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THE ROLE OF *PHYTOPHTHORA INFESTANS* OOSPORES IN PRIMARY INFECTION OF POTATO FOLIAGE IN POLAND

ABSTRACT

The study on oospore formation ability under natural conditions was carried out on the base of comparative characterization of four local populations of *P. infestans*. It was found that oospores can be formed in potato tissues under natural conditions. The comparison of proportion of both mating types isolates, high level of race complexity and racial diversity indicates, that oospores in experimental fields could play a role as a source of primary inoculum in three experimental localities in Boguchwała, in Przychojec and in Olesno Śląskie.

The oospores were produced also in all combinations of pot and field experiments. They were formed most abundantly, when the potato plants were inoculated with mixed inoculum consisted of A1 and A2 spores, in 1:1 ratio and significantly less abundantly, when the inoculum was dominated by one of mating type isolate. The effect of time of subsequent inoculation with A1 or A2 isolates on oospore formation was observed: significantly more oospores were formed when the plants were inoculated with both mating types at the same time.

Key words: oospores, *Phytophthora infestans*, potato, primary inoculum, race characteristics.

INTRODUCTION

In 1988–1998 a tendency towards the occurrence of more complex *Phytophthora infestans* pathotypes has been observed in Poland, which exhibited a broad spectrum of virulence and very high level of aggres–siveness to potato (Sujkowski *et al.* 1994, Zarzycka and Sobkowiak 1996, 1997a). A tendency towards the increase of expression of rare virulence factors was also found. The A2 mating type isolates of *P. infestans* have been detected in Poland since 1988 (Sujkowski *et al.* 1994). In the last years the occurrence of A2 mating type isolates was reported on the whole territory of our country, however its frequency was lower than that of A1 isolates and was variable in succeeding years (Zarzycka and Sobkowiak 1997b, 1997c). The comparison of virulence spectrum and the aggressiveness level of both mating types showed that A1 and A2 isolates did not differ significantly (Zarzycka and Sobkowiak 1997b).

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A2 mating type appearance was not more threatening for potato, than A1 did, but it can play a role in sexual reproduction of the pathogen. It was found that oospores were formed after artificial inoculation in potato leaves and stems both under laboratory and field conditions. After overwintering in the field the oospores were viable and infectious to potato leaves in laboratory bioassay (Zarzycka and Sobkowiak 1997a). The existence of both mating types can permit sexual reproduction and formation of oospores, which might be, besides the diseased tubers, a second source of primary inoculum. It is still unknown to what extent surviving oospores contribute to the development of late blight epidemics. It is considered, that sexual reproduction of *P. infestans* is connected with the ratio near 1:1 of both mating types in the population and with high genetic and phenotypic race diversity and complexity (Turkensteen *et al.* 1996, Hermansen and Amundsen 1996, Rivera–Peńa 1995).

Goodwin (1997) searched the mating biology of heterothallic *Phytophthora* species: why both mating types can apparently coexist in some areas without sexual recombination. He supposed that clones of an opposite mating type were genetically incompatible and had different host preferences what limited their contact.

The aim of the presented research is:

1). The assessment of oospore formation ability under natural conditions on the base of comparative characterization of local populations of *P. infestans*.

2). The search on determination of non–genetic factors, which can limit sexual recombination: the effect of various ratios of A1: A2 isolates and the effect of time of subsequent inoculation with A1 or A2 mating types.

MATERIAL AND METHODS

Comparative characterization of local populations of *P. infestans*

Experiments were carried out in 1999–2000 on four experimental potato fields on which Black's differentials possessing single genes from R1 to R11 were planted:

– in Boguchwała, podkarpackie province,
– in Przychojec, podkarpackie province,
– in Olesno Śląskie, śląskie province,
– in Młochów, mazowieckie province.

Observations of infection on crop plants and differentials were performed six times during the epidemics, this beeing evaluated according to scores of Cruickshank *et al*. (1982), starting at the moment when the first late blight symptoms appeared. From each field 20 samples of infected plants were collected twice: at the beginning and at the end of epidemics. The collected isolates of *P. infestans* were characterized with respect to virulence, aggressiveness and mating type. A range of virulence of isolates was assessed using leaflet test (Zarzycka and

Sobkowiak 1997b) on leaflets of Black's differentials possessing single genes of specific resistance from R1 to R11. The average complexity of races was presented as C_i index (Andrivon 1994a) expressed by a mean number of virulence factors per one isolate and calculated according to the formula:

$$C_i = \sum_j (p_j v_j), \quad j = 1 \ldots N_p$$

where:
v_j – number of virulence factors for a given race,
p_j – frequency of occurrence of race j within the population,
N_p – number of races in population

A significance of differences related to complexity of races was determined by analysis of variance on data transformed to angular degrees corresponding to binomial proportion, according to Freeman–Tukey transformation.

Race diversity was determined using Shannon index (Andrivon 1994a) calculated according to formula:

$$H_s = -\sum_j (p_j \ln p_j), \quad j = 1 \ldots N_p$$

where:
p_j – frequency of occurrence of race in the population,
N_p – number of races identified in the population,
ln – natural logarithm.

The level of aggressiveness was identified using leaflet test on cultivars Bintje and Tarpan not possessing R–genes. A 9–grade scale of infection described by Zarzycka and Sobkowiak (1997b) was used, where grade 1 corresponds with a totally infected leaflet. The level of aggressiveness of isolates collected at different time was compared by analysis of variance.

The mating types were determined after pairing tested isolates on rye agar together with known isolate A1 and A2.

The samples of diseased potato leaflets and stems collected at the end of epidemics were placed on the wet wood wool at 10°C, under illumination intensity of about 1600 luxes. The oospore formation was assessed microscopically.

Effect of various ratios of A1: A2 isolates in an inoculum on oospore formation

Two laboratory experiments on oospore formation were carried out on detached leaflets of susceptible cultivar Tarpan in March and June 1999. Three isolates of *P. infestans* from Młochów collection and one American clonal lineage US–8 (characterized by Goodwin *et al.* 1995) were used in the experiments. Characterization of *P. infestans* isolates is presented in Table 1.

Table 1
Characteristics of *P. infestans* isolates used in experiments on oospore formation on detached leaflets of cultivar Tarpan

Collection number	Mating type	Virulence	Aggressiveness
MP 322	A1	1.2.3.4.6.7.8.10.11	1.5[1]
MP 382	A1	1.2.3.4	1.0
MP 417	A2	1.2.3.4.7.10.11	1.0
US8	A2	1.2.3.4.6.7.8.10.11	1.0

[1] aggressiveness according to 1–9 grade score where 1 = most aggressive

The following ratios of A1: A2 isolates – 1:1, 1:10, 1:20, 1:30 and opposite combinations 10:1, 20:1 and 30:1 were used for mixed inoculum. The final concentration of inoculum was 50 sporangia/1mm^3. Two combinations of pairing isolates MP 322 x US–8 and MP 382 x MP 417 were applied in mixed inoculum. The inoculated leaflets were placed on Petri dishes on wet wood–wool, two leaflets per dish, in two replications. The intensity of oospore formation was assessed microscopically after 20 days of incubation at 10˚C under light intensity of about 1600 luxes, using 3–grade scale as single, abundant and very abundant.

In the pot experiments carried out in 1999–2000, two plants of susceptible cultivar Tarpan and resistant cultivar Bzura were sprayed with mixed inoculum at the rations 1:1, 1:5, 1:10, 1:30 (and opposite combinations: 5:1, 10:11 and so on) for both A1 and A2 isolates. The mixed inoculum at rations 1:80 and 80:1 were used additionally in 2000. Two combinations of pairing isolates MP 322 × US–8 and MP 322 × MP 417 were applied. Plants after inoculation were covered with plastic tent for 12 hours and incubated at 16˚C.

In the field experiments cultivars Tarpan and Bzura were planted in four plant plots in two replications at Młochów in 1999–2000. The plants were sprayed with mixed inoculum at the following ratios: 1:1, 1:5, 1:10, 1:20, 1:40, 1:80 (and opposite combinations: 5:1, 10:11 and so on) for both A1 and A2 isolates. The combination MP 322 × US–8 was used. The final concentration of inoculum was 50 sporangia/1mm^3. The inoculated plants were water sprayed until the disease symptoms appeared.

The intensity of infection was estimated according to a method described by Fry (1978). At the end of the season the samples of infected plant tissue in four replications were collected from each combination. After incubation on wet wood–wool each standardized sample was pulped in a mortar, then mixed with the same amount of sterile water and the oospores were counted in haemocytometer (in two reps). Differences in the reaction of the combinations were estimated by the Duncan test, the highest oospore concentration in each experiment was taken as a maximum value and the data were transformed into Freeman–Tukey degrees.

Effect of subsequent inoculations with A1 or A2 isolates on oospore formation

The pot and field experiments were carried out in 1999–2000 on susceptible cultivar Tarpan and resistant cultivar Bzura. The way of experiment carrying, infection assessing and results estimating were the same as described above. In field experiments 11 combinations of subsequent inoculation were used: simultaneous inoculation with A1 and A2 isolates, inoculation with A2 followed inoculation with A1 isolate after 1, 2, 4, 8 and 16 days and inoculation with A1 followed inoculation with A2 isolate after 1, 2, 4, 8, and 16 days. The concentration of inoculum was 50 sporangia/1mm^3.

RESULTS AND DISCUSSION

Comparative characterization of local populations of *P. infestans*

Table 2

Characteristics of local *P. infestans* population in four experimental fields

Criterion of assessment	Boguchwała		Przychojec		Olesno Śl.		Młochów	
	1999	2000	1999	2000	1999	2000	1999	2000
Number of isolates	30	43	33	40	30	29	20	40
C_i index [1]	7.7	6.1	7.7	5.5	7.8	6.5	5.3	8.0
Mean aggressiveness [2]	2.3	1.8	2.9	2.2	3.3	1.2	2.9	1.1
H_s index [3]	2.6	3.7	2.7	3.7	2.5	2.9	2.7	1.8
Frequency of A2 mating type	0.27	0.37	0.27	0.42	0.33	0.50	0.15	0.10
Frequency of A1 mating type	0.73	0.63	0.73	0.58	0.67	0.50	0.85	0.90

[1] Ci index = the mean number of virulence factors per isolate
[2] aggressiveness according to 1–9 grade score where 1 = most aggressive
[3] Shannon index of race diversity

The intensity of natural infection by *P. infestans* was similar in all potato fields. Virulence testing in four selected fields revealed that the race spectrum was found to be variable and ranked from race with single virulence factor (virulent to R2, R4 or R7) to race with eleven virulence factors (1.2.3.4.5.6.7.8.9.10.11) which was the most complex one detected at Przychojec in 1999. The mean number of virulence factors per isolate varied in succeeding years from 5.3 to 8.0 (Table 2). Polish *P. infestans* races detected in 1999–2000 seemed to be more complex than races discovered in Poland by Sujkowski *et al.* (1996) for the period of 1985–1988 that were characterised by the presence of 5.5 to 7.7 specific virulence factors per isolate. The pathotypes isolated in our country between 1992–1995 were also less complex as they had from 5.7 to 7.6 virulence factors per isolate (Zarzycka and Sobkowiak 1997b). According to Rivera–Peńa (1995) the high complexity of races, especially found at the beginning of the season might indicate that the pathogen survived by

oospores in the soil and could be a source of primary infection. In our study the complexity of races was significantly higher at the end of late blight epidemics, than at the beginning of the season (Table 3). The greatest differences between these values were observed at Przychojec and the least – at Młochów. The significantly greater late blight infec–tion appeared at Olesno and Młochów, than at Boguchwała and Przychojec.

Table 3

Comparison of virulence and aggressiveness of *P. infestans* isolates collected both at the beginning and at the end of late blight epidemics in four experimental fields within the years 1999–2000

Time of assessment	Boguchwała		Przychojec		Olesno Śl.		Młochów		Mean value for time of assess–ment[2]
	1999	2000	1999	2000	1999	2000	1999	2000	
Virulence									
1. C_i index [1]									
B [3]	6.5	5.4	6.1	4.0	6.9	5.5	5.3	7.5	
E [4]	8.5	6.7	8.8	7.4	8.5	7.4	5.3	8.7	
2. Freeman–Tukey degrees									
B	48.6	44.3	46.6	36.7	51.3	44.2	43.8	54.4	46.3 b
E	53.9	50.2	55.0	54.3	56.2	54.0	39.3	61.1	53.8 a
Experimental fields mean[2]	49.1 y		47.9 y		51.3 x		53.0 x		
Aggressiveness [5]:									
B	2.9	1.7	4.1	2.3	3.8	1.6	2.0	1.5	2.41 m
E	1.8	2.0	1.8	2.1	2.8	2.0	3.8	1.8	2.15 m
Experimental fields mean[2]	2.08 x		2.48 y		2.15 y		2.06 x		

[1] C_i index = the mean number of virulence factors per isolate
[2] The data marked with the same letters do not differ significantly according to Duncan test
[3] B = beginning of epidemics
[4] E = end of epidemics
[5] aggressiveness according to 1–9 grade scale, where 1 = most aggressive

The racial complexity expresses in virulence factors per isolate in Pol–ish local populations was higher than in other European populations e. g. 4.35–5.78 in France (Andrivon 1994b) in 1991–1993, 4.68 in the Netherlands in 1988 (Schöber, Turkensteen 1992) or 4.98 in Western Germany in 1987 (Rudlich and Schöber 1988). According to Świeżyński *et al.* (2000) frequency of single races reached to 36.4% in Great Britain and the frequency of complexed ones consisting of more than five viru–lent factors was noticed up to 90.7% (in Equador).

The frequency of particular virulence factors were similar to those which were observed in 1992–1995 (Zarzycka and Sobkowiak (1997b). The most frequently occurring specific virulence factors were virulent to resistance genes R1, R3, R4 and R7. Virulence to R5 were rare. Viru–lence to R9 was detected in the last years but this virulence factor was expressed sporadically and its expression was not stable. The increased frequency of virulence factors 7 and 8 were observed in comparison to result obtained in 1985–1988 (Sujkowski *et al*. 1996).

Virulence diversity in *P. infestans* population is the highest in central Mexico, where sexual reproduction of the pathogen is known phenomenon. Drenth *et al.* (1993 and 1994) found the *P. infestans* population in the Netherlands to be highly diverse and concluded that generative reproduction was the driving force behind the generation of new genotypes. The oospores generated initial inoculum under field conditions in Dutch allotment gardens, but not in commercial fields (Turkensteen *et al.* 1996). The isolates collected in allotment gardens showed higher genetic variation than isolates from commercial potato crops (Drenth *et al.* 1993).

The race diversity in our investigations was high and the values of Shannon index increased from year to year in the field examined, except the field at Młochów (Table 2). The values of race diversity obtained in Poland in 1999–2000 were higher than those, which were obtained by Sujkowski *et al.* (1996) in 1985–1991 (from 0.37 to 0.74). The virulence diversity in our country was also higher than in France, where Shannon index of race diversity ranged from 1.67 in 1991 to 2.22 in 1992. This progressive increasing of variation in race composition of Polish *P. infestans* populations may be influenced by sexual recombination.

The level of aggressiveness was high in all experimental fields, significantly higher at Boguchwała and Młochów than at Przychojec and Olesno, and severity of late blight infection was related to aggressiveness of local *P. infestans* populations (Table 3). The mean levels of aggressiveness at the beginning and at the end of epidemics did not differ significantly. These results confirmed the information obtained earlier (Zarzycka and Sobkowiak 1997b) that isolates from Polish pathogen population have been highly aggressive since 1992 (when mean degree of aggressiveness was 1.85).

Table 4

The frequency of specific virulence factors in local population of *P. infestans* within the years 1999–2000

Number of isolates	Boguchwała	Przychojec	Olesno Śląskie	Młochów
	73	73	59	64
Virulence factors	Frequency			
1	0.88	0.89	0.97	0.92
2	0.78	0.83	0.80	0.70
3	0.85	0.89	0.92	0.87
4	0.92	0.92	0.92	0.89
5	0.25	0.10	0.29	0.03
6	0.71	0.71	0.76	0.73
7	0.89	0.88	0.93	0.94
8	0.41	0.44	0.27	0.58
9	0	0.05	0	0.02
10	0.27	0.47	0.49	0.59
11	0.59	0.64	0.78	0.73

Isolates of A2 mating type of *P. infestans* were found in all four experimental fields (Table 2). The frequency of A2 isolates in local populations varied from 0.10 at Młochów to 0.50 at Olesno Śląskie in 2000. In others localities the frequency of A2 was found to be near 0.3. The optimal ratio for sexual reproduction is 1:1 between both mating types (Hermansen and Amundsen 1996, Turkensteen *et al.* 1996). In the Netherlands isolates collected in allotment gardens showed mating type ratios close to 1:1 (Drenth *et al.* 1993). In our investigations the proportion of both mating types in four local populations seems not to be most favourable to sexual propagation. On the other hand sexual reproduction may be considered, on the base of results from the experiments presented, that oospores were formed in wide spectrum of ratios of both mating types of isolates used for inoculation, fluctuated from 1:1 to 1:80.

Oospore formation by *P. infestans* was found to occur in plants grown on all tested potatoe fields. They are detected abundantly from July to August in infected tissues of stems and leaves of collected blighted potato plants. The oospores formed in leaves were spherical, loose in oogonium, with colourless thick walls. The oospores detected in potato tissues were smaller than the oospores formed on rye agar (Zarzycka and Sobkowiak 1998) and their mean size was 13.6±3.3 μm (range 7.8–23.3 μm).

Effect of various ratios of A1: A2 isolates in an inoculum on oospore formation

In laboratory experiments the intensity of oospore formation in potato leaflets was influenced by the time of test realization: more oospores were produced in June than in March. In the test carried out in June abundant production of oospores were observed in all combinations of inoculum applied except one with highest domination of A1 mating type in inoculum (30:1). In the pairing test done in March isolates combination MP 322 × US–8 was more productive than MP 382 × MP 417 one. In this isolates combination the oospore formation was fairly abundant for the most ratio combinations of mixed inoculum except two with highest domination of A2 mating type in inoculum (1:20 and 1:30). In the latter isolate combination the single oospores were only formed.

In pot experiments results were also different for other pairs of A1 and A2 isolates. Much more oospores were produced in tissues of potato plants inoculated with MP 322 × US–8 than with MP 322 × MP 417. The results obtained confirm the study of Cohen *et al.* (1997) that field isolates of *P. infestans* varied in their reproductive ability and some combinations produced abundant oospores, whereas others produced only a few on the same host plant. Drenth (1994) discovered, that more oospores were formed in the leaves of moderately resistant cultivar, than in leaves of a highly susceptible cultivar. But in our investigation there was no significant difference in intensity of oospore production between resistant cultivar Bzura and susceptible Tarpan. The most oospores

were formed after inoculation with inoculum consisted of equal portions of A1 and A2 sporangia and in combinations with small domination of A2 (5:1 and 10:1) (Table 5). Significantly less oospores were formed in another combinations of A1 and A2 isolates in an inoculum. In 2000 the inoculum with high domination of one mating type (1:80 and 80:1) was applied additionally, but only a few oospores were formed in diseased potato tissues. No correlation between late blight development and intensity of oospore production was observed.

Table 5

Effect of A1:A2 mating type isolates ratio in an inoculum on oospore formation in potato tissues

A1:A2 isolates ratio	Intensity of oospore formation			
	Pot experiments		Field experiments	
	Number of oospores in 1 mm^3	Freeman–Tukey degrees	Number of oospores in 1mm^3	Freeman–Tukey degrees
1:1	228	17.5a[1]	172	36.1a
1:5	46	8.4b	52	20.8bc
1:10	64	10.0b	64	22.3b
1:20	–	–	47	19.0cd
1:30	183	15.6ab	–	–
1:40	–	–	25	13.4cd
1:80	–	–	23	12.1d
5:1	197	16.3a	76	23.5b
10:1	287	19.2a	72	22.1b
20:1	–	–	28	14.1cd
30:1	69	11.0b	–	–
40:1	–	–	23	12.6d
80:1	–	–	23	12.6d

[1] The data marked with the same letters do not differ significantly according to Duncan test

In field investigations the oospores were formed after inoculation with all possible combinations of A1 and A2 isolates mixtures applied (Table 5). The significantly most oospores were formed when the ratio of A1 and A2 isolates 1:1 in the inoculum was applied, and significantly less, when the inoculum was dominated by one of mating type isolate, especially by A1 (40:1 and 80:1). According to Cohen *et al.* (1997) the ratio between A1:A2 sporangia in the sporangial inoculum had a minor effect on the number of oospores produced. It seems that their results were influenced by a small differentiations among ratios A1:A2 isolates, which were applied (1:1 – 1:19).

Effect of time of sequent inoculations with A1 or A2 isolates on oospore formation

In pot experiments the oospores were formed in all combinations, but significantly more oospores were produced, when potato plants were in–

oculated with A1 and A2 at the same time. Also in field investigations more oospores were formed in combinations with subsequent inoculation (Table 6), but numerous oospores were produced also in combinations with short interval between inoculations (one day). But it ought to be taken in consideration that under field conditions the oospore formation process might be disturbed by the natural infection with field isolates of *P. infestans*. It is probably especially possible during long intervals between subsequent inoculations. The formation of abundant oospores in combinations when inoculations with A1 followed A2 after two, four or eight days may be influenced by infections done under natural field conditions by A1 mating type, which is dominating in Polish population of *P. infestans*.

In these experiments no significant differences were observed in intensity of oospore production between resistant cultivar Bzura and susceptible cultivar Tarpan. No correlation was found between late blight development and intensity of oospore production.

Table 6

Effect of subsequent inoculation with A1 or A2 mating types isolates on oospore formation in potato tissues

Time of inoculation with A1 and A2	Intensity of oospore formation			
	Pot experiments		Field experiments	
	Number of oospores in 1 mm^3	Freeman–Tukey degrees	Number of oospores in 1 mm^3	Freeman–Tukey degrees
Simultaneous inoculation with A1 and A2	309	20.2 a[1]	102	26.6 a
A2 one day after A1	120	13.1 bc	58	20.3 a
A2 two days after A1	–	–	28	14.3 bc
A2 four days after A1	197	16.2 b	18	12.2 c
A2 eight days after A1	212	16.8 b	39	16.1 bc
A2 sixteen days after A1	–	–	28	14.5 bc
A1 one days after A2	156	14.9 bc	42	17.6 a
A1 two days after A2	–	–	56	19.7 a
A1 four days after A2	215	16.9 b	56	19.7 a
A1 eight days after A2	153	14.7 bc	40	16.9 ab
A1 sixteen days after A2	–	–	28	14.5 bc

[1] The data marked with the same letters do not differ significantly according to Duncan test

The role of *P. infestans* oospores as a source of primary inoculum

The existence in Poland the both mating types of the pathogen permits for its sexual reproduction and formation of oospores overwintering in the soil in plant debris. This oospores can become the second source of primary inoculum for development of late blight epidemics. Indeed mating type A1 was dominating as well in local populations as in whole

our country (Zarzycka and Sobkowiak 1997b), but a small proportion of an opposite mating type was enough for oospore production in infected potato tissue under conditions of high humidity (Cohen *et al.* 1997). Oospore formation has been found to occur in all experimental fields. The presence of oospores in naturally infected potato plants may be an indication that in all investigated fields there was a possibility of occurrence of a generative propagation of *P. infestans* under favourable weather conditions. Sexual recombination also would generate new genotypes, some of which may have a greater fitness in Poland. It can play a role in variation of race compositions in *P. infestans* population. Our investigations demonstrated a high level of race complexity, race diversity and aggressiveness to potato, especially in three experimental fields. Generative recombination may causes a role in the rapid breakdown of potato cultivar resistance. Cultivar Bronka, whose resistance was broken some years ago was intensively blighted by *P. infestans* isolates occurred in populations in Boguchwała and Przychojec (Zarzycka and Sobkowiak 1997c). On the basis of presented results it can be concluded that there was a possibility of occurrence of a generative propagation of *P. infestans* in Boguchwała, in Przychojec and in Olesno Śląskie, however confirmation of this phenomenon by genetic studies of *P. infestans* is desired.

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