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Facilitation of *Paenibacillus amyloliquefaciens* on *Morchella angusticeps*

Chao-Nan Li[1], Lin-Hui Li[1]*, Hai-Jun Liao[1], Ting He[1], Yan Sheng[1] and Bin-Han Li[2]

[1]College of Life Science, China West Normal University, NanChong, Sichuan - 637 002, China.
[2]TongHui Institute of Bio-Science and Technology and Information, NanChong City, Sichuan - 637 002, China.

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The study about the effect on *Morchella angusticeps (M. angusticeps)* caused by *Paenibacillus amyloliquefaciens, P. amyloliquefaciens* isolated from the stem base soil of *M. angusticeps* was performed and significant facilitation on mycelia growth and exoenzyme secretion of *M. angusticeps* happened. The result of experiments showed that the growth rate of *M.angusticeps* mycelia peaked on 5th day, reaching 21.92%, when mycelia were treated with 20% fermentation broth of *P. amyloliquefaciens* (FB*Pa*). Further studies conclusively pointed out that the facilitation was involved with extracellular enzyme of *M. angusticeps* according to its soluble protein concentration, keeping high-level during the 5 days. High-level activities were detected in laccase and protease determination when FB*Pa* concentration increased to the range of 10% to 20%, and in amylase and cellulase determination when it reached 20% on 5th day.

Key words: *Morchella angusticeps, Paenibacillus amyloliquefaciens*, Rhizobacteria, facilitation, exoenzyme.

The species of edible medicinal macrofungi, *Morchella esculenta, M.esculenta*, belong to the genus of *Morchella*, including black *Morchella* and yellow *Morchella*. Recently, increased attention has been focused on *M.esculenta* because of their potential applications in fields such as antioxidant, antimicrobial and hepatoprotective activities[1-3] as well as components of protein, amino acid, polysaccharide, vitamin, fatty acid and various minerals, which can be used as a new kind of base material health foods and a flavoring agent[4-5]. Modern medical studies pointed out that *M.esculenta* can decrease the cerebellar clinical symptoms[6] and play a potential role on immunomodulation and treating related diseases[7]. Therefore, It's necessary to research *M.esculenta* in depth for its medicinal functions and value as food under the condition of wild resources starvation. Although many authors have attempted to find critical factors associated with artificial culture of *M.esculenta*, any obvious progress have not been reported hitherto. In spite of the report that *Populus rotundifolia Griff*, rather than other *Populus*, could significantly improve *M. esculenta* production[8], *M. esculenta* on the market mainly from wild resources. And they had not considered the interaction between *M.esculenta* and its rhizobacteria. Thus, some rhizospheric microorganisms may be regarded as one of the critical factors (*Populus rotundifolia Griff* was replaced with *Populus bonatii Levl* in our experiments) based on ours previous experimental data. The purpose of this paper is to

* To whom all correspondence should be addressed.
Tel.: +86-18990825076;
E-mail: lilinhui6890@126.com

find the microorganism beneficial to the growth of *M.esculenta* and its rough action mechanism which provide theoretical basis and technical guidance for the culture of *M.esculenta* as well.

MATERIALS AND METHODS

Microorganisms and chemicals

Sporocarp samples were randomly collected at Wujiao nature preserve in Sichuan province, China. Samples were placed in sterilized polythene bags and then taken to the laboratory. *M.angusticeps* was separated and purified from those samples and stored at 4 °. The bacterium was isolated from stem base soil of *M.angusticeps* and designated A strain.

Primers were synthetized by Sangon, Shanghai,China; dNTP Mix and LA Taq were purchased from TaKaRa, Dalian, China; Long Taq Reaction Buffer‘°and TIANgel Midi Purification Kit were purchased from TIANGEN, Beijing,China. ABTS,2,2’-Azinobis-(3-ethylbenzthiazoline-6-sulphonate)ÿwas purchased from Sigma, USA. And all biochemic reagents free of inscription are domestic analytical reagent (AR).

Microorganisms indentification

The morphological and biochemical characters indentification of A strain were carried out with the method described by Dong[9]. The phylogenetic analyses based on 16S rDNA sequences comparisons were performed as follows.

The bacterium was coated on LB plates and cultured overnight. Then, the monoclonal colony was selected to perform PCR. The 16S rDNA was amplified using PCR with the universal primers 27F and 1492R[10-11]. PCR was performed in a thermocycler by subjecting the samples to 1 cycle of 94° (10 min), 30 cycles of 94° (1 min), 55° (45 sec), 72° (45 sec) followed by final extension at 72° for 10 min. PCR products were then removed using a PCR Clean up kit (TIANgel Midi Purification Kit). Purified PCR products of approximately 1400 bp were subsequently sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The sequence of isolate was identified using the BLAST program (http://www.ncbi.nlm.nih.gov/Blast/).

The effect of bacterium on growth speed of *M.angusticeps*

To determinate the effect on mycelia, the bacterium was agitated at 37° and 180 rmp for 3 days in synthetic medium($(NH_4)_2SO_4$ 0.1%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.07%, NaCl 0.05%, poplar powder 5%),pH 7.4.Then, the fermentation broth was centrifuged at 12000 rpm for 20 min and the supernatant was stored at 4°. *M.angusticeps* was previously cultivated at 25° until mycelia covered PDA medium surface(potato 20%, glucose 2%, agar 1.5%-2.0%). Next, the culture was cut out to φ8 mm size of block and inoculated into new PDA medium in petri dish containing 125μg/L ceftriaxone sodium and different concentration of fermentation broth supernatant. Then, the mycelia in petri dish were cultured at 25° for 5 days. The growth speed of *M.angusticeps* was determined as follows.

Selected as the measurement, the diameter of mycelia were determined using electronic digital caliper(Shanghai Tools Factory Co., Ltd., China)every day and the data were analysed by SPSS software. Five replicates were run for each treatment and averages were calculated as results showing via relative growth rate which was defined using the following equation:

Facilitation(%)=$[(R_i-r)-(R_0-r)]/(R_0-r)$ (i=5,10,15, 20 ……45,50)

Where, R_i is the diameter of the fungal colony when the mycelia were treated with fermentation broth supernatant. R_0 is the diameter of *M.angusticeps* mycelia when the mycelia were treated without fermentation broth supernatant. r is equal to eight.

The effect of bacterium on soluble protein of *M.angusticeps*

To determinate the effect of bacterium on soluble protein, the φ8mm size of block as above were inoculated into liquid medium(potato 20%, glucose 2%, 125μg/L ceftriaxone sodium and different concentration of fermentation broth supernatant) Then, they were agitated at 25° and 150 rpm for 5 days. In the course of next week, we took samples every day which were centrifuged at 5000 rpm for 10 min to detect the concentration of soluble protein existed in supernatant. The soluble protein concentration was determined using spectrophotometer, which was carried out with the method reported by Chen[12].

The effect of bacterium on exoenzyme activities of *M. angusticeps*

The medium, experimental methods and fermentation broth supernatant are the same as that described in the effect of bacterium on soluble

protein of *M.angusticeps* in this section. Besides, the supernatant denatured at 100° water-bath heating for 10 min was selected as the control group.

Laccase activity

Spectrophotometric assays of laccase activity were performed with the method described by Mariana and Hong[13-14].

Amylase activity

The determination of amylase activity was completed with spectrophotometer. When DNS was the substrate, the absorbance was monitored at 520 nm. The enzymatic reaction mixture contained 1.5% soluble starch solution and fermentation broth supernatant(supernatant 1 mL, soluble starch solution 1.5 mL). After the adding of supernatant at 60° for 5 min, soluble starch solution dissolved in sodium acetate, pH 6.0, was added to reaction system. Then, the reaction mixture was placed into water bath at 60° for 30 min to ensure the stability of enzymic reaction. After that, the enzymic reaction system was water-bath heated at 100° for 10 min to terminate reaction. The reagent of DNS(2mL per enzymic reaction) was mixed with reaction liquid at 100° water-bath heating for 5 min afterwards. The reaction liquid was assayed with spectrophotometer to detect amylase activity at last. One U of enzyme activity is defined as the amount of enzyme releasing 1 mg maltose from 1.5% soluble starch solution every hour at 60°.

Cellulase activity

Cellulase activity also was assayed spectrophotometrically. As the hydrolysis of cellulose to glucose, the absorbance was monitored at 550 nm (The substrate is DNS).The enzymatic reaction mixture contained 0.5% sodium carboxymethylcellulose (CMC-Na) solution and fermentation broth supernatant (CMC-Na 3mL, supernatant 1mL). Supernatant and CMC-Na dissolved in 100 mM sodium acetate buffer, pH 4.5, was mixed together and water-bath heated at 50° for 30 min. Next, the reaction was terminated by the choice of heating at 100° for 10 min. Then, DNS(3mL) was added into the reaction system after the reaction liquid temperature depressed to room temperature. After that, the mixture was transfered into the condition of 100° water-bath heating for 10 min and assayed by the choice of spectrophotometer immediately when reaction system temperature depressed. One U of enzyme activity is defined as the amount of enzyme releasing 1 mg glucose from 0.5% CMC-Na solution every hour at 50°.

Protease activity

The enzyme activity of protease was analysed with reagent of Folin. When Folin was the substrate, the absorbance was monitored at 660 nm. The enzymatic reaction mixture contained 2.0% casein solution and fermentation broth supernatant(supernatant 1mL, casein solution 1 mL). Supernatant was added into reaction system at 40° water-bath heating for 2 min to attain maximal enzyme activity. Then, casein solution dissolved in phosphate buffer, pH 7.2, was mixed with supernatant and water-bath heated for 10 min. Next, 0.4M trichloroacetic acid(TDA)(1mL) was added into the system for the termination of enzyme reaction and the mixture continued to be water-bath heated at 40° for 20 min. After that, the reaction mixture was centrifuged at 5000 rpm for 5 min to isolate denatured protein from the reaction mixture liquid. Then, the supernatant from reaction mixture was added to another reaction system and mixed with 0.4M Na_2CO_3(5mL) and Folin(1mL) successively at 40° for 20min. The reaction liquid was assayed with spectrophotometer. One U of enzyme activity is defined as the method described by Hu at 40° [15].

RESULTS

Morphological and biochemical characters of the bacterium indentification

The result observed under oil lens microscope showed that A strain was bacillus according to the character that a swollen and oval spore located at its centre, and that the gram reaction was positive. Biochemical characters experiments showed that A strain, belonging to genus of *Paenibacillus* according to Bergey's Manual of Determinative Bacteriology, was a facultative anaerobe which can grow using starch as its sole carbon source. It has characteristics of hydrolyzing lignin , D-xylose and mannitol. But it neither hydrolyzed Tween 80, nor producted indole and lecithinase. The catalase test is positive and the oxidase test is negative.

The phylogenetic analyses based on 16S rDNA sequences comparisons

Sequencing result showed that the 16S

rDNA of A strain contained 1464 base pairs. We got some 16S rDNA sequences which was similar to the sequence of A strain from GenBank Database by BLAST program. Phylogenetic analysis of 16S rDNA sequences using MEGA 5.0 software **indicated A strain was *Paenibacillus amyloliquefaciens* (Fig. 1).**

The scale bar indicates 0.02 substitutions per nucleotide position. In the brackets after each bacterial name are 16S rDNA accession numbers in GenBank Datebases.

Facilitation on M.angusticeps mycelia

An obvious facilitation has been demonstrated that the growth rate of *M.angusticeps* mycelia reached 21.92% when the mycelia were treated with 20% fermentation broth of *P. amyloliquefaciens*(FB*Pa*) for 5 days (Fig. 2 and Fig. 3). Maximal level of soluble protein in the synthetic medium was detected only when FB*Pa* increased to 20% in the 5 days(Figure 4).This could indicate that the facilitation was related to exoenzyme activities of *M.angusticeps* induced by *P.amyloliquefaciens*. To confirm this, experiment to detect 4 extracellular enzyme activities(Laccase, Amylase, Cellulase and Protease), were performed in PDA medium under various concentration FB*Pa*.

The numbers(1 to 11) on the picture stand for each treatment(0% to 50%) respectively and the concentration gradient is 5%.

Facilitation on extracellular enzyme activities of *M. angusticeps*

Laccase activity

Laccase activity in each treatment keeped a low level yet after mycelia being cultured for 2 days and increased on the following day, that is 3rd day, peaked on the Day 4 except 30% treatment

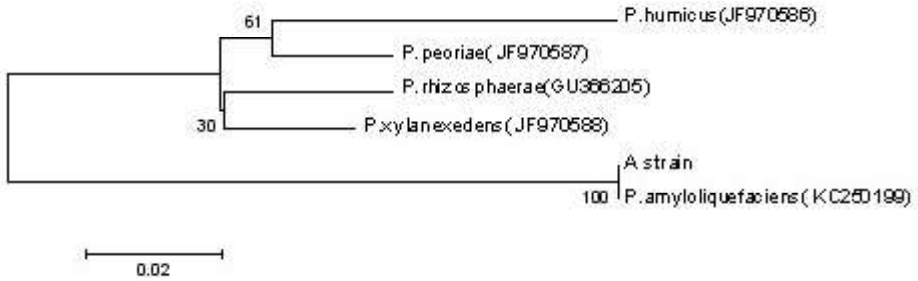

Fig. 1. Phylogenetic tree of A strain and related bacteria based on 16S rDNA

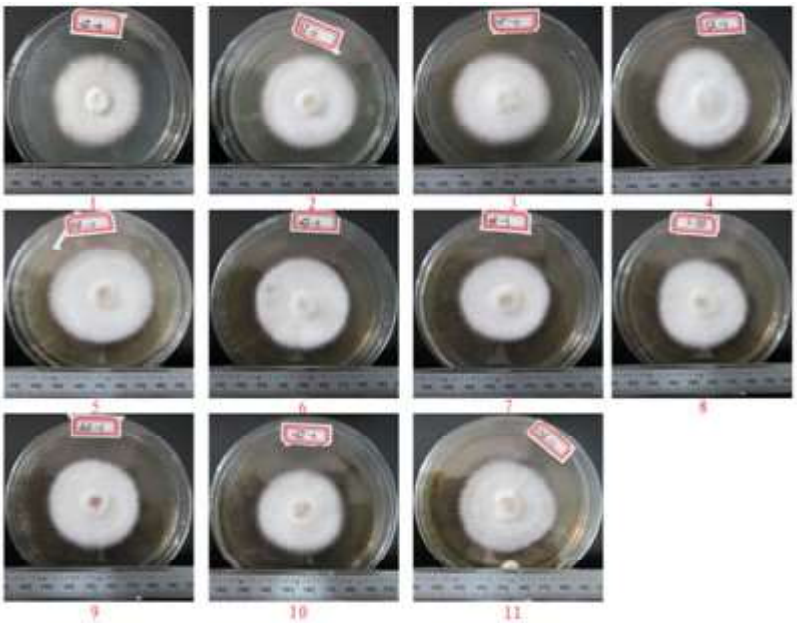

Fig. 2. The change of mycelia diameter with different FB*Pa* concentration

which need to be researched furthermore. Biggish activity was detected on 4th and 5th day when FB*Pa* reached the range of 10% to 20%.(Fig. 5).

Amylase activity

Maximal activity of amylase was detected on the 3rd day when the mycelia were treated without FB*Pa*, reaching 3.7525 U/mL. However, the activity declined rapidly on the following day. Besides, The variation of activity was unpredictable in the 5 days when FB*Pa* increased to 10%. This need to be researched furthermore. Amylase activity reached a maximum(1.3036 U/mL) on 5th day when FB*Pa* increased to 20% compared with activities under other treatment(Figure 6).

Cellulase activity

Cellulase activity peaked on 2nd day, reaching 1.0398 U/mL when the mycelia were treated with 30% FB*Pa* and declined in next couple of days(0.7629 U/mL on 5th day). Moreover, biggish activity was detected in the 5 days when FB*Pa* increased to 30%. In addition, cellulase activity keeped a low level in the 5 days when the mycelia

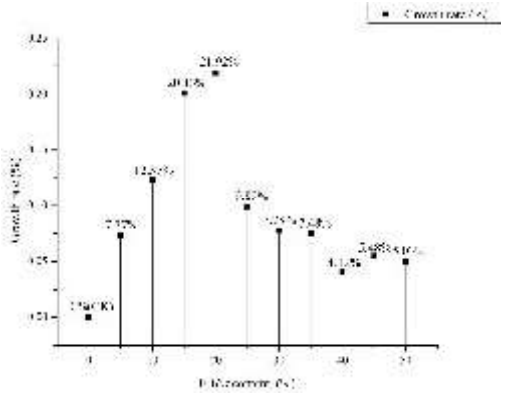

Fig. 3. The change of mycelia growth rate with different FB*Pa* concentration

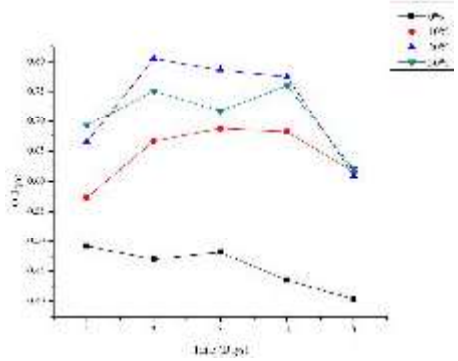

Fig. 4. The change of gross protein with different FB*Pa* concentration

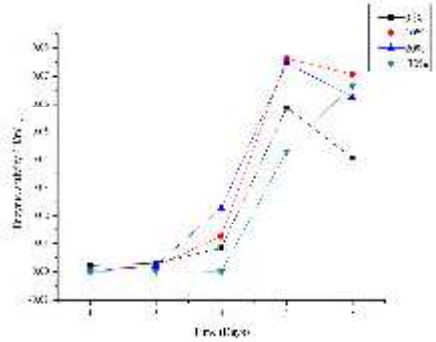

Fig. 5. The effect of different FB*Pa* concentration on the activity of laccase

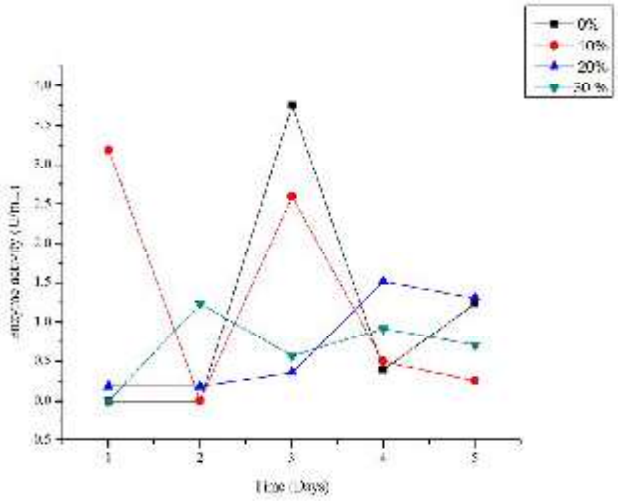

Fig. 6. The effect of different FB*Pa* concentration on the activity of amylase

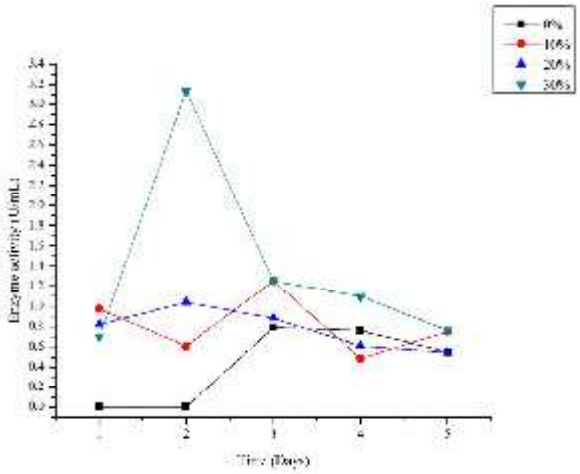

Fig. 7. The effect of different FB*Pa* concentration on the activity of cellulase

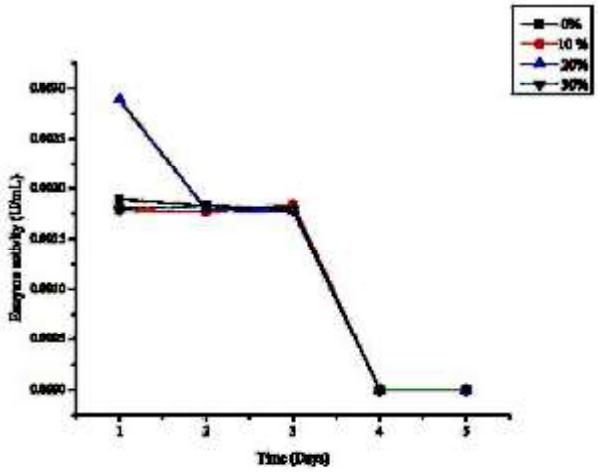

Fig. 8. The effect of different FB*Pa* concentration on the activity of protease

were treated without FB*Pa* (Fig. 7).

Protease activity

Protease activity peaked on 1st day, reaching 0.00189 U/mL when the mycelia were treated with 20% FB*Pa* .However, the maximal activity(0.00189 U/mL) declined on the following day and the activity under each treatment was nearly consistent in next days (Fig. 8).

DISCUSSION

The exoenzyme variation of *M.esculenta* previously had been reported to be related to different growth phases in different *M.esculenta*[12]. However, if they had considered the interaction between some microorganism and *M.esculenta*, their results might be better. Our results implied that some extracellular metabolite from secretion of *P.amyloliquefaciens* facilitated the growth of *M.angusticeps* mycelia, which related to exoenzyme activities induced by the strain of *P.amyloliquefaciens*(the suitable FB*Pa* concentration is 20% in our experiment.).

Laccases belong to the family of multi-copper oxidases that catalyze the oxidation of a range of inorganic and aromatic substances such potassium ferrocyanide, diphenols, arylamines, or aminophenols and can be classified to plant laccase and fungal laccase[16-18]. This implied that abundant lignin and other inorganic or aromatic substances were utilized by *M.angusticeps* on the condition of mass laccase from secretion of mycelia in our experiments. Certainly, the utilization capacity was related to enzyme activity and concentration of substrate. Both enzymic activity and substrate concentration (ligin and its analogue existed in poplar powder.) increased rapidly with the increase of concentration of FB*Pa*. Biggish laccase activity was detected when mycelia were treated with the range of 10% to 20% FB*Pa*. Therefore, despite of the increase of substrate concentration, utilization capacity of lignin and its analogue remained very low by reason of low-level activity of laccase when the concentration of FB*Pa* over 20%. This could indicate that the facilitation was indeed involved with high-level activity of laccase induced by strain of *P.amyloliquefaciens*.

Although the data showed that amylase remained a biggish activity on 5th day when FB*Pa* increased to 20% and the maximal activity of amylase was detected on 3rd day only when the mycelia were treated without FB*Pa*, we can't consider the facilitation was involved with amylase activity induced by the bacillus because of the illogical variation trend of amylase activity in our several repetitive experiments. When the mycelia were treated with 30% FB*Pa*, however, we believed that cellulase played a critical role in the facilitation which was regarded as the consequence that high-level cellulase activity catalyze the hydrolysis of cellulose with concomitant reduction of glucose which can be utilized immediately by mycelia of *M.angusticeps* based on the result that cellulase activity remained a high level in the 5 days. This indirectly confirmed that cellulase activity is related to production of edible mushroom[19].

Protease activity peaked on 1st day, reaching 0.00289 U/mL when mycelia were treated with 20% FB*Pa* and decreased on the following day. And the difference of protease in each treatment was negligible after the mycelia being cultured 2 days. It's hard to believe that the activity of protease was 0 U/mL on 4th and 5th day in each treatment. This implied that protease was induced in PDA medium in early growth phase of *M.angusticeps*. However, the decrease of protease activity could due to that various exoenzyme was degraded in later period to fulfil the requirement of nitrogen for growth of *M.angusticeps*. Thus, we concluded that some facilitation had been displayed in early growth phase of *M.angusticeps* based on the data that maximal activity of protease was detected after the mycelia being cultured 1 day when FB*Pa* increased to 20%, that is on 1st day.

CONCLUSION

Results of the experiment pointed out that the facilitation was involved with the strain of *P.amyloliquefaciens* and the mechanism was briefly described that some metabolite of *P.amyloliquefaciens* come from the zymosis (The substrate is *Populus bonatii Levl*) improved exoenzyme activities such as laccase, cellulase and protease which catalyze the oxidation of lignin, cellulose, protein and other inorganic and aromatic substances to facilitate the growth of *M.angusticeps*. This fits with the habitat of *M.angusticeps*.Therefore, more attention should

be focused on the interaction between microorganisms existed in stem base soil of *M.angusticeps* and itselves. Unfortunately, We can not find the facilitation substance and its detailed mechanism. However, these problems could be solved in our next studies.

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