The Influence of Some Sources of Nitrogen on the Growth and Development of the *Phomopsis Incarcerata* Pathogen (SACC.) Hohnel

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Phomopsis incarcerata, known as the pathogen which caused the dieback of Rosa branches was detected in many orchards in Romania. Our investigations have approached a series of bio-ecological aspects of this pathogen: isolation, purification and obtaining the pathogen; determination and identification of the pathogen; establishing in vitro parameters of fungal development (nitrogen source). The isolate used in this study was obtained from Rosa spp. branches and was cultivated on three culture media: potato dextrose agar (PDA), malt extract agar and water agar which included five amino-acids: cysteine, glycine, beta-alanine, leucine and tryptophan. Leucine and glycine were favorable for the dynamics of the fungus.When the fungus grew on water agar, the sporulation was completely inhibited. The optimum pH values for the growth and creation of the Phomopsis incarcerata are in the range of 4.4-7, so weak to neutral acid.

Keywords: Phomopsis, nitrogen sources, Rosa, pathogen

The genus *Phomopsis* Sacc. belongs to the *Deuteromycota* phylum (Mitosporic fungi), *Coelomycetes* class, *Sphaeropsidales* order, *Sphaeropsidaceae* family, *Phyalostromatineae* group [5,8]. The taxonomy and the nomenclature of the genus have caused many controversies, never until now completely elucidated, despite the major interest from the mycologists [9].

The *Phomosis incarcerata* (Sacc.) Hohnel pathogen is often reported on weak branches of different species of *Rosa*, therefore, due to the practices considered to be respected, it was selected to be presented in the study. The conidiomes are of the picnidial type; picnids are stromal, subperidermal, isolated, unilocular, multilocular or convoluted, globular, ostiolate, unpapilated; peridia is pseudoparenchymatic, brown with angular texture, with a diameter over 250 μm. Conidiophores are filiform, hyaline, branched, 1-2 splitted, of 18 - 20x2 μm [1,2] (20 x 1 μm, 15.5 - 18 x 1 - 2 μm). The paraphyses are missing. Conidiogenesis is enteroblastic, monophialidic. α-phialospores are hyaline, fusiform, sharp at both ends, straight, aseptic, 2-gutulated or multigutulated, of 6 - 8 x 2 μm (8 x 2 μm, 6.5 - 10x2.5 - 4, Uecker, 1988; 7 - 8x2 μm) . β-phialospores are hyaline, haematite, filiform, aseptic, sharp, of 16-21 μm long [1,2]

The pathogen is biotrophic and necrotrophic on branches of *Rosa canina, Rosa bankiana, Rosa sp.* In Romania the species has been reported in Alba - Iulia - the surroundings of Albac, Or'ova - the area of hydro-energy and navigation system Iron Gates [2], and on globe in Italy, France, Germany, England, Portugal and France - Toulouse [3].

Other species of *Phomopsis* reported worldwide on the *Rosa* genus [9] are *Phomopsis gulabii* Lal. & Arya on *Rosa indica* in India - Uttar Pradesh. (conidiophores 17.2 - 22.2 x 3.3 μm; α-phialospores 6.6 - 8.9 x 2.2 -3.3 μm; β-phialospores 15.5 - 22.2 x 1.11 μm ; c- phialospores 11 - 13 x 1.6 - 3.3 μm); *Ph. Piceata* (Sacc.) Grove on *Rosa* in the United Kingdom (conidiomes 200 μm, conidiophores 15 - 18 x 2 μm, α-phialospores 7 - 9 x 2 - 2.5 μm), *Ph. rhodophila* (Sacc.) Buchwald in Moller on *Rosa* in the Faeroe Islands (conidiomes 180 - 360 μm, α- fialospores 4 - 7 x 1.5 - 2 μm), *Ph. rosae* (Schulzer & Sacc.) Traverso & Spessa on *Rosa* in Yugoslavia- Vincovce (α- fialospori 6 - 10 μm), *Ph. rosarum* (Durieu & Mont.) Camara on *Rosa scandens, Rosa sempervirens* in Portugal - Coimbra, Algeria (fig. 2) [10].

The nitrogen source used in growing the mycelium influences directly its use as nutraceutical and its bioactive properties. Fungi have a wide range of enzymatic activities and the ability to grow at relatively low water activity level enabling them to inhabit, change and/or decay various organic and inorganic materials used for objects of cultural heritage in museums' displays or storages, or outdoors [13]. Mushrooms consumption has become increasingly important in recent years, these ones being considered a good source of proteins, essential amino acids, carbohydrates, fibers, vitamins, minerals and antioxidants [6].

Experimental part

Material and methods

Our laboratory experiments aimed at establishing the growth and development rates of the *Phomopsis incarcerata* fungus, different amino acids being tested, which were included separately in standard culture media [7,11]. In the experimental protocol, various amino acids such as cysteine, alanine, leucine, glycine and tryptophan were used as nitrogen sources. The nutrient groups and their concentration were chosen taking into account the information in the literature.

The results were interpreted statistically by analyzing the significance of the differences between variants with LSH and Duncan tests in the SPSS program for Windows.

As material it was used the biological isolate of *Phomopsis incarcerata* obtained from branches of *Rosa* sp., purified and isolated on CGA medium.

The establishment of *in vitro* biological parameters of the fungus was determined by cultivating it on CGA medium and agarized water. For each medium, 5 amino acids were tested - cysteine, glycine, beta-alanine, leucine and tryptophan. All variants had at least four repetitions. The observations consisted of measurements of the diameter of the colonies at established time intervals, the

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occurence of the first picnidial buttons, respectively of the waxes.

Results and discussions

The main distinguishing feature of fungi in terms of nutrition is their inability to fix inorganic carbon. The simplest component that most fungi use as an energy source is glucose. Unlike other carbohydrates, glucose degradation does not require the presence of adaptive enzymes, as being a simple compound it is readily degraded and absorbed. As far as nitrogen sources are concerned, no fungus can fix atmospheric nitrogen, nitrates being the most commonly used compounds. In contrast, urea, amino acids, various polypeptides and proteins are accessible only to certain fungi [4].

Interestingly, no fungus is able to use agar as a carbon source for growth and development, although it is a galactose polymer, made up of galactoside residues esterified at C6 with a sulfonic group. Since there is a multitude of organic substrates *in situ*, the ability of fungi to degrade them cannot be reconstituted in laboratory experiments, which are often monofactorial.

Studies on the influence of carbon sources *in vitro* on some *Phomopsis* species have been carried out in Romania by Tatiana [a]esan and collaborators on the *Phomopsis occulta* pathogen isolated from *Larix deciduas* seeds [12]. Following the investigations, they concluded that the optimal sugars for *in vitro* fungal development are mannit, glucose, arabinose, dulcita, sucrose and starch.

PR2 Isolate - Phomopsis incarcerata (Sacc.) Hohn

The mycelium has a centrifugal growth, after 4 days reaching the diameter of 6/6.5 cm. At the beginning the mycelium is stubborn-white ; after a month the aged mycelium becomes dark-gray. Picnids are numerous, formed in groups, rarely isolated to the periphery of the mycelium, with concentric arrangement from the point of inoculation, obviously pointed, covered entirely by a gray mycelium, stromatic. In the picnids, there occured α-phialospores hyaline, fusiform, sharp at both ends, straight, aseptic, 2-gutulated or multigutulated, of 7 - 8 x 2 μm, and β-phialospores hyaline, haematous, filiform, aseptic, sharp, of 19-31 x 1 μm. The waxes are yellow. (Fig. 1).

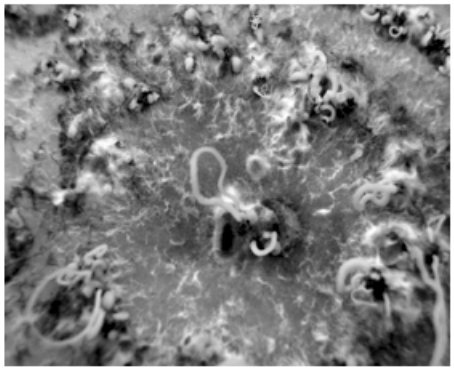

Fig. 1. Pathogen waxes on the CGA environment (culture of 21 days old)

The development of the fungus is richer, the thicker the nutrient substrate is, the number of picnids and their size gradually increasing. At a small amount of substrate, the mycelium is frail, thin, the picnidial primordia appearing later, and the picnids are smaller, most often spores not differentiating inside them. This fact is explainable because abundant food is required for their formation which is provided by a thicker substrate.

On the CGA medium, after the first 3 days of observations the most favorable amino acids for growth were leucine (4.075 cm) and glycine (3.225 cm), followed by tryptophan (2.675 cm) and beta-alanine (2.325 cm). In the variant containing cysteine the fungus did not grow. Subsequently, after 7 days, the amino acids that stimulated growth were leucine (6.950 cm) and glycine (6.425 cm), followed by tryptophan (4.525 cm), beta-alanine (4.425 cm) and cysteine (3.075 cm). After 10 days, in all the experimental variants, the diameter of the colonies was around 7 cm.

The first picnidial buttons appeared after 10 days in leucine and tryptophan variants.

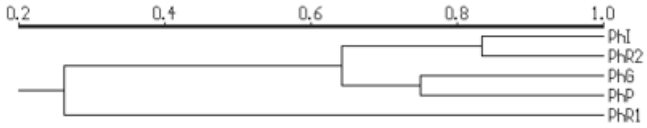

Fig. 2. The degree of similarity of the different species of *Phomopsis* of the genus *Rosa* PhI - *Phomopsis incarcerata*; PhR2 - *Phomopsis rosae*; PhG - *Phomopsis gulabii*; PhP - *Phomopsis piceata*; PhR1 - *Phomopsis rhodophila*;

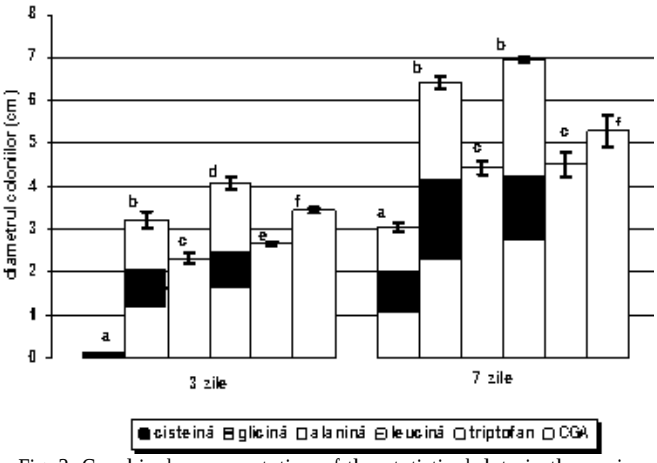

Fig. 3. Graphical representation of the statistical data in the amino acid variants, compared to the CGA witness

The same amino acids added in agarized water favor a very weak growth of the mycelium, which is barely noticeable. Unlike carbohydrates, conidiomes did not form even two months after the initiation of the cultures (Figs. 3, 4).

By analyzing the graph in fig. 3, there can be defined, after three days of observation, six different groups which are significantly different from each other: group a - cysteine , group b-glycine, group c- alanine, group d - leucine, group e - tryptogene and group f - CGA. After 7 days, the significance groups acquire the following configuration: group a - cysteine, group b-glycine, leucine, group c-alanine, tryptophan and group f - CGA. The restriction of diversity is due to the fact that, after a certain period of time *in vitro*, the diameter of the colonies becomes uniform in the variants favorable for the growth of the fungus.

The standard deviations are very small, grouped around the average values, which indicates in this case a uniform grouping of the sample values around the average.

Regarding the fungal sporulation, the first picnidial buttons appeared after 10 days in leucine and tryptophan variants. Statistical data show significant differences between all variants compared to the witness at the observations made after 3 and 7 days.

The reaction of the environment influences, in the case of pathogens in plants, their parasitic properties as well as their life cycle. Both subliminal and supraliminal values create stress conditions that negatively influence the intimate mechanisms of cell membranes, the expression of certain genes, as well as the conformation of certain enzymes (epigenetic modifications).

The optimum pH values for the growth and development of the *Phomopsis incarcerata* fungus are in the range 4.4-7, therefore weak to neutral acid, the mycelium reaching

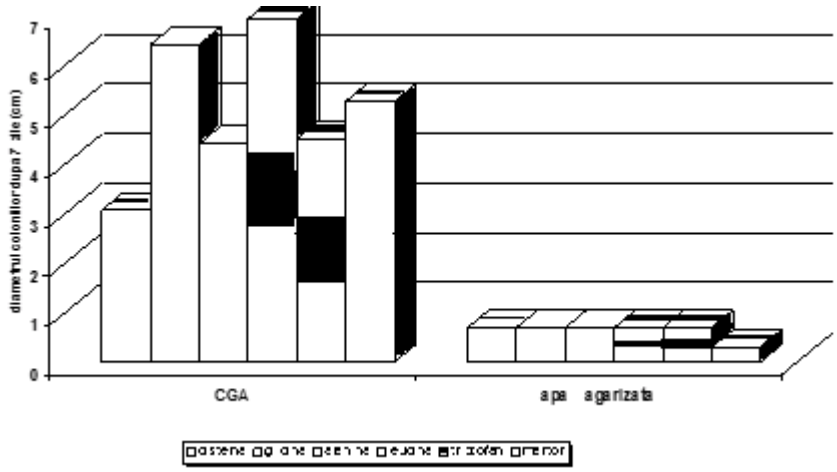

Fig. 4. The influence of amino acids on the growth and development of the *Phomopsis incarcerata* fungus depending on the culture medium

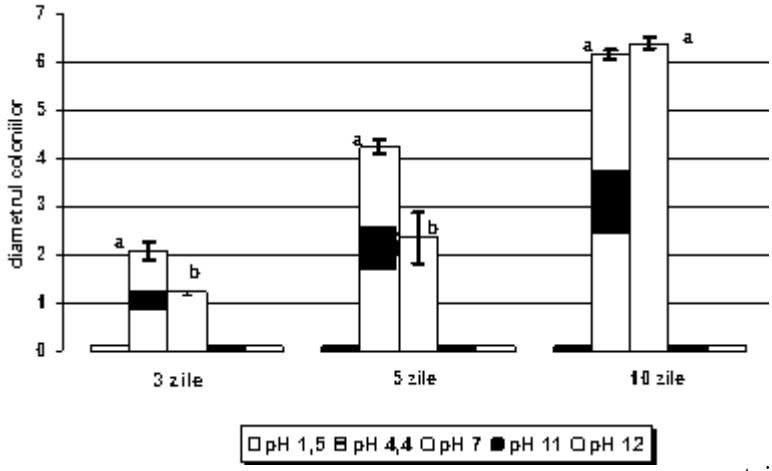

Fig. 5. The influence of pH on the growth and development of the *Phomopsis incarcerata* fungus

only after 5 days the diameter of 4.25 cm with *p*H 4,4 variant, compared to the *p*H 7 variant, where the diameter of the colony was 2.35 cm. In a strongly acid or alkaline environment the fungus did not grow (fig. 5).

The analysis tests revealed that, after 3-5 days, there are two distinctly significant groups: group a - *p*H of 4.4 and group b - *p*H of 7. Later, after 10 days the differences disappear, due to a uniform growth in the respective variants. The results presented here are also confirmed by those obtained by Tatiana Sesan (2001) in the *Phomopsis occulta* pathogen, where the optimum pH of *in vitro* development was between 4 and 6.5 [12].

In all the experimental variants presented above, we performed biometric measurements on both types of phialospores, where they were formed. It is worth noting that the variation of the composition of the culture medium did not influence the spore morphometry, but only the á/â ratio.

Conclusions

Phomopsis incarcerata is a highly virulent wound pathogen. It can infect fresh wounds throughout the vegetation period by drying out young stems and perennial ulcers on old ones. In most cases, the lesions become visible during the following spring. Other secondary pathogens, especially fungi of the genera Nectria, Melanconis, Cytospora and Diplodia [7], which continue the drying process of the stems, are frequently installed on the weak branches. The development of the fungus is richer, the thicker the nutrient substrate, the number of picnids and their size gradually increasing. The dynamics of the pathogen was correlated with the progressive administration of amino acids in the culture medium. Of these, leucine and glycine stimulated the growth of mycelial hyphae in the first days after sowing. In the variant containing cysteine the fungus did not grow. Regarding the fungal sporulation, the first picnidial buttons appeared after 10 days in leucine and tryptophan variants. The same amino acids, added successively in agarized water, cause a very weak growth of the mycelium, barely noticeable, but without the formation of conidiomes, and implicitly of phialospores.

The optimal *p*H values for the growth and development of the *Phomopsis incarcerata* fungus are in the range 4.4-7, therefore weak to neutral acid.

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