Indian J. Hort. 69(1), March 2012: 121-124

Short communication

Comparison of fluorescein- and ^{32}P-labelled probes for safe detection of potato spindle tuber viroid in potato

Priyanka Kaundal, Jagesh K. Tiwari*, Nimisha Kaushal, Deep Kumar, J. Gopal, S.K. Pandey and I.D. Garg

Central Potato Research Institute, Shimla 171 001, Himachal Pradesh

ABSTRACT

Two methods namely fluorescein- and ^{32}P-labelled probes were tested for detection of *Potato Spindle tuber* viroid in potato. Out of 94 potato microplants tested, 82 were found positive, while 12 were virus-free. On comparison with NASH, it could be seen that fluorescein-labelled probe was quite cost effective.

Key words: Fluorescein, ^{32}P-labelled, probe, potato spindle tuber viroid.

Viroids, the smallest known independently replicating pathogens, occur in a wide range of economically important crops. Potato spindle tuber viroid (PSTVd) was probably originated in the temperate world and reported in potato in 1922 (Khurana, 2). PSTVd is transmitted through contact, true potato seed (TPS) and tubers. Losses caused by PSTVd range from 20-70% depending up on the cultivar, strain (mild/severe), environmental conditions. Owing to highly contagious nature and transmission though true seeds/pollens, it is a pathogen of quarantine significance (Khurana, 2). Therefore, elimination of PSTVd from infected seed stocks is widely recognized as a challenge in potato. Selection of PSTVd-free potato seed stocks necessitates intensive research on reliable detection techniques. Unlike viruses, viroids lack antigenic coat proteins, therefore, they cannot be detected serologically. In recent years, molecular diagnostic techniques such as nucleic acid spot hybridization (NASH) have helped the construction of radioactive/ non-radioactive probes for the detection of PSTVd (Querci and Salazar, 6). One of the most sensitive molecular diagnostics techniques is based on ^{32}P-labelled probe (Owens and Diener, 4). But, the use of ^{32}P-labelled probes has several problems like short shelf-life, storage, handling, waste disposal and health hazards. To overcome these problems associated with the use of ^{32}P-labelled probe, our study aimed at validation of non-radioactive (fluorescein) labelled probe NASH technique for PSTVd detection which is as sensitive, safe and reliable as ^{32}P-labelled probe.

Ninety four potato microplants, comprising 20 each of germplasm accessions CP No. 1463, 2010, 2022 and 2101 and 14 microplants of CP No. 1683 obtained from Division of Crop Improvement, CPRI, Shimla during June 2008, were used for the PSTVd detection. Microplants were transplanted in glass house under optimum temperature range of 20-30°C for 3-4 weeks for bulking of the microplants materials. In this paper, procedure of NASH to detect PSTVd was followed as described by Singh *et al.* (12) for ^{32}P-labelled probe and Verma *et al.* (17) for fluorescein-labelled probe, is briefly described. One gram leaf tissue per plant was obtained from apical part of the plant and crushed in 1.5 ml extraction buffer (200 mM K_2HPO_4 + 10 mM DICA + 5 mM DTT and 0.1% Triton X-100) in a polythene bag. Extracted sap was subjected to low speed centrifugation at 7000 rpm for 5 min followed extraction of the supernatant with equal volume (200 µl) of 1:1 v/v mixture by of tris-saturated phenol and chloroform. The mixture was then centrifuged as above. The clear supernatant (5 µl) obtained was spotted on to nitro-cellulose membranes (10 × 10 cm) (Hybond N+) one for fluorescein and one for ^{32}P-labelled probe. These membranes were pretreated with distilled water for 5 min and 20X SSC (NaCl- 175.3 g and sodium citrate dehydrate- 88.2 g, pH 7.0) twice for 10 min each before spotting. Healthy and known PSTVd controls were also spotted on the membranes. Then membranes were baked in hybridization chamber at 80ºC for 2 h.

Plasmid ($pSP65B_2$) with full length cDNA of PSTVd insert, obtained from International Potato Center (CIP), Lima during 1995 and thereafter being maintained in *Escherichia coli* for the routine diagnosis of PSTVd, was used for the probes preparation. The plasmid was isolated from *E. coli* using Qiagen plasmid isolation kit as per manufacturer's instructions. Then, plasmid was linearised (isolated plasmid- 5 µl, BSA buffer- 3 µl, *Pst* I- 1.5 µl, distilled water- 11.5 µl) and purified with phenol-chloroform solution. Subsequently, purified

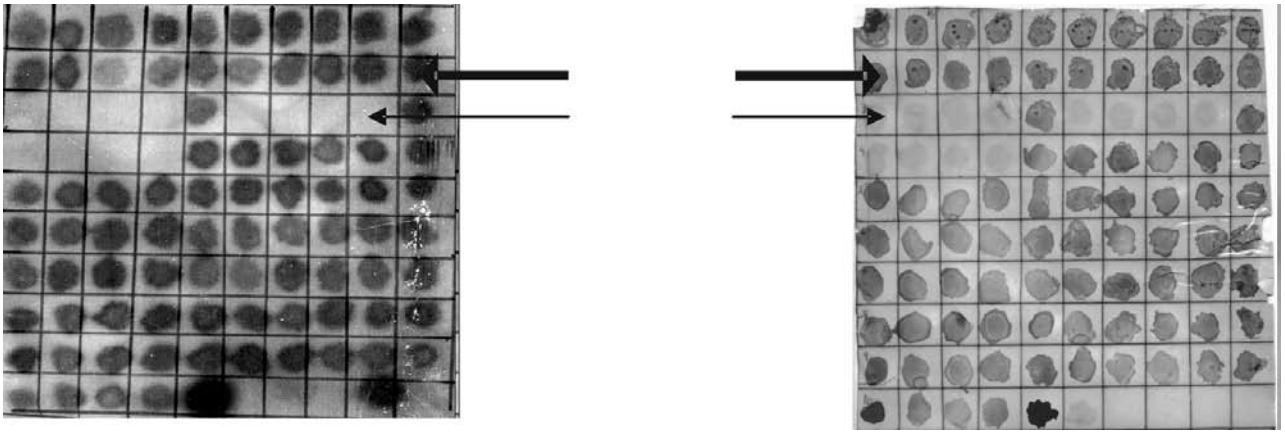
*Corresponding author's present address: Division of Crop Improvement, Central Potato Research Institute, Shimla 171 001; E-mail: jageshtiwari@gmail.com

plasmid was used for the preparation of fluorescein- and ^{32}P- labelled cRNA probe, separately.

Fluorescein-labelled probe was prepared through *in vitro* transcription (purified plasmid- 5 µl, fluorescein RNA labeling mix- 2 µl, 5X transcription buffer- 4 µl and SP6 RNA Polymerase- 2 µl) by incubation at 37°C for 2 h. Then, DNA templates of the probe were destroyed with DNase I (2 µl) by incubation at 37°C for 15 min followed by addition of 0.2M EDTA (2 µl) to terminate the reactions. ^{32}P-labelled probe was also prepared through *in vitro* transcription (purified plasmid- 7 µl, DTT- 2 µl, RNAsin- 1 µl, GTP- 1.5 µl, ATP- 1.5 µl and CTP- 1.5 µl, ^{32}P UTP- 2 µl, 5X transcription buffer- 4 µl, and SP6 RNA polymerase- 2 µl) by incubation at 37°C for 2 h followed by purification of the probe as described for the plasmid purification. Pre-hybridization was done by transferring the baked membrane in a cylindrical glass bottle with 20X SSC (3 ml), 20% SDS (60 µl), liquid block (600 µl) and 50% dextran sulphate- 1.2 ml at 60°C for 30 min in hybridization chamber followed by addition of fluorescein-labelled probe and incubation at 60°C for 18 h (overnight). Other membrane was hybridized with ^{32}P-labelled probe and 9.6 ml hybridization buffer (50% deionized formaldehyde- 20 ml, 200 mM sodium cocodilate- 5 ml, 20% SDS- 250 µl, distilled water- 14.75 ml) in a another glass bottle by incubation at 55°C for 10 min. Then, 1 ml calf-thymus DNA (denatured at 100°C for 5 min) was added and incubated at 55°C for 10 min. Finally, 2.4 ml dextran sulphate (pre-warmed) was added and incubated at 55ºC for 18 h (overnight).

Membrane was washed in 100 ml washing buffer I (20X SSC- 5 ml; 20% SDS- 500 µl) for 10 min at 60ºC and in 100 ml washing buffer II (20X SSC- 2.5 ml; 20% SDS- 500 µl) for 5 min at 60ºC. Membrane was then kept in blocking solution (90 ml diluent buffer: Tris- 7.26 g and NaCl- 10.52 g in 500 ml distilled water, pH 9.5 and blocking agent- 10 ml) for 1 h with gentle mechanical agitation followed by addition of 10 µl antifluorescein alkaline phosphatase enzyme conjugate, 50 ml diluent buffer and 0.25 g bovine albumin serum (BSA). Final washing was done in 0.3% Tween-20 (1.35 ml) and diluent buffer (450 ml) thrice for 10 min. each with 150 ml. Membrane was washed twice in 100 ml washing buffer I [0.36 M NaCl (21 g/l), 20 mM Tris base (2.4 g/l), 37% HCl (1.48 ml/l), 20% SDS (5 ml/l)] for 20 min at room temperature, once in 100 ml washing buffer II (0.1X SSC, 0.1X SDS) for 30 min at 65°C and twice in 100 ml washing buffer III (2X SSC) for 3 min. at room temperature. Subsequently, washing was done in 100 ml washing buffer III with 20 µl RNAse (stock: 10 mg/ml) (final concentration: 2 µg/ml) for 20 min. at room temperature. Final washing was done in 100 ml washing buffer II for 20 min at 50ºC.

Spots were detected by adding 20 ml detection buffer (0.1M Tris HCl and 0.1M NaCl, pH 9.5) and 200 µl NBT (Nitro blue tetrazolium chloride)/ BCIP (5-Bromo, 4-chloro, 3-Indolyl Phosphate) with hybridized and washed membrane in a plastic box and incubated in the dark at room temperature for 2-4 h. Thereafter, reaction was stopped by washing with tap water and air-dried the membrane. Membrane was detected through autoradiography by wrapping the hybridized and washed membrane with saran wrap; and X-ray film (Kodak X-OMAT AR) was placed on to it in a cassette in a dark room and stored at -80°C



1 2

Fig. 1. Detection of PSTVd by NASH based on ^{32}P- (1) and fluorescein-labelled probes (2) in potato microplants. Dark spots (thick arrow) showing PSTVd positive samples. Light spot (thin arrow) indicating PSTVd free samples.

for 24 h. Then, exposed X-ray film was developed. Dark spots on X-ray film indicated the PSTVd positive samples.

The study show that out of 94 potato microplants tested for PSTVd, 82 microplants were found positive for PSTVd and 12 microplants were found free from PSTVd with both the fluorescein- and ^{32}P-labelled probes by NASH. Positive samples produced purple-blue spots with fluorescein-labelled probe (Fig. 2) and dark black spots with ^{32}P-labelled probe (Fig. 1). Since viroid lack coat proteins, they cannot be detected by immunological approaches such as enzyme-linked immunosorbent assay (ELISA) or immuno electron microscopy. Till almost late 1970s, PSTVd was detected either biologically by inoculation on indicator hosts such as tomato (cv. Rutger) (Raymer and O'Brein, 7) or through polyacrylamide gel electrophoresis (PAGE) (Schumacher *et al.*, 11). Both of these methods were less sensitive, required higher concentration of viroid for the detection and unable to detect large number of sample in short time. To overcome these problems, the molecular diagnostics technique such as nucleic acid spot hybridization (NASH) was first developed by Owens and Diener (4) for the mass detection of PSTVd by ^{32}P-labelled probe in short time. They suggested that NASH is about 10 times more sensitive than PAGE for PSTVd detection (Owens and Diener, 4). Salazar *et al.* (9) also demonstrated the high sensitivity of NASH technique over PAGE for PSTVd detection in tuber sprouts of potato clones. Detection of mild strain of PSTVd was also possible through NASH by growing the plants under warmer field conditions (Singh *et al.*, 12). Thus, NASH based on ^{32}P-labelled probe has advantages of high sensitivity, less labour and very small sample size requirements for testing a large number of samples. But, ^{32}P-labelled probe has problems of potential health hazards, storage, handling and waste disposal because of being a beta (β) emitter and short half-life (14.3 days). Therefore, ^{32}P radio-labelled diagnostics methods were refined subsequently by other authors and attempts were made to replace hazardous ^{32}P radio-labelled probe with non-hazardous probes based on fluorescein/ biotin/ digoxygenin. In the new non-radioactive based NASH approach biotin-labelled (Candresse, 1) and fluorescein-labelled (Verma *et al.*, 17) probes were used for PSTVd detection in potato. Glass slide hybridization NASH methods based on fluorescein labelled probe showed the four times more sensitivity over dot blot hybridization techniques for the PSTVd detection in potato (Zhiyou Du, 21). Our similar results of PSTVd detection in potato microplants by NASH with both the probes, *i.e.* fluorescein- and ^{32}P-labelled confirm the high sensitivity and reliability of fuorescein-labelled probe as that of ^{32}P-labelled probe. Additionally, the fluorescein-labelled probe (non-radioactive) has advantage of safe, longer shelf-life and easy handling over ^{32}P-labelled probe (radioactive). Hence, this paper validates the sensitivity of fluorescein-labelled probe and suggests for its mass application for the safe and routine diagnosis of PSTVd in potato to ensure viroid-free seed stock.

ACKNOWLEDGEMENT

Authors are grateful to Director, Central Potato Research Institute, Shimla for providing the research facilities.

REFERENCES

1. Candresse, T., Macquaire, G., Brault, V., Monsion, M. and Dunez, J. 1990. ^{32}P- and biotin-labelled *in vitro* transcribed cRNA probes for the detection of potato spindle tuber viroid and chrysanthemum stunt viroid. *Res. Virol.* **141**: 97-107.

2. Khurana Paul, S.M. 1992. *Potato Viruses and Viral Diseases*. *Tech. Bull.* No. **35**, Central Potato Research Institute, Shimla, pp. 29-33.

3. Mukherjee, K., Singh, M.N., Paul Khurana, S.M. and Sandhu, S.K. 1999. Status of potato spindle tuber viroid in the field-grown germplasm collection determined by cRNA. **In**: *Potato Global Research & Development*, Vol. I. S.M. Paul Khurana, G.S. Shekhawat, B.P. Singh and S.K. Pandey (Eds.), pp. 487-90.

4. Owens, R.A. and Diener, T.O. 1981. Sensitivity and rapid diagnosis of spindle tuber viroid disease by nucleic acid spot hybridization. *Science*, **213**: 670-72.

5. Owens, R.A., Paul Khurana, S.M., Smith, D.R., Singh, M.N. and Garg, I.D. 1992. A new mild strain of potato spindle tuber viriod isolated from *Solanum* spp. in India. *Plant Dis.* **76**: 527-29.

6. Querci, M. and Salazar, L.F. 1998. Molecular probes for detection of viruses/viroid in potato. **In**: *Comprehensive Potato Biotechnology,* S.M. Paul Khurana, R. Chandra and M.D. Upadhya (Eds.), Malhotra Publishing House, New Delhi, pp. 187-206.

7. Raymer, W.B. and O'Brein, K.J. 1962. Transmission of potato spindle tuber virus to tomato. *American Potato J.* **39**: 401-8.

8. Salazar, L.F., Balbo, I. and Owens, R.A. 1988. Comparison of four radioactive probes for the diagnostics of potato spindle tuber viroid by nucleic acid spot hybridization. *Potato Res.* **31**: 431-32.

9. Salazar, L.F., Owens, R.A., Smith, D.R. and Diener, T.O. 1983. Detection of potato spindle tuber viroid by nucleic acid spot hybridization: Evaluation with tuber sprout and true potato seed. *American Potato J.* **60**: 587-97.

10. Salazar, L.F., Hammond, R.W., Diener, T.O. and Owens, R.A. 1988. Analysis of viroid replication following *Agrobacterium*-mediated inoculation of non-host species with potato spindle tuber viroid cDNA. *J. Gen. Virol.* **69**: 879-89.

11. Schumacher, J., Randles, J.W. and Riesner, D. 1983. A two-dimensional technique for the detection of circular viroid and virosoids. *Anal. Biochem.* **135**: 288-95.

12. Singh, M.N., Mukherjee, K., Paul Khurana, S.M., Gopal, Jai and Querci, M. 1999. Detection of potato spindle tuber viroid by NASH in exotic potato germplasm. **In**: *Potato Global Research & Development*, Vol. I, S.M. Paul Khurana, G.S. Shekhawat, B.P. Singh and S.K. Pandey (Eds.), pp. 491-94.

13. Singh, R.P. and Boucher, A. 1988. Comparative detection of mild strains of potato spindle tuber viroid from the dormant potato tuber by return-polyacrylamide gel electrophoresis and nucleic acid hybridization. *Potato Res.* **31**: 159-66.

14. Singh, R.P., Boucher, A. and Seabrook, J.E.A. 1988. Detection of the mild strain of potato spindle tuber viroid from single true potato seed by return gel electrophoresis. *Phytopath.* **78**: 663-67.

15. Singh, R.P., Boucher, A., Somerville, T.H. and Coleman, S. 1996. Detection of potato viruses A, M, S, X, Y and leafroll and potato spindle tuber viroid from tissue culture plantlets using single leaf discs. *American J. Potato Res.* **73**: 101-12.

16. Singh, Rudra P. 1999. Development of the molecular methods for potato virus and viroid detection and prevention. *Genome*, **42**: 592-604.

17. Verma, Y., Shukla, Arti, Paul Khurana, S.M. and Garg, I.D. 2006. Detection of potato spindle tuber viroid (PSTVd) using fluorescein labelled probe. *Potato J.* **33**: 122-25.

18. Welnicki, M. and Hiruki, C. 1992. Highly sensitive digoxigenin-labelled DNA probe for the detection of potato spindle tuber viroid. *J. Virol. Methods*, **39**: 91-99.

19. Welnicki, M., Skrzeczkowski, J. , Soltynska, A. , Jonczyk, P., Markiewicz, W., Kierzek, R., Imiolczyk, B. and Zagorski, W. 1989. Characterization of synthetic DNA probe detecting potato spindle tuber viroid. *J. Virol. Methods*, **24**: 141-52.

20. Zekanowski, C., Welnicki, M., Skrzeczkoski, J. and Zagorski, W. 1990. Detection of potato spindle tuber viroid (PSTV) in dormant potato tubers by contameric cDNA probe. *J. Virol. Methods*, **30**: 127-30.

21. Zhiyou Du, Bo Jin, Wenhong, Liu, Liang, Chen and Jishuang, Chen. 2007. Highly sensitive fluorescent-labeled probes and glass slide hybridization for the detection of plant RNA viruses and a viroid. *Acta Biochimica et Biophysica Sinica*, **39**: 326-34.

Received: February, 2011; Revised: December, 2011; Accepted : January, 2012