Identification of No. 8 spoilage bacteria and study on biological properties of areca taro

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Abstract. You should leave 8 mm of space above the abstract and 10 mm after the abstract. The heading Abstract should be typed in bold 9-point Times. The body of the abstract should be typed in normal 9-point Arial in a single paragraph, immediately following the heading. The text should be set to 1 line spacing. The abstract should be centred across the page, indented 17 mm from the left and right page margins and justified. It should not normally exceed 200 words. In order to explore the effects of fungus infestation on areca taro during cold storage period, a black morphologic fungus (No. 8) was isolated and identified as Curvularia lunata by morphological observation and 18sRNA gene sequencing. The study on its biological properties showed that the growth and development temperature of Curvularia lunata were 15-40 ℃, the optimal temperature was 25-30 ℃, the optimal pH was 6-8, the optimal light condition was natural light, the optimal carbon source was sorbitol, maltodextrin and mannitol, and the optimal nitrogen source was glycine. The optimum germination temperature, pH and humidity of conidia were 25 - 40℃, 6 - 9 and 100% + water drop, respectively, under the promotion of 40% areca taro solution. The aim of this study was to provide a theoretical basis for the prevention and treatment of areca taro rotting in cold storage period.

1 Introduction

Areca taro, also known as lipu taro, fuding taro and fragrant taro, is a high starch aquatic vegetable belonging to araceae taro[1].After removing the rough skin, the stem block is milky white with brown or purple areca grain. The pulpis sweet and fragrant, and becomes more intense after cooking.

The diseases and the species and quantity of spoilage bacteria during the growth of areca taro directly affect the storage effect after harvest. He Yan and others [2] systematically investigated the occurrence characteristics and rules of taro diseases and insects in Hezhou, Guangxi and found that taro was seriously harmed by taro blight, taro soft rot, taro blotch, spodoptera litura, taro aphid and underground pests during planting. Pan Feng-rong and others [3] discussed a variety of field control measures through the occurrence characteristics of areca taro diseases and insect pests. Chen Xue-rong and others[4] found in the control of taro anthracnose and blight caused by seed dressing with bactericide that 300 times liquid dressing with 58% metalaxyl manganese zinc wettable powder had a better disease prevention effect and could effectively improve the commerciality and yield of taro. Liu Qiong-ying and others [5] found in the efficacy test on the control of taro blight that 68% Redomir manganese zinc water dispersible granule, 72% metalaxyl manganese and zinc wettable powder, and 58% metalaxyl manganese zinc wettable powder all had significant effects on the control of taro blight, but 68% Redomir manganese zinc water dispersible granule had the best effect. Jiao Qing-qing and others[6] found that 72% frost urea · manganese zinc wettable powder had better control effect in the control of Taro blight form Taixing-xianghe. In conclusion, the current research on taro disease is still mainly focused on the damage and control measures of pests and diseases during the planting period, but less attention is paid to the related research on the spoilage microorganisms that cause the decay of areca taro during the storage period.

In this study, a series of spoilage molds were isolated from the rotten areca taro during cold storage. Among them, the typical black mold No. 8 was taken as the research object to identify and study the biological properties of this fungus, in order to provide theoretical basis for the prevention and treatment of rotten disease and preservation methods of areca taro during storage.

2 Materials and methods

2.1 Materials and reagents

No. 8 spoilage strain was isolated and purified from rotten areca taro collected from HeZhouJinTai Grain and

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Oil Market. PDA culture medium was purchased from Guangdong Huankai Biotechnology Co., Ltd. Basic medium 1 (yeast extract 1 g, peptone 2 g, KH_2PO_4 1 g, AGAR 20 g, water 1000 mL, puted into 250 mL triangle flask, 150 mL per flask, routine sterilization for 15min). Basic medium 2 (without peptone, the preparation was the same as that of basic medium 1). 40% areca taro culture solution (200g peeled stems of areca taro added with 1000mL water were heated to boil for half an hour, and then added the heating water to 1000mL again, filtered in a triangular flask while hot by multilayer gauze, and sterilized at 121℃ for 20 minutes). Sodium hydroxide, hydrogen chloride and other reagents are produced by Sinopharm Chemical Reagents Company. The reagents such as carbon source and nitrogen source were all analytical pure.

2.2 Instruments and equipment

Microscope (Motic China Group Co., Ltd), DL-CJ-2N type super clean table (Beijing Donglianhaer Instrument Manufacturing Co., Ltd.), Vertical pressure steam sterilizer 510C (Chongqing Yamatuo Technology Co., Ltd.), PH-070A drying oven/incubator (Shanghai Yiheng Scientific Instrument Co., Ltd.), Mold incubator (Shanghai Boxun Industrial Co., Ltd.), PXZ type (multi-segment programming) intelligent artificial climate box (Ningbo Jiangnan Instrument Factory), Precision pH meter (Shanghai Hongyi Instrument Meter Co., Ltd.).

2.3 Morphological characteristics observation and preliminary identification of pathogenic bacteria

Using the slide culture method, PDA culture solution was dropped on the sterile glass slides in the plane petri dishes. After condensation, it was inoculated from the surrounding areas and covered with the cover glass. Then, an appropriate amount of sterile water was dropped into the petri dishes, and the plates were covered, and the incubations were placed at 28℃ for 2-3 days to observe and describe the morphological characteristics of each pathogen under the light microscope [7].

2.4 Genomic DNA extraction, PCR amplification and sequencing

Sangon Bioengineering (Shanghai) Co., Ltd. was commissioned to conduct DNA extraction and sequencing.The identified gene sequences were analyzed with NCBI database for homology analysis to determine the species and genus relationship of pathogenic bacteria.

2.5 The affect on the growth, sporulation and relative quantity of mycelium by different temperatures

The small bacterial blocks were transplanted into the PDA plate with a 5mm diameter perforator, and cultured at 5, 10, 15, 20, 25, 30, 35 and 40℃ with a gradient of 5℃ for 4 days. The "+" method was used to measure the diameter of the colony, and the average value was taken. The colony was observed and the thickness and density of mycelium were visually estimated [8]. After the test, adding 10mL sterile water to each petri dish, the conidia with a sterile spoon was gently scraped to make the suspension fluid into a snowball observation counting plate (25*16). The sporulation amount was measured under a microscope, and each counting plate was repeated for 3 times.The blood count yield was calculated as follows:

Spore production quantity（cells/mL）=(spore number in 80 cells)/80×400×1000×Diluted multiples （1）

2.6 Effects of different pH values on the growth and sporulation of pathogenic bacteria and relative quantity of mycelium

With 1 moL/L NaOH and 1 moL/L HCL, pH of PDA culture medium was adjusted into 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, respectively. After routine sterilization, the PDA medium was poured on the plate. The inoculated plate was placed in a 28 ℃ incubator. After 4 days, the colony diameter, mycelium relative amount and sporulation amount were measured according to the method of 1.5, and each treatment was repeated 3 times.

2.7 Influence of different light treatments on spoilage bacteria

The same bacteria block were inoculated on PDA plate and cultured under 5 different light conditions, including continuous light, natural light (12h light dark alternation), 12h UV light (12h UV + 84h natural light), 6h UV light (6h UV + 90h natural light) and complete darkness. The growth indexes were measured according to the method of 1.5, and each treatment was repeated 3 times.

2.8 Utilization of different carbon and nitrogen sources by spoilage bacteria

Nine kinds of carbon were tested (Table 4) and five kinds of nitrogen were tested (Table 5).1g of each carbon and nitrogen were added to the basic medium 1 and 2, then the basic medium 1 and 2 were sterilized for 10 min, poured into petri dishes, then transplanted into small bacterial blocks, and incubated at 28℃ for 4 days [8]. The colony diameter and sporulation yield were measured according to the method of 1.5, and the growth was observed. Each treatment was repeated for 3 times.

2.9 Effects of culture medium, temperature, pH and humidity on spore germination

2.9.1 Influence of culture medium on spore germination

The amplified culture petri dishes were added with 10mL sterile water and 40% culture solution of taro, conidia were scraped off, and conidia suspension with a

concentration of about 5*105 /mL was prepared. The conidia suspension was dropped and evenly coated on a clean glass slide (about 50 conidia per unit field of vision) and placed in a moisturizing petri dish. The spores were cultured at different temperatures ranging from 5℃ to 45℃, and the germination of spores was examined under microscope after 24h. Each treatment was repeated three times. Conidial germination rate was calculated as follows [8-9]:

Conidial germination rate (%)=(Number of spores per unit field of view)/(Total number of spores per unit field of view) ×100% (2)

2.9.2 Influence of temperature on spore germination rate

The spore suspension was prepared with 40% taro culture solution and dropped on clean slides. The spore suspension was cultured at the above 9 temperatures for 2h and the germination was examined under microscope. Each treatment was repeated three times.

2.9.3 Effect of pH on spore germination rate

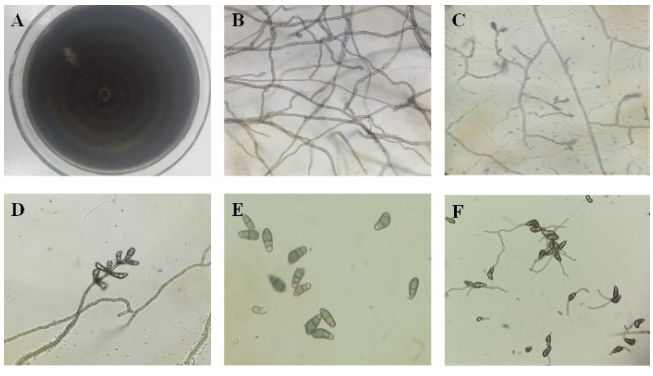
The pH value of the culture solution of 40% taro was adjusted to 1 ~ 11, a total of 11 grades, and the corresponding spore suspension was prepared according to the grade. The spore suspension was coated on the moisturizing slide and placed in a clean petri dish to moisturize and germinate for 3 repetitions and cultured at 28℃ for 2h [9], and the observation contents were the same as 1.9.1.

2.9.4 Affection on spore germination rate by humidity

The spore suspension above was coated on the slide, dried in the shade and put into an incubator with humidity of 60%, 70%, 80%, 90%, 100%, 100% + water drops. The incubator was cultured at 28℃, and the germination was examined under microscope every 2h, with 3 replicates for each treatment.

3 Results and analysis

3.1 Morphological characteristics obse-rvation and gene identification results



A. Colony morphological characteristics; B substrate mycelium; C basal surface mycelium; D conidia and conidia pedicel; E conidia; F spore

Fig. 1. Morphological observation of No. 8 spoilage bacteria

No. 8 pathogen was growing well. The colony was round, composed of concentric circles with black and white spacing, with neat edges, and was navy blue to black. The substrate mycelium was very well developed, initially navy blue, and gradually black to varying degrees, with septum and many branches. The basal surface mycelium was white, single or several root clusters, had septum, and be piled pressure after with the black conidia increase, not easy to see. Conidia pedicels was brown, single or branched, had sporulation at the apex. The conidia are gryish-brown, fusiform and crescent in shape. The cells are divided into different cells by three septa. The third cell from the base up was larger, darker in color, and the cells at both ends were relatively shallow. The results were highly similar to Zheng Nan's description of Curvularia lunata [10]. The conidia germinated, and the buds grown from both ends of the conidia, and the buds grown preferentially from the base. By 18sRNA identification, the similarity between the gene fragment and the gene registration number JN941608.1 reached 99%. Combined with morphological observation, the strain was identified as Curvularia lunata.

3.2 Study results of biological characte-ristics on No.8 spoilage bacterium (Cur-vularia lunata) of Areca taro

3.2.1 Effects of different temperatures on mycelial growth, sporulation yield and relative quantity of mycelia

The results in Table 1 showed that the hypha of Curvularia lunata could grow at 15-40 ℃, and the optimal growth temperature was 25-30 ℃. The mycelia did not grow at a temperature lower than 15℃, and the growth ability of mycelia was weakened higher than 30℃. Conidium began to generate at 15 ℃, the optimal temperature of spores is 20 - 30 ℃, and 30 ℃ was highest for sporulation yield, up to 9.58×10^5.The low temperature and high temperature were not suitable for the growth, development and reproduction of this strain.

3.2.2 Effects of different pH on mycelial growth, sporulation and relative quantity of mycelia

The results in Table 2 showed that Curvularia lunata could grow in the pH range of 4-11, and the optimum pH value for mycelium growth was 6-8, and corresponding sporulation yield was also the highest.When pH was lower than 4, the mycelia did not grow at all, and when pH was higher than 8, the mycelia of pathogenic bacteria slowly tended not to grow.Conidia began to form at a pH of 4, and the optimal pH for sporulation was 7.It could be

seen that the growth, development and reproduction of Curvularialunata tended to be neutral and alkaline environment (Table 2).

Table 1. Effects of different temperatures on the growth and sporulation yield of Curvularia lunata

Biological characteristics	Temperature（℃）							
	5	10	15	20	25	30	35	40
Colony diameter（mm）	0	0	27.93	46.00	57.00	83.47	21.50	1.63
Significance of difference	eE	eE	cC	bB	bB	aA	cC	dD
Relative amount of mycelium	-	-	+	++	+++	+++	+	+
sporulation yield（1x10^5）	0	0	0.53	5.68	7.32	9.58	0.38	0.15
Significance of difference	abE	abE	bcC	bBC	aAB	aA	bcC	cdD

Table 2. Effects of different pH on the growth and sporulation yield of Curvularia lunata

Biological characteristics	pH										
	1	2	3	4	5	6	7	8	9	10	11
Colony diameter（mm）	0	0	0	51.17	55.57	58.17	58.33	57.33	56.33	54.50	53.00
Significance of difference	dD	dD	dD	cC	bcB	abA	abA	abA	bcB	cdC	cdC
Relative amount of mycelium	-	-	-	+	++	+++	+++	+++	++	++	+
sporulation yield（1x10^5）	0	0	0	2.25	2.55	3.07	3.17	2.35	1.75	1.37	1.07
Significance of difference	dD	dD	dD	adBC	bB	abA	abA	acBC	bcC	cdC	bdC

3.2.3 Effect of light on the growth of Curvularia lunata

Table 3. Effects of different light on the growth of Curvularia lunata

Light processing	Colony diameter（mm）	Sporulation yield (1×10^{5}A/mL)	Relative amount of mycelium
Continuous light	62.33 adA	2.70 cC	++
Natural light	61.50 bdB	5.30 aA	+++
Ultraviolet (uv) 12 h	61.00 bdBC	3.93 bB	++
Ultraviolet (uv) 6 h	57.50 cC	1.97 dD	+
Complete darkness	55.17 adD	2.20 cCD	+

The results in Table 3 showed that the clods of Curvularia lunata were inoculated in the center of the plate and cultured under five different light conditions. The colony diameters were all large, but there was no significant difference between natural light and 12h ultraviolet light. The hypha diameter of continuous light was the largest, the second was the light from the darkness alternation and the highest sporulation yield was 5.3×10^5/mL. The colony diameters of complete darkness had the smallest diameter. The colony diameter and sporulation yield of 12h UV irradiation were better than that of 6h UV irradiation, and proper amount of UV irradiation could promote the growth of Curvularia lunata. According to the results of colony diameter and sporulation yield, light conditions of light dark alternation was more suitable for the growth, develop-ment and reproduction of Curvularia lunata.

3.2.4 Utilization of different carbon and nitrogen sources by Curvularia lunata

Table 4. Utilization of different carbon sources by Curvularia lunata

Carbon source	Colony diameter（mm）	Sporulation yield (1×10^5 A/mL)	Relative amount of mycelium
Maltodextrin	70.00 acB	4.18 acF	+++
Mannitol	69.50 acC	5.95 eE	+++
Sucrose	54.50 efF	38.67 bB	+
Glucose	54.33 efF	36.42 cC	+
Gum Arabic	65.67 bdD	3.38 adG	++
Soluble starch	65.83 bcD	41.33 aA	++
Lactose	64.67 cdD	34.83 abD	++
D-Fructose	60.67 eE	4.05 fF	++
Sorbitol	71.00 abA	3.08 gG	+++

According to the results in Table 4, there were significant differences in the carbon sources required by the growth stage and the conidia production stage of Curvularia

lunata. During mycelium growth, sorbitol, maltodextrin and mannitol, as carbon sources, could effectively promote mycelium growth, followed by soluble starch, gum acacia, lactose, and the worst were sucrose and glucose. During spore production, soluble starch, sucrose and glucose were carbon sources to promote spore production, followed by lactose, but maltodextrin, mannitol, gum Arabic, D-fructose and sorbitol were worst. Sorbitol produced the least amount of spores.

Table 5. Utilization of different Nitrogen sources by Curvularia lunata

Nitrogen source	Colony diameter (mm)	Sporulation yield (1×10^5 A/mL)	Relative amount of mycelium
Glycine	54.17 acC	3.05 abA	+
Methionine	54.67 abC	1.82 acC	+
Ammonium nitrate	60.00 bB	1.22 cC	++
L-histidine	67.17aA	2.45 acB	+++
Ammonia water	5.00 dD	0.00 dD	-

The results in Table 5 showed that L-histidine was used as the nitrogen source for the mycelia of Curvularia lunata growing the fastest, followed by ammonium nitrate, glycine and ammonium nitrate were slower, and the bacteria could not use ammonia water.Glycine was the nitrogen source with the highest sporulation yield, followed by L-histidine.

3.3 Effects of different conditions on spore germination rate of Curvularia lunata

3.3.1 Effects of different culture solution and temperature on spore germination

According to the results in Table 6, conidia of Curvularia lunata also had a good germination effect in distilled water, but it was weaker than that in 40% areca taro solution, that is, 40% areca taro solution promoted the conidia germination of this bacterium.The conidia could germinate at 5-45 ℃ , and the optimal germination temperature was 25-40℃.In addition, it was found that the conidia of Curvularialunata had strong germination ability. Even if it was cultured for a short time (2h) at a low temperature (5℃), it could also have a very high germination rate, which was also an important reason for the gradual infection of areca taro by this bacterium in the process of cold storage.

3.3.2 Effect of pH on spore germination

According to the results in Table 7, conidia of Curvularia lunata could germinate at pH 3-11 after 2 hours, and the optimal pH was 6-9. The germination rate was the highest when the pH was 7-8, and it did not germinate at all when the pH was less than 3, while the germination decreased when the pH was higher than 9. When the pH value was less than 3, it still did not germinate after 4 hours. The germination rate of other conidia that could germinate at 2h was nearly 100%. The relative germination length was the longest when the pH value was 6-9. Therefore, the optimal pH value for conidia germination was 6-9.

3.3.3 Effect of humidity on spore germination rate

The results in Table 8 showed that different humidity and culture time had significant effects on conidia germination.The germination rate of conidia increased gradually with the humidity, and the greater the humidity, the better the germination effect of conidia. When there was condensation, the germination rate was the best, even if it was cultured for 2h, the germination rate could reach 100%. At the same time, with the extension of culture time, the germination rate was higher and higher.Therefore, the optimal germination humidity of conidia o Curvularia lunata was 100% + water drop.

Table 6. Effects of culture solution and temperature on spore germination

Biological characteristics	Temperature (℃)								
	5	10	15	20	25	30	35	40	45
Sterile water (24h)	-	+	+	+	++	+++	+++	++	+
40 %Areca taro solution (24h)	+	++	++	+++	+++	+++	+++	+++	++
40 % Areca taro solution(2h)	+	+	+	++	+++	+++	+++	+++	++

Note: Eye observation of spore germination could be divided into 3 levels (+, ++, +++) according to growth length. "-" refers to short mycelium, which is the same in the following table.

Table 7. Effect of pH on spore germination

Biological characteristics	pH										
	1	2	3	4	5	6	7	8	9	10	11
2h conidia germination rate(%)	0	0	62.22	67.16	72.92	80.48	91.38	90.90	81.82	70.00	64.29
Significance of difference	fF	fF	eE	adD	cC	acB	abA	aA	bB	cC	dD
4h conidia germination rate(%)	0	0	100	100	100	100	100	100	100	100	100

Relative germination length	-	-	+	+	++	+++	+++	+++	+++	++	++

Table 8. Effect of different humidity on spore germination

Biological characteristics	**Humidity(%)**					
	60	**70**	**80**	**90**	**100**	**100+dew**
2h conidial germination rate(%)	0bB	0bB	0bB	0bB	0bB	100aA
4h conidial germination rate(%)	0fF	0.12eE	0.36dD	1.24cC	18.64abB	100aA
6h conidial germination rate(%)	2.12adF	10.64eE	11.0acD	17.64abC	26.45bB	100aA
8h conidial germination rate(%)	10.67eE	16.0adD	25.32cC	36.62acB	40.13abB	100aA
10h conidial germination rate(%)	15.86dC	30.55bA	50.78cB	71.36aA	89.54abA	100aA

4 Conclusion

The compound infection of multiple fungi is the main cause of areca taro's mildew and decay during storage. According to colony morphology, microscopic observa-tion and gene sequencing, the strain was identified as Curvularia lunata. Curvularia lunata is the main pathog-en causing leaf spot disease, but there are differences due to different parasitic environments and biological characteristics of plants.The biological characteristics of Curvularia lunata showed that the growth and development temperature and pH were 15-45 ℃ and 3-11, the optimum temperature was 25-35 ℃, the optimum pH was 6-8, the optimum light condition was natural light, the optimum carbon source was dextrin, mannitol, D-sorbitol, and the optimum nitrogen source was glycine, but ammonia water was not available.Conidia of this fungus could also germinate in low temperature water. 40 % areca taro solution could significantly promote the germination of conidia. The optimal germination temperature was 25-40 ℃, the optimal pH value was 3-9, and the optimal humidity was 100 % + water drop. The humidity had a significant effect on spore germination.

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