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In vitro production of pycnidia by Septoria tritici

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Abstract

The in vitro production of pycnidia by *Septoria tritici* was examined on six media reported to induce the formation of fruiting bodies. Among 26 freshly isolated cultures from various parts of the world, consistent differences in growth type were found which were only partially influenced by nutritional and environmental conditions. Cultures with yeast-like growth produced hardly any pycnidia or pseudopycnidia, while cultures with intermediate or mycelial growth types produced them frequently. Incubation in continuous darkness induced intermediate to mycelial growth types rather than yeast-like growth types in some cultures, and concomitantly the production of more pycnidia. Potato-dextrose agar induced intermediate to mycelial growth types and production of (pseudo)pycnidia more often than V8 agar and wheat leaf extract agar, which had previously been reported to be especially beneficial to (pseudo) pycnidium formation by *S. tritici*. Isolates with a consistently yeast-like growth type, producing (virtually) no fructifications under any of the experimental conditions, were slightly stimulated to form pseudopycnidia on water agar supplemented with sterile pieces of maize, wheat or carnation leaves.

Additional keywords: speckled leaf blotch, wheat, growth type, pseudopycnidia.

Introduction

The fungus *Septoria tritici* Rob. ex Desm. (perfect state: *Mycosphaerella graminicola* (Fückel) Schroeter) is the causal organism of speckled leaf blotch, one of the most important diseases of wheat (Eyal et al., 1987; King et al., 1983; Shipton et al., 1971). Under natural conditions the fungus reproduces by means of sexual and asexual spores which are formed in fruiting bodies embedded in the host tissue and named pseudothecia and pycnidia, respectively (Eyal et al., 1987). On artificial media, both ascospores and pycnidiospores may give rise to either yeast-like or mycelial colonies (Pierobom, 1983). Pink, yeast-like colonies develop through a continuous budding process with the formation of conidia and short hyphae, whereas mycelial colonies consist of dark filamentous mycelium, which only occasionally supports the formation of conidia (Annone, 1984; Hilu and Bever, 1957; Scott et al., 1988; Shearer et al., 1974; Weber, 1922).

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Unlike other species of *Septoria*, *S. tritici* has never been reported to produce pseudothecia on artificial media, and in general does not form pycnidia. It rather produces secondary conidia from germinating pycnidiospores or from very young mycelium (Hilu and Bever, 1957). Therefore, the preservation of *S. tritici* on artificial media, such as malt-yeast agar or potato-dextrose agar, is not convenient since continual subculturing of the fungus may change the characteristics of an isolate (Shearer et al., 1974) which may eventually lead to a reduction in virulence (Eyal et al., 1987; Zelikovitch and Eyal, 1989). To avoid this problem, *S. tritici* isolates may either be maintained by lyophilization, in a preservative mix of conidia with sterile soil (Shearer et al., 1974) or by means of dried wheat leaves bearing pycnidia (Eyal et al., 1987).

Infrequent production of pycnidia and pycnidia-like or stroma-like structures by isolates of *S. tritici* on various artificial media has been reported previously (Djerbi et al., 1974; Hilu and Bever, 1957; Lee and Jones, 1974; Weber, 1922). Abundant production of pycnidia by *S. tritici* on Czapek Dox-V8 and plain V8 agar was observed by Cooke and Jones (1970) and Benedict (1971), respectively. Water agar supplemented with sterile plant material was used to stimulate the in vitro formation of fructifications of *S. nodorum* and *Fusarium* spp. (M. Cunha Fernandez, EMBRAPA, Passo Fundo, personal communication; Fisher et al., 1982). The addition of wheat leaves or leaf extracts is reported to be beneficial to pycnidium formation by *S. tritici* (Pierobom, 1983; Zelikovitch and Eyal, 1989). Maintenance on wheat leaf extract agar (WLE) was considered therefore to be an easy and safe alternative for the long-term preservation of *S. tritici* isolates. Loss of virulence by subculturing and the time-consuming procedures of the aforementioned preservation methods could then be avoided (Zelikovitch and Eyal, 1989).

The present study was performed to evaluate some of the media and environmental conditions reported to be conducive to the abundant production of fruiting bodies by *S. tritici* and other fungi.

Materials and methods

Isolates. Twenty-six isolates, originating from Argentina (5), Ethiopia (7), Israel (2), Kenya (1), the Netherlands (1), Syria (1), Turkey (8) and Uganda (1), were included in this investigation. Isolate IPO8042 was kindly provided as a slant culture by Prof. Z. Eyal from Tel Aviv University, Israel. The other isolates were obtained from the IPO-collection, which is maintained in dry wheat seedling leaves, colonized with monopycnidial cultures.

Media. Six artificial media were compared; (1) Campbell's® V8 vegetable juice agar (V8; juice : water = 1 : 1, v/v; 2.5% Oxoid L13 agar), (2) potato-dextrose agar (PDA; Oxoid-CM 139), (3) wheat leaf extract agar (WLE1: 30 g and WLE2: 240 g wheat leaf extract prepared from ca. 120 g seedling leaves of cv. Lakhish, blended in 300 ml demineralized water; Zelikovitch and Eyal, 1989), (4) water agar (Oxoid L13) supplemented with sterile pieces of maize leaves (WA-M) (M. Cunha Fernandez, EMBRAPA, Passo Fundo, personal communication), (5) water agar with sterile pieces of wheat leaves (WA-W) (Pierobom, 1983) and (6) water agar with sterile pieces of carnation leaves (CLA) (Fisher et al., 1982).

Experimental procedures and conditions. Inocula were obtained from wheat seedling leaves bearing pycnidia, after an incubation period of approximately one hour in Petri dishes containing wet filter paper, ensuring the high relative humidity necessary to induce oozing of spores by the pycnidia. For each isolate, a cyrrhus from a single pycnidium was collected under a stereo microscope, and subsequently transferred to malt-yeast agar. The cultures were grown for five days in small plastic Petri dishes (5 cm ∅) at 18-20 °C under continuous exposure to fluorescent light (1.10^3 Lux) prior to inoculation of the media under study. The inoculated plates were incubated at 18 °C and either exposed to light (L; 1.10^3 Lux provided by two Philips TL 8W/33 H8 fluorescent tubes) for 16 h per day, or kept in continuous darkness (D). All isolates were investigated in duplo on V8, WLE1 and PDA under both conditions in two experiments (eight isolates were tested three times). Additionally two sets of nine isolates were used in an experiment which included either WA-M and WA-W or WLE1, WLE2 and CLA.

Evaluation of colony morphology and occurrence of pycnidia. Development of the colonies and pycnidium production was examined regularly for six weeks, with a final observation after several months. Pycnidia were only identified as such if they oozed cyrrhi containing pycnidiospores. True pycnidia were generally found along with non-oozing structures that resembled them. Such structures are at present considered to be developmentally arrested pycnidia and will be referred to as pseudopycnidia (Pierobom, 1983; Djerbi et al., 1974). The occurrence of pycnidia and pseudopycnidia was classified in four categories: absent, scarce, moderate or abundant. The growth types of cultures were assessed as yeast-like, intermediate or mycelial. We considered the growth type to be yeast-like if the fungus produced pinkish, smooth and shiny colonies with, at the most, scarce hyphal development at its periphery. Intermediate growth types were characterized by a dark, smooth surface with extensive hyphal development from the edges of the colonies. Mycelial growth types were characterized by dark grey colonies with extensive hyphal development all over the surface (sometimes culminating in aerial mycelium).

The influence of the media, exposure to light and continuous darkness on the growth type and production of pycnidia and pseudopycnidia was examined with non-parametric statistical analyses, viz. the tests of Kruskal-Wallis and Wilcoxon corrected for ties (Sokal and Rohlf, 1969).

Results

Eight of the 26 isolates were consistent in growth type, regardless of the applied experimental conditions (Table 1). The growth type of IPO87023, IPO88004, IPO88013, IPO88021, IPO88016, IPO88017 and IPO88038 was always yeast-like, and that of IPO88039 mycelial. However, the growth type of the majority of the isolates depended on the media and the applied light conditions in a generally consistent way. Growth on PDA induced intermediate and mycelial growth types of more isolates than growth on V8 and WLE1, while growth in continuous darkness significantly increased the frequency of intermediate, rather than yeast-like colonies (Table 2). The formation of fructifications was not consistently the same for all isolates under any of the experimental conditions (Table 1). The occurrence of (pseudo)pycnidia was strongly asso-

Table 1. Growth types of *Septoria tritici* isolates on PDA, V8, WLE1 under intermittent light exposure (L, 16 h) and in continuous darkness (D), and the occurrence of pycnidia and pseudopycnidia in colonies of these isolates.

Isolates	Growth type[1]						Occurrence of (pseudo)pycnidia											
	PDA		V8		WLE1		PDA				V8				WLE1			
	L	D	L	D	L	D	L		D		L		D		L		D	
							py[2]	*ps*	*py*	*ps*	*py*	*ps*	*py*	*ps*	*py*	*ps*	*py*	*ps*
IPO86063	i(y)[3]	m(i)	y	i(m)	y(i)	y(m	0[4]	2	2	2	0	1	1	1	0	0	1	1
IPO86068	i	i	y	y(i)	y(m)	y(m)	0	0	1	2	1	0	0	0	0	0	0	0
IPO86078	y(m)	i(y)	y	y	y	y	0	0	1	1	0	0	0	0	1	1	0	0
IPO87022	y	i(m)	y	y	y	y	0	0	0	1	0	0	0	0	0	0	0	0
IPO87023	y	y(i)	y	y	y	y	0	0	0	0	0	0	0	0	0	0	0	0
IPO88004	y	y	y	y	y	y	0	0	0	0	0	0	1	0	0	0	0	0
IPO88010	y	i	y	y	y	y	0	0	2	0	0	0	0	0	0	1	0	1
IPO88012	y	i	y	y	y	y	0	0	0	3	0	0	0	0	0	0	0	0
IPO88013	y	y(i)	y	y	y	y	0	0	0	0	0	0	0	0	0	0	0	1
IPO88018	i	y(i)	y	y	y(m)	y	0	0	1	1	0	0	0	0	0	0	0	0
IPO88021	y	y	y	y	y	y(i)	0	0	0	0	0	0	0	0	0	0	1	2
IPO88022	y	i	y	y	y	y	0	0	0	0	0	0	0	0	0	0	0	0
IPO86036	i	i	y	y(i)	y(m)	y(m)	0	0	1	1	0	0	0	0	0	0	0	0
IPO 8042	y	m	y	y	y	y	0	1	0	0	0	0	0	0	0	0	0	0
IPO86026	y	i	y	y	y	y	0	0	1	3	0	0	0	0	0	0	0	0
IPO89011	i	i	y	y	y	y	0	0	0	0	0	0	0	0	0	0	0	1
IPO88039	m	m	m	m	m(i)	m(i)	1	3	1	3	1	1	1	1	2	1	2	1
IPO86009	m	i(m)	y	y(i)	y	y	0	1	0	1	0	0	0	0	0	0	0	0
IPO86010	m	i(m)	y	i(y)	y(i)	y	1	3	2	3	1	0	2	1	0	0	0	0
IPO86013	y	i(y)	y	y(i)	y	y	0	0	0	0	0	0	0	0	0	0	0	0
IPO86022	i	i(m)	y	i(y)	y	y(i)	0	3	1	2	0	0	0	1	0	0	0	1
IPO86023	m	m	y	i	y(m)	y(m)	0	3	0	3	0	0	0	1	0	0	0	1
IPO88014	i	i(m)	y	y	y	y	0	0	0	2	0	0	0	0	0	0	0	0
IPO88016	y	y(i)	y(i)	y(i)	y	y	0	0	0	0	0	0	0	1	0	0	0	0
IPO88017	y	y	y	y	y	y	0	0	0	0	0	0	0	0	0	0	0	0
IPO88038	y	y	y	y	y	y	0	0	0	0	0	0	0	0	0	0	0	0

[1] y: yeast-like; i: intermediate; m: mycelial growth type.
[2] *py*:pycnidia; *ps*: pseudopycnidia.
[3] Less commonly occurring growth type between parentheses.
[4] 0: absent; 1: scarce; 2: moderate; 3: abundant.

ciated with intermediate and mycelial types of growth (Table 3). A similar association was found for growth on PDA compared with growth on V8 and WLE1, as well as incubation in continuous darkness versus incubation with light exposure (Table 2). This could be expected since these conditions were conducive to the development of colonies with an intermediate or mycelial type of growth. Cultures grown on PDA in darkness indeed produced larger amounts of (pseudo)pycnidia than cultures incuba-

Table 2. Frequency distributions of 26 *S. tritici* isolates over three cultural growth types and over four abundancy categories of pycnidia and pseudopycnidia, in relation to the applied media (calculation irrespective of light conditions), and light conditions (calculation irrespective of media). Similar frequency distributions are followed by the same letter (columnwise comparison only; $\alpha = 0.05$).

	Growth type[1]			Occurrence of pycnidia[2]				Occurrence of pseudopycnidia[2]			
	y	i	m	0	1	2	3	0	1	2	3
Medium											
PDA	23	21	8 b	40	9	3	0 b[3]	31	7	5	9 b
V8	46	4	2 a	45	6	1	0 ab	44	8	0	0 a
WLE1	50	0	2 a	47	3	2	0 a	41	10	1	0 a
Light conditions											
16 h exposure	65	7	6 a[4]	71	6	1	0 a	66	7	1	4 a
0 h exposure	54	18	6 b	61	12	5	0 b	50	18	5	5 b

[1] y: yeast-like; i: intermediate and m: mycelial growth type.
[2] 0: absent; 1: scarce; 2: moderate; 3: abundant.
[3] Based on Wilcoxon test, although the overall Kruskal-Wallis test was not significant.
[4] $P = 5.88\%$.

Table 3. Frequency distributions of 26 *S. tritici* isolates over four abundancy categories of pycnidia and pseudopycnidia in relation to the cultural growth types of the isolates under the experimental conditions (application of PDA, V8 and WLE1 under intermittent light exposure or continuous darkness). Similar frequency distributions are followed by the same letter (columnwise comparison only; $\alpha = 0.001$).

Growth type[1]	Occurrence of pycnidia[2]				Occurrence of pseudopycnidia[2]			
	0	1	2	3	0	1	2	3
y	112	7	0	0 a	106	11	1	0 a
i	16	6	3	0 b	9	9	4	4 b
m	4	5	3	0 b	1	5	1	5 b

[1] y: yeast-like; i: intermediate and m: mycelial growth type.
[2] 0: absent; 1: scarce; 2: moderate; 3: abundant.

ted under any other condition (Table 1). Pycnidia were mainly observed at the periphery of the colonies, but in some isolates with an intermediate growth type cyrrhi also occurred on top of large stromata as observed by Djerbi et al. (1974). Pseudopycnidia generally outnumbered pycnidia and showed a similar association with the growth types of the isolates, the light conditions applied and the media involved in this study (Tables 2 and 3).

Table 4. Frequency distributions of nine *S. tritici* isolates over four abundancy categories of pycnidia and pseudopycnidia in relation to the applied media (calculation irrespective of light conditions). Similar frequency distributions are followed by the same letter (columnwise comparison only; $\alpha = 0.001$).

Medium	Occurrence of pycnidia[1]				Occurrence of pseudopycnidia[1]			
	0	1	2	3	0	1	2	3
PDA	14	3	1	0 a	14	2	1	1 a
WA-M	12	5	1	0 a	0	14	4	0 b
WA-W	13	4	1	0 a	0	17	1	0 b

[1] 0: absent; 1: scarce; 2: moderate; 3: abundant.

In our experiments WLE1 was no more beneficial to pycnidium formation than the other media. Neither was a more concentrated variant (WLE2; data not shown). Finally, nine isolates (IPO86068, IPO86078, IPO87022, IPO88010, IPO88021, IPO86026, IPO89011, IPO88017 and IPO88038) were also tested on WA-W and WA-M. The last two had a consistently yeast-like growth type on PDA, V8 and WLE, which never favoured pycnidium formation. The production of pseudopycnidia on WA-W and WA-M was scarce but significantly better than on PDA; pycnidium production was not stimulated, however (Table 4). Similar tests on CLA revealed comparable results (data not shown).

Discussion

In the present study the growth types of isolates could only be slightly influenced by the conditions used. The production of pycnidia and pseudopycnidia was strongly associated with the growth type. Cultures with a yeast-like growth type hardly, if ever, produced pycnidia or pseudopycnidia in contrast to isolates with an intermediate or mycelial type of growth. This observation is supported by the data of Cooke and Jones (1970) and Scott et al. (1988). The former authors used an isolate with a blackish grey stroma-like growth type and obtained pycnidia on PDA, malt-extract agar and oatmeal agar. However, since no cyrrhi were noticed, these structures were most probably similar to what we defined as pseudopycnidia. True pycnidia, exuding cyrrhi were only observed on Czapek Dox-V8-agar. This medium was also used by the latter authors who described a yeast-like growth type for the five studied isolates, without any pycnidium formation. Cooke and Jones (1970) exposed the aforementioned isolate to continuous near-ultraviolet (NUV) light in order to induce the formation of fruiting bodies, whereas Scott et al. (1988) did not expose their isolates to NUV. The observed pycnidium formation in Cooke and Jones' study was probably not at all due to exposure to NUV, however, but merely to the growth type of the isolate involved. Benedict (1971) used V8-agar, exposed his cultures, as in the present study, to fluorescent light (1.10^3 Lux, 16 h day^{-1}) and obtained pycnidia just like Cooke and Jones (1970). This is not surprising, however, since the latter authors supplied the isolate, which was most probably identical to the isolate with the blackish grey stroma-like

growth type used in their own experiments. Polymorphism of *S. tritici* isolates has indeed been reported previously (Djerbi et al., 1974; Pierobom, 1983), and manipulation of the growth types appears possible only to a limited degree (present data). This provides an explanation for our observation that pycnidium production was not consistently high under any of the experimental conditions in our study.

The observed stimulative effect of growth in darkness on (pseudo)pycnidium production is in accordance with Pierobom (1983) who noticed a slightly better formation of these structures under intermittent than under continuous exposure to light. Zelikovitch and Eyal (1989) also did not expose their experiments to light. Unlike the results of Benedict (1971) and Cooke and Jones (1970), isolates on V8 generally had a yeast-like growth type in our study, particularly when exposed to light, and V8 thus appeared not to be at all beneficial to pycnidium formation. This is in accordance with Hilu and Bever (1957) and Shearer et al. (1974) who found plain V8 agar, or mixtures of it, to be most successful for regular spore production, simply because of the development of yeast-like colonies.

Contradictions between our data and the results of others are probably largely due to the limited number of isolates involved in those investigations. Hence, natural variation of *S. tritici* as observed in the present study could not be taken into consideration. Zelikovitch and Eyal (1989) studied the preservation of virulence with three isolates, of which IPO86036 and IPO8042 were also included in the present study. Abundant pycnidium formation by these isolates on WLE1 could not be confirmed, however, and only occurred to a limited extent in four other isolates. The application of WLE as an aid in preserving *S. tritici* isolates therefore seems only to be valid for particular isolates.

Although intermediate and mycelial growth types were strongly associated with the development of pycnidia, such growth types did not assure production of these structures (e.g. IPO89011 and IPO86023). Compared with PDA, WA-M and WA-W appeared to have an aspecific stimulative effect on the formation of pseudopycnidia in particular, but pycnidium production was not significantly better on these media. Since we considered pseudopycnidia to be developmentally arrested true pycnidia, optimalization of WA-M and WA-W might still offer prospects for enhanched pycnidium formation by *S. tritici* in vitro. In general, however, this approach is severely restricted by the limited possibilities for manipulating the growth type of *S. tritici* isolates.

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