JOURNAL OF PLANT PROTECTION RESEARCH Vol. 52, No. 4 (2012)
DOI: 10.2478/v10045-012-0077-5

THE INFLUENCE OF LONG-TERM STORAGE IN LIQUID NITROGEN ON SURVIVAL AND PATHOGENICITY OF *PHYTOPHTHORA INFESTANS* ISOLATES

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Received: April 4, 2012
Accepted: October 24, 2012

Abstract: A set of 14 aggressive *Phythophthora infestans* (Mont.) de Bary isolates with a wide range of virulence, was stored for eight years (2002–2010) in liquid nitrogen at –196°C (209 samples) and under paraffin oil at 7°C (70 test tubes). The survival rate of samples stored in liquid nitrogen was scored as 88%, 45 days after thawing. The revived isolates were passaged through potato tissues four times and tested for virulence on a set of 11 Black's differentials. The mean number of virulence factors per isolate, of these *P. infestans* isolates evaluated before storage, was 7.4. The isolates stored in liquid nitrogen and under paraffin oil and passaged four times through potato tissues showed a similar mean number of virulence factors per isolate, 7.3 and 6.9, respectively. Isolates stored under paraffin oil showed no expression of factor *avr5*, but expression of this factor occurred in six isolates after liquid nitrogen treatment. The initial expression of this factor occurred in four isolates. Before storage, the average aggressiveness of the tested isolates was assessed as 1.7, on a 1–9 scale, where 1 means the most aggressive. After storage and after four subsequent passages through potato tissues, the mean aggressiveness of isolates stored in liquid nitrogen and under paraffin oil reached the level of 1.5 and 2.1, respectively. The mean aggressiveness was not significantly different from the level of initial assessment.

Key words: aggressiveness, liquid nitrogen, long-term storage, *Phytophthora infestans*, survival, virulence

INTRODUCTION

Phytophthora infestans (Mont.) de Bary is the most important pathogen of potato and tomato worldwide. A significant amount of scientific research is devoted to this organism from the Oomycetes group. Its genome is big and fast-mutating. Large variation within this species is observed worldwide (Haas *et al.* 2009). A reliable and long-term preservation of *P. infestans* diversity in collections of isolates is essential for studies aiming at a better understanding of its biology, population genetics, migrations, interactions with host plant, and adaptation.

The maintenance of *P. infestans* cultures on potato, tissues is the best way to preserve the pathogenicity (Świszczewska *et al.* 1971). However, the necessary procedure of passaging these cultures every seventh day on fresh potato leaves or tuber slices is very laborious (Zarzycka 1995) and not feasible for long term-storage.

Another method of *P. infestans* preservation is based on slowing down its metabolism in low temperature and under limited oxygen access. The cultures maintained under paraffin oil at 7°C, survive for three years (Zarzycka 1996). This method has been applied in our *P. infestans* collection so far (Sobkowiak *et al.* 2004b). The growing size of the collection, though, has demanded growing amounts of space, and work on refreshing cultures every three years.

Cryopreservation is a method of storing frozen biological materials at a very low temperature (–196°C). The method of deep freezing *P. infestans* cultures in liquid nitrogen has been applied in the USA since 1960 (Dahnen *et al.* 1983). The *P. infestans* cultures were stored in liquid nitrogen at temperatures from –172 to –196°C and all metabolic processes were fully inhibited (Meryman 1956).

Studies on freezing of *P. infestans* isolates in liquid nitrogen at Plant Breeding and Acclimatization Institute – National Research Institute (Instytut Hodowli i Aklimatyzacji Roślin – Państwowy Instytut Badawczy, IHAR – PIB) Młochów began over 10 years ago (Sobkowiak and Zarzycka 1999). According to these studies, the best survival rates were observed at the variant of initial gradual freezing: for 15 min with –7°C, for 30 min with –18°C and for 30 min with –70°C, when the diameter of culture piece was 10 mm and DMSO (dimethyl sulfoxide) was used as a cryoprotectant.

The evaluation of the pathogenicity of *P. infestans* isolates stored long term in liquid nitrogen in comparison to the same isolates stored under paraffin oil was the aim of the presented work. We chose aggressiveness and virulence against a set of 11 Black's differentials as described by Zarzycka (2001), as the measures of *P. infestans* pathogenicity that were assessed before and after storage. The

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survival was assessed only for the *P. infestans* isolates stored in liquid nitrogen.

MATERIALS AND METHODS

P. infestans isolates

We used 14 *P. infestans* isolates collected from 1994 to 2001. Their origins as well as mating type, virulence, and aggressiveness assessed after isolation are shown in table 1. These *P. infestans* isolates were highly aggressive and of a wide spectrum of virulence. Only one of them came from the USA, the others were isolated from various locations in Poland. Most of the isolates were of A1 mating type, with two A2, and a self-fertile one (Table 1). Isolates were stored as pure cultures on rye A agar slopes (Caten and Jinks 1968) covered with paraffin oil at 7°C, and refreshed every three years, until the beginning of the storage experiment in 2002.

Table 1. Initial characteristics of *P. infestans* isolates used in the experiments

No.	*P. infestans* isolate	Year of isolation	Isolated from cultivar	Place of collection	Virulence[1]	Aggressiveness[1]	Mating type
1	US-8	1994	unknown	USA	1.2.3.4.6.7.8.10.11	1.8	A2
2	MP322	1997	Mila	Bonin	1.2.3.4.6.7.11	1.8	A1
3	MP324	1997	Gloria	Bonin	1.2.3.4.6.7.10.11	2.8	A1
4	MP419	1997	Van Gogh	Bonin	1.2.3.4.5.6.7.10.11	1.0	A1
5	MP420	1997	Gloria	Bonin	1.2.3.4.5.6.7.11	2.3	A1
6	MP421	1997	Mila	Bonin	1.2.3.4.6.7.11	2.0	A1
7	MP423	1998	Bryza	Częstochowa	2.6.7	2.8	A2
8	MP424	1999	Oda	Młochów	1.2.3.4.5.6.7.11	1.0	A1
9	MP425	1999	Dunajec	Przychojec	1.2.3.4.5.6.7.10.11	1.0	A1
10	MP426	1999	Fala	Stare Olesno	1.2.3.4.6.7.11	1.0	A1
11	MP427	1997	Tarpan	Młochów	1.2.3.4.6.7.10.11	1.0	A1
12	MP428	2001	Dunajec	Jadwisin	1.2.3.4.6.7.11	1.5	A1
13	MP429	2001	Lawina	Przychojec	1.2.3.4.6.7.11	2.0	SF[2]
14	MP430	2001	Tarpan	Chociwel	1.2.3.4.6.7.11	1.5	A1

[1] virulence spectrum and level of aggressiveness were estimated on 27 March 2002, before storing isolates in liquid nitrogen and under paraffin oil

Virulence: assessed on single Black's differential from R1 to R11

Aggressiveness: on a 1–9 scale, where 1 means the highest aggressiveness

[2] SF – self fertile

Storage in liquid nitrogen

Before freezing in liquid nitrogen, *P. infestans* isolates stored at 7°C under paraffin oil were passaged on fresh rye agar medium (Zarzycka 2001). Then the *P. infestans* isolates were acclimated for two weeks at 4°C, according to Sobkowiak *et al.* (2004a). Next, for each isolate, 18 fragments which were 5 mm in diameter, were cut out from medium covered with mycelium. Before freezing, the samples were immersed in the 15% solution of DMSO in cryovials. The temperature was gradually reduced to –70°C by treating the samples for 15 min with –7°C, for 30 min with –18°C, and for 30 min with –70°C according to the best variant described by Sobkowiak *et al.* (2004a). Then, the samples were transferred into liquid nitrogen.

The *P. infestans* isolates have been stored in liquid nitrogen at –196°C for eight years (deep frozen 5 May 2002) from 2002 to 2010 (thawed 18–25 May 2010).

Storage under paraffin oil

Pieces cut from two-week old *P. infestans* cultures growing on rye A agar medium on Petri dishes were transferred to rye A agar slopes. Then, the slopes were incubated in darkness at 16°C for 10 days and when the mycelia grew, they were flooded with sterilised paraffin oil. The cultures were stored in a dark chamber at 7°C.

Within the storage experiment, 14 *P. infestans* isolates had been stored under paraffin oil at 7°C from 7 May 2002 to 18–25 May 2010. Each isolate was stored under paraffin oil in five separate test tubes. The isolates had not been refreshed for eight years.

Survival of *P. infestans* cultures after storage

The *P. infestans* cultures stored in liquid nitrogen were thawed at room temperature for 15 min and washed three times in sterile distilled water. Then, they were immersed in semi-fluid rye A agar medium (standard rye A medium with agar contents reduced to 2.5 g/l) on Petri dishes. The experiment was done in two replications per isolate. Each replication consisted of six to nine pieces of mycelium frozen in liquid nitrogen. The number of mycelium pieces forming the colonies on medium was scored on the 15th, 30th and 45th day after thawing.

P. infestans cultures stored under paraffin oil were passaged on Petri dishes containing semi-fluid rye A agar medium. Each isolate was passaged on five Petri dishes with six mycelium pieces on each dish. The survival of *P. infestans* cultures stored under paraffin oil was not assessed. These cultures had been stored for eight years. Without refreshing, the cultures had grown slowly. Nevertheless, it was possible to assess their virulence and aggressiveness after storage.

Aggressiveness and virulence tests

The virulence and aggressiveness of *P. infestans* isolates were assessed before storage, and directly after revival of the samples from storage by both methods, and after one, two, and four passages through potato tissues.

Both traits were evaluated in the laboratory detached leaflet test as described by Zarzycka (2001). Virulence was assessed on 11 potato genotypes, with specific resistance genes (*R1-R11*) from *Solanum demissum* (Black's differential set was obtained from the Scottish Agricultural Science Agency, Edinburgh, UK). Two detached leaflets of each tester per *P. infestans* isolate were inoculated with a suspension of $5x10^4$ sporangia/ml (Zarzycka and Sobkowiak 1997). The inoculum was incubated for 2 h at 7°C, to enhance zoospore release. Two drops of inoculum were placed on opposite sides of the leaf vein. Each drop contained about 2 000–2 500 sporangia. Inoculated leaflets were placed in trays, covered with glass, and incubated for six days in an illuminated chamber at 2 100 lx, at 16°C and high humidity. The isolate was considered virulent, when it caused a typical disease spot with sporulation on leaflets of a given differential.

The level of aggressiveness of each isolate was assessed on two leaflets of two *P. infestans* potato cultivars; Craigs Royal and Tarpan (with no identified R-genes) which are susceptible to *P. infestans*. The inoculum was prepared in the same way as in the virulence test, inoculation and incubation conditions were also the same. Aggressiveness was evaluated six days after inoculation on a 1–9 scale, where 1 means a completely infected leaflet and the strongest aggressiveness (Lapwood 1963 and Zarzycka 2001).

Data analysis

The survival scores were transformed into Freeman-Tukey degrees. The differences of survival were compared by Tukey's test (MSTAT-C, version 2.00).

The significance of differences in the aggressiveness level was estimated by analysis of variance using Tukey's test.

RESULTS AND DISCUSSION

Survival of *P. infestans* cultures after storage

The viability of 14 *P. infestans* isolates, which have been stored in liquid nitrogen for eight years, was examined after thawing. A total of 209 samples were thawed; from 12 to 18 samples per isolate. Number of mycelium fragments forming colonies on rye A agar medium on the 15th, 30th and 45th day after thawing in relation to a total number of fragments of each isolate is shown in table 2. The total survival rates on these three dates were 62, 81, and 88%, respectively. On the 15th day after thawing, the number of samples forming colonies varied from 0 to 17, depending on the isolate. All tested mycelium fragments of isolates US-8, MP419, MP427, and MP430 already formed colonies on the rye agar medium (Table 2).

All *P. infestans* isolates assessed 30 days after thawing developed some growing colonies. However, only 2–5 from 16 mycelium fragments of isolates MP429, MP428, and MP426 formed colonies until this date. Studies conducted by Sobkowiak *et al.* (2004a) showed that *P. infestans* isolates can be kept in liquid nitrogen for many months. In that work the survival of *P. infestans* isolates stored for three months in liquid nitrogen ranged from 7.5 to 20.0% on the 10th day after thawing and from 27.5 to 82.5% on the 30th day after thawing.

The evaluation of the *P. infestans* survival, 45 days after thawing showed increasing percentage of survival. After storage in liquid nitrogen, 15 samples started growing slowly only between the 30th and 45th day after thawing. All the samples of the 10 *P. infestans* isolates out of the 14 tested were growing on the 45th day after thawing. In the case of isolates MP428 and MP429, only 7 and 4 samples, respectively, survived out of 16 samples tested per isolate. The development of the colonies of isolates MP322 and MP426 was observed in 13 from 14, and 13 from 16 samples, respectively.

The number of colonies produced until the first observation was significantly smaller than on the second and the third date. The number of colonies noted during the second and third observation did not differ significantly.

Cryogenic methods are suitable for the preservation or storage of *P. infestans* culture collections, although their efficacy may vary with individual isolates (Webb *et al.* 2011). The average survival rate of 88% of *P. infestans* isolates frozen in liquid nitrogen – obtained in this study, is within the range described by other authors. According to Tooley (1988), the best freezing variant (initial freezing for 24 min at –20°C, and 60 min at –80°C) resulted in a 99% survival rate of cryopreserved *P. infestans* isolates. In another study (Nagai *et al.* 2000), the percentage of isolates recovering after one year in liquid nitrogen reached 77%. Almost a 100% survival was obtained when the cultures were kept at a low temperature for 30 min (Caten and Jinks 1968, Tooley 1988). The survival rate obtained in this study was similar to the 82.5% rate described earlier by Sobkowiak *et al.* (2004a).

The effect of storage of *P. infestans* on their aggressiveness

Storage in liquid nitrogen

The aggressiveness level of *P. infestans* isolates directly after revival from liquid nitrogen was very weak and therefore these data were excluded from analysis. After a single passage through potato tissues, the mean aggressiveness of the 14 tested isolates was 3.4 (Table 3). Aggressiveness of some *P. infestans* isolates (MP324 – 8.0, MP419 and MP423 – 7.0), after a single passage, was still weak – which had an influence on the overall average value. After two passages, the average aggressiveness of the isolates reached a level of 2.1, and after four passages of 1.5. After two passages, only the isolate MP423 was weakly aggressive (5.5). The *P. infestans* isolates frozen in liquid nitrogen were significantly more aggressive after two passages on potato tissues than after a single passage, which was in accordance with the experiments described before (Sobkowiak *et al.* 2004a). All the isolates after four passages, had reached a degree of aggressiveness within a range of 1.0–3.0, This range of aggressiveness was not significantly different from the level of aggressiveness scored before freezing (Table 3).

Table 2. Number of surviving samples (mycelium fragments) of *P. infestans* isolates from liquid nitrogen observed 15, 30 and 45 days after thawing

No.	*P. infestans* isolate	Total number of samples	Number of samples forming colonies, days after thawing		
			15th	30th	45th
1	US-8	14	14	14	14
2	MP322	14	4	12	13
3	MP324	12	11	12	12
4	MP419	14	14	14	14
5	MP420	14	11	14	14
6	MP421	14	13	14	14
7	MP423	14	12	14	14
8	MP424	16	12	16	16
9	MP425	16	6	16	16
10	MP426	16	0	5	13
11	MP427	16	16	16	16
12	MP428	16	0	3	7
13	MP429	16	0	2	4
14	MP430	17	17	17	17
	Total	209	130 b[1]	169 a	184 a

[1] data in the line designated with the same letters indicate lack of significant differences (at $\alpha = 0.05$) according to Tukey's test

Table 3. The effect of storing *P. infestans* cultures in liquid nitrogen and under paraffin oil on their level of aggressiveness after one, two, and four passages on potato tissues – infection of leaflets of r-gene potato cultivars according to 9-degree scale, where 1 = completely infected

No.	Name of isolate	Aggressiveness after storage						Initial level of aggressiveness[1]
		in liquid nitrogen			under paraffin oil			
		Number of passages						
		1	2	4	1	2	4	
1	US-8	1.3	1.3	1.0	2.0	2.0	1.5	1.8
2	MP322	2.3	2.0	2.0	8.0	3.5	3.5	1.8
3	MP324	8.0	4.0	3.0	1.8	1.0	1.0	2.8
4	MP419	7.0	3.0	2.5	3.8	2.5	2.0	1.0
5	MP420	2.5	2.3	1.5	2.0	1.3	1.0	2.3
6	MP421	1.0	1.0	1.0	3.3	2.0	1.8	2.0
7	MP423	7.0	5.5	1.5	5.3	1.5	1.3	2.8
8	MP424	5.5	1.3	1.0	2.0	1.8	1.8	1.0
9	MP425	1.0	1.0	1.0	7.3	4.0	3.8	1.0
10	MP426	4.0	1.5	1.5	2.8	2.0	1.8	1.0
11	MP427	2.0	1.8	1.8	6.0	2.5	1.8	1.0
12	MP428	1.8	1.5	1.0	2.8	2.0	1.8	1.5
13	MP429	1.0	1.0	1.0	3.3	2.8	2.3	2.0
14	MP430	3.3	2.4	1.6	7.0	5.0	4.0	1.5
	Mean	3.4 b[2]	2.1 cd	1.5 e	4.1 a	2.4 c	2.1 cd	1.7 de
	Range	1.0–8.0	1.0–5.5	1.0–3.0	1.8–8.0	1.0–5.0	1.0–4.0	1.0–2.8

[1] initial assessment of the level of aggressiveness was assessed on 27 March 2002

[2] homogenous groups – significant at $\alpha = 0.05$ according to Tukey's test (data in the row designated with the same letters indicate lack of significant differences among interaction storage methods by passages)

LSD ($\alpha = 0.05$) for interactions:

II/I – LSD for comparison of mean aggressiveness of isolates within a passage: 1.99

I/II – LSD for comparison of mean aggressiveness of passages within an isolate: 1.53

Table 4. Influence of storage in liquid nitrogen and under paraffin oil on number of *P. infestans* isolates with respective virulence factors (*avr1-11*)

Virulence factors (*avr*)	Number of isolates at the initial assessment[1]	Number of isolates with virulence factor after storage							
		in liquid nitrogen				under paraffin oil			
		not passaged	number of passages			not passaged	number of passages		
			1	2	4		1	2	4
avr1	13	3	10	13	14	6	8	12	13
avr2	14	4	11	13	13	5	9	12	13
avr3	13	5	12	14	14	3	10	13	14
avr4	13	6	10	14	14	4	10	14	14
avr5	4	0	2	4	6	0	0	0	0
avr6	14	2	12	12	13	5	10	13	14
avr7	14	5	12	14	14	6	9	11	14
avr8	1	0	0	0	0	0	0	0	0
avr9	0	0	0	0	0	0	0	0	0
avr10	5	3	3	3	4	3	3	3	4
avr11	13	6	5	5	10	2	7	9	10
Mean number of virulence factors per isolate	7.4	2.4	5.5	6.6	7.3	2.4	4.7	6.2	6.9

[1]the initial evaluation of expression of virulence factors of *P. infestans* isolates was assessed on 27 March 2002

Storage under paraffin oil

In our study, some *P. infestans* isolates (US-8, MP324, MP419 MP420, MP421, MP424, MP426, MP428, and MP429) stored for eight years under paraffin oil were aggressive at the level of 1.8 to 3.8 degree after a single passage through potato slices (Table 3). To obtain high aggressiveness, the isolates MP322, MP423, MP425, and MP427 (range from 1.5 to 4.0) had to be passaged twice through fresh potato tissue, and the isolate MP430 – even four times. The average aggressiveness after storage under paraffin oil was still significantly different from the initial score after the first and the second passage of the isolates through potato tissues. Only after the fourth passage, this trait returned to the level noted before storage (Table 3). After a long storage of *P. infestans* cultures under paraffin oil, a decrease in the aggressiveness level was already observed. However, high levels of this characteristic can be restored by 1–2 passages of the pathogen through potato tissues (Sujkowski 1992). In a different study, the cultures passaged twice and those passaged many times through potato tuber slices, were significantly more aggressive in comparison with the ones that were passaged only once (Sobkowiak *et al.* 2004a).

The effect of storage of *P. infestans* isolates on their virulence

In the initial assessment done before storage, the mean number of virulence factors per isolate of the 14 tested *P. infestans* isolates was 7.4. After recovery from an eight-year storage by two methods, the same isolates were tested again for virulence on 11 Black's differentials directly, and after one, two, and four passages through potato tissues (Table 4). *P. infestans* isolates recovered after storage were less virulent than before storage. The average number of virulence factors per isolate was 2.4 for both storage methods. A reduction of *P. infestans* virulence after storage in liquid nitrogen has been described before (Sobkowiak *et al.* 2004a). In our present study, passages through living potato tissues caused an increase of virulence expression. In the case of isolates stored in liquid nitrogen, the mean number of virulence factors per isolate was 5.5, 6.6 and 7.3 after one, two, and four passages, respectively (Table 4). After storage under paraffin oil, the increase in the mean number of virulence factors per isolate was smaller and reached 4.7, 6.2 and 6.9 after one, two, and four passages, respectively. While after the fourth passage, most of the virulence factors were scored in approximately the same number of isolates as before storage, but an *avr5* was not. Before storage, this virulence factor was present in four isolates out of 14 tested, but after storage under paraffin oil and four passages through potato tissues it was not detected in any of them. In contrast, after storage in liquid nitrogen and four passages through potato tissues, *avr5* came out in six *P. infestans* isolates (Table 4). These data correspond well with the data obtained by a consortium that tested the stability of *P. infestans* virulence in 12 different European laboratories. The results of the ring test indicated that virulence to R5 is rather variable and sensitive to environmental conditions (Andrivon *et al.* 2011). However, virulence to R2, also described by Andrivon *et al.* (2011) as variable, was stable in our study and it was detected on a similar level before and after storage (Table 4).

The stability of virulence before and after storage and four passages through potato tissues varied depending on the isolate (Fig. 1). The virulence of the most stable *P. infestans* isolates US-8 and MP424 was not affected by long-term storage. The isolate MP423 before storage was virulent only on three differentials, and after storage on eight of them (Table 1, Fig. 1). This gain of virulence could possibly be explained by an unreliable initial assessment of this isolate, in which only few virulence factors were identified.

Thurston (1957) and Hodgson and Sharma (1967) found that *P. infestans* isolates maintained on artificial medium, lost pathogenicity. Subsequent passages of the

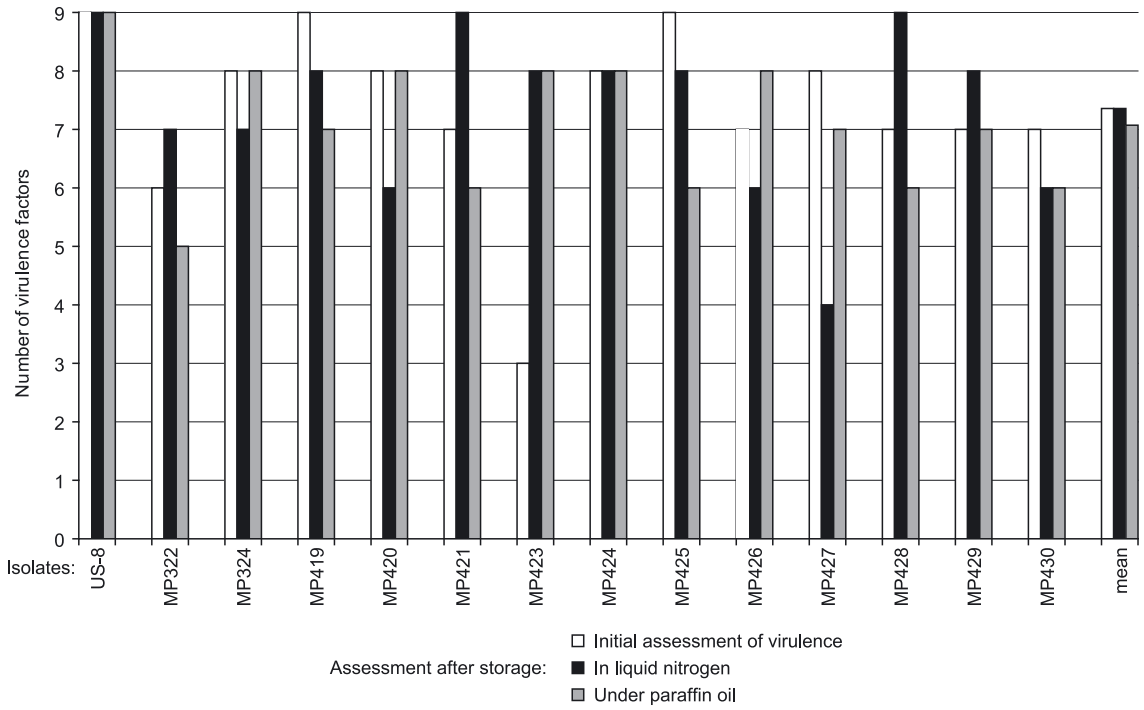

Fig. 1. Virulence of *P. infestans* isolates stored for eight years in liquid nitrogen and under paraffin oil and passaged four times on potato tissues

Oomycete on the leaves of potato genotype, from which the pathogen isolate originated, restored its original characteristics (Sujkowski 1983). Zarzycka observed (1995) the decrease of the virulence factors detectability in *P. infestans* cultures to 71% after its maintenance on rye agar medium at 7°C for eight months, while Pietkiewicz (1972) supposed the greater loss (70–80%) of the virulence spectrum in isolates stored on tuber slices for five months. However, in our study four passages through potato tissues were sufficient to recover virulence of isolates to the levels observed before storage. The discrepancies were within the range of error in the scoring of this trait noted by others (Andrivon *et al.* 2011).

CONCLUSIONS

1. The survival rate of *P. infestans* isolates stored in liquid nitrogen for eight years was a high 88%.
2. The isolates stored in liquid nitrogen and under paraffin oil and passaged four times through potato tissues showed a similar mean number of virulence factors per isolate and did not differ from cultures evaluated before storage.
3. Isolates stored under paraffin oil showed no virulence on *R5* potato differential, but this virulence factor was detected in isolates after storage in liquid nitrogen.
4. The passages through potato tissue enhance aggressiveness and virulence of *P. infestans* isolates after storage and at least two passages are needed to recover the before-storage qualities of the isolates.

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