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Assessment of Possibilities of Microtuber and in vitro Plantlet Seed Multiplication in Field Conditions. Part 1: PVY, PVM and PLRV Spreading

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Abstract Currently in vitro plantlets and microtubers provide the basis for pre-base production of potato seeds, from which minitubers are produced under covers – they serve later as seed material to be planted in the field. The aim of the research was to determine the possibility for multiplication of material produced in vitro directly in field conditions. The research assessed PVY, PVM and PLRV infection of potato tubers derived from plants grown directly from in vitro plantlets, microtubers, minitubers and traditional seed potatoes planted in the field at different times. Moreover, testing in laboratory conditions, the susceptibility of these plants to virus infection was determined for the case of artificial inoculation of *Myzus persicae* and *Aphis nasturtii*. It was found that the infection of tubers derived from in vitro plantlets and microtubers was greater than that of seed potatoes and minitubers. Yet it seems that the reason for their higher infection level resulted not from the plant's sensitivity or its greater attractiveness to aphids but from a largely unknown cause. Earlier planting of microtubers and in vitro plantlets in the field in case of the more resistant cultivar and certainly later in relation to the main time of planting had an impact on limiting the PVY and PVM infection of potato tubers. Hence multiplication of microtubers and in vitro plantlets in field conditions could be very economical using cultivars which are relatively resistant to viruses. However, adopting a later than usual planting period (end of June) and applying an additional protective cover (such as non-woven agricultural fabric) in the first period of a plant's growth, promotes multiplication of microtubers and in vitro plantlets in field conditions for cultivars with low resistance levels.

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Resumen Actualmente, las plántulas in vitro y los microtubérculos suministran el sustento para la producción de semillas pre-básicas de papa, de las cuales se producen los minitubérculos bajo cubierta, que después sirven como material de siembra para ser plantado en el campo. El propósito de esta investigación fue determinar la posibilidad para multiplicación de material producido in vitro directamente bajo condiciones de campo. La investigación analizó la infección por PVY, PVM, y PLRV en tubérculos derivados de plantas que crecieron directamente de plántulas in vitro, microtubérculos, minitubérculos y semilla-tubérculo tradicional de papas sembradas en el campo en diferentes tiempos. Además, en pruebas de laboratorio se determinó la susceptibilidad de estas plantas a la infección viral mediante inoculación artificial de *Myzus persicae* y *Aphis nasturtii*. Se encontró que la infección de tubérculos derivados de plántulas in vitro y microtubérculos era mayor que la del tubérculo-semilla y minitubérculos. Sin embargo, parece ser que la razón para su mayor nivel de infección fue el resultado, no de la susceptibilidad de la planta o por su mayor atractivo a los áfidos, sino por otra causa mayor desconocida. La siembra temprana de microtubérculos y las plántulas in vitro en el campo, en el caso de la variedad más resistente y seguramente más tarde en relación con la fecha principal de siembra, tuvieron impacto en la limitación de la infección de tubérculos por PVY y PVM. De aquí que la multiplicación de microtubérculos y de plántulas in vitro, bajo condiciones de campo, pudiera ser muy económica usando variedades relativamente resistentes a los virus. No obstante, la adopción de una fecha de siembra más tarde de lo normal (a fines de junio) y aplicando una cubierta adicional de protección (como una malla agrícola) en el primer período de crecimiento de la planta, estimula la multiplicación de microtubérculos y de plántulas in vitro en condiciones de campo en variedades con bajos niveles de resistencia.

Keywords Minitubers · Microtubers · PLRV · PVM · PVY · in vitro plantlets · Potato · *Myzus persicae* · *Aphis nasturtii*

Introduction

Seed production is a key element in potato production. Nowadays, in many countries globally, including in Poland, in vitro plantlets, microtubers and minitubers are the basis for pre-base material production (Struik and Wiersema 1999; Halterman et al. 2012; Wang et al. 2011). The application of seed material from in vitro permits:

- commencing seed production on the basis of possessing entirely healthy base material (base material) (no virus infection and quarantine diseases);
- rapid multiplication, irrespective of the season, in heated and lighted glasshouses or foil tunnels;
- storage and transportation of the seed material in a relatively easy way because of their small bulk;
- wide international exchange.

The introduction of micro- and minitubers into seed production has revolutionized production, resulting in a shortening of the field production cycle to obtain an adequate number of seed potatoes and hence guaranteeing a high level of healthiness of base materials. Microtubers are produced in laboratory conditions. They are made as a result of tuberization in controlled conditions on in vitro plantlets. The production of micro-tubers takes about 80 days including 60 days in darkness. In terms of their size they are reminiscent of a bean seed, their weight ranges between 24 to 273 mg and their diameter from 4 to 7 mm, their length 10–12 mm (Ranalli 2007).They constitute base material for the production of mini-tubers which can also be made from in vitro plantlets planted directly into the soil, e.g. in glasshouses. Much research is concerned with improving efficacy and increasing the size of produced micro-tubers e.g. by cyclical overflowing of plants with fluid nutrient during tuberization (Etienne and Berthouly 2002), and providing with nutrient diversified doses of potassium (Naik and Sarkar 1998), or with Jasmonic acid (Zhang et al. 2006), etc. A lot of researchers have examined the possibility to plant microtubers directly in field conditions (Wattimena et al. 1983; Leclerc and Donnelly 1990; Rannalli et al. 1994; Kawakami et al. 2003). In the research, concerned mainly with the plants' growth and their harvesting, the researchers found lower and uneven tuber yield during the research years in comparison with conventional crops using traditional seed potatoes.

Minitubers are small tubers produced on in vitro plantlets or microtubers. Depending on the cultivar and density of planting their size ranges from 10 to 50 mm. Therefore, the seed value of minitubers (apart from healthiness) determines their size. From one in vitro plantlet or microtuber it is possible to obtain from 2 to 10 minitubers, and using the most modern methods, as many as 40 minitubers (Struik and Wiersema 1999), though they are very small tubers with little seed value. It largely depends on plant density per m^2. In the global production of seed, minitubers presently constitute a bridge between fast methods of in vitro reproduction based on passage of in vitro plantlets production and field reproduction. Moreover, currently applied methods for their controlled multiplication ensure a high level of healthiness in the collected tubers. Minitubers are usually reproduced by breeders looking to obtain a high level of seed potato healthiness or with the intention of selling on to specialized seed producers to reproduce subsequent generations. Seed potato production from minitubers requires a much greater control, sometimes employing covers in field conditions, especially when the onset or end of the season is frosty or involves heavy aphid infestation (Struik 2007). In the case of minitubers planted directly into the field, their size matters. Lommen and Struik (1994, 1995) and Rykaczewska (2007) found that the larger the minitubers, the more equal are the emergences and the higher the yield and contents of the dry mass.

Within the literature, there is minimal information addressing the possibility to plant in vitro plantlets and microtubers in field conditions with respect to seed reproduction. In particular how they react to virus infections, the impact of the level of plant infestation by aphids on the virus infection of progeny tubers or the efficacy of diversified times of planting in the field in order to protect the young plants against first (in the case of an earlier planting) or peak (at a delayed time of planting in field) aphid flights. From the only available paper (McDonald 1987) it follows that an attempt to use in vitro plantlets in field production carried a risk of *Potato virus Y* (PVY) and *Potato virus S* (PVS) infection. In the case of PVY significant differences in comparison with the control – constituted by traditional seed potatoes occurred only in 1 year. In my own research it was observed that plants grown in a field from in vitro plantlets were more numerously settled on by wingless aphids, *Aphis nasturtii* Kalt., than for plants produced from traditional seed potatoes (Wróbel 2009).

There is therefore a need to find out precisely the reaction of materials derived from in vitro (in vitro plantlets, micro- and minitubers) to virus infections and study the difficulties of such multiplication in the field. In order to assess the possibility for seed production in the field directly from these materials, three basic research aims were defined:

1. A comparison of PVY, *Potato virus M* (PVM) and *Potato leafroll virus* (PLRV) infection of tubers derived from in vitro plantlets, microtubers, minitubers and seed potatoes in field conditions.

2. An assessment of different planting times for microtubers and in vitro plantlets in the field with respect to virus infection of progeny tubers.
3. An assessment of the susceptibility of plants derived from different forms of seed materials (in vitro plantlets, microtubers, minitubers, traditional seed potatoes) to PVY, PVM and PLRV infection under conditions of artificial inoculation with aphids: *Myzus persicae* Sulz. and *A. nasturtii* (laboratory conditions).

Material and Methods

Field experiments were conducted between 2006–2012 in the north of Poland (Department of Potato Protection and Seed Science in Bonin near Koszalin, 54°09' N, 16°15' E). We compared the PVY, PVM and PLRV infection of progeny tubers in material grown from in vitro plantlets, microtubers and minitubers and traditional seed potatoes from cultivars of different resistance levels to viruses (Table 1). Between 2010–2012 we also assessed the impact of diversified times of planting on the in vitro plantlets and microtubers in field conditions for the infection of progeny tubers with the above mentioned viruses.

On each occasion, the seed material (in vitro plantlets, microtubers and minitubers) was derived from the Bank of Germplasm by the Department of Potato Protection and Seed Science in Bonin near Koszalin. Seed potatoes were purchased annually, directly from the breeder of the cultivar. To make sure that they were not infected with viruses prior to planting they were assessed in terms of healthiness using the DAS ELISA in an eye test.

Table 1 Index of potato cultivars used in the field experiment

Year	Cultivar (maturity – mid-early)	Resistance to†		
		PVY	PLRV	PVM
2006	Adam	3–4	5–6	-††
2008	Adam	3–4	5–6	-
2009	Quincy	3–4	3–4	-
	Tajfun	7	7	2,5
2010	Quincy	3–4	3–4	-
	Tajfun	7	7	2,5
2011	Quincy	3–4	3–4	-
	Tajfun	7	7	2,5
2012	Quincy	3–4	3–4	-
	Tajfun	7	7	2,5

† ratio 1–9, where 1 refers to no resistance, and 9 to total resistance

†† resistance was not assessed, no data

Preparation of the Material and Planting

Because of the large number of microtubers and in vitro plantlets as well as the difficulties in obtaining them, the whole experiment was carried out over three replications. One small plot of land comprised 4 ridges (spacing 75 ×35 cm), each of which was randomly planted with seed material of different origin totaling 50 items: traditional seed potatoes, minitubers, microtubers and in vitro plantlets. Additionally, potatoes secondarily infected with viruses were planted around the plots (with a single ridge on each side) in order to increase virus infection pressure. In total, for each cultivar, 6 plots were created, out of which half were additionally protected with Sunspray 850 EC mineral oil in intensity 2 or 4 % (2006 and 2007), usually in 7-day-long intervals subsequent to 90 % of emergences appearing. The plots were additionally distributed at random within a larger plot of land, both protected and unprotected with mineral oil.

The major period for planting in the field was during the fourth week of April, referred to as the 2nd term (Table 2) within the study. At this time only seed potatoes (35–45 mm diameter) and minitubers (15–30 mm diameter) were planted manually. However, in the case of minitubers, planting was done on the previously profiled ridges due to their size. The in vitro plantlets and microtubers were not planted directly in the field due to their delicacy and sensitivity to early spring weather conditions (low temperatures) but the seed material was first prepared in a glasshouse. For this reason, about 7–10 days after planting the traditional seed potatoes and minitubers in the field, microtubers with a 5–10 mm diameter were planted in the glasshouse into pots containing peat substrate. Seven days later the in vitro plantlets were treated in the same way. At the point at which full emergences of seed potatoes and minitubers were achieved on the plots (this took place around 23–39 days after planting, depending on the year of the research and the cultivar), microtubers and in vitro plantlets were well developed (average height about 10–15 cm) and rooted and could therefore be planted manually in the field. These were planted sufficiently deep so as to not make them stand out over the top of the ridges by more than 3–7 cm. Planting of in vitro plantlets and microtubers was delayed in order to equalize the size of all seed materials growing in the field at the time of a threat with the first virus infections.

Between 2010–2012, the impact of an earlier term (1st term – second week of April) and a much later one (3rd term – last week of June/first week of July) on the planting of microtubers and in vitro plantlets in the field was also assessed. For these studies the plants were planted in pots and prepared as described above, prior to planting in the field. A single plot comprised two ridges, on one of which microtubers were planted and, on the other one, in vitro plantlets, in total

Table 2 Dates of planting and treatments used in 2006–2012

Treatments	2006	2008	2009	2010	2011	2012
Date of planting in the field						
1st term (II decade† of April)	-	-	-	16.04	12.04	13.04
2nd term (III decade of April)	28.04††	28.04	22.04	26.04	21.04	20.04
3rd term (III decade of June/I decade of July)	-	-	-	29.06	1.07	2.07
Number of fungicide/insecticide treatments						
1st term	-	-	-	2/1	2/2	3/1
2nd term	2/2	4/1	7/1	3/1	5/2	61
3rd term	-	-	-	4/0	4/0	4/0
Date of haulm damage						
1st term	-	-	-	30.07	13.07	20.07
2nd term	27.07††	6.08	11.08	12.08	12.08	14.08
3rd term	-	-	-	24.09	15.09	25.09
Number of mineral oil treatments						
1st term	-	-	-	4	3	4
2nd term	8	9	9	9	10	11
3rd term	-	-	-	5	6	7

† the first decade of month is from 1 to 10 day, the second decade is from 11 to 20 day, and the third decade is from 21 day to end of month

†† day of month, e.g. "28.04" means 28 April

approximately 50 plants. Consistent with the main time of planting (2nd term) potato tubers secondarily infected with viruses were planted on the neighboring ridges. Plants from the pots were planted onto profiled ridges which one day earlier were sprayed with the herbicide Plateen 41.5 WG (metribuzin 17,5 %+flufenacet 24 %) in a dose of 2 kg ha^{-1}. After planting, the plants were watered with a water solution of seed treatment, Prestige 290 FS (imidachloprid 140 g l^{-1}+ pencycuron 150 g l^{-1}), with a concentration 0.1 %, dose 100 ml per plant. The seed treatment was required to protect plants against Colorado potato beetle, which emerged from the soil during an earlier term of planting and to protect against potential aphids in a later planting. Both ridges together with plants were subsequently covered with non-woven agricultural fabric of width 3.2 m and weight 19 g $(m^2)^{-1}$, providing enough space for the plants to grow freely during the following weeks. The cover aimed to protect delicate plants during an early period of growth against cold weather and in a later period it provided a mechanical barrier against aphids.

The duration of the plants' growth under covers was dependent on the weather across particular years of the research. In the case of the first term of planting it ranged from 62 to 77 days, and in the case of the third term, 36–44 days. During this period the plants were neither uncovered nor additionally protected against diseases and pests. Subsequently the fabric was removed and the plants protected with mineral oil Sunspray 850 EC in 2 % concentration and with fungicides against *Phytophtora infestans*.

Protection and Observations During Growth Season

During the entire growth season full chemical protection against *P. infestans* and *Colorado potato beetle* was carried out. From 2 to 7 treatments were made annually against potato disease and a maximum of 2 treatments against *Colorado potato beetle*.

Every 10 days, observations on plant settling by aphids were carried out using a method of "100 leaves" for each assessed combination. For this purpose, out of 100 plants selected at random, a single leaf was picked from a middle internode (in total 100 leaves) and all the aphids were counted, with a division into species and developmental forms.

Assessment of Virus Infection

When potato progeny tubers achieved adequate size (seed potato domination) and the skin was mature, the leaves were damaged mechanically-chemically. This method involves mechanical damage of the plants' overground parts leaving only 15 cm of the stalk (for this purpose we used the potato haulm topper Grimme KS 75–2). This is followed by spraying with a dessicant in a 50 % lower dose, in this case Reglone 200 SL (ion of diquat 200 g l^{-1}), applied dose – 2.5 l ha^{-1}. After around 14 days, in order to assess the infection of the progeny tubers with viruses, a single tuber was picked from each plant at random. The tuber's diameter was approximately 40–50 mm and, in total, depending on the year, the number of collected tubers ranged from 1200 to 3600. PVY, PVM and

PLRV infection of the collected tubers was evaluated after 8–9 months of storage during the spring of the following year (April-May), in an eye test using DAS ELISA. The evaluation procedure was performed as follows: the cut out fragments of tubers containing the eye were planted into pots with a soil substrate in a glasshouse. Around 4 weeks after emergence from the middle internode of each plant, 2–3 leaves were picked from which sap was extracted. The presence of PVY, PVM and PLRV was evaluated from the sap (diluted with extraction buffer in a 1:10 ratio) using a modified procedure of DAS ELISA, as described by Wróbel (2013). Polyclonal antibodies from Neogen Europe Ltd. (http://plant.neogeneurope.com) and microtiter plates from Greiner Bio One (Product no. 655101) were applied. In total during the 6 years of field research about 15 600 tubers were analyzed.

Laboratory Research

In terms of laboratory research carried out in glasshouses throughout 2010–2012, artificial inoculations were made, inoculating potato plants with PVY (strain: $PVY^{N}W$ – tobacco veinal necrosis Wilga isolate, PVY^{NTN} – potato tuber necrotic ring spot disease) and PVM using winged forms of aphids *M. persicae* and *A. nasturtii*, and with PLRV – using *M. persicae*. Young (few days old) plants which grew out of in vitro plantlets, microtubers, minitubers, and traditional seed potatoes were also inoculated.

Test Plants

Seed material (in vitro plantlets, microtubers and minitubers) came from the Bank of Germplasm at the Department of Potato Protection and Seed Science in Bonin. Seed potatoes were bought directly from the cultivar breeder. In order to make sure that they were not infected with viruses, each time following their purchase, their health was tested using DAS ELISA and an eye test.

Every 7–10 days, in vitro plantlets and microtubers of approximately 0.5–1 cm diameter, minitubers of about 1.5–2 cm diameter and seed potatoes of approximately 2–3 cm diameter were planted into pots filled with peat substrate, in a series of 20–30 plants. Plants grown out of traditional seed potatoes and minitubers were inoculated in stage 2 of developed leaves (height about 5–7 cm), while plants derived from microtubers were inoculated having reached the height of 5 cm. As far as in vitro plantlets are concerned, after their adequate rooting, the inoculation was made 7–10 days following their planting in the glasshouse.

For microtubers and in vitro plantlets they were sizes which during the experiment enabled for their transfer from the glasshouse to the field.

For each of the assessed viruses, plants of two potato cultivars with different resistance to PVY, PVM and PLRV were infected (Table 3).

Aphids

For PLRV artificial inoculation, winged specimens of M. *persicae* were used as they are considered to be the most effective vectors of this virus. For PVM inoculation winged specimens of *A. nasturtii* were used, whereas for the inoculation with PVY, both aphid species were used: *M. persicae* as the most effective vector and *A. nasturtii* as the most common species on potato plantations during growth season. The large number of these aphids, in spite of their lower effectiveness in PVY transfer, pose a great threat in practice. To ensure adequate aphid numbers, they were constantly bred in a phytotron (special insect incubation chamber). A 16-h-long day was established during which the plants on which aphids were bred were artificially lit with additional light of intensity 13 300 lx, and a 8-h-long night (with no lighting). Temperature during the day did not exceed 25 °C with relative humidity approximately 40 %, and, at night, 15 °C and 60 % respectively. The population of each aphid species was introduced from one specimen on each occasion. Beijing cabbage *(Brassica pekinensis* Rupr) was used as host plants for *M. persicae*, whereas potato plants (*Solanum tuberosum*), free from viruses, were used for *A. nasturtii*.

Virus Sources

The sources of particular viruses were potato plants secondarily infected with PVY^{NTN}, $PVY^{N}W$, PVM or PLRV. Each of these viruses was present on its own on the plant. In order to ensure an adequately high concentration of the virus in the plant, plants were analyzed every few days for their presence using DAS ELISA. Only those plants which had high absorbance value were selected for the experiment. Higher absorbance value guaranteed higher concentration of the virus in the plant.

Table 3 Potato cultivars used in the laboratory experiment

Virus assessed	Cultivar	Resistance to virus[†]
PVY	Quincy	3-4
	Tajfun	7
PVM	Rosalind	2–3
	Korona	7
PLRV	Quincy	3–4
	Tajfun	7

[†] in ratio 1–9, where 1 denotes no resistance, and 9 total resistance

Method Employed for Inoculation of Plants with Viruses

The whole experiment was divided into approximately a dozen trials, performed between 2010–2012. Each time (per day) 10–20 plants of each type of seed material and each cultivar underwent artificial inoculation. It was planned that a minimum of 140 plants grown out of in vitro plantlets, microtubers, minitubers and traditional seed potatoes would be infected with each virus. In some cases (e.g. PLRV) the number of inoculated plants was increased due to the difficulties with its transfer, with a sample involving a 24-h-long feeding. In others, because of the lack of test material ($PVY^{N}W$ – *M. persicae*), the number was smaller. In total 6 928 potato plants grown from various seed materials underwent artificial inoculation (Table 4 and 5).

For inoculation, the fittest insects were selected with no visible damage. They were placed in glass test-tubes protected with density gauze. Aphids placed in this manner were starved for approximately 2 h to increase their voracity. After that time they were transferred onto plants which contained the virus source for acquisition feeding. In the case of PVM and PVY strains, this feeding took around 2–4 min (Kostiw 1976), whereas for PLRV it took 48 (Kostiw 1987) or 72 h. Following virus acquisition, feeding aphids were transferred by their wings using tweezers onto test plants for inoculation feeding. The inoculation stage took 2–4 min for PVY and PVM respectively and 48 or 96 h for PLRV. For inoculation of each plant, in order to increase the likelihood of infection, 2 aphids were transferred onto each test plant. After inoculation, insects were removed and destroyed while the plants, after being watered with 0.1 % of Prestige 290 FS seed treatment solution (for protection against potential uncontrolled aphid appearance), were placed in a glasshouse, where they were kept until the end of the growth season in order to start producing tubers. Since, in the case of PLRV the feeding time was relatively long, the plants which were the sources of viruses together with aphids were placed in special isolators (net cages) for the duration of the acquisition feeding. After the aphids were transferred onto test plants, the plants were placed inside finely perforated polyethylene bags and kept in a cold but bright place to ensure they would not overheat. In randomly tested bags, the temperature was 14–25 °C, while relative humidity was 48–99 %. According to Singh et al. (1988) these are the best conditions for PLRV and PVY infection. In the case of PLRV the prolonging of the acquisition feeding time from 48 to 72 h, and of inoculation feeding time from 48 to 96 h was a result of an unsatisfactory result of transferring the virus at the first attempt.

Assessment of Virus Infection

Following the growth season, tubers were collected separately from each plant, and placed in independent, adequately-marked containers and placed in storage for a few months. The effectiveness of inoculation of potato plants – the infection of progeny tubers with viruses – was assessed in the spring of the following year (April–May) in an eye test using DAS ELISA. From the collected tubers for each individual plant, two tubers were selected at random to decrease the possibility of an error associated with unequal distribution of viruses among progeny tubers. Smaller tubers were planted directly into the pots containing soil substrate in a glasshouse, or in a special plant growth chamber if the spring was cold. From larger tubers, a piece with a single eye was removed using a semicircular spoon and planted as described above. Further actions were analogous, as in the case of samples taken from the field. However, the medium of infection from two planted tubers was not analyzed, only the infection or its lack of was assessed.

Results Analysis

The results concerning the virus infection of tubers underwent Bliss transformation according to the following equation (Wójcik et al. 1976):

$$y = \arcsin\sqrt{x}$$

in which

y value after transformation
x percentage values of virus infection

Table 4 Number of potato plants treated with artificial inoculation with viruses using winged forms of *M. persicae*

Seed material	PLRV		PVY^{NTN}		$PVY^{N}W$	
	cv. Quincy	cv. Tajfun	cv. Quincy	cv. Tajfun	cv. Quincy	cv. Tajfun
Traditional seed potatoes	154/104[†]	179/105[†]	211	197	58	85
Minitubers	205/100	177/93	232	202	81	90
Microtubers	189/112	164/105	226	201	74	88
In vitro plantlets	158/108	160/104	207	204	82	87

[†] the first value denotes the number of plants treated with artificial inoculation for acquisition feeding and inoculation feeding lasting 24 h each, whereas the second concerns the longer time of aphid feeding, 72 and 96 h respectively

Table 5 Number of potato plants treated with artificial inoculation with viruses using winged forms of *A. nasturtii*

Seed material	PVM		$PVY^{N}W$	
	cv. Rosalind	cv. Korona	cv. Quincy	cv. Tajfun
Traditional seed potatoes	150	148	166	156
Minitubers	150	150	152	150
Microtubers	155	151	140	144
In vitro plantlets	152	143	131	148

Subsequently, the obtained values underwent statistical analysis using an ANOVA. To assess the significance of differences between the studied combinations, mean values were tested using Tukey's test at the significance level $p=0.01$ in order to increase the reliability of the obtained results. Statistical calculations were made using Statistica 10.0 (StatSoft, Inc. 2011). Having made the analyzes, the obtained values were retransformed to percentages and are presented in this form in the paper.

Results and Discussion

Aphids Settling on Plants

The dynamics of plant settling by aphids varied across the years. The greatest number were recorded in 2008 (Wróbel 2009), and also in 2012, when very early and numerous colonies of aphids on leaves were registered at the end of May, which meant that their flights from winter hosts onto potatoes were very early. Such a state translated to significantly higher values of virus infection of tubers in comparison with the remaining years of the research. Although in previous years some increased tendencies were observed among aphids to settle on potato plants grown from microtubers and in vitro plantlets (Wróbel 2009), especially in 2008, i.e. during the season in which high aphid pressure was prevalent, in subsequent years of research 2009–2012 such dependencies were not observed (Fig. 1). Slight differences in aphid numbers, on plants grown out of traditional seed potatoes, minitubers, microtubers and in vitro plantlets, recorded in particular seasons, were not confirmed statistically. This can result partially from high aphid pressure. However, Boiteau et al. (2000) did not observe differences in laboratory studies of behavior and preferences among winged *M. persicae* to colonize plants grown out of traditional seed potatoes, minitubers, and in vitro plantlets. One can, therefore, assume that the origin of seed material did not influence aphid settlement preferences for potato plants.

It was claimed that a later planting (3rd term) and application of non-woven agricultural fabric for the first 36–44 days significantly limited aphid settling on these plants. The cover was removed around 10th August – after the peak flight. After the non-woven agricultural fabric was removed, not a single aphid was observed on potato leaves during observations carried out until the end of September. In addition, Kostiw and Robak (2012) in their systematic catches into yellow traps registered a significantly decreasing number or a sporadic occurrence of winged aphids in that period.

PVY, PVM and PLRV Infection Pressure

Particular growth seasons were significantly diverse in terms of the infection pressure of the assessed viruses (Fig. 2). PVY spread the most extensively, reaching its highest pressure in 2012. PVM spread less intensively in spite of there being

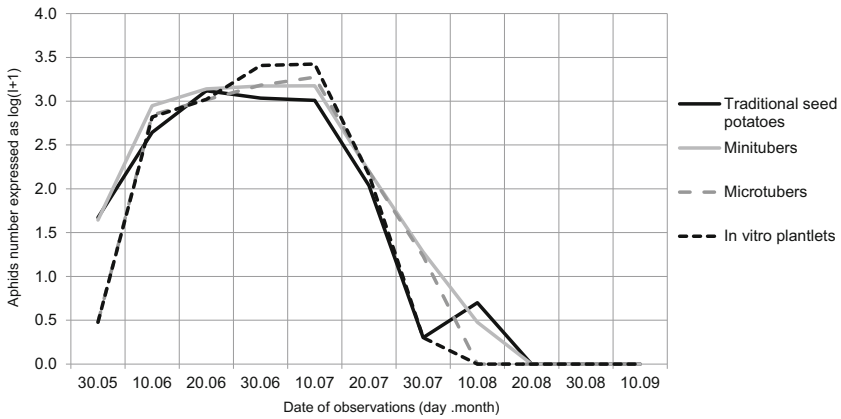

Fig. 1 Dynamic of aphids *M. persicae, A. nasturtii* i *A. frangulae* numbers on potato plants during growth season (total number of specimens on 100 leaves, 2006-2012)

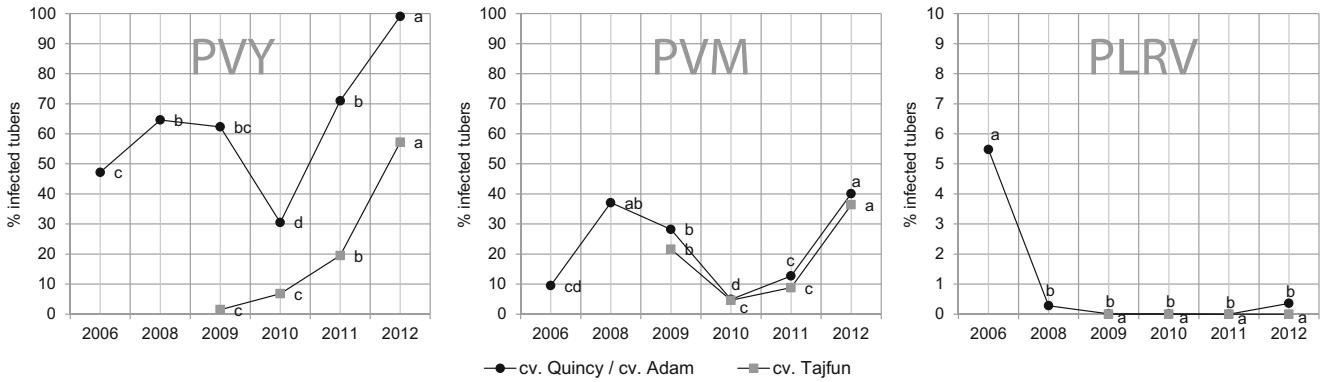

Fig. 2 Infection pressure of PVY, PVM and PLRV expressed using mean percentage of infected tubers from all tested seed materials in particular research years (sample size - 2400 tubers from each varieties in each year, means with the same letters do not differ significantly according to the Tukey test (p=0.01))

several sources of infection nearby. Similar peak intensities of PVM were recorded in 2008 and 2012. One can see that in the case of both potato cultivars the intensity of PVM was similar across the study years. This affirms the fact that although there is no information regarding the resistance of Quincy cultivar to PVM, one can certainly assert that the cultivar has a similar level of resistance to this virus to that of the Tajfun cultivar. A similar, strongly varying potato virus pressure was also observed by Kostiw and Robak (2010), Kostiw (2011) and Wróbel (2012).

PLRV was essentially not recorded in the assessed material. Though in 2006 about 5.5 % of tubers were recorded as having been infected with this virus, in the remaining years of the research the virus was not recorded. This confirms a tendency which has been observed for some time now for PLRV to disappear in Poland (Kostiw 2011; Kostiw and Sekrecka 2009; Wróbel and Wąsik 2013).

Susceptibility to Viruses

Analysis of the collected material clearly indicates that there is a markedly more frequent PVY infection of tubers derived from plants grown out of microtubers and in vitro plantlets than in the case of traditional seed potatoes and minitubers (Table 6). The differences were clearer in the case of the Tajfun cultivar, which is more resistant. Along with a fall in the resistance level these differences were smaller yet are still statistically significant. It is worth noting that in the case of a resistant cultivar, the maintenance of adequate healthiness of the collected tubers, likewise for minitubers, would be difficult. Thus seed multiplication of very susceptible cultivars at the stage of pre-base materials will pose difficulties. McDonald also mentions this (1987) in his paper when describing one of the first field experiments on in vitro plantlets.

PVM, just like PVY was stronger in infecting seed material from in vitro plantlets than traditional seed potatoes (Table 7). In spite of there being small plots of land around the experimental field – providing numerous sources of PLRV, the spread of PLRV was marginal or was not recorded at all. Hence no dependencies were found (Table 8).

Mineral oil protection was very effective – the share of tubers infected with PVY was 40–50 % smaller in relation to unprotected plants (Table 9), while that of PVM infected – 52–60 % smaller (Table 10). Nevertheless no statistical differences were observed regarding the effect of protection with mineral oil according to the kind of seed material. The effectiveness of protection was on a similarly high level both in the case of traditional seed potatoes and the remaining seed materials. Kurppa and Hassai (1989), Milošević (1996), Turska and Wróbel (1999), Rolot et al. (2008), Boiteau et al. (2009),

Table 6 Mean percentage of share of progeny tubers infected with PVY (the main – 2nd term of planting)

Seed material	Susceptible cultivar†	Moderately resistant cultivar††
Traditional seed potatoes	57.0 b	7.1 b
Minitubers	65.0 ab	15.4 ab
Microtubers	70.9 a	24.0 a
In vitro plantlets	69.0 ab	24.1 a

† cv. Adam and Quincy, rating to PVY – 3–4 in ratio 1–9, where 1 denotes lack of resistance, and 9 extreme resistance, mean of years 2006–2012

†† cv. Tajfun, rating to PVY – 7 in ratio 1–9, where 1 denotes lack of resistance, and 9 extreme resistance, mean of years 2009–2012

means within each column with the same letters do not differ significantly according to Tukey test (p=0.01)

Table 7 Mean percentage of share of progeny tubers infected with PVM (the main – 2nd term of planting)

Seed material	Susceptible cultivar†	Moderately resistant cultivar††
Traditional seed potatoes	6.2 b	15.2 b
Minitubers	16.1 a	23.0 a
Microtubers	19.0 a	21.7 ab
In vitro plantlets	25.8 a	21.7 ab

† cv. Tajfun, rating to PVM – 2.5 in ratio 1–9, where 1 denotes lack of resistance, and 9 extreme resistance, mean of years 2009–2012

†† cv. Adam and Quincy, rating to PVM – unknown, mean of years 2006–2012

means within each column with the same letters do not differ significantly according to Tukey test (p=0.01)

Wróbel (2006) also reported on the high efficacy of the mineral oil in the protection of traditional seed potatoes against PVY and PVM.

The Impact of Planting Times

The differentiation of planting times of microtubers and in vitro plantlets in the field had an significant impact on limiting the infection of the tubers with PVY and PVM. Irrespective of the resistance of the cultivar, the most effective period was the delayed 3rd term of planting (Table 10 and 11). Delayed planting influenced a significantly lower level of infection of progeny tubers with PVY and PVM both in the case of in vitro plantlets and microtubers. During this term, with a lower resistance level of the cultivar to PVY, the plants grown from microtubers turned out to be slightly more susceptible to a viral infection than the plants grown out of in vitro plantlets. Moreover, such a low resistance to PVY also mattered at an earlier term of planting which proved to be ineffective in limiting the infection of tubers with this virus: there were no significant differences between the 1st and the 2nd term. However, along with an increase of resistance of the cultivar to PVY, the share of infected tubers decreased significantly. This explains why for the more resistant cultivar (Tajfun), an earlier planting significantly limited the PVY infection level of the tubers. In relation to PVM, in spite of a low resistance level of both cultivars, both terms of planting (1st and 3rd) led to healthier tubers (Table 11).

Considering the numerous sources of viruses around the little plots, i.e. creating provocative conditions of multiplication, which is unusual in seed production, the delay in microtuber and in vitro plantlet planting in field conditions seems the most favorable from a practical point of view.

Table 8 Mean percentage of share of progeny tubers infected with PLRV (main – 2nd term of planting)

Seed material	Susceptible cultivar†	Moderately resistant cultivar††
Traditional seed potatoes	0.3 a	0.0 a
Minitubers	0.5 a	0.0 a
Microtubers	0.2 a	0.0 a
In vitro plantlets	0.6 a	0.0 a

† cv. Adam and Quincy, rating to PVY – 3–4 in ratio 1–9, where 1 denotes lack of resistance, and 9 extreme resistance, mean of years 2006–2012

†† cv. Tajfun, rating to PVY – 7 in ratio 1–9, where 1 denotes lack of resistance, and 9 extreme resistance, mean of years 2009–2012

means within each column with the same letters do not differ significantly according to Tukey test (p=0.01)

Table 9 Mean percentage of share of progeny tubers infected with PLRV (main – 2nd term of planting)

Oil protection	Susceptible cultivar†			Moderately resistant cultivar†		
	PVY	PVM	PLRV	PVY	PVM	PLRV
No	79,0 a	23,8 a	0,4 a	24,5 a	28,2 a	0,0 a
Yes	50,5 b	9,6 b	0,4 a	10,6 b	13,4 b	0,0 a

† explain see table 6, 7 and 8

Laboratory Experiments

This stage of the research aimed to help explain the reasons for a higher infection level in field conditions of in vitro materials (microtubers and in vitro plantlets). It was assumed that one reason can be a greater delicacy of these plants in relation to those grown from traditional seed potatoes – hence their greater susceptibility to infections. It was attempted to infect a large group of materials with viruses using aphids. Yet the obtained results (Table 12, 13 and 14) are not entirely unequivocal and do not point to clear causes of heightened infection levels in the field. However, one can clearly see a highly significant difference in the susceptibility of the cultivars. In the case of PLRV the Quincy cultivar was infected several times more than the more resistant Tajfun cultivar. However, no significant differences in the infection level

Table 10 Impact of planting terms on percentage share of tubers infected with PVY (mean values from the years 2010–2012)

Planting terms	Susceptible cultivar†		Moderately resistant cultivar†	
	microtubers	in vitro plantlets	microtubers	in vitro plantlets
1.	52.9 a	52.7 a	4.1 b	4.6 b
2.	58.6 a	58.8 a	25.7 a	27.1 a
3.	12.5 b	2.3 b	1.6 b	0.2 b

† explain see table 6

means within each column with the same letters do not differ significantly according to the Tukey test (p=0.01)

Table 11 Impact of planting terms on percentage share of tubers infected with PVM (mean values from the years 2010–2012)

Planting terms	Susceptible cultivar†		Moderately resistant cultivar†	
	microtubers	in vitro plantlets	microtubers	in vitro plantlets
1.	5.4 ab	9.1 a	1.3 b	2.8 b
2.	13.2 a	11.6 a	8.0 a	13.4 a
3.	1.5 b	0.5 b	0.4 b	0.1 b

† explain see table 7

means within each column with the same letters do not differ significantly according to the Tukey test (p=0.01)

between the tested seed material were registered. This virus proved difficult to transfer. Applying a 24-h-long acquisition feeding time and inoculation feeding time only in the case of microtubers, the progeny tubers were infected much more than in the remaining materials but no statistical differences. After increasing the acquisition feeding time up to 72 h and the inoculation feeding time to 96 h the share of infected plants increased several times. Yet for microtubers it was in total 50 % lower than for the remaining seed materials. No clear replications and statistically significant differences make it difficult to draw conclusions. It is also difficult to relate these results to observations in the field because of a lack of spreading of this virus in recent years (Kostiw 2011; Kostiw and Sekrecka 2009; Wróbel and Wąsik 2013).

In the case of PVY, a higher infection of the cultivar susceptible to the virus was found only when PVY^{NTN} was transferred by *M. persicae*, yet the dependence was not statistically significant (Table 13). Tubers derived from seed potatoes of this cultivar were infected more than in the case of the remaining material, irrespective of the PVY strain and the vector used, yet this dependence was not proven statistically. Moreover, the results were not compatible with the results of the field experiments in which, during each growth season, there was a significantly lower share of infected tubers grown

Table 12 Percentage share of virus infected tubers after an artificial inoculation using winged forms of *M. persicae*

Seed material	PLRV 24/24†		PLRV 72/96†	
	Quincy	Tajfun	Quincy	Tajfun
Traditional seed potatoes	2.6 a	0.0 a	20.2 a	0.0 a
Minitubers	1.9 a	1.1 a	24.0 a	0.0 a
Microtubers	11.1 a	2.4 a	12.5 a	0.0 a
In vitro plantlets	3.8 a	1.3 a	24.1 a	0.0 a
Mean	4.9 a	1.2 b	20.2 b	0.0 a

† time of acquisition and inoculation feeding in hours

means within each column (without "Mean" position) with the same letters do not differ significantly according to the Tukey test (p=0.01)

Table 13 Percentage share of tubers infected with strains of PVY following artificial inoculation using winged forms of *M. persicae* and *A. nasturtii*

Seed material	*M. persicae* PVY^{NTN}		*M. persicae* PVY^{N}W		*A. nasturtii* PVY^{N}W	
	Quincy	Tajfun	Quincy	Tajfun	Quincy	Tajfun
Traditional seed potatoes	33.6 a	5.6 a	11.4 a	4.5 a	46.6 a	14.1 a
Minitubers	7.8 a	2.0 a	8.6 a	2.7 a	2.5 a	11.1 a
Microtubers	8.8 a	3.0 a	5.7 a	6.3 a	29.7a	26.1 a
In vitro plantlets	5.3 a	4.9 a	1.5 a	4.1 a	13.4 a	18.4 a
Mean	13.9 a	3.9 a	4.3 a	4.4 a	23.1 a	17.4 a

means within each column (without "Mean" position) with the same letters do not differ significantly according to the Tukey test (p=0.01)

out of traditional seed potatoes than those grown from microtubers and in vitro plantlets. There was a slightly greater efficacy of transfer in the case of PVY^{NTN} when *M. persicae* was used as a vector. Kostiw and Trojanowska (2011) previously observed similar differences. Although *M. persicae* is considered to be the most effective vector in PVY transfer (Kostiw 1987; Radcliffe and Ragsdale 2002; Verbeek et al. 2010), in fact it was *A. nasturtii* which clearly proved to be more effective in the PVY^{N}W strain transfer. Thus earlier observations concerning a greater settling of plants grown out of microtubers and in vitro plantlets during growth seasons mainly by this aphid species (Wróbel 2009) can in some ways explain higher values of infection as recorded in the material from field multiplications. However, the subsequent years of the research (2009–2012) failed to confirm aphids' increased colonization of in vitro material. Furthermore, Boiteau et al. (2000) did not record differences in preferences for settlement of plants grown out of in vitro plantlets, minitubers and traditional seed potatoes. At present it is difficult to clearly explain an increased susceptibility of microtubers and in vitro plantlets in field conditions to viral infections and, therefore, this problem requires further research.

Table 14 Percentage share of tubers infected with PVM following artificial inoculation using winged forms of *A. nasturtii*

Seed material	Rosalind†	Korona††
Traditional seed potatoes	6.0 a	0.0 a
Minitubers	2.0 a	1.3 a
Microtubers	3.9 a	1.3 a
In vitro plantlets	3.9 a	1.4 a
Mean	4.0 a	1.0 b

† cultivar susceptible to PVM – 3,5

†† cultivar medium-resistant to PVM – 7

means within each column (without "Mean" position) with the same letters do not differ significantly according to the Tukey test (p=0.01)

Significant diversification in resistance of cultivars to PVM (Table 14) was confirmed. Moreover, a higher share of tubers infected with PVM was recorded in the case of traditional seed potatoes, mainly in the case of a susceptible cultivar, yet this dependence was not proven statistically. Plants grown out of traditional seed potatoes were infected 1.5–3 times more frequently than the remaining seed material, yet infection values were very low even in a susceptible cultivar.

Conclusions

- Reproduction of microtubers and in vitro plantlets of cultivars relatively resistant to viruses in the field is possible.
- Later planting (end of June) and use in the first term of plant growth of an additional cover of non-woven agricultural fabric allows for the reproduction of microtubers and in vitro plantlets of cultivars with low resistance to viruses in field conditions.
- It is difficult to clearly explain an increased susceptibility of microtubers and in vitro plantlets to viral infections in field conditions. It seems that it is not their delicacy that is the reason for a higher infection level but another, not entirely known cause and, therefore, this issue requires further research.

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