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EFFECT OF DEEP FREEZING OF *PHYTOPHTHORA INFESTANS* CULTURES ON THEIR SURVIVAL AND PATHOGENICITY

ABSTRACT

The maintenance of *Phytophthora infestans* cultures isolates from blighted potato, frozen in liquid nitrogen, was studied in two experiments. The effects of deep freezing and acclimatization pre-treatment at different temperatures on culture survival and stability of virulence and aggressiveness were evaluated. The best survival of cultures maintained for three months in liquid nitrogen was expressed, when cultures were acclimatized before freezing at 7°C. The survival of frozen cultures was significantly worse in comparison with control combinations stored on rye-agar and rye-agar under paraffin oil for short time (in experiment I – 40 days, in experiment II – three months). The virulence spectrum of frozen cultures after thawing was more narrow than that one observed before culture freezing. However this virulence spectrum did not differ significantly from virulence range of control cultures. The level of aggressiveness of culture stored in liquid nitrogen did not differ significantly from control cultures as well.

The virulence spectrum and aggressiveness level of cultures frozen in liquid nitrogen were significantly higher after two passages of thawed cultures on potato tissues than after single passage.

Key words: acclimatization, aggressiveness, liquid nitrogen, maintenance, *Phytophthora infestans*, survival, virulence

INTRODUCTION

Phytophthora infestans isolates have been collected at the Młochów Center of the Plant Breeding and Acclimatization Institute (IHAR) since 1986. The storage of *Phytophthora infestans* cultures creates problems, especially for cultures maintained for many years. Up to day, *P. infestans* cultures have been stored at IHAR Młochów on potato tissues and on rye agar medium. The cultures stored on potato tissue preserve the higher level of aggressiveness and better stability of their virulence in comparison with cultures stored on rye agar. But this method of cultures maintenance is very troublesome because the cultures must be passaged on fresh tissue every week (Świszczewska *et al.* 1971, Zarzycka 1995 and 1996). The aggressiveness level and virulence spectrum of cultures maintained on rye agar for long period may be reduced especially when they are kept

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at room temperature. At IHAR Młochów, the cultures stored for long time are kept on rye agar medium covered with paraffin oil. The cultures maintained this way might survive for tree years, particularly at 7°C (Zarzycka 1996).

Recently research on long storage of *P. infestans* cultures in liquid nitrogen has been started (Sobkowiak and Zarzycka 2000). The essence of cryopreservation is to effect cell dehydration and concentration of the cytosol in a controlled and minimally injurious manner so, that ice crystallisation in the cytosol is precluded or minimised during quenching in liquid nitrogen (Steponkus *et al.* 1992). The *P. infestans* cultures stored in a liquid nitrogen were kept in the temperature from -172 to -196°C and all metabolic processes were fully inhibited (Meryman 1956). According to Caten and Jinks (1968) the specimens treated with temperature -80°C for 30 min., before putting them into liquid nitrogen, have been expressed the satisfactory survival. The best effect of survival was observed, when the cultures, before freezing in liquid nitrogen, were immersed into DMSO and then gradually frozen (Sobkowiak and Zarzycka 2000). A specimens prepared unsuitable, before putting them in a liquid nitrogen, can lose totally their vitality of organs (Steponkus *et al.* 1992). In this work the effects of deep freezing of *P. infestans* cultures and different culture pre-treatments on their survival, stability of virulence and aggressiveness were studied.

This publication presents preliminary results obtained.

MATERIALS AND METHODS

Phytophthora infestans isolates characterized by high level of aggressiveness and wide spectrum of virulence were used in two experiments. These isolates were stored at 7°C under paraffin oil and passaged on fresh rye agar. Fragments of thallus (5 mm size) were cut out from the colony developed on rye agar and immersed at the solution of DMSO (dimethyl sulphoxide). The time of gradually cooling of *P. infestans* cultures, before putting them into liquid nitrogen was chosen according to the best variant described by Sobkowiak and Zarzycka (2000). Using this variant of cooling the temperature was reduced to –70°C gradually by keeping the samples for 15 min. at –7°C, for 30 min. at –18°C and for 30 min. at –70°C. After thawing at ambient temperature for 30 minutes, the pieces of thallus were washed in sterile water and put on rye agar medium.

1. The effect of freezing of *P. infestans* cultures on their survival

The survival ought to be accepted as a most important problem of culture storage. This problem was examined in the two experiments. In the first experiment the effect of different pre-treatment temperatures before freezing on culture survival was examined. Two *P. infestans* isolates were tested. Two-week old cultures grown on rye agar medium have been placed at temperatures 4°C, 7°C, 16°C and 22°C for 40 days. Then discs of

thallus were cut out from colonies and frozen as described above. The cultures were kept in liquid nitrogen for three hours. In each combination 20 thallus discs were used. The cultures acclimatized at the respective temperatures for 40 days and not frozen in liquid nitrogen were used as the control combinations. The survival of *P. infestans* cultures was assessed as a percent of thallus pieces forming the colonies on rye agar. The evaluations have been done at 10^{th} and 30^{th} day after thallus thawing.

As the best survival in the first experiment was presented by cultures acclimatized at 7°C, so this temperature was used in the second experiment for culture pre-treatment. In this experiment nine isolates of *P. infestans* were examined. The cultures were stored in liquid nitrogen for three months. Each combination was done in five replications. The control cultures were stored at 7°C for three months on rye agar medium (control 1) and on rye agar medium under paraffin oil (control 2).

Cultures survival has been evaluated as in the first experiment. The differences of survival were compared by Tukey's test.

2. The effect of freezing of *P. infestans* cultures on their virulence and aggressiveness

The stability of virulence of *P. infestans* cultures was assessed in the second experiment only. Nine *P. infestans* isolates characterized with high level of aggressiveness and wide spectrum of virulence were used in the experiments (Table 1). The cultures after thawing were once or twice passaged on potato tuber slices. The range of virulence was evaluated on leaflets of Black's differentials possessing genes of specific resistance (from R1 to R11) (Zarzycka and Sobkowiak 1997). The level of aggressiveness was assessed on leaflets of potato cultivars Bintje and Tarpan (not possessing R-genes), according to 1-9 score, where 1 designates total infection (Zarzycka and Sobkowiak 1997).

Table 1

Initial characteristics of *P. infestans* isolates used in the first and second experiments

No	Number of isolate	Year of isolation	Spectrum of virulence	Level of aggressiveness	Mating types
1	US-8	1994	1.2.3.4.5.6.7.10.11	1.0	A2
2	MP 322	1997	1.2.3.4.6.7.8.10.11	1.0	A1
3	MP 324	1997	1.2.3.4.5.6.7.8.10.11	1.0	A1
4	MP 421	1997	1.2.3.4.5.6.7.8.10.11	1.0	A1
5	MP 419	1997	1.2.3.4.5.6.7.8.10.11	1.0	A1
6	MP 423	1998	1.2.3.4.5.6.7.8.10.11	1.0	A2
7	MP 422	1998	1.2.3.4.5.6.7.8.10.11	1.0	A1
8	MP 426	1999	1.2.3.4.5.6.7.8.10.11	1.0	A1
9	MP 427	1999	1.2.3.4.5.6.7.8.10.11	1.0	A1

assessed on single Black's differential from R1 to R11; in 1-9 scale, where 1 means total infection

The evaluations of virulence spectrum and aggressiveness level were done after single and two passages of cultures. The difference of virulence and aggressiveness were compared by Tukey's test.

RESULTS AND DISCUSSION

1. The effect of storage of *P. infestans* cultures in liquid nitrogen on their survival

The effect of cold acclimatization on survival of *P. infestans* cultures stored in liquid nitrogen was examined in the first experiment. The best survival was presented by the cultures acclimatized at 7°C before placing them into liquid nitrogen. At 30th day after thawing 82.5% of thallus fragments formed the colonies on rye agar medium. It was significantly better survival in comparison with cultures pre-treated at 4°C (67.5%), at 16°C (42,5%) and at 22°C (27,5%) (Table 2). Many authors investigated the effect of cold acclimatization on survival of organism fragments immersed in liquid nitrogen. Dowgert and Steponkus (1983) and Suguwara and Steponkus (1990) studied the influence of cold acclimatization on ice formation and on positive modification of the plasma membrane lipid composition in isolated protoplast. According to results of their experiments each organism has to be acclimatized in low temperature during suitable time before storage in liquid nitrogen (Gordon-Kamm and Steponkus 1984, Sugawara and Steponkus 1990, Fuikawa and Steponkus 1990).

Table 2

The effect of storing *P. infestans* cultures pre-treated at various temperatures on their survival in liquid nitrogen (assessed at 10 and 30 day after thawing)

Method of storage		Mean percent of thallus fragments forming colonies					
		Experiment I					Experiment II
		Colonies pre-treated					
		4°C	7°C	16°C	22°C	mean	7°C
On rye agar	At 10 day	35.0de	32.5de	80.0bc	97.5a	61.3	89.9abc
	At 30 day	100.0a	100.0a	100.0a	100.0a	100.0	93.3ab
On rye agar under paraffin oil	At 10 day	-	-	-	-	-	76.6c
	At 30 day						98.8a
In liquid nitrogen	At 10 day	7.5g	17.5fg	15.0fg	20.0ef	15.0	28.9d
	At 30 day	67.5c	82.5b	42.5d	27.5ef	55.0	82.2bc
Mean	At 10 day	37.9n	-	-	-	-	65.1y
	At 30 day	77.5m					91.4x

control 1; control 2; Values in the same row and column followed by different letters are significantly different

In our experiment the number of thallus fragments forming colonies after 10 days fluctuated from 7.5 to 20% and the survival decrease was greatest in the higher temperatures. In control combinations 100% of thallus fragments formed colonies after 30 days, but after 10 days the best survival was observed at 22°C.

No considerably differences were noted between two isolates of *P. infestans* used in the experiment in relation to its survival. Isolates stored in liquid nitrogen grown slowly and as late as 30 days after thaw-

ing were needed to achieve by them the level of 80% of thallus pieces growing into colonies. The thallus fragments of isolates MP 423 and MP 426 treated with liquid nitrogen did not formed the colonies before 30th day after thawing, when 40 percent and 60 percent (respectively) of thallus pieces began to produce the mycelium (data not shown).

The cultures of *P. infestans* stored for three month in liquid nitrogen grown more slowly, after thawing, than control ones (experiment II). At 10th day after thawing only 28.9% thallus fragments formed colonies on rye agar, but at 30th day the number of thallus pieces producing colonies reached 82.2% (Table 2). In control combinations colonies developed from thallus fragments reached the level 76.6% or 89.9% and 93.3% or 98.8% respectively. The fungus cultures usually began to lose their ability to survive after one year's storage on agar media (Jinks and Grindle 1963). Three months maintenance of the control cultures on rye agar was too short to observe the decrease of survival as well as the decrease of virulence spectrum and level of aggressiveness.

The differences in survival of particular isolate cultures were observed. The isolates US-8, MP 421 and MP 427 were the best surviving ones (data not shown).

2. The effect of storage of *P. infestans* cultures in liquid nitrogen on their virulence

The means of virulence factors expressed over three variants of storing of nine *P. infestans* isolate cultures (experiment II) tested in the experiment did vary significantly, when virulence was compared after single (6.3 factors expressed) and twice (7.8 factors) passaged cultures through potato tissue (Table 3). Similar significant difference was found between range of virulence expressed after single and double passaging of cultures maintained in liquid nitrogen. No significant differences were found in range of expressed virulence factors between two control variants and isolates stored in liquid nitrogen, for both single and double passage of cultures before checking their virulence expression. Virulence factors with incomplete expression (only one out of two inoculated leaflets of respective R- genotype was infected) were more often noted after single passage of isolates (24 cases) than after two passages (16 cases).

The decrease of virulence expression in all combinations after three months of maintenance was observed in comparison with the initial virulence spectrum factors. The tested isolates expressed full potential of its virulence, described before the experiment (Table 2), in a few cases only (US-8, MP 324, MP 419, MP 422) after double passaging, and mainly after storing in liquid nitrogen. Zarzycka observed (1995) the decrease of virulence factors detectability in *P. infestans* cultures to 71% after its maintenance on rye agar medium at 7°C during eight months of storing, while Sujkowski (1992) recorded the greater loss (70-80%) of virulence spectrum in isolates maintained on tuber slices after five

Table 3

The effect of storing of *P. infestans* cultures for three months in liquid nitrogen on expression of virulence spectrum

Black's differentials		Name of isolate																	
		US-8						MP 322						MP 324					
		Single passage			Double passage			Single passage			Double passage			Single passage			Double passage		
		A	O	N	A	O	N	A	O	N	A	O	N	A	O	N	A	O	N
R1			(+)	+	(+)		+	+		+	+	+	+	+	+	+	+	+	+
R2			+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
R3		(+)		+	+	(+)	+	+	(+)	+	+	+	+	+	+	+	+	+	+
R4				(+)	+		+	+	+	(+)	+	+	+	+	+	+	+	+	+
R5			+				(+)						(+)	+			(+)	+	+
R6			+	(+)	+	+	+	+	+		+	+	+	+	+	+	+	+	+
R7				+	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+
R8													+						
R9													+						
R10							+						+	+	(+)		+		+
R11				+		(+)	+	+		(+)	+		+	+	(+)	+	+		+
	V	1	4	7	6	4	9	7	5	6	7	6	11	9	8	7	9	7	9
	I	1.2.3.4.5.6.7.10.11						1.2.3.4.6.7.8.10.11						1.2.3.4.5.6.7.8.10.11					

Table 3

Continued

Black's differentials	Name of isolate																	
	MP 421						MP 419						MP423					
	Single passage			Double passage			Single passage			Double passage			Single passage			Double passage		
	A	O	N	A	O	N	A	O	N	A	O	N	A	O	N	A	O	N
R1	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+
R2	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+
R3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R4	+	+	+	+	+	+	+	+		+	+	+	+	+		+	+	+
R5			(+)			(+)	(+)	+			+	(+)	(+)			+	(+)	
R6	+		+	+	+	+	+	+		+	+	+	+	+	(+)	+	+	+
R7	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+
R8							(+)					(+)						
R9																		
R10	+					+	+	+		(+)	+	+	+	(+)		+		
R11	+		+	+	+	+	+	+	+	+	+	+	+	(+)	(+)	+	+	+
V	8	5	8	7	7	9	10	9	5	8	9	10	9	8	3	9	8	7
I	1.2.3.4.5.6.7.8.10.11						1.2.3.4.5.6.7.8.10.11						1.2.3.4.5.6.7.8.10.11					

Table 3

Continued

Black's differentials	Name of isolate																	
	MP 422						MP 426						MP 427					
	Single passage			Double passage			Single passage			Double passage			Single passage			Double passage		
	A	O	N	A	O	N	A	O	N	A	O	N	A	O	N	A	O	N
R1	+	+	+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+
R2	+	+	+	+	+	+	(+)	+	+	+	+	(+)				+	+	+
R3	+	+		+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+
R4	+	+	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+
R5	+	+			+										+		(+)	
R6	+	+		+	+					+	+				+	+	+	+
R7	+	+	+	+	+	+	(+)	+	+	+	+	+	+	(+)	+	+	+	+
R8					(+)					+					+			
R9															+			
R10	+	+		(+)	+						+				+	+	+	
R11	+	+		+	+					(+)	+				+	+	+	+
V	9	9	4	8	10	5	5	4	5	8	8	5	4	4	6	8	9	7
I	1.2.3.4.5.6.7.8.10.11						1.2.3.4.5.6.7.8.10.11						1.2.3.4.5.6.7.8.10.11					

Passages on potato tissues	Mean number of virulence factors			
	Cultures stored:			
	on rye-agar at 7°C	under paraffin oil at 7°C	in liquid nitrogen	Mean
single	6.9 abc6	6.2 bc	5.7 c	6.3 y
two	7.8 ab	7.6 ab	8.0 a	7.8 x

A = Cultures stored on rye-agar medium at 7°C (control 1)
O = Cultures stored under paraffin oil at 7°C (control 2)
N = Cultures stored in liquid nitrogen
Number of expressed virulence factors
Factors with incomplete expression (one from two tested leaves was affected) are shown in brackets
Values in the same row and column marked with different letters are significantly different
I = Initial virulence of *P. infestans* isolate

Table 4

The effect of storing of *P. infestans* cultures on their aggressiveness (in 1-9 scores), after single and two passages trough potato tissues

The cultures stored	Name of isolate																	
	US-8		MP 322		MP 324		MP 421		MP 419		MP 423		MP 422		MP 426		MP 427	
	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D
	Aggressiveness (grades)																	
on rye agar at 7°C (control 1)	7.0	2.8	2.3	1.3	1.3	1.0	1.3	1.0	1.0	1.5	1.0	1.0	1.3	1.0	2.3	1.3	3.0	1.0
under paraffin oil at 7°C (control 2)	6.0	6.0	4.8	2.8	1.5	1.0	3.5	2.5	2.0	1.3	4.0	1.8	1.3	1.5	3.0	1.0	3.8	1.0
in liquid nitrogen	1.3	1.5	3.3	1.8	1.8	1.0	1.8	1.0	2.0	1.8	6.5	1.0	4.0	1.5	2.5	1.0	1.8	1.3
I	1.0		1.0		1.0		1.0		1.0		1.0		1.0		1.0		1.0	

	Mean aggressiveness			
After thawing of culturespassages on potato tissues	Cultures stored (grades)			
	on rye-agar at 7°C	under paraffin oil at 7°C	in liquid nitrogen	Mean
single	2.3 bc	3.3 a	2.6 ab	2.8 x
double	1.3 c	2.0 bc	1.3 c	1.6 y

The isolates single passaged after thawing through potato tissues
The isolates twice passaged after thawing through potato tissues
3 Values in the same rows and columns marked with different letters differ significantly
Initial level of agressiveness of *P.infestans* isolates

months. In our experiment virulence differences between storing variants were not significant, what might be explain by short period of storage treatment, especially for control combinations stored on rye agar medium.

3. The effect of storage of *P. infestans* cultures in liquid nitrogen on their aggressiveness

The way of pathogen multiplication after thawing had significant influence on level of aggressiveness of stored cultures (experiment II). The overall mean intensity of leaflet infection was higher after two passages in comparison with single passage (1.6 and 2.8 grades in 1-9 scale respectively) (Table 4). Storage of *P. infestans* isolates in liquid nitrogen did not influence significantly on their aggressiveness after thawing in relation to controls. However the level of aggressiveness of single passaged cultures was rather high and fluctuated from 3.3 to 2.3 grades for cultures kept on rye agar medium and 2.8 for cultures frozen in liquid nitrogen. The level of aggressiveness of maintained cultures increased after two passages on potato tissues to 2.0 and 1.3 (in two controls) and 1.3 grades (in frozen cultures). The level of aggressiveness, after two passages in relation to single passage was higher for all combination tested and for all isolates used in the study except the isolate US-8. (Table 4).

On the base of presented results it can be concluded that the two passages trough potato tissues had better effect on aggressiveness level of cultures stored in liquid nitrogen in comparison with single passage. In our investigations the decrease of aggressiveness in all combinations after three months of storing was insignificant in comparison with initial aggressiveness. According to Zarzycka (1996) the maintenance of *P. infestans* cultures over 10 months on rye agar had no effect on aggressiveness, also, when isolates were passaged trough potato tissue before testing.

The stability of pathogenicity of *P. infestans* cultures treated for 40 days (experiment I) in different temperatures, before storing them in liquid nitrogen were also examined and no significant differences were observed.

CONCLUSIONS

1. The best survival of *P. infestans* cultures maintained for three months in liquid nitrogen was received, when the cultures were acclimated before freezing at 7°C for 40 days.
2. The survival of *P. infestans* cultures frozen in liquid nitrogen was significantly worse in comparison with not frozen control combinations maintained on rye agar and on rye agar under paraffin oil for 40 days or three months.

3. The differences in virulence spectrum and aggressiveness level between cultures stored in liquid nitrogen and control cultures were not significant.
4. The virulence spectrum and aggressiveness level expressed by cultures frozen in liquid nitrogen were significantly higher after two passages of thawed cultures through potato tissues than after single passage.

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