PRELIMINARY STUDIES REGARDING THE *Potato Virus Y* (NECROTIC STRAINS) EXTRACTION AND PURIFICATION

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Abstract. The objective of this research was to study if the frozen material could be used to obtain a PVY suspension with a proper concentration for antisera preparation. The virus extraction and purification was achieved from frozen leaves and fresh tobacco leaves (cv. 'Samsun' and 'White Burley') mechanically inoculated with a PVY^N infected material (cv. 'Record' secondary infection with this pathogen). The experiment followed a schema used in other virology laboratories with some modifications particulary quantitative. Results of this preliminary studies indicated that frozen PVY^N infected material is not advisible for the preparation of a virus suspension solution to be used for antisera production.

Keywords: potato virus Y, necrotic strains, purified preparation

Abbreviations: PVY = potato virus Y; PVY^N=PVY necrotic strains

INTRODUCTION

Potato Virus Y (PVY) is one of the most economically important virus of *Solanum tuberosum* L. plants, because his frequency and damaging potential. The virus Y occurs worldwide and the plant production with secondary infection can be diminuated with 33-90%, depending on the variety and the virus strains [3]. De Bokx ans Huttinga state that the PVY infections can reduce yields 10-80% [9]. Rykbost et al. [20] reported reduction in yield of number one tubers of cv. Russet Norkotah by 12-40%. Similary, a reduction in marketable yield of 65% in Russet Norkotah was reported by Hane and Hamm [11]. Nolte et al. studyied the effect of tuber borne PVY infection on Russet Bubank, Russet Norkotah and Shepody and reported a yield loss of 0.18 tones/ha for each 1% increase in PVY infection [19].

Several variants or strains of PVY have been found and originally classified in three main groups PVY^O, PVY^N, PVY^C, depending on the symptoms produced in tobacco and differential potato cultivars [8, 25]. PVY^N isolates produce mild mosaic in many potato cultivars and sever systemic veinal necrosis in tobacco. The damage caused by this pathogen can be both quantitative (significant reduction of production) and qualitative (commercial depreciation of tubers). In case of cultivation of sensitive varieties under favorable conditions, financial losses can be important both for potato consumption (it can become unmarketable) as for seed potatoes (it will be downgraded or rejected from certification). PVY^{NTN} strains produce symptoms on tubers, causing the so-called necrotic ring staining of potato tubers (PNRTD). Being very aggressive, these strains can overcome existing resistance to infection with other PVY strains [1,2].

Potato virus Y was situated on the 5th place in top 10 plants virus list for molecular Plant Pathology [23]. One of the strains of this pathogen is PVY^N, responsible for the necrosis of infected plants. In spite of the latest improvements in detection and molecular characterization methods [16, 17, 21, 24] routinely applied procedures are unable to accurately characterize isolates responsible for tuber necrosis. There is no efficient means to manage the risks of epidemics caused by emerging necrotic variants. In the present state of a competitive international potato market, PVY necrotic strains are potentially responsible for huge agronomic and economic losses.

Most PVY isolates are detectable by policlonal antibodies produced by injecting rabbits with a purified virus preparation [18]. The policlonal antibodies detect all known strains of the virus, but are incapable in differentiating among O and N groups. With the application of monoclonal antibody technology to PVY [22], additional antibodies produced in mice were capable of differentiating between viruses O and N serotypes. The PVY^N and PVY^{NTN} isolates are of the N serotype whereas the O and N:O isolates are of the O serotype [7]. Because the N:O isolates are of the O serotype, possibly led to its relatively rapid spread due to missidentification as an ordinary isolate [15].

The objective of this research was to study if a frozen PVY^N infected material could be used in the purpose to obtain a PVY suspension with a proper concentration for antisera preparation.

In Romania, the virus tests were made initially only with antiserum (IgG and conjugate) from import. For reducing the financial effort of the potato virus sampling, researches have been initiated at the National Institute of Research and Development for Potato and Sugar Beet Brasov, in the aim to obtain antiserum, IgG and conjugate for the X and S potato viruses [4, 5]. Then, the research activities have been extended for obtaining antiserum for PVY [6].

MATERIAL AND METHODS

The virus multiplication. This activity was carried out in the insect proof greenhouse. For the PVY^N multiplication, the tobacco plants (varities 'Samsun' and 'White Burley') have been mechanical inoculated in the 2-4 leaves phase (using Carborundum). After 4

weeks from inoculation, the biological material was tested using DAS ELISA (PVY^N antisera and conjugates from Bioreba, Switzerland). The tobacco leaves were harvested only from infected plants. For the inoculation, a secondary PVY^N infected material (cv. 'Record' from the institut collection) was used.

The viruses extraction. The virus extraction and purification has been made from frozen leaves and fresh tobacco leaves. The material homogenization has been done in McIlvaine buffer (phosphate, citric acid) 0.18 M, pH 7 containing as additive stabilizer sodium DIECA (0.02 M) and sodium thioglycol (0.01 M).

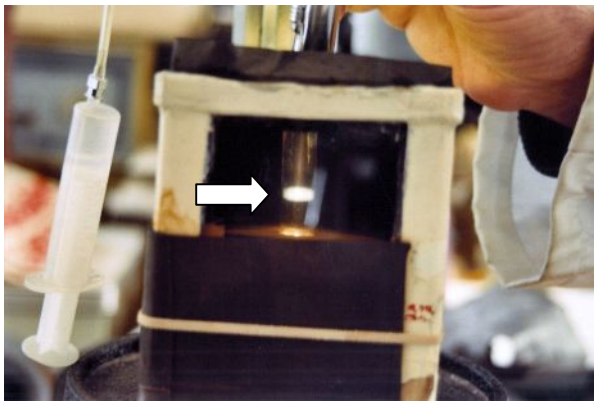

Figure 1. Virus area extraction after the centrifugation stage in differential CsCl gradient (the virus area is signaled by the white arrow) [6]

The purification methodology. The purification methods has been established using the schema used in the virology labs from Germany and Holland (specified in [6]) with some modifications particulary quantitative, as well one regarding the centrifugation in density gradient [6]. The mean stages of the purification schema were the following:

1. The leaves were grinded up and homogenizated (4 probes of 100 g leaves and 200 ml extraction buffer 0.2M). Then, a Triton X-100 solution (20%) has been added and the suspension was incubated over night at 4°C with continuous agitation.
2. The homogeneity filtration through a dense filter was performed (Multilab 1242, pores 7-9 μm) and the filtrate was centrifugated for 15 min at 7000 rpm (Janetzki centrifuge model K23, Heinz Janetzki K.-G. Mashinenbau Leipzig, Germany).
3. The suspension was centrifugated 90 min at 2400 rpm and 10°C (Janetzki centrifuge model K23, Germany).
4. The sediment was resuspended in 380-400 ml extraction/homogenization buffer (McIlvaine buffer), at least 60 min at 40°C has been shaked and then centrifugated as point 2.
5. The supernatant centrifuged again during 120 min at 20000 rpm and 10°C (Janetzki centrifuge model K23, Germany).
6. The resuspension of the sediment in 6-7 ml McIlvaine buffer, minimum agitation 60 min at 4°C and 15 min centrifugation at 7000 rpm and 10°C (Janetzki centrifuge model K23, Germany).
7. The supernatant was centrifuged (1 ml virotic suspension/tube), in sucrose gradient of 10-40%, in McIlvane buffer, during 120 min at 25000 rpm and 10°C with SW41 (Beckman centrifuge, model L7-55, classified R with all Beckman preparative rotors except the Type 15 rotor and zonal and continious-flow rotors, Beckman USA).
8. The viruses area extraction 1:1 dilution with McIlvane buffer and centrifugation during 150 min at 25000 rpm and 10°C (Beckman centrifuge, model L7-55, classified R with all Beckman preparative rotors except the Type 15 rotor and zonal and continious-flow rotors, Beckman USA).
9. The sediment resuspension in 10 ml McIlvaine buffer (without additive), short homogenization and 30-60 min shaking with low speed, centrifugation at 5000 rpm (if necessary).
10. The viruses suspension (10 ml) is transferred in a 50 ml cylinder and CsCl is added (12.15 g CsCl in 15 ml bidistiled water, brought up to 30 ml and mixed well). The gradient is centrifuged for 22 h at 36000 rpm and 10°C SW55 (6x5ml) (Beckman centrifuge, model L7-55, Beckman USA).
11. The viruses area was extracted (Fig. 1) and then, there was dialyse in McIlvaine buffer. A sample is taken (200μl) for the electron microscopy evaluation [13]. The virus from the purified preparation was stained with 1% uranil acetat (UA) and examined in a Philips EM 300 electron microscope [13]. For preservation of virus suspension, glycerine (1:1, v/v) was added and the purified virus preparation was stored at -18°C.

There were performed two series of virus purification. For each series, 400 g infected leaves were used. In the future, for the following methodological steps, there will be used the schema for the X and S antiserum [4-6] for the antiserum purification, in the purpose of the ballast globulins elimination, from the antiserum (nonimune) and the separation of the most active fraction IgG, as well for the antibodies marking with alkaline phosphatase [6, 10]. The testing of optimal dilution of solution is also forseen.

RESULTS

The analysis of the purified preparation show a good purity, usefull for obtaining antiserum with high specificity, and without positive reactions with sap from healthy plants.

The virus concentration in the preparations was also high, in case of purified potato virus Y^N from the fresh harvested leaves from tobacco 'Samsun'cv. (Fig. 2A) and from cv. 'White Burley', respectively (Fig. 2B). Both the purity and concentration of the preparations constituted the premise to obtain antiserum for DAS ELISA technique.

In the preparation of the PVY purified suspension obtained from the frozen leaves (a mixture from tobacco 'Samsun' and 'White Burley'cv) approaching 12 months, the virus was almost absent, only several particles baing still present (Fig. 2C). Regardig the use of frozen material, the loss of the virus could be caused

in particular, by the solid aggregation of the viruses particles as a result of the following causes:

- tissue congelation, pointed out by Derrick and Brlansky (specified by [12]);
- virus elimination together with the sediment as a result to the centrifugations.

Also, there is suggested that the PVY^N extraction should be accomplished from fresh harvested leaves (before the homogenization).

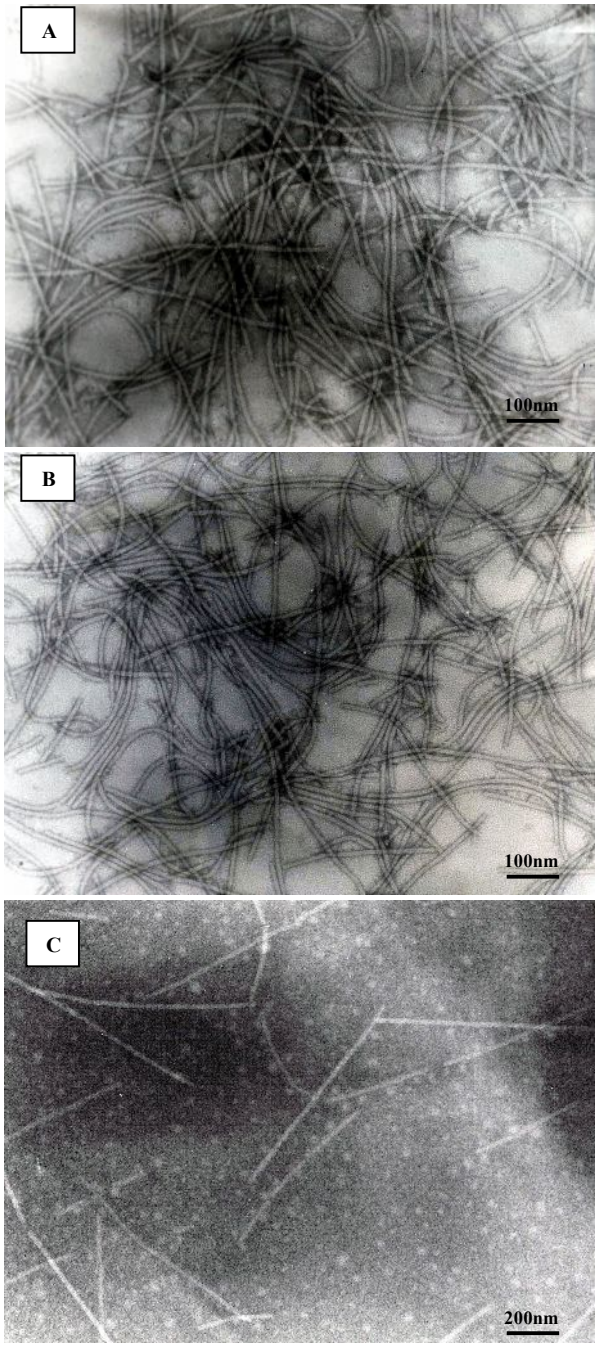

Figure 2. Electron-micrographs - particles of virus Y – virus suspension obtained from fresh tobacco leaves cv. 'Samsun' (**A)** and cv.'White Burley' (**B)** and from frozen leaves using a mixture of tobacco cvs. 'Samsun' and 'White Burley'(**C**). The extraction and purification methodologies used are presented above. The virus from the purified preparation was stained with 1% uranil acetat (UA) and examined in a Philips EM 300 electron microscope [13].

DISCUSSION

In Romania, the volume of material tested in seed potato production is high (more than 30,000-35,000 samples/year) [6] and therefore, the seed potato indexation (sampling) involve high costs for buying the antiserum from import. Obtaining the antiserum, antibodies (IgG) and conjugates (IgG-AP) needed for PVY^N detection by ELISA could reduce the financial effort of seed potato indexation. To initiate these researches, there is need to obtain a good purified PVY^N suspension.

In other similar study [14], the potato virus Y^N was purified by homogenizing and clarifying tobacco infected leaves in a mixture of 0.1 M Tris thioglycolic acid buffer, carbon tetrachloride and chloroform, followed by differential centrifugation applying moderate centrifugal forces. The method of our preliminary studies used a mixture 0.2M Tris-thioglicolic acid buffer and only chloroform, without carbon tetrachloride for virus extraction. At the same time, compared with other studies [10, 14, 22, 24], as virus infected material, we tried to use frozen leaves from tobacco cultivars 'Samsun' and 'Whyte Burley' (12 months, -20^{o}C). In other research papers, to obtain virus infected material, the necrotic strains of PVY (PVY^N) were multiplied only on *Nicotiana tabacum* 'Xanthy' variety [22,24] using specific isolates. We obtained good results regarding the level of PVY^N infection (with high ELISA signals for most of them) using another tobacco cultivars that Rose et al. (1987) [22] and Schubert et al. (2008) [24].

As in our previous research work regarding the purification of a mixture of PVY isolates [6], the methods used for the purification of PVY^N were usefull to obtain clean and concentrated preparations of virus particles (about 10^6-10^{10} particles/ml), corresponding to specific antiserum achievement.

The PVY^N purification from the frozen material was not succesfull. Therefore, only a fresh PVY^N infected material is advisible for preparing a virus suspension efficient for antisera induction. Such observations as well as the characteristics of this virus suspension in the achievement of specific antiserum for PVY^N, deserve further investigations.

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