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DEVELOPMENT OF REPRODUCTIVE STRUCTURES OF *Phomopsis helianthi* Munt.-Cvet. *et al.* AND *Phoma macdonaldii* Boerema ON SUNFLOWER SEEDS

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SUMMARY

We investigated possibilities for the formation of reproductive structures of important sunflower pathogens, *Phomopsis helianthi* and *Phoma macdonaldii*, on the husk and kernels of naturally infected and inoculated seeds.

Pycnidia were typically formed at the wider part of the husk in all sunflower lines and hybrids infected by *P.helianthi* and/or *P.macdonaldii*, partly immersed in the epidermis. The pycnidia of *P.macdonaldii* were larger than the pycnidia of *P.helianthi*.

The kernels infected by *P.helianthi* showed slight structural changes in comparison with the non-infected achenes. An exception were the seeds of a line which, on infection by *P.helianthi*, showed a complete disintegration of the central part of the cotyledon while numerous pycnidia formed in the outer layers of the parenchyma. Seeds of lines infected by *P.macdonaldii* were considerably damaged. The cotyledonary tissue was disintegrated and pycnidia arranged in rows formed in the outer layers of the parenchyma.

Key words: **seed, *Phoma macdonaldii*, *Phomopsis/Diaporthe helianthi*, pycnidia, sunflower**

INTRODUCTION

Among the fungal pathogens of the sunflower, *Diaporthe/Phomopsis helianthi* Munt.-Cvet. *et al.* and *Phoma macdonaldii* Boerema are the major parasites of seed. They may considerably affect crop performance and bring in question the economy of production of this important oil crop. *D./P.helianthi* is known as a causative agent of the stem canker (Mihaljčević *et al.*, 1980; Muntañola-Cvetković *et*

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al., 1981), while *P.macdonaldii* is a causative agent of the black stem disease (Fayzalla and Marić, 1981).

On the vegetative sunflower parts, the anamorph, *P.helianthi*, forms pycnidia with β-conidia, while the telemorph, *D.helianthi*, is formed on the detritus (Muntañola-Cvetković *et al.*, 1988). The source of infection are ascospores, while histological investigations showed that the infection route is leaf-petiole-stem (Muntañola-Cvetković *et al.*, 1989; 1991).

P.macdonaldii also forms pycnidia on the sunflower plants during growing season, which, after three years of storage, lose their normal content and the capacity to transmit the disease. Perithecia of the teleopmorph, *Leptosphaeria lindquisti*, form on the detritus (Marić *et al.*, 1981). Pycnospores and ascospores are responsible for the spread of infection. The sympotoms of the black stem disease, in the form of large oval or eliptical spots, are most obvious on leaves, petioles, stems and necks of the stem (Maširević, 1988).

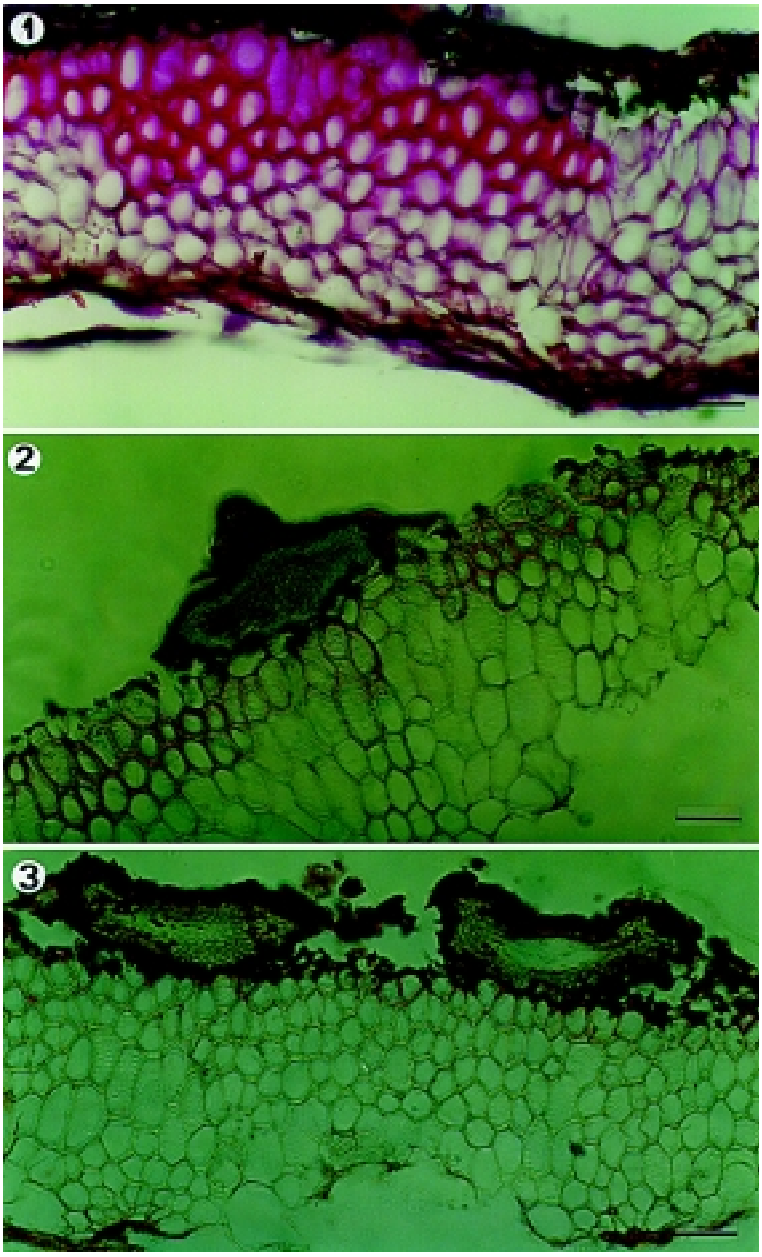
The sunflower fruit in a specific nutlet (with the flying appendix, pappus) - achene. The achene is composed of lignin-cellulose pericarp and kernel (Denis *et al.*, 1994). The pericarp of mature fruit is dry and hard and it can be easily separated from the kernel. It is composed of: epidermis, hypodermis, sclerenchyma and parenchyma. The kernel is composed of: seed coat, endosperm and embryo (Pustovoit, 1975).

In this work, we investigated the formation of reproductive structures of the two pathogens on the pericarp and kernels of naturally and artificially infected sunflower seeds.

MATERIALS AND METHODS

The seeds of 27 lines and 2 sunflower hybrids naturally or artificially infected by the fungal pathogens *Diaporthe/Phomopsis helianthi* and *Phoma macdonaldii*, were collected from 12 locations in the Vojvodina Province and from one location in each, Croatia, Bosnia and Romania (Table 1). Seed samples were coded as follows: line-L, hybrid-H; pathogens: *P.helianthi*-Ph, *P.macdonaldii*-Pm; locations: Novi Sad-NS, Vukovar-V, Dunavac-D, Irmovo-I, Rakovac-Ra, Neštin-N, Banoštor-Ba, Nova Topola-NT, Sremski Karlovci-SK, Bečej-B, Ledinci-L, Čerević-Č, Romania-R, Futog-F, Bijeljina-Bi; year of sampling: 97, 98, 99.

A modified paraffin method was employed (Johansen, 1940). The pericarp and kernels were fixed in FAA for 14 days (because of slow permeation) instead of for 24 h. Sections of 20 μm thickness were counterstained using Safranin O and Light green SF Yellowish and mounted in Canada balsam. The sections were then microscopically analyzed and photographed using Leica DMRB.



Cross section of the pericarp

Figure 1: Healthy achenia (Bar=500 µm)
Figure 2: Pycnidium of Phomopsis helianthi
Figure 3: Pycnidium of Phoma macdonaldii

The collection of histological sections of pericarp and kernels and their photographs are kept at the Institute of Botany and Botanical Garden "Jevremovac" in Belgrade.

RESULTS AND DISCUSSION

A: Changes of the pericarp caused by *P.helianthi* and *P.macdonaldii*

Samples from 1997

The seeds of H1.NS.97. were treated with the fungicide Lekinol and used as control for the morphological and anatomical changes in infected seeds. The seeds of L1.Ph.V.97., L2.Ph.D.97., L3.Pm.I.97. and L4.Ph.I.97. were naturally infected and the seeds of H1.Ph.NS.97. were inoculated.

The seeds of H1.NS.97. were free of pycnidia (Figure 1) which was expected considering their treatment with the fungicide. Pycnidia of characteristic were formed in the seeds of the other samples. The percent of infection by *P.helianthi* varied from 2% (L1.Ph.V.97.) to 2.4% (L2.Ph.D.97.). The percent of infection by *P.macdonaldii* was 1.0% (L3.Pm.I.97.).

In the naturally infected material, *P.helianthi* pycnidia typically formed at the wider part of the pericarp. The pycnidia on pericarp surface were slightly immersed in the epidermis, pear shaped, and they had very short necks (Figure 2). The dimensions of the pycnidia were 57.12-228.48 x 38.08-142.80 μm, the length of the necks was 47.60 to 76.16 μm. The pycnidia were not abundant, only one or two per one half of the pericarp, and they were completely or partially filled. Histological sections frequently showed exudate oozing from the ostiole.

In the case of seeds inoculated by *P.helianthi*, pycnidia covered the entire surface of the pericarp. They were slightly immersed in the epidermis. They were sparse, mostly round, and if the neck was present, it was very short. The dimensions of the pycnidia were 114.24-257.04 x 66.64-95.20 μm, of the necks 28.56-47.60 μm.

Samples from 1998

We tested naturally infected seeds of 21 lines: (L5.Ph.Ra.98., L6.Ph.B.98., L7.Ph.B.98., L8.Ph.B.98., L9.Ph.Pm.SK.98., L10.Ph.Pm.Ra.98., L11.Ph.Pm.Ra.98., L12.Ph.Pm.F.98., L13.Ph.Č.98., L14.Ph.Č.98., L15.Ph.Pm.SK.98., L16.Ph.Pm.N.98., L17.Ph.Pm.Ba.98., L18.Ph.NT.98., L20.Ph.Č.98., L21.Ph.L.98., L22.Ph.Bi.98., L23.Ph.SK.98., L24.Pm.SK.98., L25.Pm.Č.98.) and two hybrids (H2.Pm.R.98., H2.Ph.R.98.) (Table 1).

The percent of infection by *P.helianthi* varied from 0.25% (L12.Ph.F.98.) to 5.5% (L7.Ph.B.98.). In the samples infected by both pathogens, the percents of infection by *P.helianthi* varied from 0.25% (L8.Ph.Pm.B.98., L16.Ph.Pm.N.98.) to

9.75% (L11.Ph.Pm.Ra.98.), and by *P.macdonaldii* from 0.25% (L8.Ph.Pm.B.98., L9.Ph.Pm.SK.98., L16.Ph.Pm.N.98.) to 3.25% (L10.Ph.Pm.Ra.98.).

Table 1: Samples of sunflower seeds

Hybrid/line	Pathogen	Location	Year of sampling	Code
NS-H-26		Novi Sad	1977	H1.NS.97.
H-26	*P.helianthi*	Vukovar	1997	L1.Ph.V.97.
PH-BC-13	*P.helianthi*	Dunavac	1997	L2.Ph.D.97.
Line	*P.macdonaldii*	Irmovo	1997	L3.Pm.I.97.
Line	*P.helianthi*	Irmovo	1997	L4.Ph.I.97.
NS-H-26	*P.helianthi*	Novi Sad	1997	H1.Ph.NS.97.
Line	*P.helianthi*	Rakovac	1998	L5.Ph.Ra.98.
Line	*P.helianthi*	Beèej	1998	L6.Ph.B.98.
Hybrid	*P.macdonaldii*	Romania	1998	H2.Pm.R.98.
Hybrid	*P.helianthi*	Romania	1998	H2.Pm.R.98.
Line 1	*P.helianthi*	Beèej	1998	L7.Ph.B.98.
Line 2	*P.helianthi* and *P.macdonaldii*	Beèej	1998	L8.Ph.Pm.B.98.
Line 3	*P.helianthi* and *P.macdonaldii*	Sr. Karlovci	1998	L9.Ph.Pm.SK.98.
Line 4	*P.helianthi* and *P.macdonaldii*	Rakovac	1998	L10.Ph.Pm.Ra.98.
Line 5	*P.helianthi* and *P.macdonaldii*	Rakovac	1998	L11.Ph.Pm.Ra.98.
Line 6	*P.helianthi*	Futog	1998	L12.Ph.F.98.
Line 7	*P.helianthi*	Èereviæ	1998	L13.Ph.È.98.
Line 8	*P.helianthi* and *P.macdonaldii*	Èereviæ	1998	L14.Ph.Pm.È.98.
Line 9	*P.helianthi* and *P.macdonaldii*	Sr. Karlovci	1998	L15.Ph.Pm.SK.98.
Line 12	*P.helianthi* and *P.macdonaldii*	Neštin	1998	L16.Ph.Pm.N.98.
Line 20[3]	*P.helianthi*	Banoštor	1998	L17.Ph.B.98.
Line 21	*P.helianthi*	Nova Topola	1998	L18.Ph.NT.98.
Line 22	*P.helianthi*	Neštin	1998	L19.Ph.N.98.
Line 23	*P.helianthi*	Èereviæ	1998	L20.Ph.È.98.
Line 25	*P.helianthi*	Ledinci	1998	L21.Ph.L.98.
Line 27	*P.helianthi*	Bijeljina	1998	L22.Ph.Bi.98.
Line	*P.helianthi*	Sr. Karlovci	1998	L23.Ph.SK.98.
Line	*P.macdonaldii*	Sr. Karlovci	1998	L24.Pm.SK.98.
Line	*P.macdonaldii*	Èereviæ	1998	L25.Pm.È.98.
Line	*P.macdonaldii*	Rakovac	1999	L26.Pm.Ra.98.
Line	*P.helianthi*	Rakovac	1999	L27.Ph.Ra.98.

Pycnidia could be seen in the histological sections of the pericarp infected by *P.helianthi*. The pycnidia on the pericarp surface were slightly immersed in the epidermis. The number of the pycnidia per one half of the pericarp was small (2-3). Only those pycnidia which were full were seen to ooze the exudate. The dimensions of the pycnidia were 95.20-285.60 x 47.60-123.76 µm. The pycnidia were mostly round, and their necks, when present, were short, from 19.04 to 57.12 µm.

The pycnidia of *P.macdonaldii* were larger, 152.32-476.00 x 28.56-152.32 µm on the seeds of the lines and 133.28-323.68 x 38.08-104.72 µm on the seeds of the hybrid.

Samples from 1999

Seeds of the lines L26.Pm.R.99. and L27.Ph.R.99. were naturally infected with the pathogens. The percents of infection by *P.helianthi* and *P.macdonaldii* were 0.75% and 0.25%, respectively.

The position and dimensions of *P.helianthi* and *P.macdonaldii* pycnidia on the seeds collected during winter 1999 were similar to the samples from 1998, except that empty pycnidia of *P.helianthi* dominated in the former samples.

The percents of seeds infected by *P.helianthi* and *P.macdonaldii* were low, but not insignificant, considering thair destructivness. According to Lačok and Mihaljčević (1998), the percents of infection by *P.macdonaldii* and *P.helianthi* in different hybrids were 0-1.75% and 0-2%, respectively. The probability of simultaneous occurence of both pathogens was relatively low, for example, 5.50% in hybrid NS-H-27RM. In our experiments, the lines were more sensitive to the pathogens in comparison with the hybrids, the percent of infection by *P.helianthi* reaching 9.75% (L11.Ph.Ra.98.), and the percent of infection by *P.macdonaldii* reaching 3.25% (L10.Ph.Ra.98.).

The variability in the percent of infection among the samples seems to be due to genetic factors, because the morphological characteristics of seeds, according to Thomison *et al.* (1989), showed little reaction to the intensity of infection. They established, in experiments with soybean seeds, that the infection by *Phomopisis* was more frequent in seeds with etched coats than in the other types of seed, but the difference was only 5%. Thus they concluded that seed coat etching and the increased electrolyte leakage associated with it had little or no effect on the incidence or severity of seed infection by *Phomopsis*.

The histological sections showed that the pericarp was not considerably damaged at the places of formation of the reproductive structures of the pathogens. All layers remained unchanged, only the epidermis was damaged at the places of the pycnidia formation. This is in accordance with the results of Singh *et al.* (1977) who reported that all pericarp layers were completely formed by the time of infection occurrence and that the infection came from the outside.

Intensity of seed infection by *P.helianthi* is also influenced by the presence of phytomelanin layer in the pericarp. The chemical nature of this layer is not yet known, but it has been established that deposition of phytomelanin in the pericarp proceeds from the achene apex to the equator, the consequence being that the apex is more resistant to mechanical penetration of pathogens (Stafford *et al.*, 1984). The presence of phytomelanin in the apex explains the occurrence of pycnidia of the studied pathogens on the wider part of the seed.

The diameter of the pycnidia in the pericarp of the seeds infected by *P.helianthii* varied from 228.48 to 285.60 µm. These pycnidia were larger than those formed on the stem, which were 200 µm in diameter according to previous results (Muntañola-Cvetković *et al.*, 1989).

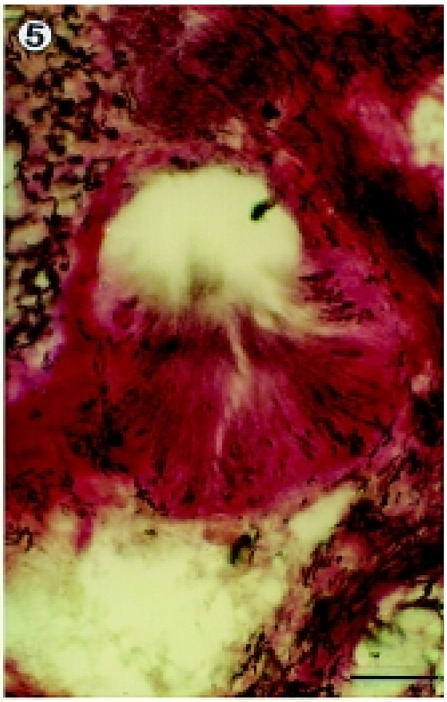
B: Changes of the kernel caused by *P.helianthi* and *P.macdonaldii*

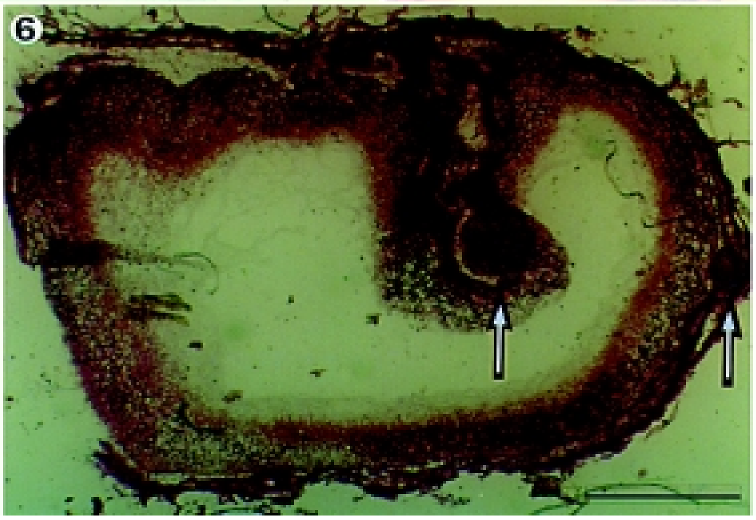
The kernels of all investigated samples showed changes in shape and color in the presence of the pathogens. The intensity of the changes varied in dependence of pathogen and susceptibility of the host.

The kernels infected by *P.helianthi* showed insignificant changes, *i.e.*, the tissue at the place of pycnidia formation became slightly darker. Histological sections showed that neither layer of the kernel suffered significant damage (Figure 4). The kernels of the lines were more damaged than the kernels of the hybrids. In the wider part of the infected cotyledons, parenchyma cells containing coagulated protoplasma in the center could be seen. Cell membranes and organelles were partly or completely destroyed by the pathogen, enabling the pathogen to ingest the simple and soluble compounds (Šutić, 1995). This process led to the formation of cytoplasmatic coloid particles and their gathering in the central part of the cell. In consequence to the presence of the pathogen in the upper and inner epidermis and in the outer layers of the cotyledon parenchyma, there occurred cells with black content. The dark or melanoid pigments are complex organic compounds formed by chinones binding with other cell compounds when plant cells or tissues are damaged, or by flavonoles from vacuoles of epidermal cells. Cell necrosis has a protective role, because the necrosed cells prevent the pathogen from further development (Šutić, 1995).

In the case of the line L6.Ph.B.98., round or pear shaped pycnidia containing β-conidia formed in the surface layers of cotyledon parenchyma, very rarely deeper (Figures 5 and 6). The pycnidia were usually in groups of 3 to 5, and their dimensions were 96-252 x 88-200 µm.

The kernels infected by *P.macdonaldii* were wrinkled and dark. Histological sections showed that the parenchyma and palisade tissue, especially of the cotyledons infected by the fungus, were extensively disintegrated (Figure 7). Numerous spherocrystals of inulin could be seen in the infected cotyledons. Inulin is a characteristic polyhexose present in the cytoplasm of plants belonging to the *Asteraceae* family. When water is removed from the cells, inulin acquires the form of spherocrystals with concentric layers and radial cracks (Figure 9). The presence of large spherocrystals is the consequence of the prolonged treatment of the seeds in FAA fixer, which contains 70% ethyl alcohol. Pycnidia arranged in rows could be seen in the outer layers of the cotyledon parenchyma (Figure 8). They differed in shape, number and the length of necks.



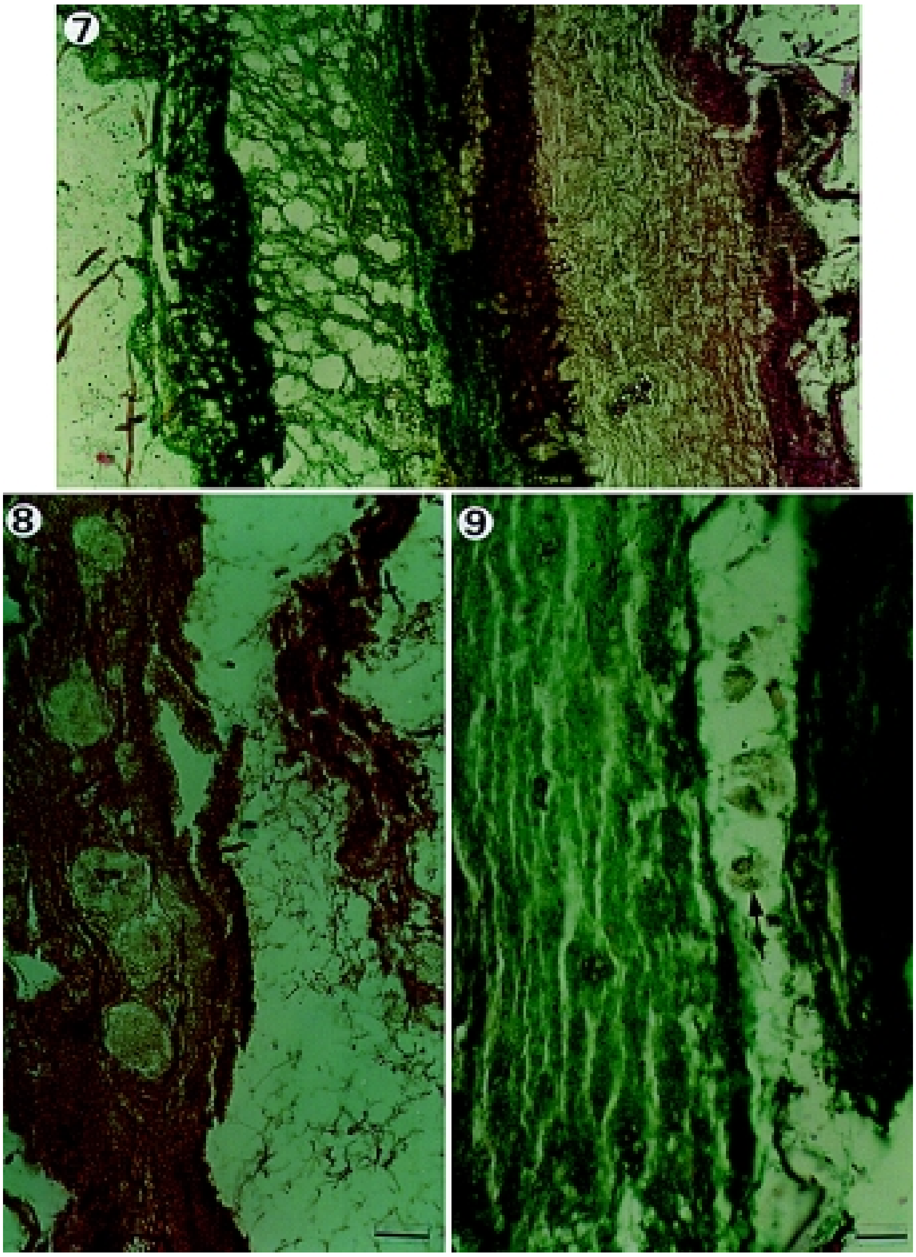


Cross section of seed infected by Phomopsis helianthi

Figure 4: Morphological change of cotyledon at the level of pycnidium formed at the pericarp (Bar=500 µm)

Figure 5: Half-empty pycnidium (Bar=500 µm)

Figure 6: Full pycnidia formed deep in the cotyledon and pycnidia below seed coat (Bar=500 µm)



Cross section of seed infected by Phoma macdonaldii

Figure 7: Desintegrated cotyledone tissue (Bar=500 µm)

Figure 8: Pycnidia in cotyledone (Bar=500 µm)

Figure 9: Spherocrystals of inulin (Bar=500 µm)

CONCLUSIONS

1. Pycnidia were formed on the pericarp of the seeds of all analyzed lines and hybrids infected by *P.helianthi* and/or *P.macdonaldii*. They were partly embedded in the epidermis, mostly at the wider part of the seed.
2. The pycnidia of *P.macdonaldii* were larger than those of *P.helianthi*.
3. The seeds infected by *P.helianthi* showed very slight structural changes. The damages were noticed as dark pigmentation, *i.e.*, cell necrosis, protoplasmatic coagulation and the formation of pycnidia. The exception were the seeds of line L6.Ph.B.98. which, on infection by *P.helianthi*, showed a complete disintegration of the central part of the cotyledon while numerous pycnidia formed in the outer layers of the parenchyma.
4. The seeds infected by *P.macdonaldii* were considerably damaged. The cotyledon tissue was disintegrated and pycnidia arranged in rows were formed in the outer layers of the parenchyma.

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DESARROLLO DE ORGANOS REPRODUCTIVOS DE *Phomopsis helianthi* Munt.-Cvet. *et al.* Y *Phoma macdonaldii* Boerema EN LAS SEMILLAS DE GIRASOL

RESUMEN

La investigacion de la posibilidad de desarrollo de organos reproductivos de los patogenos importantes del girasol, *Phomopsis helianthi* y *Phoma macdonaldii*, en el pericarpio y las semillas de las plantas de girasol naturalmente y artificialmente.

En el pericarpio de semillas de las lineas analizadas y los hibridos infectados por los hongos *P.helianthi* y/o *P.macdonaldii* se creaban picnidias en mas grande parte de semillas, parcialmente mojadas en la epidermis. Las picnidias de la especie de *P.macdonaldii* formadas en el pericarpio eran mas grandes que las picnidias de la especie *P.helianthi*.

Las pipas de semillas infectadas por la especie de *P.helianthi* mostraron menos grandes cambios estructurales con respecto a las pipas de semillas no infectadas. La excepcion eran las semillas de una linea infectada por la especie *P.helianthi* en la cual ocurrio la destruccion total de la parte central de cotiledon, y las picnidias numerosas se formaron en las capas externas. Las semillas de las lineas infectadas por la especie de *P.macdonaldii* eran considerablamente dañadas. El tejido de cotiledon fue destruido, y las filas de picnidias se formaron en las capas externas del parenquima.

DÉVELOPPEMENT DES ORGANES REPRODUCTEURS DU *Phomopsis helianthi* Munt.-Cvet. *et al.* ET DE *Phoma macdonaldii* Boerema SUR LES ACHÈNES DE TOURNESOL

RÉSUMÉ

Les possibilités de développement des organes reproducteurs du *Phomopsis helianthi* et du *Phoma macdonaldii*, importants pathogènes du tournesol, ont été étudiées sur le péricarpe et les semences de plantes de tournesol infectées naturellement et artificiellement.

Sur le péricarpe des achènes de toutes les lignes et hybrides infectés par le *P.helianthi* et/ou le *P.macdonaldii* analysés les pycnidies se sont formées dans la plus large partie des achènes et ils étaient en partie enfoncées dans l'épiderme. Les pycnidies du *P.macdonaldii* formés sur le péricarpe étaient plus grandes que les pycnidies du *P.helianthi*.

Les graines d'achènes infectées par le *P.helianthi* montraient moins de changements structuraux que les achènes non infectés. Il y a une exception: la partie centrale du cotylédon a été complètement désintégrée dans les achènes d'une ligne infectée par le *P.helianthi* et de nombreuses pycnidies se sont formées sur les couches extérieures. Les graines des lignes infectées par le *P.macdonaldii* ont été considérablement endommagées. Le tissu du cotylédon a été désintégré et des rangées de pycnidies se sont formées sur les couches extérieures du parenchyme.