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CONSISTENCY OF β-CONIDIA FORMATION BY *Phomopsis helianthi*

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SUMMARY

In this publication we presented the latest results of a four-year research of the anamorph *Phomopsis helianthi*. In the past 4 years we isolated about one hundred cultures from the pycnidia of the vegetative *Helianthus annuus* plants and debris. In all pycnidia, both on diseased sunflower plants in the field and those cultivated on substrates, no conidial type but β-conidia has been found. Nutritive factor did not influence the type of conidia formed.

Key words: Sunflower, *Phomopsis helianthi*, pycnidia, β-conidia

INTRODUCTION

Ever since the outbreak of the disease caused by *Phomopsis/Diaporthe helianthi* there has been continuance in our research which refers to the morphology, physiology, cytology and pathology of the fungi. Muntañola-Cvetković et al., (1988) reviewed the history of the disease. A special challenge in our study, for years, has been to examine the structure, function and consistency of β-conidia formation in the pycnidia (Muntañola-Cvetković et al., 1985). Although some literature sources (Pezet, 1994; Wehmeyer, 1975) claim that β-conidia are unable to germinate, the problem of their persistence and intensity of forming remains open.

Literature data exist, which, among others, were reported by Aćimović and Štraser (1982), Marić et al., (1982), Ivanović (1992), showing that *P. helianthi* pycnidia contains also α-conidia, which are contrary to our results. Contradictory reports in connection with this matter can also be seen in publications of foreign authors. Yang et al., (1984), who were first to report the existence of *P. helianthi* in North America, have found exclusively β–conidia in conidiomata of isolates gathered from sunflower stems in Texas. Herr et al., (1983) have found, on sunflower from Ohio, a *Phomopsis* for which they have suggested that could it be *P. helianthi.* Field sunflower plants have formed pycnidia which contained α-conidia at first, however, in late summer and in autumn only β- or α- and β-conidia could be found. Assemat and Fayret (1988) reported the results of an experiment in which they noticed α- and β-conidia depending upon culture age; regardless of breeding conditions, at the beginning of conidia production, in all isolates, only few α-conidia were formed; after 11 days, a massive differentiation of α-conidia started, and, after 48^h of coexisting with α-conidia, β-conidia were left as only conidial type. At the beginning of their research, Muntañola-Cvetković et al., (1980) and Petrov et al.(1981) have found, in individual cases, small numbers of α-conidia on stalks, leaf bases and the substrate made up of debris of sunflower leaves. These results have never been repeated, so, in this work, we did one more check.

When describing the characteristics of *P. sojae*, Morgan-Jones (1984), have considered that β-conidia could be relict spermatia, i.e., that the exhaustion of nutrients could provoke substitution of real conidia (α) with spermatia (β-conidia). Intrigued by this opinion we used, as mediums, even sterilized parts of different plants in order to get more diverse substrates.

The purpose of the present work was to examine in detail not only the spontaneous occurrence of β-conidia, but also the influence of different parameters, such as nutritive factors, growth conditions, age of culture, etc., on this process.

MATERIALS AND METHODS

1) Fungal isolates

For experiments in which we have examined the formation of conidia in the pycnidia of *P.helianthi* (H1), we used isolates obtained from host pycnidia (*Helianthus annuus*) collected during the summer and autumn of 1989, 1990, 1991 and 1992 (H1-P.89, H1-P.90, H1-P.91, H1-P.92, respectively). Isolations were done from pycnidia from stems, which were at different stages of development, as well as from over wintered sunflower stems (debris).

2) Media

We used: (I) artificial nutritive media and (II) plant material prepared as media.

(I) **Artificial nutritive media** were: malt-streptomycin-agar (MSA), malt-agar (MA), potato-dextrose-agar (PDA) and water-agar (WA) (Booth, 1971).

(II) **Plant material** prepared as media were: a) green host stems (*H. annuus*) which were obtained from a field at Rimski Šančevi, Vojvodina; b) debris of *H.annuus* stems gathered through winter at Vojvodina fields; c) autoclaved plant material of cultivated and weed plants: as seeds (*Triticum aestivum*), flakes (*Glycine max*) or stem parts (*H. annuus, Rubus* sp., *Achillea millefolium, Artemisia vulgaris, Arctium lappa, Amaranthus retroflexus, Cichorium intybus, Cirsium arvense, Daucus carota, Lactuca serriola, Melilotus albus, Pulicaria vulgaris, Sonchus arvensis, Tanacetum vulgare, Tripleurospermum maritimum* and *Xanthium italicum*). Flakes and seeds were put directly in petri dishes, autoclaved for 25 min. at 120°C and pressure of 0.98 bar, and then WA was added up to the middle of petri dish; stems were cut in 6-8 cm pieces, 2-3 of them put in petri dishes, and then treated in the same way.

3) Inoculation

The artificial nutritive media (MSA, MA, PDA) were inoculated with the isolates H1-P.89, H1-P.90, H1-P.91, H1-P.92 cultivated for 7 days on PDA. Small parts of mycelia, of each isolate, were transferred to 10 petri dishes with the same media.

Plant materials (four petri dishes of each medium) were inoculated with parts of agar (approximately $2mm^2$) from H1-P.89, cultivated for 7 days on PDA, on the surface of stems, flakes or seeds, without previously wounding them.

4) Breeding conditions

Cultures inoculated in different natural and artificial nutrient media were cultivated in 2 regimens of light and temperature, laboratory conditions and thermostat. Laboratory conditions: natural light, day-night rhythm, temperature 19-22°C. Thermostat: white neon light, 7.78 $\mu Mm^{-2}s^{-1}$, PAR 400-700 nm, 12 hours light- 12 hours dark, temperature

22-24°C. Humidity was provided by adding of distilled water (2-5 ml every 5-7 days), depending on state of culture.

5) Preparations

Preparates for routine research, inspection of species, number and maturity of reproductive structures and spores, were made by the standard microscopy of host stems and cultures, they were also observed under a light microscope. Structure of pycnidia was more precisely analyzed by histological sections.

RESULTS

A massive development of pycnidia was observed on the diseased tissue of sunflower in the field as well as in the culture on the media used. Pathogenesis at the histological level was monitored from the occurrence of black spots on infected sunflower stems (Muntañola-Cvetković et al.,1989). Full life cycle and changes inside cavities of pycnidia were followed below the epidermis but not deeper than the outer endoderm of the primary parenchyme. Rape pycnidia have been found on debris during all winter months, and even on plant remains one year old. Peritecia in mass have formed on debris over the studied years.

In culture, pycnidie formed separately, or grouped in badly developed stromata 170-320 μm in diameter (Figure 2), partially immersed, dark brown in color, unilocular, with walls in layers (texture angularis) (Figure 3) and ostiola on the top from which the white-yellowish exudate runs always in drops (Figures 1, 2). In many cases pycnidia were overgrown by mycelia. The development of pycnidia from primordia to the release of conidia, was followed at the histological level *in vitro* as well as *in vivo*. Singular peritecia

Table 1. Type of conidia formed in the pycnidiaof *Phomopsis Helianthi* developed on live sunflower plants and debris, as well as on artificial nutrient media

Sampling time		Sunflower	Conydial type formed on:											
			MSA				MA				PDA			
			7d	15	21	50	7d	15	21	50	7d	15	21	50
1989	Avgust	β	β	β	β	β	β	β	β	β	β	β	β	β
	Septembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Decembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Mart	β	β	β	β	β	β	β	β	β	β	β	β	β
1990	Avgust	β	β	β	β	β	β	β	β	β	β	β	β	β
	Septembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Decembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Mart	β	β	β	β	β	β	β	β	β	β	β	β	β
1991	Avgust	β	β	β	β	β	β	β	β	β	β	β	β	β
	Septembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Decembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Mart	β	β	β	β	β	β	β	β	β	β	β	β	β
1992	Avgust	β	β	β	β	β	β	β	β	β	β	β	β	β
	Septembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Decembar	β	β	β	β	β	β	β	β	β	β	β	β	β
	Mart	β	β	β	β	β	β	β	β	β	β	β	β	β

Table 2. Type of fructification of *Phomopsis/Diaporthe helianthi* on different substrates

P helianthi (H1–P.89) on MA or PDA: Pycnidia (β–conidia)	
Inoculated on	
Sterilized plant material	Fructification type
1. *Achillea millefolum* L.	Pycnidia (β–conidia) Perithecia (ascospore)–PDA: pycnid. (β–conidia)
2. *Amaranthus retroflexus* L.	Pycnidia (β–conidia)
3. *Arctium lappa* L.	Pycnidia (β–conidia) Perithecia (ascospore)–PDA: pycnid. (β–conidia)
4. *Artemisia vulgaris* L.	Pycnidia (β–conidia)
5. *Cichorium intybus* L.	Pycnidia (β–conidia) Perithecia (ascospore)–PDA: pycnid. (β–conidia)
6. *Cirsium arvense Scop.*	Pycnidia (β–conidia)
7. *Daucus carota* L.	Pycnidia (β–conidia)
8. *Glycine max* (L.) Merr.	Pycnidia (β–conidia) Perithecia (ascospore)–PDA: pycnid. (β–conidia)
9. *Helianthus annuus* L.	Pycnidia (β–conidia)
10. *Lactusa serriola* L.	Pycnidia (β–conidia)
11. *Meliolotusalbus* Medic.	Pycnidia (β–conidia) Perithecia (ascospore)–PDA: pycnid. (β–conidia)
12. *Pulicaria vulgaris* Gaertner	Pycnidia (β–conidia) Perithecia (ascospore)–PDA: pycnid. (β–conidia)
13. *Rubus* sp.	Pycnidia (β–conidia)
14. *Sonchus arvensis* L.	Pycnidia (β–conidia)
15. *Tripleurospremum maritimum* schulth.-Bip.	Pycnidia (β–conidia) *Perithecia (ascospore)–PDA: pycnid.* (β–conidia)
16. *Triticum aestivum* L.	Pycnidia (β–conidia)
17. *Xanthium italicum* Mor.	Pycnidia (β–conidia)

formed very rarely on the artificial nutritive media. On sterilized plant materials, peritecia formed on most of the substrates used, in different intensity, separately or in groups.

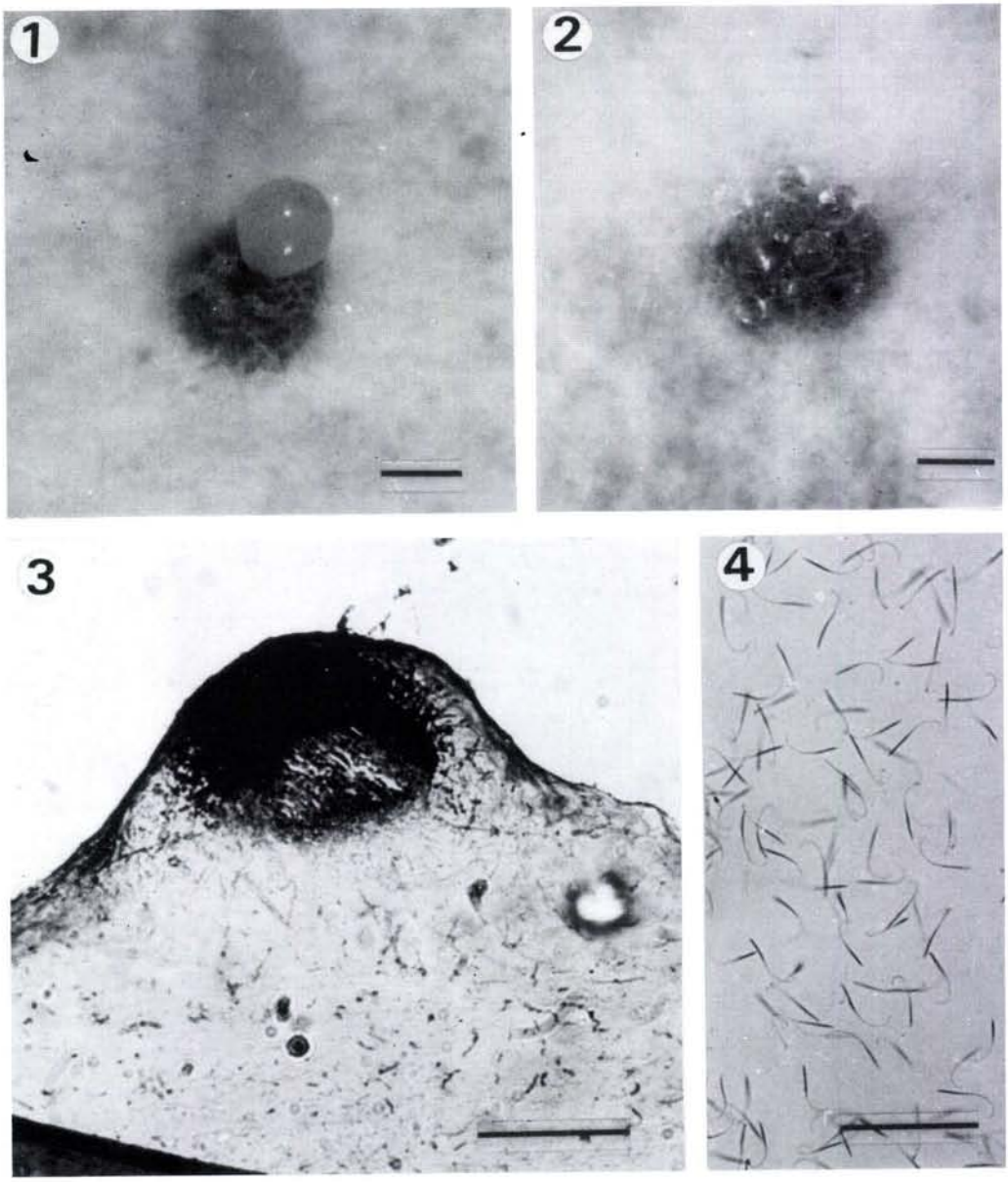
Routine slides were made of pycnidia on infected sunflower stem in different phases of development, with purpose to follow the type of conidia formed. We followed approximately a hundred of randomly chosen pycnidia from each sample, which makes, at least, 6000 randomly taken samples over the 4 year period of the present research. The same research was done at the same time on the debris of *H. annuus* stems. In pycnidia, β-conidia of *P. helianthi* were found in one hundred percent of the cases (Table 1).

Table 3. Type of fructification of *Phomopsis/Diaporthe helianthi* on different substrates

P helianthi (H1–D.89/Am) on MA or PDA: Pycnidia (β–conidia)	
Inoculated on	
Sterilized plant material	Fructification type
1. *Achillea millefolum* L.	Pycnidia (β–conidia) Perithecia (ascospore)
2. *Amaranthus retroflexus* L.	Pycnidia (β–conidia) Perithecia (ascospore)
3. *Arctium lappa* L.	Pycnidia (β–conidia) Perithecia (ascospore)-PDA: pyncid. (β-conidia)
4. *Cichorium intybus* L.	Pycnidia (β–conidia) Perithecia (ascospore)-PDA: pyncid. (β-conidia)
5. *Daucus carota* L.	Pycnidia (β–conidia) Perithecia (ascospore)
6. *Glycine max* (L.) Merr.	Pycnidia (β–conidia) Perithecia (ascospore)-PDA: pyncid. (β-conidia)
7. *Helianthus annuus* L.	Pycnidia (β–conidia) Perithecia (ascospore)
8. *Lactusa serriola* L.	Pycnidia (β–conidia) Perithecia (ascospore)
9. *Meliolotus albus* Medic.	Pycnidia (β–conidia) Perithecia (ascospore)
10. *Rubus* sp.	Pycnidia (β–conidia)
11. *Sonchus arvensis* L.	Pycnidia (β–conidia) Perithecia (ascospore)
12. *Tanacetum vulgare* L.	Pycnidia (β–conidia) Perithecia (ascospore)
13. *Triticum aestivum* L.	Pycnidia (β–conidia)
14. *Xanthium italicum* Mor.	Pycnidia (β–conidia) Perithecia (ascospore)

In experiments with nutritive media after inoculation, with isolates H1-P.89, H1-P.90, H1-P.91 and H1-P.92, cultures were maintained at the same time under lab conditions and in the thermostat. First checks were made after 10 days, and then successively each five days till the cultures dried out (approximately between 50-60 days). When the slides were made from these pycnidia, it was found that they contained β-conidia only, regardless of the age of the pycnidia, type of medium or the cultivation conditions (Table 1).

The culture H1-P.89 was used for the inoculation of plant material. Experiments were made both under lab conditions and in the thermostat. First checks were made after 7 days. It was found that the growth of the mycelia was dependent on substrate, and it varied from very weak, on *A. millefolia* to very intensive, on *H. annuus*. Frequency of pycnidia varied also depending on substrate from very low, on *A. millefolia*, to very intensive, on *H. annuus*. Most of the pycnidia were in their first phases of development, except those on *A. lappa* which were already ripe with a great number of β-conidia.



Figures 1–4. Phomopsis helianthi

1. Mature conidiomata, solitary, with drop-like exudate from a 15-day

2. Conidiomata on a stroma from 15-day old colony on PDA (Bar= 400 μm).

3. Longitudinal section of a conidioma from a 30-day old colony on MA (Bar=200 μm).

4. β-conidia (Bar= 40 μm).

In our next check, 14 days after inoculation, we noticed that the formation of pycnidia continued progressively, their number enlarged and they matured. We found the presence of the β-conidia on all slides.

We continued the checks up to 60 days of inoculation of the cultures. In the pycnidia of *P. helianthi* we never found any other spores except the _*b*-conidia (Table 1).

Isolates from the ascospores that formed on sterilized stems of *A. millefolia* (H1-D.89/AM) after inoculation with H1-P.89, were tried with a great number of media and plant materials. Checks were made in the same rhythm and the results were in concordance with the previous experiment (Table 3).

DISCUSSION

The results obtained in the last 4 years are in agreement with the results already published (Muntañola-Cvetković et al., 1988) stating that *P. helianthi* forms strictly β-conidia, either in pycnidia *in vivo* during the vegetative life of the host plant, or in those developed *in vitro* regardless of the medium type.

Muntañola-Cvetković et al., (1985a) proved that, except for the pycnidia of *P.helianthi*, the pycnidia of some other *Phomopsis* species could exist on sunflower debris quite rarely. That, could probably explain the earlier reports of some authors, which refer to the early years of the research when the parasite had not been very well known, or the theories based on those reports (Marić et al., 1988; Smith et al., 1988 and Ivanović, 1992). Still, we shall not exclude the possibility of different biotypes existing in some geographical regions.

Morgan-Jones (1984) opinion about the effect of nutrients on the special conidia type production, was in agreement with our results when we did our experiments with *P. sojae* but it was not so in the *P. helianthi* case. Considering the fact that even when the nutritive media were varied, only _*b*-conidia formed in *P.helianthi* pycnidia, our opinion is that in the *P. helianthi* case, the composition of media or the nutrient itself are not as important as the genetic factor could be.

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CONSISTENCIA DE FORMACION DE CONIDIAS POR *Phomosis Helianthi*

RESUMEN

En esta publicación se presentaron los últimos resultados de cuatro años de investigación del anamorfo *Phomosis helianthi*. En los últimos cuatro años fueron aislados alrededor de cien cultivos a partir de picnidios y de partes vegetativas de *Helianthus annuus* y residuos. En todos los picnidios, tanto sobre plantas enfermas en el campo como sobre plantas cultivadas sobre sustratos, solo se han encontrado conidias tipo β. El factor nutritivo no influenció el tipo de conidia formado.

UNIFORMITÉ DE LA FORMATION DE β CONIDIES CHEZ *Phomopsis Helianthi*

RÉSUMÉ

Dans cette publication nous présentons les derniers résultats des quatro années de recherche sur l'anamorphe *Phomopsis helianthi*. Au cours de ces quatre dernières années, nous avons isolé environ une centaine de cultures issues de pycnides présentes sur des plantes d'*Helianthihus annuus* en phase végétative et sur débris de culture. Dans toutes les pycnides, tant celles observées sur plantes infectées que celles obtenues sur substrat, seules des conidies de type β ont été trouvées. Les facteurs nutritifs n'ont pas d'influence sur le type de conidies formées.