JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Enantioselective Separation and Phytotoxicity on Rice Seedlings of Paclobutrazol

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ABSTRACT: The environmental significance of enantioselectivity in chiral insecticides and herbicides has been widely studied. However, little information is currently available on the enantioselective behavior of chiral plant growth regulators. In this study, paclobutrazol enantiomers were resolved and prepared by chiral high-performance liquid chromatography with a Sino-chiral OJ column. The relationship among absolute configuration, optical activity and circular dichroism of paclobutrazol enantiomers was established. The enantioselective behavior of paclobutrazol, including enantioselective effect of paclobutrazol on the growth of rice seedlings and cyanobacteria and enantioselective loss of paclobutrazol in rice seedling growth media, in rice culture system was studied. The (2*S*,3*S*)-(−)-enantiomer was almost 3.1 times more active than the (2*R*,3*R*)-(+)-enantiomer toward shoot growth as measured by 7 day EC50 values. Enantioselectivity could not be determined with respect to root growth of rice seedlings because a typical dosage response was not observed in the range of the concentrations studied. The dissipation of paclobutrazol in rice growth medium is not enantioselective. Enantiomers and diastereoisomer of paclobutrazol all facilitated the growth of cyanobacteria, which increase the effectiveness of rice biofertilizers. The (2*S*,3*S*)-(−)-enantiomer showed stronger stimulatory activity on *Microcystis aeruginosa* cyanobacteria than the (2*R*,3*R*)-(+)-enantiomer, whereas the latter was a more potent stimulator of *Anabaena* sp. growth. These observations indicate that application of the (2*S*,3*S*)-(−)-enantiomer of paclobutrazol and *Microcystis aeruginosa* in rice cultivation is a good strategy for improving rice seedling performance.

KEYWORDS: enantioselectivity, paclobutrazol, toxicity, rice, cyanobacteria

INTRODUCTION

The enantioselectivity of pesticides is an important consideration for reasons of activity and environmental safety. Enantiomers often exhibit differences in effects or toxicity: the "active" enantiomer of a chiral pesticide has the desired effect on a target species, whereas the other enantiomer may not.[1] Therefore, research on enantioselectivity is necessary to promote the use of pure active enantiomers in order to reduce waste and undesirable effects.

Enantioselectivity of chiral insecticides and herbicides in environmental metabolism, ecotoxicity and phytotoxicity has been recognized in recent years.[2] However, the enantioselective ecological effects and toxicities of chiral plant growth regulators have not received as much attention as those of chiral insecticides and herbicides. Paclobutrazol [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol] (Figure 1), a triazole plant growth regulator, is a chiral compound that contains two stereogenic centers, and therefore consists of two diastereoisomers, each composed of a pair of enantiomers.[3] The diastereoisomer composed of (2*R*,3*R*)- and (2*S*,3*S*)-enantiomers has a very high fungicidal and plant-growth regulatory activity, while the other diastereoisomer is very much less active.[4] The present process of paclobutrazol production gives only the diastereoisomer composed of (2*R*,3*R*)- and (2*S*,3*S*)-enantiomers.[5] Several works at the resolved enantiomer level have shown that paclobutrazol could produce significant enantioselective effects on plant growth.[6] The (2*S*,3*S*)-enantiomer is a potent inhibitor of apple seeding growth, whereas the (2*R*,3*R*)-enantiomer is less effective.[4] In contrast, the (2*R*,3*R*)-enantiomer had higher potency for inhibiting cell proliferation and sterol composition in celery than the corresponding enantiomer.[7] The (2*S*,3*S*)-enantiomer inhibited shoot growth more effectively than root growth in wheat seedlings, whereas the opposite was seen with the (2*R*,3*R*)-enantiomer.[8]

Rice, the most important staple food for the human population, is grown on all continents and is the grain with the second highest worldwide production. The most used method for cultivating rice is flooding the fields during or after setting the young seedlings. This irrigation is favorable for the formation and growth of cyanobacteria, which make a significant contribution as natural biofertilizers in rice paddy fields.[9,10] A study of agricultural practice indicates that paclobutrazol is an effective plant growth regulator that improves rice seedling quality when applied by spraying or immersing.[11] It is thought that a large proportion of paclobutrazol would fall into flooded rice soil in the processes of spraying, and thus affect the growth of cyanobacteria.

In order to gain insight into the role of enantioselectivity of paclobutrazol in rice cultivation, this work investigated the enantioselective effects of paclobutrazol on the growth of rice seedlings and cyanobacteria. *Xiushui 63*, a rice strain widely cultivated in China, was used to examine the enantioselective activity of paclobutrazol on the growth of rice. *Microcystis aeruginosa* (*M. aeruginosa*) and *Anabaena* sp., two important cyanobacteria species found in rice paddy soils, were used to

Received: December 21, 2010
Accepted: February 28, 2011
Revised: February 27, 2011
Published: March 11, 2011

evaluate possible enantioselective effects of paclobutrazol on populations of natural biofertilizer microbes.

■ MATERIALS AND METHODS

Chemicals, Rice Seeds and Cyanobacteria. Paclobutrazol (99% purity, only containing (2*R*,3*R*)- and (2*S*,3*S*)-enantiomers) was purchased from Jiangsu Sevencontinent Green Chemical Co., Ltd. (Zhangjiagang, China). Solvents including *n*-hexane, ethanol (EtOH), isopropanol (IPA) and ethyl acetate were HPLC grade from Tedia (Fairfield, OH, USA). The pesticide stock solution was prepared by dissolving paclobutrazol in ethanol at 2000 mg/L and stored at 4 °C in the dark. Rice seeds (*Xiushui 63*) were provided by The National Rice Research Institute of China (Hangzhou, China). The two types of cyanobacteria, *M. aeruginosa* and *Anabaena* sp., were obtained from Institute of Hydrobiology of Chinese Academy of Sciences (Wuhan, China).

Chromatographic Separation and Analysis. Enantiomers of paclobutrazol were resolved on a Jasco LC-2000 series high performance liquid chromatography (HPLC) system with a variable wavelength CD-2095 circular dichroism detector (CD) and an OR-2090 plus optical rotary dispersion detector (ORD) as used in previous studies.[10] The detection wavelength of the CD detector was 230 nm. The column was Sino-chiral OJ (250 × 4.6 mm, Funsea Technology Inc., Beijing, China) with chiral stationary phase (CSP) [cellulose tris(4-methylbenzoate)] coated onto a 5 μm silica gel substrate. A volume of 20 μL was injected for enantiomeric separation in a normal-phase eluent of hexane/isopropanol (85/15) at a flow rate of 1.0 mL min^{-1}. To obtain enantiopure paclobutrazol for phytotoxicity, the resolved enantiomers were individually collected at the HPLC outlet of the ORD during the observed responses.[12] The purity and concentration of the recovered enantiomers were determined by chiral HPLC and GC analysis, respectively. The enantiomeric fraction was found to be higher than 0.99 for the two enantiomers in the study. An Agilent 6890N GC (Agilent Inc. USA) equipped with an electron capture detector (μ-ECD) and a HP-1701 capillary column (30 m × 0.32 mm × 0.25 μm, Agilent Inc. USA) were used to quantify the concentration of each enantiomer. The inlet and detector temperatures were 250 and 300 °C, respectively. The flow rate of carrier gas (nitrogen) was 1.0 mL min^{-1}. The column was held at 60 °C for 5 min, ramped at 20 °C min^{-1} to 140 °C (first ramp) and then at 5 °C min^{-1} to 260 °C (second ramp) and held at 260 °C for 10 min.

The enantiomeric fraction of paclobutrazol was determined on an Agilent 6890N gas chromatography with a BGB-172 column (30 m × 0.25 mm × 0.25 μm, 20% *tert*-butyldimethylsilyl-β-cyclodextrin dissolved in 15% dimethylpolysiloxane, BGB Analytik, Anwil, Switzerland). The GC conditions were as follows: oven temperature 90 °C for 1 min, from 90 to 160 at 15 °C min^{-1}, from 160 to 200 at 2 °C min^{-1} held for 3 min, from 200 to 230 at 15 °C min^{-1} and 230 °C held for 20 min; flow rate of carrier gas (nitrogen) 1 mL min^{-1}; ECD temperature 300 °C, injector temperature 250 °C; injection mode, splitless.

Enantioselective Effect of Paclobutrazol on Rice Seedling Growth Tests. Before being used in tests, the rice seeds were pretreated by sterilization in 10% sodium hypochlorite for 10 min, washing with intensive distilled water, immersion in distilled water for 1−3 days and a final wash with distilled water. The treated seeds were placed on moist gauze in growth medium for germination until seedlings could be chosen for the growth inhibition test. The growth medium was prepared according to the International Rice Research Institute's nutrient solution protocol.[13] Seedlings of all experiments were kept in a climatic chamber under controlled environmental conditions with light/dark alternation (18,000 lx, 14 h/10 h), a 25 ± 1 °C temperature regime and 60% relative humidity.

Studies of the effect of paclobutrazol on rice seedlings growth inhibition tests were carried out according to OECD guidelines for the testing of chemicals.[14] Seven day old seedlings which had primary roots and shoots 2−3 cm in length were selected and transferred to glass beakers containing the two separated enantiomers and diastereoisomer of paclobutrazol solutions of various concentrations (0, 0.125, 0.25, 0.5, 1, 2, 4, and 8 mg/L). Ethanol was used as assist additive, and the final concentration was <1% (v/v), which did not produce a significant effect on rice seedling growth. Three replicates were used in each treatment, and every replicate contained 10 seedlings. The relative inhibition rate of root or shoot elongation was determined after 7 days. The relative inhibition rate (*I*%) was calculated by the following equation:

$$I = [1 - (X_t - X_0)/(A_t - A_0)] \times 100$$

where A_0 and X_0 represent the shoot (or root) length in the control (CK) and treatments at zeroth day, respectively. A_t and X_t are the shoot (or root) length in CK and treatments at *t* days, respectively.

Dissipation of Paclobutrazol in Rice Growth Medium. The loss of paclobutrazol in rice growth cultures was determined as follows. The rice seedlings (as above) were transferred to a container with 2.5 L of fresh growth medium. The initial concentrations of paclobutrazol solution in the growth medium were 1.0 mg/L. Samples were prepared in triplicate, and another set of samples without rice seedlings served as control. All containers were kept in a climatic chamber under the conditions described for rice seedling growth inhibition studies. Three replicate samples were extracted from each treatment at 0, 1, 2, 3, 5, 7, 11, 21, 30 days after pesticide addition, respectively. Growth media (2 mL) were sampled at room temperature, filtered through a 0.45 μm membrane filter, and then transferred to 10 mL glass centrifuge tubes. The samples were mixed with anhydrous sodium sulfate, shaken with 2 mL of ethyl acetate for 10 min by mechanical shaker and then centrifuged at 5000 rpm for 10 min. The same extraction step was repeated a total of three times, and the solvent extracts were filtered through 5 g of anhydrous sodium sulfate for dehydration. The combined extract was evaporated to semidryness at 35 °C under nitrogen and reconstituted in *n*-hexane for gas chromatographic (GC) analysis. The extract (1 μL) was injected into an Agilent 6890 GC equipped with ECD for concentration determination.

Two sets of samples without the pesticide were used to validate the method, among which one contained rice seedlings and the other did not. The samples were extracted at scheduled intervals. The pesticide was spiked in samples and then analyzed by the method. The detection limit and recovery of the method were 10 μg L^{-1} and 85 ± 5.0%, respectively.

Enantioselective Effect of Paclobutrazol on Cyanobacteria Growth Tests. The cyanobacteria, *M. aeruginosa* and *Anabaena* sp., were maintained in growth medium BG 11 at 25 ± 0.5 °C in a climatic incubator under continuous illumination of 2000 lx with a daily cycle of 12 h light and 12 h dark.[15] Each culture was shaken four times per day to prevent settling of the cyanobacteria and ensure optimal growth. The cyanobacteria were periodically inoculated into fresh medium to keep cells in the logarithmic growth phase. The cell density of *M. aeruginosa* and *Anabaena* sp. in growth medium were monitored at 685 and 680 nm by using a Jasco V-550 UV/vis spectrophotometer. The regression equations of cyanobacteria between cell density ($y \times 10^5$ cells L^{-1}) and OD (x) were calculated as $y = 437.5x - 41.0$ ($R^2 = 0.98$) for *M. aeruginosa* and $y = 306.5x - 7.5$ ($R^2 = 0.98$) for *Anabaena* sp.

Study of the enantioselective effect of paclobutrazol on cyanobacteria growth test was conducted in accordance with the updated OECD guideline.[16] Cyanobacteria in the exponential growth period were inoculated into flasks containing the test solutions at a density of approximately 10^6 cell mL^{-1}. The concentration of diastereoisomer and enantiomers of paclobutrazol was 0.05, 0.1, 0.5 and 1.0 mg/L. All flasks were repositioned daily within the climatic chamber to minimize

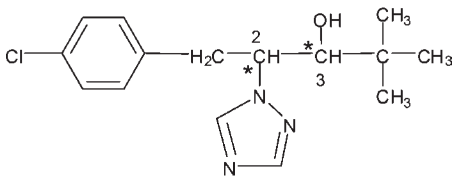

Figure 1. The structure of paclobutrazol (* chiral center).

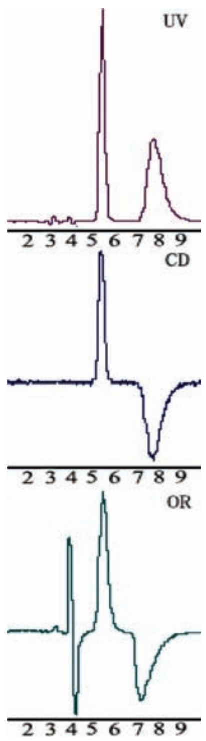

Figure 2. Chiral HPLC chromatography of paclobutrazol separated on the Sino-chiral OJ column.

possible spatial differences in illumination and temperature. The effect of paclobutrazol on cyanobacterial growth was determined according to the linear equation between direct cell counts and optical density.

Data Analysis. Half-maximal effective concentration (EC50) values were determined using the LD50 Data Processing Program (Version 1.01) (Blue Cosmos Studio, Guangzhou, China), based on the Probit analysis method. Statistical analysis for significance in the effect of these compounds on rice seedlings and cyanobacteria growth was performed using Origin 6.0 (Microcal Software, Inc., Northampton, MA, USA). Values were considered to be significantly different when $p < 0.05$.

RESULTS AND DISCUSSION

Enantiomeric Separation and Analysis. Paclobutrazol enantiomers were baseline resolved on a Sino-chiral OJ column with a mobile phase of n-hexane/isopropanol (85:15, v/v) at a flow rate of 1.0 mL min^{-1} at room temperature (Figure 2). The OR chromatogram indicated that (+)-paclobutrazol was eluted on the column prior to its (−) form. The separation could be achieved in less than 10 min. According to Liu,[17] the absolute configuration of dextrorotatory paclobutrazol is (2*R*,3*R*), whereas the levorotatory isomer is (2*S*,3*S*). The CD chromatogram further indicated that (2*R*,3*R*)-(+)-paclobutrazol had a positive signal and the (2*S*,3*S*)-(−)-enantiomer had a negative signal at 230 nm. Enantiomeric separation of paclobutrazol enantiomers was also previously achieved on amylopectin-tris-(phenylcarbamate) and amylose tris-(*S*)-1-phenylethylcarbamate CSP by HPLC.[18,19] However, the absolute configuration and optical activity of enantiomers were not distinguished in these studies, and the separation time was greater than 15 min. The newly developed method has obvious advantages in confirming the absolute configuration of the enantiomers and collecting quantities sufficient for phytotoxicity assays in a short time.

Table 1. 7d-EC50 Values of Paclobutrazol for Shoot Length of Rice Seedlings

compound	r^2	p	7d-EC_{50} (mg/L)
(2*S*,3*S*)-(−)-paclobutrazol	0.98	<0.001	0.66 ± 0.31
rac-paclobutrazol	0.97	<0.001	1.48 ± 0.24
(2*R*,3*R*)-(+)-paclobutrazol	0.93	<0.001	2.03 ± 0.33

Enantioselective Effects of Paclobutrazol on the Growth of Rice Seedlings. Root length and shoot length are two important agronomic traits for measuring plant growth. Thus, these parameters were used to evaluate enantioselective activity of paclobutrazol in rice seedlings in this study. As shown in Table 1 and Figure 3a, the 7 day EC50 (7d-EC50) values of (2*S*,3*S*)-(−)-, (2*R*,3*R*)-(+)- and *rac*-paclobutrazol for shoot length of rice seedlings were 0.66 ± 0.30 mg/L, 2.03 ± 0.33 mg/L and 1.48 ± 0.24 mg/L (95% fiducial limits), respectively. Statistical analysis of the 7d-EC50 values revealed that (2*S*,3*S*)-(−)-enantiomer was almost 3.1 times more active than the (2*R*,3*R*)-(+)-enantiomer on shoot growth. The 7d-EC50 values of enantiomers and diastereoisomer of paclobutrazol for root length of rice seedlings were not determined because a typical dose response was not observed in the range of paclobutrazol concentrations in the present study. Low concentrations (0.125−0.5 mg/L) of paclobutrazol and its enantiomers induced hormesis on root growth of rice seedlings, whereas high concentrations (4−8 mg/L) had retardant effects (Figure 3b). This result is consistent with previous observations that paclobutrazol can increase crop and tree root development.[20−23] The goal of paclobutrazol application was to improve seedling quality by inhibiting leaf elongation and facilitating root development. According to the application rate of paclobutrazol in rice,[24] it is suspected that the concentration of paclobutrazol in paddy field is generally less than 0.5 mg/L. Although both the enantiomers and diastereoisomer of paclobutrazol could improve rice seedling quality at low concentrations, pure (2*S*,3*S*)-(−)-enantiomer was more effective than the others. Thus, application of pure (2*S*,3*S*)-(−)-enantiomer instead of diastereoisomer of paclobutrazol is recommended for rice cultivation so as to reduce the environmental load. Stimulation of root development by paclobutrazol did not always occur in prior tests; for example, in a study of the enantioselective activity of paclobutrazol in wheat seedlings, Lenton et al. found that the paclobutrazol and its enantiomers all had inhibitory effects on leaf growth and root elongation.[8] This could be due to insufficient concentrations of paclobutrazol and/or to a lack of responsiveness of wheat seedling roots to any concentration of paclobutrazol. Thus, both concentration and species must be considered in attempting to improve seedling quality through the use of pure enantiomers or diastereoisomer of paclobutrazol.

Dissipation of Paclobutrazol in Rice Cultures. The enantiomer fractions (EFs) of paclobutrazol in rice cultures were

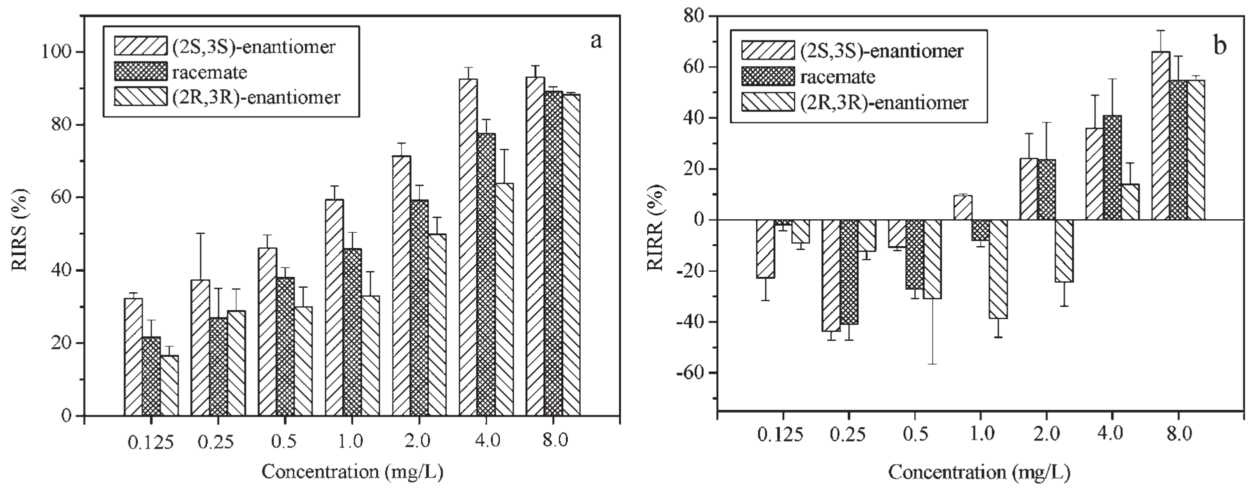

Figure 3. Enantioselective effect of paclobutrazol on root and shoot elongation of rice. (a) Relative inhibition rate of shoot (RIRS). (b) Relative inhibition rate of root (RIRR).

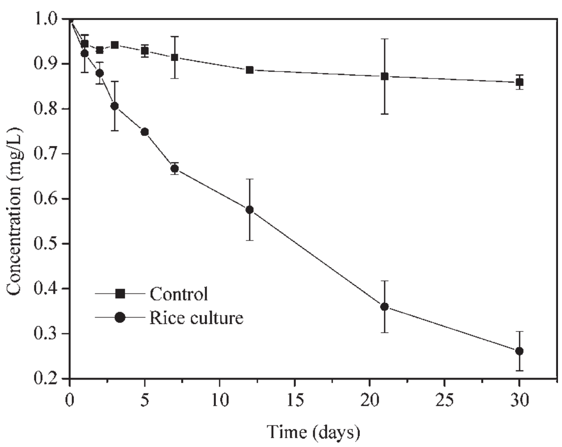

Figure 4. Loss of paclobutrazol from control and rice cultures.

measured at predetermined intervals. EFs of the samples ranged from 0.489 to 0.504, which are not significantly different from that of the diastereoisomer. This indicates that the loss of paclobutrazol in rice growth medium is not enantioselective. The result is consistent with a previous study, which showed that the dissipation of diclofop in rice growth medium was nonenantioselective.[25] Figure 4 shows changes in the residual concentration of paclobutrazol with exposure times in rice seedlings and control medium. After a 30 day, long-term hydroponic experiment, almost 74% of the *rac*-paclobutrazol was dissipated in the rice seedling growth medium. However, the *rac*-paclobutrazol was barely degraded in the control medium throughout the 30 day experiment period, with more than 86% of the initial concentration still present on the 30th day. These results suggested that hydrolysis was not the leading dissipation pathway of paclobutrazol in the rice seedling growth medium, while degradation and/or uptake by rice seedlings might predominate the dissipation of paclobutrazol.

Enantioselectivity in Aquatic Toxicity of Paclobutrazol on Cyanobacteria. Aquatic toxicity tests showed that paclobutrazol had significant effects on the growth of *M. aeruginosa* (Figure 5a) and *Anabaena* sp. (Figure 5b). Specifically, paclobutrazol could stimulate the growth of cyanobacteria at concentrations ranging from 0.05 to 1 mg/L. The growth of *Anabaena* sp. was more highly stimulated by paclobutrazol than that of *M. aeruginosa*. The enantioselective effect of paclobutrazol on *M. aeruginosa* and *Anabaena* sp. was related to concentration of paclobutrazol. For *Anabaena* sp., there were no significant differences in stimulation between (2*S*,3*S*)-(−)-paclobutrazol and (2*R*,3*R*)-(+)-paclobutrazol at low concentrations (Figure 5b). Also 1.41- and 1.47-fold differences were observed between (2*R*,3*R*)-(+) and (2*S*,3*S*)-(−)-enantiomers of paclobutrazol at concentrations of 0.5 and 1.0 mg/L, respectively. For *M. aeruginosa*, (2*S*,3*S*)-(−)-paclobutrazol gave 1.3−1.7 times more potent stimulation than (2*R*,3*R*)-(+)-paclobutrazol, with the diastereoisomer having an intermediate effect between the two enantiomers (Figure 5a). Cai et al. reported that the (*S*)-(−)-enantiomer of the chiral herbicide diclofop-methyl had a stronger toxicity to both *Chlorella ulgaris* and *Scenedesmus obliquus* than the (*R*)-(+)-enantiomer.[26] However, in the present study, the enantioselectivity of paclobutrazol on two freshwater species differed. We found that the (2*S*,3*S*)-(−)-enantiomer had a more potent stimulatory effect on *M. aeruginosa* than the (2*R*,3*R*)-(+)-enantiomer, whereas the (2*R*,3*R*)-(+)-enantiomer stimulated the growth of *Anabaena* sp. more than the (2*S*,3*S*)-(−)-enantiomer. Although the mechanism for differential enantiomer-specific responses in these species is uncertain, it is clear that enantioselectivity has specific effects on different organisms.

This study demonstrated enantioselectivity for improving rice seedling quality and stimulation of cyanobacteria growth by paclobutrazol. The (2*S*,3*S*)-(−)-enantiomer was more effective in improving rice seedling quality than either the (2*R*,3*R*)-(+)-enantiomer or the diastereoisomer. The loss of paclobutrazol in rice growth medium might be mainly due to degradation and/or uptake by rice seedlings, which is not enantioselective. Although the two enantiomers and the diastereoisomer of paclobutrazol all significantly stimulated the growth of cyanobacteria, the stimulus ability of the enantiomers was different. The (2*S*,3*S*)-(−)-enantiomer showed a stronger stimulatory effect on *M. aeruginosa* than the (2*R*,3*R*)-(+)-enantiomer whereas the latter showed a stronger stimulatory effect on *Anabaena* sp. Therefore, when using cyanobacteria as natural biofertilizer in rice paddies, the application of pure (2*S*,3*S*)-enantiomer instead of diastereoisomer of paclobutrazol and inoculation with *M. aeruginosa* is

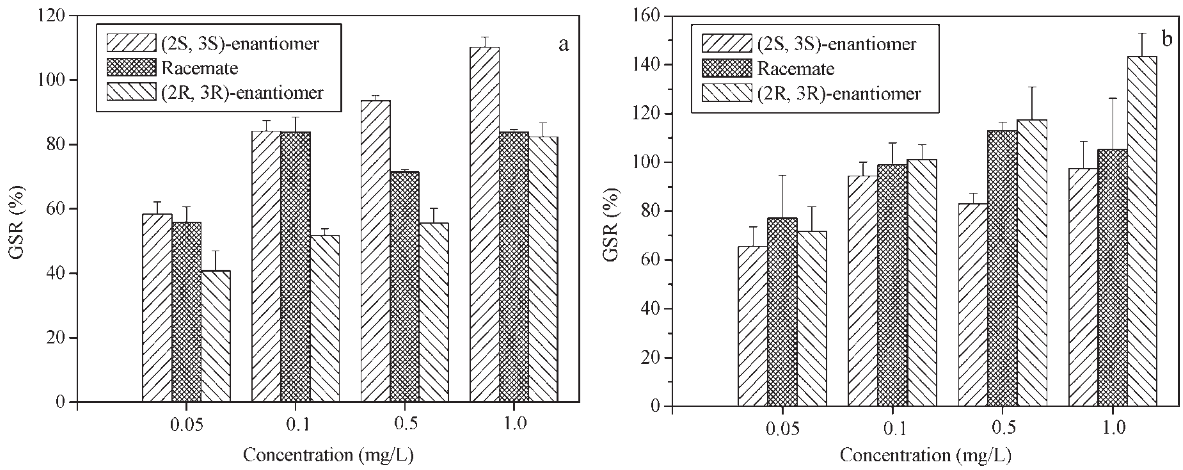

Figure 5. Enantioselective effects of paclobutrazol on growth of *M. aeruginosa* (a) and *Anabaena* sp. (b). GSR, growth stimulation rate.

recommended so as to reduce the environmental load and improve the performance of rice seedlings. In addition, because paclobutrazol is relatively soluble in water and has obvious stimulus effects on growth of cyanobacteria, contamination of paclobutrazol from rice paddies to aquatic systems through leaching must be taken into consideration.

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Funding Sources

This study was supported by the National Natural Science Foundation of China (30771255 and 41073090), the National Basic Research Program of China (2009CB421603), and the Natural Science Foundation of Zhejiang Province, China (Y507220).

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