

26. Basu, A. and Saxena, N. D., Bathymetry computation from free-air anomaly data. *Mar. Geodesy*, 1993, **16**, 325–336.
27. Watts, A. B., An analysis of isostasy of the world's ocean. 1. Hawaiian Emperor seamount chain. *J. Geophys. Res.*, 1978, **83**, 5989–6004.
28. NGDC/NIO ship bathymetry data over the study area as obtained from NIO, Goa, pers. commun.
29. Udintsev, G. B., Fisher, R. L., Kanev, V. F., Laughton, A. S., Simpson, E. S. W. and Zhiv, D. I., *Geological and Geophysical Atlas of the Indian Ocean*, Academy of Sciences of USSR, Moscow, 1975.
30. ETOPO5 website address: <http://www.ngdc.noaa.gov/mgg/image/2minrelief.html>

ACKNOWLEDGEMENTS. We thank Dr C. Hwang, National Chiao Tung University, Taiwan for help in software/data during various stages of development of this activity. Thanks are also due to the two anonymous referees for their critical comments which have helped in improvement of the manuscript. We also thank Dr R. R. Navalgund, Director, SAC, Dr K. L. Majumder, Dy. Director, RESIPA, and Dr S. R. Nayak, Group Director, MWRG for their keen interest during the course of this study. Ship bathymetry over the study area as obtained from NIO, Goa on personal request is acknowledged. R.B. acknowledges fellowship from CSIR, New Delhi.

Received 27 August 2004; revised accepted 8 September 2005

Reverse transcription polymerase chain reaction-based detection of *Arabidopsis mosaic virus* and *Strawberry latent ringspot virus* in vector nematodes

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In a survey of rose and lily plantations in experimental fields of the Institute of Himalayan Bioresource Technology, Palampur, a number of symptoms characteristic of nepoviruses were observed. Symptomatic rose and lily leaves were tested for the presence of nepoviruses. *Strawberry latent ringspot virus* (SLRSV) and *Arabidopsis mosaic virus* (ArMV) were detected by ELISA and RT-PCR using virus-specific antibodies and prim-

ers respectively, in rose cultivars Raktgandha, Landora and Sonia. Only SLRSV was detected in lily cultivars Galeili, Star Gazer Max and White Merostar. To ascertain the presence of viruliferous nematodes, soil samples were collected from around the roots of rose and lily cultivars. *Xiphinema diversicaudatum* and *Longidorus macrosoma* isolated from soil were placed separately into 5–10 µl each of RNAlater™ and sterile water. Total RNA from these nematodes was isolated using three different RNA isolation kits, viz. RNAqueous™, RNeasy Plant mini kit and RNAwiz™. RT-PCR was performed using virus-specific primers for SLRSV and ArMV. Out of the three RNA isolation kits used, only RNAqueous™ gave reproducible results. Nematodes isolated and stored in RNAlater™ gave amplification of expected size (over a period of one month storage at –20°C) for both the viruses, while nematodes stored in water did not give any amplification (even after one week of storage) with RT-PCR. *Cucumis sativus* was used as bait plant for confirming the nematode transmission of these viruses. In the present study RNAlater™ was found to be the best nematode storage solution and RNAqueous™ the best RNA isolation kit for virus detection in viruliferous nematodes by RT-PCR.

Keywords: Lily, Nepoviruses, RNA isolation, rose, RT-PCR.

ROSE and lily are important commercially grown flower crops and are susceptible to a number of viruses including *Strawberry latent ringspot virus* (SLRSV) and *Arabidopsis mosaic virus* (ArMV)^{1,2}. SLRSV and ArMV are both nematode-transmitted polyhedral (nepo)viruses and are transmitted by *Xiphinema diversicaudatum* and *Longidorus macrosoma* respectively³. While *Tobacco rattle virus* (TRV) and *Grapevine fan leaf virus* (GFLV) have been detected in nematodes^{4,6}, there exist few reports for PCR-based detection of nepoviruses in nematodes.

During the survey of rose and lily plantations in experimental fields of the Institute of Himalayan Bioresource Technology (IHBT), Palampur, a number of symptoms characteristic of nepoviruses were observed. Rose and lily leaves were tested for the presence of SLRSV and ArMV by ELISA and RT-PCR using virus-specific antibodies and primers. Rose cultivars (Raktgandha, Landora and Sonia) were found to be positive for ArMV and SLRSV, while Oriental hybrid lily cultivars (Galeili, Star Gazer Max and White Merostar) were found to be positive only for SLRSV. The present study was carried out to isolate the viruliferous nematodes from rose- and lily-grown field soil, to confirm virus transmission using bait plants and to standardize the detection protocol for viruses in nematodes. Various commercially available RNA isolation kits were compared for total RNA isolation from viruliferous nematodes for consistent results in RT-PCR.

DAS-ELISA was performed for the initial survey and detection of viruses from rose and lily cultivars using antibod-

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ies specific for SLRSV and ArMV (DSMZ, Germany). Positive and negative controls provided with the kits were used. The protocols used for DAS-ELISA were same as those described by Clark and Adams⁷. A total of ten plants each of rose cultivars, viz. Raktgandha, Landora, Sonia and Oriental Hybrid lily cultivars, viz. Galeili, Star Gazer Max and White Merostar (either symptomatic and non-symptomatic) were tested for the presence of SLRSV and ArMV.

Soil samples were collected from around the roots of rose and lily cultivars. A bulk sample consisting of 20 cores (diameter 1.9 cm; depth 0–20 cm) was taken from each of the rose and lily cultivars. Nematodes were isolated from properly mixed 200 ml soil samples separately, using the method of Whitehead and Hemming⁸. Soil was kept in a tissue paper inside a plastic sieve and Millipore water was added into the collection tray. Nematodes were collected on a 325-mesh sieve after 24 h. Nematodes were counted in a Doncaster counting dish⁹ using dissecting microscope and were identified using light microscope at 400X magnification^{1–12}. Nematode population varied from 145 to 160 per 200 ml soil samples collected from the rose and lily fields.

Four hundred each *X. diversicaudatum* and *L. macrosoma* identified/isolated from rose and *X. diversicaudatum* from lily soil were collected and distributed among two separate Eppendorf tubes (in four sets, 50 individual nematodes each), one containing a drop of RNAlaterTM (Ambion, USA) and the other containing a drop of water. The nematodes in RNAlaterTM and water were stored in deep freezer (–20°C) for a period of up to one month and total RNA was isolated from the stored nematodes at weekly intervals to perform RT-PCR.

Cucumis sativus, used as bait plants were grown in autoclaved soil and watered with sterile water periodically. For transmission studies, 50 freshly isolated nematodes from soil around rose (*X. diversicaudatum* and *L. macrosoma* separately) and lily (*X. diversicaudatum*) plants were kept in 5 ml sterile water in separate beakers. On emergence of cotyledonary leaves of bait plants, the nematode suspension was inoculated near the root zone. Inoculated plants were kept at 20–25°C for 30 days for symptom development. Bait plants inoculated with 5 ml sterile water with nematodes free from SLRSV and ArMV served as control.

Total RNA from viruliferous and non-viruliferous nematodes (maintained on healthy *C. sativus* plants) was isolated using commercially available kits, including RNAqueousTM (Ambion, USA), RNAwizTM (Ambion, USA) and RNeasy Plant mini kit (Qiagen, Germany) according to the manufacturers' instructions. Total RNA isolated using RNAqueousTM from SLRSV- and ArMV-infected rose cultivars was used as positive control. Total RNA from healthy *C. sativus* was used as negative control in RT-PCR detection.

RT-PCR amplification was performed using primers specific for SLRSV and ArMV, as described by Bertolini *et al.*¹³ and MacKenzie *et al.*¹⁴ respectively, which are specific for the amplification of a part of their coat protein

gene (approximately of 200 bp for SLRSV and 520 bp for ArMV). For RT reaction, 20 µl RNA was used with 0.2 µg downstream primer, 2 µl of 30 mM dNTP mix, 20 units of human placental RNAase inhibitor, 4 µl of 5X RT buffer and 200 units M-MLV reverse transcriptase (USB, USA). The reaction mixture was incubated at 37°C for 1 h followed by 70°C for 5 min. Further amplification was carried out in Mastercycler (Eppendorf, Germany) with 50 µl of reaction mixture containing 10 µl cDNA product, 0.2 µg downstream primer, 0.2 µg upstream primer, 5 µl of 10X PCR buffer, 1 µl of 30 mM dNTP mix and 1.5 units of Taq DNA polymerase (Genei, India). For SLRSV, 45 PCR cycles of denaturation at 94°C for 30 s, annealing at 50°C for 2 min and extension at 72°C for 1 min followed by one cycle of final extension at 72°C for 10 min was given. For ArMV, 35 PCR cycles of denaturation at 94°C for 30 s, annealing at 50°C for 20 s and extension at 72°C for 1 min followed by one cycle of final extension at 72°C for 10 min were given. PCR product (15 µl) was run on 2% TAE agarose gel, stained with ethidium bromide and photographed under UV.

In ELISA, all the three rose cultivars tested were found to be positive for ArMV and SLRSV and three lily cultivars for SLRSV only. The viruses were detected only in the symptomatic plants of rose (70%) and lily cultivars (60%). ArMV and SLRSV were present together in all the three rose cultivars.

Fifteen to eighteen days after inoculation (with nematodes isolated from rose and lily soil), bait plants showed chlorotic and necrotic lesions on the new emerging leaves. The presence of virus in inoculated *C. sativus* plants was also confirmed by ELISA and RT-PCR. The viruses were not detected by ELISA or RT-PCR in the nonsymptomatic control plants.

Total RNA from nematodes isolated from soil around rose cultivars stored in RNAlaterTM gave specific amplification of ~520 bp for ArMV (Figure 1) and ~200 bp for SLRSV (Figure 2). The total RNA from nematodes isolated from soil around Oriental hybrid lilies stored in RNAlaterTM gave specific amplification of ~200 bp for SLRSV (Figure 2) even after one month of storage at –20°C, while no amplification was observed for ArMV and SLRSV from nematodes stored in water at –20°C (Figures 1 and 2). Out of the three kits used, only RNAqueousTM gave reproducible results for detection of ArMV and SLRSV from nematodes isolated from rose and for SLRSV, from nematodes isolated from lily. The other two kits used did not give reproducible results with respect to virus detection from nematodes, even when they were stored in RNAlaterTM.

The three rose cultivars tested for ArMV and SLRSV and the three lily cultivars tested for SLRSV by ELISA were also found to be positive. This indicates that the rose cultivars under study were infected with ArMV and SLRSV and lily cultivars were infected only with SLRSV.

The nematodes isolated from rose and lily cultivars and stored in RNAlaterTM gave amplification of expected size

even after one month of storage at -20°C , while nematodes stored in water did not give any amplification even after one week of storage at -20°C . This indicates that RNAlaterTM,

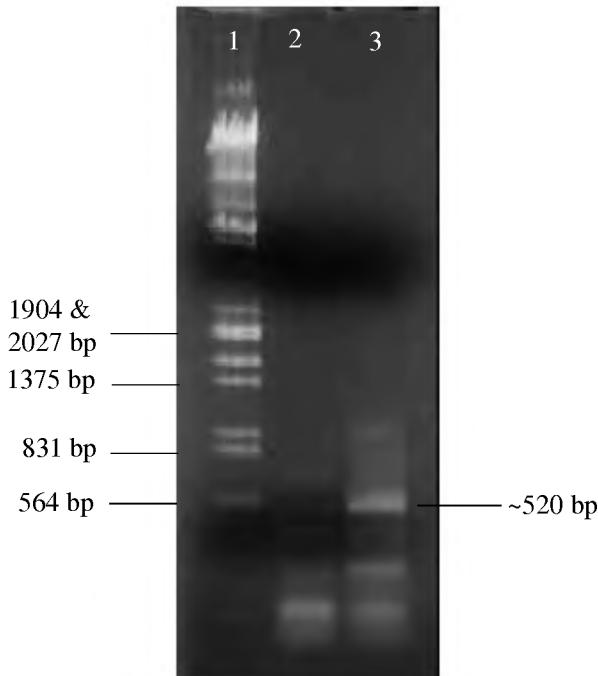


Figure 1. Gel photograph showing amplification of *Arabidopsis mosaic virus* (ArMV) from nematode stored in water and in RNAlaterTM after one month of storage. Lane 1, λ DNA *EcoRI/HindIII* digest; lane 2, No amplification of ArMV (rose) from nematodes stored in water; lane 3, Amplification of ArMV (rose) from nematodes stored in RNAlaterTM.

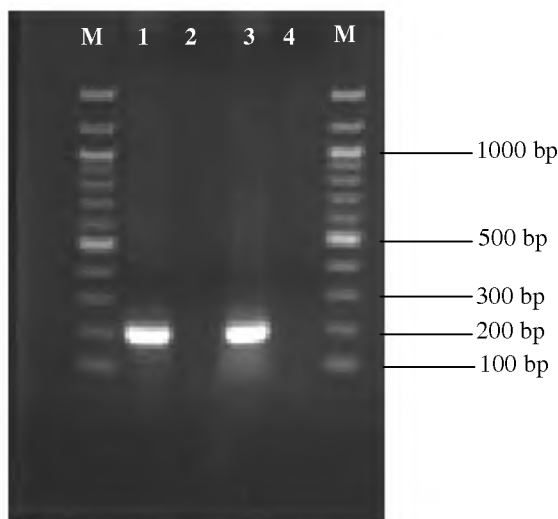


Figure 2. Gel photograph showing amplification of *Strawberry latent ringspot virus* (SLRSV) from nematode stored in water and in RNAlaterTM after one month of storage. Lane M, 100 bp DNA marker; lane 1, Amplification of SLRSV (rose) from nematodes stored in RNAlaterTM; lane 2, No amplification of SLRSV (rose) from nematodes stored in water; lane 3, Amplification of SLRSV (lily) from nematodes stored in RNAlaterTM; lane 4, No amplification of SLRSV (lily) from nematodes stored in water.

being a RNA-stabilizing solution, stabilized the RNA of the stored nematodes much more efficiently than water. Out of the three kits used for RNA isolation, only RNAqueousTM (Ambion, USA) gave reproducible results for detection of viruses from nematodes. These results indicate that for virus detection from nematodes, they should be stored in some RNA-stabilizing solution like RNAlaterTM (Ambion, USA) prior to RNA isolation and not in water. Further, RNAqueousTM kit should be used for RNA isolation from nematodes.

C. sativus plants inoculated with nematodes isolated from rose and lily cultivars showed virus-like symptoms on new emerging leaves. Presence of viruses was further confirmed by ELISA and RT-PCR. It showed that *X. diversicaudatum* and *L. macrosoma* (in case of rose) and *X. diversicaudatum* (in case of lily) were viruliferous and transmitted the virus to bait plants. These results are consistent with earlier findings that ArMV and SLRSV are transmitted by nematodes³. Earlier reports on virus detection from nematodes are TRV in *Trichodorus* spp. and GFLV in *Xiphinema index* by RT-PCR using the different procedures for RNA isolation and RT-PCR⁴⁻⁶. These reports are on RNA isolation from nematodes by phenol:chloroform extraction method which is tedious and time-consuming. The technique described in the present communication is easy, less time-consuming and nematodes isolated in RNAlaterTM can be stored for at least one month without RNA degradation. In the present study, a protocol has been standardized for specific detection of two different nematode-transmitted viruses from two different crops, which can be applied for the detection of other nematode-transmitted viruses. It is a definitive report on transmission and detection of ArMV and SLRSV in *X. diversicaudatum* and *L. macrosoma* using molecular techniques like RT-PCR.

1. Derks, A. F. L. M., Lily. In *Virus and Virus-like Diseases of Bulb and Flower Crops* (eds Loebenstein, G., Lawson, R. H. and Brunt, A. A.), John Wiley, United Kingdom, 1995, pp. 313-321.
2. Loebenstein, G., Rose. In *Virus and Virus-like Diseases of Bulb and Flower Crops* (eds Loebenstein, G., Lawson, R. H. and Brunt, A. A.), John Wiley, United Kingdom, 1995, pp. 426-438.
3. Brunt, A. A., Crabtree, K., Dallwitz, M. J., Gibbs, A. J. and Watson, L., In *Viruses of Plants*, CAB International, University Press, Cambridge, 1996, pp. 678-688.
4. Demangeat, G., Komar, V., Cornuet, P., Esmanjand, D. and Fuche, M., Sensitive and reliable detection of *Grapevine fan leaf virus* in a single *Xiphinema index* nematode vector. *J. Virol. Methods*, 2004, **122**, 79-86.
5. Esmanjand, D. and Abad, P., Detection of a region of the coat protein gene of *Grapevine fanleaf virus* by RT-PCR in the nematode vector *Xiphinema index*. *Plant Dis.*, 1994, **78**, 1087-1090.
6. Van der Wilk, F., Korsman, M. and Zoon, F., Detection of *Tobacco rattle virus* in nematodes by reverse transcription and polymerase chain reaction. *Eur. J. Plant Pathol.*, 1994, **100**, 109-122.
7. Clark, M. F. and Adams, A. N., Characterization of the micro figure method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.*, 1977, **34**, 475-483.

RESEARCH COMMUNICATIONS

8. Whitehead, A. G. and Hemming, J. R., A comparison of quantitative methods of extracting small vermiform nematodes from soil. *Ann. Appl. Biol.*, 1965, **55**, 25–38.
9. Doncaster, C. C., A counting dish for nematodes. *Nematologica*, 1962, **7**, 334–337.
10. Aboul-eid, H. Z. and Coomans, A., Intersexuality. In *Longidorus macrosoma*. *Nematologica*, 1966, **12**, 343–345.
11. Goodey, J. B., Peacock, F. C. and Pitcher, R. S., A redescription of *Xiphinema diversicaudatum* (Micoletzky, 1923 and 1927), Throné 1939 and observation on its larval stages. *Nematologica*, 1960, **5**, 127–135.
12. Mai, W. F. and Lyon, H. H., Pictorial key to genera of plant-parasitic nematodes. Department of Plant Pathology, New York State College of Agriculture, a Unit of the State University of New York, Cornell University, Ithaca, New York, 1960.
13. Bertolini, E., Olmos, A., Martinez, M. C., Gorris, M. T. and Carinra, M., Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA virus in olive trees. *J. Virol. Methods*, 2001, **96**, 33–41.
14. MacKenzie, D. J., McLean, M. A., Mukerji, S. and Green, M., Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription–polymerase chain reaction. *Plant Dis.*, 1997, **81**, 222–226.

ACKNOWLEDGEMENTS. S.K. thanks the Council of Scientific and Industrial Research, New Delhi for awarding Senior Research Fellowship. O.K.A. thanks Third World Academy of Sciences, Trieste, for awarding postdoctoral fellowship. We thank the Director, IHBT, Palampur, India for providing necessary facilities. This is IHBT communication no. 346.

Received 21 February 2005; revised accepted 27 June 2005
