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The influences on conidiophore pleomorphism in *Clonostachys rosea* and RAPD analysis to the mutant producing only verticillate conidiophores

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Abstract - Being an effective biocontrol agent, *Clonostachys rosea* possess morphologically two different kinds of conidiophore structures, verticillate and penicillate respectively. However, the factors that influenced this morphological pleomorphism and its related genetic background remains to be elucidated. In this study, single-spore isolates were obtained from these two types of conidiophores and observed for their cultural and morphological characteristics. The stability of pleomorphism was confirmed through three-months of continuous inoculation and incubation. Additionally, the influences on conidiophore's morphology under different cultural conditions were also recorded. Our data indicated that the fungal inhibitor thiabendazole changed the formation of conidiophores with only verticillate type produced, which subsequently had effects on its abilities to infect the plant pathogenic fungus *Rhizoctonia solani* and nematodes *Panagrellus redivivus* in our bioassay. However, the genomic analysis by Random Amplified Polymorphic DNA (RAPD) assay showed no obvious differences detected in the mutant with only verticillate conidiophores and its parent strain, suggesting the change in conidiophore's type should be attributed to different transcriptional patterns.

Key words: *Clonostachys rosea*; conidiophore pleomorphism; RAPD analysis.

INTRODUCTION

Pleomorphism generally refers to the phenomenon that a fungus contains both the sexual and asexual reproductive structures at the same time and that it produces more than one kind of spores. In the narrow sense, it refers to having two or more types of asexual reproductive structures (Liang and Roland, 1998). Carmichael (1981) classified the asexual pleomorphism into three categories and included a large number of examples. Gams (1982) summarized Carmichael's points using more data and reclassified it into two categories. Recent elaboration on the investigation of fungal pleomorphism demonstrated that there were other pleomorphisms of the chlamydospore, fungus nuclear and the hypha besides the spore asexuality pleomorphism (Liang, 1997). Among them, conidiophore pleomorphism in fungi is currently considered as belonging to the following three categories: (1) conidiophores containing more than two types of conidia with dissimilar appearances grow on the same strain; examples of this group include *Fusarium* sp. that has both big spores and small ones at the same time (Pitt and Hocking, 1997); (2) conidiophores that have more than two different growth types, such as *Hirsutella pichilinguensis* parasitizing the larva of Lepidoptera; it has been described as having not only conidiophores that are transparent, smooth, thin-walled, pouroveal or obpyriformis, but also conidiophores with big, narrow-rod phragmospore with thick wall diaphragm, at the same time; (3) conidiophores having a variety of sporonts, for example, it has been reported that entomogenous fungi *Paecilomyces farinosus*, *Paecilomyces tenuipes* and *Hirsutella stibelliformis* could all produce conidiophores that were peduncle and gleba in coremium and mycelium at the same time. In another species, *Paecilomyces odonatae,* there are two types of conidiophores, with the conidiophore of *Acremonium* first, followed by the production of penicillate conidiophores (Liu *et al*., 2000). The fungus *Paecilomyces militaris* generates verticillate and penicillate conidiophores successively (Liang and Roland, 1998).

Clonostachys rosea, an important biocontrol agent against pathogenic fungi as well as nematodes, has also been described as having an asexual pleomorphism (Hoopen *et al*., 2003; Dong *et al*., 2004). Its conidiophores contain the morphological characters of *Verticillium* sp., *Penicillium* sp. and *Trichothecium* sp., and this pleomorphism had contributed to the confusion in anamorph nomenclature and taxonomy. Often, such polymorphic conidiophores

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may also be mistakenly identified as contaminants of other fungal species. Today, despite the solution on taxonomic status, the conditions influencing conidiophore types and its related genetic background are still unclear. In this study, the stability of pleomorphism was confirmed by studying single-spore isolates. In addition, several potential factors that could affect the generation of conidiophores were also investigated. Furthermore, we compared the genetic background in the mutant with only verticillate conidiophores to its parent strain.

MATERIALS AND METHODS

Strains and media. The fungus *Clonostachys rosea* 176 was isolated from the larvae of nematodes in Jianshui County, Yunnan Province, and deposited in the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University.

The media used in the experiments were Corn Meal agar (CMA), Potato Dextrose agar (PDA) (Yu *et al*., 2007) and Basic medium. The ingredients of Basic medium include (%, w/v): 1 maltose, 0.2 $NaNO_3$, 0.05 $MgSO_4 \cdot 7H_2O$, 0.05 KH_2PO_4, 0.065 Na_2HPO_4, 0.05 KCl, 2 agar, pH 6.5.

Isolation of single spores and morphological observation. The isolation of single spores was performed based on the modified method previously used (Li *et al.*, 2006b). Briefly, when *C. rosea* grew with diameter of 1-2 cm on PDA medium, samples were examined using a dissecting microscope. Single spore was isolated with a sterilized toothpick and then cultivated on fresh PDA again at 28 °C. This process would be repeated for at least three months. Morphological properties of the conidiophores were observed by using light microscopy (BH 2; Olympus).

Influence of different cultural conditions on conidiophores formation and the growth in *Clonostachys rosea.* The effect of pH was assayed by cultivating the fungus on PDA at pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, and 14.0. The fungus was inoculated on the culture media using an inoculumn of 5 mm in diameter. After ten days of incubation at 28 °C, the fungus was observed both microscopically and macroscopically.

The effect of temperature was assayed by cultivating the fungus on PDA at 10, 15, 20, 25, 28, 30, 33, 35, and 37 °C for ten days.

To test the effect of light, *C. rosea* was cultivated in poor or good light conditions, respectively on PDA at 28 °C for ten days.

To test the effect of the carbon source, 1, 2, 3 and 4% (w/v) glucose, sucrose, glycerol was added to the Basic medium to substitute the original source of carbon individually. The fungus was cultivated at 28 °C for ten days and then observed under microscope.

To investigate the influence of nitrogen source on *C. rosea*, 0.2, 0.5 and 1% (w/v) $NaNO_3$, aspartic acid, peptone and $(NH)_2SO_4$ were added to the Basic medium individually, and then cultivated at 28 °C for ten days.

Additionally, the effects on the formation of conidiophores were also assayed by adding different fungal inhibitors: thiabendazole (TBZ) 0.13 ml/L, dimethachlon 0.25 ml/L, pingyangmycin 0.25 ml/L, carbendazim 45 mg/L, kasugamycin 0.9 mg/L, thiran 60 mg/L, tricyclazole 45 mg/L, tricyclazole 60 mg/L, hymexazol 114 mg/L, polyoxin 210 mg/L, and then cultivated at 28 °C for ten days.

All experiments above were performed with at least 3 repeats.

The bioassay of *Clonostachys rosea.* The bioassay against nematodes was performed according to the method reported by Liu and Chen (2000). The tested strains of *C. rosea* (including wild strain and the mutant) were inoculated to the centre of the PDA plates and cultivated at 25 °C for 7 days, while the same culture media without *C. rosea* were employed as the control. After washing the nematode *Panagrellus redivivus* thoroughly with a 10 mM sterilized PBS (phosphate buffered saline, pH 7.0), a suspension containing 500 nematodes (about 100 µl) was added in the culture media. The culture plate was exposed to sterilized air for half an hour to vaporize the redundant water. Then the culture plates were incubated for 48 h. The nematodes were then washed with 5 ml of 0.1% Tween-20, and about 200 nematodes were selected randomly and observed under the microscope. The numbers of dead and living nematodes were counted, and the percentage of dead was calculated:

Fixation percentage = [the number of dead nematodes - the total number of the nematodes x (1 - the mortality of control)] / (the total number of the nematodes) x 100

In the experiment to test parasitic activity of *C. rosea* against *Rhizoctonia solani*, both of them were co-cultured on 1% PDA at 28 °C, with about 2 cm of overlap between the two colonies. The parasitic capacity was determined based on the relative numbers of sclerotia formed by *R. solani*. The number of the sclerotia produced by *R. solani* in the overlapped colony and the number of the sclerotia formed in the absence of *C. rosea* at the opposite end on the same media were counted, taking into account the size of the area where sclerotia was counted. The latter treatment was used as the negative control.

The parasitic capacity = (the sclerotia formed in the control - the sclerotia formed in the overlapped section) / (the sclerotia of the control) x 100

Based on the percentage of sclerotia reduction, relative parasitism were classified to five grades 0, 1, 2, 3, 4 corresponding to the percent of sclerotial reduction in the overlapped colony by 0-25%, 25-50%, 50-75%, 75-100% respectively.

These bioassay experiments above were repeated at least ten times.

Genomic DNA extraction. DNA extraction was performed by CTAB method. Briefly, 200 mg fungus mycelium was ground to a fine paste in approximately 500 µl of CTAB buffer. CTAB/fungi extract mixture was added to a microfuge tube, and then incubate for about 15 min at 55 °C in a water bath. After that, centrifuge was performed at 12000 *x g* for 5 min to remove cell debris. The supernatant would be transfer to clean tubes followed by adding 250 µl of chloroform:iso amyl alcohol (24:1) to each tube. After mixing by inversion, the mixture was spun at 13000 rpm for 10 min, and then the upper aqueous phase only (contains the DNA) was transfer to new tubes. By adding 50 µl of 7.5 M ammonium acetate and 1 ml of ice cold 100% ethanol, the genomic DNA was precipitated at 13000 rpm for 30 min. Twice wash to DNA was performed using 500 µl of ice cold 75% ethanol. At last, the pellet of DNA would be dried and resuspended in 50 µl sterile DNase free water.

TABLE 1 - The primers used in RAPD analysis

Primer No.	Sequences of primer (5′-3′)	Primer No.	Sequences of primer (5′-3′)
OPA-02	TGCCGAGCTG	S-22	TGCCGAGCTG
OPC-15	GGAACTGCTT	S-23	AGTCAGCCAC
OPD-05	TGAGCGGACA	S-28	GTGACGTAGG
OPL-02	TGGGCGTCAA	S-36	AGTCAGCCAC
OPE-20	AACGGTGACC	S-51	AGCGCCATTG
OPN-14	TCGTGCGGGT		

RAPD analysis. The RAPD reaction was performed in a total volume of 50 µl consisting of 5 µl template DNA, 10X PCR buffer, 3.5 mM MgCl2, 200 µM deoxynucleoside triphosphates, 1.25 U of Taq DNA polymerase (Fermentas, Lithuania), and 1 µM of primer. PCR cycling procedures were as follows; 45 cycles of 94 °C for 1 min, 37 °C for 1 min and 72 °C for 1 min. A final step of extension was applied at 72 °C for 10 min. After PCR reaction, 5 µl products were analyzed by electrophoresis in a 1.5% agarose gel with TBE (tris-borate-EDTA) buffer.

Eleven random primers (Table 1) were used to determine the genetic differences between the mutant and its parent strain. Besides that, two additional wild isolates from some other origins were employed in our RAPD analysis as controls.

Statistical analysis. All data from repetitive experiments were expressed as mean ± SE. The results were analyzed using SPSS13.0. Significant differences among experimental time points were analyzed within a level using a one-way ANOVA. Differences were considered to be statistically significant at the level of $p < 0.05$.

RESULTS

Stability of conidiophores pleomorphism in *Clonostachys rosea*

Asexual single spore isolates were continuously inoculated and cultivated for three-months. It was found that each isolate whether from verticillate or penicillate conidiophores would stably produce two types of conidiophores. In detail, the elementary (verticillate) conidia could be observed after two days of incubation on PDA at 25 °C followed by penicillate conidiophores from the fourth day. When 5 microscopic fields were randomly selected to every single spore isolate and both conidiophores were counted, our results showed that the single spore isolates from penicillate conidiophores would generate 83.6 ± 7.1 verticillate type and 337.0 ± 23.2 penicillate type respectively; while the single spore isolates from verticillate conidiophores would generate 92.7 ± 11.3 verticillate type conidiophores and 330.0 ± 24.6 penicillate type respectively. The statistical analysis revealed that all the strains from different single spore isolates had the similar percentages of two types of conidiophores (Fig. 1).

The influence on the morphology of conidiophores in *Clonostachys rosea*

In all of the factors we tested, only TBZ, a kind of fungal inhibitor, could significantly influence the production of conidiophores' types in *C. rosea*. Medium containing TBZ produced a mutant with only verticillate conidiophores when the concentration of TBZ was 0.13 ml/L. Furthermore, the microscopic observation illustrated that those verticillate conidiophores in the mutant had no obvious difference in the morphologic characters compared with the wild strain (Fig. 2B). It was also revealed in our data that this phenotype generating one type of conidiophores would keep stable in the several successive inoculations if this mutant continued to grow on medium with the same TBZ concentration. But when this mutant was transferred to the normal medium and then the repeated inoculations (3-5) went on, it would at last revert to generate both types of conidiophores.

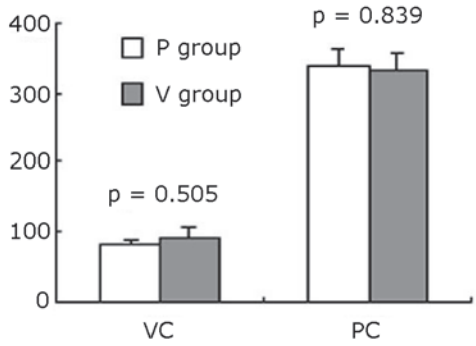

FIG. 1 - Comparison of the formation of penicillate and verticillate conidiophores between P and V group. P group: single spore isolates from penicillate conidiophores; V group: single spore isolates from verticillate conidiophores; PC: represent the formation of penicillate conidiophores; VC: represent the formation of verticillate conidiophores. Number of strains tested (n = 9). Error bars represent +SE.

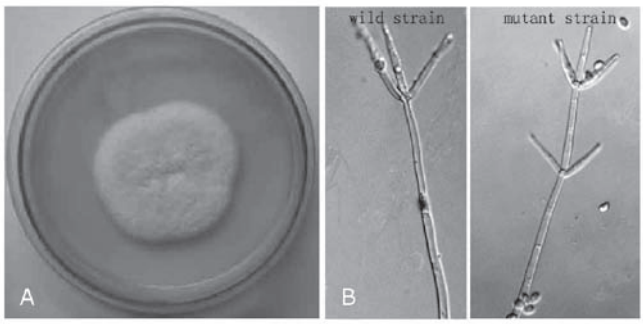

FIG. 2 - Growth of the mutant strain producing only penicillate conidiophores on PDA medium containing fungal inhibitor thiabendazole TBZ (A), and the microscopic observation to verticillate conidiophores of the wild strain and the mutant strain (B).

Meanwhile, our experimental data indicated that medium pH, growth temperature, illumination, as well as our tested carbon or nitrogen sources primarily affect the growth of mycelium and the numbers of conidia in *C. rosea*, but not the formation of conidiophores pleomorphism. When the pH of the culture medium was adjusted to ≥ 3.0, *C. rosea* produced both verticillate and penicillate conidiophores, with different pH within this range showing variable effects on the growth rate of *C. rosea*. When the pH of the culture medium was between 4.5-11.0, this fungus grew normally and the growth reached the maximum when pH was 6.0. After incubation for ten days on the media of pH 11.0-14.0, the fungus displayed light snuffcoloured with yellow drips at the centre of colonies. No obvious growth of *C. rosea* could be observed on the culture medium of pH < 3.0.

Clonostachys rosea grew well at temperatures between 20 and 34 °C, and the optimum growth temperature was 28 °C. At lower temperatures or the temperature exceeded 35 °C, the aerial mycelium was loose with fewer conidiophores and mostly the verticillate type. Illuminations could also influence the pigment of spores but with relatively little effect on conidiophores pleomorphism as well as the growth rate of *C. rosea*. All examined carbon or nitrogen sources supported the growth of *C. rosea* with two types of conidiophores, and they only show an effect on the growth of hypha. In the sugar concentration gradient experiment, the greatest hyphal growth rate was observed when the sugar concentration was 4% glucose. Among all of the tested nitrogen sources, $NaNO_3$ was comparatively fit for the growth of *C. rosea* while sparse mycelium could be found when the nitrogen source became $(NH_4)_2SO_4$.

Comparisons of the parasitic capacity of the *Clonostachys rosea* mutant and wild type strains against nematodes *Panagrellus redivivus* and phytopathogenic fungus *Rhizoctonia solani*

To understand if the phenotype of conidiophore pleomorphism has effects on the abilities to biocontrol nematodes or phytopathogenic fungi, we compare the infection efficacies of the mutant and wild strains against *P. redivivus* and *R. solani*. In our bioassay against *P. redivivus*, we counted the nematode mortalities after 48 h treatment. It was shown that in the mutant producing only the verticillate conidiophores, the infection capacity to nematodes was much weaker (47.1% ± 5.4) than that of wild strain with two types of conidiophores (74.6% ± 8.9; $p < 0.05$). However, PDA culture medium without *C. rosea*, as the negative controls, had less than 10% of nematocidal activities for the same time.

Our bioassay against *R. solani* revealed that the wild strain of *C. rosea* could significantly inhibit the sclerotia formation by *R. solani* ($p < 0.05$). On the overlapped colonies, most mycilia of *R. solani* were parasitized by the *C. rosea*. Our statistic data showed 57.9% ± 10.2 parasitic capacity, corresponding to the Parasitism grade 3. To the mutant strain producing only verticillate conidiophores, the parasitic capacity decreased to 38.6% ± 7.4, corresponding to the parasitism grade 2.

Genetic analysis to the mutant and its parent strain by RAPD

In order to know if there are any genetic differences between the mutant and its parent strain of *C. rosea*, RAPD typing was used in our study. The mutant strain producing only verticillate conidiophores and three strains were all successfully typed using eleven primers in RAPD method, and the examples of the electrophoretic profiles were shown in Fig. 3, respectively. Data analysis showed

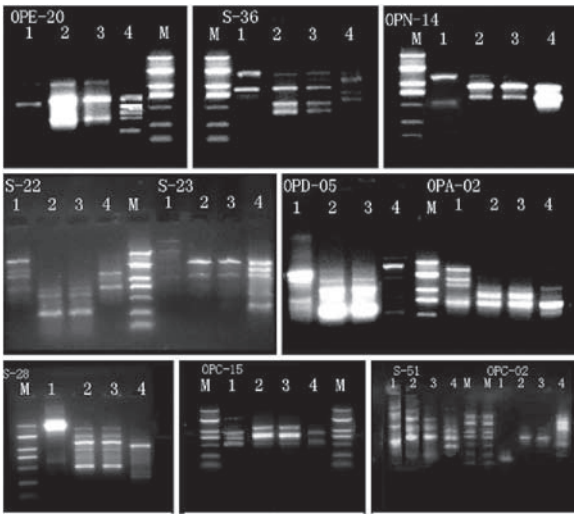

FIG. 3 - The agar electrophoresis of PCR products in our RAPD analysis. Lanes 2 and 3 represent the parent strain and its mutant; lanes 1 and 4 represent the other two wild strains with different origins. Lane M represent the DNA marker, including 2000, 1600, 1000, 750, 500, 250 and 100 bp. OPE-20, S-36, OPN-14, S-22, S-23, OPD-05, OPA-02, S-28, OPC-15, S-51 and OPC-02 represent the random primers used in our analysis, respectively.

that there were no obviously different profiles found in all of the PCR products between the mutant and its parent strain, suggesting that they have no detected differences in genome. Contrarily, the other two wild strains with different origins had significantly distinct profiles at the genomic level compared to the mutant as well as its parent strain.

DISCUSSION

Fungi not only have pleomorphism of sexuality, but also have pleomorphism in asexual structures. The same fungus can have several anamorphic names because of pleomorphism of their asexual structures (Henrebert, 1971). As a result, the classification and identification of such fungi can be problematic. Mycologists have made several proposals to solve the taxonomic problem, but a unified solution remained elusive. *Clonostachys rosea* has different kinds of asexual conidiophores characteristic of those in the genera *Verticillium*, *Penicillium*, and *Trichothecium*. In addition, its colony morphology and conidiophore structure can change dramatically among cultural conditions. Therefore, it is necessary to observe the morphologic characteristics of different types of conidiophores and the potential changes of colony morphology under different culture conditions. Meanwhile, obtaining the mutants with only one type of conidiophores can also supply an alternative for comparing their genetic background, and further understand the molecular mechanisms of formation of conidiophores plemorphism.

Our study revealed that most of the common growth conditions, including pHs, temperatures, illuminations and the nutrition in culture medium, only showed variable effects on hyphal growth rates on *C. rosea* except that the fungal inhibitor TBZ could change the formation of conidiophores' type. *Clonostachys rosea* on medium with

a proper concentration of TBZ produced only verticillate conidiophores and this phenotype could be kept stable on TBZ cultural medium. However, when the mutant mycelia were transferred to normal media without any TBZ, two types of conidiophores gradually re-appeared after repeated inoculation. Based on the transitions between two types of conidiophores when different cultural mediums were used, we hypothesized that the underlying mechanisms for conidiophores pleomorphisms are mostly attributed to the differential gene expression. Thus, we further compared the genetic background between the mutant strain producing only verticillate conidiophores and its parent strain by RAPD method. PCR products from eleven primers confirmed no obvious differences in genome between both of the strains, but with great genetic diversity among those isolates with different origins. All of the experimental data suggested that the conidiophores pleomorphisms in *C. rosea* should result from different transcription of genes rather than the changes of genome, which can be resolved by other research method such as SSH or mRNA microarray.

Clonostachys rosea is found around the roots of plants and has been found capable of enhancing the growth of the plants, likely due to its antagonistic effect against plant pathogens. It has been reported as a hyperparasite *that* can parasitize some pathogenic fungi and it is widely used as a biocontrol agent. For example, *C. rosea* could effectively prevent and cure cacao diseases caused by *Pseudomonas aeruginosa and Cladobotryum amazonense* (Vakili, 1992). Recently, there have been several investigations of successfully using *C. rosea* to prevent and cure crop diseases caused by the *Rhizoctonia solani* (Mo *et al*., 2002). Aside from being an important natural antagonist to fungal pathogens, *C. rosea* have also been found capable of parasitizing the grub and plant parasitic nematodes (Hay and Skipp, 1993). On the mechanisms of infection, the virulence factors that have been previously reported included hydrolytic enzymes and several secondary metabolites. An N-acetyl-beta-d-glucosaminidase gene was recently identified to be up-regulated in antagonistic interactions with *Fusarium culmorum* (Mamarabadi *et al*., 2008). Meanwhile, *C. rosea* could produce an extracellular protease belonging to the serine protease family that could hydrolyze proteins of the purified nematode cuticle in *vitro* and of immobilized nematodes in bioassays (Li *et al*., 2006a). Besides, a variety of nematocidal metabolites from *C. rosea* have also been reported (Dong *et al*., 2005). However, in our current study, it was shown that environments with predominantly the verticillate conidiophores were less effective at immobilizing nematodes and parasitizing phytopathogenic *R. solani*, supplying an illumination that some potential virulence factors involved the process of infecting hosts in *C. rosea* might be influenced synchronously in the process of generating only conidiophores.

In conclusion, we firstly found that the condition of TBZ contained cultural medium could switch the wild strain with both conidiophores to the mutant forming only verticillate type. Furthermore, the genetic analysis by PAPD verified the changes of gene transcription not in genome contributing for the differences of conidiophores' type, and thus this mutant offered a good foundation for understanding the mechanisms of cornidiophore pleomorphism by comparing the differential gene expression with wild strain.

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