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SHORT COMMUNICATION

Evidence of *Grapevine leafroll associated virus-1–3, Grapevine fleck virus* and *Grapevine virus B* Occurring in Himachal Pradesh, India

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Abstract During a survey conducted in the grapevine orchards of Himachal Pradesh, variety of symptoms ranging from leaf yellowing, vein greening, reduced leaf size, downward rolling/cup shaped leaves to reduced fruit bearing were observed. Symptomatic leaf samples were collected and analyzed by serological (DAS-ELISA) and molecular methods (RT-PCR, PCR) for viruses and phytoplasma known worldwide on grapevine. DAS-ELISA was used for detection of *Grapevine leafroll associated virus 1, 2 and 3* (GLRaV-1, 2 & 3), *Grapevine virus A* (GVA), *Grapevine fan leaf virus* (GFLV), *Grapevine fleck virus* (GFkV) and successfully detected GLRaV-1 & 3 and GFkV. All these samples were complemented with RT- PCR along with GVb and phytoplasma (additional to ELISA) using specific primers. Specific amplification in RT-PCR for GLRaV-1 (~232 bp), GLRaV-3 (~300 bp), GFkV (~179 bp) and GVB (~440 bp) confirmed the presence of these pathogens. Overall, ELISA and RT-PCR results confirmed the presence GLRaV-3 (66.7 %), GLRaV-1& GFkV (50 %), and *Grapevine virus B* (GVB) (12.5 %) in symptomatic plants. None of the samples were found positive for GFLV, GLRaV-2 and phytoplasma. Mixed infection was common and none of the plants were found virus free. To the best of our knowledge this is the first report of detection of GFkV and GVB in India.

Keywords GLRaV · GFkV · GVA · GVB · RT-PCR · DAS-ELISA

Grape (*Vitis vinifera*) is important cash crop grown worldwide. A large number of viruses have been reported to infect grapevine causing huge losses [3, 5, , 16, 17]. Among these, Grapevine leaf roll associated viruses (GLRaV's), *Grapevine fleck virus* (GFkV), *Grapevine fan leaf virus* (GFLV) and *Grapevine virus A & B* (GVA & GVB) were frequently encountered in vineyards all over the world and were of major concern [6–10, 11, 18, 19].

In India, grape is an important crop grown in sub-tropical (Haryana, Punjab, Uttar Pradesh, Delhi), hot tropical (Maharashtra, Hyderabad, Karnataka, Andhra Pradesh), and mild tropical regions (some parts of Bangalore and Kolar districts of Karnataka; Chittoor district of Andhra Pradesh and Coimbatore, Madurai and Theni districts of Tamil Nadu) and in some sub-temperate regions of Himachal Pradesh (HP), Jammu and Kashmir (J&K) and Uttrakhand (UK). However, not much is known about infecting viruses in vineyards in India, only GLRaV-1 and GLRaV-3 have been reported [12, 13]. In order to study the health status of grapevine in HP, survey was conducted in the month of May and June, 2011 in different grape growing areas in districts Kullu (Bajaura, and Bhuntar) and Kinnaur (Reckong Peo and Rogi) in HP.

During survey, symptoms related to viral infection were frequently observed in fields, ranging from leaf yellowing, vein greening, reduced leaf size, downward rolling/cup shaped leaves to reduced fruit bearing (Fig. 1). Samples were collected from symptomatic plants. To evaluate the

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prevalence of viral pathogens in this region both serological (ELISA) and molecular (RT-PCR, cloning and sequencing) detection methods were used for detection of all the viruses (except GVB and grape phytoplasma which were analyzed by RT-PCR and PCR respectively). Primary aim of this study is to investigate the presence of different grapevine viral pathogens and phytoplasma which were not reported to occur in India. Accurate and timely identification of new pathogens and their prevalence will ultimately lead to the development, implications of strategies for their control and eradication.

Commercially available DAS-ELISA kits (Bioreba, Switzerland and Agdia, USA) were used for GLRaV-1, GLRaV-2, GLRaV-3, GVA, GFLV and GFkV [2]. Samples were tested in duplicate and OD_{495} reading $\geq 3 \times$ the negative control was taken as positive. RT-PCR was performed for the above viruses. Virus and phytoplasma specific primers were used in this study (Supplementary Table 1). Total RNA was extracted using RNeasy Plant Mini kit (Qiagen, Germany). For DNA extraction CTAB method was used [14, 15]. Reverse transcription was carried out in a 25 µl reaction mixture containing 7 µl total RNA (1–1.5 µg), 1.0 µl of 0.2 µg/µl of random hexamer primers, 1.0 µl of 40 mM dNTP mix (Fermentas, Lithuania), 5 µl of 5× RT buffer and 100 Units of M-MuLV Reverse Transcriptase (USB Corporation, USA). The cDNA synthesis reaction mixture was incubated at 42 °C for 1 h followed by 80 °C for 5 min. PCR was performed in a 50 µl reaction mix containing 5 µl of 10× *Taq* buffer A (Genei, India), 1.0 µl dNTP mix (10 mM), 1 µl each of forward and reverse primers (10 pmol), 1.5 U of *Taq* DNA polymerase (Genei, India) and 5 µl cDNA. PCR was performed in a thermal cycler (G-Storm GS2, Gene Technologies, UK). To obtain desired amplicons, amplification was carried out at 72 °C for 40 s for all pathogens. For detection of phytoplasma, reaction components and conditions as described previously were used [14]. PCR products were electrophoreses in 1 % agarose gel with 1 µg/ml of ethidium bromide in 1× TAE buffer.

For cloning and sequencing, PCR amplified products were eluted from the gel (Promega, Madison, USA). The eluted product were ligated in pGEM-Teasy cloning vector (Promega, Madison, USA) and mobilized into *E. coli* DH5α. Sequencing was performed using ABI prism Big Dye^{TM} Terminator v3.1 Ready Reaction Cycle sequencing Kit (Applied Biosystems, USA). Nucleotide and amino acid similarity was determined by BLASTN and BLASTX.

Results of DAS-ELISA showed the presence of viral infections in 23 out of 24 tested samples. Specifically, GLRaV-1, GLRaV-3 and GFkV were detected in 50 % (12/24), 66.7 % (16/24) and 50 % (12/24) of analyzed samples, respectively. None of samples tested was found positive for GLRaV-2, GFLV, GVA. RT-PCR confirmed these results, indicating a viral titer in plant tissues up to the detection limit of ELISA, and also revealed the presence of GVB in three out of 24 tested samples. However, no evidences were found for GLRaV-2, GVA, GFLV (both in ELISA and RT-PCR) and grapevine phytoplasma (in PCR). Amplicons of the expected size for GLRaV-1 (~232 bp), GLRaV-3 (~340 bp), GFkV (~179 bp) and GVB (~460 bp) were obtained in RT-PCR

Fig. 1 Symptoms observed in vineyards during survey. **a** Leaves displaying characteristic symptoms of grapevine leafroll associated viral disease on white grapes which includes leaf yellowing, reduced leaf size, downward rolling/cup shaped leaves and reduced fruit bearing. **b** Symptoms on red grapevine. Leaves became red whereas main veins remain green. **c** Reduced leaf size and their yellowing or mosaic pattern however main veins remain green. **d** A row showing viral symptoms as described earlier at Bajuara (Kullu). In all these samples GLRaV-1 & 3 was detected. Beside these in **b** & **c** GVB and GFkV was also detected respectively

(Fig. 2). Overall, among positive samples: 62.5 % (15/24) were infected with more than one virus and 37.5 % with single virus. Combination of GLRaV-1 and -3 was prevalent and diagnosed in 29.2 % (7/24) of analyzed samples. Mixed infections of GLRaV-1 + GLRaV-3 + GFkV were detected in 3 out of 24 samples whereas infection of GLRaV-1 + GFkV + GVB was found only in one sample. These findings were very similar as reported previously by many workers from different parts of world. Nucleotide sequences for GLRaV-1, GLRaV-3, GVB, and GFkV were obtained and submitted to EMBL database (Accession numbers HE649961, HE649962, HE649963, HE649964 respectively). When blasted, sequences of GLRaV-1 and GLRaV-3 showed 99 % similarity with US isolates (JF811849, GU983863) whereas GVB and GFkV nucleotide sequences showed 88 and 98 % similarity with Chinese (JF927940) and Italian (AJ309022) isolates, respectively. At amino acid level, a sequence similarity of 99, 100, 90 and 100 % with acc. no. ACT31733 (China), AAR02009 (Czech), ACX30795 (China) and NP542613 (Italy) was found for GLRaV-1, GLRaV-3, GVB and GFkV respectively.

Previously many workers determined these pathogen incidence in different parts of world as 15.27 % incidence of GLRaV in Anatolia region of Turkey, of which GLRaV-1 8.36 %, GLRaV-3 5.78 %, GLRaV-7 3.86 % and GLRaV-2 2.41 % were present [1]; 6.36 % GFLV; 4.67 % GLRaV-1; 16.05 % GLRaV-2; 6.41 % GLRaV-3; 0.26 % GLRaV-7; 14.99 % GFkV; 5.57 % GVA, 0.78 % GVB infection incidence was determined in Chile [4]. In Napa valley 62 % infection incidence of grapevine leafroll associated virus complex (8.8 % GLRaV-1, 46.8 % GLRaV-2, 9.1 % GLRaV-3, 12.3 % GVA, 30.7 % GFkV and 9.6 % GFLV) was accessed [20]. Difference in rate of viral incidence as reported by many workers is mainly due to different vector population in respective areas, sampling choice/method and, above all, orchard management practices of respective country or region. In this study we, provided the evidences for presence of GFkV and GVB first time from Indian grapevine yards. In these symptomatic samples incidence of GLRaV-3 is very high i.e. 66.7 %, followed by GLRaV-1 & GFkV 50 %, and GVB 12.5 %. Mix infection was found commonly in field and there is reduction in crop yield. Results indicate the dominance of virus infection in grapevine yards as in other parts of world. Based on observed field symptoms and results of the analysis, infection rate in field must be much higher than expected, as large number of plant were displaying symptoms related to viral infection. If we consider grapevine growing areas where virus free planting material is preferred from certified nurseries and good orchard practices are followed the production is generally high. Keeping in mind the loss that can be caused by these viral pathogens, there is a need for immediate attention towards disease management, with particular regards to the production of virus free planting material and its distribution to farmers, control of vector population which helps in virus spread from plant to plant and good orchards management practices. Also as mixed viral infection was common also in other crops there is need of development of multiplex RT-PCR system, which can detect these major pathogens in one reaction, saving time, cost and labour.

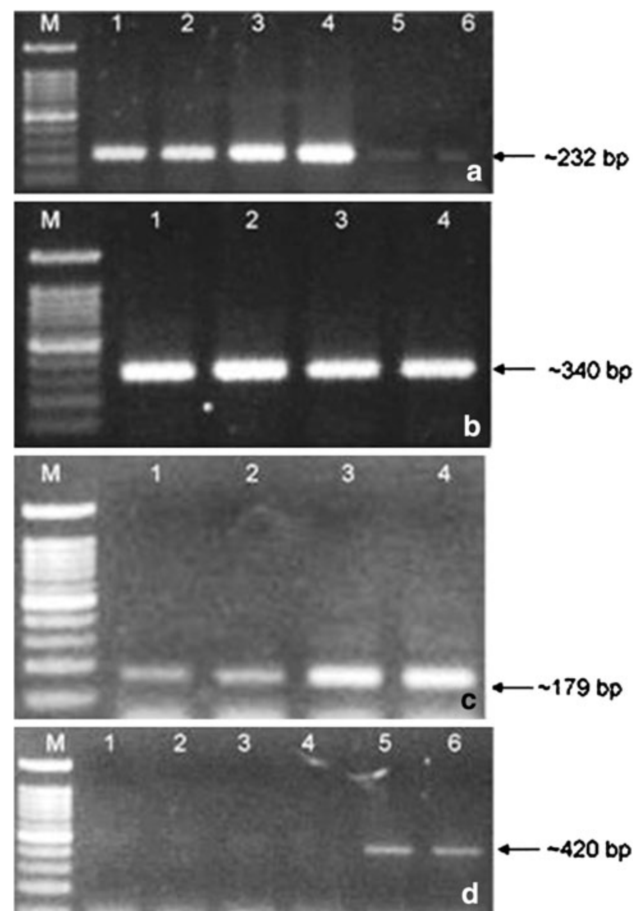

Fig. 2 RT-PCR based detection of grape viruses in Himachal Pradesh during 2011. **a** Amplicons of GLRaV-1 *Lanes 1–6* amplified products of S1, S3, S5, K7, K8 and K9; *lane M* marker (100 bp). **b** Amplicons of GLRaV-3 *Lane 1–4* samples S1, S2, K1 & K2. **c** Amplicons of GFkV *Lanes 1–4* amplified products of samples S5, S6, S7 & S8. **d** Amplicons of GVB samples S1, S2, S3, S4, S5 & S6 was loaded in *lanes 1–6* respectively

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