity of chiral insecticides and herbicides has been widely recognized because of its environmental significance.[1] The different biological activities of enantiomers of chiral pesticides displayed in many research areas, such as toxicity, endocrine disruption effect, and environmental fate, have enhanced interest in the enantioselectivity and environmental safety of pesticides.[2−4] The previous studies indicated that chiral plant growth regulators would exhibit enantioselectivity in regulating the physiological processes of plants and causing toxicities or ecotoxicities to plants.[5,6] However, compared with chiral pesticides, there is an obvious lack of study on enantioselective activity and ecological effects of chiral plant growth regulators. For example, uniconazole [(*E*)-(*p*-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol] (Figure 1), a widely used chiral triazole plant growth retardant with an asymmetric carbon, was scarcely considered at the enantiomeric level in the investigation of biological activity.[7]

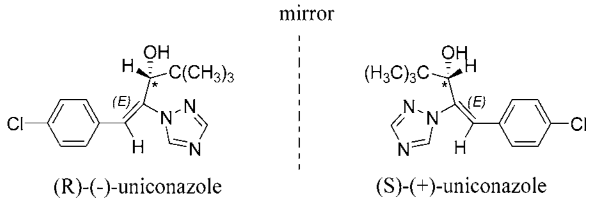

Figure 1. Chemical structures of enantiomers of uniconazole (∗ indicates stereogenic center).

Rice is the world's single most important staple food and a primary food source for more than a third of the world's human population.[8] The effective method of cultivating rice is flooding fields during or after setting of the young seedlings and then inoculation with cyanobacteria as natural biofertilizers in rice paddy fields.[9,10] Additionally, to improve the lodging resistance and the yield of rice, uniconazole was commonly used to control the growth of rice by seed soaking or leaf spraying.[11,12] No matter which application method of uniconazole in rice cultivation was adopted, it would be inevitably introduced into flooded rice water bodies and thus produce effects on the biomass of cyanobacteria. Therefore, the regulation role of uniconazole should be investigated from two aspects: one is direct interaction between uniconazole and rice, and the other is indirect influence, which was derived from the effect of uniconazole on cyanobacteria biomass. Furthermore, the research should be extended at the enantiomeric level, because enantiomers were known to selectively interact with biological systems and behaved as drastically different compounds.[1,13] Unfortunately, enantioselectivity was not involved in all studies that focused on regulation of rice by uniconazole.

In an attempt to fill in the knowledge gap, the enantioselective effects of uniconazole on the growth of rice seedlings and cyanobateria were studied in the present work.

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Rice Xiushui 63, a strain widely cultivated in China, was chosen to investigate the enantioselective activity of uniconazole on the growth of rice. To elucidate the underlying mechanism of enantioselective effects of uniconazole enantiomers on rice seedlings, the binding mode of enantiomers to multifunctional enzyme gibberellin (GA) 20-oxidase was performed. Two important cyanobacteria species, *Microcystis aeruginosa* and *Anabaena* sp., were used to evaluate possible enantioselective effects of uniconazole on biomass of natural biofertilizer microbes. Investigation into the role of enantioselectivity of uniconazole in rice cultivation is helpful to facilitate the proper application of the chiral plant growth regulator and potentially protect nontarget organisms in the aquatic environment.

■ MATERIALS AND METHODS

Chemicals, Rice Seeds, and Cyanobacteria. The analytical standard of uniconazole (racemate, purity of 99%) was provided by Jiangsu Sevencontinent Green Chemical Co., Ltd. (Zhangjiagang, China) and dissolved in ethanol at 2000 mg/L before use. All solvents and chemicals used in this study were of HPLC or pesticide-residue grade. Seeds of rice (Xiushui 63) were generously supplied by The National Rice Research Institute of China (Hangzhou, China). The two types of cyanobacteria, *M. aeruginosa* and *Anabaena* sp., were obtained from the Institute of Hydrobiology of Chinese Academy of Sciences (Wuhan, China).

Chromatographic Separation and Analysis. Enantiomers of uniconazole were resolved and isolated on a Jasco LC-2000 series high-performance liquid chromatograph (HPLC) (Jasco, Inc. Tokyo, Japan) with a Chiralpak AD column (250 × 4.6 mm, chiral stationary phase (CSP) [amylase tris(3,5-dimethylphenylcarbamate)], Daicel Chemical Industries, Tokyo, Japan). The two chiral detectors, a circular dichroism (CD) detector and an optical rotatory (OR) dispersion detector, were employed to assign the corresponding enantiomers of peaks in the chromatography. The processes of enantiomeric separation and collection were briefly introduced as follows. A volume of 20 μL of solution was injected into the HPLC in normal-phase mode with a flow rate of 1.0 mL/min of *n*-hexane/isopropanol (85:15) at room temperature (25 ± 2 °C). The resolved enantiomers were individually collected at the HPLC outlet of the OR detector according to the response variation.[14] The purity of the two collected enantiomers was found to be >99% by chiral HPLC analysis.

Quantification of enantiomer concentration was performed on an Agilent 6890N gas chromatograph (Agilent Inc., Santa Clara, CA) equipped with an electron capture detector (μ-ECD) and a HP-5 capillary column (30 m × 0.32 mm ×0.25 μm, Agilent Inc.). Samples were injected splitless (split opened after 1.0 min) at an initial temperature of 60 °C. After a 5 min hold, the oven was ramped at 20 °C/min to 140 °C and at 5 °C/min to 260 °C and then held for 10 min. The carrier gas was nitrogen at 1.0 mL/min. Injector and detector temperatures were 250 and 300 °C, respectively. After the qualification of purity and concentration of the collected enantiomers, the enantiomers were reconstituted in ethanol and stored at 4 °C in the dark.

Enantioselective Activity of Uniconazole on Rice Seedling Growth Tests. The activity of enantiomers and the racemate of uniconazole toward rice seedlings was investigated according to the procedure developed in our previous work.[6,15] Briefly, the rice seeds, which were treated by 10% sodium hypochlorite and distilled, were germinated in moist gauze. Uniformly germinated seedlings were selected and transferred into glass beakers containing growth medium spiked with known concentrations of the two enantiomers and the racemate of uniconazole (0, 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mg/L). The glass beakers were kept at 25 ± 1 °C in a climatic chamber with light/dark alternation (18000 lx, 14/10 h) and 60% relative humidity. Three replicates were prepared for each concentration level, and each replicate contained 10 seedlings. Ethanol was used as an assist additive, and the final volume in growth medium was <1% (v/v), which did not obviously affect rice seedling growth.

Homology Modeling. The amino acid sequence of GA 20-oxidase (GenBank ID no. AAM56041.1) was used for structural construction. The tertiary structure of GA oxidase was constructed with the I-TASSER server following the reported protocol.[16] The generated structures were finally validated by Prosa web server.[17]

Molecular Docking. The chemical structures of (*R*)- and (*S*)-uniconazole isomers were manually built and docked into the structure of GA 20-oxidase using the molecular docking soft MVD with the default protocols (version 3.2.0). The binding pocket covers a site with a user-defined origin and a radius of 15 Å. Twenty independent MolDock SE searching algorithms were applied for the docking. The energetic evaluations of the complexes were performed using the MolDockScore function. Ten poses were finally generated, and the best one with the highest score was chosen for the analysis.

Enantioselectivity of Uniconazole on Cyanobacteria Growth Tests. The enantioselectivity of uniconazole toward cyanobacteria growth was evaluated by relative growth rate of *M. aeruginosa* and *Anabaena* sp. in BG 11 growth medium with different concentrations of enantiomers and the racemate of uniconazole at 25 ± 0.5 °C. The tests were carried out in accordance with the method described in Zhang[6] and were briefly introduced as follows. Cyanobacteria in the exponential growth period were inoculated into BG 11 growth medium containing the racemate and enantiomers of uniconazole and then incubated in a climatic chamber under continuous illumination of 2000 lx with a daily cycle of 12 h light/12 h dark.[18] The concentration of racemate and enantiomers of uniconazole ranged from 0.01 to 0.5 mg/L. Biomass of cyanobacteria was determined by using a Jasco V-550 UV−vis spectrophotometer (Jasco, Inc., Tokyo, Japan) according to the linear equation between direct cell counts and optical density at 685 nm for *M. aeruginosa* and 680 nm for *Anabaena* sp. in Zhang.[6]

Data Analysis. Origin 6.0 (Microcal Software, Inc., Northampton, MA) was used to analyze the significance in the effect of these compounds on rice seedlings and cyanobacteria growth. Values were considered to be significantly different when the probability (p) was <0.05.

■ RESULTS AND DISCUSSION

Enantiomeric Separation and Analysis. Optimal separation of uniconazole enantiomers was achieved on the Chiralpak AD column by using *n*-hexane/IPA (85:15 by volume) as the mobile phase at 1.0 mL/min at room temperature (Figure 2). The OR chromatogram of the enantiomers successfully designated the first and second eluted compounds as (+)-uniconazole and (−)-uniconazole, respectively. According to Liu, the absolute configuration of dextrorotatory uniconazole is *S*, whereas the levorotatory isomer is *R*.[19] The CD chromatogram further indicated that *S*-(+)-uniconazole had a positive signal and the *R*-(−)-uniconazole had a negative signal at 250 nm. The uniconazole enantiomers were also successfully resolved on other chiral stationary phases by HPLC under normal or reversed phase conditions.[20] CD was often used to assign the chiral character of uniconazole enantiomers in these studies. However, OR and absolute configuration were the common configuration assignments of uniconazole enantiomers in early previous works that investigated chiral separation and enantioselective activity of uniconazole.[19] The inconsistency in assignments of configuration to uniconazole enantiomers led to fracture in finding the relationship of results involved in the studies. The relationship among OR, CD, and absolute configuration established in the present work could supply the useful information to resolve the difficulty. Moreover, the developed method warranted the sufficient purity of the enantiomers collected at the outlet of HPLC for activity assays in a short time, because excellent enantioseparation could be achieved in <10 min.

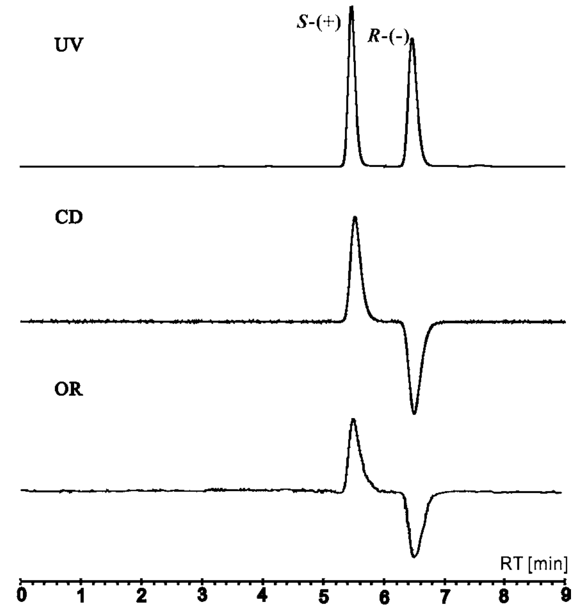

Figure 2. Chiral HPLC chromatography of uniconazole separated on the Chiralpak AD column.

Enantioselective Effects of Uniconazole on the Growth of Rice Seedlings. Two important agronomic traits for measuring plant growth, root length and shoot length, were chosen to evaluate the enantioselective activity of uniconazole in rice seedlings in this study. Retardant effects of uniconazole on shoot and root growth were observed in this work. Therefore, the relative inhibition rate (RIR%) was used to describe the retardant effects of uniconazole on the growth of rice seedlings and calculated by the equation described in Zhang[6]

$$\mathrm{RIR\%} = [(\mathrm{LT}_t - \mathrm{LT}_0)/(\mathrm{LC}_t - \mathrm{LC}_0) - 1] \times 100\% \qquad (1)$$

where LT_t and LT_0 are the shoot (or root) length in treatments at t and 0 days, respectively. LC_t and LC_0 represent the shoot (or root) length in the control (CK) at t and 0 days, respectively.

As shown in Tables 1 and 2, the inhibitory effect increased with the increase of concentration. This was a little different from the effect of paclobutrazol on the growth of rice seedlings; that is, hormesis on root growth of rice seedlings was induced by low concentrations of paclobutrazol and its enantiomers (0.125–0.5 mg/L), whereas retardant effects were produced by high concentrations (4–8 mg/L).[6] Moreover, uniconazole could achieve a higher inhibition rate than paclobutrazol to rice seedlings growth at the same concentration level. Therefore, it was concluded that uniconazole was more active than paclobutrazol in retarding the growth of rice seedlings.

Comparison of the two enantiomers revealed significant differences for shoots and roots at concentrations ranging from 0.0625 to 0.125 mg/L and from 0.0625 to 4 mg/L, respectively. The enantioselectivity is consistent between shoots and roots of rice seedlings; that is, the *S*-(+)-enantiomer always had higher activity than the *R*-(−)-enantiomer in retarding shoot and root growth of rice seedlings in the above range of concentrations. The observation was similar to the results of retarding rice seedlings by paclobutrazol, which indicated that (2*S*,3*S*)-(−)-paclobutrazol was always more effective than (2*R*,3*R*)-(−)-paclobutrazol toward the growth of rice seedlings.[6] However, the consistent enantioselectivity between shoots and roots of rice seedlings was not a general rule for chiral pesticides. For example, our previous work demonstrated the *R*-diclofop acid enantiomer was more active toward root growth, whereas the *S*-enantiomer was more active toward shoot growth.[13] Thus, enantiomer-specific responses in shoots and roots must be considered in evaluating the enantioselective effect of chiral pesticides on the growth of rice seedlings. A further statistical analysis showed that there were no significant differences of retardation activity between the *S*-(+)-enantiomer and the racemate. The above observations indicated the activity of the *rac*-uniconazole was due to to the *S*-(+)-enantiomer. Therefore, application of the pure *S*-(+)-enantiomer instead of the racemate of uniconazole is recommended for rice cultivation to reduce unknown environmental risk from the *R*-(−)-enantiomer.

Interactions between Uniconazole with GA 20-Oxidase. The plant hormone gibberellin has long been known to modulate developmental processes, including seed germination, elongation growth, flowering, and fruit development.[21] Plant growth retardants are used to control the stature of cereals through inhibiting GA biosynthesis. GA 20-oxidase, a key and multifunctional enzyme in the biosynthesis of GAs, can catalyze the conversion of the nonactive carbon-20 GAs to the physiologically active carbon-19 GAs.[22]

To explore the mechanism of enantioselective effects of uniconazole, it is essential to probe the interactions between uniconazole and GA 20-oxidase at the atomic level. Molecular docking was applied to investigate the binding mode of uniconazole to GA 20-oxidase. The *R*-(−)- and *S*-(+)-enantiomers were separately docked into the active site of GA 20-oxidase (Figure 3).

Uniconazole enantiomers were located at the active site of GA 20-oxidase and were surrounded by the following amino

Table 1. Enantioselective effect of Uniconazole on Shoot Elongation of Rice Seedlings

	relative inhibition rate of root (%) at a concentration of						
compound	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L
S-(+)-uniconazole	57.5 ± 2.3	62.9 ± 2.3	65.5 ± 0.7	69.9 ± 6.9	73.4 ± 5.1	76.1 ± 1.5	79.3 ± 3.8
rac-uniconazole	53.2 ± 2.1	62.9 ± 1.9	64.9 ± 0.9	68.4 ± 7.1	72.5 ± 6.1	74.9 ± 6.5	77.9 ± 5.9
R-(−)-uniconazole	37.4 ± 5.2	43.7 ± 3.6	64.4 ± 5.0	68.7 ± 4.6	69.4 ± 3.9	74.0 ± 4.1	76.2 ± 0.8

Table 2. Enantioselective Effect of Uniconazole on Root Elongation of Rice Seedlings

	relative inhibition rate of root (%) at a concentration of						
compound	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L
S-(+)-uniconazole	40.2 ± 4.1	44.6 ± 1.7	44.5 ± 3.2	50.1 ± 5.1	56.5 ± 7.2	59.5 ± 4.9	70.0 ± 3.8
rac-uniconazole	25.7 ± 5.6	38.0 ± 4.3	39.3 ± 9.9	50.2 ± 2.8	57.1 ± 2.8	59.0 ± 6.6	69.8 ± 7.8
R-(−)-uniconazole	13.6 ± 8.2	26.2 ± 4.7	32.1 ± 4.7	35.4 ± 3.6	38.2 ± 5.9	55.2 ± 4.4	55.3 ± 5.6

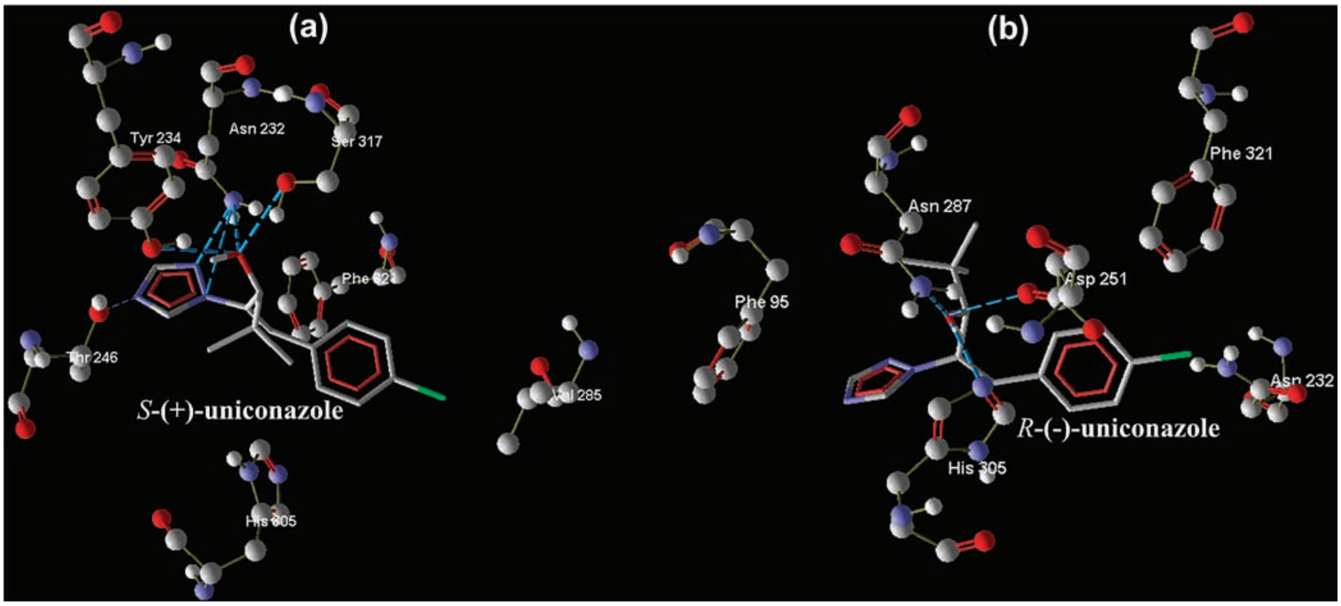

Figure 3. Binding mode of *R*-(−)- and *S*-(+)-isomers to GA 20-oxidase. The dashed line indicates the hydrogen bond formed between uniconazole isomers and GA 20-oxidase. The uniconazole isomers are shown in stick and the residues of GA 20-oxidase in licorice representation.

Table 3. Enantioselective Effect of Uniconazole on Growth of *M. aeruginosa*

	growth stimulation rate of *M. aeruginosa* (%) at a concentration of			
compound	0.01 mg/L	0.05 mg/L	0.1 mg/L	0.5 mg/L
S-(+)-uniconazole	15.8 ± 2.8	17.7 ± 2.0	27.5 ± 0.2	30.6 ± 0.5
rac-uniconazole	15.2 ± 4.6	16.8 ± 5.4	26.7 ± 1.6	27.3 ± 6.1
R-(−)-uniconazole	9.2 ± 4.4	9.3 ± 1.1	18.7 ± 3.4	21.0 ± 3.4

Table 4. Enantioselective Effect of Uniconazole on Growth of *Anabaena* sp.

	growth stimulation rate of *Anabaena* sp. (%) at a concentration of			
compound	0.01 mg/L	0.05 mg/L	0.1 mg/L	0.5 mg/L
S-(+)-uniconazole	18.2 ± 9.6	30.7 ± 6.7	37.3 ± 3.1	41.6 ± 0.5
rac-uniconazole	21.3 ± 4.4	32.9 ± 0.1	45.6 ± 6.6	50.1 ± 2.3
R-(−)-uniconazole	38.1 ± 1.8	62.0 ± 3.7	79.0 ± 6.7	84.3 ± 7.6

acid residues: Asn 287, Asn 232, Asp 251, His 305, Phe 95, Phe 321, Tyr 234, Ser 317, Thr 246, and Val 285. *S*-(+)-Uniconazole (Figure 3a) was located at the active site of GA 20-oxidase and formed six hydrogen bounds with GA 20-oxidase, including three hydrogen bonds formed with Asn 232 by its functional nitrogen-containing heterocyclic ring. However, *R*-(−)-uniconazole (Figure 3b) has only three hydrogen bonds with Asn 287, Asp 251, and His 305. Our experiment on retarding shoot and root growth of rice seedlings showed that the *S*-(+)-enantiomer always had higher activity than the *R*-(−)- enantiomer. *S*-(+)-Uniconazole has more specific hydrogen bond interactions with GA 20-oxidase, which has an effect on the function of GA 20-oxidase, resulting in reduced biosynthesis of GAs; consequently, the growth of plant was retarded.

Enantioselective Effects of Uniconazole on Growth of Cyanobacteria. Different from the inhibitory effect of enantiomers and racemate of uniconazole toward rice seedling growth, *S*-(+)-, *R*-(−)-, and *rac*-uniconazole all could significantly stimulate the growth of *M. aeruginosa* and *Anabaena* sp. at concentrations ranging from 0.01 to 0.5 mg/L (Tables 3 and 4). The growth stimulation rate (GSR) was used to quantitatively describe the effect of uniconazole on the growth of cyanobacteria and was calculated by the equation

$$\mathrm{GSR\%} = [(\mathrm{ODT}_t - \mathrm{ODT}_0)/(\mathrm{ODC}_t - \mathrm{ODC}_0) - 1] \times 100\% \tag{2}$$

where ODT_t and ODT_0 are the optical density of cyanobacteria in treatments at *t* and 0 days, respectively. ODC_t and ODC_0 represent the optical density of cyanobacteria in the control (CK) at *t* and 0 days, respectively.

GSRs increased with concentration of uniconazole within the investigated concentration range (0.01−0.5 mg/L) for both *M. aeruginosa* and *Anabaena* sp. The stimulation was very helpful to rice cultivation, because rapid growth of cyanobacteria could produce more neutron nutrients to rice in the paddy. For *S*-(+)-uniconazole, the active enantiomer to rice seedlings, the stimulation of growth of *M. aeruginosa* was not obviously different from that of *Anabaena* sp. Therefore, the choice between *M. aeruginosa* and *Anabaena* sp. was not important to rice cultivation in which *S*-(+)-uniconazole was used as a growth retardant.

Enantioselectivity was also observed in the stimulation effect of uniconazole on the growth of the two cyanobacteria. For *M. aeruginosa*, differences ranging from 1.5- to 2-fold were exhibited between the *S*-(+)- and *R*-(−)-enantiomers. For *Anabaena* sp., *R*-(−)-uniconazole gave about 2 times more potent stimulation than *S*-(+)-uniconazole, with the racemate having a bit higher effect than the latter. It is worth noting that the enantioselectivity is reversed between *M. aeruginosa* and *Anabaena* sp. *S*-(+)-Uniconazole had a more potent stimulatory effect on *M. aeruginosa* than *R*-(−)-uniconazole. On the contrary, *R*-(−)-uniconazole was more active than *S*-(+)-uniconazole in stimulating the growth of *Anabaena* sp. The similar enantioselectivity had also been observed for stimulation of *M. aeruginosa* and *Anabaena* sp. by paclobutrazol. Although the mechanisms still could not be thoroughly elucidated by the present knowledge, it again definitely proved that enantioselectivity of certain chiral compounds was closely related with the species.

R-(−)-Uniconazole had potential to enter the water body through leaching and/or runoff because of its high solubility in water and could strongly stimulate the growth of *Anabaena* sp. Moreover, *Anabaena* sp. was the first dominant species in cyanobacteria bloom.[23] Therefore, *R*-(−)-uniconazole was highly likely to lead to cyanobacteria blooming and should be eliminated from rice cultivation.

The results of the present study first demonstrated the enantioselectivity of uniconazole in retarding rice seedling growth and stimulation of cyanobacteria growth. *S*-(+)-Uniconazole was more effective in retarding rice seedling growth than *R*-(−)-uniconazole. The application of pure *S*-(+)-uniconazole instead of the racemate of uniconazole was thus recommended to improve rice seedlings. The enantiomers and racemate of uniconazole all significantly stimulated the growth of cyanobacteria. *S*-(+)-Uniconazole showed a stronger stimulatory effect on *M. aeruginosa* than *R*-(−)-uniconazole, whereas the latter showed a stronger stimulatory effect on *Anabaena* sp. The differences of stimulatory effect of *S*-(+)-uniconazole between *M. aeruginosa* and *Anabaena* sp. were not obvious. Therefore, both *M. aeruginosa* and *Anabaena* sp. could be used as natural biofertilizers in rice paddies. Because *R*-(−)-uniconazole had a potent stimulatory effect on *Anabaena* sp. growth, pure *S*-(+)-uniconazole instead of the racemate of uniconazole was needed to reduce cyanobacteria bloom and other unknown environmental risks posed by *R*-(−)-uniconazole.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86-571-8898-2740. Fax: +86-571-8898-2341. E-mail: wliu@zju.edu.cn.

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