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Accumulation of Potato Virus Y- in *Nicotiana tabacum* Callus Culture to Obtain a Virus Preparation

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ABSTRACT

The accumulation of potato virus Y- (PVY) in tissue culture of *Nicotiana tabacum* (*N. tabacum*) was studied. Plants of *N. tabacum* variety, Samsun, inoculated with PVY-infected sap of potato variety, Cherie, were used for the virus accumulation. According to enzyme-linked immunosorbent assay (ELISA) results, virus showed the highest optical density (OD) on the 25th day of inoculation. Murashige and Skoog medium containing kinetin 2 mg/l, 2.4-D 0.5 mg/l, indole-acetic acid 1 mg/l sucrose 2% agar 0.7%, in addition to the standard components, was used to induce callus culture from *N. tabacum* leave explants. ELISA results showed that the callus culture was able to maintain viral infection during four transplantations. Slightly and highly purified (Y-Cherie) virus preparations were obtained from the PVY-infected tissue culture. The slightly-purified antigens showed an OD approximately equal to the positive control in sandwich ELISA. The Y-Cherie antigen was detected as PVY necrotic strain. Specific to the virus polyclonal antibodies that reacted with a maximum 1/3200 titer of antigen in indirect ELISA were obtained in the result of the laboratory mouse immunisation.

Keywords: Potato virus Y- (PVY), callus tissue, ELISA, antigen, polyclonal antibodies

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INTRODUCTION

Potato is one of the major crops in Kazakhstan; it takes the second place in terms of its importance, after wheat. Potato productivity mainly depends on the stability and resistance of its varieties to their fungal, viral and bacterial diseases (Hooks et al., 2007; Karasev et al., 2012). Viruses, as intracellular pathogens, are able to change plant's metabolism and cause degeneration, as characterised by a big decline in potato productivity and significant decrease in tubers nutritional and raw values (Whitworth et al., 2006; Rusevski et al., 2013).

Potato virus Y is one of the pathogenic viruses, which causes mostly wrinkled and banding mosaic on leaves in potato (Pallas et al., 2011). PVY virions have thread-like form, with 750 x 12 nm size, and their inactivation temperature is around 55-65ºC and infected-sample sap can be diluted up to 10^{-2} - 10^{-4}. The virus is transmitted to host plants via mechanical contacts and insect-vectors. Diseases caused by PVY are the most harmful as they can dramatically reduce potato production efficiency (Gray et al., 2010). In order to obtain high yield for potatoes, application of sensitive diagnostic methods in potato seed production is very important. The key element in the development of modern diagnostic methods and their implementation in agricultural practice is the availability of qualitative antigen. It is known that highly purified viral antigens are used to produce specific antibodies (Gnutova, 1993). At the same time, specific antibodies are indispensable components of immunoassay diagnostic tests. In vitro, technology allows for production of environmentally clean raw materials throughout the year to increase the content of biologically active substances and regulate virus mass and accumulation in tissue culture (Simakov et al., 2000; Hooks et al., 2007; Islam et al., 2014). Individual authors have used callus tissue as the source of virus antigens (Kogovšek et al., 2011; Rusevski et al., 2013). Growing potato callus tissue with simultaneous virus accumulation has paved way for *in vitro* PVY as mono-infection for a long time (Ding et al., 1998). Callus tissue allows us to obtain highly purified virus antigen as it lacks many specific proteins. The callus culture can be used to extract sufficient antigen and get homogeneous infectious material that is free from contamination by other viruses and pigments all year round (Gnutova, 1993).

The purpose of this study was to analyse PVY accumulation dynamic in *N. tabacum* tissue culture for its further purification and obtain specific antibodies.

MATERIALS AND METHODS

Plants Samsun a variety of *N. tabacum* were used as the accumulators of potato virus Y. Potato banded mosaic is caused by PVY ordinary strain (PVY^{O-F}) and manifested in the form of necrotic veins and dark-brown necrosis in leafstalk. Necrosis is clearly visible from the lower side of the leaf. Severely infected leaves have dark-brown necrosis on their petioles and stems. By the end of the growing season, almost all leaves, firstly the lower ones, dry up and hang on potato stems. Under natural conditions, banded leaf mosaic is accompanied by wrinkliness (Sohair et al., 2007). Test plants were grown from seeds in bio humus "Terra Vita" and soils at a ratio of 1:1. Plants were grown under constant lighting, with 1000 luxes intensity of light and 24-25°C of temperature. Plants were inoculated using standard methods (Kotzampigikis et al., 2009). PVY-infected potato variety Cherie plants were used to inoculate *N. tabacum* test-plants. The inoculated plants were shaded for 24 hours a day. After that, they were contained in diffused light After 20-25 days of inoculation, and the upper young leaves containing viral antigen were separated, washed with distilled water and disinfected, before they were sequentially incubated in 20% C_2H_5OH (1 min), 7% Ca(Cl)OCl (15 min), and 5% NaOCl (20 min) (Gnutova, 1993). Then, the leaves were washed 3 times with sterile water and cut into square shaped segments of 0.5-0.7 mm size. Explants were planted in petri dishes on agar nutrient medium based on mineral Murashige and Skoog containing in addition to the standard components: kinetin 2 mg/l, 2.4-D 0.5 mg/l, indole-acetic acid 1 mg/l, sucrose 2%, agar 0.7%. Incubation of callus over the entire period was performed under constant illumination (1500 lux) at the temperature of 25-26°C. Accumulation of viral antigen in the test plants and callus tissue was monitored by enzyme-linked immune-sorbent assay (Malyshenko et al., 1993). "Sandwich" ELISA commercial diagnostic kits were used to detect potato virus in test-plants (Salim Khan, 2003). The presence of the virus in the test-plants samples was registered using a spectrophotometer of wavelength 490 nm and light vertical flow (ASYS Expert-96, Austria).

Virus antigen was obtained from the non-pigment tissue culture of *N. tabacum* (grown in darkness) after the 4th transplantation. Virus purification was conducted using two different methods. Using the first method, slightly-purified PVY preparation was obtained after homogenizing tissue culture, squashing through two layers of gauze and centrifuging at 3000 rotations per minute for 10-15 minutes. The immunogenic supernatant was used to immunise mice for polyclonal antibodies production. PVY-Cherie antigen received from *N. tabacum* using the second method was obtained in the Russian Academy of Agricultural Sciences (RAAS named after Lorkh A.G.). Purification of the virus was carried out according to the procedure adopted at the Department of Biotechnology and Immunodiagnostic of the Institute (Atabekov, 2002). In this method, *N. tabacum* callus tissue was homogenised by adding 0,1 M buffer of K_2PO_4, 1% 2-mercaptoethanol and 0,01 M Na_2EDTA into the sample in a ratio of 1:5. The prepared sap was clarified by centrifuging it at 12000 rotations per minute for 20 minutes. After centrifugation, 0.5% of nonionic-detergent triton-X-100 was added into the solution, followed by precipitation of the virus by PEG-6000 and low-speed centrifugation. The final purification was carried out by ultracentrifugation through a 25% sucrose pad, followed by suspending the virus in solution and low-speed centrifugation at 10000 rotations per minute for 15 min. Concentration of the virus was determined using the spectrophotometer (SmartSpec plus BioRad), 260 nm, USA, extinction coefficient 2.35 (Schubert et al., 2004). To study immunogenic property of the slightly-purified-PVY and produce antibodies, the following scheme of mice immunisation was used: 100 µL of a 1 µg/ml the virus was injected intraperitonealy. Then on the 7th and 19th days of immunisation, the same patterns were repeated with buffered solution pH 7,2-7,4 (Gnutova, 1993; Čeřovská et al., 2003).

RESULTS AND DISCUSSION

In total, 47 plants of *N. tabacum* variety Samsun were inoculated in the juvenile phase of growth. After 14 days of inoculation, the first symptoms of viral infection were observed in the form of veins lightening, leaves deformation and mottling. Injured leaves were transparent and shrivelled (Christopher, 2001). Results presented in Table 1 show that a positive reaction was found in six lines of *N. tabacum*.

ELISA optical density for the samples № 38, 42, 43 exceeded the commercial positive control on the 15th day of inoculation, which was earlier than expected. It should be noted that OD markedly decreased on the 25th day of inoculation.

This decline in OD corresponded to the literature data, according to which, PVY could be defined only 15 days after inoculation. The high concentration of virus in the culture was short (15 day) and after

Table 1
PVY infected Nicotiana tabacum *plants optical density in ELISA*

Lines №	Plant, variety	OD values in ELISA, units		
		7th day	15th day	25th day
10	*N. tabacum,* Samsun	0,008	0,826	1,335
38	*N. tabacum,* Samsun	0,003	1,313	0,767
42	*N. tabacum,* Samsun	0,571	1,211	0,936
43	*N. tabacum,* Samsun	0,497	1,070	0,977
44	*N. tabacum,* Samsun	0,102	0,571	0,232
1000	*N. tabacum,* Samsun	0,180	0,412	0,343
-	Positive	0,865	1,021	0,912
-	Negative	0,019	0,023	0,021

Table 2
Test results for the callus of N. tabacum *Samsun variety in ELISA*

№	PVY- infected plant samples	ELISA, OD, units		
		Sample	Positive	Negative
1	callus tissue obtained from the line *N. tabacum* №10, №1	0.709	0.375	0.014
2	callus, (*N. tabacum* № 10), the sample №2	0.256	0.375	0.014
3	callus, (*N. tabacum* № 10), the sample №3	0.291	0.375	0.014
4	callus, (*N. tabacum* № 10), the sample №4	0.578	0.317	0.015
5	callus, (*N. tabacum* № 10), the sample №5	0.371	0.375	0.014

a few weeks, it decreased dramatically, whereas on the 60th day of inoculation, no virus was captured by specific antibodies in ELISA (Gnutova, 1993).

However, *N. tabacum* line № 10 showed a maximum accumulation of PVY exactly on the 25th day of inoculation. Primary callus was transplanted onto fresh nutrient medium of the same composition. Callus transplantation was repeated four times (once in 3-4 weeks) during the experiment (Figure 1).

N. tabacum callus average growth-rate throughout the research was 107%. ELISA results showed PVY presence in all the tested callus cultures derived from the infected plant of *N. tabacum*, variety Samsun № 10 (Singh et al., 1983; Salim Khan et al., 2003) (Table 2).

Samsun № 10 optical density always remained at the level of positive control, though sometimes exceeded it. In the next step of our research, PVY was purified from non-pigment tissue culture of *N. tabacum*. Supernatant obtained as the result of callus homogenisation and low-speed centrifugation was used in the research without any further dilution. The purified antigen was comparatively studied in sandwich-ELISA with commercial PVY antigen. Table 3 presents results of the test.

Table 3
Sandwich-ELISA results of PVY antigens

ELISA, OD, units		
Sample	Positive	Negative
0,922	1, 087	0,033

Table 3 presents the ELISA results of slightly-purified virus antigen. Optical density of the virus in the sample was at the same level with positive control. Purification of PVY from *N. tabacum* callus tissue was done using the second method that included multiple steps (Table 4).

From Table 4, it is evident that after each step of purification, virus concentration reduced significantly in the extract. The greatest loss occurred after clarification

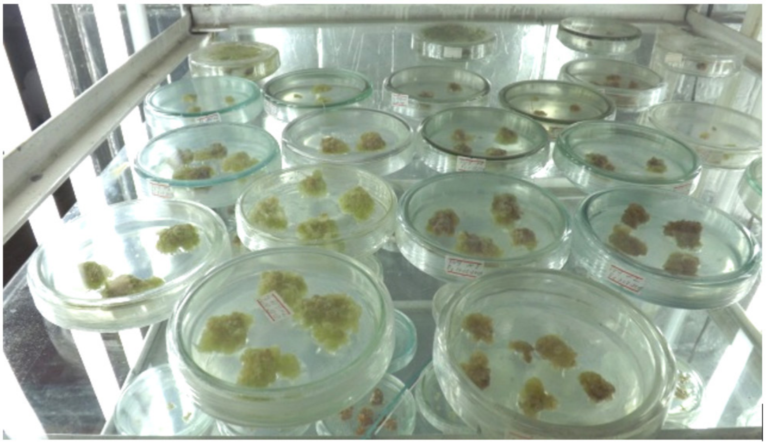

Figure 1. PVY-infected *in vitro* callus of *Nicotiana tabacum*

and extraction of the virus, precipitated with PEG. At the same time, the virus concentration increased significantly as the volume of virus containing-extract reduced multiple times (Syller, 2014). In order to study optical density of the virus, PVY-Cherie was scanned in spectrophotometer SmartSpec Plus, wavelength 240-360 nm (Atabekov I.G.). The results showed that the virus has the same absorption specter (minimum 240 and maximum 278 nm) as threadlike RNA potyvirus.

Potato virus Y is characterised by emergence of new strains, which causes tubers necrosis and reduces their quality (Friemel, 1987; Al-Ani et al., 2011). Taking into account strains diversity, the next step of our research was to study PVY-Cherie for strains identification. For this purpose, ELISA "sandwich" was conducted to test PVY-Cherie in comparison with commercial-collection strains PVY^{o}-F and PVY^{N}-L (Tribodet et al., 2005; Nasir et al., 2012) (Table 5).

The PVY-Cherie showed to be of necrotic strain as it reacted weakly with antibodies specific to the PVY "ordinary" strain. Nowadays, polyclonal antibodies as specific immunological reaction component are used widely for potato viral diseases diagnosis (Cojocaru et al., 2009). Based on this, white mice were immunised with PVY antigens (slightly-purified and highly-purified Y-Cherie) to produce specific polyclonal antibodies and determine viral preparations antigenicity (Fridlyanskaya, 1987). Titre of specific antibodies is main indicator of antivirus-diagnostic sera efficiency (Clark et al., 1977). In our research, indirect ELISA was conducted to test the antibodies (Table 6).

The data presented in Table 6 show that the obtained antibody reacted with a 1/3200 titre of PVY-Cherie (the same as commercial PVY) and with a 1/200 titre of slightly-purified antigen.

Polyclonal antiserum, specific to PVY with a maximum titre of 1/3200 in indirect ELISA, was received from the laboratory white mice (Zulaykha et al., 2014). Thus, PVY accumulation and maintenance in plants and tissue culture of *N. tabacum* indicate the possibility of obtaining viral antigens suitable for mice immunisation and PVY-specific antibodies production.

CONCLUSION

PVY was accumulated and maintained in the test-plants of *N. tabacum*, growing in environmental chamber. Plant of *N. tabacum* variety Samsun, line №10 showed maximum optical density in sandwich-ELISA on the 25th day of inoculation. *N. tabacum* callus tissue average growth-rate was 107%. Slightly-purified and highly-purified (PVY-Cherie) antigens were obtained from the callus culture. PVY-Cherie was detected as PVY "necrotic" strain. Antibody received in answer to PVY-Cherie antigen, showed a titre 16 times higher than in answer to slightly-purified antigen.

Table 5
Highly-purified virus Y-Cherie strains identification-test results

Virus concentration ng/ml	ELISA, OD, units, $A_{450,}$					
	Group of ordinary strains			Group of necrotic strains		
	PVY^0-F	PVY^N-L	Y-Cherie	PVY^0-F	PVY^N-L	Y-Cherie
500	1,936	1,317	0,737	0,679	1,113	0,959

Table 6
Mouse antibody test in indirect ELISA

Serum specific PVY antigens	Titers						
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Slightly-purified viral antigen	+	+	-	-	-	-	-
Highly-purified Y-Cherie antigen	+	+	+	+	+	+	-
Commercial PVY (positive control)	+	+	+	+	+	+	-

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