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The technique for visualization of hyphal interaction zones of *Botrytis cinerea* monoconidial isolates

Technika wizualizacji stref interakcji strzępek grzybni monokonidialnych izolatów *Botrytis cinerea*

SUMMARY

The visualization technique of hyphal interaction zones of *Botrytis cinerea* monoconidial isolates is described. The isolates were collected from the natural environment. The 10 isolates were obtained as pure cultures: 8 isolates were taken from a grape host, 2 were taken from a sunflower host. Splicing was carried out on glass slides. The specimens were stained with Safranin O. Transparent lacquer was applied onto the perimeter of the slides for prolongation of the temporary storage of the specimens. The main morphological filamentous components, such as cell walls of hyphae, septae (intercellular interstices), nuclei, microconidia, anastomoses and other structures (interlacements and non-unions) were clearly visualized by the light microscope.

Keywords: *Botrytis cinerea,* filamentous fungi, heterocaryosis, monoconidial isolates, vegetative incompatibility, Safranin O

STRESZCZENIE

W pracy opisano technikę wizualizacji stref interakcji strzępek grzybni monokonidialnych izolatów *Botritis cinerea.* Izolaty pobrano ze środowiska naturalnego. Otrzymano10 izolatów w postaci czystych kultur: 8 izolatów pobrano z winorośli, 2 pochodziły ze słonecznika. Przebieg interakcji obserwowano na szkiełkach. Próbki barwiono safraniną O. W celu przedłużenia czasu przechowywania preparatów na obwodzie szkiełek położono przezroczysty lakier. Morfologiczne elementy strzępek, takie jak ściany komórkowe, przegrody, jądra, mikrokonidia, anastomozy i inne struktury były wyraźnie widoczne w mikroskopie świetlnym.

Słowa kluczowe: *Botritis cinerea*, grzyby nitkowate, heterocaryosis, izolaty monokonidialne, wegetatywna niezgodność, safranina O

INTRODUCTION

The production of hyphal anastomoses is a general property of filamentous fungi, it means we observe fusion of mycelium cells of one and the same species in case of physical contact. Hyphal anastomoses play an important role in the process of adaptation of fungi to the environmental conditions. If fungal hyphae contain genetically different nuclei it can form heterocaryons (6). The next mitoses in fungal cells support the proliferation of different allelic states of separate genes in the heterocaryon. Thus, heterocaryosis replaces diploidisity in fungi with haploid nuclei, while in the heterocaryotic cell the same allelic relations (dominant, recessive, codominant) are expressed phenotypically, which is characterystic of heterozygotic cells too (4).

Anastomoses that occur between the hyphae with subsequent nuclei migration, result in disappearance of intrapopulation units – individuals and they provide their integration into a uniform network of a mycelium that results in their higher adaptive abilities (3).

In many species of filamentous fungi, heterocaryosis is supported by rare actions of nucleus fusions in vegetative cells and the formation of heterozygotic diploids. If that diploidic nucleus gets into spore then diploidic clone is formed. We can observe the genetic recombination (parasexual process) based on such nuclear processes as haploidization, nondisjunction of chromosomes during mitosis, mitotic crossing-over in nuclei of such clones (4). However, researches on a great number of strains collected from nature suggest that the majority of isolates in paired crops do not form heterocaryons because of vegetative incompatibility (19). For the first time, this phenomenon was revealed during population research of modelling saprotrophical fungi – *Aspergillus nidulans* (12), *Podospora anserine* (2) – (15), and then, the phenomenon of vegetative incompatibility was described while investigating many phytopathogenic mushrooms including *Botrytis cinerea* (8, 16). As the vegetative incompatibility that blocks the formation of heterocaryons as well as the adaptive benefits resulting from it, the presence of strong natural selection favoring incompatibility has forced experts in the field of mycology and population genetics to pay undivided attention, both to the phenomenon itself and to its evolutionary consequences (6, 13).

The subject of vegetative incompatibility is extremely interesting and insufficiently known by domestic geneticists (5). Detaching groups of vegetative incompatibility is rather actually in researches of population genetics of fungi and *B. cinerea*, in particular. This species is a serious and widespread pathogen of many agricultural crops (20), and studying biological features of *B. cinerea* is useful for understanding the process of circulation of genetic material of phytopathogenic fungi (10). Furthermore, vegetative incompatibility is interesting as it is an original isolating mechanism of speciation which can be observed everywhere. The vegetative incompatibility has difficult genetic regulation and has extremely important population and evolutionary value because, according to some researches, it serves as a mechanism of protection from independently-replicated cytoplasmatic elements (14).

Nowadays, there are many techniques for the investigation of vegetative incompatibility: visual examination, microscopy of borderline zones, genetic control by using auxotrophic, pigmental and fungicide-resistant mutations, using microinjection and fusion of protoplasts, and sex crossing (6). But none of them is simultaneously simple, rapid and informative enough.

At present, the techniques of studying filamentous interactions are still insufficiently developed. Thus, the purpose of the study is to work out a technique of visualization of the interaction zones of two isolates of *B. cinerea* that would allow us to analyze their filamentous interactions at the cytological level.

MATERIALS AND METHODS

The technique description

First we detached monoconidial isolates of *B. cinerea* in Petri dishes on a thin layer (1–1.5 sm) of agar nutrient medium.

For preparation of the initial material, i.e. the isolates of *B. cinerea*, we applied a method of pure cultures (9). The isolates of *B. cinerea* were detached from a plant material with obvious signs of development of grey mould. The identification of fungi of the genus *Botrytis* detached from sunflowers and grapes was carried out by the use of a determinant of phytopathogenic fungi (17). With the help of a sterilized needle conidium samples, defined as *B. cinerea*, were transferred to the sterile Petri dishes containing 7–10 ml of the stiffened potato-glucosic nutrient medium (by Shcherbakov) (9).

Composition of nutrient medium:

Potato……..20 gr.

Glucose……...10 gr.

Agar……..2–2.5 gr.

Water……...100 gr.

The monoconidial cultures of a mycelium were detached for maintenance of relative homogeneity of isolates. Looking through a binocular some conidia located on one conidiophore were transferred from Petri dishes to test tubes containing the nutrient medium (preliminary cooled to 47–55° C) by a thin needle. After that, the test tubes were scrolled between palms – to provide splitting the conidia head up into separate conidia. The contents of the test tubes were poured in a sterile Petri dish. The sprouting mycelium was, thus, a derivative of one conidia and it was homogeneous according to genomic characteristics, i.e. it was genetically similar in all the cells. The thickness of a layer of a firm nutrient medium in a Petri dish was not to exceed 1.5 mm. The homogeneous isolates were incubated in Petri dishes during 7 days at the temperature 23–24° C.

Next, agar blades with a mycelium of the corresponding isolates were picked out by using a sterile metal tube (d=5 mm) and placed in pairs on slides in sterile damp chambers at a distance of 4–5 mm, with the mycelium upwards.

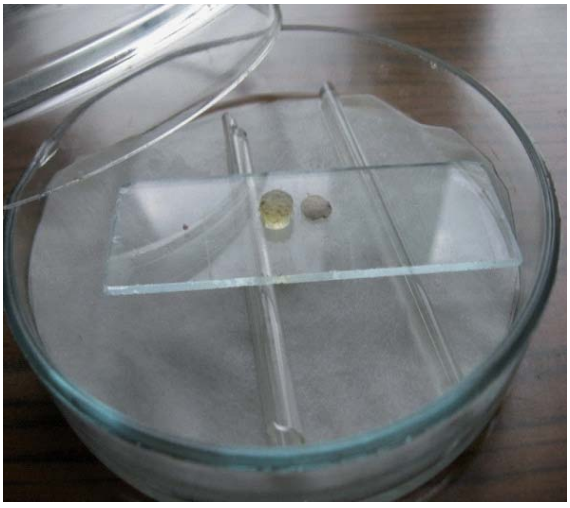

Fig. 1. Moist chamber

The sterile damp chambers were Petri dishes with a double layer of filtering paper at the bottom on which there were slides pressed against glass sticks (Fig. 1). The glass sticks prevented direct contact of glasses with the filtering paper. The filtering paper was moistened with distilled water (2 ml per cup), for maintaining conditions of increased humidity which are necessary for intensive growth of *B. cinerea* (7).

The third step involved staining specimens with the dye Safranin O. Safranin O had been chosen as it is one of the fastest (2–7 min.) and the most intensive dyes that can stain fungal nuclei, walls and entocyte.

The composition of solution (1):

Distilled water……..79 ml
0.5% Safranin O in distillate…… 6 ml
3.0% the KOH in distillate……10 ml
Glycerin……5 ml

Before staining, the interaction zones of two mycelia, agar blades were preliminary removed from slide. A thin layer of Safranin O solution was poured on the hyphae remaining on the slide. After 4–7 minutes of staining the specimens were covered with the cover slip. For temporary storage of the specimens, a thin layer of transparent varnish was put at the edge of the cover slips.

The fourth and the last stage included observation of the received specimens in a microscope and the analysis of the visible structures – nuclei, septae, anastomoses, micro- and macroconidia. We used the light microscope MBI-3 with resolution limit X 900 (40x15x1.5). Pictures of the interaction zones of isolates were made by means of Canon A530 camera.

RESULTS AND DISCUSSION

During the approbation of the consecutive four stages of technique for visualization of interaction zones of *B. cinerea* isolates, 16 specimens of temporary storage were created. They were isolates interacting in pairs, detached from two kinds of the host plants – grapes and sunflower. In total, 10 monoconidial isolates of *B. cinerea* were detached for temporary storage. Eight isolates were detached from grapes, and two of them were derived from sunflowers. The monoconidial isolates from two kinds of host plants were paired. In the process of merging isolates in pairs we received 16 slides with the sites of interacting hyphae (Fig. 2).

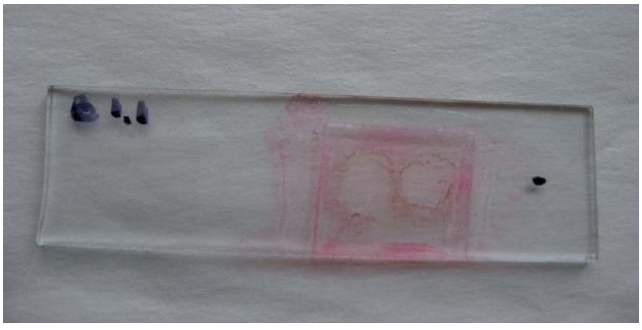

Fig. 2. The stained specimen

The specimens were analyzed taking into account the degree of staining of the basic morphological structures that are important for cytological definition of different types of hyphae interaction, namely: cellular walls, septum, and nuclei. As a result, after 5–7 min. of staining, on 16 received specimens the basic morphological filamentous components were clearly visualized. They were hyphal walls, septae (intercellular partitions), nuclei, microconidia, anastomoses and not anastomoses (other interactions – interlacings, nonunions). It is possible to hold that the technique approbation has passed successfully; the received specimens can be exposed to a further cytological analysis.

Besides visual registration of the reaction of vegetative incompatibility based on the characteristic morphological structures in the accretion zone of isolates, the important stage in studying the types of interaction of fungal mycelia is the amount of cytological transformations in a contact zone. The criteria confirming the type of interaction are cytological and morphological transformations of the basic mycelium structures (hyphae, conidiophores, formation of conidia). The technique was developed taking into account peculiarities of vegetative mycelium growth of *B. cinerea* isolates.

An important condition for studying the type of interaction of the hyphae isolates, collected from the nature, is the maintenance of relative homogeneity of the isolates, i.e. cultivation of monoconidial isolates. The homogeneity of isolates assumes the uniformity of mycelium properties in its different sites that allows to extrapolate the obtained cytological data concerning interaction character of hyphae of a specific site of the mycelium (d=5 mm) to the properties of the whole mycelium of the investigated isolates.

It should be noted that the radius of growth of a hyphae on the periphery of an agar blade is limited. Therefore, the distance between two blades on the slide is an important parameter. For calculation of optimal disposition of agar blades in relation to each other, the intensity of isolate growth on the surface of glasses has been estimated (Tab. 1).

Tab. 1. Index of the radius of mycelium overgrowth on the surface of slide glasses; min – minimal radius, max – maximal radius, med – medium radius; B, ZK – the shorthand of isolates

No. Petri dish	Isolates	Description	R, мм
1	ZK 1	intense Floccus	min 2, max 4
	B 1.1	weak Floccus	min 1, max 2
2	ZK 1	intense Floccus	min 2, max 5
	B 2.1	weak Floccus	min 2, max 4

3	ZK 1	intense Floccus, sporiferous	med 3
	B 3.1	medium Floccus, sporiferous	med 4
4	ZK 1	intense Floccus	med 3
	B 4.1	medium Floccus	med 4
5	ZK 2	medium Floccus	med 4
	B 1.1	weak Floccus	med 3
6	ZK 2	intense Floccus, sporiferous	med 6
	B 2.1	weak Floccus	med 1
7	ZK 2	intense Floccus, sporiferous	med 5
	B 3.1	intense Floccus	med 4
8	ZK 2	intense Floccus, sporiferous	med 3
	B 4.1	weak Floccus	med 2

Thus it is shown that the optimum distance between the blades is 5 mm, and the thickness of an agar layer in a Petri dish should vary within 1–1.5 mm. The given parameters of an agar layer are an optimum for the growth of filamentous structures on the glass (Fig. 3).

Fig. 3. Radius measurement of overgrowth of mycelium on the surface of slide glasses

During the preparation of the materials for staining, special attention should be paid to the removal of agar disks from the surface of glass. This step should be

conducted very carefully to avoid damage of the thin layer of interacting hyphae of both isolates because in this case preservation of the natural structure of hyphae location of the investigated isolates is important.

The use of Safranin O as a dye was justified as it is quite simple in preparation and is characterized by high-speed. Safranin O stains the genetic material of fungal hyphae and the cytoplasm most intensively, hyphae partitions become distinctly visible too, i.e. septum of mycelium is visualized. This is quite important for observation of hyphae anastomoses in vegetative compatible strains. Hence, the cytological and morphological characteristics of the material can be analyzed. The technique is suitable both for defining the character of hyphae interaction of different isolates, and for the description of morphological features of vegetative growth of *B. cinerea*.

The main advantage of the technique is the possibility to maintain the natural structure of interacting hyphae for further cytological analysis. Hyphae interaction between different isolates occurs directly on the slides, during the growth of hyphae on the perimeter of an agar disk caused by the temperature, humidity level in chambers and presence of a primary substratum (directly agar disk).

We also examined microconidia in the specimens in a microscope. These structures may function as spermatia in the sexual process of *B. cinerea* (11) (Fig. 4).

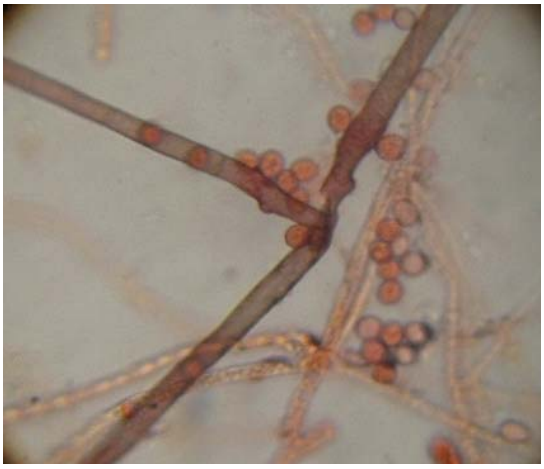

Fig. 4. Reaction of hyphae interaction against the background of microconidium

Advantages of the method:

– maintenance of homocaryonations of the investigated isolates, for maintaining standardization of the features of vegetative growth;

– convenience while receiving interaction zones which are in the focus of observation directly on slides;

– fast and simple staining of the main morphological structures with Safranin O;

– preparation of temporary storage specimens for the analysis of quantitative parameters of vegetative growth (quantity of conidia, anastomoses, nuclei).

Disadvantages of the method:

– maximum control over sterility at each stage;

– high price of Safranin O;

– unfixed time for preparation of the specimens which depends on specific features of the growth of isolates on slides.

The proposed technique for visualization of interaction zones between monoconidial isolates of *B. cinerea* allows to obtain and analyze the data about the processes proceeding in interacting hyphae at the cytological level. It is easy to apply, in contrast to other methods (allocation of groups of incompatibility with the help of auxotrophic or pigmentary mutants), because the preparatory stage, i.e. direct deducing and selecting of the initial material (mutants), does not demand essential expenses (18). The method of simple visual fixing of reactions of incompatibility does not allow to divide interaction reactions into such groups as semiincompatibility and semicompatibility (16).

The proposed methodical work can be used in courses of laboratory research on studying vegetative incompatibility and can be used for further research of other species of filamentous fungi.

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