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Potato Virus Y (PVY) Purification and Antiserum Preparation

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Abstract: This study included identification, purificationand antiserum preparation of potato virus Y (PVY). Positive reaction of PVY diagnosis using DAS-ELISA was characterized by yellow color. The results of spectrometrical tests indicated the good purification of the virus using differential centrifugation, precipitation and filtration techniques. The purity of PVY was 0.483 mg per gram of fresh plant tissue.A_{260}/A_{280} value of PVY purity was1.116. Antiserum preparation was done by immunization albino New Zealand male rabbit through four injections of purified PVY at interval of one week, intravenously and intramuscularly. The results of agar double diffusion and chloroplast agglutination tests indicated the successful preparation of the virus antiserum.

Keywords: *PVY, DAS-ELISA, Purification, Antiserum.*

I. Introduction

Potato virus Y (PVY) is the most dangerous virus in potato fields (Nascimento *et al.*, 2003 and Biswas *et al.*, 2005). This virus belongs to *Potyvirus* genus from Potyviridae family (Posada and Crandall, 2001). PVY virions are non-enveloped, filamentous, linear and flexuous rods with a length and width about 730-740 and 11-12 nm in diameter respectively and helical symmetry (Kerlan and Moury, 2008). Its symptoms on potato ranged between mild mosaic to severe necrosis and death of plants depending on cultivar and viral strain (Robert *et al*., 2000). PVY is widespread in Iraq on potatoes (Al-Sameae, 2000; Kassim and Mohammad, 2002 and Kassim and Younis, 2003).

To separate virus particles from plant constituents, isolation and purification of plant viruses are performed. Purified virus preparation is a prerequisite for studying the virus properties as well as for raising an antiserum against the virus. Antiserum against plant viruses is produced in a suitable warm blooded animal such as rabbits, rats, mice and chicken (Dijkstra and de Jager, 1998). This study aims to purify PVY,using differential centrifugation, precipitation and filtration process using Minisart filter, and to prepare an antiserum against this virus using albino New Zealand male rabbit.

II. Materials And Methods

Plant Material

Potato (*Solanum tuberosum* L. cv Santa) tubers provided by the Agricultural Directorate of Semmel city have been used throughout this investigation. The sprouted tubers were cultured in pots containing a mixture of sterilized sand and peatmoss (2:1) in a plastic house.

Propagation of the Virus Isolate

The virus isolate was propagated in *S. tuberosum* cv Santa plants grown in pots left in a plastic house. The developed plants were inoculated mechanically with PVY isolates.The infected frozen leaves were grounded in 0.01M phosphate buffer (KH_2Po_4) (7.0 pH) using blender machine. The primary leaves of plants were dusted with carborundum (600 mesh) and gently rubbed with the inocula usingforefinger. Inoculated leaves were rinsed with tap water for 10 seconds (Albrechtsen, 2006).

PVY Detection

Serological Assay

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was used for PVY diagnosing according to Koenig *et al.* (2008) and DSMZ manual. The PVY polyclonal antiserum set was requested from DSMZ Company, Germany. The procedure of DAS-ELISA was as follows. The purified immunoglobulin G (IgG) was diluted in coating buffer at 1:1000. ELISA plate was coated with 100 µl antibodies solution to each well of the plate using micropipette and incubated for 2-4 hrs at 37 °C and then washed with phosphate buffered saline (PBS)-Tween using washing bottle, soaked for a few minutes. Washing was repeated twice. The plate was coated with 100 µl of the test sample juice to duplicate wells and incubated overnight at 4 °C and then washed three times as mentioned before. 100 µl of the conjugate antibodies solution

added to the plate and incubated at 37 °C for 4 hrs and then washed. Then, 100 µl of freshly prepared substrate was added to each well and incubated at room temperature for 30-60 minutes. 100 ml of NaOH solution (3N) was added to each well to stop the reaction. The results were assessed by visual observation.

PVY Purification

Modified procedures of Dijkstra and de Jager (1998) and Al-ani *et al.* (2011) were used to purify PVY using differential centrifugation, precipitation and filtration technique, as follows. In extraction process, 300 g of the systemically infected potato leaves, which kept frozen, mixed with 0.01 M phosphate buffer (pH 7.0) in 1:2 ratio. The mixture was grounded with blender machine for 3-4 minutes. The prepared suspension was filtrated and placed in an icebox. Then 0.1 M ascorbic acid and ethylene diamine tetra acetic acid (EDTA) were added to the suspension.

In clarification Process, 40% of the chloroform was added to suspension with 2 minutes strong mixing using blender machine. Prepared suspension was poured into centrifuge tubes and spun at 10000 rpm at 4 °C for 20 minutes using cooling centrifuge. The supernatant was taken and placed in an icebox. In final purification step including precipitation and centrifugation, ammonium sulphate was added to 1 liter suspension at half saturated point. Centrifuge tubes were filled with suspension and spun at 14000 rpm at 4 °C for 2 hrs. The pellet was took and resuspended in 0.01 M phosphate buffer (pH 7.0). The prepared suspension dialyzed in a cool environmentagainst 1 liter sterilized distilled water with continuous slow stirring. In the last step of final purification, the purified suspension of PVY was passed through minisart filter (0.45 µm) for further purification (Al-ani *et al.*, 2011).

Spectrometrical tests were used to assess the purity of PVY suspension and the efficiency of procedure used to purify the virus. This test was done using UV-spectrophotometer for scanning the purified solution at a wave length ranged between 200-300 nm, at 10 nm intervals (Dijkstra and de Jager, 1998) to plot the absorbency curve, estimate A_{260}/A_{280} ratio and PVY concentration in potato tissues.

Production of PVY Antiserum

Antiserum was produced according to that of Noordam (1973), as follows. 2 ml of the purified viral preparation was injected intravenously at interval of one week into the ear vein of albino New Zealand male rabbit. After two weeks, the injection was repeated at the same quantity (booster injection) intramuscularly into the muscle of rabbit, after mixing the purified virus solution with sterilized alum solution (1:1) (Kassim and Ali, 2004). 7 days after final injection, 15 ml of the blood was taken from the injected rabbit. The antiserum was obtained and purified as reported by Clark and Adams (1977). Blood was allowed to clot at room temperature, and the clot was removed. Clotted blood was centrifuged at 5000 rpm for 10 minutes. The clear antiserum (supernatant) was collected and discarded the pellet. The antiserum (serum plus antibodies) was poured into a small vial and kept frozen at -18 °C.

Assay of the Efficiency of Prepared Antiserum Using Agar Double Diffusion and Chloroplast Agglutination Tests

a.) Agar Double Diffusion Test

The procedure of this test was according to that of Purciffull and Batchelor (1977), as follows. 1.2 g of pure agar was dissolved in 100 ml of 0.01 M phosphate buffer (7.0pH) on a magnetic stirrer hot plate. The dissolved agar was sterilized using autoclave (at 121 ºC and 1.5 kg cm^{-2} for 15 minutes). The agar solution was cooled down to approximately 60 °C, and added 1% sodium azide to it and then the agar solution was poured to Petri-dish which filled about 2/3 of petri-dish size. Seven wells (7 mm in diameter) were punched in the agar plate using cork borer. The distance between them was 10 mm. The agar plugs were removed from the wells. The central well was filled with prepared antiserum. Two of the surrounding wells were filled with healthy plants' extract, while another with PVY infected plants' extract mixed with 5% sodium dodecyl sulphate (SDS) to disrupt virus particles. While filling the wells with the virus and antiserum suspensions, spilling outside the wells was avoided. The dish was covered and incubated for 24 hrs at room temperature, and the result was observed visually.

b.) Chloroplast Agglutination Test

The used procedure of this test was according to that of Noordam (1973), as follows. The infected and healthy frozen leaves were grounded in 0.01M phosphate buffer (KH_2Po_4) (7.0 pH) using mortar and pestle. Draw two circles, one on each side of a clean slide by using a paraffin pen to prevent the distribution of solution. Some drops of prepared antiserum solution put into each circle using syringe and then some drops of healthy and diseased leaves juice into the first and second circle separately. The results were assessed by visual observation.

III. Results And Discussion

PVY Detection

Serological Assay

Positive reactions were observed in diagnosis of PVY in infected potato plants using DAS-ELISA test which was characterized by the yellow color of the substrate solution.

PVY Purification

PVY purification depends on separation of virus particles from plant constituents. In extraction process, use of phosphate buffer, ascorbic acid and EDTA help to keep the pH at neutral, prevent virus oxidation and keep the homogenecity of virus diffusion in suspension, respectively. Generally, use of chloroform in clarification process helps to remove all cell membranes which contain lipid and lipoprotein and their precipitation at low-speed centrifugation with less virus loss. Also in final purification step including precipitation and centrifugation, ammonium sulphate helps to precipitate the virus particles at high-speed centrifugation (Dijkstra and de Jager, 1998). The successful purification procedures of PVY were proved in criteria.

Criteria of Purity

Spectrometrical Test

a.) Plotting the Absorbency Curve

The UV-spectrum of the purified virus gave maximal absorption (1.382) at 260 nm, while the absorbency at 280 nm was 1.238 (Fig. 1). The nucleic acid and protein gave the maximal absorption at 260 and 280 nm, respectively. So, this result indicated the good and successful virus purifying. This result was in agreement with that of Dijkstra and de Jager (1998).

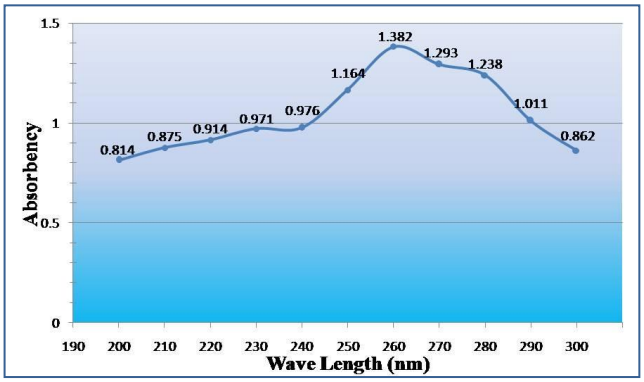

Figure (1): Absorbency of Purified PVY Solution at a Wave Length Ranged Between 200-300 nm Using UV-Spectrophotometer.

b.) Estimation of PVY Using A_{260}/A_{280} Ratio

The purity of PVY solution was indicated by the value of A_{260}/A_{280} ratio which was 1.116. This result showed that the used procedures were successful for separating and purifying PVY from infected potato plant tissues. In this aspect, Lorenzen *et al.* (2006) found that the value of PVY purity was between 1.20 and 1.37. Dijkstra and de Jager (1998) stated that the value of A_{260}/A_{280} ratio of elongated viruses is around 1.2 and for those with isometric particles around 1.7.

c.) PVY Concentration in Potato Tissues

The results showed that the PVY concentration (C) in potato tissues was 0.483 mg virus for each gram of fresh plant tissue. This concentration was calculated by dividing the absorbency at 260 nm (A_{260}) to Extinction coefficient ($E^{0.1\%}_{1cm}$) of PVY (2.86). This result indicated the presence of high concentration of the virus particles in diseased plant tissues. The result was in agreement with that of Stace-Smith and Tremaine (1970). On the other hand, the results indicated the successful PVY purificationusing the procedures mentioned before, so this is an effective method for plant virus purification.

Production of PVY Antiserum

Rabbit was chose to produce antiserum against PVY antigen because the amount of serum that can be obtained from a single animal is good. The relative ease of keeping and handling of this animal leads to select it to produce antiserum. Antiserum was prepared by injecting rabbit with purified PVY solution intravenously then

intramuscularly using alum with booster injection which slows the movement of the virus particles into animal blood circulation. Alum activates the immune system of animal and helps to increase the rate of antibody production (Dijkstra and de Jager, 1998). Intravenous and intramuscular injections had a good result to give antiserum. This result was in agreement with that of Kassim and Ali (2004). Agar double diffusion and chloroplast agglutination tests were used to assay the efficiency of the prepared antiserum.

Assay of the Efficiency of Prepared Antiserum Using Agar Double Diffusion and Chloroplast Agglutination Tests

a.) Agar Double Diffusion Test

In agar double diffusion test, the prepared antiserum reacted positively with PVY-diseased potato leaves juice. The positive test is characterized by the production of a white colored line in agar gel around the wells contain diseased plant leaves juice. SDS helps to disrupt the virus particles and diffuse radially into the gel easily and react with diffused antibodies. The reaction occurs between virus particles and antibodies where they meet each other (Dijkstra and de Jager, 1998). The formed precipitate, as a white line, was visible in the agar gel between the wells contain diseased juice and was not form around the wells of healthy juice. This assay is more suitable for isometric viruses, but tubular viruses like PVY need to an adjuvant to diffuse easily between agar gel pores (Dijkstra and de Jager, 1998). Sodium dodecyl sulphate (SDS) is the adjuvant chemical which helps in tubular virus particles fraction. Succeed of this assay depends on SDS which caused PVY particle fraction and its easy diffusion between the gel pores (Purciffull and Batchelor, 1977).

b.) Chloroplast Agglutination Test

In chloroplast agglutination test, the prepared antiserum reacted positively with PVY-diseased potato leaves juice. The positive test is characterized by the production of precipitate within the center of solution with a darker color tending to brown, compared to the negative test. The results of agar double diffusion and chloroplast agglutination tests indicated the purity and efficiency of the prepared antiserum for PVY detection using rabbit immunization. These results showed the successful preparation of antiserum through the used procedure using albino New-Zealand male rabbit. These results were in agreement with Gibbs and Harrison (1980) and Hull (2002).

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