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Dichlorvos Degradation by REMI Mutants of *Trichoderma koningii*

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Abstract: The dichlorvos degradation abilities of *Trichoderma koningii* mutants obtained by REMI (restriction enzyme mediated integration) and the optimal conditions for effective degradation were studied. Results showed both the wild type (T30) and mutants (TK-1, TK-2, TK-3, TK-5, TK-7, TK-8, TK-21, TK-38, TK-53, TK-30, TK-42) were able to degrade dichlorvos. TK-3 could reach 98% dichlorvos decomposition, which is the most efficient among mutants. Dichlorvos degradation rate was closely associated with glucose, dichlorvos initial concentration and pH of the medium. It was found that glucose at 1 000 μg/mL, pH 7.0 and dichlorvos at 500 μg/mL were the optimal conditions for effectively degrading dichlorvos by the mutant TK-3. The degradation efficiency of TK-3 would decline if dichlorvos concentration exceeded 1 000 μg/mL.

Key words: *Trichoderma koningii*; restriction enzyme mediated integration (REMI); dichlorvos; degradation
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木霉菌 REMI 突变菌株在降解敌敌畏中的作用

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摘 要:本研究对木霉菌 T30 野生株和限制性内切酶介导的 DNA 整合技术(REMI)构建的突变株进行降解敌敌畏能力和最佳条件的研究。结果表明,野生株和几个 REMI 突变株均能降解有机磷农药敌敌畏,但以突变株 TK-3 降解活性最高,其降解效率达 98%。TK-3 突变株降解有机磷农药效率取决于葡萄糖剂量、敌敌畏初始浓度、初始 pH 等。突变株降解敌敌畏的葡萄糖最佳浓度为 1 000 μg/mL,最适 pH 为 7.0,敌敌畏浓度 500 μg/mL。敌敌畏浓度超过 1 000 μg/mL 则会抑制木霉菌降解能力。

关键词:康氏木霉; 限制性内切酶介导基因合技术(REMI); 敌敌畏; 降解

Trichoderma, as a bio-control microbe, has been widely applied in the control of soil

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borne diseases[1-2], which is a resource-rich antagonistic organism as well as a useful soil remediation microorganism. As people pay more attention to agricultural environment pollution control, the *Trichoderma* strain is required not only with antimicrobial activity but also with potential to reduce pollutants[3-6]. Therefore, in the future, multi-function *Trichoderma* agents would be highly valuable for comprehensive application in agriculture[7].

In the past decades, numerous microorganisms have been genetically modified for improving degradation activity of organophosphorus pesticide. Lorz et al.[8] successfully used protoplast transformation to construct a multi-functional genetically engineered strain. Woloshuk et al.[9] transferred insect detoxifying gene into microbial, creating a new pesticide degrading strain. Sun et al.[10] took advantage of gene recombination in different microbe and finally obtained a strain with better dichlorvos degradation performance.

Nowadays, studies on the degradation of organophosphorus pesticides by *Trichoderma* are increasingly emphasized because of extensive concerns over environment pollution[11]. However, as most wild type strains from *Trichoderma* seemed not effective enough to reduce organophosphorus pesticide residues in environment, genetic modification should be a rational choice to improve their degradation ability. REMI(Restriction Enzyme Mediated Integration) is a relatively new approach to create diversified molecular mutations by random insertion of DNA fragment into chromosome, elite functional strain may be obtained from mutated population. Liu et al.[12] used REMI to obtain *Trichoderma viride* T21, which works better than Ttrm31, Ttrm34 and Ttrm55 in bio-control tomato grey mold caused by *Botrytis cinerea*. The three REMI mutants produce higher chitinase and β-1, 3 glucanase activity than wild type, suggesting the bio-control activity is significantly improved. Zhou[13] had studied cyanides degradation efficiency of different *Trichoderma* REMI mutants. It is found that the mutants T30, TKB6 and TaK1 show higher rhodanese activity than wild type strain and other mutants. Therefore, REMI is a promising method to effectively create genetic variation to improve theagro-chemicals residue degradation ability ofTrichoderma. Huang[14] also used REMI to get plenty of transformants, it is proved that there are significant improvement of *Trichoderma* in the inhibition against barnyard grass and tall fescue grass growth, which means that selected mutants of *Trichoderma* are useful to inhibit the growth of some herbs. Taken together, *Trichoderma* REMI mutants can be used in many fields.

To date, however, reports on the role of REMI *Trichoderma* mutants in the degradation of dichlorvos are pretty limited. Previous studies demonstrated microorganisms that can degrade dichlorvos are mainly focused on bacteria. In this study, we intend to evaluate the activity of *Trichoderma koningii* REMI mutants in dichlorvos degradation, and find out the optimal conditions for REMI transformants to degrade DDV efficiently, eventually open a new way to treat harmful pollutants present in agricultural environment.

1 Materials and methods

1.1 Strains

T. koningii wild type T21, T23, T30, and REMI mutants TK-1, TK-2, TK-3, TK-5, TK-7, TK-8, TK-21, TK-38, TK-53, TK-30, TK-42, were stored in School of Agriculture & Biology, Shanghai Jiaotong University.

1.2 Medium

Potato dextrose agar(PDA), Potato dextrose

(PD)，Burk media(g/L：KH_2PO_4，0.8；$MgSO_4 \cdot 7H_2O$，0.2；$CaSO_4 \cdot 2H_2O$，0.1，$Na_2MoO_4 \cdot 2H_2O$，0.0033；$FeSO_4 \cdot 7H_2O$，0.005；$(NH_4)_2SO_4$，1.0；glucose，1.0)(pH 7.0).

1.3 Reagent

80% dichlorvos，100 μg/mL dichlorvos acetone solution (purchased from Beijing Zhongwei Food Hygienic Technology Company)，10%(*V*/*V*)ethanol-water solution，1%(m/*V*) resorcinol aqueous solution，0.5%(m/*V*)NaOH solution，etc.

1.4 Primary screen

The Trichoderma *koningii* strains stored with silicone beads were cultured on PDA for 3-4 days until the spores were produced. With hole punchers，the colony were inoculated into Burk solid media containing dichlorvos (100 mg/mL)，incubated at 28 ℃ for 5 days. Tolerance of dichlorvos for different *T. koningii* mutants was compared.

1.5 Re-screen

The spores were washed by sterile water，diluted into 1×10^7 conidia/mL，and then inoculated in PD (100 mL). Flasks were shaked at 28 ℃ for 2 days. After filtration with Buchner funnel and washing with sterile water，thallus were weighed and transferred to Burk media containing dichlorvos，cultured at 30 ℃ with shaking(180 r/min) for 60 h，the concentration of dichlorvos was determined after filtration.

Resorcinol fluorescence technique was used to determine the contents of dichlorvos. The sample was diluted with culture medium containing dichlorovs to 10 mL by adding 4 mL 10% (*V*/*V*) ethanol solution，0.96 mL 0.5%(m/*V*)NaOH solution and 0.24 mL 1% (m/*V*) resorcinol aqueous solution. After blending，samples were placed in boiling water bath for 3 min，when it's cool to room temperature by water，the fluorescence intensity at excitation wave length λex 491.6 nm and fluorescent wave length λem 521.1 nm was determined. Meanwhile，media without dichlorvos was used as control. If the initial concentration of dichlorvos was C1，concentration after treatment was C2，the dichlorvos degradation rate could be represented as(C1−C2)/C1×100%.

1.6 Factors affecting dichlorvos degradation by TK-3

Three factors (pH，glucose concentration and dichlorvos concentration) were studied to evaluate their influence on dichlorvos degradation by REMI transformant TK-3. Detailed experimental procedures were as follows：

Burk media containing 300 μg/mL dichlorvos was adjusted to different pH value (pH 3-10)，inoculated with the REMI mutants of *T. koningii*，and then it was cultured shakily (120 r/min) at 28 ℃ for 60 h，the residual concentration of dichlorvos was determined to calculate the decomposition rate.

The mutants of *T. koningii* was inoculated into Burk media containing 300 μg/mL dichlorvos，and gluocose was added to different concentration (500，1 000，5 000，10 000 μg/mL)，cultured with shaking(120 r/min)at 28 ℃ for 60 h，dichlorvos degradation rate was determined as described.

Then，500 μg/mL gluocose was added to Burk media，containing different concentration of dichlorvos (50，100，300，500，1 000 μg/mL)，then the mutants of *T. koningii* were inoculated and cultured at the above mentioned conditions. Then，dichlorvos degradation rate was determined as described.

2 Results

2.1 Primary screen of strains

It showed all transformants of *Trichoderma koningii* could grow on Burk solid media containing dichlorvos，indicating REMI mutants of *T. koningii* had tolerance for dichlorvos. Clones with relatively fast

growth were subjected to re-screen.

2.2 Re-screen of strains

After further analysis, it was found there were great differences between different transformants (Fig. 1). Among the transformants from T30, TK-3 had the highest degradation rate (up to 96.9%). Spore suspension, fermentation broth and the mycelium broken by ultrasonication of TK-3 were studied separately when exposed to dichlorvos to identify the degradation process. It indicated the mycelium of the mutants of Trichoderma *koningii* played an important role in degrading dichlorovs.

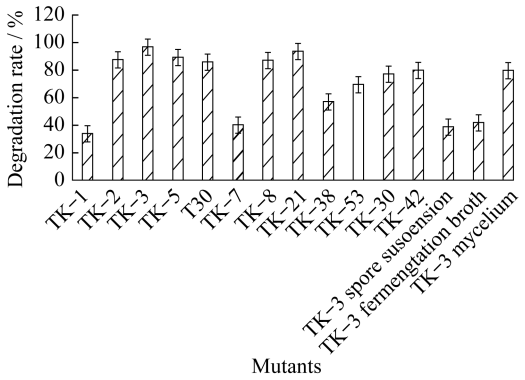

Fig. 1 Degradation rate of dichlorovs by different *Trichoderma* mutants

2.3 Factors affecting dichlorvos degradation by TK-3

TK-3 could grow well at different pH(6-9), when pH was 7, the degradation rate of TK-3 reached maximum(Fig. 2). As pH rose, the degradation rate gradually decreased.

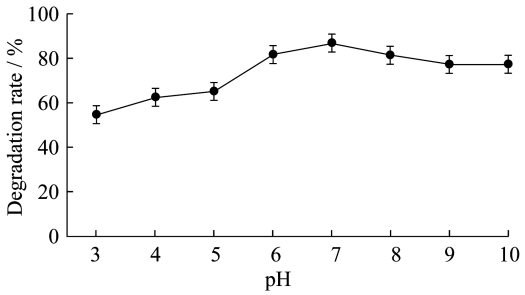

Fig. 2 Degradation efficiency of TK-3 under different pH

Fig. 3 showed that at different glucose concentration, the degradation rate was different. When it was 1 000 μg/mL, the degradation rate could reach up to 95.9%. However, if the glucose concentration was reduced, the degradation rate was dropped down as well. But if glucose concentration was over 1 000 μg/mL, the mutants of *T. koningii* were induced to use glucose in priority and the degradation rate decreased as a result.

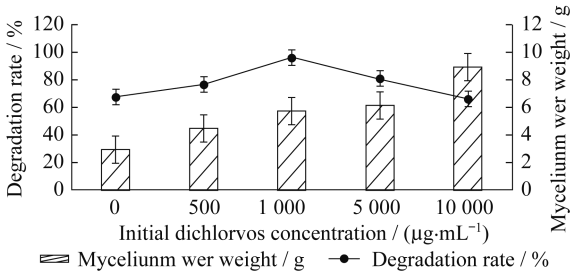

Fig. 3 Degradation efficiency of TK-3 under different glucose concentrations

Fig. 4 indicated when dichlorvos concentration was 500 μg/mL, the degradation rate was up to the highest. The degradation rate increased linearly with initial dichlorvos concentration differed from 50 μg/mL to 500 μg/mL. When increased to 1 000 μg/mL, the degradation rate declined. It declared that the tolerance dichlorvos concentration for transformant of *T. koningii* was 500 μg/mL, if the concentration was too high, it may poison the transformant and make the degradation rate decreased.

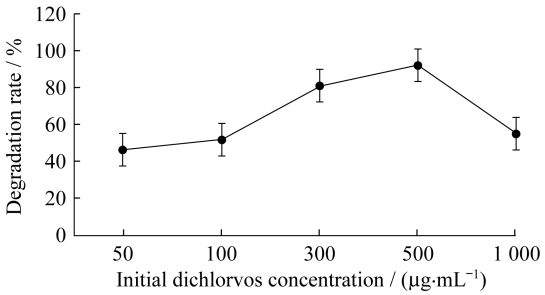

Fig. 4 Degradation efficiency of TK-3 under different initial dichlorvos concentrations

3 Discussion

To date little work has been reported on

degradation of dichlorvos by *Trichoderma*. The major target pesticides for being investigated on microbial degradation includes parathion, monocron and dimethoate and the majority of investigated microbe are bacteria rather than fungi. Even so those bacteria are not effective enough in dichlorvos degradation. Tang et al.[15] reported that *Trichoderma* had a relatively high degradation rate for dichlorvos. Similarly, in this study, we researched the degradation of dichlorvos by *T. koningii* and its REMI transformants. The results indicated that both the wild type and mutants had ability to degrade dichlorvos. In general, there was a closely connection between dichlorvos degradation and experimental conditions including amount of glucose, dichlorvos initial concentration and pH of the medium.

As to organophosphate pesticide degradation mechanism, some authors have reported the significance of co-metabolism.

Sun et al.[16] has studied *T. atroviride* on degrading pesticides and found that degradation was closely related to its growth, indicating co-metabolism degradation. When glucose concentration was 5 g/L, the mycelium grew quickly while the degradation rate of methamidophos decreased. However, when glucose concentration was increased to 10 g/L, degradation of methamidophos was fully inhibited, suggesting that substrate may play an important role in pesticide degradation by microbes which means the excess of nutrients may inhibit the degrading activity of enzymes. Therefore, researches on the type and proportion of carbon, nitrogen and phosphorus were needed to clarify dichlorvos degradation mechanism by *Trichoderma*. In this study, it was demonstrated that the degradation rate of REMI mutants of *T. koningii* was lower when glucose concentration was 500 μg/mL and 5 000 μg/mL than 1 000 μg/mL, which implied presence of co-metabolism between REMI mutants of *T. koningii* and dichlorvos, and depended on the concentration of carbon source. In addition, based on our previous experiment, we hypothesis that higher concentration of glucose over 1 000 μg/mL may influence the function of some transporters that pump dichlorvos out of cells.

Dichlorvos concentration is also a major factor impacting biodegradation of dichlorvos. When the concentration of dichlorvos is too high, that would be toxic to the microbe, leading to microbial population dropped significantly. To the contrary, if the concentration of dichlorvos is too low, degradation rate is also declined as lacking of carbon and nitrogen source to support microbe growth. Zhao et al.[5] confirmed when the concentration of dimethoate was 0.2%, the degradation by microorganism was the best, while lower in degradation at 0.1% and 0.5%. Here, in this article, we've got the similar results. When dichlorvos concentration was 500 μg/mL, the degradation rate was the highest, but declined at 100 μg/mL and 1 000 μg/mL. Thus, it is not a linear relation between pesticide concentration and degradation level by microbe. In other words, the biodegradation relied on specific concentration could provide a scientific basis to face up to serious pollution incited by over release of pesticide into environment. Meanwhile, studies on degradation of low concentration pesticides could provide theoretical basis and reference to bioremediation on environmental pollution caused by pesticide residues.

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