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Artigo

MICROCHEMICAL TECHNIQUES ON *BOTRYTIS CINEREA*: CONTRIBUTION TO IDENTIFICATION OF PRIMARY AND SECONDARY METABOLITES

TÉCNICAS MICROQUÍMICAS EM *BOTRYTIS CINEREA*: CONTRIBUIÇÃO PARA A IDENTIFICAÇÃO DE METABOLITOS PRIMÁRIOS E SECUNDÁRIOS

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ABSTRACT: Histochemical techniques comprise several staining methods in microscopy for investigation of chemical groups or pathologies regarding tissues in plants and fungi and have been widely used for detection of groups of substances. Thereby, our work demonstrates the application of histochemical techniques recognized for detection of secondary metabolites in plants to detect substances in the fungus *Botrytis cinerea*. Our findings are compatible with previous reports on metabolites identified in *B. cinerea*, and show proteins stored in vesicular structures (0.3-0.9µm), accumulated in small groups inside of developed mycelia, and in large quantity in spores (0.2-0.7µm). Alkaloids were only detected in mycelium containing spores, not influenced by their size. Terpenoids were detected in spores, and with lighter color for mycelia vesicles. However, in younger mycelium we verified terpenoids in storage structures in the mycelia segments, demonstrating higher quantity in early development. The presence of neutral polysaccharides and terpenoids was observed around the mycelia containing younger spores and, in some storage, structures close to the spores. Our results corroborate reports on the presence of substances of the secondary metabolism in *B. cinerea* and could be utilized in other species of fungi to screen substances of pharmacological and nutraceutical interest.

KEYWORDS: Histoquímica, Metabólitos Secundários, Alcaloides, Micélio, *Botrytis cinerea*.

RESUMO: As técnicas histoquímicas compreendem vários métodos de coloração em microscopia para investigação de grupos químicos ou patologias em relação a tecidos em plantas e fungos e têm sido amplamente utilizados para a detecção de grupos de substâncias. Assim, nosso trabalho demonstra a aplicação de técnicas histoquímicas reconhecidas para detecção

de metabólitos secundários em plantas para detectar substâncias no fungo *Botrytis cinerea*. Nossos achados são compatíveis com relatórios anteriores sobre metabólitos identificados em *B. cinerea*, e mostram proteínas armazenadas em estruturas vesiculares (0,3-0,9µm), acumuladas em pequenos grupos dentro de micélios desenvolvidos, e em grande quantidade em esporos (0,2-0,7µm). Alcaloides foram detectados apenas em micélio contendo esporos, não influenciados por seu tamanho. Terpenoides foram detectados em esporos, e com cor mais clara para vesículas micelianas. No entanto, no micélio mais jovem, verificamos terpenoides em estruturas de armazenamento nos segmentos miceliais, demonstrando maior quantidade no início do desenvolvimento. A presença de polissacarídeos neutros e terpenoides foi observada em torno da micela contendo esporos mais jovens e, em algum armazenamento, estruturas próximas aos esporos. Nossos resultados corroboram relatórios sobre a presença de substâncias do metabolismo secundário em *B. cinerea* e podem ser utilizados em outras espécies de fungos para rastrear substâncias de interesse farmacológico e nutracêutico.

PALAVRAS-CHAVE: Histoquímica, Metabólitos Secundários, Alcaloides, Micélio, *Botrytis cinerea*.

1. Introduction

The genus *Botrytis* is highly diversified, with species standing out for differences regarding characteristics of biology, ecology, morphology and host [1]. One of the most studied species in the areas of pathology, chemistry and pharmacy is *B. cinerea*, for presenting a high number of substances of different metabolic classes. Furthermore, this species causes the gray mold, a disease affecting fruits, capable to kill cells of the hosts due to toxins through increased levels of reactive oxygen species [2].

Among the secondary metabolites produced by *B. cinerea*, two classes of non-selective phytotoxins stand out: one belonging to the class of sesquiterpenes, with basic chemical structures, known as botrydians, mainly

botrydial and dibotrydial, and another class belonging to the polyketides, represented by botcinolides (botcinic acid and botcinin A) and botrylactones [3-8].

Histochemistry applied to microchemical tests has the objective to pinpoint *in situ* the main chemical groups occurring in tissues, associating the histology to a chemical aspect and determining the nature of substances present and their location in tissues. In all applied methods, it is necessary to avoid the artificial displacement of the substance to be characterized during the treatments preceding the histochemical reaction, detected under the microscope by specific stain, or by radiation emission, whereby the determinations are only qualitative [9].

Furthermore, techniques for the detection of lipids and acid phosphatases were already utilized on mycelium of *B. cinerea* [10], demonstrating the feasibility of the adaptation of techniques utilized in plants or tissues, aiming the detection of substances from primary and secondary metabolism. Based on this premise, our objective was to determine by qualitative methods, the presence of some classes of substances derived from the metabolism of *B. cinerea*, as a contribution to the localization of substances present in this species.

2. Materials and Methods

2.1 Material

The isolate of *B. cinerea* was obtained from saplings of *Pinus taeda* L, deposited in the Collection of Forest Phytopathogenic Fungi of the Brazilian forest research agency (Embrapa Florestas), identified as *Botrytis cinerea* Pers. *ex* Pers. (= *Botryotinia fuckeliana* (de Bary) Whetzel) by sequence analysis, deposited in the GenBank (KJ476441).

2.2 Culture of *B. cinerea* for Morphological Study

For the evaluation of the morphology and histochemical tests, the fungus was pricked out and cultivated in Petri dishes, containing medium of potato dextrose agar - PDA (KASVI®) and kept in BOD chamber at temperature of 22°C, in the dark [11], until a complete production of hypha, mycelium and conidium.

2.3 Preparation of Slides for Microchemical Analysis

Fragments of mycelium and conidia were enclosed in histological slides and submitted to chemical treatment according to the protocols described by Figueredo *et al*. [9]. The assay for detection of proteins was based on the protocol of Bradford [12]. The Bradford reagent was added to the material during 3min. Next, the slide was washed with solution of acetic acid 7% for 10 min, followed by washing with ultra purified water. As positive control, we utilized a filter paper disc impregnated with bovine albumin (SIGMA®), followed by Bradford reagent, to verify the blue color. In the negative control, the material was treated with solution of acetic anhydride 10% in solution of pyridine during 4h, and then the material was washed with distilled water and stained with Coomassie Brilliant Blue dye.

The detection of alkaloids was performed following two protocols. The first utilized the Dragendorff reagent [13], considered effective in the detection of alkaloids. Nonetheless, because fungi generally contain nitrogenate substances in some peptide bonds, we applied a protocol complement with Wagner reagent [14], to confirm the presence of alkaloid or of an artefact arisen from the reaction [15]. In this assay, the Dragendorff reagent was applied on the material and next, a drop of solution of sodium nitrate at 5%, during 5min, then the material was washed three times with ultra purified water. In the negative control, the material was treated with

tartaric acid 5% during 48h. Thereafter, the slide was washed with water, and the absence of color was observed. The Wagner reagent was applied on the slide with material during 10min. Next, the material was washed with ultra purified water and covered with a cover glass. Some negative control steps described for the Dragendorff reagent were utilized for this procedure.

Lipids were investigated by the visible light method. The slide material was treated with two drops of Sudan IV dye, for a period of 5 to 15min [16]. Next, the slide was washed with ethanol 70%, to remove excess of dye. In the negative control, the material was treated with solution of chloroform, hydrochloric acid and methanol (4:33:66 v/v), for one hour. Hereupon, the Sudan VI reagent was applied and after 15min washed and the absence of color was observed.

In the assay for detection of neutral polysaccharides, the material was treated with Schiff's reagent, in the dark, for 45min. Then, the slide was washed three times with ultra purified water. In the negative control, the material was treated with solution of periodic acid at 1%, during 30min, followed by washing with ultra purified water, then the Schiff's reagent was applied and kept in the dark for 45min, and washed with ultra purified water [17].

In the assay for detection of terpenoids, we utilized the method of 2,4-dinitrofenilhydrazine [18]. Thereby, three drops of reagent were applied on the slide containing material, during 10 min, and then the material was washed. In the negative control, the material received a solution of sodium tetra hydroborate at 1% during 10min. Thereafter, the material was washed three times with ultra purified water, and we applied the reagent 2.4-dinitrofenilhydrazine.

After application and washing of each specific reagent, each slide received a glycerin solution at 1% and cover glass for observation under optic microscope at 4x, 10x, 40x and 100x magnification. All assays of confirmation tests were run in triplicate.

After the observation of color, the materials were photographed under photonic microscope (BX40, Olympus®), coupled with a photographic camera (BX40, Olympus, Japan), at 4x, 10x, 20x and 40x magnification. The observed structures were measured utilizing Software ImageJ®. After the acquisition and analysis of images, they were treated with gaussian pre-filtering and color inversion [19-21], aiming to obtain the best image quality for visualization of spots with possible chemical groupments and of the structures present.

3. Results

3.1 Microscopic Aspects of *B. cinerea*

The analysis of structures on images acquired by treatment with filtering of Gaussian blur and the contrast demonstrate that, beside the known morphology and septa present in the mycelium (figs 1A, 1B, 1C), there are small structures of rounded shape measuring between 3 and 9μm, here described as vesicles and storage bodies with irregular shape, containing storage substances. The vesicles are covered by a thick membrane, varying between 2 and 4μm diameter (figs 1D, 1E, 1F). The storage bodies beside their irregular shape, are larger, with 3 to 6 μm diameter, containing more storage substances (fig 1 B). The size of the conidia varied between 2 and 7μm (fig 2A), with a thick membrane cover, in some cases being a layer of dense material located between this membrane and the internal constitution of the conidium, with shape of small purses which may have storage function (fig 2B). The presence of storage bodies is also observed inside the conidia, with rounded shape measuring 2-7μm, covered by a myelin membrane (figs 2A, 2B).

3.2 Microchemical Analysis

The microchemical tests revealed a wide diversity of chemical groups from primary and secondary metabolisms. The presence of proteins was detected in vesicles of the mycelium and conidia, with intensification of color where the storage bodies are found, being possible to distinguish small granules of irregular shape dispersed inside (figs 3A and 3C). The images acquired after treatment demonstrated spots with intense color in the center of the vesicles of the mycelium, involved by a sheath like the myelin sheath (figs 3B and 3D).

Alkaloids were found in the cell wall covering the mycelium and conidia (figs 4A, 4B, 4C and 4D), being positive for both tested reagents. The presence of lipids can be visualized by the color intensity inside young mycelia, where substances of lipid character completely fill the mycelium, being intercalated by septa (figs 5A and 5B), and in conidiophores inside the vesicles, being possible to visualize the presence of lipids in the conidia, mostly placed in their center (figs 5C, 5D, 5E and 5F). Neutral polysaccharides were visualized as constituents of the cell wall of the most developed mycelium and conidia (Figs 6A, 6B and 6C). And in some mycelia containing vesicles of smaller size (1.0-2.0μm) it was possible to visualize in the conidiophore, the presence of polysaccharides in storage bodies with irregular shape inside the hypha (fig 6C). These polysaccharides are just limited to the conidia wall and were not found in the storage bodies. Similarly, to the observed for polysaccharides, the terpenoids were detected in the wall of hyphae and conidia, with some storage structures in more developed mycelium (figs 6D, 6E and 6F).

4. Discussion

The observations on the morphology of *B. cinerea* obtained under

photonic microscope do not allow to detail the storage structures, and respective chemical groups present. Our studies can notably help the characterization and isolation of chemical groups present in this species, also helping in the qualification of the fungal mass in biotechnological research with emphasis in production of metabolites.

The description presented in our study, as well as the presence of storage structures, allow a better understanding on the production and storage of substances from the primary and secondary metabolism. The presence of dense cell wall and of inclusions called storage bodies in both mycelium and conidia, plus spherical structures, granules and inclusions present in intact hyphae were reported only in hyphae treated with uranyl acetate and submitted to transmition electron microscopy [10, 22].

The presence of such inclusions and dense walls in the spores were also observed by Pârvu *et al*. [23], who described the dense cell wall covering the spores, and the presence of lipid granules, and our results corroborate this, as demonstrated by the microchemical tests for *B. cinerea*. Elad *et al*. [1] and Weber *et al*. [10] reported that a striking characteristic of the spores of *B. cinerea* is that they resemble the inclusions of animal and plant cells, for containing a great quantity of lipids and proteins, which function is the vacuolization during the spore germination.

Under normal conditions, the size and number of protein bodies increase over the development of sclerotia in fungi, particularly when fully developed [24]. Under a transmission electron microscope, the protein bodies are round or elongated, bound to the membrane and are completely full of materials moderately dense in electrons [25].

The endoplasmic reticulum, in form of long and parallel bodies being formed, suggests its possible role in protein synthesis, as occurs in other animal and plant systems [26]. Sclerodia of *Paxillus involutus* (Batsch) Fr. [27] and *Typhula incarnata* (Lasch) Fr. [25] were observed containing polyphosphates embedded in the matrix of the protein bodies. Such mature

sclerodia and protein bodies are the major reserve of cytoplasmic storage [10]. González et al. [28] identified the presence of 158 *O*-glycosylate proteins in *B. cinerea*, and that the glycosylation of these proteins is what helps to maintain them in the cell wall or in the extracellular matrix.

The presence of lipids in granules was also described by Mercer [29] and Chou & Meek [30] for structures with crenated edges and dark interiors. Our study evidenced the presence of lipids by the technique of staining with Sudan IV. The origin of storage of the protein bodies in conidia of *B. cinerea*, as well as evidence shown by Buckley *et al*. [22] upon the function of the endoplasmic reticulum in the production of lipids and their stock in the storage bodies, suggest that conidia are source of substances of lipid origin, important for the cell development, structure and function.

Instead of special storage bodies, Backhouse & Willets [31] suggested that the lipid bodies in the sclerodia of *B. cinerea* are normal constituents of the mycelium, and that beside being dependent on the species, the lipid content of sclerodia can also be partially attributed to the culture medium [32]. The main lipids present in the constitution of *B. cinerea* are the myristic, palmitic, stearic, oleic and linoleic acids [33].

Although the isolation of alkaloids had not been reported for species of the genus *Botrytis,* our results presented positive stain for alkaloids in both utilized methods, what opens the possibility for isolation studies, with the aim to identify which classes of alkaloids are present in the cell wall of the conidia and hyphae. Hayashi [34] demonstrated that alkaloids of the class of okaramines are widely produced by fungi, with *c.* 18 isolated substances, five being produced by *Penicillium expansum* (Link.) Thom., as well as the production of asperparalines A, B and C, isolated from *Aspergillus japonicus* Saito.

B. cinerea is rich in polysaccharides, being composed mainly of glucans and ramno-galacto-mannans, recognized for immunomodulatory activities [35] hypocholesterolemia, as well as in the reduction to glycemic and insulin

response [36]. The cell wall of fungi and yeasts is rich in polysaccharidic structures, such as chitin, mannans, glucans and notably alpha and beta (1,3) glucans [37], as well as the presence of polyanionic polymers such as glucuronanate [38]. In this context, the identification of structures of novel and original polysaccharides leads to new field of applications, even if just a very small number of known polysaccharides is explored. Knowledge on chemical constitution of the fungi wall, mainly regarding presence of polysaccharides, is important for biotechnological use of fungi, since such molecules can be utilized in several industrial processes, or moreover, are useful for taxonomic identification of such organisms [39].

The presence of terpenoids in *B. cinerea* evidences the richness of different groups such as botryane sesquiterpenoids [7], secobotrytriendiol, methyl botryoloate, 15 α-metoxy-O-methyl dihydrobotrydial, secobotrytriendiol, dihydrobotrydialone, dehydrobotrydienol, 11-hydroxydehydrobotrydienol, and 12-hydroxydehydrobotrydienol [40, 41].

Although there are no reports on the use of *B. cinerea* in traditional medicine, this species presents important metabolites of primary and secondary origin, of great interest for several fields of chemistry, pharmacy, agronomy and biology.

5. Conclusions

Overall, this study shows the microchemical profile of *B. cinerea*, as well as the use of techniques of Gaussian pre-filtering and color inversion, for visualization of images. *B. cinerea* presents storage bodies located in the hyphae, with rectangular or irregular shape, containing proteins, lipids and terpenoids. These hyphae also have in their wall, large quantities of polysaccharides, alkaloids, lipids and terpenoids. Visually, the conidia present mostly proteins inside, alkaloids and polysaccharides in the cell wall, as well as terpenoids distributed in the interior and in the cell wall, either.

Futurely, the application of these techniques could help other researchers in the isolation of the classes of described substances, as well as in microchemical and morphological studies on other species of fungi.

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References

1. Elad Y, Williamson B, Tudzynski P, Delen N. *Botrytis* spp. and diseases they cause in agricultural systems – an introduction. In: Elad Y, Williamson B, Tudzynski P, Delen N, eds. *Botrytis*: biology, pathology and control. Springer, Dordrecht; 2007: 1-8.

2. Choquer, M.; Fournier, E.; Kunz, C.; Levis, C.; Pradier, J.; Simon, A.; Viaud, M. *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. FEMS Microbiol Lett. 2007; 277(1): 1-10. pmid: 17986079.

3. Welmar, K.; Tschesche, R.; Breitmaier, E. Botrylactone, a new antibiotic from the culture solution of the fungus *Botrytis cinerea*. Chemische Berichte. 1979; 112(11): 3598- 3602.

4. Colmenares, A.J.; Aleu, J.; Duran-Patron, R.; Collado, I.G.; Hernandez-Galan, R. The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea*. Journal of Chemical Ecology. 2002; 28(5): 997-1005. pmid: 12049236.

5. Tani, H.; Koshino, H.; Sakuno, E.; Nakajima, H. Botcinins A, B, C, and D, metabolites produced by *Botrytis cinerea*, and their antifungal activity against *Magnaporthe grisea*, a pathogen of rice blast disease. Journal of Natural Products. 2005; 68(12): 1768-1772. pmid: 16378371.

6. Tani, H.; Koshino, H.; Sakuno, E.; Cutler, H.G.; Nakajima, H. Botcinins E and F and botcinolide from *Botrytis cinerea* and structural revision of botcinolides. Journal of Natural Products. 2006; 69(4): 722-725. pmid: 16643065.

7. Reino, J.L.; Duran-Patron, R.M.; Daqubi, M.; Collado, I.G.; Hernandez-Galan, R. Biosynthetic studies on the botcinolide skeleton: new hydroxylated lactones from *Botrytis cinerea*. The Journal of Organic Chemistry. 2006; 71(2): 562- 565.

8. Moraga, J.; Pinedo, C.; Durán-Padrón, R.; Collado, G.I.; Hernández-Gálan, R. Botrylactone: a new interest in an old molecule – rewiew of its absolute configuration and related compounds. Tetrahedon. 2011; 67(2): 417- 420.

9. Figueredo, A.C.; Barroso, J.G.; Pedro, L.G.; Ascenção, L. Histoquímica e citoquímica de plantas: Princípios e protocolos, Lisboa, 1ª edição.

Faculdade de Ciências da Universidade de Lisboa, Centro de Biotecnologia Vegetal, 2007:80.

10. Weber, R.W.; Wakley, G.E.; Pitt, D. Histochemical and ultrastructural characterization of vacuoles and spherosomes as components of the lytic system in hyphae of the fungus *Botrytis cinerea*. Histochem J. 1999; 31(5): 293-301.

11. Alfenas, A. C.; Mafia, R. G. Métodos em fitopatologia. Viçosa, MG: Universidade Federal de Viçosa, 2007: 382.

12. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 7(72): 248-254. pmid: 942051.

13. Svendsen AB, Verpoorte R. Cromatography of alkaloids. New York: Elsevier Scientific Publishing Company, 1983.

14. Furr, M.; Mahlberg, P. G. Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa*. Journal of Natural Products. 1981; 44(2): 153-159.

15. Brechú-Franco, A.E.; Laguna-Hernández, G.; De la Cruz-Chacón, I.; González-Esquinca, A.R. In situ histochemical localisation of alkaloids and acetogenins in the endosperm and embryonic axis of *Annona macroprophyllata* Donn. Sm. seeds during germination. European Journal of Histochemistry. 2016; 60 (1): 55-58. pmid: 26972713.

16. Pearse, A. G. E. Histochemistry theoretical and applied: preparative and optical technology. 4. ed. Edinburgh: Churchill Livingston, 1980: 439.

17. Clarck, M.F. Immunosorbent assays in plant pathology. Ann Rev.Inc.1981; 19 (1): 83-106.

18. Ganter, P.; Jollés, G. Histologie normale et pathologique. Paris: Gauthier– Villars, 1969, 1970. v. I e II.

19. Murray, S.; Bex, P.J.Perceived blur in naturally contoured images depends on phase. Frontiers in Psychology. 2010; 1: 1-12.

20. Louvard, Y.;Thomas, M.; Dzavik, V.; Smith, D.H.; Galassi,A.R.; Pan, M. et al. Classification of coronary artery bifurcation lesions and treatments: time for a consensus. Catheterization and cardiovascular interventions. 2008; 71:175–183. pmid: 17985377.

21. Mazzoleni, P.; Matta, F.; Zappa, E.; Sutton, M.A.; Cigada, A. Gaussian pre-filtering for uncertainty minimization in digital image correlation using numerically-designed speckle patterns. Optics and Lasers in Engineering. 2015; 66: 19–33.

22. Buckley, P.M.; Sjaholm, V.E.; Sommer, N.F. Electron microscopy of *Botrytis cinerea* conidia. Journal of Bacteriology. 1966; 91(5): 2037–2044.

23. Pârvu M, Pârvu AE, Crăciun C, Barbu-Tudoran L, Tămaş M. Antifungal activities of *Chelidonium majus* extract on *Botrytis cinerea in vitro* and ultrastructural changes in its conidia. J. Phytopathol. 2008; 156: 550-552.

24. Esterio, M.; Munoz, G.; Ramos, C. Characterizations of *Botrytis cinerea* isolates present in Thompson seedless table grapes in the central valley of Chile. Plant disease. 2011; 95(6): 683- 690.

25. Willetts, H.J.; Bullock, S.; Begg, E.; Matsumoto, N. The structure and histochemistry of sclerotia of *Typhula incarnata*. Canadian Journal of Botany, 1990. 68(10): 2083-209.

26. Jorgensen, L.B.; Beinke, H.D.; Mabry, T.J. Protein-accumulating cells amd dilated cisternae of the endoplasmic reticulum in three glucosinolate-containing genera: *Armoracia*, *Capparis*, *Drypetes*. Planta. 1977; 137: 215-224. pmid: 24420656.

27. Moore, A.E.P., Ashford, A.E., and Peterson, R.L.: Reserve substances in *Paxillus involutus* sclerotia determined by histochemistry and X-ray microanalysis. Protoplasma. 1991; 163: 67-81.

28. González, M.; Brito, N.; Gonzalez, C. Identification of glycoproteins secreted by wild-type *Botrytis cinerea* and by protein O-mannosyltransferase mutants. BMC Microbiology. 2014; 14: 254.

29. Mercer, E. H. The evolution of intracellular phospholipid membrane systems. In R. J. C. Harris [ed.]. The interpretation of ultrastructure. Academic Press, Inc., New York. 1962: 369- 384.

30. Chou, J. T. Y.; G. A. Meek. The ultrafine structure of lipid globules in the neurones of *Helix aspersa*. Quart. J. Microscop. Sci. 99:279-284. 1958

31. Backhouse, D.; Willetts, H.J. Development and structure of infection cushions of *Botrytis cinerea*. Transactions of the British Mycological Society 1987; 89(1): 89-95.

32. Kohn, L.M.; Grenville, D. Some Factors Influencing Lipid Storage in Sclerotia of Sclerotinia minor. Mycologia. 1987; 79(6): 907-909.

33. Griffiths, Robert G., et al. Lipid composition of *Botrytis cinerea* and inhibition of its radiolabelling by the fungicide iprodione. The New Phytologist, 2003; 160(1): 199–207.

34. Hayashi, H. Bioactive alkaloids of fungal origin. Studies in Natural Products Chemistry. 2005; 32: 549-609.

35. Fanizza, G.; Bisignano, B.; Pollastro, S.; Faretra, F. Effects of polysaccharides from *Botryotinia fuckeliana* (*Botrytis cinerea*) on in vitro culture of table and wine grapes (*Vitis vinifera*). Vitis. 1995; 34(1).

36. Rondanelli M, Opizzi A, Monteferrario F. The biological activity of beta-glucans. Minerva Med. 2009; 100(3): 237- 45. pmid: 19571787.

37. Laroche, C.; Michaud, P. New developments and prospective applications for beta (1,3) glucans. Recent Pat Biotechnol. 2007; 1(1): 59-73. pmid: 19075833.

38. De Ruiter, G. A.; Josso, S. L.; Colquhoun, J.; Voragen, A. G. J.; Rombouts, F. M. Isolation and characterization of p (1-4)- D-glucuronans from extracellular polysaccharides of moulds belonging to Mucorales. Carbohydrate Polymers. 1992; 18: 1-7.

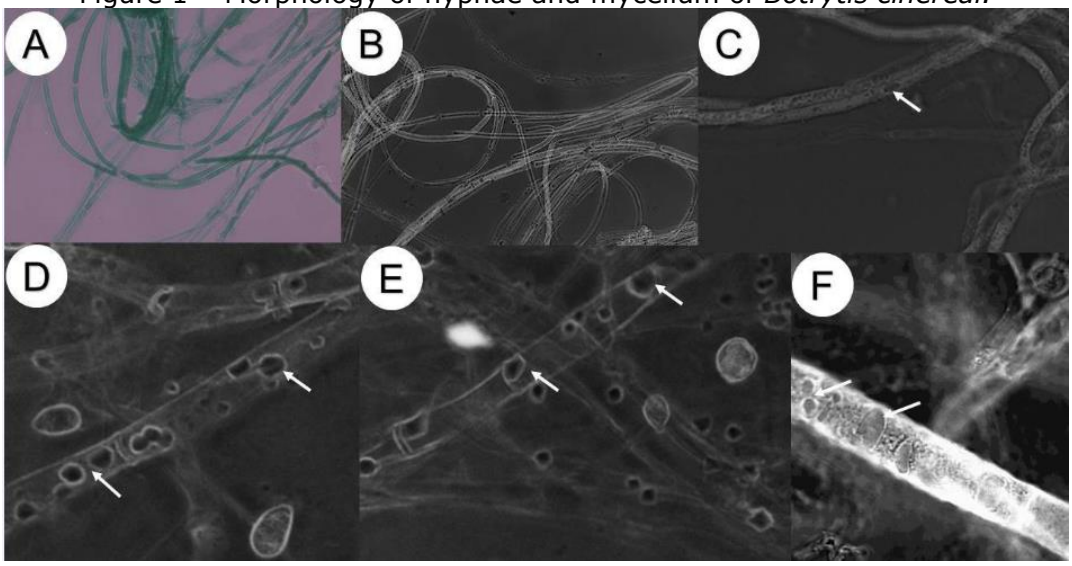
39. Madigan, M. T.; Martinko, J. M.; Parker, J. Microbiologia de Brock. 10. ed. São Paulo: Prentice Hall, 2004.

40. Collado I. G., Hernandez-Galan R., Duran-Patron R., and Cantoral J. M. Metabolites from a shake culture of *Botrytis cinerea*. Phytochemistry. 1995; 38: 647- 650.

41. Durán-Patrón, R.; Aleu, J.; Hernández-Galán, R.; Collado, I.G. Biotransformation of (4E,8R)-caryophyll-4(5)-en-8-ol by *Botrytis cinerea*. J Nat Prod. 2000; 63(1): 44-7.

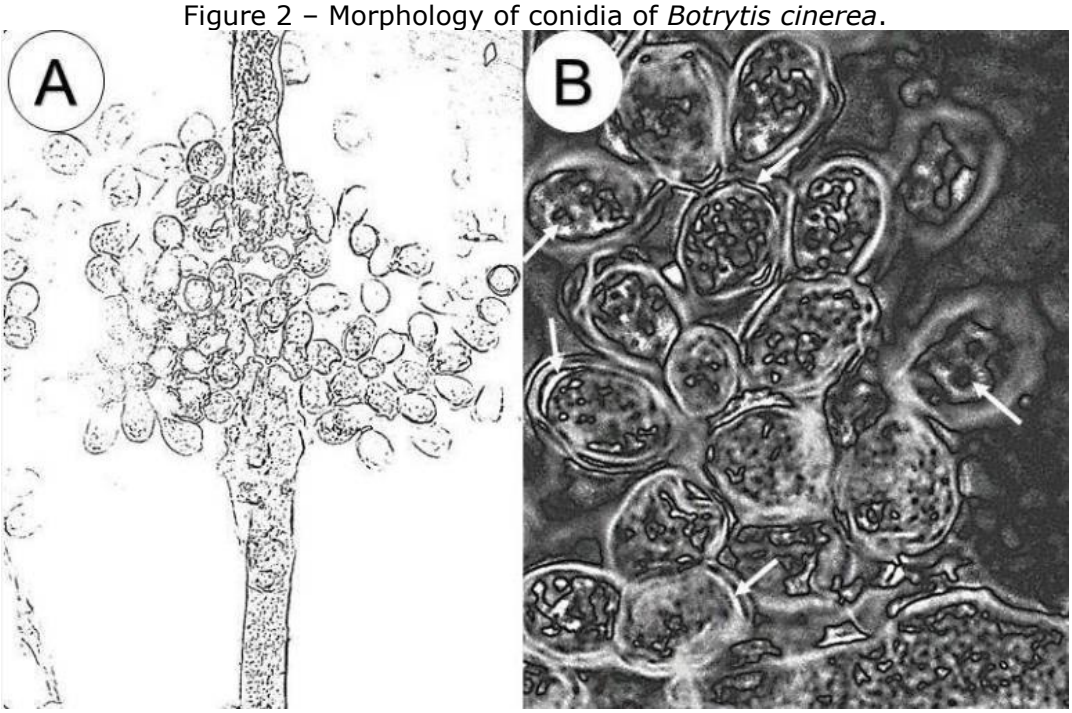
List of Figures

Figure 1 – Morphology of hyphae and mycelium of *Botrytis cinereal.*



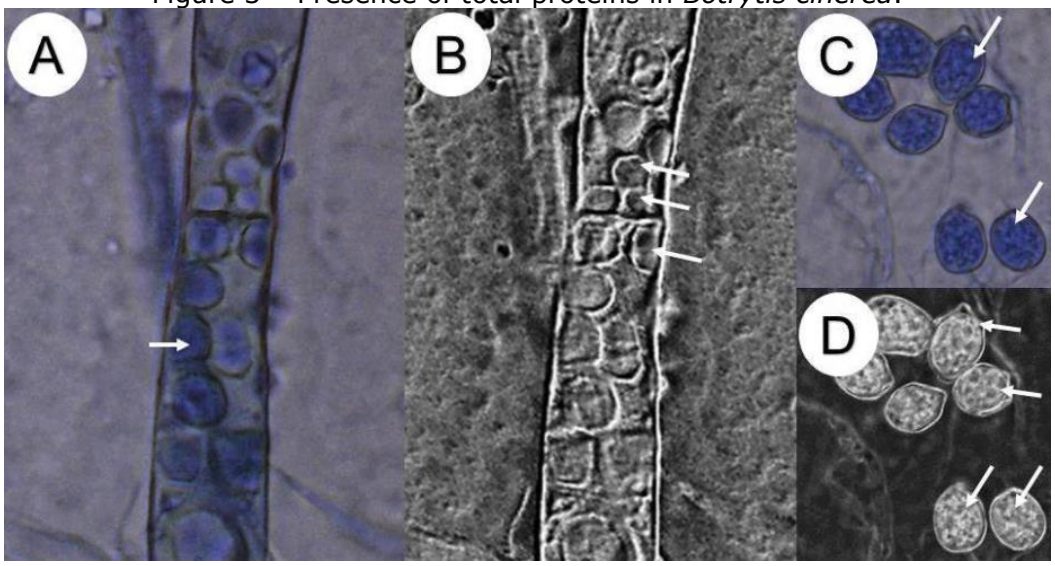
A - Aspect of young mycelium. B - Application of the technique of Gaussian blur and color inversion. C - Presence of storage bodies of rectangular shape, inside of young hyphae (arrow). D, E, F – Presence of storage bodies of rounded shape and in higher quantity in more developed hyphae (arrow).
Source: Authors.

Figure 2 – Morphology of conidia of *Botrytis cinerea*.



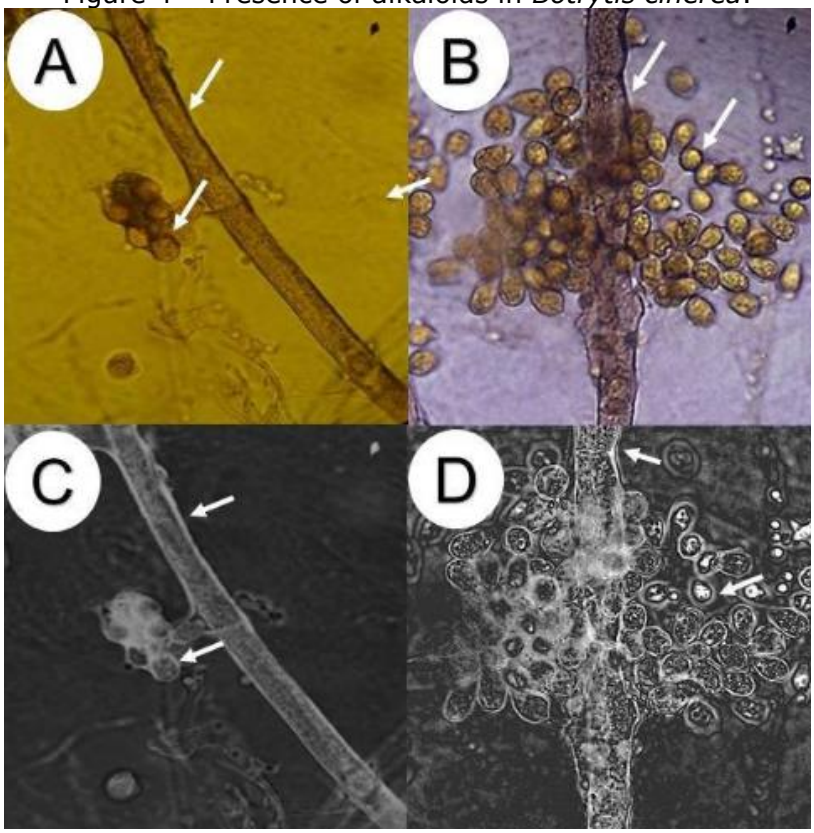
A - Technique of Gaussian blur. B – Detail of conidia containing storage bodies inside the spores (arrows) and storage membranes of purse shape (arrows).
Source: Authors.

Figure 3 – Presence of total proteins in *Botrytis cinerea*.



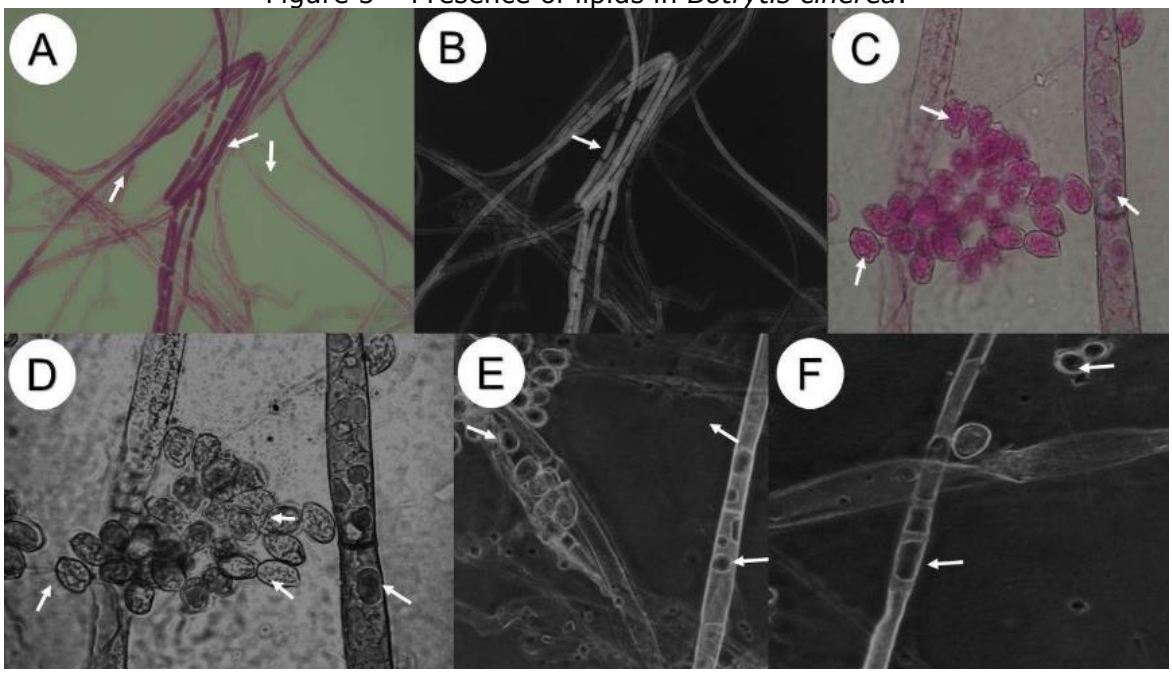
A - Proteins in the conidia (arrow). B - Storage bodies containing proteins in the central region of the conidia (arrows), evidenced by Gaussian blur and color inversion. C, D – Rounded aspect of the protein bodies present inside the conidia (arrows).
Source: Authors.

Figure 4 – Presence of alkaloids in *Botrytis cinerea*.



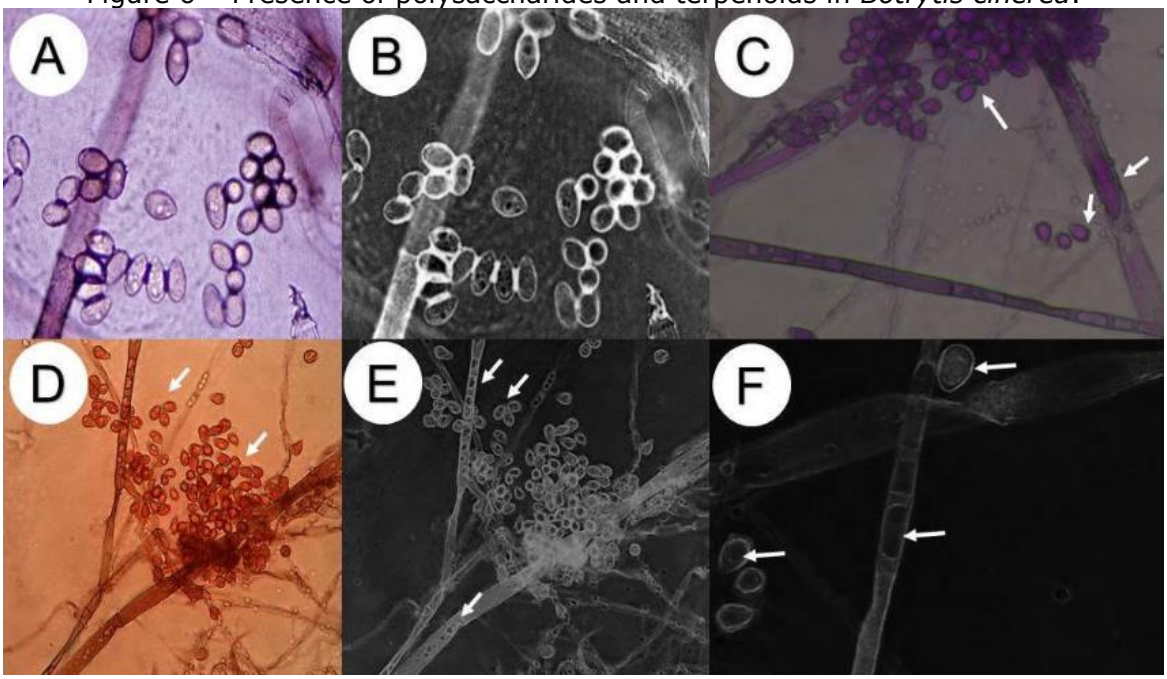
A, C. Alkaloids in the cell wall of hyphae and conidia (arrows) by the Dragendorff method. B, D - Alkaloids in the cell wall of hyphae and conidia (arrows) by the Wagner method.
Source: Authors.

Figure 5 – Presence of lipids in *Botrytis cinerea*.



A, B - Young hyphae containing storage bodies of quadrangular shape. C, D, E, F, G - Presence of lipids in storage bodies (larger and irregular units) and vesicles (smaller units) of hyphae and in conidia.
Source: Authors.

Figure 6 – Presence of polysaccharides and terpenoids in *Botrytis cinerea*.



A, B - Polysaccharides in the wall of hyphae and conidia. C – Storage bodies with irregular shape close to the conidia (arrows). D, E, F- Terpenoids in the cell wall of hyphae and conidia (arrows).
Source: Authors.