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INFLUENCE OF NUTRITION MEDIA ON FORMATION OF PERITHECIA OF *Diaporthe helianthi* (*Phomopsis helianthi*) Munt.-Cvet.

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SUMMARY

Diaporthe helianthi isolates were grown on various media (water agar with various plant tissues, potatoes dextrose agar, malt agar and V-8 juice agar) with the aim to examine media influence on the development of reproductive structures. Isolates were distinguished by whether they form teleomorphic stage or not, and by the number of formed pycnidia and perithecia. Water agar with various plant parts was proved as a suitable media for perithecia production.

Key-words: Diaporthe helianthi, production of reproductive structures

INTRODUCTION

Artificial infections are a necessary method in breeding program and testing of hybrid resistance to sunflower cancer caused by *Diaporthe helianthi* Munt.-Cvet. et al. While choosing a method of artificial infection it is also necessary to determine pathogen reproductive structures the infection will be performed with. Phytopathologists and breeders put an emphasis on *in vitro* production of perithecia and ascospores for *D. helianthi* because the anamorphic stage produce only B conidia whose role in disease epidemiology still remains undefined. While studying ultrastructures of A and B conidia of *Phomopsis* species, Muntanola-Cvetković et al. (1985) determined that germination of A conidia was a common process for many *Phomopsis* species. Studying germination of B conidia within first two days of experiment, morphological changes were noticed in around 30% of conidia characterized by irregular enlargement along the conidia. In the most cases enlarged conidia disintegrated, but some of them exhibited mycelial threads on the fifth day of trial. Only in exceptional cases, those threads developed to form normal colonies. Over four-year long experiments only a small number of colonies were obtained from B conidia, therefore it is considered to be an exception. Comparison of ultrastructures A and B conidia showed that A conidia had numerous long cristae present in mitochondria, while B conidia had those in a small number. The presence of polysaccharides and reserve proteins in form of granules in vacuoles of A conidia and their absence in B conidia can be understood as a cause of their quick disintegration.
The best method to test the resistance of sunflower genotypes according to Mihaljčević and Muntanola-Cvetković (1989) was applying suspension of ascospores *D. helianthi* on intact tissue. As it is very hard to produce perithecia in greater amounts on artificial media, Vukojević et al. (1995) carried out experiments on various substrates. The authors used water agar (WA) with stems of 19 plants and Pseudomonas agar F

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(PAF) medium. PAF medium was also used in experiments by Assemat and Fayret (1987). The most abundant production of perithecia was obtained on WA with autoclaved stems of *Cichorium intybus*, *Lactuca serriola* and *Pulicaria vulgaris*. Perithecia occurred after 32 to 42 days.
Assemat and Fayret 1987 (cit. Vukojević et al., 1995) produced perithecia *D. helianthi in vitro* after microelements (Cu, Fe, Mg, Mn, Zn), amino acids (L-alanine, L-serine) and vitamins (thiamin and biotin) had been added to nutrition media. The aim of the present study was to determine influence of various substrates on the formation of pycnidia and perithecia of *D. helianthi* and to compare fertility potential of our isolate.

MATERIAL AND METHODS

Influence of nutrition media on the development of reproductive structures of *D. helianthi* isolates (23 isolates) were studied through growing fungus on various media. Autoclaved parts of various plants (green beans and soybean seeds, stems of sunflower, soybean, *Abutilon theophrasti*, *Xanthium strumarium, X. italicum*, *Arctium lappa*, *Achillea millefolium* and bark Elm) were added to water agar (WA). Length of plant tissue was 5-7cm. Plant material was autoclaved at 115ºC, under the pressure of 1.2 bar for 25 min. Autoclaved plant parts were put to WA. Inoculum of *D. helianthi* 15-days-old cultures grown on potato dextrose agar (PDA), 4 x 4 mm in size, was placed on plant parts. Cultures were kept in a thermostat at 24±1ºC under 12 h light/dark regime. Development of fungi was monitored over an 80-day-long period, over which abundance of pycnidia and perithecia, were assessed on the 6^{th}, 12^{th}, 20^{th}, 30^{th} and 40^{th} day. On WA with bark Elm media pycnidia and perithecia abundance was assessed on the 45^{th} day. PDA, malt agar (MA), and V-8 juice agar (V-8) were also used for determination of the above stated parameters.

RESULTS AND DISCUSSION

Isolates differed according to whether they formed teleomorphic stage or not, according to number of formed pycnidia and perithecia. Table 1 presents overview nutrition media influence on the development of fructification structures for three isolates *D. helianthi* because of their similar expression regarding fertility. These data refer to the period up to 45 days as that period exhibited the most intensive changes.
Number of days necessary for perithecia formation on particular media for all examined isolates (23) was presented in Table 2.
Characteristics of *D. helianthi* in a culture varied depending on nutrition media used for fungi growing. In a pure culture on PDA, MA and on V-8, fungus firstly formed sparse aerial mycelium which filled out Petri dish of 9 cm in diameter for 7-9 days. Later on, mycelium became lanate**,** white or dirty white in color, except on MA, where it was white and olive green, and in the course of its ageing did not change significantly. Mycelium was more compact around stromatic structures. In the beginning of their development cultures looked wet in their central part.
Mycelium on the WA was very sparse except on the WA with stems of *A. millefolium* where it was more compact. Mycelium was more dense on plant parts. The most abundant mycelium was determined on stems of *A. theophrasti* and sunflower, while it was weaker on stems of *A. millefolium*.
On PDA, MA and V-8 media, pycnidia occurred 5-12 days after inoculation, depending on the isolate. On WA with plant parts pycnidia started to form from 8^{th} to 12^{th} day. The highest number of pycnidia was determined on the following media: MA, soybean stems, *A. theophrasti* and soybean pods added to the basic substrate (WA). Pycnidia were solitary or in groups, and inside of them we found only B conidia. On PDA, MA and V-8 media, pycnidia was developed mostly in groups inside of stromatic formations. They were densely concentrated around inoculation place.
Thirty days after inoculation perithecia occurred on WA with stems of *Xanthium* sp. (6 isolates), soybean (4 isolates), and *A. lappa* (5 isolates). According to our results, isolates of *D. helianthi* (12 of 23 isolates examined) formed perithecia 30-50 days after inoculation regardless of the type of substrate. The highest number of isolates (11) formed perithecia on WA with bark Elm and it was the only substrate on which

Su11/04 and Su12/05 isolates formed perithecia. Isolates Su5/04, Su12/04 and Su8/05 formed perithecia 40 days after inoculation on PDA and MA, however, number of perithecia was very low.
Perithecia were formed mostly in groups, with exception of the WA with bark Elm and sunflower stems where they were formed separately. Average number of perithecia for all tested isolates was presented in Table 3. Regardless isolate, the highest number of perithecia was determined on soybean stems (Photo 2), *A. lappa* and *Xanthium* spp.

Table 1. Influence of nutrition media on development fructification structure for *D. helianthi* (Su15/05, Su35/05 i Su36/05)
Tablica 1. Utjecaj hranjive podloge na razvoj fruktifikacijskih organa za izolate D. helianthi (Su15/05, Su35/05 i Su36/05)

Nutrition media *Hranjiva podloga*	Day *Dan*	Mycelia colour *Boja micelija*	Presence of pycnidia *Nazočnost piknida*	Presence of perithecia *Nazočnost peritecija*
PDA	6. 12. 20. 30. 40.	white, dirty white */bijela, prljavo bijela*	+ + + + + + + + + + + +	- - - - -
MA	6. 12. 20. 30. 40.	white some parts olive green /*bijela, pojedini djelovi maslinasto zeleni*	+ + + + + + + + + + + + +	- - - - -
V-8	6. 12. 20. 30. 40.	white/*bijela*	+ + + + + + + + + + + +	- - - - -
WA with *Xanthium* sp. stems *VA sa stabljikama Xanthium sp.*	6. 12. 20. 30. 40.	white/*bijela*	- + + + + + + + + + +	- - - + + + + + +
WA with sunflower stems *VA sa stabljikama suncokreta*	6. 12. 20. 30. 40.	white/*bijela*	- + + + + + + + + + +	- - - - + +*
WA with soybean seeds *VA sa sjemenom soje*	6. 12. 20. 30. 40.	white/*bijela*	- + + + + + + +	- - - - -
WA with soybean pods *VA s mahunama soje*	6. 12. 20. 30. 40.	white/*bijela*	- + + + + + + + + + + +	- - - - +
WA with soybean stems *VA sa stabljikama soje*	6. 12. 20. 30. 40.	white/*bijela*	- + + + + + + + + + + + + +	- - - + + + + + +

Table 1. Continued
Tablica 1. Nastavak

Nutrition media *Hranjiva podloga*	Day *Dan*	Mycelia colour *Boja micelijskog pokrivača*	Presence of pycnidia *Nazočnost piknida*	Presence of perithecia *Nazočnost peritecija*
WA with bark Elm VA s korom brijesta	6. 12. 20. 30. **45**.	white/*bijela*	- - + + + + + +	- - - - + +
WA with *A. theophrasti* stems *VA sa stabljikama A. theophrasti*	6. 12. 20. 30. 40.	white/*bijela*	- + + + + + + + + + +	- - - - + +
WA with *A. millefolium* stems *VA sa stabljikama A. millefolium*	6. 12. 20. 30. 40.	white/*bijela*	- - + + + + + + + +	- - - - + +
WA with *A. lappa* stems *VA sa stabljikama A. lappa*	6. 12. 20. 30. 40.	white, dirty white/*bijela, prljavo bijela*	- + + + + + + + +	- - - + + + + + +

Legend:		*Legenda:*	
-	**not present**	-	*nema pojave*
+	**very few present**	+	*vrlo slaba pojava*
+ +	**weakly present**	+ +	*slaba pojava*
+ + +	**moderately present**	+ + +	*srednja pojava*
+ + + +	**abundantly present**	+ + + +	*jaka pojava*

***- isolate Su15/05 formed perithecia on the fortieth day, isolate Su35/05 formed perithecia on sunflower stem on the seventieth day and isolate Su36/05 did not form perithecia on that media**
**- izolat Su15/05 formirao je peritecije četrdeseti dan, izolat Su35/05 formirao je peritecije na agaru sa stabljikama suncokreta sedamdeseti dan, a izolat Su36/05 nije formirao peritecije na ovom supstratu*

Mihaljčević et al. (1980), Muntanola-Cvetković et al. (1981) determined perithecia formation on PDA when *D. helianthi* was isolated for the first time. Later on, Muntanola-Cvetković et al. (1988, 1996) stated that *D. helianthi* did not form perithecia *in vitro* on PDA. In our experiment, three isolates (Su5/04, Su12/04 and Su8/05) formed perithecia on PDA. However, only a small number of perithecia were formed on PDA and MA and they could not be used for perithecia production at artificial infections. Vukojević et al. (1995) reported that three isolates of *D. helianthi* produced perithecia on WA with stem parts of cultivated plants and weeds (19 plant species). However, there were isolates that did not form perithecia on artificial media under laboratory conditions (Muntanola-Cvetković et al. 1988, Viguie et al. 1999). Out of 23 isolates of *D. helianthi* in our experiment, 11 did not form perithecia on either substrate. Assemat and Fayret 1987 (cit. Vukojević et al. 1995), reported that they produced perithecia *in vitro* on media with microelements. Muntanola-Cvetković et al. (1988) and Vukojević et al. (1995) considered that this medium seems to be unsuitable for perithecia production. Aćimović (1998) also failed to obtain perithecia on nutrition media with microelements.
Aćimović and Štraser (1982) settled pycnidia formation for the Bački Petrovac isolate on the agar with various plant parts (fresh plants, stems and leaves of sunflower and soybean and soybean pods). Perithecia

formation was not determined, probably because the experiment period of 12 days was too short for development of teleomorfic structure.

Photo 1. Perithecia of *D. helianthi* on autoclaved *A. theophrasti* stem
Slika 1. Periteciji D. helianthi na autoklaviranim stabljikama A. theophrasti

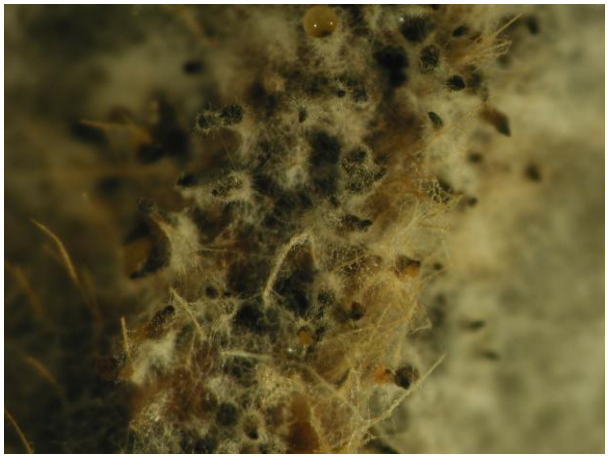

Photo 2. Perithecia of *D. helianthi* on autoclaved soybean stem
Slika 2. Periteciji D. helianthi na autoklaviranim stabljikama soje

Table 2. Time taken for perithecia production depending on isolate and nutrition media
Tablica 2. Broj dana potrebnih za formiranje peritecija D. helianthi ovisno o izolatu i supstratu- (not determined)
- (nisu utvrđeni)

Isolate Izolat	WA with plant parts /*VA uz dodatak biljnih dijelova*								
	stems/ *stabljike Xantium* sp.	sunflower stems/ *stabljike suncokreta*	soybean seeds/ *sjeme soje*	soybean pods/ *mahune soje*	soybean stems/*stabljike soje*	bark Elm/*kora brijesta*	stems/ *stabljike A. theophrasti*	stems/ *stabljike A. millefolium*	stems/ *stabljike A. lappa*
Su35/05	30	70	-	40	30	45	35	38	30
Su36/05	30	-	-	40	30	45	38	38	30
Su44/05	40	-	-	-	38	45	40	40	38
Su42/05	-	-	-	-	-	-	-	-	-
Su43/05	-	-	-	-	-	-	-	-	-
Su8/05	30	38	-	38	30	42	38	40	30
Su12/05	-	-	-	-	-	50	-	-	-
Su7/05	30	38	-	40	30	42	35	35	45
Su4/05	-	-	-	-	-	-	-	-	-
Su20/05	-	-	-	-	-	-	-	-	-
Su14/05	-	-	-	-	38	-	-	38	38
Sj Su	-	-	-	-	-	-	-	-	-
Su3/04	38	-	-	40	45	45	38	45	38
Su11/04	-	-	-	-	-	50	-	-	-
Su4/04	-	-	-	-	-	-	-	-	-
Su7/04	-	-	-	-	-	-	-	-	-
Su5/04	30	38	-	40	38	42	38	35	30
Su8/04	-	-	-	-	-	-	-	-	-
Su9/04	-	-	-	-	-	-	-	-	-
Su12/04	35	-	-	40	38	45	40	38	35
Su15/05	30	38	-	40	30	45	35	38	30
Su3/06	-	-	-	-	-	-	-	-	-
Su11/06	-	-	-	-	-	-	-	-	-

Table 3. Number of perithecia depending on nutrition media
Tablica 3. Brojnost peritecija ovisno o supstratu

Grade/*Ocjena*	WA with plant parts /*VA s biljnim dijelovima*
3	stems /*stabljike*: *A. lappa*, *Xanthium* sp. , soybean /*soja*
2	stems /*stabljike*: *A. millefolium* , *A. theophrasti*, soybean pods/ *mahune soje*
1	bark Elm/*kora brijesta*, sunflower stem /*stabljike suncokreta*

1-small number of perithecia (less than 5 perithecia on plant part)
2-moderate number of perithecia (perithecia irregularly covered plant parts)
3-abundant number of perithecia (plant parts uniformly covered with great number of perithecia)

1-mali broj peritecija (manje od 5 peritecija po biljnom dijelu)
2-osrednji broj peritecija (periteciji neravnomjerno prekrivaju biljne dijelove)
3-veliki broj peritecija (biljni dijelovi ravnomjerno prekriveni velikim brojem peritecija)

Vukojević et al. (1995) assumed usage of autoclaved stem parts to be simple and efficient method in perithecia production, which is also confirmed in our experiment. Previous studies (Vukojević, 1989, cit. Vukojević et al., 1995) resulted with the conclusion that perithecia did not form on WA with pounded stems fragment. It was assumed that intact stems, on which mycelia developed, stimulated formation of perithecia. Since in our experiment the highest number of perithecia was obtained on autoclaved stems of soybean, *A. lappa* and *Xanthium* sp., usage of that plant parts is recommended for production of perithecia, however, it should be kept in mind that there are some isolates which do not form perithecia *in vitro*.

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UTJECAJ HRANJIVE PODLOGE NA FORMIRANJE PLODIŠTA IZOLATA *Diaporthe helianthi* (*Phomopsis helianthi*) Munt.-Cvet.

SAŽETAK

Izolate Diaporthe helianthi uzgajali smo na različitim supstratima (vodeni agar uz dodatak tkiva različitih biljnih vrsta, krumpir dekstrozni agar, maltz agar i podloga s dodatkom soka rajčice) kako bismo ispitali utjecaj supstrata na razvoj reproduktivnih struktura. Izolati se razlikuju prema tome formiraju li teleomorfni stadij ili ne te prema broju formiranih piknida i peritecija. Vodeni agar s dodatkom pojedinih biljnih dijelova pokazao se kao pogodan medij za produkciju askusa i askospora.

Ključne riječi: Diaporthe helianthi, produkcija reproduktivnih struktura

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