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WHAT COLOUR SUITS YOU BEST? A GENERAL OVERVIEW OF METHODS FOR FUNGAL ORGANISMS STAINING AS POST-VITAL MICROSCOPICAL PREPARATIONS USED IN PLANT PROTECTION

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Abstract

Staining fungi or anatomical parts of them (spores, conidiae, conidiophores, mycelia etc.) is still one of the most used study method in phyto- or zoo- or anatomopathology. In order to fix, dehydrate and clarify them, lactophenol remains the best reagent. Even if various modern techniques are used today, some of the nowadays neglected „classical ones" can sometimes provide an alternate investigation method which can led to interesting morphological and systematic data, or, at least reveal in more specific ways microscopical structural details.

Key words: *Fungi staining, dyes, spore staining, mycelia staining.*

INTRODUCTION

In plant (as in animal or human) pathology, staining of microbial and fungal tissues, isolated from the host or inside it are a valuable method of investigation, we can say a fundamental one. From the discovery of the lactophenol, its use in association with various stains (from which the water-soluble aniline blue, still called „Cotton blue" by the mycologists) represented the standard method of primarily investigation in micology and the starting point for various modifications, which help us to understand how do microscopical fungi infect their hosts, how do they develop, reproduce and can spread the disease to another plant (or animal) host.

Because in many cases, the microscopical fungi are +/- transparent, their refraction indices having values near the ones of glass or water (around 1,00, the coloured ones being not taken into account here) they need in order to contrast them to be stained.

MATERIALS AND METHODS

The presented methods have been validated in the laboratory by staining a conidial suspension and mycelia of *Beauveria bassiana* and *Fusarium* sp. mycelia, both cultivated on potato-dextrose-agar medium (PDA). The stains used, their Colour Index number, class and synonims are presented in the Table 1 (Gabe, 1970, Llewellyn, B.D., 2019).

The preparation methods for the mountants and iodine reagents (Table 2), simple stains (Table 3) and double stains (Table 4) as well as the bibliographical indications the results and (where it was the case) photographs (Figures 1-5) of the stained fungi are also presented.

The inspection of the preparations and the doccumentaion was performed by using a NOVEX-B series microscope, with a total magnification of 1000x and captions were acquired via a MicroQ S series camera with ToupView V 3.2 software.

The mycelia were recolted with the aid of a lanceolate needle previously flame-sterilised.

Saline solution spreading of the material: A small amount of biological material was suspended in a drop (2-3 mm diameter) of Ringer-Locke solution on a alcohol-degreased microscopical slide previously flame-sterilised (the heating of the slide increases the surface's hydrophobicity helping the formation of small, almost spherical liquid droplets, thus enhancing the homogenous distribution of the biological material between the slide and the coverslip).

A alcohol-degreased coverslip is placed with care (in order to not include air bubbles) onto the droplet containing the biological material.
A small pice of blotting paper is placed over the coverslip, after a little pressure being exerced by gently tapping onto the coverslip with a match. The Ringer-Locke solution excess is absorbed by the blotting paper. Care should be exerced in order to not smash excessively the biological material. Staining was performed by the following method: A drop of the staining solution is placed at one of the coverslip margins. A piece of blotting paper is placed at the opposite margin of the coverslip, which will absorb the Ringer-Locke solution, permitting the access of the staining solution by capilarity under the coverslip.
Dye solution spreading of the material: A small amount of biological material is suspended in the same conditions as above, but in a staining solution.
Heating the preparation after adding the staining solution isn't always necessary, but can be helpful when dealing with mycelia which don't „catch" the stain at room temperature. In any case, care must be exerced in order to avoid the boiling of the solution, which can led to the formation of small air bubbles between the slide and the coverslip. The best methods are: i) „step" onto a small, blue burner flame some two to five times with the preparation (coverslip side up) for one to two seconds. The preparation must be warm, not hot (check by touching with the preparation the back of the hand – the slide must be supportably warm; and ii) heat the preparation on a hot plate setted at about 50 degrees Celsius for 5 to 10 seconds, then check the temperature as previously. The entire procedure must not be repeated more than two times, and the preparations must be examined as soon as possible, but not later than 5 to 10 minutes.
The margins of the coverslip and its' superior surface are gently wiped with blotting paper and the preparation can be examined. The doccumentation will take place as soon as possible, because the aqueous solutions can evaporate in less than half an hour and some of the preparations transferred in lactophenol did not reatin the stain in a satisfactory manner, because the dye dissolves in the lactophenol.

Table 1. Name, Colour Index recommended name and number, chemical category and synonims of the stains quoted in the text.

Name	Colour Index recommended name and number	Category	Synonims
Aniline Blue, water soluble (Mixture of Methyl Blue * and Water Blue **	* Acid blue 22 (42.755) ** Acid blue 93 (42.780)	Acid, Triarylmethane	*China Blue, Cotton Blue, Methyl Blue ** Water Blue, Aniline Blue, acid
Sudan III	Solvent Red 23 (26.100)	Azo, Lysochrome	Sudan Red, Sudan Red BK
Eosin B	Acid Red 91 (45.400)	Acid, Fluorone, Xanthene	Eosin Red, aqueous, Eosine bluish, Imperial Red
Toluidine Blue	(52.040)	Basic, Thiazine	Toluidine Blue
Trypan Blue	Direct Blue 14 (23.850)	Acid, Azo, Vital	Niagara Blue 3B
Phloxine B	Acid Red 92 (45.410)	Acid, Fluorone	Phloxine
Acid Fuchsin	Acid fuchsin (42.685)	Acid, Triarylmethane	Acid Magenta, Acid Rosein, Acid Rubin, Acid Violet 19, Rubin S
Janus Green B	Diazine green (11.045)	Basic, Phenazine, Azo, Vital	IDEM!
Congo Red	Direct Red 28 (22.120)	Acid, azo	Direct red, Cotton red
Chrysoidin	Basic Orange 2 (11.270)	Acid, azo	Chrysoidin G, Chrysoidin Y

Table 2. The preparation methods for the mounting media and the iodine-based reagents

Reagent	Quantity	Function	Compounding procedure
Amann's lactophenol (Amann, 1896; Langeron, 1949; Langeron & Vanbreuseghem, 1952; Constantinescu, 1974)			
Phenol	10 g	Clarifying and fixing agent	The liquids must be weighed, not measured. When the mixutre is homogenous, add the phenol. Freshly prepared is transparent, but with time it becomes yellow than deep brown.
Lactic acid	10 g	Fixing agent and solvent	
Glycerol	20 g	Clarifier and solvent	
Distilled water	10 g	Solvent	
Results	Claryfies and permanentises the microscopical preparations. In time it can get brown due to the oxydation, leading to a possible permanent darkening of the preparation, making it impossible to be examined.		
Amann's chloral-lactophenol (Langeron & Vanbreuseghem, 1952)			
Phenol	10 g	Clarifying and fixing agent	As the previous solution
Chloral hydrate	10 g	Clarifying and fixing agent	
Lactic acid	10 g	Fixing agent and solvent	
Results	Claryfiing agent more effective than the „classical" lactophenol (n = 1..49), but also softens and inflates the material more.		
Berlese mounting medium modified acc. to M.A. Ionescu (Ionescu, 1937; Toma & Anghel, 1976)			
Chloral hydrate	40 g	Clarifying agent	See below the „Observations" rubric
Gum acacia (Gum arabic)	10 g	Density enhancer	
Glucose syrup	5 g	Density enhancer and solvent	
Glacial acetic acid	5 g	Acidifying agent and solvent	
Anhydrous glycerol	5 g	Solvent	
Observations	Weigh all the ingredients. Dissolve the gum acacia (15 g) in 20 g of water at room temperature for one day with occasional stirring. After all the impurities sedimented, filter through glass wool. Dissolve the glucose (15 to 20 g) in the minimum amount of water (about 15 mL) with stirring and heating on water bath, in order to obtain a honey-like consistency. The chloral hydrate is put in a flask, adding the solutions of gum acacia, glucose, the acetic acid and the glycerol. The flask is heated on a water bath until the mixture liquefies. The solution is decanted the next day, in order to permit the impurities eventually present to sediment. The solution must be kept in a dark glass-stopped bottle.		
Results	Claryfies and permanentises the microscopical preparations. The degree of clarification is higher compared to the lactophenol. Also, the medium does not darkens due to oxydation.		
Lugol's iodine solution			
Potassium iodide	2 g	Increases the iodine solubility	Dissolve the potassium iodide the necessary quantity of water (approx. 10 mL), then add the iodine and disolve it. Add the rest of the water and homogenise.
Iodine	1 g	Reagent for the amyloid	
Distilled water	100 mL	Solvent	
Results	The amyloid components of the fungi stains blue, the non-amyloid stains dark yellow to brown.		
Melzer's iodine reagent (Melzer, 1924)			
Chloral hydrate	100 g	Clarifying and fixing agent	Prepare the iodine-iodide solution as previously indicated (Lugol's solution). Add the chloral hydrate and homogenise. Use as such.
Potassium iodide	5 g	Increases the iodine solubility	
Iodine	1,5 g	Reagent for the amyloid	
Distilled water	100 mL	Solvent	
Results	Same results as the Lugol, but the material is clarified due to the chloral hydrate.		

Table 3. Preparation of the simple stains solutions

Reagent	Quantity	Function	Compounding procedure
Sudan Red – Lactopenol (saturated solution) (Langeron & Vanbreuseghem, 1952, Constantinescu, 1974)			
Sudan III	q.s.	Lipophyle stain	Dissolve the stain in the lactophenol in small portions, until a parcel remains undisolved. Filter and use as such.
Lactophenol	100 mL	Solvent, clarifying and fixing agent	
Results	The lipohyle parts of the fungi stains reddish-orange against the almost clear background.		
Observations	Even if 0,1 g of Sudan III are used, the dye dissolves poorly, resulting a supersaturated solution which precipitates when in contact with serum or distilled water. Better results are obtained when mycelia is placed directly in the stain than when placed in saline which is replaced by the stain.		
Acetic Cotton Blue (Langeron & Vanbreuseghem, 1952)			
Cotton Blue C4B	0,5 g	Stain	Dissolve the stain in the distilled water. Add the acetic acid and homogenise. Use as such.
Glacial Acetic acid	3 mL	Acidifier	
Distilled water	100 mL	Solvent	
Results	The fungi stains blue against the transparent ground. This aqueous acidified solution is comparable in almost all aspects with the lactophenol one, with the exception that it doesn't clarify the examined material.		
Toluidine blue O (Sangeetha & Thangaduray, 2013)			
Toluidine blue O	0,1 g	Stain	Staining solution (see Observaqtions)
Distilled water	3 g	Solvent	
37% Hydrochloric acid	0,1 mL	Acidifier	
Absolute ethanol	7 mL	Solvent	
Glacial acetic acid	4,5 mL	Solvent	Sulfatiomn reagent (See Observations)
98% Sulphuric acid	1,5 mL	Sulfation reagnet	
Results	The fungi stains reddish-blue against the pale blue ground.		
Observations	Staining solution: Dissolve the stain in the water, add the acid, homogenise, ad the etahnol and homogenise. Sulfation reagent: Pour dropwise carefully the sulphuric acid in the acetic acid, mix well and let cool. Add the sulfation reagent to the wet preparation: 10 mins. Replace with water (4-5 times). Add the stain solution: 3 mins. Rinse sucessively with 95% Ethanol: 10 sec., Absolute ethanol: 10 sec., Xylene: 10 sec., mount and examine		
Trypan blue (Constantinescu, 1974, Schunert et al., 1987)			
Trypan blue	0,1 g	Stain	Dissolve the stain in the buffer solution, check the pH and homogenise. Use as such.
Acetic acid acid – sodium acetate buffer solution acc. to Walpole, pH = 6,4	9,9 mL	Solvent and buffer component	
Results	The fungi stains greyish-blue against the transparent ground.		
Observations	According to Schubert et al., (1987), the stain can be dissolved at 0,05% in lactophenol. The aqueous buffered solution did not worked accordingly, the lactophenolic one sucessfully stained the fungi.		
Eosin (Langeron & Vanbreuseghem, 1952)			
Eosin	1 g	Stain	Dissolve the stain in the solvent. Use as such.
Distilled water	100 mL	Solvent	
Result	The fungi stains vivid pink against the transparent ground.		
Observation	This is a personal modification of the Phloxine staining method, as the two dyes have very similar structures (also indicated by their C.I. numbers). Also, Langeron & Vanbreuseghem, 19XX claim that the stain to be dissolved in a phenol solution.		
Lactophenolic Rubin S (Langeron & Vanbreuseghem, 1952; Constantinescu, 1974)			
Rubin S	0,1 g	Stain	Dissolve the stain in the solvent. Use as such.
Lactophenol	99,9 g	Solvent and clarifying agent	
Results	The fungi stains vivid reddish-violet against the transparent ground.		
Mayer's Mucicarmine (Gabe, 1968; Sangeetha & Thangaduray, 2013)			
Mucicarmine	0,30 g	Stain	Dissolve the satin in the alcohol. Dilute 1:4 (v/v) with distilled water before use.
Ethanol 50%	20 mL	Solvent	
Results	The mycelia stains violet		

Observations	The fungi species (or at least the strains used in this work) stained poorly with this solution, even when the preparation was heated.		
Janus Green (Constantinescu, 1974)			
Janus green	0,005 g	Stain	Mix the two buffer solutions, homogenise and check the pH. Dissolve the dye in the solution and mix well. Use as such.
0,2 M Acetic acid	25,5 mL	Buffer solution acc. to Walpole, pH = 4,6	
0,2 M Sodium acetate	24,5 mL		
Result	The fungi stains green-bluish against the transparent ground.		
Congo Red (Langeron & Vanbreuseghem, 1952; Constantinescu, 1974)			
Congo Red	Stain	5 g	Mix the two components.
Distilled water	Solvent	100 mL	
Results	The fungi stains red against the pale ground.		
China ink (Langeron & Vanbreuseghem, 1952; Rapilly, 1968; Constantinescu, 1974; Sangeetha & Thangaduray, 2013)			
China ink	1 mL	Contrast substance	Mix the two components.
Distilled water	5 to 10 mL	Solvent	
Results	As this is not a stain *per se*, this solution will provide a black background against which the colourless fungi can be observed.		
Observations	The China ink can be used as such (without dilution). Better results are obtained when mycelia is placed directly in the China ink than when placed in saline which is replaced by the China ink.		
Nigrosin, water soluble (Langeron & Vanbreuseghem, 1952; Constantinescu, 1974)			
Nigrosin	5 g	Contrast stain	Mix the two components.
Distilled water	100 mL	Solvent	
Results	Incolor fungi on a black ground.		
Dosch's Chrysoidine (Langeron & Vanbreuseghem, 1952)			
Chrysoidine	2 g	Stain	Dissolve the stain in water. Use as such.
Distilled water	100 mL	Solvent	
Results	The fungi stains bright orange against the transparent ground.		
Observations	Use only 0,1 g of stain. Homogenise and use as such.		

Table 4. Preparation of the double stains solutions

Reagent	Quantity	Function	Compounding procedure
Cotton Blue C4B - Sudan Red – Lactophenol (Langeron & Vanbreuseghem, 1952)			
Saturated Sudan Red solution in lactophenol	100 mL	Staining, clarifying and fixing agent	Prepare the saturated Sudan Red solution in lactophenol as indicated below (Sudan red in lactophenol). Add the Cotton blue and homogenise.
Cotton Blue C4B	0,5 g	Stain	
Results	The hyphae stains blue, some parts of them stains light orange due to the presence of lipophyle structures.		
Observations	Even if 0,1 g of Sudan III are used, the dye dissolves poorly, resulting a supersaturated solution which precipitates when in contact with serum or distilled water. Better results are obtained when mycelia is placed directly in the stain than when placed in saline which is replaced by the stain.		
Picro-Nigrosin – lactophenol (Langeron & Vanbreuseghem, 1952; Constantinescu, 1974)			
Nigrosin	0,2 g	Stain	Dissolve the nigrosin in the picric acid saturated solution. Use this solution instead of water to prepare the lactophenol: The liquids (minus the stain solution) must be weighed, not measured. When the mixutre is homogenous, add the phenol.
Picric acid, saturated aqueous solution	10 mL	Stain and solvent	
Phenol	10 g	Clarifying and fixing agent	
Lactic acid	10 g	Clarifying and fixing agent	
Glycerol	20 g	Clarifier and solvent	
Results	The fungi stains yellowish – light green with darker parts agains the pale ground.		
Observations	Better results are obtained when mycelia is placed directly in the stain than when placed in saline which is replaced by the stain.		

RESULTS AND DISCUSSIONS

The vital or post-vital staining methods and colour reactions used were compiled and adapted from various authors (see bibliography). Most of the methods are merely "citations of other citations", as in some cases is almost impossible to find the original source

(even if the author's name is known, some of the periodicals in which the paper was published are not available, not even in electronic format, also being quite difficult to find a physical copy. Thus, four major works have been used, two classical ones (Langeron, 1949, Langeron & Vanbreuseghem, 1952), a modern one (Constantinescu, 1974) and a recent one (Sangeetha & Thangadurai, 2013).

In the investigated literature are quoted more techniques, which at the time they have been developed and played an important role in mycology. Amann (1896) prepared and used for the first time a mixture which later becomed one of the most precious aid in fixing, clarifying, observing and mounting fungi – the "lactophenol" ("the mycological reagent by excellenece", as Langeron (Langeron, 1949, Langeron & Vanbreuseghem,1952) classified it).

The original recipe suffered some modifications in order to increase its' refractive index, originally n = 1,44 ("hydrated lactophenol") to 1,48 ("anhydrous lactophenol"). So, by adding chloral hydrate, the refractive index raises up to n = 1,49 or even more as in the case of the Berlese's medium modified acc. to Ionescu (1937), quoted by Toma & Anghel (1976) which provides a permanent mounting medium which claryfies the material more effective than the lactophenol. Due to the fact that the phenol itself auto-oxydises in time in contact with the atmospheric oxygene, its' colour changes gradually from translucent to cherry-red, due to a multitude of oxydation products with complex formulae, thus the lactophenol also brunishes with time, becoming yellow then dark brown, process which is accelerated in the slide preparations, even if the coverslip borders have been sealed.

Various staining methods, more or less specific, from which ones have been specially developed (e.g. lactophenolic acid aniline blue, which the mycologists use under a older chemical synonim – Cotton blue) (Şerbănescu-Jitariu et al., 1983) or adapted from the botanic or zoological histology protocols (e.g. eosin) have been used in order to evidentiate specific morphological and anatomical structures pertaining to the fungi, both free or inside their plant or animal hosts.

Some of them lack the precise preparation or clearly defined structure as required by chemistry, as in the case of some stains which are used in saturated or even supra-saturated solutions. In some cases, the stain concentration in the specific solvent – in this case the lactophenol - need to be raised in order to obviate in some cases the low permeability of the fungal cell wall.

But, in some cases, empirical formulations have been used as such (apparently succesfull), as in the case of a sulfanylamido- conjugated stain (sulphanylamidochrysoidine), initially developped as a chemoterapeutic agent (Rubiazol™, "Prontosil soluble") by I.G. Farbeindustrie and later used by Dosch to stain fungal structures. At the time, probably the indicated concentration (2%) of the sulphanilamido-chrysoidine worked, but the actual water solubility of the stain itself (chrysoidine) is way lower. In this work, a sample of the stain dissolved at 0,1 to 0,5%, thus, the original concentration was recalculated, a final 0,5% aqueous chrysoidine solution giving far better results. But, when used at the prescribed 2% concentration, even if as such, (so not even in contact with the lactophenol) it began to crystallise under the coverslip, making the preparation quite embarassing to examine due to a multitude of small dye crystals floating in the solution.

The same happened when using Janus green (as a vital stain) and partially with aqueous eosin. Some other stains quoted in the literature (e.g. lactophenol-saturated Sudan III solution) didn't worked simply because the stain didn't disolved completely, even at 0,1%, resulting a solution of the stain which contained small particles of undisolved dye. From four different batches tested, none of them dissolved at concentrations higher than 0,025 to 0,04 %. The conservation of the stain by the examined structures in lactophenol was poor, the dye dissolving in the mounting medium, with the exception of some of the staining solutions in which the solvent was the lactophenol itself, thus making almost impossible to make permanent preparations – the almost immediate doccumenting (microphotography) being necessary.

The China ink, which when used (according to one of the quoted references) diluted 1:1,5 with saline or distilled water formed a solution, which even if appeared as black, at the microscope was light grey. The China ink was

used undiluted, with good results. Instead of using a saturated solution of Cotton blue in lactophenol, a 0,5% solution gave same (even better) results.

Same problem apply to othe staining solutions, which at the time they were developped little or not at all was known about the best concentration to be used or to the method of progressive staining. In all those cases, the anatomy of the investigated fungi and the impediment of the host's +/- lignified tissues (with some exceptions) the use of concentrated solutions was necessary. Afterwards, by routine, those concentrated solutions were used for staining even thin-walled fungi. The author does not doubt in any moment the level of expertise of the researchers who in a brilliant manner discovered a multitude of fungal species and unveiled large areas of their unknown universe. He can only simply give some advices concerning the concentrations of the staining solutions, which, in his opinion, can be adjusted so as their staining properties remain the same, without dispensing quite significative amounts of dyes which only role is to assure a "saturated" solution prepared "according to the literature".

The dyes (with some exceptions, as in the case of the stabylised diazonium salts or of some compound dyes, as is the mucicarmine), due to their complex structure, are stable and conserve their staining properties over long periods of time. This fact is important, as the named substances can be sucessfully conserved and used (it's important to test their staining properties prior to use, though) over long periods of time. Also, by using more diluted solutions (progressive staining) or preparing smaller volumes it can improve: i) the laboratories' working costs (by avoiding useless spills of over-saturated solutions), ii) the environment protection, by minimising the amount of stains used and therefore the quantity of waste solutions resulted which need proper collection and withdrawal, and iii) the worker's health, as more of the stains and other chemicals used (e.g. phenol) are, if not carcinogenic or mutagenic, at least irritant or simply harmful.

Some of the staining and mounting methods are not currently used today, some of them are not widely known, but in some cases they can led to interesting results (e.g. the modification of the Berlese's chloral-lactophenol mounting medium modified acc. to Ionescu) which provides a permanent mounting medium which claryfies the material more effective than the lactophenol. In some cases, the lactophenolic solutions of stains gave good results in the author's hands, but the aqueous solutions of the same ones used for the staining, when replaced with lactophenol under the coverslip didn't conserved the coloration, because the dye used simply dissolved in the mounting medium.

The use of the China ink diluted 1:3 v/v with saline serum or distilled water (or water-soluble nigrosin) in order to obtain a black fundal on which the fungi would appear transparent didn't worked, but the undiluted China ink worked accordingly.

Some of the methods which were developed for the study of the fungal organisms inside their hosts could not be tested simply because they have been developed for working on sections fixed on slides. Even if some of the spores or hyphae affixing methods on slides have been elaborated, the proportion of the organisms sucessfully retained thorough all the stining process is quite low. When using a 0,1% w/w collodion solution (nitrocellulose dissolved in a 1:1 v/v absolute ethanol-dry diethyl ether) film in order to obtain a transparent membrane to hold in place the fungi as in the animal histology, the affixing were good, but the structure conservation was affected.

It may be possible that the quoted methods would work with another fungi species, or at least with other strains or cultivation methods of the two species used hereby by the author. The colleagues which kindly provided to the author the cultivated fungi have done their best to obtain cultures comparable in any aspect to the ones they use.

As for the stains used in this study, their tinctorial properties are fully conform with the literature and the results obtained by using them are perfectly comparable with the expected ones, at least when they were used in the histological study of other organisms by the author.

CONCLUSIONS

The 0,5% lactophenolic Cotton blue, the 0,1% lactophenolic Rubin S (Figure 1), the 0,05% lactophenolic Trypan blue (Figures 2, 3 and 4), the lactophenolic Picro-Nigrosin (Figure 5), the 0,5% aqueous Eosin (used instead of Phloxine, due to their very similar structures) (Figure 6), the 0,5% aqueous Chrysoidine (Figure 7), and the buffered Janus green solution stained well (Figure 8).

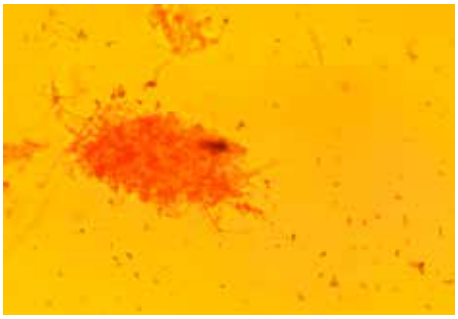

Figure 1. *Fusarium* sp. – 0,1% lactophenolic Rubin S – 200x

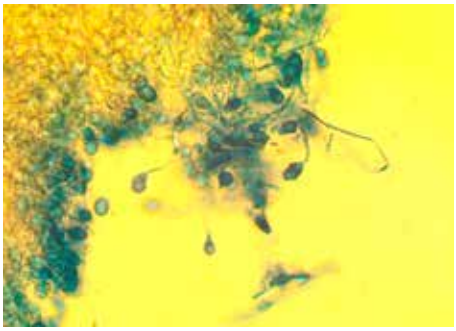

Figure 2. *Fusarium* sp. – 0,05% lactophenolic Trypan blue – 400x

Figure 3. *Fusarium* sp. – 0,05% lactophenolic Trypan blue – 1000x

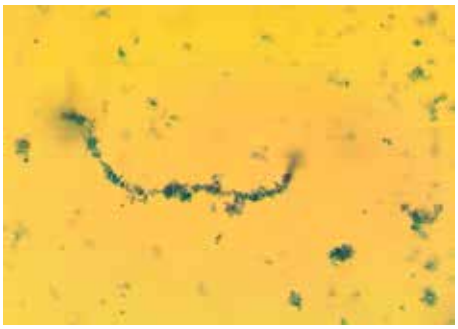

Figure 4. *Beauveria bassiana* – 0,05% lactophenolic Trypan blue – 1000x

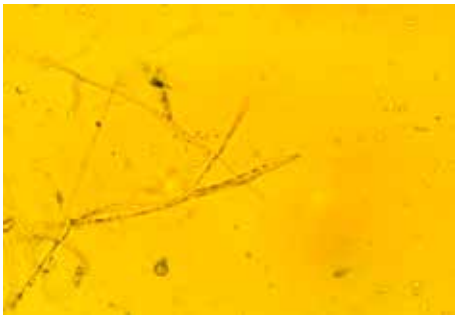

Figure 5. *Fusarium* sp. – 0,1% lactophenolic Picro-nigrosin – 400x

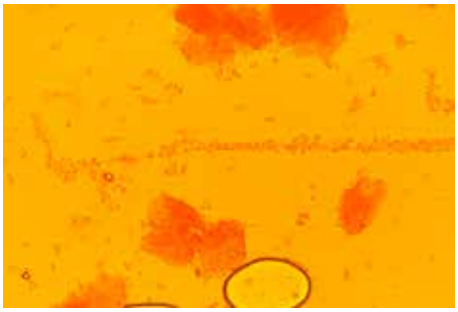

Figure 6. *Beauveria bassiani* – 0,5% aqueous Eosin – 400x

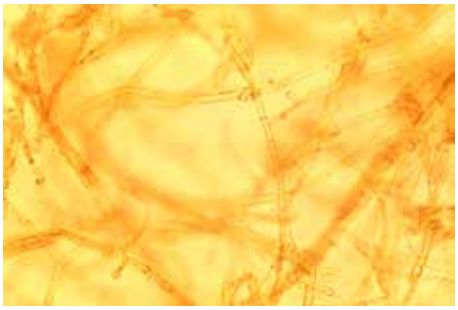

Figure 7. *Fusarium* sp. – 0,5% aqueous Chrysoidine – 400x

Figure 8. *Beauveria bassiani* – 0,1% aqueous buffered Janus green – 400x

The buffered Trypan blue solution didn't worked accordingly, the tissue stained lightly. The Mayer's Mucicarmine solution did'n worked at all. The Sudan III-lactophenol solution, used on saline-suspended hyphae precipitated the stain in small crystals. When hyphae were placed in the same solution, they stained light yellowish, but the solution also crystalised. The Cotton blue – Sudan III in lactophenol stained deep blue the hyphae (at least at the margin of the examined sample), only a small central portion becoming faint yellow.

The structures didn't retained any stain when mounted in lactophenol (even if they were examined one day later), with the exception of Cotton blue and Trypan blue (100% colour intensity conserved), Eosin red (75 to 100%) and Rubin S (roughly 50%).

The staining methods (from which the cotton blue-lactophenol appears to be the preferred one nowadays) currently in use are well-established and well known. But, what can appear in this work as „personal preferences" can be of interest in studying a more broad array of fungi that the two species that the author had at hand.

By using some of the techniques detailed here, it would be possible to gain some interesting structural and morphological criteria of identification, or at least some well evidentiated taxonomic characteristics which could be helpful in our comprehension of the representants of such a diverse phylum as Mycota is.

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REFERENCES

Amann, J. (1896). Konservierungsflüssigkeiten und Einschlussmedien für Moose, Chloro- und Cyanophyceen, Zeitschrift fü r wissenschäftliche Mikroskopie, XIII, 18.

Andrei, M., Rădulescu, D. (1972). *Caiet pentru tehnica preparării şi conservării materialului biologic – tehnica obţinerii preparatelor microscopice*, Centrul de multiplicare al Universităţii din Bucureşti, 87 pp.

Constantinescu, O. (1974). *Metode şi tehnici în micologie*, Editura Ceres, Bucureşti, 215.

Gabe, M. (1968). *Techniques histologiques*, Masson, Paris, 1113

Ionescu, M.A. (1937). Notă asupra montării artropodelor şi pieselor lor în preparate fixe, *Buletinul Societăţii Naturaliştilor din România*, 10, 12-15

Langeron, M. (1949). *Précis de microscopie*, Ed. 7, Masson, Paris, 1340.

Langeron, M., Vanbreuseghem, R. (1952). *Précis de mycologie*, Ed. 2, Masson, Paris, 703.

Llewellyn, B.D., 2019, http://stainsfile.info. Retrieved March 14, 2022.

Melzer, F. (1924). L'ornementation des spores des russules, *Bulletin de la société mycologique de France*, XL, 78-81.

Rapilly, F. (1968). „Les techniques de mycologie en pathologie végétale", *Annales des Épiphytities*, 19 (numéro hors série), 102.

Sangeetha, J., Thangaduray, D. (2013). Staining techniques and biochemical methods, 237-257, in: GUPTA, V.K. et al., *Laboratory protocols in fungal biology - Current methods in fungal biology*, Fungal Biology, Springer Sciences + XXXX, LLC, 2013.

Schubert, A., Marzachi, C., Mazzitelli, M., Cravero, M.C., Bonfante-fasolo, P. (1987). Development of total and viable extraradical mycelium in the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* Nicol.. & Schenck. *The new phytologist*, 1987(107), 183-190.

Serbănescu-Jitariu, G., Andrei, M., Rădulescu-Mitroiu, N., Petria, E. (1983). *Practicum de biologie vegetală*, Întreprinderea Poligrafică Iaşi, 296 pp.

Toma, N., Anghel, I. (1976). Tehnica întocmirii preparatelor microscopice botanice, pp. 69-106, in: Anghel., I., Untu, C., Tudor, C., Meşter, L., Năstăsescu, M., Toma, N., Gregorian, L., Nitescu, E., *Practicum de biologie*, Tipografia Universiţăţii din Bucureşti, 354 pp.