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參展科別　植物學

作品名稱　綬草根部共生真菌的多樣性探討

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關鍵詞　綬草 Spiranthes sinensis、共生真菌 Mycorrhizal fungus

作者簡介

大家好，我們是吳品昕、王恩婧和賴奕儒，目前就讀國立宜蘭高級中學科學班。由於對生物的共同興趣讓我們加入了生物專題，做專題研究是一件辛苦卻充滿收穫的事，我們也在其中感受到做實驗的樂趣與完成一研究的困難。很高興在高三時有幸能入選國際科展，也感謝悉心指導的馮淑卿老師、李勇毅教授和學長，讓我們有一次很棒的體驗。

中文摘要：
蘭科植物的種子細微如沙塵然而胚發育不完全，亦無胚乳。在自然條件下，蘭科植物需仰賴菌根真菌方能順利完成其生活史。本研究欲探討綬草(*Spiranthes sinensis*)與根部共生真菌（root-originated mycorrhizal fungi）的關係，將實驗分為四個部分，一為觀察綬草根部、種子和共生真菌。二為探討綬草的根部共生菌種。三為測試共生菌絲與萌發所需物質的關係。四為探討共生真菌與綬草萌發的關係。結果發現綬草的根部共生菌種類會受環境影響，並且綬草根部的真菌種類不少，其中包含角擔菌*Ceratobasidiaceae*、膠膜菌*Tulasnellaceae*、傘菌科*agaricomycetidae*等，且能分解環境中的澱粉與蛋白質。

The Diversity of Symbiotic Mycorrhizal Fungi in the Roots of *Spiranthes sinensis*

Abstract.
The seeds of orchids are as fine as dust and numerous, their embryos are not fully developed, and no endosperm. Under natural conditions, orchids rely on mycorrhizal fungi grown in root to provide nutrients for seed germination and grow to complete its life cycle. In this study, *Spiranthes sinensis* was selected as the experimental object to study the relationship to its symbiotic fungus. The experiments are divided into four parts: to examine microscopically the structure of roots, seeds and symbiotic fungi of *S. sinensis*; to identify the species of root symbiotic fungus; to test the relationship between the symbiotic hyphae and the substances required for germination and to explore the relationship between symbiotic fungi and seed germination of *S. sinensis*. It was found that the types of symbiotic mycorrhizal fungus in the roots of S. *sinensis* affected by the environment . There are many types of fungi in the roots of *S. sinensis* , including *Ceratobasidiaceae, Tulasnellaceae*, *Agaricomycetidae*, etc., which can decompose starch and protein in the environment.

Ⅰ.Introduction

Orchidaceae is the second largest family of flowering plants after *Asteracea*e. It has great value on biodiversity, species conservation and the production of medicinal products or as decorative plants. Many orchids are difficult to germinate due to human interference, such as over-collection to meet economic and horticultural needs which leads to low regeneration rates. Because the seeds of orchids are as fine as sand, they do not have endosperm, and the embryos develop only into spherical embryos stage (protocorm), the embryo and radicle were not fully differentiated. Most of the seeds fail to germinate after the capsules ripening and crack in the wild environment. Only a few seeds float to a suitable environment and encounter fungi to achieve a stable symbiosis that help their germination, seedlings growth and development of flower and fruit. Therefore,to explore the relationship between symbiotic fungi and orchid germination would be an important topic.

Spiranthes sinensis is the smallest native orchid in Taiwan. It is a perennial orchid plant. It blooms during the Ching Ming Festival , so it is called "chingming grass", the inflorescences spiral upwards like a ribbon, and have white and thick fleshy roots, shaped like ginseng, also known as "Panlong"Ginseng. *Spiranthes sinensis* is one of the ground orchids whose

seeds mature quickly. The average time from pollination to seed maturity is no more than 15 days.The seed has no endosperm and is composed of undifferentiated embryo and membranous seed coat. Germination in natural environment is heterotrophic and requires symbiotic fungi to supply nutrients. and the average germination rate is extremely low.

This plant has medicinal properties to treat many diseases, its economical value is quite high. However, in recent years, its numbers are largely reduced,caused by over-exploitation, excessive collection and the lack of attention in weeding.

After referring to the literature, it was found that many orchid symbiotic fungi were involved in its seed germination and seedling growth, but there is still a lot of research space for the relationship between the two (Galih et al.,2020), and *S. sinensis* is no exception. Therefore, we hope that the study by exploring the symbiotic fungi (root-originated mycorrhizal fungi of *S. sinensis*) and the germination of its seeds would be helpful to restore its ecological role.

Ⅱ.Research objectives
(1) To observe the structure of root symbiotic fungi (rOMF) and seed with microscope.
(2) To identify the species of symbiotic fungus on the roots of *S. sinensis*.
(3) To explore the relationship between the symbiotic fungi on the roots of *S. sinensis* and the nutrients required for germination
(4) To explore the relationship between symbiotic fungi in the roots of *S. sinensis* and seed germination

Ⅲ.Material
1. Experimental objects: *Spiranthes sinensis*
Bought from the Internet - Pingtung Meizai Orchid Garden Codonopsis
2.Seeds
◎Provided by Hualien Agricultural Reform Farm
◎Collected from the wild, Tainan's Barclay Memorial Park, National Hualien Vocational High School of Agriculture,
◎HOYAO BIOTECH CORPORATION HOYAO BIOTECH CORPORATION
3. symbiotic fungus
Separated from roots of *Spiranthes sinensis*
Equipments

electronic balance	Petri dish	Thermometer	Alcohol lamp	beaker

glass rod	graduated cylinder	Microcentrifuge tubes	Micropipettes	PCR Reaction Tubes
scalpel	Mortar and pestle	Recording paper	blade	ruler
Compound Microscope	Dissecting Microscope	Digital Camera	High-speed centrifuge	autoclave
Larminal flow	Water bath	Vacuum pump	Zeiss AX10 microscope	autoradiography

culture medium

Agar powder (agar), potato, glucose, distilled water, gauze, petri dish, oats,Chloramphenicol, hydrochloric acid (HCL)

PCR

bleach、10x Buffer、d NTP、F primer、R primer、Bio Taq DNA polymerase、MGCL2、 BSA、ddH2O、TBE

Histology

15%, 30%, 50%, 70%, 75%, 80%, 95%, 100% alcohol, 2.5% glutaraldehyde, 1% superphosphoric acid, Toluidine blue O (TBO), PAS (Periodic Acid-Schiff stain), polyethylene glycol (PEG-400), sodium tetraborate (Borax solution), Schiff reagent (Scihff reagent), phosphate buffer (Phosphate buffer), infiltration solution (infitration solution) (dissolve 0.5g activation, solidification Hardener, periodic acid, Technovit® 7100 (Kulzer Technik) resin

DNA Extraction

Extraction Solution A、Extraction Solution B、RNase A solution、Precipitation Solution、Binding Solution-Ethanol、Wash Solution、Elution Solution

DNA electrophoresis

TBE buffer、agarose、easysafe、loading dye、marker

tryptone、coomassie blue、Tween-20

starch、 iodine solution

PDA (for culturing)	Agar, Potato Extract, Glucose, Distilled Water, Chloramphenicol
OMA (for succession)	Oats, agar, hydrochloric acid (HCl)
Starch + agar	starch（0.5g/1 L、0.8g/1L、1.0g/1L）、agar
protein + agar	Tryptone、agar

Ⅳ.Methods

1. Preliminary work

(1) Planning

The experiments are divided into four parts,

(a) To observe the structure of root symbiotic fungi (rOMF) and seed with microscope.(referred to as experiment 1)

(b) To identify the species of symbiotic fungus on the roots of *S.sinensis*. (experiment 2)

(c) To explore the relationship between the symbiotic fungi on the roots of *S. sinensis* and the nutrients required for germination . (experiment 3)

(d) To explore the relationship between symbiotic fungi in the roots of *S. sinensis* and seed germination. (experiment 4)

(2)Preparing 500ml PDA medium

(a) Peel and dice 100g of potatoes, add 500ml of distilled water and boil until soft.

(b) Add 10g of glucose and 15g of agar, heat until dissolved, put them into a high-temperature sterilizer, and sterilize for 20 minutes.

(c) Take it out and pour it into a petri dish.

(3)Preparing 500ml OMA medium

(a) 1.5 g of oats were ground with a mortar.

(b) After adding 4.5g agar and heating, put it into a high-temperature sterilizer for sterilization at 1.2kg/cm2, and sterilize for 20 minutes.

(c) Take it out and pour it into a petri dish

(4) Fungus separation experiment on the roots of *S. sinensis*

(a) Take a section of root microscopic examination to confirm the presence of fungus and disinfect it with sodium hypochlorite solution (available chlorine is about 0.5%,Water:sodium hypochlorite=9:1).

(b) Enter the aseptic operation table and sterilize the equipment with an alcohol lamp.

(c) Drop high-temperature sterilized chloramphenicol solution (20mg/1L distilled water) on five places of the PDA medium.

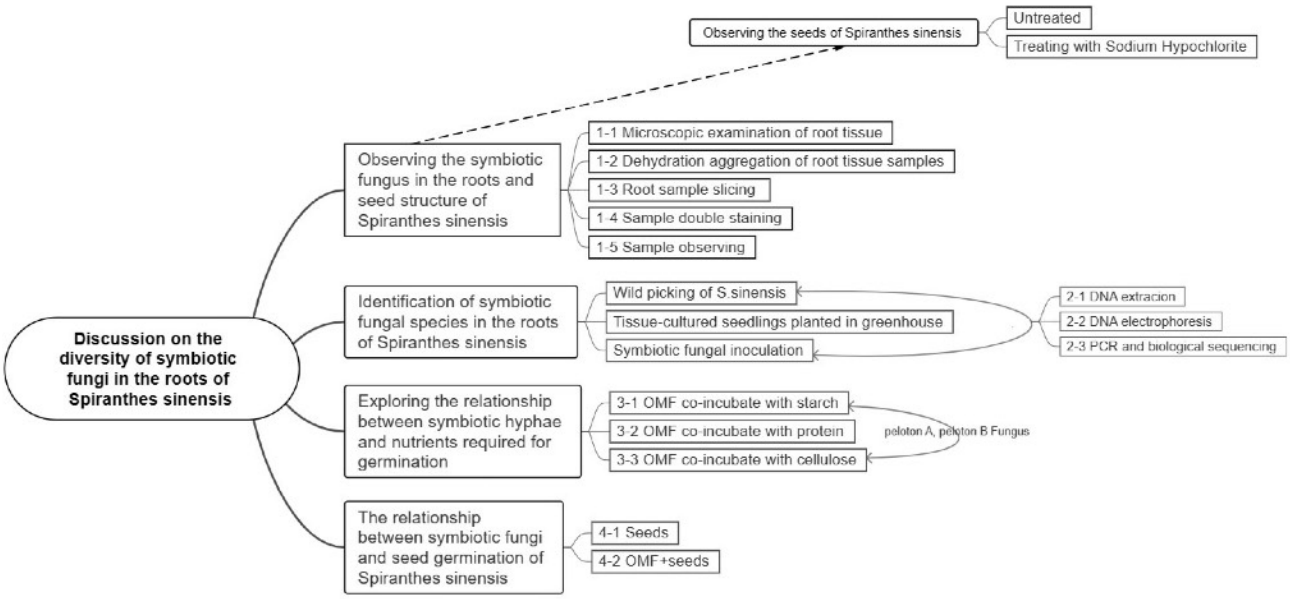
(d) The roots are peeled and sliced, and put into chloramphenicol solution respectively.
(e) After sealing the culture dish with a paraffin film, put it into a 25oC incubator.
(f) Name the hyphae cultured by bacteria at different times, and divide them into pelotonA and pelotonB

(5)Subculture
(a) Put toothpicks in pepper jars for high temperature sterilization.
(b) Enter the aseptic operation table, dig out a small piece of culture medium on the edge of the previously separated fungus with a toothpick.
(c) Put in OMA medium, seal the petri dish with a paraffin film, and put it into a 25^{o}C incubator.

(6) Preparing coomassie blue dye
(a) Dissolve 0.1g of coomassie blue in 10ml of glacial acetic acid and 50ml of 95% alcohol, then add distilled water to make the volume 100ml.
(b) bottle for later use.

(7) Preparing iodine solution
(a) Use 1.2g I2 and 12g KI, dissolved in 1 liter of 0.1N HCL solution.
(b) Pack into a bottle that can avoid light for subsequent use.

2. The experimental structure is as follows



1. Histological observation of the roots and symbiotic fungi of Saffron

● Sample dehydration, aggregation

(1) Add glutaraldehyde fixative to the root slices and vacuumize.

(2) Wash three times with phosphate buffer solution (Phosphate buffer), 10 minutes each time.

(3) Dehydration with alcohol (15%, 30%, 50%, 70%, 80%, 90%, 100%), 30 minutes each time.

(4) Use 100% alcohol and infitration solution (dissolve 0.5g activator into 50ml alkaline
Resin) is divided into three ratios (2:1, 1:1, 1:2) to remove alcohol, each time more than three hours.

(5) After mixing the immersion solution with polyethylene glycol (PEG-400), add a hardener.

(6) Use the infiltration solution to cover the sample and put it into a capped capsule.

(7) Absorb the infiltration solution again, and add embedding medium (embedding medium)

● Sample slices

(1) Use a glass knife making machine to make glass knives for later use.

(2) Take out the sample that has been polymerized and carve it with a length and width smaller than the size of a glass knife.

(3) Insert the glass knife and start slicing.

(4) The excised samples are soaked in ddH2O for later use.

(5) After slicing, pick up the complete slice and put it on a glass slide to dry for later use.

● Double staining of samples

(1) Soak the dried sample in 1% periodic acid for 10 minutes.

(2) Rinse with water continuously for 5 minutes, after rinsing, tap vigorously to dry.

(3) Use PAS to avoid light and soak for 15 minutes.

(4) Repeat step 2.

(5) Use TBO to soak for 2 minutes.

(6) Repeat step 2.

(7) After drying the dried samples, observe them with a microscope.

2. Observation of *S. sinensis* seeds

(1) Take the seeds out of the flower holder.

(2) Prepare 0.5% bleach, add 1 drop of Tween, and add some seeds for 20 minutes.

(4) Clean the treated seeds with a funnel and high-temperature sterilized ddH2O on a sterile operating bench.

(3) The treated and untreated seeds were observed with a microscope respectively.

(2)Experiment 2:

1. DNA extraction

(1) After slicing with bare hands to confirm the presence of mycelium clusters at the root, take 100 mg per portion and sterilize with sodium hypochlorite solution

(Available chlorine is about 0.5%, water:sodium hypochlorite=9:1).

(2) Put the sample in the mortar, add liquid nitrogen and grind it into powder.

(3) Put the powdered sample into a 1.5mL centrifuge tube, and add 360μL Extraction Solution , 40μL Extraction Solution B, 4μL RNase A Solution

(Shake well and put it in a 65-degree water bath for 20 minutes).

(4) Add 130 μL Precipitation Solution (centrifuge for 5 minutes after ice bath).

(5) Pour the supernatant into the spin-filter connected to the 2mL Collection Tube (centrifuge for 2 minutes).

(6) Pipette the liquid in the Collection Tube to a 1.5mL centrifuge tube.

(7) Add Binding Solution-Ethanol 1.5 times the volume of the liquid in the tube, and use the pipette to suck and spit evenly.

(8) Transfer 650 μL of the mixture into the spin-column-collection tube (discard the liquid after centrifugation for 1 minute),

This step is repeated twice.

(9) Add 700 μL Wash Solution to the spin-column-collection tube (centrifuge for 1 minute).

(10) Repeat step (9), pour off the liquid in the collection tube (centrifuge for 5 minutes).

(11) After the spin column is connected to a new 1.5mL centrifuge tube, add 100μL Elution Solution

(centrifuge for 1 minute), and the final liquid is the purified DNA.

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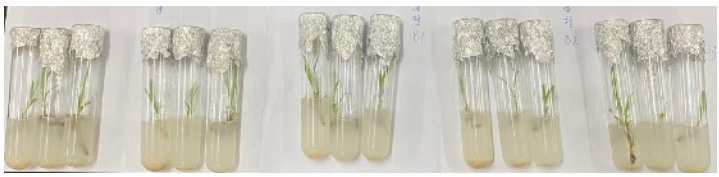
(centrifuge for 1 minute), and the final liquid is the purified DNA.
3. PCR and sequencing
(1) Add the following drugs 10x Buffer 2.5μL, dNTP 1μL to 2.5μL of purified DNA, F primer (10μM) 0.2μL, R primer (μM), 0.2μL, MgCL2, BSA 0.2μL, ddH2O 18μL, and finally add 0.2μL of refrigerated Bio Taq DNA polymerase.
(2) After adding all the reactants, tap the tube wall with your fingers to mix evenly, and centrifuge for a few seconds.
(3) Put the sample into the temperature cycler to set and react.
(4) After the reaction, take out the sample for electrophoresis analysis.
4.Inoculation of pelotonA and B1 ,B2, B3 into the root of *S. sinenesis*
Inoculation of hyphae A and B to test the growth of peloton in the root of *S. sinensis*, the inoculation media was divided into two groups, soil and agar
1.Preparing soil
(1)Mix peat, pearl stone, vermiculite in 2:1:1 proportion.
(2)Add water until moist (like a towel soaked and wrung out).
(3)Fill into test tube (about 5 cm) and seal with two layers of aluminum foil.
(4)Autoclave for thirty minutes.
2.Preparing ager
(1)Prepare 500ml lower concentration OMA medium.
(2)Pour into a test tube (about five centimeters) and wait for cooling.
3.Seedling vaccination
(1)Inoculate the fungus(A,B1,B2,B3) into the test tube in a sterile environment.
(2)Seal the seedlings after inoculation.



(3)Experiment 3:
1. The relation of symbiotic fungi and starch
(1) Prepare 1L of three different concentrations of medium, and pack them

into petri dishes after high-temperature sterilization.
(2) Put pelotonA and pelotonB fungi into the culture medium with a concentration of 0.5g/L by subculture.
(3) 62uL iodine solution was dripped in different days, and the color change was observed by measuring the data with a spectrophotometer (610nm).

starch concentration	group	Days of co-cultivation with fungi
500mg/L	A-1	0
	A-2	3
	A-3	7
	A-4	10

starch concentration	group	Days of co-cultivation with fungi
500mg/L	B1-1	0
	B1-2	3
	B1-3	7
	B1-4	10

starch concentration	group	Days of co-cultivation with fungi
500mg/L	B2-1	0
	B2-2	3
	B2-3	7
	B-4	10

2. The relation of Symbiotic fungi and protein
(1) Prepare 1L of culture medium, after high-temperature sterilization, dispense it into petri dishes.
(2) Put the pelotonA and pelotonB fungi into the culture medium with a concentration of 25 mg/L by subculture.
(3) After dripping 50 ul coomassie blue in different days, the culture medium was heated, and the spectrophotometer was used to (595nm) Measure the data and observe the color change.

protein concentration	group	Days of co-cultivation with fungi
500mg/L	A-1	0
	A-2	3
	A-3	7
	A-4	10

protein concentration	group	Days of co-cultivation with fungi
500mg/L	B1-1	0
	B1-2	3
	B1-3	7
	B1-4	10

3. The relation of symbiotic fungi and cellulose
(1) Prepare 1L of culture medium, after high-temperature sterilization, dispense it into petri dishes.
(2) The pelotonA and pelotonB fungi were subcultured into medium with different concentrations.
(3) After heating the culture medium, drop 50 ul coomassie blue in different days respectively, and use a spectrophotometer to
(595nm) Measure the data and observe the color change.

Dietary Fiber Concentration	Group	Days of co-cultivation with fungi	Dietary Fiber Concentration	Group	Days of co-cultivation with fungi	Methylcellulose concentration	Group	Days of co-cultivation with fungi	Methylcellulose concentration	Group	Days of co-cultivation with fungi
1g/L	A1-1	0	1g/L	B1-1	0	800mg/L	B1-1	0	800mg/L	A1-1	0
	A1-2	3		B1-2	3		B1-2	3		A1-2	3
	A1-3	7		B1-3	7		B1-3	7		A1-3	7
	A1-4	10		B1-4	10		B1-4	10		A1-4	10
2g/L	A2-1	0	2g/L	B2-1	0	1.6g/L	B2-1	0	1.6g/L	A2-1	0
	A2-2	3		B2-2	3		B2-2	3		A2-2	3
	A2-3	7		B2-3	7		B2-3	7		A2-3	7
	A2-4	10		B2-4	10		B2-4	10		A2-4	10
3g/L	A3-1	0	3g/L	B3-1	0	2.4g/L	B3-1	0	2.4g/L	A3-1	0
	A3-2	3		B3-2	3		B3-2	3		A3-2	3
	A3-3	7		B3-3	7		B3-3	7		A3-3	7
	A3-4	10		B3-4	10		B3-4	10		A3-4	10

(4) Experiment 4:germination environmental testing

1. Co-cultivation of seeds and fungi

(1) Prepare 0.5% bleach, add 1 drop of Tween-20, then add some seeds and time it for 20 minutes.

(2) Clean the treated seeds with a funnel and high-temperature sterilized ddH2O on a sterile operating bench.

(3) After rinsing the sterilized seeds with sterile water for 3 times, use a dropper to absorb and drip the seeds at five places on the culture medium.

(4) After sealing the petri dish with a paraffin film, put it into a 25^{o}C incubator.

2.New formula - test the possibility of germination without the presence of fungi

(1) Dosage per liter of medium: Contains 20 g of Huabao No. 1, 20 g of sucrose, 20 g of mashed potatoes, and half a cent

pellets, 6 g of agar, pH 5.6

Experimental design for testing seed germination is as below

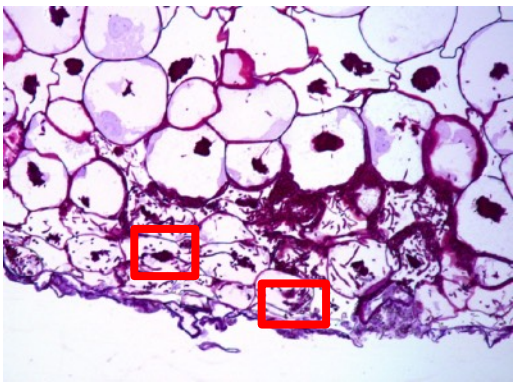
groups	Starting date	Seed sources	medium
1	8th. April	(1) Tainan (2) Hoyao	oma
2	4th.April	Tainan	oma
3	4th.May	hualien	New formula
4	25th.May	Tainan	New formula
5	20th.July	Taipei	Oma(coincubated with

V. Research Results

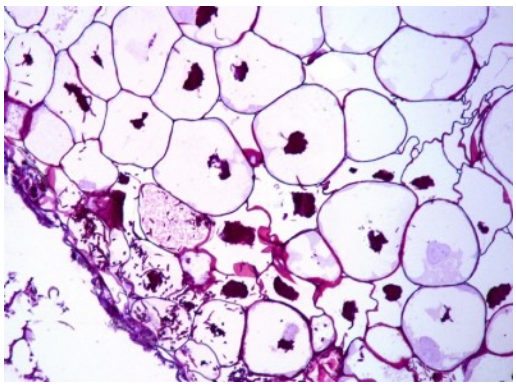
1. Structure of the roots of *S. sinensis*

Symbiotic fungi and seed.

(1)the base of the root



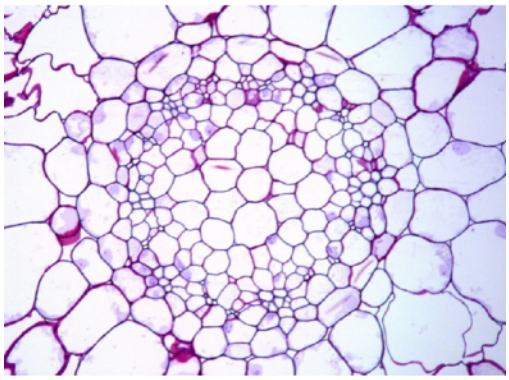
▲Figure 1



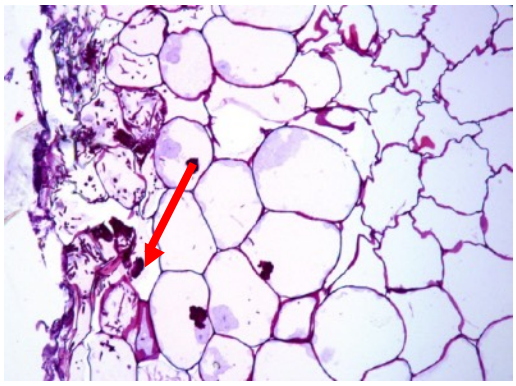
▲Figure 2

▲Figure 1: It can be seen that there are many mycelium clusters close to the epidermis

▲Figure 2: It can be found that the number of fungi near the epidermis is small, but cannot be sure that all are symbiotic fungi. (100x), but with obvious mycelial invasion. (100x)



▲Figure 3

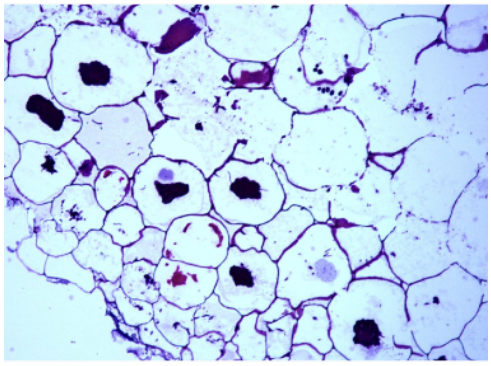


▲Figure 4

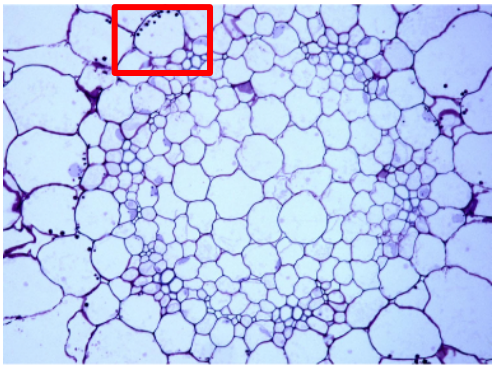
▲Figure 3: The cortex at the base of the root has no starch granules or Hyphae . (100x)

▲Figure 4: It can be seen that the epidermis is slightly damaged after treatment.And that the mycelium clusters are not evenly distributed. (100x)

(2)the middle section

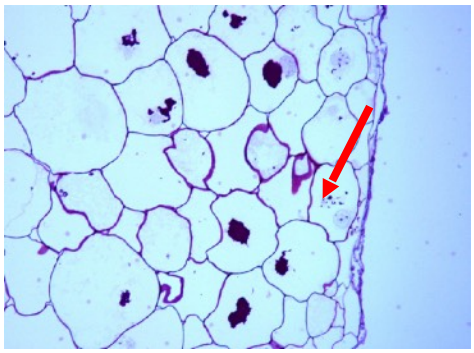


▲Figure 5

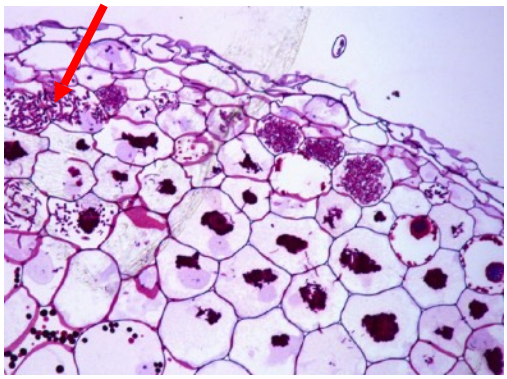


▲Figure 6

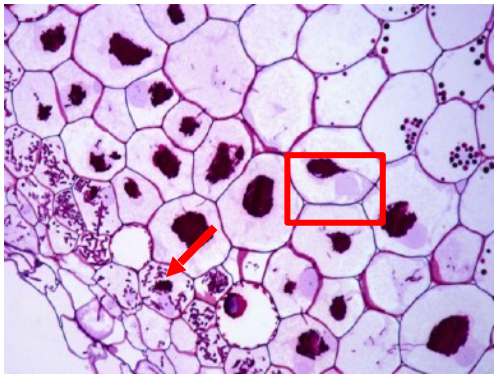
▲Figure 5: The roots of the middle section are relatively symbiotic
Less hyphae. (100x)
▲Figure 6: A little bit can be found in the cortex of the middle ro ▲Figure 7: Mycelium clusters are obvious. Cell nucleus can be observed(100x)
(3)root apex



▲Figure 7

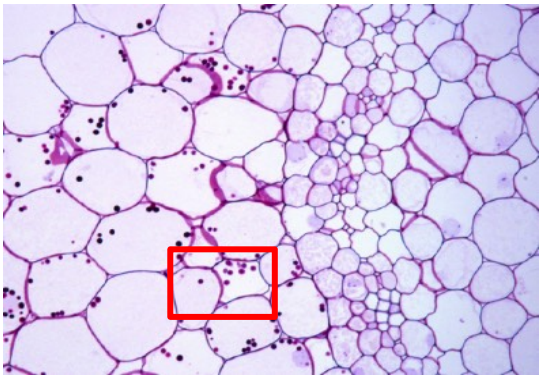


▲Figure 8

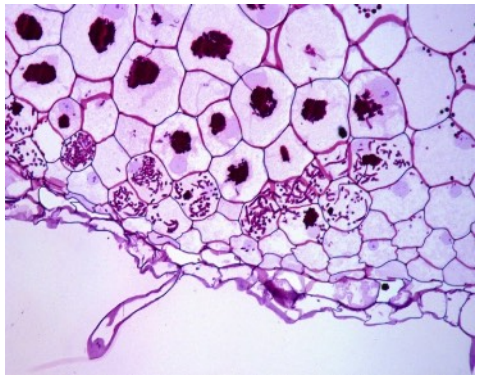


▲Figure 9

▲Figure 8: The root hairs on the epidermis are obviously extended to increase the contact area. (100x)
▲Figure 9: It can be seen that the mycelium mass is invading inwardly,
And the hyphae can pass through the cell wall (100x)



▲Figure 10



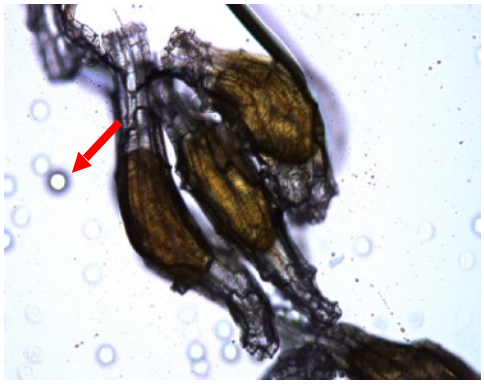
▲Figure 11

▲Figure 10: It can be clearly seen that there are many starch particles in the roots . (100x)

▲Figure 11: Many cells have not yet formed mycelium but just the mycelial mas. (100x)

(4) Observation of *S. sinensis* seeds

1. Untreated seeds

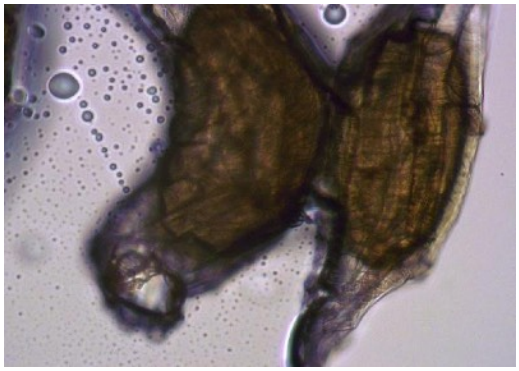


▲Figure 12

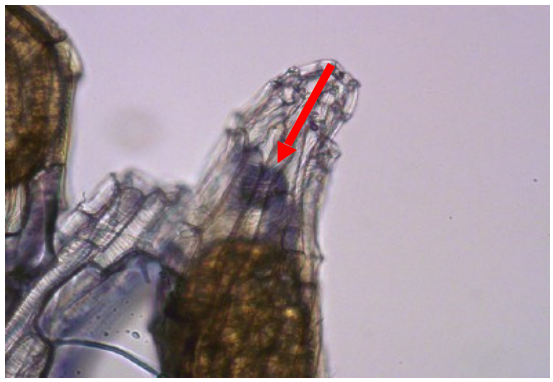
▲Figure 13

▲Figure 12: The seed coat of the untreated Spiranthes sinensis is hydrophobic, it can be found that there are many air bubbles beside the seeds

▲Figure 13: Air can be seen to form obvious cavities in the seed coat due to the hydrophobicity of the seed coat. It can also be found seeds vary in size. (100x)



▲Figure 14

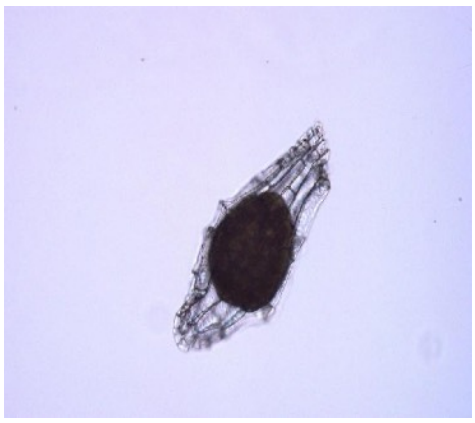


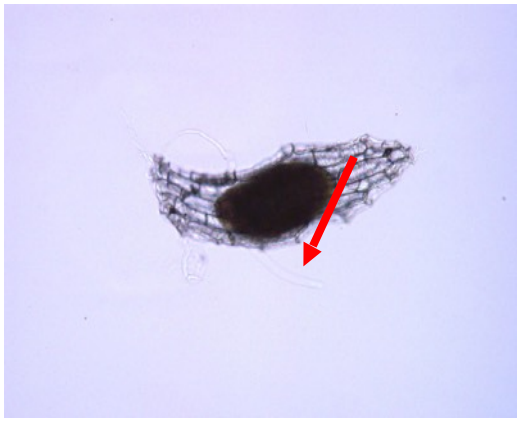
▲Figure 15

▲Figure 14: From this picture, it can be clearly seen that the seeds are filled with air bubbles.

▲Figure 15: There are obvious air bubbles in the seed coat, and it is found that the seeds of *S. sinensis* do not have the endosperm. (200x) .

2. Treated seeds

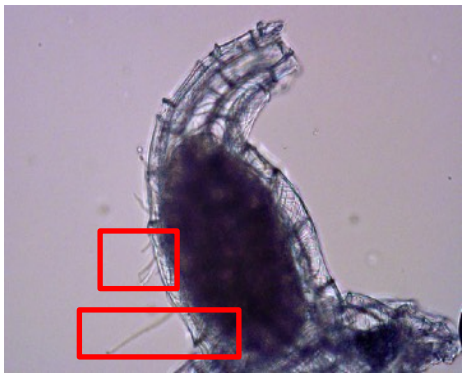


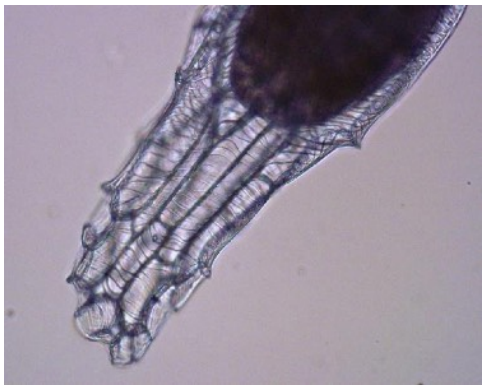


▲Figure 16 ▲Figure 17

▲Figure 16: Small air bubbles around the seeds after treatment has disappeared, indicating that the hydrophobicity of the seed coat is reduced after processing (100x)

▲Figure 17: It can be found that the seeds have been infected by unknown hyphae. (100x)





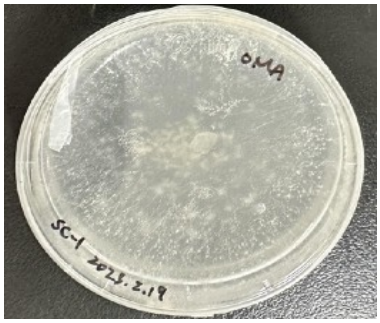
▲Figure 18 ▲Figure 19:

▲Figure 18: After zooming in, the presence of mycelia can be seen more clearly, and it is known that more than one seed is infected by unknown mycelium. (200x)

▲Figure 19: In this picture, the structure of cellulose can be clearly seen, and the cavity has disappeared after treatment. (200x)

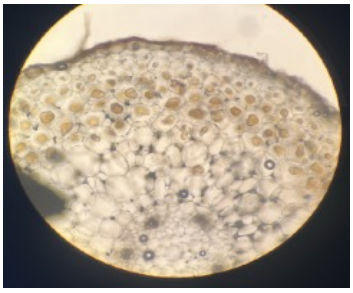
3. The result of the fungi separation

There are two results of the fungus separation of *S. sinensis*: the first fungus separation experiment was operated in about mid-December, and the mycelia separated are called "pelotonA" below , the second fungus separation experiment was operated about mid-February, and the hyphae separated are called "pelotonB" below



▲Figure 20


▲Figure 21


▲Figure 22

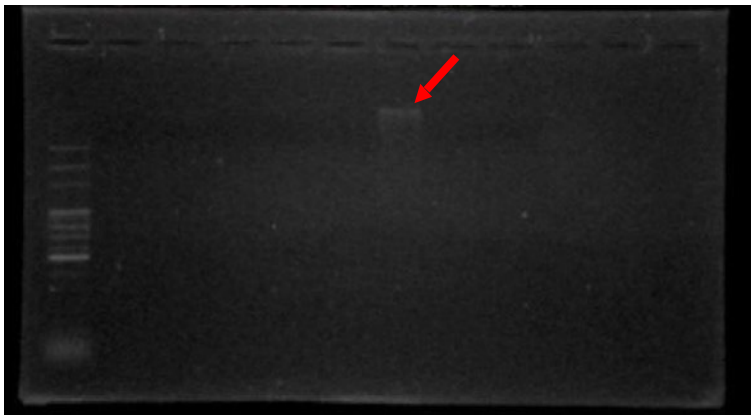
▲Figure 20: fungi isolated from pelotonA in the university laboratory
▲Figure 21: Peloton B was isolated in the school
▲Figure 22: Fungi in the mycelial mass during microscopic examination

2. Experiment 2

The following results were sampled from the roots of *S.sinensi*s

(1)DNA extraction (marker is loading dye)

1. The first one

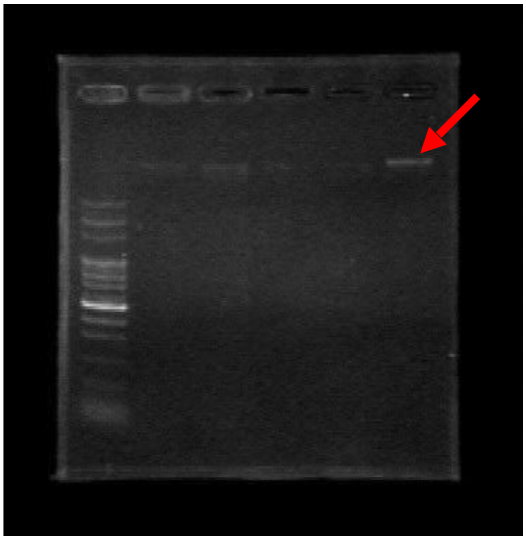


▲Figure 23

It can be seen from the figure that only the seventh channel has a lot of DNA extracted, but there is a bit of tailing, which represents the specificity of nucleic acid is not high.

2. The second experiment

It can be observed from the figure that the sixth track is more obvious than the other tracks, which also means that we have extracted DNA.



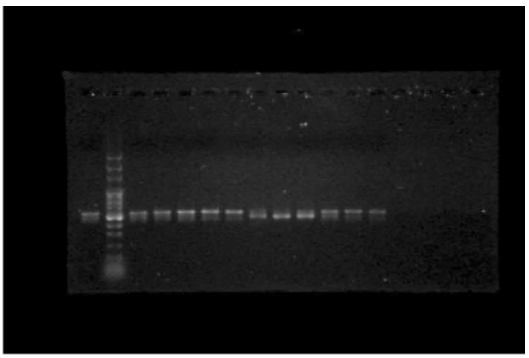
▲Figure 24

(2) PCR (marker is loading dye)

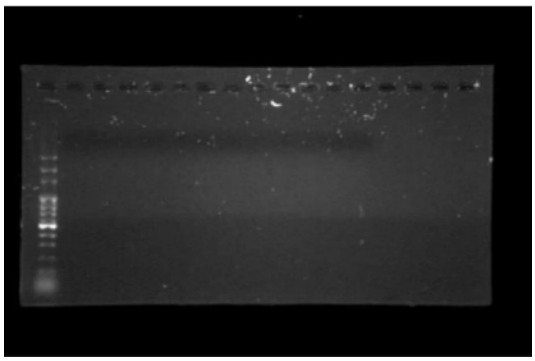
From these two figures, it can be observed that different primers have great differences in the effect of PCR on *S. sinenesis*. Although the left figure is somewhat smeared, compared with the results of DNA extraction, more and more concentrated DNA can be found

1. Use different primers

(3) Gene Sequencing (Sequencing: Kiron Mix Biotechnology Company & Nucleic Acid Sequence Alignment: NCBI Website)



▲Figure 25



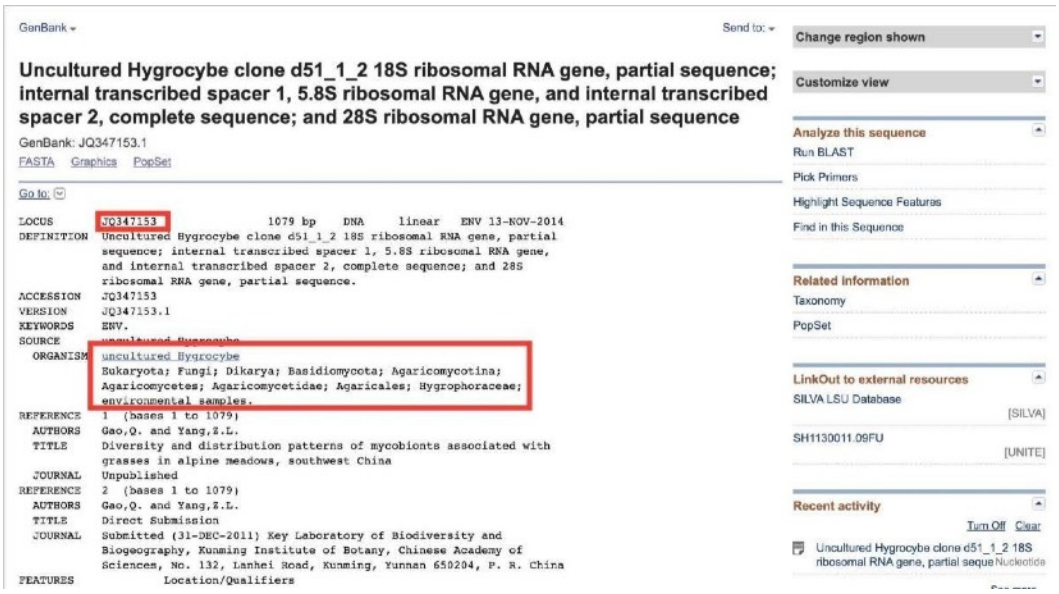
▲Figure 26

After sequencing, the first six species of fungi with high similarity to the target sequence in this paragraph (Figure 27) are all unknown fungi, but the similarity with certain fungi of the family *Agaricomycetidae* is as high as 90.10% (Figure 27)

GGGCCCTTGTATGCGATTGCAGATTCAGTGAATCATCAAATCTTTGAACGCAAATGGCGCTTCTGGGATAGGCCTAGGAG
CATGCCTCTCTGAGTATCGGTTCTATCTATCAACGACAGCGTGCTTGTAAAAGAGCAACACTGGGCAGTTGGAAAATGAG
CGTCTGATTGCGCAAGCAAATCAGTCACTTTAAACAGATAGAAGCTTGAATCGCACTAGAGGAATGTTTCGACGCAAGTC
GAAGACGAGTTGTTATGGCTCCATTCTATATGCTAGTGACGTGGATAGAAGTACCCTTGTGGTCTTTCAAAACGTCTGCT
AGTTGAATGTGTTTGGCTGTTATGACTTCGTGTAAGCTCTGGTTAAGTGGATTCAGTATAGTTTCTGACTCGAATTCCCC
TTGTCTCCGGACTTGGGTTGTTTGAATTAGATCTCAGATGAAGCAAGAGGACCCCCTGAATTTAAGCATATTATTAAGGG
GAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	902	902	97%	0.0	98.82%	2021	OU942108.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	752	752	97%	0.0	93.35%	2032	OU942790.1
☑	uncultured soil eukaryote genomic DNA sequence contains 5.8S rRNA gene, ITS2, 28S rRNA gene, clone df58e...	uncultured soil e...	743	743	97%	0.0	93.09%	1612	HG996286.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	737	737	97%	0.0	93.16%	2017	OU939371.1
☑	uncultured Basidiomycota genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 26S r...	uncultured Basid...	721	721	97%	0.0	92.40%	2019	HG995975.1
☑	Uncultured Hygrocybe genes for 18S rRNA, ITS1, 5.8S rRNA, ITS1, 28S rRNA, partial and complete sequence, ...	uncultured Hygro...	706	706	96%	0.0	92.12%	1048	LC440288.1
☑	Uncultured Hygrocybe clone d51_1_2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	uncultured Hygro...	652	652	97%	0.0	90.10%	1079	JQ347153.1
☑	uncultured stramenopile genomic DNA sequence contains 5.8S rRNA gene, ITS2, 28S rRNA gene, clone 79cc78...	uncultured stram...	647	647	97%	0.0	89.84%	1604	HG995913.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	636	636	97%	1e-177	89.84%	1993	OU940551.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	636	636	97%	1e-177	89.82%	1993	OU939534.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	630	630	97%	6e-176	89.69%	2010	OU942099.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	625	625	97%	3e-174	89.51%	2006	OU942643.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	617	617	97%	5e-172	89.06%	2018	OU939341.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	348	348	97%	7e-91	79.92%	2123	OU939744.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	342	342	97%	3e-89	80.11%	1976	OU941815.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	340	340	97%	1e-88	79.74%	2113	OU941394.1
☑	uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge...	uncultured fungus	339	339	97%	4e-88	79.81%	1937	OU941498.1

▲Figure 27



GenBank ▾ Send to: ▾

Uncultured Hygrocybe clone d51_1_2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: JQ347153.1

FASTA Graphics PopSet

Go to:

```
LOCUS       JQ347153                1079 bp    DNA     linear   ENV 13-NOV-2014
DEFINITION  Uncultured Hygrocybe clone d51_1_2 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,
            and internal transcribed spacer 2, complete sequence; and 28S
            ribosomal RNA gene, partial sequence.
ACCESSION   JQ347153
VERSION     JQ347153.1
KEYWORDS    ENV.
SOURCE      uncultured Hygrocybe
  ORGANISM  uncultured Hygrocybe
            Eukaryota; Fungi; Dikarya; Basidiomycota; Agaricomycotina;
            Agaricomycetes; Agaricomycetidae; Agaricales; Hygrophoraceae;
            environmental samples.
REFERENCE   1  (bases 1 to 1079)
  AUTHORS   Gao,Q. and Yang,Z.L.
  TITLE     Diversity and distribution patterns of mycobionts associated with
            grasses in alpine meadows, southwest China
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1079)
  AUTHORS   Gao,Q. and Yang,Z.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-DEC-2011) Key Laboratory of Biodiversity and
            Biogeography, Kunming Institute of Botany, Chinese Academy of
            Sciences, No. 132, Lanhei Road, Kunming, Yunnan 650204, P. R. China
FEATURES             Location/Qualifiers
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Change region shown

Customize view

Analyze this sequence
Run BLAST
Pick Primers
Highlight Sequence Features
Find in this Sequence

Related information
Taxonomy
PopSet

LinkOut to external resources
SILVA LSU Database [SILVA]
SH1130011.09FU [UNITE]

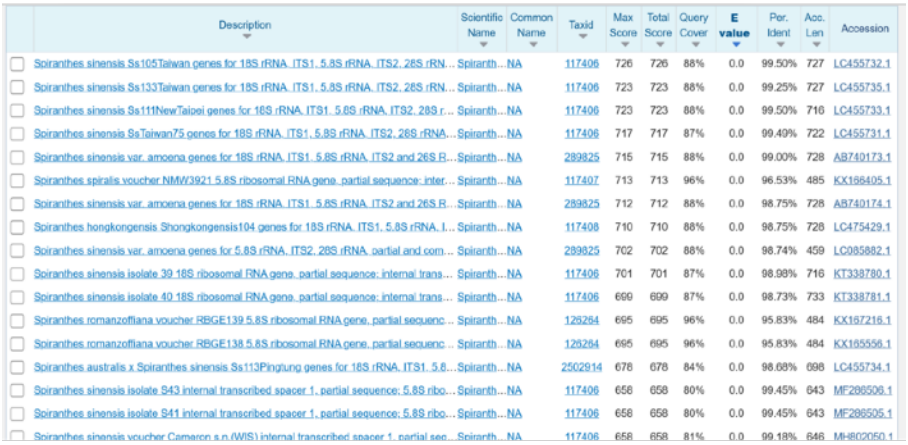
Recent activity
Turn Off Clear
Uncultured Hygrocybe clone d51_1_2 18S ribosomal RNA gene, partial seque Nucleotide

▲Figure 28

2. Result two

After comparison of the nucleic acid sequences, it was found that the target sequence had a high probability of being the *S. sinensis* itself.

GGGAATGTGGTGTGATTGCAGATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAGGCCAATTGGCTGAGGG
CACGTCCGCCTGGGCGTCAAGCATTACATCGCTTCATCCCGCGCCACCTCGCAGGCGGGGTGATTGTGCCGGGTTGAATG
TGGAGATTGGCCCTTCGTGCAAACATTTGTGCGGCGGGTTGAAGCGCGGATGGCTCTCCTCTCGGCCGATGCGTGACAAA
GGGGTGGTTGGATGCTGCCGCTTGGCCCACATTGTCATGTCATTGGCCTTGAGGATGGCGTGCACACACATCGATAATAG
GTCACCCGACGGATTCGTTGTAGGTGGCACATCGAAATGCGACCCCAGGATGGGCGGGATGACCCGCTGAGTTTAAGCAT
ATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCCTAGTAACA



Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Spiranthes sinensis Ss105Taiwan genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRN...	Spiranth...	NA	117406	726	726	88%	0.0	99.50%	727	LC455732.1
Spiranthes sinensis Ss133Taiwan genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRN...	Spiranth...	NA	117406	723	723	88%	0.0	99.25%	727	LC455735.1
Spiranthes sinensis Ss111NewTaipei genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S r...	Spiranth...	NA	117406	723	723	88%	0.0	99.50%	716	LC455733.1
Spiranthes sinensis SsTaiwan75 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA...	Spiranth...	NA	117406	717	717	87%	0.0	99.49%	722	LC455731.1
Spiranthes sinensis var. amoena genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S R...	Spiranth...	NA	269825	715	715	88%	0.0	99.00%	728	AB740173.1
Spiranthes spiralis voucher NMW3921 5.8S ribosomal RNA gene, partial sequence; inter...	Spiranth...	NA	117407	713	713	96%	0.0	96.53%	485	KX166405.1
Spiranthes sinensis var. amoena genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S R...	Spiranth...	NA	269825	712	712	88%	0.0	98.75%	728	AB740174.1
Spiranthes hongkongensis Shongkongensis104 genes for 18S rRNA, ITS1, 5.8S rRNA, I...	Spiranth...	NA	117408	710	710	88%	0.0	98.75%	728	LC475429.1
Spiranthes sinensis var. amoena genes for 5.8S rRNA, ITS2, 28S rRNA, partial and com...	Spiranth...	NA	269825	702	702	88%	0.0	98.74%	459	LC085882.1
Spiranthes sinensis isolate 39 18S ribosomal RNA gene, partial sequence; internal trans...	Spiranth...	NA	117406	701	701	87%	0.0	98.98%	716	KT338780.1
Spiranthes sinensis isolate 40 18S ribosomal RNA gene, partial sequence; internal trans...	Spiranth...	NA	117406	699	699	87%	0.0	98.73%	733	KT338781.1
Spiranthes romanzoffiana voucher RBGE139 5.8S ribosomal RNA gene, partial sequenc...	Spiranth...	NA	126264	695	695	96%	0.0	95.83%	484	KX167216.1
Spiranthes romanzoffiana voucher RBGE138 5.8S ribosomal RNA gene, partial sequenc...	Spiranth...	NA	126264	695	695	96%	0.0	95.83%	484	KX165556.1
Spiranthes australis x Spiranthes sinensis Ss113Pingtung genes for 18S rRNA, ITS1, 5.8...	Spiranth...	NA	2502914	678	678	84%	0.0	98.68%	698	LC455734.1
Spiranthes sinensis isolate S43 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Spiranth...	NA	117406	658	658	80%	0.0	99.45%	643	MF286506.1
Spiranthes sinensis isolate S41 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Spiranth...	NA	117406	658	658	80%	0.0	99.45%	643	MF286505.1
Spiranthes sinensis voucher Cameron s.n.(WIS) internal transcribed spacer 1, partial seq...	Spiranth...	NA	117406	658	658	81%	0.0	99.18%	646	MH802050.1

▲Figure 29-30

3. Result three

After comparison of the nucleic acid sequences, it was found that the target sequence had a high probability of being the *Spiranthes sinensis* itself.

AAGTGGTGTGATTGCAGATCCCGTGACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAGGCCAATTGGCTGAGGGCACGT
CCGCCTGGGCGTCAAGCATTACATCGCTTCATCCCGCGCCACCTCGCAGGCGGGGTGATTGTGCCGGGTTGAATGTGGAG
ATTGGCCCTTCGTGCAAACATTTGTGCGGCGGGTTGAAGCGCGGATGGCTCTCCTCTCGGCCGATGCGTGACAAAGGGGT
GGTTGGATGCTGCCGCTTGGCCCACATTGTCATGTCATTGGCCTTGAGGATGGCGTGCACACACATCGATAATAGGTCAC
CCGACGGATTCGTTGTAGGTGGCACATCGAAATGCGACCCCAGGATGGGCGGGATGACCCGCTGAGTTTAAGCATATCAA
TAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCCTAGTAACA

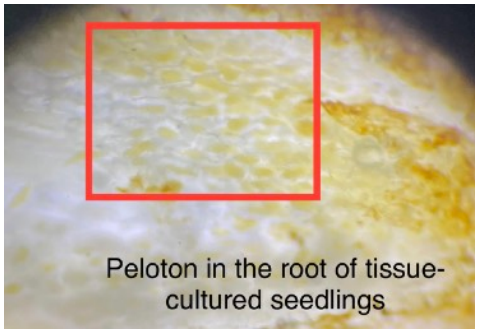
Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accessior
Spiranthes sinensis Ss105Taiwan genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRN...	Spiranth...	NA	117406	719	719	89%	0.0	99.25%	727	LC455732.
Spiranthes sinensis Ss133Taiwan genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRN...	Spiranth...	NA	117406	715	715	89%	0.0	99.00%	727	LC455735.
Spiranthes sinensis Ss111NewTaipei genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S r...	Spiranth...	NA	117406	715	715	89%	0.0	99.25%	716	LC455733.
Spiranthes sinensis SsTaiwan75 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA...	Spiranth...	NA	117406	710	710	88%	0.0	99.24%	722	LC455731.
Spiranthes sinensis var. amoena genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S R...	Spiranth...	NA	269825	708	708	89%	0.0	98.75%	728	AB740173.
Spiranthes spiralis voucher NMW3921 5.8S ribosomal RNA gene, partial sequence; inter...	Spiranth...	NA	117407	706	706	97%	0.0	96.30%	485	KX166405.
Spiranthes sinensis var. amoena genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S R...	Spiranth...	NA	269825	704	704	89%	0.0	98.50%	728	AB740174.
Spiranthes hongkongensis Shongkongensis104 genes for 18S rRNA, ITS1, 5.8S rRNA, I...	Spiranth...	NA	117408	702	702	89%	0.0	98.50%	728	LC475429.
Spiranthes sinensis var. amoena genes for 5.8S rRNA, ITS2, 28S rRNA, partial and com...	Spiranth...	NA	269825	702	702	89%	0.0	98.74%	459	LC085882.
Spiranthes sinensis isolate 39 18S ribosomal RNA gene, partial sequence; internal trans...	Spiranth...	NA	117406	693	693	87%	0.0	98.72%	716	KT338780.
Spiranthes sinensis isolate 40 18S ribosomal RNA gene, partial sequence; internal trans...	Spiranth...	NA	117406	691	691	88%	0.0	98.48%	733	KT338781.
Spiranthes romanzoffiana voucher RBGE139 5.8S ribosomal RNA gene, partial sequenc...	Spiranth...	NA	126264	689	689	97%	0.0	95.60%	484	KX167216.
Spiranthes romanzoffiana voucher RBGE138 5.8S ribosomal RNA gene, partial sequenc...	Spiranth...	NA	126264	689	689	97%	0.0	95.60%	484	KX165556.
Spiranthes australis x Spiranthes sinensis Ss113Pingtung genes for 18S rRNA, ITS1, 5.8...	Spiranth...	NA	2502914	671	671	85%	0.0	98.42%	698	LC455734.
Spiranthes sinensis isolate S43 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Spiranth...	NA	117406	651	651	81%	0.0	99.17%	643	MF286506.
Spiranthes sinensis isolate S41 internal transcribed spacer 1, partial sequence; 5.8S ribo...	Spiranth...	NA	117406	651	651	81%	0.0	99.17%	643	MF286505.
Spiranthes sinensis voucher Cameron s.n.(WIS) internal transcribed spacer 1, partial seq...	Spiranth...	NA	117406	651	651	81%	0.0	98.91%	646	MH802050.

▲Figure31-32

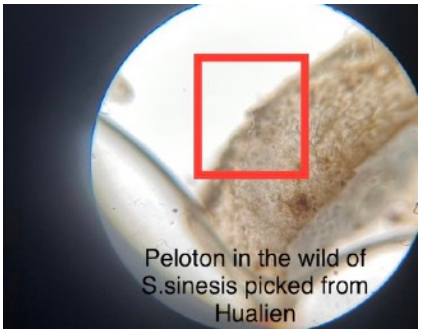
4.Comparison of root symbionts collected from wild grasslands or tissue culture seedlings planted in grasslands or greenhouses

The results show that the root symbionts of the wild *S. sinenesis* collected from the National Taiwan University campus or Hualien Agricultural School

and tissue-culture seedlings planted on campus are all *Ceratobasidiaceae*, while the root symbionts of the *S. sinenesis* tissue culture seedlings grown in the greenhouse are *Tulasnellaceae*.
Pictures below are planted in the wild and in the greenhouse



▲Figure33



▲Figure34

CGCTTCGGTAGGTGACCTGCGGAAGGATCATTATTGAATTTAATGTAGAGGTTGGTTGTAGCTGGCCCAGATGTTAATAT
CTGGGCATGTGCACACCTTCTCTTTCATCCACACACACCTGTGCACCTGTGAGACAGAAGTGGGACTTTTAATTTAATTT
AATTGGACCCTCTGTCTACTCAATCCACATAGACTCAATTTATTTTAAAATGAATGTAACTGGATGTAACGCATCTAATA
CTAAGTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCTTGGAGCATGCCTGTTTGAGTATCATGAA
ATCTTCAAAGTAAATCTTTTGTTAACTCAATTGGTTCTGCTTTGGTATTGGAGGTTATTGCAGCTTCACACCTGCTCCTC
TTTGTGCATTAGCTGGATCTCAGTGTTATGCTTGGTTCCACTCAGCGTGATAAGTATCTATCGCTGAGGACACTGTAACA
GGTGGCCAAGGTAAACGCAGATGAACCGCTTCCAACAGTCCATTCACTTGGACAAAACTCTTATGATCTGATCTCAAATC
AGGTAGGACTACCCGCTGAACTTAAGCATATCTGAAGGGGGGGAGAGAGAGAAAA

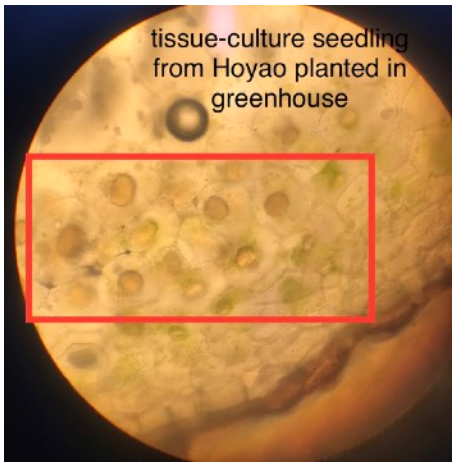
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Rhizoctonia sp. isolate R77 internal transcribed spacer 1, partial sequence: 5.8S ribosomal RNA gene and internal t...	Rhizoctonia sp.	1134	1134	94%	0.0	98.01%	885	MK084684.1
Rhizoctonia solani culture CBS 594.81 strain CBS 594.81 small subunit ribosomal RNA gene, partial sequence; inte...	Rhizoctonia solani	1134	1134	96%	0.0	97.31%	894	MH861382.1
Uncultured Ceratobasidiaceae A2-6 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete s...	uncultured Cerat...	1133	1133	95%	0.0	97.59%	676	LC458753.1
Uncultured Ceratobasidiaceae isolate 767 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	uncultured Cerat...	1133	1133	96%	0.0	97.16%	792	HM141030.1
Uncultured Ceratobasidiaceae isolate 140 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	uncultured Cerat...	1131	1131	93%	0.0	98.01%	850	HM141027.1
Uncultured Ceratobasidiaceae genes for 18S rRNA, ITS1, 5.8S rRNA, ITS1, 28S rRNA, partial and complete seque...	uncultured Cerat...	1129	1129	96%	0.0	97.16%	891	LC440227.1
Uncultured Thanatephorus genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence, clone: M1280	uncultured Thana...	1129	1129	96%	0.0	97.17%	718	AB712280.1
Uncultured Ceratobasidiaceae isolate 168 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen...	uncultured Cerat...	1127	1127	93%	0.0	98.00%	649	HM141028.1
Rhizoctonia solani isolate AG-8_Carling 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Rhizoctonia solani	1123	1123	95%	0.0	97.15%	671	KX118332.1
Thanatephorus cucumeris isolate 10RS SV5 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Thanatephorus c...	1123	1123	95%	0.0	97.29%	677	OR471296.1
Uncultured Ceratobasidiaceae genes for 18S rRNA, ITS1, 5.8S rRNA, ITS1, 28S rRNA, partial and complete seque...	uncultured Cerat...	1120	1120	96%	0.0	96.88%	892	LC440225.1
Thanatephorus sp. ANOF4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosom...	Thanatephorus s...	1120	1120	96%	0.0	96.88%	725	KJ495966.1
Uncultured Ceratobasidiaceae clone TANOF8 18S ribosomal RNA gene, partial sequence; internal transcribed spac...	uncultured Cerat...	1118	1118	96%	0.0	96.87%	691	KJ495978.1
Fungal sp. strain pw-13 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S rib...	fungal sp.	1114	1114	96%	0.0	96.72%	681	MG592691.1
Fungal sp. strain pw-6 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribo...	fungal sp.	1114	1114	96%	0.0	96.72%	882	MG592686.1
Thanatephorus cucumeris isolate 10RS SV3 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Thanatephorus c...	1112	1112	95%	0.0	96.99%	677	OR471294.1
Rhizoctonia sp. Tm1-4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal R...	Rhizoctonia sp. T...	1109	1109	94%	0.0	96.98%	687	AY433813.1

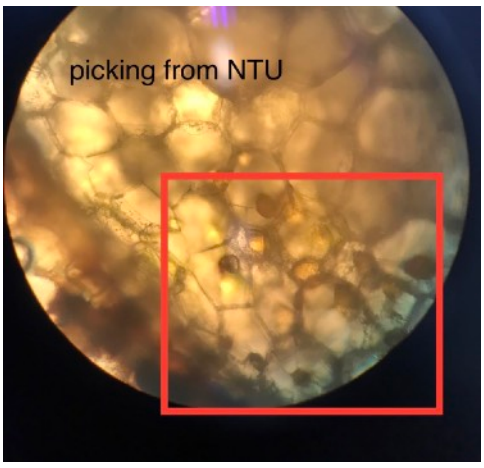
▲Figure35

TCCGGTAGGTGACCTGCGGAGGATCATAGTAATCGTCTTTGACGTTCTATTCCGTCGTCCTCGGGACGTTAAGGCGCTCC
GGTCGAGGATAAACGACCCCTCTGACCGAGGTTAAACGGTCGCTGCCTGTGTTACCTCTTCGGAGGCACACGTTAAAGAT
CGTTCCGCGTTGTGAGTCTAACACCAGTTGTAACTTTTTTACAACCGGCAGCGCTGGATCCCTTGGCACGTCATTCGATG
AAGACCGTTGCAAATTGCGATAAAGTGATGTGATGCGCAAGTCCACCACTTATACGTGAATCATCGAGTTGTTGAACGCA
TTGCACCGCGCCCTAATCCGGCTGCGGTATGCCCCTTTGAGCGTCATTGTATTCCTTCGGGAGTCTTTCCTTGCGAAAGA
CCCGAGTTCGGAGTCCTCGGTCTTCGGATCGTGTTCTCTCAGATGCGTCGCGCCGATCGCCTGATGGGTACTCTAATGCC
TGAGCGTGGAGTCCCTCGGGGTTTGAGACGCGCTTGACCGGCCGTTGGGCTCGCGTCACCAAGTCCGCGTCCTTTGGGAC
GGCGGTACTACAACGCATGACCTCATCGGGGTAGGACAACCCGCTAGACTTAAGCATATTAATCAGCGGAGGAAAAGAAA
CTAACAAGGTCCCCCCAAAAAAAAC

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Uncultured Tulasnellaceae voucher HuPc19R small subunit ribosomal RNA gene, partial sequence; internal transcri...	uncultured Tulas...	1105	1105	97%	0.0	97.40%	695	OP537809.1
Uncultured Tulasnellaceae clone OTUA5_PlateKaro_1_F7 18S ribosomal RNA gene, partial sequence; internal tran...	uncultured Tulas...	1105	1105	97%	0.0	97.40%	745	JX649082.1
Tulasnellaceae sp. isolate 67.1.1a.9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ...	Tulasnellaceae sp.	1105	1105	91%	0.0	99.51%	611	MN545129.1
Tulasnellaceae sp. isolate 67.1.1a.1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ...	Tulasnellaceae sp.	1105	1105	91%	0.0	99.51%	611	MN545109.1
Uncultured Tulasnellaceae voucher DAp7R small subunit ribosomal RNA gene, partial sequence; internal transcribe...	uncultured Tulas...	1103	1103	97%	0.0	97.24%	696	OP537796.1
Uncultured Tulasnellaceae voucher DAp3R small subunit ribosomal RNA gene, partial sequence; internal transcribe...	uncultured Tulas...	1101	1101	97%	0.0	97.24%	699	OP537795.1
Uncultured Tulasnellaceae voucher HuPc15R small subunit ribosomal RNA gene, partial sequence; internal transcri...	uncultured Tulas...	1099	1099	97%	0.0	97.24%	694	OP537808.1
Uncultured Tulasnellaceae voucher DAp18R small subunit ribosomal RNA gene, partial sequence; internal transcrib...	uncultured Tulas...	1099	1099	97%	0.0	97.24%	690	OP537797.1
Uncultured Tulasnellaceae voucher DAp19R small subunit ribosomal RNA gene, partial sequence; internal transcrib...	uncultured Tulas...	1098	1098	97%	0.0	97.09%	710	OP537798.1
Uncultured Tulasnellaceae clone C3C-2A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	uncultured Tulas...	1098	1098	96%	0.0	97.52%	661	KC243935.1
Uncultured Tulasnellaceae voucher HuPc9R small subunit ribosomal RNA gene, partial sequence; internal transcrib...	uncultured Tulas...	1098	1098	97%	0.0	97.09%	693	OP537805.1
Uncultured Tulasnellaceae voucher DAp27R small subunit ribosomal RNA gene, partial sequence; internal transcrib...	uncultured Tulas...	1096	1096	97%	0.0	97.09%	691	OP537799.1
Uncultured Tulasnellaceae clone OTUA5_PLATEAGA_10_H12 18S ribosomal RNA gene, partial sequence; internal ...	uncultured Tulas...	1094	1094	97%	0.0	97.09%	746	JX649081.1
Uncultured Tulasnellaceae clone PM418 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	uncultured Tulas...	1094	1094	95%	0.0	97.80%	635	GQ241663.1
Tulasnellaceae sp. isolate 67.1.1a.7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ...	Tulasnellaceae sp.	1092	1092	90%	0.0	99.18%	610	MN545131.1
Tulasnellaceae sp. isolate pf41.1-ITS4-Tul_culture small subunit ribosomal RNA gene, partial sequence; internal tr...	Tulasnellaceae sp.	1088	1088	96%	0.0	97.21%	716	MZ129307.1
Uncultured Tulasnellaceae clone Dmpl_Aga_10A3 18S ribosomal RNA gene, partial sequence; internal transcribed ...	uncultured Tulas...	1088	1088	97%	0.0	96.94%	744	JX024736.1
Tulasnellaceae sp. isolate 65.1.1b.1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ...	Tulasnellaceae sp.	1088	1088	89%	0.0	99.50%	607	MN545127.1
Tulasnella calospora isolate Pch-QS-0-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Tulasnella calosp...	1086	1086	97%	0.0	96.79%	690	GU166407.1
Tulasnellaceae sp. isolate pf38.m3-ITS4-Tul_culture small subunit ribosomal RNA gene, partial sequence; internal t...	Tulasnellaceae sp.	1085	1085	97%	0.0	96.79%	660	MZ129303.1

▲Figure36



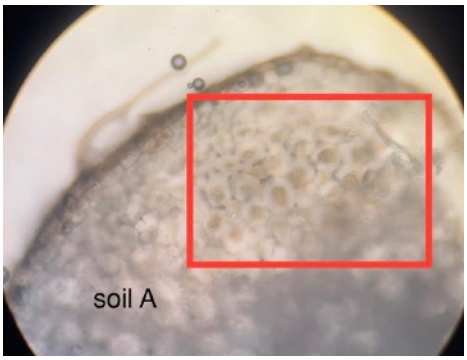


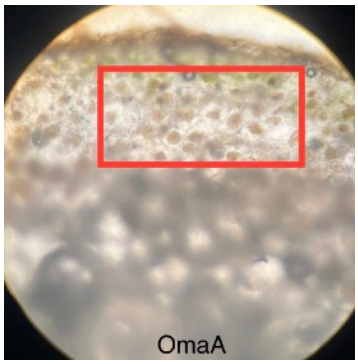
▲Figure37 ▲Figure38

5.The results of inoculating showed that peloton A was inoculated successfully , and the peloton could be seen obviously at the roots of the *S. sinenesis*.

The DNA sequencing results showed that the fungus is *Ceratobasidiaceae*. Although the results about inoculation of peloton B can be seen , the type of fungus still can not be identified through liquid culture or extraction of root DNA for sequencing.

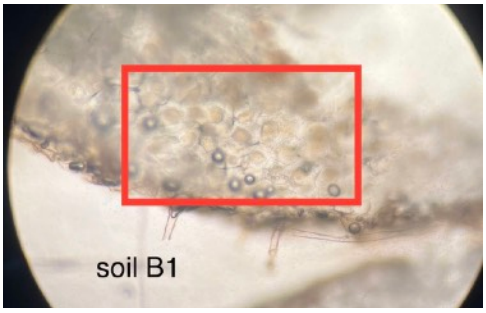
(1)A

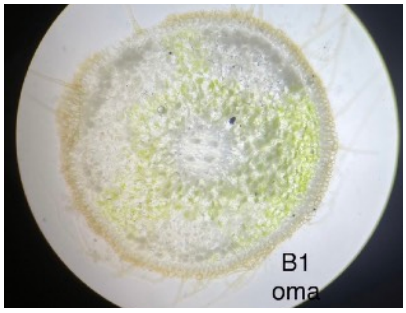




▲Figure39 ▲Figure40

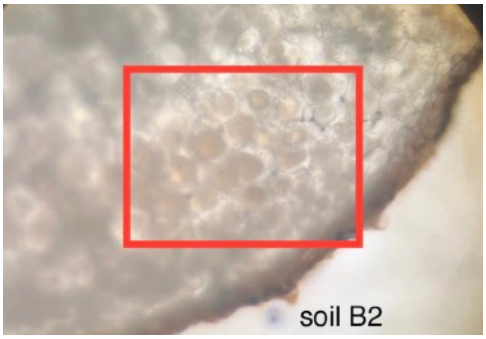
(2)B1



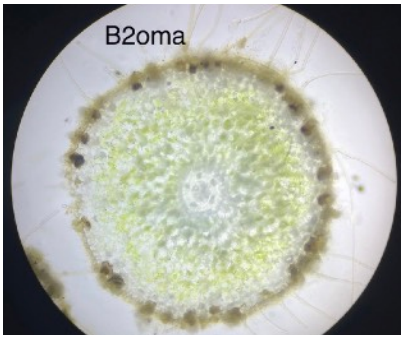


▲Figure41 ▲Figure42

(3)B2

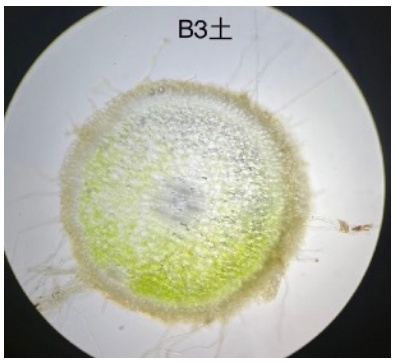


▲Figure43

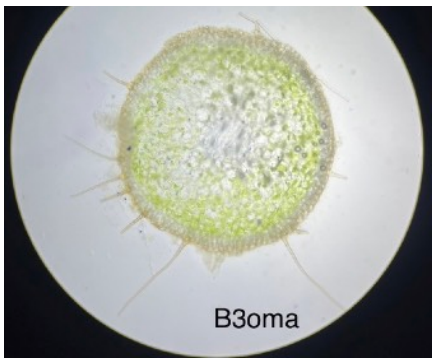


▲Figure44

(4)B3

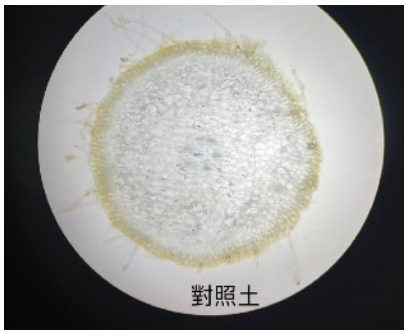


▲Figure45

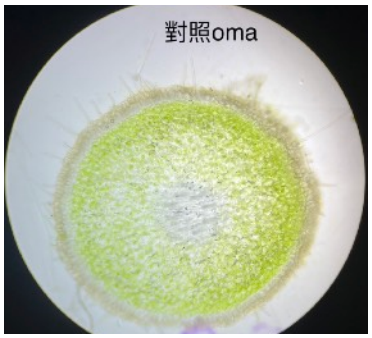


▲Figure46

Control



▲Figure47



▲Figure48

3. Experiment 3: Test whether the symbiotic hyphae have the ability to decompose the substances needed for germination.
(1) The result of the interaction between symbiotic fungi and starch
Starch concentration: 500mg/L

starch concentration	group	Days of co-cultivation with fungi
500mg/L	A-1	0
	A-2	3
	A-3	7
	A-4	10

starch concentration	group	Days of co-cultivation with fungi
500mg/L	B1-1	0
	B1-2	3
	B1-3	7
	B1-4	10

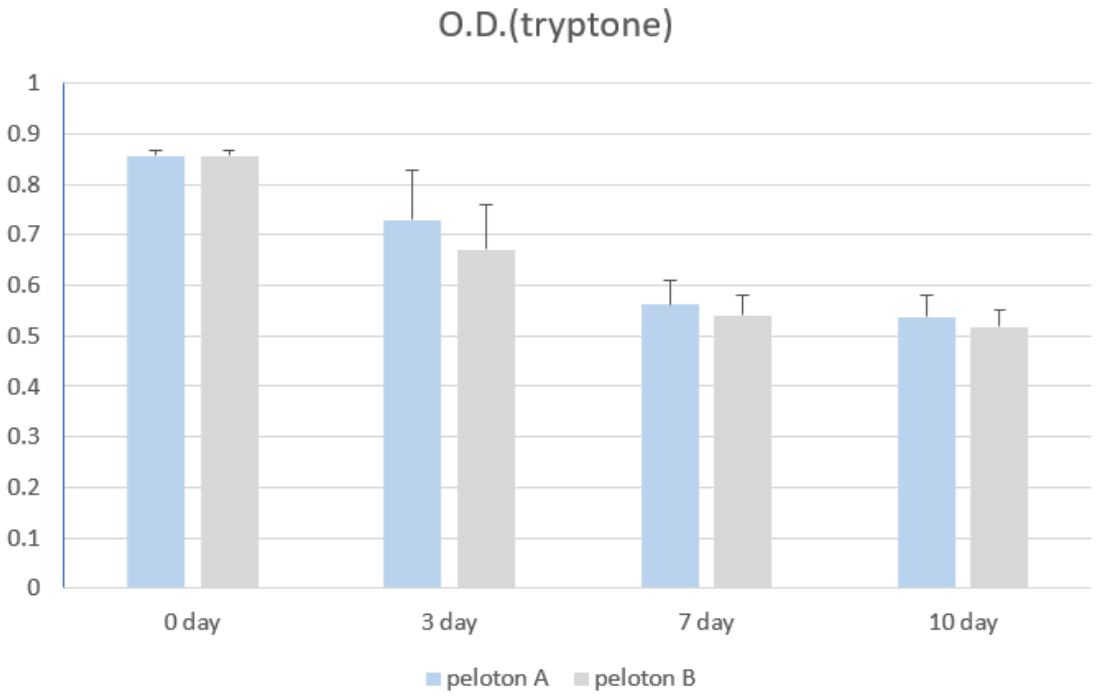
starch concentration	group	Days of co-cultivation with fungi
500mg/L	B2-1	0
	B2-2	3
	B2-3	7
	B-4	10

(2) The results of the interaction between symbiotic fungi and proteins

Protein concentration:

Dietary Fiber Concentration	Group	Days of co-cultivation with fungi	Dietary Fiber Concentration	Group	Days of co-cultivation with fungi	Methylcellulose concentration	Group	Days of co-cultivation with fungi	Methylcellulose concentration	Group	Days of co-cultivation with fungi
1g/L	A1-1	0	1g/L	B1-1	0	800mg/L	B1-1	0	800mg/L	A1-1	0
	A1-2	3		B1-2	3		B1-2	3		A1-2	3
	A1-3	7		B1-3	7		B1-3	7		A1-3	7
	A1-4	10		B1-4	10		B1-4	10		A1-4	10
2g/L	A2-1	0	2g/L	B2-1	0	1.6g/L	B2-1	0	1.6g/L	A2-1	0
	A2-2	3		B2-2	3		B2-2	3		A2-2	3
	A2-3	7		B2-3	7		B2-3	7		A2-3	7
	A2-4	10		B2-4	10		B2-4	10		A2-4	10
3g/L	A3-1	0	3g/L	B3-1	0	2.4g/L	B3-1	0	2.4g/L	A3-1	0
	A3-2	3		B3-2	3		B3-2	3		A3-2	3
	A3-3	7		B3-3	7		B3-3	7		A3-3	7
	A3-4	10		B3-4	10		B3-4	10		A3-4	10

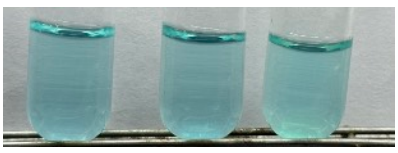
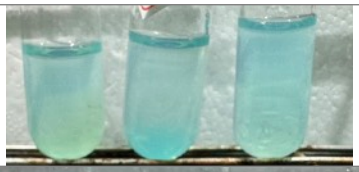
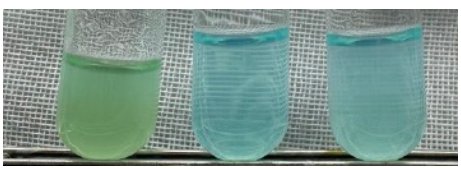
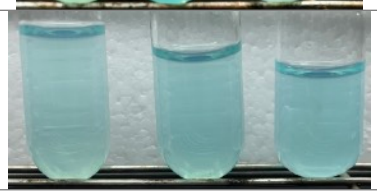
the results of co-cultivation with fungi					
PelotonA	O.D.	O.D.	O.D.	average	standard deviation
0 day	0.848	0.854	0.867	0.8563	0.00971
3 day	0.615	0.782	0.792	0.7297	0.09943
7 day	0.572	0.507	0.605	0.5613	0.04986
10 day	0.583	0.496	0.536	0.5383	0.04355
PelotonB	O.D.	O.D.	O.D.	average	standard deviation
0 day	0.848	0.854	0.867	0.8563	0.00971
3 day	0.580	0.683	0.752	0.6717	0.08656
7 day	0.549	0.497	0.575	0.5403	0.03972
10 day	0.524	0.482	0.548	0.5180	0.03341



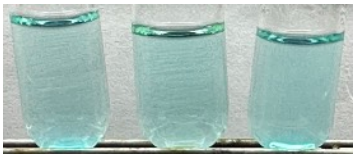
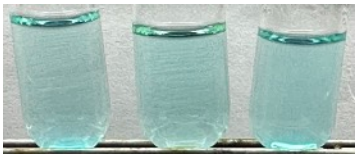
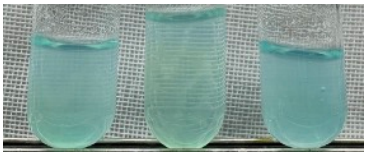
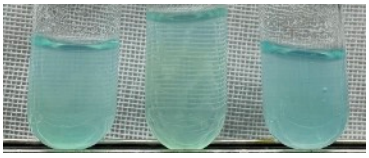
(3) Results of interaction between symbiotic fungi and cellulose

1. Peloton A

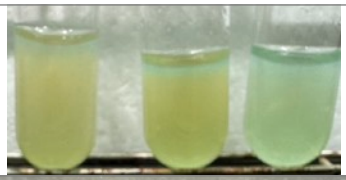
(1)methylcelloluse concentration：1g/L

0day		7day	
3day		10day	

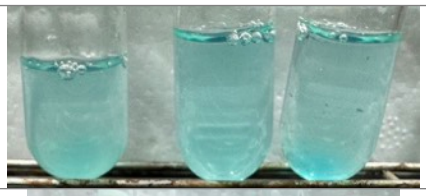
(2)methylcelloluse concentration：2g/L

0day		0day	
3day		10day	

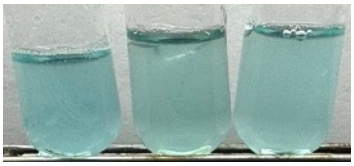
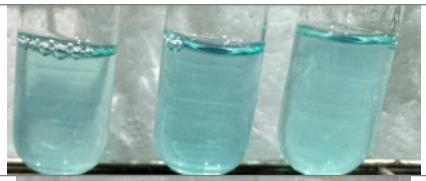
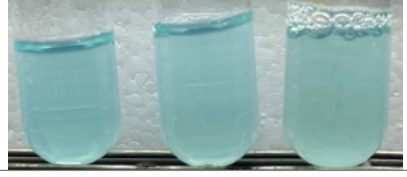
(3)methylcelloluse concentration：3g/L

0day		7day	
3day		10day	

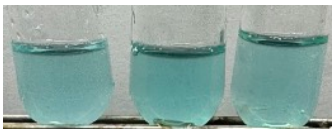
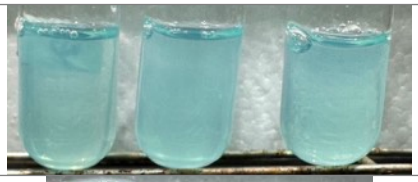
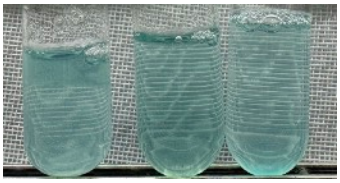
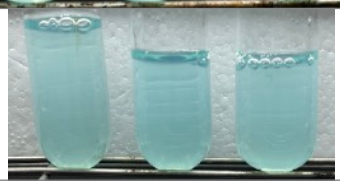
(4)dietary fiber concentration：0.8g/L

0day		7day	
3day		10day	

(5)dietary fiber concentration：1.6g/L

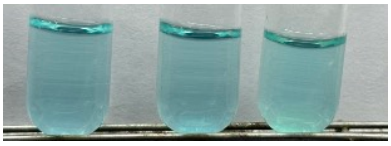
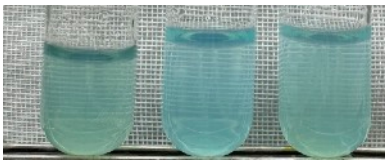
0day		7day	
3day		10day	

(6)dietary fiber concentration：2.4g/L

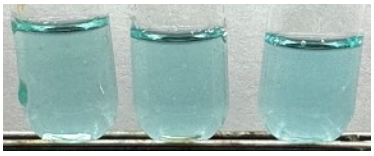
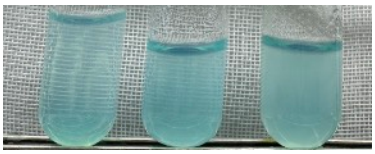
0day		7day	
3day		10day	

2. Peloton B

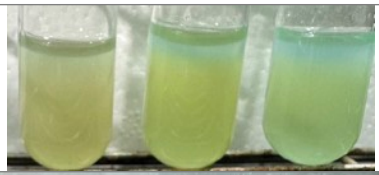
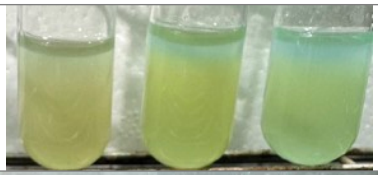
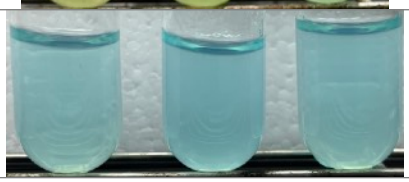
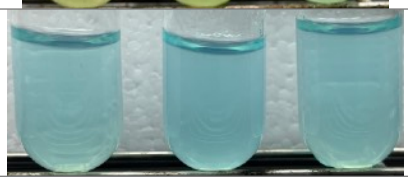
(1)methylcelloluse concentration：1g/L

0day		7day	
3day		10day	

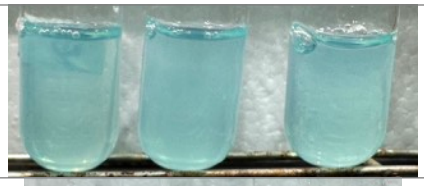
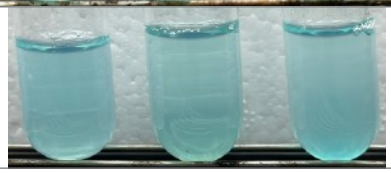
(2)methylcelloluse concentration：2g/L

0day		7day	
3day		10day	

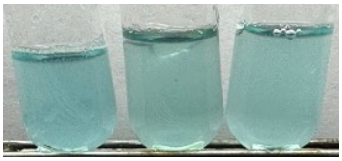
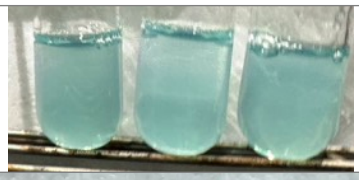
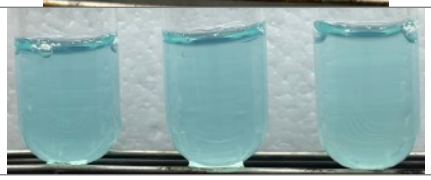
(3)methylcelloluse concentration：3g/L

7day		7day	
10day		10day	

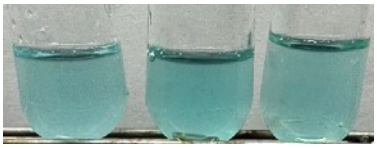
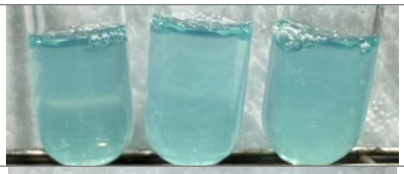
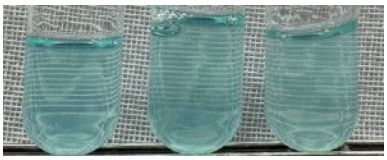
(4)dietary fiber concentration：0.8g/L

0day		7day	
3day		10day	

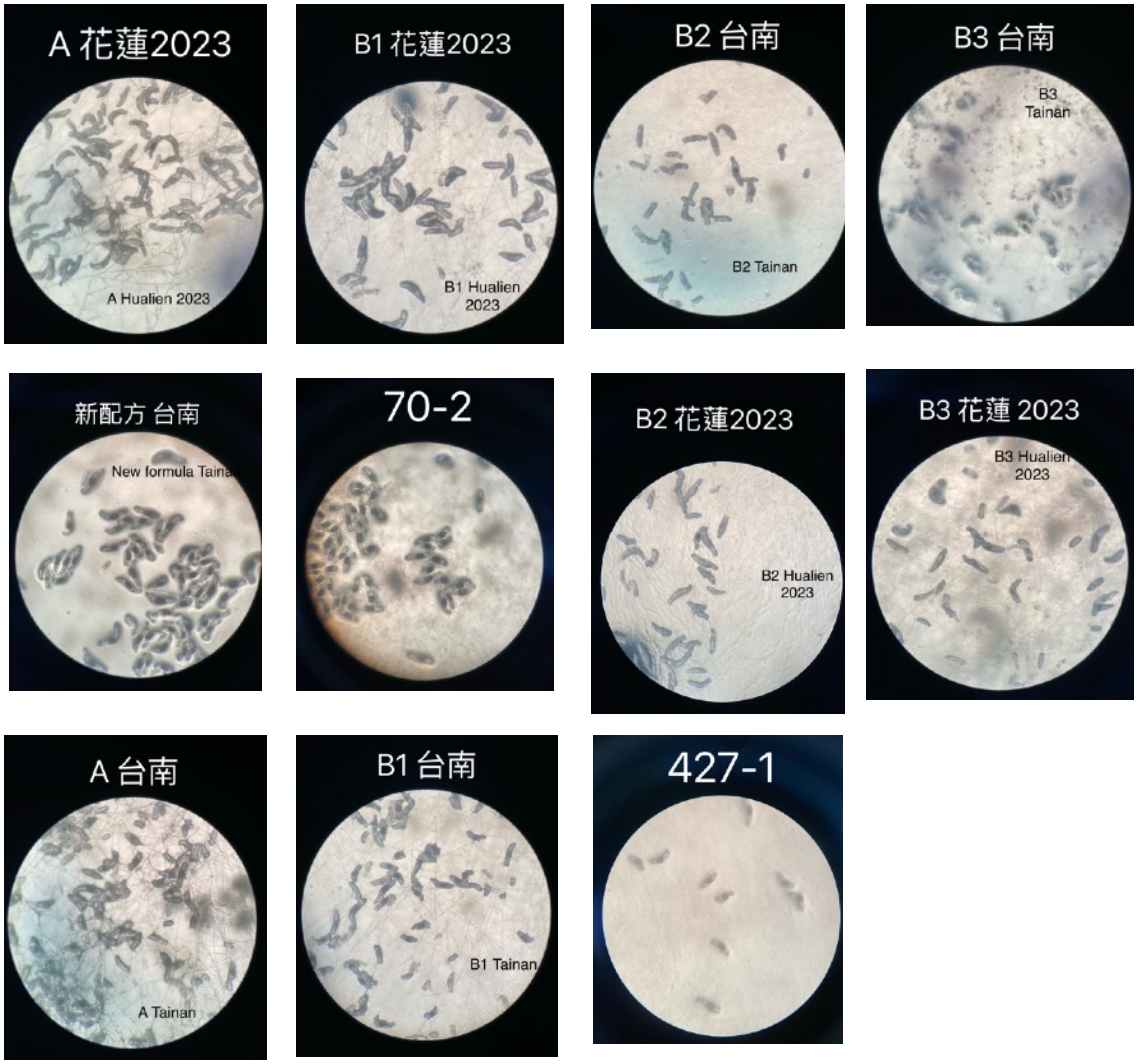
(5)dietary fiber concentration：1.6g/L

0day		7day	
3day		10day	

(6)dietary fiber concentration：2.4g/L

0day		7day	
3day		10day	

4. Experiment 4: To explore the relationship between symbiotic mycorrhizal fungi and the germination of *S. sinensis* seeds in progress. According to the literature, it can be known that symbiotic fungi are needed for seeds to germinate, but the length of time required is not certain. Although the seeds in this experiment have not yet germinated, it can be seen from the results of Experiment 3 that the hyphae we isolated can decompose starch and protein, so the next thing we want to test is whether the seeds can germinate in a specific culture environment and whether the germination is related to root-symbiotic mycorrhizal fungi.So far, all these seeds have not germinated.



VI.Discussion

1. According to the literature, most of the orchid root symbiotic fungi will take a long time to grow mycelium in the in vitro separation experiment. Our first fungus separation experiment took almost two months to grow, but the second separation experiment, however, the mycelia were grown in only five days after the secondary separation, so they were named A and B respectively, but A and B were also co-cultured with starch or protein, and both have the effect of gradually decomposing starch or protein. After inoculation experiments, it was found that peloton A was *Ceratobasidiaceae*, while peloton B could not be identified in either liquid culture or inoculation experiments? According to the waiting room theory in the literature (Marc-André Selosse 2022), Rhizoctonia and other Rhizoctonia fungi may have evolved from a branch of ancient orchid symbiotic fungi. Fungal symbiosis in plant roots is very common. These endophytic fungi will not lead to morphological differentiation of roots or provide nutrients to plants, but Rhizoctonia is recruited to evolve a symbiotic relationship (mycorrhizal symbiosis). The number of original endophytes is small, so it is difficult to form peloton, which increases the

difficulty of identification. Peloton B might be an endophyte or not.It is worth further discussion.

2. The literature shows that the symbiotic fungi of the same species of orchids may be different at different development stages or in different growth environments. What are the possible environmental factors that affect the time to grow into hyphae ? Worth revisiting.
3. The results obtained in the DNA sequencing experiment on the hyphae of the root mycelium of *Spiranthes sinensis* showed that the mycelium contained many unknown species, including the family *Agaricalaceae*,but there is no literature talking about this so far.
According to other literature reports on the root symbiosis of orchids, it shows that the specificity between orchids and mycorrhizal fungi is not very strict (Meng et al., 2009) and different bacteria play different roles in different growth stages of orchids (Chen et al. et al., 2008), we speculate that these may be endophytes, but what role do they play in the growth of *S. sinensis* ? Our findings are worthy of further investigation.
4. Whether the wild *S. sinensis* plants from the National Taiwan University campus or Hualien Agricultural School or tissue culture seedlings collected were planted in the grassland, the symbiotic fungi in the roots of the three *S. sinensis* were all *Ceratobasidiaceae*, but if the *S. sinensis* was Tissue culture seedlings grown in a greenhouse (the medium is mainly peat soil), the symbiotic fungi in their roots are *Tulasnellaceae*. This shows that the growth environment will affect the types of symbiotic fungi in the roots of *S. sinensis*. What are the environmental factors that affect it? How to detect different environmental factors and what is the detailed mechanism of establishing contact or symbiosis between fungi and *S. sinensis* ? It is worth further exploration.
5. Regardless of the isolated hyphae A or B, the trend of starch and protein being gradually decomposed over time can be seen, which shows that our isolated hyphae can produce certain starch-decomposing enzymes and proteases. Our discoveries are consistent with the views mentioned in other literatures , that is, the symbiotic fungi on the roots of *S. sinensis* may promote seed germination by decomposing organic nutrients in the environment. (Galih et al., 2020)
6. To explore the effect of symbiotic mycelia on the germination of seeds is in progress. The literature shows that the co- cultivation of symbiotic fungi and seeds of ç is helpful for germination, but the time required varies greatly. The shortest time is two weeks, and the longest is Several months or even one to two years, Our experimental results show that there is still no germination after 4-6 months of co-cultivation of the isolated hyphae A or B and *Tulasnellaceae* with the seeds of *S. sinensis* .

This is still worthy of further discussion.

7. It is mentioned that there are three major families of Rhizoctonia, Our research found that there are two families of symbiotic fungi in the roots of *S.sinenesis,* ie *Ceratobasidiacea* and *Tulasnellaceae* , whether *Serendipitaceae* also have a symbiotic relationship with *S. Sinensis* or just as endophyte is worth exploring.
8. We will continue to explore whether the symbiotic hyphae in the roots of *S. sinensis* can also decompose lipid and cellulose to promote seed germination.
9. Whether orchids release signal substances to attract symbiotic fungi or make symbiotic fungi decompose nutrients to promote germination remains to be explored.

Ⅶ.Conclusion

1. The symbiotic mycorrhizal fungus in the roots of *S. sinensis* are *Ceratobasidiaceae* when grown in the wild or cultivated, but they are *Tulasnellaceae* when cultivated in greenhouses.
2. There are more than one kind of endophyte in the root of *S. sinensis* , and one of them is *Agaricaceae*. This is a new discovery.
3. The symbiotic mycorrhizal fungi in the roots of *S. sinensi*s can decompose starch, protein and cellulose in the environment.

Ⅷ.References

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【評語】060019

1. 本研究由緩草根部分離得多株共生菌種，主要目的在對綬草(Spiranthes sinensis)根部共生真菌的多樣性進行探討。結果發現綬草的根部共生菌種類會受環境影響，其中包含角擔菌 Ceratobasidiaceae、膠膜菌 Tulasnellaceae、傘菌科 agaricomycetidae 等，且能分解環境中的澱粉與蛋白質。

2. 本研究主要結果在找出綬草的根部共生真菌，實驗設計及研究過程單一，建議可以收集緩草根部滲出物並與所分離出之共生菌共同培養。尋找可影響共生真菌生長發育所需之植物次級代謝物。

3. 以綬草根部共生菌探討綬草種子萌發，宜多收集蘭科植物之文獻資料。