Microstructure characterization of different types of chlamydospores in Duddingtonia flagrans

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June 16, 2023

Abstract

The morphological and structural differences of different types of chlamydospore of Duddingtonia flagrans, a nematophagous fungus, were studied under light microscope and electron microscope to provide reference for the biological control of parasitic nematodiasis. In this study, D. flagrans isolate F088 dormant chlamydospore and non-dormant chlamydospore were selected as the research objects. The structural differences of these spores were observed by optical microscopy through lactol cotton blue, Trypan blue and MTT staining. FUN-1, DAPI and CFW staining were used to observe the metabolic activity, cell wall and nucleus differences of the two types of spores under fluorescence microscope. Ultrastructure of the two kinds of spores was observed using scanning electron microscope (SEM) and transmission electron microscope (TEM). Since lacto phenol cotton blue, trypan blue staining cannot distinguish dormant spores from dead spores, MTT assay was performed. Fluorescence microscopy observation showed that the cytoplasmic metabolic activity of non-dormant spores was stronger than that of dormant spores. The nucleus of dormant spores was bright blue, and their fluorescence was stronger than that of non-dormant spores. The cell wall of non-dormant spores produced stronger yellow-green fluorescence than that of dormant spores. Ultrastructural observation showed that there were globular protuberances on the surface of the two types of spores, but with no significant difference between them. The inner wall of dormant spore possesses a thick zona pellucida with high electron density which was significantly thicker than that of non-dormant spores, and their cytoplasm is also changed. In this study, the microstructure characteristics of dormant and non-dormant chlamydospores of D. flagrans fungi were preliminarily clarified, suggesting that the state of cell wall and intracellular materials were changed after spores entered to dormancy.

Microstructure characterization of different types ofchlamydospores in*Duddingtonia flagrans*

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Abstract: The morphological and structural differences of different types of chlamydospore of *Duddingtonia flagrans* , a nematophagous fungus, were studied under light microscope and electron microscope to provide reference for the biological control of parasitic nematodiasis. In this study, *D. flagrans* isolate F088 dormant chlamydospore and non-dormant chlamydospore were selected as the research objects. The structural differences of these spores were observed by optical microscopy through lactol cotton blue, Trypan blue and MTT staining. FUN-1, DAPI and CFW staining were used to observe the metabolic activity, cell wall and nucleus differences of the two types of spores under fluorescence microscope. Ultrastructure of the two kinds of spores was observed using scanning electron microscope (SEM) and transmission electron microscope (TEM). Since lacto phenol cotton blue, trypan blue staining cannot distinguish dormant spores from dead spores, MTT assay was performed. Fluorescence microscopy observation showed that the cytoplasmic metabolic activity of non-dormant spores was stronger than that of dormant spores. The nucleus of dormant spores was bright blue, and their fluorescence was stronger than that of non-dormant spores. The cell wall of non-dormant spores produced stronger yellow-green fluorescence than that of dormant spores. Ultrastructural observation showed that there were globular protuberances on the surface of the two types of spores, but with no significant difference between them. The inner wall of dormant spore possesses a thick zona pellucida with high electron density which was significantly thicker than that of non-dormant spores, and their cytoplasm is also changed. In this study, the microstructure characteristics of dormant and non-dormant chlamydospores of *D. flagrans* fungi were preliminarily clarified, suggesting that the state of cell wall and intracellular materials were changed after spores entered to dormancy.

Keywords: *Duddingtonia flagrans* ; nematophagous fungi; dormant chlamydospore; non-dormant chlamydospore; metabolic activity; ultrastructure

Introduction

Nematophagous fungus *Duddingtonia flagrans* is a promising candidate for biological control of animal helminthic diseases. The chlamydospore of this fungus was produced very early in the growth process. Compared with other nematopgagous fungi that did not produce chlamydospore, this fungus could produce chlamydospore in three days and produced few conidia [1,2]. This fungus is the only species of chlamydospore producing among the nematopgagous fungi of the Orbiliaceae family [3]. In addition, the advantage of this fungus in the biological control of animal parasitic nematodes is that the double-walled chlamydospore added to the feed can resist the adverse environment of the digestive tract [4-7]. The spores then develop along with the worm eggs in the feces of the animal, the spores of the fungus begin to germinate, and the larvae that hatch further stimulate the mycelium of the fungus to specialize into feeding organs to feed on the larvae as nourishment for the fungus itself [6]. Due to the strong resistance of chlamydospore to harsh external environment, such as freezing and drying, chlamydospore as an effective component of feed additives, such as various formulation, lick blocks and powders, has good commercial value [8-10]. Both powder and pellet formulation can effectively reduce infectious larvae in pasture, and have obvious control effect on gastrointestinal nematodes in grazing horses, cattle, sheep, goats and other livestock [11-13].

The dormancy of spores is one of the most mysterious life forms because of their resistance to adverse conditions and their ability to remain in a state of hypometabolism (a sort of half-dead) for long periods of time, which might be the best form for the source of all life on Earth [14]. The study of fungal spore dormancy is much later than that of bacterial spore. There are still many questions to explain the phenomenon of fungal spore dormancy. *D. flagrans* is a species of fungi belonging to the Deuteromycotina. At present, no sexual stage has been found, and the chlamydospore is asexual.

These spores are often found dormant in nature or in biocontrol products [15]. And this dormancy is a deep endogenous dormancy, which is different from the exogenous dormancy of some fungi such as *Aspergillus* conidium [16]. Endogenous dormancy spore cannot germinate even in the presence of sufficient nutrients such as water, carbon and nitrogen sources. At present, researchers pay more attention to the dormancy of some species of *Aspergillus* and *Trichophyton rubrum* conidium [17-20], and these fungi are mainly op-

portunistic pathogens and cause skin diseases in humans. However, there are relatively few literatures on the observation of the ultrastructure of fungal spores. Anjo et al. observed the structural characteristics of conidia of *A. fumigatus* at different culture stages by transmission electron microscopy [21]. Some studies observed the ultrastructure of the resting and germinating conidia of*A. ochraceus* and the resting conidia of *Botrytis* cinerea [22,23]. The structure of dormant chlamydospore of fungi was less observed. Among these studies, Citiculo et al. observed the metabolic activity of chlamydospore at different days of age by fluorescence microscopy against the conditioned human pathogens *Candida albicans* and *Candida Dublin* [24]. Yan et al. observed the ultrastructure of dormant and non-dormant chlamydospore of *M. oryzae* [25]. In some reports, the interaction of *D. flagrans*with L3 of *C. contortus* was observed by SEM [26,27]. However, the morphological data of dormant and non-dormant of *D. flagrans*chlamydospore are still lacking. In the practical application of biotechnology, producers encounter the problems of chlamydospore seed material inspection and final product quality inspection. Due to the existence of structural dormancy of chlamydospore in the product, it could not germinate on the basis of normal culture, so the conventional methods of spore incubation and colony counting could not reflect the actual number of live spores. Therefore, to fix this problem, it is necessary to find an effective method to distinguish dormant spores from dead spores by morphology. Meanwhile, it is also of great scientific significance to study the morphological and structural differences between dormant spores and non-dormant spores.

In this study, we aim to elucidate the morphological differences of different types of chlamydospore under light microscopy and electron microscopy. Firstly, the structural differences of dormant, non-dormant and dead spores were observed by optical microscopy with different staining methods. Secondly, the differences of metabolic activity, cell wall and nucleus between dormant and non-dormant spores were observed by fluorescence microscopy. Finally, the ultrastructure of the two types of spores was compared by scanning electron microscope and transmission electron microscope. The data obtained in this study provide a scientific basis for the application of *D. flagrans* in the biological control of animal parasitic nematodes and for understanding the dormancy mechanism of the fungal chlamydospore.

1 Materials and methods

1.1 Test strains

The *D. flagrans* isolate used in this study was F088 (CGMCC No. 11209), the gene database entry number is KU881774.1, which was isolated and purified from sheep feces collected in Heilongjiang Province, China in 2014 by an improved spreading separation technique. The isolate was stored in refrigerator at 4 on test tube containing 2% cornmeal agar. Mycelium and spores were taken from the preserved inclined plane and inoculated on Potato Dextrose Agar (PDA, Beijing Landbridge Technology Co., LTD.) plate containing chloramphenicol and incubated at 28 for 7 days.

1.2 Strain culture and chlamydospore preparation

1.2.1 Preparation of non-dormant chlamydospore

The agar containing mycelium grown on PDA was separated into 6˜8 even small rounds with a sterilized hole punch (aperture 5×5 mm) and inoculated in the above triangular flask containing Sandburg's liquid culture medium. The flask was shocked at 28 at 200 r/min for 72 h and the culture was stopped. 10 mL of the culture containing mycelium was inoculated into a triangle flask containing 1000 mL of 200 g grains [28]. Then the culture was placed in an incubator at 28 for 21 days. When more mycelium grew on the grain and yellow powder culture was observed by naked eye, it was proved that more chlamydospore had been produced, and the culture was terminated. An appropriate amount of 0.05% sterilized Tweene-80 solution was added to the triangular flask, and the solution was swirled for 1-2 min, then mycelium and spores were washed off the grains, and the crude spore suspension was collected and placed in ultrasonic cell pulverization instrument (SCO-2500, Shanghai Shengyan Ultrasonic Instrument Co., LTD.), and subjected to ultrasonic treatment for 5 min at 150W and 20 KHZ. At an interval of 30 s, the spore suspension was filtered through a 300-mesh copper screen, the filtrate was centrifuged at 1000 r/min for 5 min, and then washed with sterilized distilled water for 3 times. The sediment (i.e. chlamydospore) was suspended with sterilized distilled water

and counted with a blood cell count plate. Finally, the concentration of the spore suspension was adjusted with distilled water to about 1x10^4 spores /mL for further use.

1.2.2 Preparation of dormant chlamydospore

With the same batch of culture incubated at 28 and relative humidity of 30-40% to 50 d, and dried at 35 for 10 d, the spore germination rate was determined to be less than 5% [28].

1.2.3 Preparation of artificially lethal chlamydospore

The purified fresh chlamydospore was placed at 121 under high pressure for 15 min.

1.3 General optical microscopy and staining observation

Suspended drops of dormant, non-dormant and dead chlamydospore were prepared and added to the slide. The slide was covered and photographed under an optical microscope. The purified spore suspension was placed in a 2 mL centrifuge tube at 1500 r/min for 5 min, 2/3 of the supernatant was removed, and the milk phenol cotton blue dye solution (Guangzhou Exon Biotechnology Co., LTD.) was added for 5 min at room temperature, and the spore suspension was absorbed, observed and photographed on a slide. 0.9 mL of the spore suspension was added to 0.1 mL of 0.4% Trypan blue dye solution, mixed evenly for 3 min, and observed under a microscope. Take a 5 mL centrifuge tube and firstly add 1 mL MTT dye solution, 1 mL PBS buffer solution, at the same time add 2.5 μL cobalt chloride solution with a concentration of 2.5 mM, take 100 μL spore suspension to be dyed and purified, swirl and mix for 1 min, and then incubate at 27 for 24-36 h. The spore fluid was absorbed and observed under an optical microscope.

1.4 Metabolic activity of dormant and non-dormant chlamydospore

To determine the level of metabolic activity of dormant and non-dormant chlamydospore, FUN-1 fluorescent probe (Shanghai Yubo Biotechnology Co., LTD.) was used for staining. The chlamydospore suspension 20 μL was placed on a clean slide, and the prepared 10 μmol/L FUN-1 (containing 2% glucose) was added to the slide and stained for 30 min at room temperature, away from light. Firstly, the fluorescence intensity was examined under a fluorescence microscope (PWS-Scan-F, Shanghai Powerscin Biotechnology Co., LTD.), then the fluorescence intensity was excited under a confocal laser scanning microscope (Leica, Beijing Derica Biotechnology Co., LTD.) with a wavelength of 488 nm laser, and then with a wavelength of 488 nm and 633 nm laser at the same time. The fluorescence changes of the two types of spores were observed and photographed after the images at the two wavelengths were superimposed.

1.5 DAPI and CFW staining of dormant and non-dormant spores

The spore suspension to be tested was centrifuged 200 μL at 1500 rpm for 5 min, and the supernatant was discarded, and then centrifuged with PBS (pH7.2) twice, and the supernatant was discarded. The washed spores were fixed with 5% paraformaldehyde at 4 for 24 h, and then gently washed with PBS for 3 min. The spores were suspended in 10 mL DAPI dye working solution (1 μg/mL, Shanghai Jizhi Biochemical Technology Co., LTD) for 20 min. The stained samples were placed on slides, and the fluorescence was analyzed under confocal laser scanning microscope with maximum excitation wavelength of 340 nm and maximum emission wavelength of 488 nm. According to the instructions recommended by the reagent manufacturer (Wuhan Purity Biotechnology Co., LTD.), the CFW dye drops were added to the chlamydospore fixed with 5% paraformaldehyde, the fluorescence intensity was adjusted, and the staining of the cell wall was observed and photographed under a fluorescence microscope.

1.6 Electron microscope

According to the published method [29], the above dormant and non-dormant chlamydospores were coated on cellulose film respectively, and incubated at 28 for 12 h, then fixed overnight with 2.5% glutaraldehyde solution (special for electron microscopy, Beijing Mycorrhizal Biological Company), and then treated with PBS (0.01 M, pH7.4) briefly rinsed, alcohol gradient dehydration, tert-butanol vacuum drying. Samples on

the charged rubber were gold-plated fixed, and then were observed and photographed by using scanning electron microscope (SEM) (JEOLS-3400IV, Peabody, Massachusetts) under 15 KV voltage.

These dormant and non-dormant chlamydospores were fixed in 2.5% glutaraldehyde, then washed three times with PBS buffer (pH7.4), placed in 1% osmic acid for 2 h, and washed three times with PBS buffer. After gradient dehydration with 30%˜90% acetone at room temperature, the ultrathin sections were prepared by penetration embedding and polymerization at 60 for 36 h according to the conventional process of electron microscopy. The sections were treated with lead citrate staining solution for 10 min, and then stained with uranium acetate for 30 min. After washing and drying, JEM-1230 transmission electron microscope (TEM) was used to observe and photograph.

2 results

2.1 Observation and staining of different types of chlamydospores under general light microscope

Under an optical microscope, the dormant or non-dormant chlamydospore appears round or oval and yellowish brown in the absence of staining (Figure 1A & 1B), while the staining of artificially killed spores were dark (Figure 1C). The normal mature chlamydospore was double walled, spherical, 11.3˜35 μm in diameter, and oval in size 16.3˜27.5×27.5˜67.5 μm. The average spore wall thickness under oil microscope was 2±0.1 μm. The immature chlamydospore or the spore in liquid medium has a smooth surface and has globular protuberances on the surface when mature. The above three types of spores can be stained blue by lactol cotton orchid staining (Figure 1D, 1E & 1F). The artificially killed spores were pale and dark in color. The three types of spores stained with trypan blue were all black (Figure 1G, 1H & 1I), is difficult to distinguish. In MTT assay, dormant and non-dormant spores were stained black or blue, and no matter the spores died artificially or naturally, they were not stained (Figure 1 J, 1K, 1L & 1M). Their differences were significant, suggesting that this staining method could distinguish dead and dormant spores.

2.2 Comparison of metabolic activity and cell structure of different types of chlamydospores

FUN-1 staining showed that the dormant spores could only see green fluorescence after the simultaneous excitation of 488 nm and 633 nm laser (Figure 2A), while non-dormant spores showed orange fluorescence. Non-dormant spores can be superimposed under both fluorescence conditions, while dormant spores cannot be superimposed, suggesting that non-dormant spores have stronger metabolic activity than dormant spores (Figure 2B). DAPI staining results showed that the nuclei of resting spores were regular, round and dense, showing bright blue fluorescence (Figure 2C & 2D). Non-dormant spore nucleus materials emit weak fluorescence, showing blue circles. CFW staining showed that non-dormant spores could produce strong yellow-green fluorescence, while dormant showed weak yellow-green fluorescence (Figure 2E & 2F), indicating that there was a significant difference between the binding strength of cell walls and CFW dye.

2.3 Comparison of the ultrastructure of dormant and non-dormant chlamydospore

Under scanning electron microscope, the dormant spores did not germinate after 12 h of culture (Figure 3A), but the shape of spores were round and full. The non-dormant spores had germinated (Figure 3B), the germination tubes were extended, and the surface was locally deflated. There were nodules on the surface of both types of spores but no obvious differences. The transmission electron microscope (TEM) image shows the internal structure of the spore. The spore wall is composed of outer shell, outer membrane and cortex from the outside in (Figure 3C, 3D, 3E & 3F). The white transparent part of the spore wall, with certain electron transmittance, is the inner wall of the spore, or the cortex. Due to the high electron permeability, the inner wall of the dormant spore forms a thick white zona pellucida, which is significantly thicker than that of the inner wall of the non-dormant spore, and several times the difference can be observed. The cytoplasm of non-resting spores is full of many round, regular and small liposome contents, and there are also a few glycogen particles with low electron density. As the resting spores aging process, many cytoplasmic vesicles gradually fuse to form several large nearly spherical masses (possibly liposome spheres). The nuclei are crowded to the near edge, and the nuclei are lobulated. There are still some glycogen-like particles with

low electron density in the edge of the cytoplasm.

3 Discussion

D. flagrans is a new species first reported by microbiologist Duddington in 1948 (Duddington, 1948), which was then recorded as a spherical structure with a diameter of 24-30 μm [1]. There are also elliptic ones with a size of 15˜29×28˜59 μm, which is consistent with the observed results in our study. Because of its thick wall, chlamydospores are thought to be more resistant to adverse environmental conditions than zoospores and ascospores. In some species of *Phytophthora* , lipid vacuoles and dense inclusion bodies similar those found in other resistant spores are present in aged chlamydospores [30]. It has been reported that 3 to 10 chlamyspores of *Phytophthora cinnamomi* were formed at the apex or interstream of mycelium during in vitro culture. They are formed by the expansion of the mycelium wall and its protoplasm into the expanded structure [31]. Candida albicans and Candida Dublin chlamydospores generally forms four to five beaded structures. In this study, the formation of *D. flagrans* chlamydospore is similar that of other fungi mentioned above [24].

The chlamydospore of fungi is considered to be an important survival structure and a major source of inoculation. *D. flagrans*chlamydospore is not only important in the survival cycle, but also an active component of biocontrol preparations and a component of strain preservation. Due to the widespread structural dormancy of the chlamydospore, the chlamydospore will become dormant during the preparation process. This situation precludes the use of the method of counting yield and quality by viable tests during product testing in the preparation of this bacterium, as spores cannot germinate on their normal medium within a short time after structural dormancy is formed. Therefore, in order to find a simple and effective method for detecting the product quality, various tests were carried out to prove the structural differences between resting chlamydospore and dead spore under the optical microscope. The results showed that the observation without staining under the optical microscope could not distinguish the resting spore from the dead spore. The appearance of artificially killed spores is rather dull, but it is difficult to distinguish it with natural dead spores in practice by this phenomenon alone, because the spores have thick walls and normal internal structure cannot be observed by optical microscopy. Unexpectedly, the cell wall of chlamydospore remained intact after high temperature and high pressure, and the spore remained undisturbed and could not be distinguished from live spores. Although the artificial lethal spores showed light and dark color by the cotton orchid staining of the fungus staining solution, it was proved that the dormant, non-dormant and dead spores could not be distinguished in practice. Trypan blue dye is often used to stain living cells. This method is used to detect the integrity of cell membranes and determine whether the cells are alive or not. Because the living cell membrane is complete, the dye solution cannot enter the cell, while the dead cell membrane is incomplete and the dye solution can enter the cell. However, in our study, no matter dormant spores, non-dormant spores or dead spores could be stained black by trypan blue, which made it impossible to distinguish between dead and alive spores, possibly because the permeability of fungal cell wall was different from that of animal cell membrane. MTT dye can act on mitochondria of succinic dehydrogenase reduction into blue-purple crystals deposited in cells, dead cells because of the absence of this enzyme, cannot combine with MTT dye into blue-purple crystals. In our study, MTT staining was used to incubate spores at 27˜30 for 24 h, which could distinguish live spores (including dormant spores) from natural dead spores, and artificial death was not stained. Our method is simple and reliable, and can be used to detect and count the live chlamydospore.

FUN-1 is a specific fluorescent dye for fungal metabolic activity. In order to detect the metabolic activity and vitality of chlamydospore, the resting and non-resting spores were used as experimental materials in this study. The molecular probe FUN-1 was used to detect the metabolic activity of these spores. The results showed that the non-resting spores had high metabolic activity, and the resting spores had no orange fluorescence, indicating that the metabolic activity was very weak. Studies on Candida albicans and Candida Dublin showed that chlamydospore of 5 days of culture had high metabolic activity detected by this probe. After 5 days of culture, the metabolic activity would be weakened under the condition of nutrient deficiency, and the metabolic activity could not be detected completely after 30 days of incubation. Without adding

a new medium, the spores could not recover activity after 30 days, indicating that the spores had died [24]. In our study, the non-dormant spores were cultured at the age of 25 days, which still showed strong metabolic activity compared with the spores of Candida albicans, while the dormant spores showed very weak metabolic activity but did not die, which was inconsistent with the results of the above study. The reason may be that Candida albicans is a parasitic fungus in human body rather than in nature. Its spores have a shorter life span than *D. flagrans* spores. If *D. flagrans* dormant spore is subjected to a period of cold or moderate temperature and humidity, or accompanied by a special phase of external stimulation that induces increased metabolic activity, it will re-germinate and begin a new life cycle [15].

DAPI is a fluorescent dye that binds strongly to DNA and can adhere to the sulci region of the DNA double helix. The excitation and emission wavelengths of the DAPi-DNA complex are 360 nm and 460 nm, respectively, and because DAPI can pass through intact cell membranes, it can be used for staining both living and stationary cells. DAPI has a high photo-bleach tolerance level and can be used to detect chloroplast DNA, yeast mitochondrial DNA, viral DNA and chromosome DNA. In this study, DAPI staining results showed that resting spores had stronger blue fluorescence than non-resting spores, which might be related to the DNA density of chromatin in the nuclei of resting spores. CFW can bind to cellulose and chitin of fungal cell wall, and light green fluorescence can be observed under fluorescence microscope. The results of our study showed that the non-dormant spores were stained with light green and had stronger fluorescence, while the dormant spores had weaker fluorescence, which might be because the non-dormant spores had more contents of chitin and cellulose than the dormant spores. It was proved that the types and proportion of cell wall components and their binding forms were different between the two types of spores, leading to their different permeability [14].

The ultrastructure of different types of *D. flagrans*chlamydospore in our study has not been reported so far. Some studies on the ultrastructure of the spores of two species of *M. oryzae* by scanning electron microscopy showed that the diameter of the chlamydospore of *M. oryzae* was 3˜5 μm, and the length of the spore was 200˜500 nm [25]. The surface of the dormant spore (black spore) had protuberances. The surface of non-dormant spore (yellow spore) has protuberances, the height of the former is 458˜1130 nm, the latter is 288˜721 nm, there is a significant difference between the two. In our study, *D. flagrans* chlamydospore also had protuberances on its surface, and there was no significant difference between dormant and non-dormant spores. In addition, the spores with protuberances are generally found on solid medium, and with the aging of the verruca has a tendency to increase, in liquid medium culture has not been found to have protuberances. Under transmission electron microscopy, Yan *et al.* found that the yellow chlamydospore had a thin wall, while the black chlamydospore had a thick wall, especially the inner wall, with a wide white band with high electron transmittance [25], and this finding is consistent with our results on *D. flagrans* chlamydospore. Under transmission electron microscope, the yellow chlamydospore (non-resting spore) had complete nucleus, distinct nucleoli and nuclear membrane, and some starch granules with different shapes, while the black chlamydospore had no complete cell structure, and the cytoplasm was filled with a large lipid sphere. However, no obvious nucleolus or nuclear membrane was seen in *D. flagrans*chlamydospore under TEM. Perhaps the nondormant chlamydospore we collected was so old that the liposomes filled the entire cytoplasm and covered other cellular structures, and this needs further study. Interestingly, we found that the cytoplasm of the non-dormant chlamydospore was filled with small circular liposome contents, and the liposome particles in the dormant chlamydospore further fused and became larger and less in number, which was similar to the phenomenon observed in the black chlamydospore.

The morphological and structural observation of different types of chlamydospore in this study showed that there were differences in the structure and morphology between resting and non-resting spores, which were not only reflected in the difference of staining under the optical microscope, but also, more importantly, reflected in the difference of ultrastructure. In this study, we found a staining method that can distinguish dead and dormant spores, which provides a useful strategy for determining spore survival in future quality detection and other studies. As for the differences in the ultrastructure of the two types of spores, some phenomena are still difficult to explain due to our incomplete work and limited knowledge. From the microscopic level, it may be reflected in the differences in gene expression and the chemical composition of

spores, which need to be further studied in the future.

The dormant spore in *D. flagrans* is not a "dead" state, as was previously thought, but a living structure in nature. What role do they play in the history of life under natural conditions remains unclear. The dormancy and germination of chlamydospore is a complex and mysterious phenomenon, which probably requires some complex signaling and regulatory pathways and still needs further study. In this study, it was shown that after the structural dormancy of the spores, the material state or cytoplasm of the cells changed, which led to the structural changes. This study has certain scientific significance for the study of dormancy mechanism, and the results also provide certain theoretical support for the application of chlamydospore in the production and application of biocontrol preparations.

Funding

This work was supported by the Shaanxi University Science and Technology Association youth Lifting project (20220217), Doctoral Research Project of Yan'an University (YAU202212288 and YDBK2021-07), Scientific Research Program Funded by Education Department of Shaanxi Provincial Government (Program No. 22JK0613), the Yan'an City Key Science and Technology Research Development Planning Project (No.2020ZCSY-005), and the Scientific Research Project of Shaanxi Provincial Department of Education (No.20JG041).

Compliance with ethical standards

Conflict of interest statement The authors declare that they have no financial or commercial conflicts of interest.

Ethics statement This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure 1 Light microscope observation of different types of chlamydospores. A. Dormant-spores; B. Non-dormant; C. Dead spores; D. Dormant spores stained with lactophenol cotton blue; E. Non-dormant stained with lactophenol cotton blue; F. Dead spores stained with lactophenol cotton blue; G. Dormant spores stained with trypan blue; H. Non-dormant stained with trypan blue; I. Dead spores stained with trypan blue; J. Dormant spores stained with MTT; K. Non-dormant stained with MTT; L. Naturally dead spores stained with MTT; M. Artificially dead spores stained with MTT.

Figure 2 Observation on metabolic activity, cell nucleus and cell wall of chlamydospores with fluorescent dye. A. Dormant spores stained with FUN-1; B. Non-dormant spores stained with FUN-1; C. Dormant spores stained with DAPI; D. Non-dormant spores stained with DAPI; E. Dormant spores stained with CFW; F. Non-dormant spores stained with CFW.

Figure 3 Electron microscopic images of dormant and non-dormant chlamydospores. A. Dormant spores under SEM; B. Non-dormant sporesunder SEM; C. Non-dormant spores under TEM; D. Non-dormant spores under TEM (showing spore walls and cytoplasmic vesicles); E. Dormant spores under TEM; F. Dormant spores under TEM (showing the spore wall and a part in the spore).

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