A METHOD FOR PURIFICATION OF PLANT VIRUSES BELONGING TO DIFFERENT VIRUS GROUPS

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ABSTRACT:

A method for isolation and purification of plant viruses was described. The method involved use of organic solvents, differential centrifugation and density gradient centrifugation on step and linear caesium sulphate gradients with which a sucrose cushion was used. The method was found suitable for purification of a range of plant viruses belonging to different virus groups and representing the different shapes reported for plant viruses. The plant viruses purified through this method included Potato virus Y (PVY), Tobacco etch virus (TEV), Alfalfa mosaic virus (AMV), Cucumber mosaic virus (CMV), Tobacco mosaic virus (TMV) and Tomato yellow leaf curl virus (TYLCV). Both the step and linear gradients of caesium sulphate gave similar results in isolation and purification of these viruses and no appreciable differences were observed between them when either of which was used.

Key words: *Purification, virus, density gradient centrifugation, sucrose cushion.*

INTRODUCTION:

Symptoms on diseased plants were considered among the initial indications of the infection of plants with viruses. That is usually followed by other supportive etiological studies such as transmissibility through different methods such as mechanical transmission, seed transmission, and vector enhanced transmission through the different types of virus vectors and the different modes of their transmission. An important study which researchers usually try to perform and usually encounter difficulties with is the isolation and purification of a plant virus. Obtaining virus particles in a pure form is of utmost importance for carrying out several important studies such as physico-chemical properties including virus morphology, virus composition and all the studies related to that such as molecular weight of virus protein, amino acid sequence of the protein, molecular weight and nucleotide sequence of the virus nucleic acid, in addition to the need of the purified virus for antiserum production. Reasons for the difficulties often encountered in isolation and purification of plant viruses are probably due to the host plant or to the nature of the plant viruses themselves, their different shapes and sizes, their concentrations in their host plants which is usually, according to Markham 1959, should not be lower than five to 10 mg of viral particles per kilogram of fresh plant leaves for efficient virus purification and most importantly is the virus stability.

All these factors should be taken into consideration before an efficient purification protocol can be planned.

Because of these factors many problems are involved in separating of a virus in a pure form from the plant cell constituents and these are only partially solved. These are some reasons why only relatively few viruses from the large number of the mechanically transmissible ones have been successfully purified (Smith 1977). Also probably that is the reason why different purification methods have been published (Tolin 1962, Polson 1977, Smith 1977, Albrechtsen and Heide1990, Poison 1993, Dionne et al. 2008, and Khan et al. 2009) and usually each method is generally applicable to one virus or probably to some of the members of the same virus group after some modifications in the applied method.

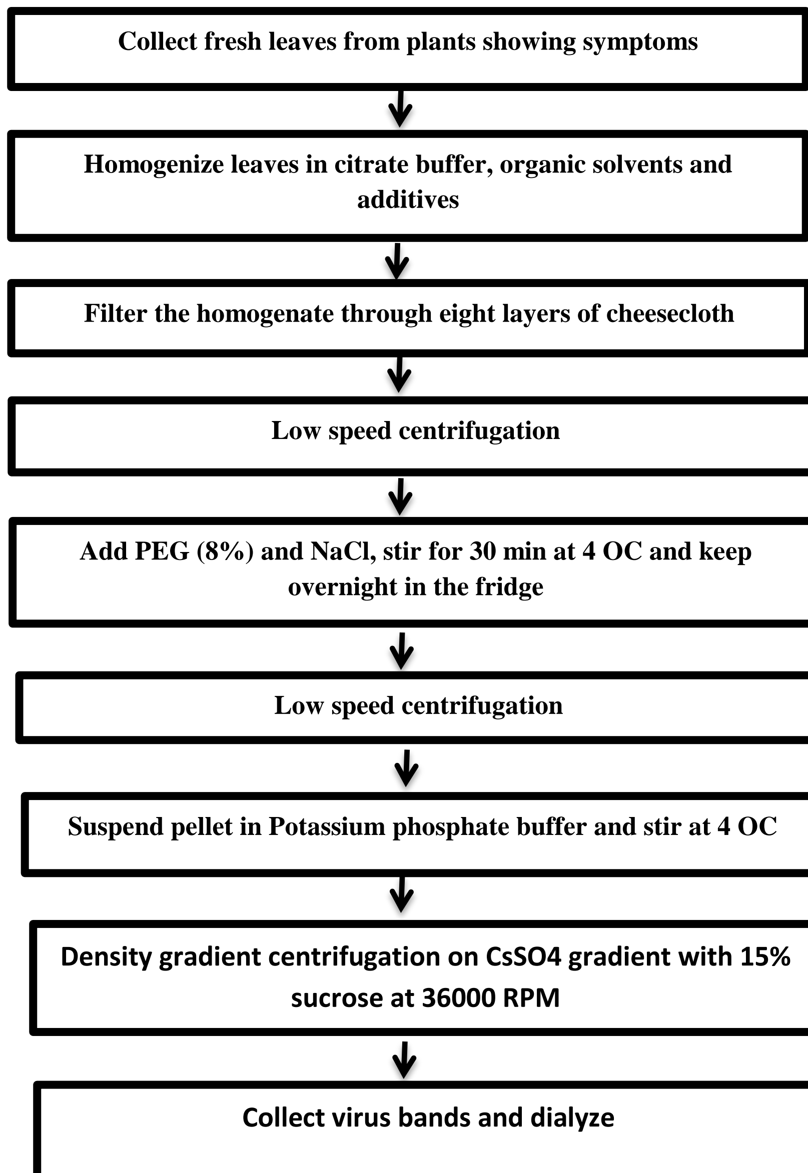
The objective of this study is to present an efficient method of virus isolation and purification which is applicable to a wider scope of plant viruses of different morphology and belonging to different virus groups.

Materials and Methods:

The purification method described below takes into consideration the three steps that are generally involved in plant virus purification which are extraction, removal of non-viral components and concentration of the purified virus, as shown below. Leaves were collected from tobacco plants showing PVY symptoms with which the plants were inoculated 3-4 weeks earlier. The leaves were homogenized in a pre-cooled warring blender in 0.05 M potassium citrate buffer, pH 7.1 in which sodium sulfite was dissolved in a ratio of 0.05 g per gram of plant tissues. Na-EDTA was also dissolved in the buffer in a concentration of 0.01 M to remove plant proteins and ribosomes and enhance extraction of the virus by chelating copper and/or by inhibition of polyphenoloxidase resulting in virus inactivation due to O-quinones (Khan et al. 2009). Chloroform and carbon tetrachloride which were used in this method disrupt plant tissues and enhance the release of virus particles. The ratio of plant tissues to the buffer to the mixture of the organic solvents was 1:2:1. Triton X-100 was added in a final concentration of 1% to help in release of virus particles from cell components. Leaves were homogenized for about two minutes in the pre-cooled warring blender. The homogenate was then filtered through eight layers of cheesecloth. Polyethylene glycol (PEG) 6000 was added to the filtrate in a concentration of 8% and NaCl in a concentration of 0.25 M. The filtrate was then stirred for about 30 minutes at 4 OC to dissolve PEG and NaCl and kept overnight in the fridge. It was then subjected to low speed centrifugation in Beckman type 19 rotor at 12000 RPM for 15 minutes. The supernatant was discarded and the pellet in each bottle was dissolved in 2 ml of 0.05 M Potassium phosphate buffer, pH 7.2. The suspended pellets were combined in a small beaker and stirred for 30 minutes at 4 OC. A sucrose cushion of 15% sucrose in 0.05 M potassium phosphate buffer, pH 7.2 was prepared and used to prepare a caesium sulphate gradient in the ratios of 0%, 15%, 22.5% and 30%. 2.5 ml of each of the four concentrations of the gradients were loaded

in Beckman ultra-clear centrifuge tubes to make a step gradient. 2 ml of the virus suspension was loaded on top of the step gradient in each tube and in tubes containing linear gradients in the same concentration for comparison. The tubes were balanced, inserted in buckets which were hooked to type SW41 Beckman swinging bucket rotor. The rotor was then placed in a Beckman Coulter Optima L-80XP ultracentrifuge and density gradient centrifugation was performed at 36000 RPM for 24 hrs. Similarly, equivalent amount of leaves were collected from tobacco plants showing symptoms for Tobacco etch virus or Alfalfa mosaic virus, or Cucumber mosaic virus or Tobacco mosaic virus or from tomato or tobacco, N. benthmiana showing symptoms of each of two strains of Tomato yellow leaf curl virus, Gezira and Yemeni strains (Alsaleh, et al., 2014) were subjected to the same purification protocol.

A Diagram Showing steps of the method of virus purification



Electron microscopy:

Drops of the virus zones for five of the purified and dialyzed viruses were separately placed on carbon-coated grids purchased from Ted Pella Inc., California, USA. Three minutes later, the drops were removed using Whatman filter paper.

A drop of phosphotungstic acid was placed on each grid to negatively stain the virus particles, left for one minute before removed with Whatman filter paper and examined in the transmission electron microscope, JEOL-JEM 1011 (JOEL ltd., Japan, www.jeol.com).

Results and Discussion:

Results indicated that, subsequent to the 24 hrs density gradient centrifugation on the CsSO4 gradient, clear virus bands were formed at about the middle of the tubes. However impurities of plant materials were separated in bands toward the bottom of the tube whereas pigments from plant material remained at the top of the tube (Fig 1). Virus zones were collected by puncturing the tubes by hypodermic syringes size 3 ml, combined for each virus separately and dialyzed against several changes of the 0.05 M Potassium phosphate buffer, pH 7.2 until all sucrose and CsSO4 were completely removed.

Viruses of different shapes and belonging to different virus groups were purified using the above-mentioned method. These were PVY, TEV, AMV, CMV, TMV and TYLCV. Pure and clear virus particles for each of five of the purified viruses were observed in the transmission electron microscope indicating the wide applicability of this method for isolation and purification of all these viruses which belong to five different groups of plant viruses that in fact represent all the known

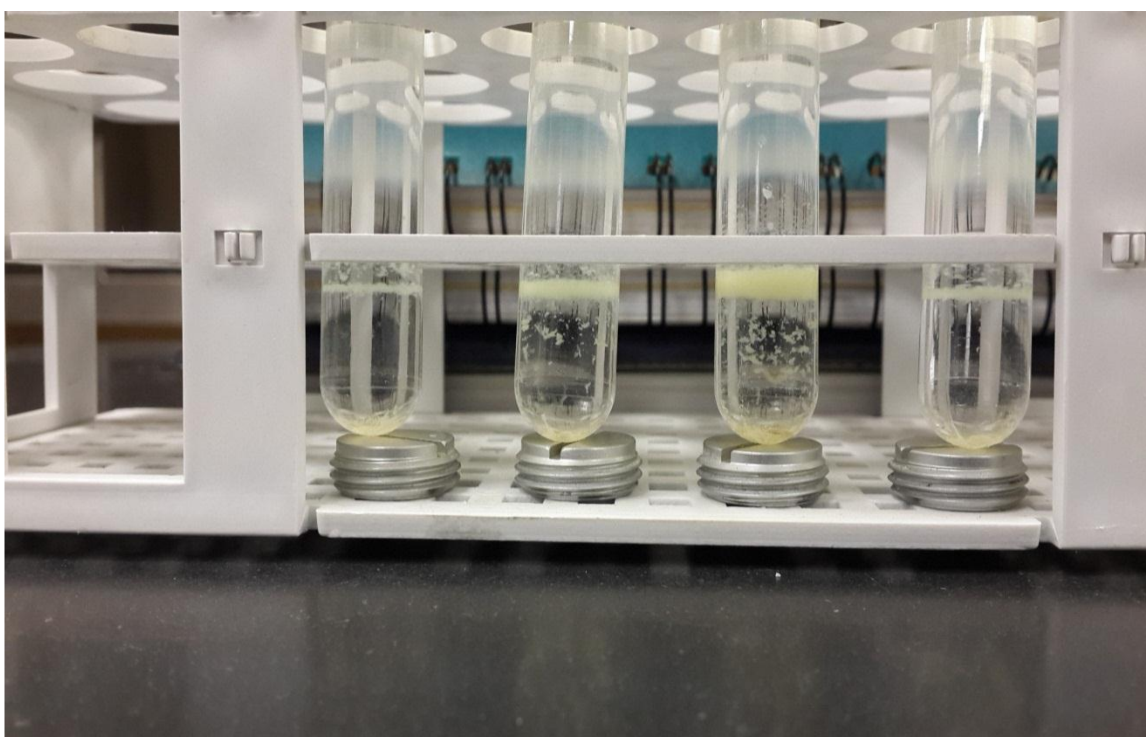


Fig 1. Separation of the virus in a pure form subsequent to density gradient centrifugation on caesium sulphate gradients.

plant virus shapes (Fig 2A & Fig 2B). Not only that, but the results also suggest the suitability of this method to purify different viruses within the same group e.g. PVY and TEV in the potyvirus group and even its suitability for purification of strains of the same virus, Gezira and Yemeni strains of TYLCV, (Alsaleh, et al., 2014).

These results indicate that this method excels the previous methods in its wide scope of applicability and its ability of isolation and purification of several different plant viruses from different virus groups that represent all reported plant virus shapes, purification of different viruses within the same group and even isolation and purification of strains of the same virus. Some of the reasons that this method yielded pure virus preparations in sufficient concentration are that it included antioxidants such as sodium sulphite to prevent oxidation that often occurs during plant virus

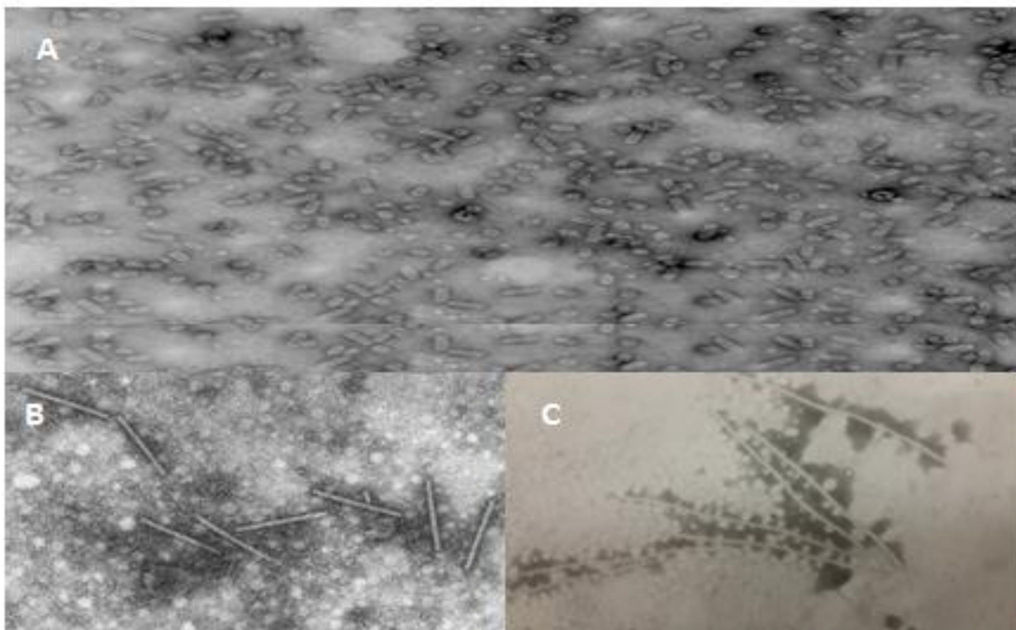


Fig 2A. **Electron micrographs of plant virus particles negatively stained with phosphotungstic acid for (A): bullet-shaped virus particles of AMV, (B): Rigid rod virus particles of TMV and (C): Elongated flexible virus particles of PVY.**

purification. It also included NaEDTA to enhance virus extraction through removal of plant proteins and ribosomes, copper chelation and inhibition of polyphenoloxidase enzyme. The use of organic solvents to disrupt plant tissues resulting in easy release of the virions from the cells and the use of Triton X-100 to free virus particles from plant tissues were also of considerable help. It is also important to mention that both of the step and linear gradients of caesium sulphate gave similar results in separation of the virus in form of pure bands in the middle of the ultra-clear centrifuge tubes and that no differences were observed when either of them was used.

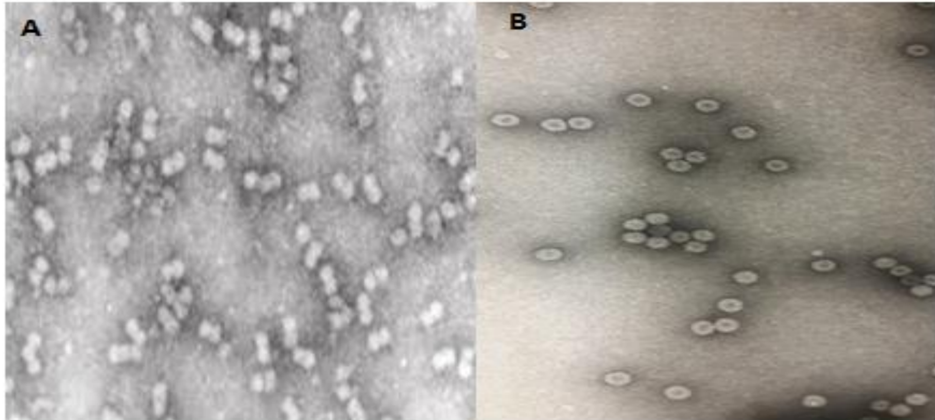


Fig 2B. **Electron micrographs of plant virus particles negatively stained with phosphotungstic acid for (A): geminate virus particles of TYLCV, (B): icosahedral virus particles of CMV.**

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الملخص العربي

A METHOD FOR PURIFICATION OF PLANT VIRUSES BELONGING TO DIFFERENT VIRUS GROUPS

طريقة لتنقية فيروسات نباتية تنتمي إلى مجموعات فيروسية مختلفة

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تم توصيف طريقة لعزل وتنقية بعض الفيروسات النباتية. تميزت الطريقة باستخدام محاليل عضوية، طرد مركزي تفاضلي وطرد مركزي متدرج الكثافة على محاليل ملحية متدرجة ومستقيمة من كبريتات السيزيوم وبإستخدام وسادة من السكروز. ثبت أن الطريقة مناسبة وجيدة لتنقية العديد من الفيروسات النباتية التي تنتمي إلى مجموعات فيروسية مختلفة تمثل الأشكال المختلفة التي تم تسجيلها لفيروسات النبات. شملت الفيروسات التي تم تنقيتها بهذه الطريقة كل من فيروس البطاطس واي، فيروس اتش في التبغ، فيروس موزاييك البرسيم، فيروس موزاييك الخيار، فيروس موزاييك التبغ وفيروس إلتفاف وإصفرار الطماطم. أعطى كل من محلولي كبريتات السيزيوم المتدرج والمستقيم نتائج متماثلة في عزل وتنقية الفيروسات المذكورة أعلاه ولم تلاحظ اختلافات كبيرة في النتائج عند استخدام أي منهما.

كلمات مفتاحية: التنقية، فيروس، الطرد المركزي لمحلول متدرج الكثافة، وسادة السكروز.