SHORT COMMUNICATION

SIMULTANEOUS DETECTION OF *ONION YELLOW DWARF VIRUS* AND *SHALLOT LATENT VIRUS* IN INFECTED LEAVES AND CLOVES OF GARLIC BY DUPLEX RT-PCR

S. Majumder[1], V.K. Baranwal[1] and S. Joshi[2]

[1]Plant Virology Unit, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110012, India
[2] Division of Vegetable Science, Indian Agricultural Research Institute, New Delhi 110012, India

SUMMARY

A duplex RT-PCR was standardized for the simultaneous detection of *Onion yellow dwarf virus* (OYDV) and Garlic latent virus (GarLV), a synonym for the recognized species *Shallot latent virus* (SLV) in garlic, which allowed the successful identification of both viruses in cloves and leaves. The titre of OYDV was higher both in leaves and bulbs compared with SLV. In the leaves, OYDV was detected up to an RNA dilution of 10^{-4} while SLV could be detected up to an RNA dilution of 10^{-3}. Duplex RT-PCR detected both viruses in ten commercial garlic cultivars. The procedure is cost-effective and sensitive and will be highly useful in quarantine and certification programmes.

Key words: *Allium sativum*, OYDV, SLV, Duplex RT-PCR, diagnosis.

Garlic (*Allium sativum* L.), one of the oldest known horticultural crops, is widely used for its antibiotic, anti-diabetic, anti-cancerous, anti-oxidant activity and lipid lowering action (Keusgen, 2002). Garlic stocks are often infected by multiple viruses that belong to different taxa and are collectively designated as the 'garlic viral complex' (Walkey and Antill 1989; Van Dijk, 1994). This complex may include potyviruses (*Onion yellow dwarf virus*, OYDV, and *Leek yellow stripe virus*, LYSV), carlaviruses (*Shallot latent virus*, SLV or its synonym Garlic latent virus, GarLV and *Garlic common latent virus*, (GarCLV) and allexiviruses. These viruses may not kill the plant but can reduce yield up to 50% over time (Lot *et al.*, 1998; Conci *et al.*, 2003). Clonal propagation leads to build up of viruses in each generation and virus-free stocks are re-infected within three to four growing seasons due to continuous influx from diseased plants growing nearby (Lot *et al.*, 1998). A sensitive virus detection method is, therefore, essential for the production of virus-free propagating material.

Corresponding author: V.K. Baranwal
Fax: +91.11.25840772
E-mail: vbaranwal2001@yahoo.com

Although ELISA has often been employed for garlic virus diagnosis (Conci *et al.*, 2003), PCR has proved to be more efficient and sensitive (Dovas *et al.*, 2001; Shiboleth *et al.*, 2001). In particular, standard RT-PCR has been used to identify individual viruses such as OYDV (Dovas *et al.*, 2001; Takaichi *et al.*, 2001; Arya *et al.*, 2006), GarLV (Tsuneyoshi *et al.*, 1998) and allexiviruses (Dovas *et al.*, 2001). Since detection of individual viruses is expensive and time consuming, a duplex RT-PCR for simultaneous detection of a potyvirus (OYDV) and a carlavirus (SLV) was standardized and evaluated.

A selected line of garlic PS-10, obtained from the Division of Vegetable Science, IARI, New Delhi, was initially used for virus detection by immunosorbent electron microscopy (ISEM). Since both viruses were observed in ISEM, a standardized duplex RT-PCR was developed for their simultaneous detection. Half of the cloves from a mother bulb were used for planting and half were used directly in the experiment. The same garlic cultivar was grown from apical meristem of clove using a modified tissue culture protocol of Dantu and Bhojwani (1992) and the plantlets obtained, if found virus-free by ISEM, were used as healthy negative controls.

Total RNA was extracted from 50 mg tissues of infected or healthy garlic cloves or leaves, using RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol. RNA from each sample was eluted in 40 µl of RNase free water.

For OYDV detection, primers previously designed on the conserved region of the polymerase gene and 3' UTR were used (Arya *et al.*, 2006). Clustal W was used to design primers from the conserved sequences of the 3' region of the coat protein (CP) gene sequences available under the name of GarLV in NCBI GenBank. The primers used for OYDV were 5' ATAGCAGAAACAGCTCTTA 3' and 5' GTCTCYGTAATTCACGC 3' whereas those used for SLV were 5' GTGGTNTGGAATTAC 3' and 5' CAACATCGATTYTCTC 3'. The BLAST programme in NCBI GenBank was used on the primer sequences of SLV to confirm their specificity.

For standard RT-PCR, the first strand of cDNA was synthesized separately for OYDV and SLV using 4 µl of total RNA and a reverse transcription (RT) mixture containing the reverse primer of OYDV or SLV at a

concentration of 0.2 µM, 20 U M-MuLV reverse transcriptase (Fermentas, USA), 4 µl of 5x reaction buffer and 0.3 mM dNTPs. The total reaction mixture of 20 µl was incubated at 42°C for 45 min. The enzyme was inactivated by heating at 70°C for 10 min.

To determine the annealing temperature for the OYDV and SLV primers, gradient PCR was performed using the temperature range 44-52°C in an Eppendorf Master Cycler. PCR was performed using a reaction mixture containing 5 µl of RT reaction mixture, 5 U of Taq DNA polymerase (Promega, USA), 5 µl of 10x reaction buffer, 1.5 mM $MgCl_2$, primers at a concentration of 0.2 µM and 0.2 mM dNTPs. The temperature profile consisted of a denaturation step at 94°C for 5 min, then 30 cycles of 45 sec at 94°C, 20 sec at annealing temperature and 1 min at 72°C and one final extension step at 72°C for 10 min. OYDV and SLV could be amplified optimally at 48°C and 46°C respectively (Fig 1a, b).

Ten microlitres of amplified product were separated by electrophoresis in a 1.2% agarose gel containing ethidium bromide at a concentration of 0.5 µg ml^{-1} and photographed under UV illumination with the Bio-Rad XR documentation system. PCR products were purified using the PCR Purification kit (Qiagen, USA). The purified PCR product was ligated into pGEM-T Easy vector (Promega, USA) and competent *Escherichia coli* (strain DH5α) was transformed by standard methods (Sambrook *et al.,* 1989). Recombinant clones were identified by colony PCR and sequenced. Nucleotide sequences of cloned DNA showed that the OYDV and SLV fragments were of 1110 bp and 308 bp, respectively. The OYDV sequence showed 99% identity with a viral sequence from India (DQ519034) and 81% to 87% with other OYDV sequences available in GenBank. SLV had 96% sequence identity with a GarLV sequence from India (EF600902) and 81 to 86% with other GarLV and SLV sequences.

To standardize the duplex PCR, 5 µl cDNA of OYDV and SLV were mixed initially and used as template for simultaneous detection. The PCR mixture contained 5 µl of both the RT reaction mixture, 5 U of Taq DNA polymerase, 5 µl of 10x reaction buffer. Based on results of standard RT-PCR for OYDV and SLV, primers were used at a concentration of 0.2 µM for OYDV and 0.4 µM for SLV. Optimized higher concentration of dNTPs (0.4 mM) and $MgCl_2$ (2.5 mM) were used in the PCR mixture. To determine the appropriate annealing temperature for the duplex PCR, a gradient PCR was set up with annealing temperatures of 46/47/48°C for 20 sec. The denaturation and other reaction steps were the same as described for standard PCR. OYDV and SLV could both be amplified by the above reaction, and amplification of both viruses was more intense at an annealing temperature of 48°C (result not shown).

To simplify the duplex RT-PCR further, cDNAs of OYDV and SLV were prepared simultaneously in a single-tube reaction using 4 µl of total RNA, and the duplex PCR was performed as above on a gradient PCR with annealing temperatures of 46/47/48°C for 20 sec. As a control, standard RT-PCR was performed for each virus separately using 5 µl of the same RT reaction mixture and 48°C as annealing temperature. cDNAs prepared simultaneously in single-tube also detected both viruses with better amplification intensity at annealing temperature of 48°C (Fig. 1C).

To determine the sensitivity of standard and duplex RT-PCR, different dilutions of RNA ranging from 10^1 (1 µl of 40 µl RNA eluted from 50 mg tissue) to 10^{-4}, corresponding to 0.313 µg to 0.0003 µg of RNA, extracted from diseased leaves or cloves was used for preparing individual as well as mixed cDNAs. Reaction conditions for cDNA preparation were as described for standard and duplex PCR. OYDV was detected up to a dilution of 10^{-4} while SLV was detected up to a dilution of 10^{-3} both in leaves and cloves (Fig. 2A, lanes 1, 2 and 2B, lanes 3, 4). A similar result was obtained in duplex PCR for both viruses in the leaves. However, in the cloves, both viruses were detected by duplex RT-PCR only up to 10^{-2} dilution, but OYDV could be detected even up to 10^{-4} dilution of RNA (result not shown).

The standardized duplex RT-PCR was used to determine the presence of OYDV and SLV in ten selection

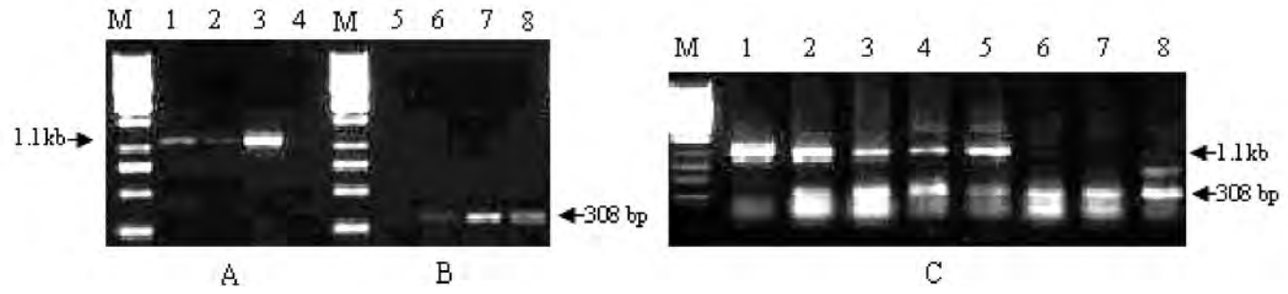

Fig. 1. Gel electrophoresis showing effect of different annealing temperatures in standard RT-PCR (A and B) and duplex PCR (C). (A) OYDV; Lanes 1 to 4 correspond to annealing temperatures of 52°C, 50°C, 48°C and 46°C respectively (B) SLV; Lanes 5 to 8 correspond to annealing temperatures of 50°C, 48°C, 46°C and 44°C, respectively and (C) Duplex RT- PCR of OYDV and SLV, lanes 2 and 3, 48°C,; 4 and 5, 47 °C; 6 and 7, 46°C; lane 1, OYDV alone, and lane 8, SLV alone, at 48°C; lane M, 1 kb marker.

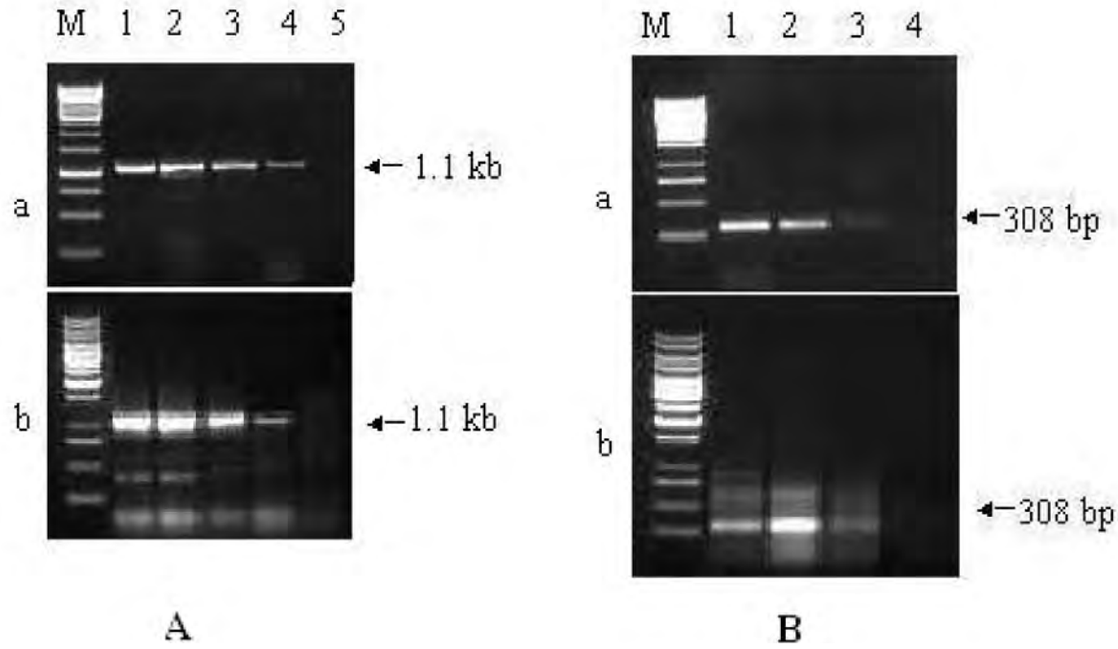

Fig. 2. RT-PCR detection of OYDV (A) and SLV (B) in leaves (a) and cloves (b) using RNA at different dilutions. Lanes: M, marker; lane 1, $10^{-1;\ lane}$ 2, 10^{-2}; lane 3, 10^{-3}; lane 4, 10^{-4}; lane 5, healthy.

lines of garlic (Table 2) available at the Division of Vegetable Science farm, IARI. Five random leaf samples were collected for each selection line, and tested at least twice. All experiments were repeated twice.

The protocol could successfully detect both viruses in all the selection lines collected from the field. Out of ten selection lines, duplex RT-PCR detected SLV in all samples, whereas OYDV, though detected in all of the ten selection lines, could be detected in 4 out of 5 samples of selection 17 and selection 34 (Table 1). Results for only five selection lines are shown here (Fig. 3). There was no amplification from healthy plants raised in tissue culture.

OYDV and the SLV isolates of SLV are two common viruses in garlic cultivars in India. It is important to develop a sensitive and reliable RT-PCR based simultaneous detection of these two viruses so that it can be used for production of virus-free garlic. It is often necessary to optimize the reaction parameters while performing multiplex PCR for simultaneous detection of viruses based on the standard PCR (Chamberlain and Chamberlain, 1994; Singh and Nie, 2003). In the present study optimization was done for the standardization of

Table 1. Detection of OYDV and SLV in field samples of different selection lines of garlic using standardized duplex RT-PCR.

	Garlic selection lines	No. of OYDV-positives out of 5 field samples	No. of SLV-positives out of 5 field samples
1	GS - 282	5	5
2	PGS - 14	5	5
3	Selection - 34	4	5
4	Accession - 9	5	5
5	Selection - 17	4	5
6	Panipat selection - 1-C	5	5
7	Agrifound Parvati	5	5
8	Selection - 9	5	5
9	Pusa selection - 10	5	5
10	G - 1	5	5

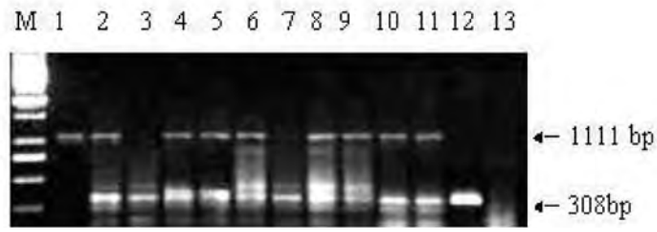

Fig. 3. Simultaneous detection of OYDV and SLV in field samples by duplex PCR Lanes: M, Marker; lane 1, OYDV positive control PS-10; lanes 2 and 3, duplex PGS-14; lanes 4 and 5, selection 9; lanes 6 and 7, Gs- 282; lanes 8 and 9, P S-10; lanes 10 and 11, Selection-34; lane 12, SLV positive control PS-10; lane 13, healthy (negative control).

duplex RT-PCR for detection of these two viruses in terms of annealing temperature and concentration of primers. A two-fold increase in dNTP concentration coupled with 1.5 fold increase in $MgCl_2$ improved the simultaneous detection of OYDV and SLV (result not shown). Generally, a higher concentration of primers can compensate for a lower concentration of template (Singh *et al*., 2000), and in our study also it was found that a two-fold increase in SLV primers, improved its detection in duplex PCR. This increase in primer concentration was critical for its amplification, though in the field samples SLV gave stronger bands than OYDV, indicating that the concentration of viruses may fluctuate (seasonally) as also observed by Dovas *et al.* (2002). The difference in the intensities of the amplified virus-specific bands was due to differences in viral RNA concentration as seen in our dilution experiment of individual viruses, and was not caused by competition, as the targets are different (Nassuth *et al.*, 2000; Hassan *et al.*, 2006). The sensitivity level of duplex PCR for detection of both viruses was not reduced, as both could be detected to the same level as in the standard RT-PCR for individual viruses. Both leaves and bulbs can be used as routine test material for duplex PCR.

ACKNOWLEDGEMENTS

The authors would like to thank Department of Science and Technology, Government of India, for financial assistance and Dr. R.K. Jain, HOD, Division of Plant Pathology IARI, New Delhi, for his valuable suggestions and providing the laboratory facilities.

REFERENCES

Arya M., Baranwal V.K., Ahlawat Y.S., Singh L., 2006. RT-PCR detection and molecular characterization of Onion yellow dwarf virus associated with garlic and onion. *Current Science* **91**: 1230-1234.

Chamberlain J.S., Chamberlain J.R., 1994. Optimisation of multiplex PCRs. In: Mullis KB, Ferre F., Gibbs R.A. (eds.), The Polymerase Chain Reaction, pp.38-46, Birkhauser, Boston, MA, USA.

Conci V.C., Canavelli A., Lunello P., 2003. Yield losses associated with virus–infected garlic plants during five successive years. *Plant Disease* **87**: 1411-1415.

Dantu P.K., Bhojwani, S.S., 1992. In vitro propagation of gladiolus : Optimisation of conditions for shoot multiplication. *Journal of Plant Biochemistry and Biotechnology* **1**: 115-118.

Dovas C.I., Hatzibukas E., Salomon R., Barg E., Shiboleth Y.M., Katis N.I., 2001. Comparison of methods for virus detection in *Allium* species. *Journal of Phytopathology* **149**: 731-737

Dovas C.I., Mamolos A.P.M., Katis N.I., 2002. Fluctuations in concentration of two potyviruses in garlic during the growing period and sampling conditions for reliable detection by ELISA. *Annals of Applied Biology* **140**: 21-28.

Hassan M., Myrta A., Polak J., 2006. Simultaneous detection and identification of four pome fruit viruses by one-tube pentaplex RT-PCR. *Journal of Virological Methods* **133**: 124-129

Keusgen M., 2002. Health and Alliums. In: Rabinowitch H.D., Currah L.(eds.) Allium Crop Science: Recent Advances, pp 357-378. CAB International, Collinwood, UK

Lot H., Chovelon V., Souche S., Delecolle B., 1998. Effects of Onion yellow dwarf and Leek yellow stripe viruses on symptomatology and yield loss of three French garlic cultivars. *Plant Disease* **82**: 1381-1385.

Nassuth A., Pollari E., Helmeczy K., Stewart S., Kofalvi S., 2000. Improved RNA extraction and one tube RT-PCR assay for simultaneously detection of control plant RNA plus several viruses in plant extract. *Journal of Virological Methods* **90**:37-49.

Sambrook J., Fritsch E.F., Maniatis T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York, NY, USA.

Shiboleth Y.M., Gal-On A., Koch M., Rabinowitch H.D., Salomon R., 2001. Molecular characterisation of *Onion yellow dwarf virus* (OYDV) infecting garlic (*Allium sativum* L.) in Israel: Thermotherapy inhibits virus elimination by meristem tip culture. *Annals of Applied Biology*, **138** :187-195.

Singh R.P., Nie X., Singh M., 2000. Duplex RT-PCR: reagent concentrations at reverse transcription stage affect the PCR performance. *Journal of Virological Methods* **86**: 121-129.

Singh R.P., Nie X., 2003. Multiple virus and viroid detection and strain separation via multiplex reverse transcription-polymerase chain reaction. *Canadian Journal of Plant Pathology* **25**:127-134.

Takaichi M., Yamamoto M., Nagakubo T., Oeda K., 2001. Mixed virus infections of garlic determined by a multivalent polyclonal antiserum and virus effects on disease symptoms. *Plant Disease* **85**: 71-75.

Tsuneyoshi T., Matsumi T., Natsuaki K.T., Sumi S., 1998. Nucleotide sequence analysis of virus isolates indicates the presence of three potyvirus species in *Allium* plants. *Archives of Virology* **143**:97-113.

Walkey D.G.A., Antill D.N., 1989. Agronomic evaluation of virus-free and virus infected garlic (*Allium sativum* L.). *Journal of Horticultural Science*. **64**: 53-60.

Van Dijk P., 1994 Virus diseases of *Allium* species and prospects for their control. *Acta Horticulture* **358**: 299-306.

Received September 21, 2007
Accepted March 10, 2008