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ORIGINAL ARTICLE

Development of polyclonal antibodies using bacterially expressed recombinant coat protein for the detection of *Onion yellow dwarf virus* (OYDV) and identification of virus free onion genotypes

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Abstract
Onion yellow dwarf virus (OYDV) belonging to the genus *Potyvirus*, family *Potyviridae,* is one of the widely distributed viruses of *Allium* species worldwide. It causes dwarfing, yellow striping, crinkling and flaccidity of the leaves of onion and garlic. To see the occurrence and incidence of OYDV on *Allium* crop, an attempt was made to develop antibody based diagnostic assay which would be useful for routine indexing and screening of the germplasm. The total RNA was isolated from the symptomatic leaves of onion and the gene encoding coat protein (CP) was cloned. The nucleotide sequencing analysis of the cloned RT-PCR product revealed ~ 774 bp amplicon (OYDV CP) and it was further cloned in pET-28a (+) expression vector which yielded ~ 30 kDa fusion protein with Histidine tag (His_6BP). The expression of fusion CP was primarily checked on SDS–PAGE and further confirmed by Western blot. The His_6BP-OYDV-CP was obtained in soluble state after purification and was used to immunize New Zealand white rabbit for the production of polyclonal antibody (PAb). The produced PAb against the purified fusion protein successfully detected OYDV from onion and garlic samples at 1:2000 dilutions in indirect-enzyme linked immunosorbent assay (DAC-ELISA). Thus, this study presents first report that Histidine tag (His_6BP) fusion OYDV-CP based antibody production and its successful application in identification of virus free onion and garlic genotypes.

Keywords OYDV (onion yellow dwarf virus) · *Allium* species · Polyclonal antibody · Coat protein · Histidine tag (His_6BP) fusion OYDV-CP · Indirect-ELISA

Introduction

Onion (*Allium cepa* L) and Garlic (*Allium sativum*) are two most important commercial vegetable crops grown worldwide and consumed in various forms. In India, they are cultivated for the last 5000 years and generally used as vegetable, spices and medicines. Onion is grown in 143 countries of the world. India leads in area (23.5%) whereas China is the major producer (24.9%) of dry onions in the world. Both these countries account for 50% of the area and production in the world. India shares 23.5% area (1.22 mha) and produced 22.81 MMT of dry onion (FAOSTAT 2019). Both the crops are affected by both biotic and abiotic stresses. Onion yellow dwarf virus (OYDV) is an important viral pathogen of onion and garlic crops throughout the world. It causes degeneration, dwarfing, yellow striping and crinkling of the leaves in onion and garlic (Krystyna et al. 2014). In India, the virus has been reported in epiphytotic proportion in Delhi region (Ghosh and Ahlawat 1997a) and the highest incidence of OYDV infection was reported from Maharashtra, Gujarat and Madhya Pradesh (Gawande et al. 2013). It has been associated with considerable losses in onion seeds as well as in bulb production (Hoa et al. 2003). The virus is reported from several countries, including Argentina, Turkey, Iran, Serbia, Poland, Italy, Nepal, Sudan, Greece, Egypt, Brazil, Czech Republic and in the USA (Sevik and Akcura 2013; Baghalian et al. 2010; Bagi et al. 2012; Chodorska et al. 2014; Manglli et al. 2014; Majumder and Johari 2014). OYDV belongs to the genus *Potyvirus* under the family

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Potyviridae (Takaki et al. 2006). Genome of OYDV consists of single stranded positive sense RNA (~ 10 kb) and has terminal untranslated (UTR) regions (Celli et al. 2013; Verma et al. 2015). The viral RNA encodes a single, large polyprotein that is cleaved by three virus-encoded proteases in to ten functional proteins.

Large numbers of serological and molecular methods are available for the detection of plant viruses. The most common methods used for the detection of OYDV are ELISA, RT-PCR, RT-qPCR assay and LAMP (Kumar et al. 2010; Arya et al. 2006; Tiberini et al. 2018, 2019). ELISA test is the most commonly used method for its accuracy, low cost and user's friendly. Traditionally, a polyclonal antibody is developed by purifying the virion from systemically infected test plants and subsequently immunizing the rabbit. However, polyclonal antibodies produced by this method have its limitations, such as the purity of the virion and low titre of virus in the infected plant tissue. Further, the procedure is quite lengthy and requires virus propagation in a suitable host, followed by purification of virion (Hull 2002). To overcome these difficulties, various bacterially expressed recombinant protein have been used as an antigen for raising polyclonal antibodies (PAb) against targeted viruses (Kokane et al. 2020; Raikhy et al. 2007; Rani et al. 2010; Pramesh et al. 2013; Kapoor et al. 2014; Rai et al. 2018). The bacterial expressing system is simple, fast and inexpensive for producing large quantities of purified protein as antigen to produce specific antibody (Hartley 2006). Since OYDV infection is most common in *Allium* crops hence an attempt was made to develop polyclonal antibody using coat protein gene of OYDV for the detection of virus free planting material.

Materials and methods

Virus source and electron microscopy

Leaf samples showing dwarfing, yellow striping and crinkling from onion (04) and garlic (02) were collected from the experimental fields of Division of Vegetable Science, Indian Agricultural Research Institute, Pusa Campus, New Delhi. Healthy samples of onion (02) were taken from seed grown plants from glass house. Initially samples were examined under leaf-dip electron microscopy (Gibbs et al. 1966). Crude extracts from leaf tissues of healthy and symptomatic infected plants of onion were homogenized in 0.07 M phosphate buffer (pH 6.5) negatively stained in 2% uranyl acetate and observed under JEOL-1011, transmission electron microscope (TEM). Digital images were recorded by Olympus CCD camera, SIS MEGAVIEW G2, attached to the EM interface. Further, immunosorbent electron microscopy (ISEM) (Ahlawat et al. 1996) was done using polyclonal antibodies to OYDV developed in the present study.

RNA isolation

100 mg of OYDV suspected onion and garlic leaf tissues were taken for total RNA isolation. Liquid nitrogen was used for fine grinding of frozen leaf tissue in mortar-pestle, followed by addition of 1 ml of TRIzol ™ Reagent (Invitrogen, Thermofisher Scientific, Wilmington, USA) and processed as per manufacturer's instructions. Later on RNA was quantified using NanoDrop™ One Spectrophotometer (Thermofisher Scientific, Wilmington, USA) and stored at − 80 °C for further analysis.

Amplification, TA cloning and construction of recombinant plasmid of OYDV-CP

A pair of primer was designed for the expression of full length coat protein gene based on OYDV-CP gene (GenBank Accession No. FR873734.1). Primer details are as follows: **OYDV RP Hind III: 5′CGCAAGCTTCATCTTGATACC GAGCAACG3′ (reverse primer) and OYDV FP BAM HI: 5′GCGGGATCCATGGCAGGTGAAGGAGAAGA3′ (forward primer)** with Hind III and Bam H I restriction sites (underlined). Reverse transcription-polymerase chain reaction (RT-PCR) was performed in two steps. In first step, cDNA was prepared using Verso cDNA synthesis Kit (Thermofisher Scientific, Wilmington, USA) according to manufacturer's instructions, i.e., a reaction mixture of 20 µl was consisted of 5 µl of total RNA (500 ng), 2 µl of dNTPs (10 mM), 1 µl of an oligo dT (500 ng), 1 µl of RT enhancer, 4 µl of 5 × cDNA synthesis buffers, 1 µl of Verso Enzyme Mix and 6 µl of nuclease free water. The cDNA reaction was performed at 42 °C for 1 h.

In second step, a PCR reaction mixture of 25 µl was prepared which consisted of 1 µl of cDNA, 2.5 µl $MgCl_2$ (25 mM), 0.5 µl each primer, 0.5 µl dNTP (10 mM), 2.5 µl Standard Taq Buffer (10×), 17 µl ddH_2O and 0.5 µl Taq DNA Polymerase (1.0 unit). The thermo cycler (Eppendorf Mastercycler Nexus GX2) conditions were as follows: one cycle of initial denaturation 94 °C for 5 min; followed by 30 cycle of denaturation at 94 °C for 45 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, and at last final extension at 72 °C for 10 min followed by 4 °C hold. The PCR amplified products were run on 0.8% agarose gel using gel electrophoresis unit and visualized under gel documentation system. The SV wizard PCR cleanup system kit (Promega, Madison, USA) was used for gel purification of the expected size of amplicon OYDV-CP (~774 bp) and TA cloning of purified PCR product was achieved using pGEM-T Easy vector (Promega, Madison, USA) following standard molecular biology protocols (Sambrook and Russell 2001).

The cloning was further confirmed through scolony PCR with specific primers, restriction digestion using Eco R1 and sequenced bidirectionally by 1st BASE, Selangor, Malaysia.

The purified pET-28a (+) expression vector (5369 bp, Novagen, San Diego, USA) and pGEM-T Easy vector containing insert (OYDV-CP gene) were simultaneously subjected to restriction digestion with their appropriate enzymes Hind III and Bam H I (Fast digest, Fermentas, Burlington, Canada). Released insert was further ligated into open pET-28a (+) vector using T4 DNA ligase (5 U/µl, MBI, Fermentas, Germany) and incubated at 16 °C for 18 h followed by transformation in *Escherichia coli* BL21(DE3) (Stratagene, La Jolla, CA USA). Recombinant clones were screened by kanamycin (30 µg/ml) selection and further confirmed by colony PCR and restriction digestion.

Expression of OYDV recombinant coat protein

In vitro expression of recombinant fusion coat protein with Histidine tag (His_6BP) at its N terminus in transformed *E. coli* BL21(DE3) were grown in Luria–Bertani (LB) supplemented with kanamycin (30 µg/ml) and chloramphenicol (100 µg/ml) for overnight incubation at 37 °C, 200 rpm. Freshly prepared 100 ml LB was re-inoculated with 10% of overgrown bacterial culture with above mentioned antibiotic selection and incubated at 37 °C, 200 rpm for the period until the optical density (OD600) value of 0.5 was reached. After that culture was induced by Isopropyl β-d-1-thiogalactopyranoside (IPTG) with 0.4 mM, 0.6 mM, 0.8 mM and 1 mM concentrations and incubated at 37 °C, 200 rpm for 1 h, 2 h and 3 h each for the standardization of optimal expression of OYDV coat protein. Culture was centrifuged at 6000 rpm for 5 min to obtained cell Pellets and these Pellets were re-suspended into Laemmli sample buffer to denature whole cell proteins prior to PAGE by heating at 95 °C for 5 min. Recombinant fusion protein (His_6BP-OYDV-CP) was analyzed on 5% stacking and 12% resolving gels using SDS–PAGE (Laemmli 1970). Electrophoresis was done at 70 V for 2 h and gel was stained with Coomassie brilliant Blue R 250 stain (Sigma, St. Louis, USA).

Western blotting

The specificity of expressed recombinant fusion coat protein with Histidine tag (His_6BP) was confirmed through western blotting using commercially available Anti-His_6 monoclonal antibody specific to His_6-tagged proteins (Sigma–Aldrich, Merck). The induced and un-induced bacterial cell cultures were sonicated followed by re-suspension into 1× binding solution and centrifugation at 6000 rpm to form soluble and insoluble fractions. The supernatant was collected as soluble protein fraction and Pellet as insoluble fraction. Samples containing loading buffer and without it were resolved separately on 12% SDS gels and analyzed. Separated fractions of crude protein on SDS–PAGE unstained gel was then transferred overnight to nitrocellulose membrane (NCM, 0.22 µm, MDI, Germany) using wet blotting system (Biorad, USA) at 15 V. NCMs were kept for blocking using 1× phosphate buffer saline (PBS) containing 5% skimmed milk for 2 h at 37 °C incubation followed by four times washing with phosphate buffer saline Tween 20 (PBS-T) at the interval of 5 min each and then NCM was probed with Anti-His_6 monoclonal antibody (1:3000) in phosphate buffer saline–Tween 20–poly-vinylpyrrolidone–ovalbumin (PBST-PO) for 2 h at 37 °C incubation. Again, NCM was washed thrice and followed by incubation with secondary antibody, i.e., goat anti-rabbit IgG alkaline phosphatase (Sigma, MO, USA 1:30,000) for 1 h at 37 °C. The visual color development of membrane was later achieved by incubating the membrane in 5-bromo-4-chloro-3-indolyl-phosphate-nitrobluetetrazolium (BCIP/NBT) substrate solution (Genei, Bangalore, India) for 37 °C until the clear visual desired intensity protein bands were obtained.

Purification of fusion protein and production of polyclonal antibody (PAb)

The quantity of expressed fusion protein (His_6BP-OYDV-CP) derived from pET-28a (+) vector construct was purified from transformed *E. coli* BL21(DE3) culture grown in 1.5 L LB broth supplemented with kanamycin (30 µg/ml) and chloramphenicol (100 µg/ml). In the purification procedure; transformed cells were first pelleted down and then re-suspended into 1× binding buffer (500 mM NaCl, 5 mM imidazole, 20 mM Tris HCL, ph 7.9) followed by sonication and again pelleted and re-suspended in 75 ml 1X binding buffer containing 6 M urea. The expressed fusion protein was purified using His-Bind Purification Kit (Novagen, San Diego, USA) and nickel charged nitrilotriacetic acid (Ni–NTA) column following manufacturer's guidelines. Desired protein eluted fractions obtained after passing through Ni–NTA column were resolved and analyzed on 12% SDS–PAGE and was further dialyzed to remove the salt impurities in PBS using Float-A-Lyzer G2 dialysis device (Spectrum laboratories, Inc., Rancho Dominguez, CA, USA), lyophilized and quantified using NanoDrop™ One Spectrophotometer (Thermo Scientific, Wilmington, USA). The production of PAb was outsourced to Abgenex Pvt. Ltd, E-5, Infocity, KIIT Post Office, Bhubaneshwar. 500 µg concentrated purified fused protein emulsified with equal volume of Freund's incomplete adjuvant (Genei, Bangalore, India) was injected intramuscularly to immunized New Zealand white rabbits weekly up to 5 weeks. One week after the last immunization; the rabbit was bled at regular interval of 7th day for 3 weeks and named as first bleed, second bleed and third

bleed, respectively. The crude antisera thus obtained on each 7th day was mixed with 100% glycerol (1:1, v/v) and stored at − 80 °C.

Evaluation of PAb titre by ISEM and DAC-ELISA

To determine the titre of raised PAb, a DAC-ELISA (Clark and Adams 1977; Clark and Bar-Joseph 1984) was performed using recombinant coat protein (positive control), healthy (negative control) and OYDV infected onion plant leaves (PCR confirmed). An ELISA plate was coated with 100 µl crude sap prepared by grinding 100 mg onion leaf tissues (1:1, w:v) in 1× carbonate coating buffer containing 2% poly-vinyl-pyrrolidone (PVP, MW 40,000) followed by centrifugation at 10,000 rpm for 2 min. 50 ng/100 µl concentration of purified recombinant coat protein was used as positive control. Incubation is carried out at 37 °C for 2 h followed by thrice washing with PBS-T. A twofold dilution of PAb from 1:64 to 1:4096 was prepared as per requirements using PBS–TPO (Phosphate buffer saline–Tween 20–polyvinylpyrrolidone–ova-albumin); 100 µl of prepared PAb were added to its corresponding wells of micro titre plate and incubated for 2 h at 37 °C. ELISA plate was washed thrice with PBS-T and loaded with secondary antibody, i.e., goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma–Aldrich, India, 1:30,000) followed by incubation at 37 °C for 1 h. Finally, ELISA plate was washed thrice, a substrate tablet (p-nitro phenyl phosphate, 1 mg/ml; Sigma–Aldrich, India) was dissolved in substrate buffer (diethanol-amine, pH 9.8) and loaded to wells of ELISA plate and readings were recorded at 405 nm after 1 h incubation at 37 °C in ELISA reader (Automatic microplate reader, Genetix, India). The titre of the antibody was also determined by immunosorbent electron microscopy (ISEM). In-house developed PAbs were standardized for DAC-ELISA of OYDV detection and later compared with a commercial ELISA Kit of OYDV (Agdia, Elkhart) using OYDV symptomatic and non-symptomatic onion and garlic leaf samples and OYDV-CP positive control.

Standardization of DAC-ELISA for the detection of OYDV and validation

The PAb was further evaluated for OYDV detection in onion and garlic leaf samples using DAC-ELISA (Clark and Bar-Joseph 1984). Total 81 samples (40 different onion genotypes, 12 onion wild varieties) and 71 samples (2 different genotypes) were tested by ELISA (1:2000 dilution). In first step, ELISA plates were coated with antigen (100 µl plants sap), recombinant coat protein, OYDV infected plant (positive control), healthy (negative control) and buffer control; incubated for 2 h at 37 °C followed by thrice washing with PBS-T and overnight blocking with BSA (bovine serum albumin) at 16 °C. ELISA plates were washed thrice with PBS-T; 100 µl PAb (1:2000 dilutions) was added to each well of a micro titre plate as per requirements and incubated for 2 h at 37 °C followed by three times washing with PBS-T. ELISA plates were then coated with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma–Aldrich, India, 1:30,000) followed by incubation at 37 °C for 1 h and washing with PBS-T. Finally, substrate was added to ELISA plates and incubated at 37 °C for 1 h; readings were noted at 405 nm after 1 h using an ELISA reader (Automatic micro plate reader, Genetix, India). Some of the samples which were found positive or negative in DAC-ELISA were further confirmed by RT-PCR using OYDV-CP specific primers.

Results

Electron microscopy and ISEM

Onion samples showing dwarfing, yellow striping and crinkling of the leaves showed flexuous filamentous particles measuring ~775 nm. These particles were further decorated with polyclonal antibodies of OYDV developed during the present study at 1:50 dilution. The antibody reacted positively and heavy antibody halo around the virus particle was readily visualized in negatively stained preparations (Fig. 1).

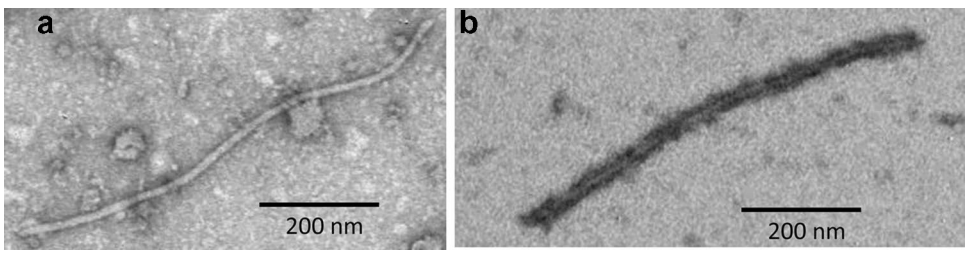

Fig. 1 **a** Electron micrograph showing flexuous particles measuring 775 nm from OYDV infected onion plant; **b** Electron micrograph showing OYDV decorated with OYDV antiserum developed in the present study (1:50 dilution)

Expression and purification of fusion coat protein of OYDV

An expression construct of OYDV-CP-pET-28a (+) was developed in *E. coli* host strain BL21(DE3), (Fig. 2a, b, c, d and e) which was initially verified by colony PCR. The right frame of cloned gene was confirmed through restriction digestion using Hind III and Bam H I enzymes (Fig. 2d) and DNA sequencing confirmed the full length coat protein of OYDV which was submitted to NCBI (GenBank Accession No. MW729705). In vitro expression of ~30 kDa abundant recombinant coat protein (His_6BP-OYDV-CP) was achieved by 3 h incubation at 37 °C after induction with 1 mM IPTG. The desired fusion coat protein was only observed in pellet (cell debris, insoluble fraction) with ~30 kDa intense protein band when resolved on 12% gel of SDS–PAGE. The fusion protein expressed by OYDV-CP-pET-28a (+) was further confirmed through Western blot using an Anti-His_6 monoclonal (Sigma–Aldrich, Merck) (Fig. 3).

After Western blot confirmation, the expressed fusion protein (His_6BP-OYDV-CP) was obtained from 1.5 L bacterial culture by repeated sonication and centrifugation at 8000 × *g* for 15 min using 6 M urea and further purified using His-Bind Purification Kit (Novagen, San Diego, USA) and nickel charged nitrilotriacetic acid (Ni–NTA) column. The ~30 kDa protein fractions were later pooled, dialyzed and lyophilized. The protein yield was ~0.332 mg/µl as determined by Nanodrop™ (Thermofisher Scientific, Wilmington, USA). Total yield of protein was 33.2 mg/10 ml.

Production, standardization and evaluation of PAb

Blood from immunized rabbits were collected at weekly intervals for 3 weeks following the last injection and named as 1st, 2nd and 3rd bleeding, respectively. Approximately 15 ml of blood was collected in each bleed and stored in 4 °C. The DAC-ELISA result showed that out of all the serial dilutions from 1:64 to 1:4096 for all the bleeds, 2nd and 3rd bleed antiserum with 1:2000 concentration worked well to distinguish OYDV-CP (50 ng/100 µl), healthy and infected plant tissue as the infected plant tissue and fusion protein always showed twice and thrice higher absorbance value, respectively, than healthy plant tissue. The potency of reactivity of antiserum showed that ~2 ng/100 µl concentration of purified fusion protein could be easily detected in DAC-ELISA with absorbance value of < 0.5, the absorbance value of 2nd, and 3rd bleed always remained 5–6 times higher than pre-bleed (Fig. 4).

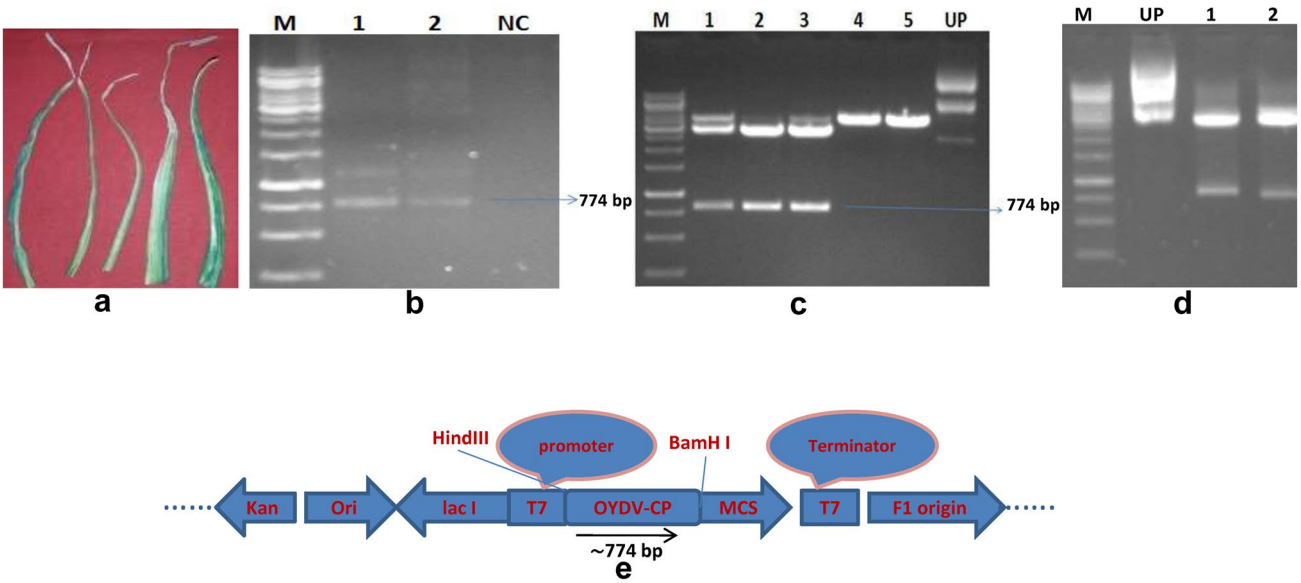

Fig. 2 Systematic representation showing the development of recombinant construct with the conserved coat protein sequence (OYDV-CP; ~ 774 bp) of OYDV fused in a sense orientation in between Hind III and BamH I of multiple cloning site (MCS). **a** Yellow striping, curling, flattening and crinkling symptoms of collected onion leaves; **b** RT-PCR amplification for coat protein of OYDV from symptomatic onion leaf; Lane M, GeneRuler™ 1 kb DNA Ladder(Thermo Scientific™); Lanes 2 and 3, Onion samples; Lane NC, negative control. **c** Restriction check for the confirmation of pGEM-TEasy vector (Promega, Madison, USA) containing Insert (OYDV-CP) using EcoR I enzyme; Lane M, GeneRuler™ 1 kb DNA Ladder (Thermo Scientific™); Lanes 1–5, restriction check to identify transformed and non-transformed *E. coli* colonies; Lane UP, uncut plasmid **d** OYDV-CP pET-28a (+) vector containing in frame insert (OYDV-CP; ~ 774 bp) were confirmed through restriction digestion with HindIII and BamH I; Lane M, GeneRuler™ 1 kb DNA Ladder (Thermo Scientific™); Lane UP, uncut plasmid; Lane 1–2, restriction check of transformed *E. coli* BL21(DE3) colonies; **e** Schematic presentation of in frame OYDV-CP (~ 774 bp) cloned in pET-28a (+) vector

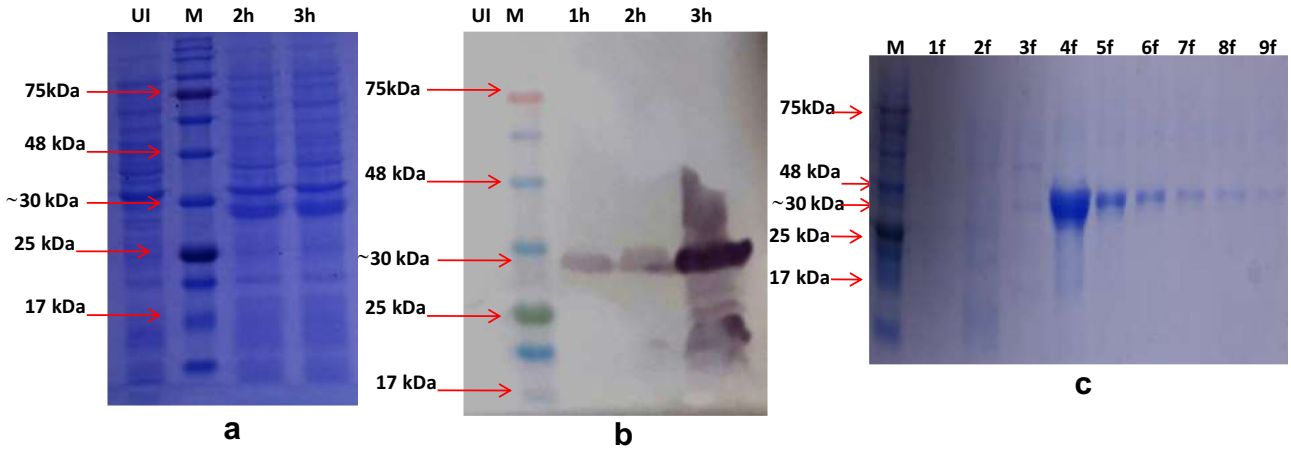

Fig.3 Poly-acrylamide gel electrophoresis (PAGE) showing in vitro expression of fusion coat protein of OYDV cloned in pET-28a (+) vector which is transformed into *E. coli* BL21(DE3) and its Western blotting followed by its purification using His-Bind Purification Kit (Novagen, San Diego, USA) and nickel charged nitrilotriacetic acid (Ni–NTA) column. **a** pET-28a (+) vector showing ambient expression of ~ 30 kDa fusion protein band on SDS—PAGE; lane 1, un-induced; Lane 2-M: BLUeye Prestained Protein Ladder (BR Biochem) lanes 3 and 4:2 h and 3 h after induction with 1 mM isopropyl-D 1, 5 thiogalactopyranoside (IPTG). **b** Western blotting results of in vitro expressed OYDV-CP; Lane 1, Un induced; Lane 2, M: BLUeye Prestained Protein Ladder (BR Biochem); Lanes 3, 4 and 5 show 1 h, 2 h and 3 h expression, respectively, of induced bacterial culture. **c** Purified protein comes after 3rd fraction; Lane1, M: BLUeye prestained protein ladder (BR Biochem); Lane 2–3, no protein observed, Lane 4–9, ~ 30 kDa fusion protein band was observed in decreasing concentrations, respectively

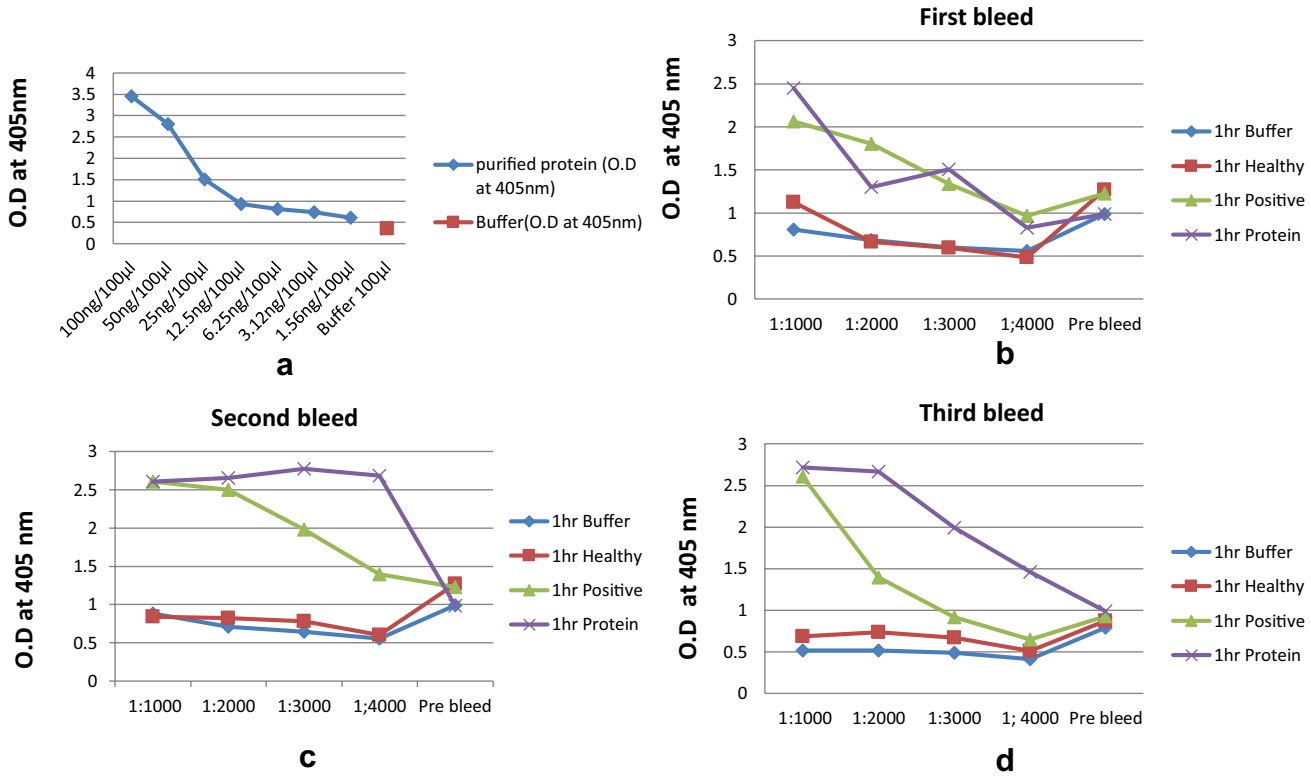

Fig. 4 **a** Comparative efficacy of PAb developed in the present investigation with different concentrations of purified CP of OYDV. **b–d** Titre of the anti OYDV—CP PAb against purified OYDV-CP was used as a immunogen in DAC-ELISA; Different dilutions of crude antiserum of 1st bleed, 2nd bleed and 3rd bleed compared with purified protein and infected sample. Micro titre plates were coated with 100 µl (50 ng of purified CP), 1:10 (W/V) of infected and healthy sample (**b–d**). Absorbance values were measured at 405 nm after 1 h of substrate addition

Field screening of onion genotypes at Division of Vegetable Science, Indian Agricultural Research Institute, onion field was carried out in the month of April 2019. Out of 81 onion samples tested, 21 samples were found positive for OYDV and out of 71 garlic samples tested, 22 samples were found positive for OYDV (Table 1). The PAbs developed in the present study was also compared with commercial antibodies (Agdia, Elkhart) (data not shown) and the results were comparable showing that the developed antibodies can be used for the indexing of onion and garlic germplasm against OYDV. The antibody produced against OYDV has shown that 29 onion genotypes viz: NHRDF Red-4, Bhima Red, Bhima Light Red, Bhima Shweta, Akola Safed, Bhima Kiran, Punjab Naroha, Bhima Raj, GJRO-11, Sukhsagar, GJWO3, JWO-1, Pusa Riddhi, B-780, Pusa Madhavi, Sel-397, PWR, Hisar-2, Arka Pithambar, HOS-4, Talaja Red, Kalyanpur Red, Round, VL Pyaz, L 28, Udaipur Local, Bhima Super, Pusa Shobha, RO-59, Bhima Safed and 12 wild onion genotypes namely North East (*Allium* sp.), MMK-125 (*Allium* sp.), *A. ampeloprasum*, MMK-122 (*Allium* sp.), *A. tuberosum, A. roylei*-1, *A. roylei*-2, *A. roylei*-3, *A. carolinianum, A. fistulosum*-1, *A. fistulosum*-2, *A. schoenoprasum* were found free from OYDV. These genotypes will be used in breeding programme.

Discussion

Onion is infected with more than 20 viruses belonging to genera *Potyvirus, Carlavirus, Allexivirus* and *Tospovirus* worldwide (Kumar et al. 2010; Majumder and Johari 2014; Barg et al. 1994, 1997; Diekmann 1994; Pringle 1998; van Dijk 1993; Walkey and Antill 1989). Amongst them, OYDV is one of the most important viruses causing severe yield loss to onion crop (Ahlawat and Varma 1997; Ghosh and Ahlawat 1997a; Ghosh et al. 1997b). The symptoms produced by OYDV in onion and garlic are more or less similar to that of symptoms produced by various other diseases and nutrient deficiencies and, therefore, it is very difficult to identify the disease on the basis of symptoms. Molecular methods such as RT-PCR are not advisable for routine tests for indexing of large quantities of samples due to its high cost and relative complexities of conducting the tests. Therefore, serological methods like ELISA has been used as most common methods for indexing large number of samples (Djelouah et al 2002). It is also cost effective than nucleic acid-based detection methods in term of large-scale screening of viruses.

As onion and garlic are often infected with multiple viruses, antibody production against individual virus by conventional method is very difficult due to complexity in separation of OYDV from mixed infection. Traditionally, the polyclonal antibodies are produced by purifying the virion from systematically inoculated test plants and then immunizing the rabbits. It has been demonstrated that the polyclonal antisera raised against purified virions may have antibodies of other viruses (Boscia et al. 1995). To overcome these difficulties, the PAbs are raised against recombinant coat protein of viruses which reduces the possibility of cross reactivity and non-specific antibodies production. The polyclonal antibody developed against the recombinant coat protein of the virus has been widely used in virus detection using different serological methods. Therefore, bacterially expressed coat protein is a better choice for the production of antibodies for the development of serological assay of OYDV. Majority of the expressed proteins are found in insoluble form which can be easily isolated, shows lower degradation, and was resistant to cellular proteases (Bowden et al. 1991). Previously many workers have used recombinant DNA technology for the production of polyclonal antibodies (Ling et al. 2004; Kamo et al. 2010; Kumari et al. 2001).

In our study, the expressed coat protein was found in insoluble form. Expression of recombinant His-fusion protein in the insoluble fraction has also been reported for number of viruses viz: cymbidium mosaic virus and odontoglossum ringspot virus (Rani et al. 2010), apple chlorotic leaf spot virus (Rana et al. 2011), cucumber mosaic virus, papaya ringspot virus, nucleocapsid protein (N) gene of groundnut bud necrosis virus (Kapoor et al. 2014) and grapevine leaf roll-associated virus (Rai et al. 2018). However, a few studies have also demonstrated expression of fusion protein in soluble fraction (Raikhy et al. 2007; Lee and Chang 2008; El-Attar et al. 2010; Khan et al. 2012; Khatabi et al. 2012; Pramesh et al. 2013).Query

The polyclonal antibody developed in the present study was able to detect the virus as low as 1.56 ng/100 µl of purified protein and detected successfully up to 1:4000 dilutions from crude sap which suggested that the antibody is suitable for the detection of virions from the infected samples. The virus free genotypes of onion and garlic identified in this study can be used in breeding programme for the production of OYDV resistant varieties.

Table 1 Detection of OYDV from different onion and garlic genotypes in DAC-ELISA using the PAb generated against its coat protein

S. No	IARI field sample	No. of ELISA + ve samples/No. of samples tested (A 405 nm*) OYDV (1:2000)
Onion genotypes		
1	NHRDF Red-4	0/3 (0.041–0.231)**
2	Bhima red	0/2 (0.012–0.023)
3	Bhima light red	0/2 (0.02–0.03))
4	NHRDF Fursungi	1/3 (0.102–0.944)
5	Bhima Shweta	0/2 (0.029–0.032)
6	Akola Safed	0/2 (0.039–0.051)
7	PRO6	2/2(0.742–0.827)
8	Bhima Kiran	0/1 (0.055)
9	Punjab Naroha	0/3 (0.089–0.198)
10	Arka Bhima	3/3 (1.402–1.704)
11	Bhima Raj	0/2 (0.017–0.060)
12	GJRO-11	0/2(0.071–0.090)
13	Sukhsagar	0/1(0.045)
14	Bhima Shakti	2/2 (1.054–1.572)
15	GJWO3	0/2(0.058–0.092)
16	JWO-1	0/1(0.005)
17	Pusa Riddhi	0/1(0.085)
18	Phursungi local	2/2 (1.179–1.356)
19	B-780	0/2(0.048–0.062)
20	Pusa Madhavi	0/1(0.054)
21	Sel-397	0/3(0.027–0.043)
22	PWR	0/1(0.018)
23	Hisar-2	0/1(0.265)**
24	Pusa Sona	3/3 (1.179–1.524)
25	Arka Pithambar	0/1(0.048)
26	HOS-4	0/1(0.073)
27	Talaja red	0/1(0.063)
28	Kalyanpur red round	0/2(0.021–0.0438)
29	VL Pyaz	0/1(0.022)
30	L 28	0/1(0.049)
31	Early Grano	1/1 (0.690)
32	Udaipur local	0/1(0.005)
33	RO-252	2/2 (0.841–0.895)
34	Bhima super	0/1(0.057)
35	Pusa Shobha	0/1(0.301)**
36	PKV white	2/2 (1.348–1.852)
37	RO-59	0/1(0.034)
38	Bhima Safed	0/2(0.098–0.151)
39	Bhima Shubra	1/3(0.214–0.852)
40	Bhima dark red	1/1(1.360)
Wild genotypes		
41	North East (*Allium* sp.)	0/1 (0.101)
42	MMK-125 (*Allium* sp.)	0/1(0.106)
43	*A. ampeloprasum*	0/1(0.093)
44	MMK-122 (*Allium* sp.)	0/1(0.105)
45	*A. tuberosum*	0/1(0.103)
46	*A. roylei*-1	0/1(0.123)
47	*A. roylei*-2	0/1(0.172)
48	*A. roylei*-3	0/1(0.156)

Table 1 (continued)

S. No	IARI field sample	No. of ELISA + ve samples/No. of samples tested (A 405 nm*) OYDV (1:2000)
49	*A. carolinianum*	0/1(0.072)
50	*A. fistulosum*-1	0/1(0.012)
51	*A. fistulosum*-2	0/1(0.394)**
52	*A. schoenoprasum*	0/1(0.194)
PCR negative onion sample		
54	Healthy control	0/1(0.175)
PCR positive onion sample		
55	Infected sample	1/1(1.782)
56	Protein	2.34
Garlic genotypes		
1	G386	14/34 (0.294–0.937)
2	PGS 204	8/37 (0.345–0.810)
	Healthy	0.353
	Garlic infected sample	1.283

Result: onion (21/81) and garlic (22/71) samples were found positive for OYDV after 1 h ELISA reading at 405 nm. O.D. value was 2–3 times higher than that of healthy plant value

*The absorbance value at 405 nm was recorded 1 h after addition of substrate with p-nitrophenyl phosphate. Samples with absorbance values more than two times of healthy control were considered to be positive

**Samples with low virus titre could not be detected by PAb

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Declarations

Conflict of interest Authors declare no conflict of interest.

Research involving in human and animal participants This manuscript does not contain any experiment involving human or animal participants.

References

Ahlawat YS, Varma A, Pant RP, Shukla A, Lockhart B (1996) Partial characterization of a badnavirus associated with citrus yellow mosaic disease in India. In Proceedings of 13th International Organisation of Citrus Virology. In: P Moreno, JV da Graca and LW Timmer (eds) Riveside California USA

Ahlawat YS, Varma A (1997) Serological detection of mixed viral infection in onion seed crop and possible measure for its management. Indian Phytopathol 50(1):137–140

Arya M, Baranwal VK, Ahlawat YS, Singh L (2006) RT–PCR detection and molecular characterization of onion yellow dwarf virus associated with garlic and onion. Curr Sci 91:1230–1234

Baghalian K, Kim OK, Natzuaki KT (2010) Molecular variability and genetic structure of the population of onion yellow dwarf virus infecting garlic in Iran. Virus Genes 41:282–291

Bagi F, Stojscaron V, Budakov D, ElSwaeh SM, GvozdanovićVarga J (2012) Effect of onion yellow dwarf virus (OYDV) on yield components of fall garlic (*Allium sativum* L.) in Serbia. Afr J Agric Res 7(15):2386–2390

Barg E, Lesemann DE, Vetten HJ, Green SK (1994) Identification, partial characterization, and distribution of viruses infecting *Allium* crops in South and South-East Asian countries. Acta Hortic 358:251–258

Barg E, Lesemann DE, Vetten HJ, Green SK (1997) Viruses of *Alliums* and their distribution in different *Allium* crops and geographical regions. Acta Hortic 433:607–616

Boscia D, Greif C, Gugerli P, Martelli GP, Walter B, Gonsalves D (1995) Nomenclature of grapevine leafroll-associated putative closteroviruses. Vitis 34(3):171–175

Bowden GA, Paredes AM, Georgiou G (1991) Structure and morphology of protein inclusion bodies in *Escherichia coli*. Biotechnology 8:725–730

Celli MG, Torrico AK, Kiehr M (2013) Striking differences in the biological and molecular properties of onion and garlic isolates of onion yellow dwarf virus. Arch Virol 158:1377–1382

Chodorska M, Paduch-Cichal E, Kalinowska E, Szyndel MS (2014) First report of onion yellow dwarf virus, garlic common latent virus and shallot latent virus on garlic in Poland. Dis Notes 98(6):858

Clark MF, Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J Gen Virol 34(3):475–483

Clark MF, Bar-Joseph M (1984) Enzyme immunosorbent assays in plant virology. In: Maramorosch K, Koprowski H (eds) Methods in virology, vol 3. Academic Press, New York, pp 51–85

Diekmann M, Frison EA, Putter T (eds) (1994) Corporate Author: Food and Agriculture Organization (FAO), Rome, Italy. International Plant Genetic Resources Instit., (IPGRI), Rome, Italy, p 128. https://www.bioversityinternational.org/e-library/publi

cations/detail/faoipgri-technical-guidelines-for-thesafe-movement-of-small-fruit-germplasm/
Djelouah K, Frasheri D, Onghia AMD (2002) Serological Diagnosis of Citrus psorosis virus and citrus tristeza virus using flower parts. Fifteenth IOCV Conference. Short communications. 363–365
El-Attar AK, Riad BY, Saad A, Soliman AM, Mazyad HM (2010) Expression of the coat protein gene of potato leafroll virus in *Escherichia coli* and development of polyclonal antibodies against recombinant coat protein. Arab J Biotechnol 13(1):85–98
FAOSTAT (2019) Onion production, area and productivity.http://www.fao.org/faostat/en/#data/QC. Accessed 1 March 2021
Gawande SJ, Chimote KP, Gurav VS, Gopal J (2013) Distribution and natural incidence of onion yellow dwarf virus (OYDV) on garlic and its related *Allium* species in India. Indian J Hortic 70(4):544–548
Ghosh DK, Ahlawat YS (1997a) Filamentous viruses associated with mosaic disease of garlic in India. Indian Phytopathol 50(2):266–276
Ghosh DK, Ahlawat YS, Gupta MD (1997b) Production of virus-free garlic (*Allium sativum*) plants by thremotherapy and meristem-tip culture. Ind J Agric Sci 67(12):591–594
Gibbs AJ, Varma A, Woods RD (1966) Viruses occurring in white clover (*Trifolium repens*) from permanent pastures in Britain. Ann Appl Biol 58:231
Hartley JL (2006) Cloning technologies for protein expression and purification. Curr Opin Biotechnol 17(4):359–366
Hoa NV, Ahlawat YS, Pant RP (2003) Partial characterization of onion yellow dwarf virus from onion in India. Indian Phytopathol 56(3):276–282
Hull R (2002) Matthews' plant virology, 4th edn. Academic Press, New York
Kamo K, Jordan R, Guaragna MA (2010) Resistance to cucumber mosaic virus in gladiolus plants transformed with either a defective replicase or coat protein subgroup II gene from cucumber mosaic virus. Plant Cell Rep 29:695–704
Kapoor R, Mandal B, Paul PK, Chigurupati P, Jain RK (2014) Production of cocktail of polyclonal antibodies using bacterial expressed recombinant protein for multiple virus detection. J Virol Methods 196:7–14
Khan S, Jan AT, Mandal B, Haq QM (2012) Immunodiagnostics of cucumber mosaic virus using antisera developed against recombinant coat protein. Arch Phytopath Plant Prot 45(5):561–569
Khatabi B, He B, Hajimorad MR (2012) Diagnostic potential of polyclonal anti-bodies against bacterially expressed recombinant coat protein of alfalfa mosaic virus. Plant Dis 96:1352–1357
Kokane SB, Kokane AD, Misra P, Warghane AJ, Kumar P, Gubyad MG, Sharma AK, Biswas KK, Ghosh DK (2020) In-silico characterization and RNA-binding protein based polyclonal antibodies production for detection of citrus tristeza virus. Mol Cell Probes. https://doi.org/10.1016/j.mcp.2020.101654
Krystyna W, Ewa S, Wojciech S (2014) Prevalence of infections with onion yellow dwarf virus, leek yellow stripe virus and garlic common latent virus in plants from the genus *Allium*. Acta Scientiarum Polonorum 13(3):123–133
Kumar S, Baranwal VK, Joshi S, Arya M, Majumder S (2010) Simultaneous detection of mixed infection of onion yellow dwarf virus and an *Allexivirus* in RT-PCR for ensuring virus free onion bulbs. Indian J Virol 21(1):64–68
Kumari SG, Makkouk KM, Katul L, Vetten HJ (2001) Polyclonal antibodies to the bacterially expressed coat protein of Faba bean necrotic yellows virus. J Phytopathol 149(9):543–550
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685
Lee SC, Chang YC (2008) Performances and application of antisera produced by recombinant capsid proteins of cymbidium mosaic virus and odontoglossum ring spot virus. Eur J Plant Pathol 122:297–306
Ling KS, Zhu HY, Gonsalves D (2004) Complete nucleotide sequence and genome organization of grapevine leafroll-associated virus 3, type member of the genus *Ampelovirus*. J Gen Virol 85:2099–2102
Majumder S, Johari S (2014) First report of onion yellow dwarf virus and garlic common latent virus infection in garlic from Nepal. J Plant Pathol. https://doi.org/10.4454/JPP.V96I4.012
Manglli A, Mohammed HS, Ali EL, Hussein A, Agosteo GE, Albanese G, Tomassoli L (2014) Molecular analysis of the 3′ terminal region of onion yellow dwarf virus from onion in southern Italy. Phytopathol Mediterr 53(3):258
Pramesh D, Singh P, Baranwal VK (2013) In vitro expression and purification of the coat protein gene of garlic common latent virus and its application in ELISA-based diagnostics. J Plant Pathol 95(2):385–389
Pringle CR (1998) Virus taxonomy-San Diego. Arch Virol 143:1449–1460
Rai R, Khurana SP, Kumar S, Gupta N, Baranwal VK (2018) Serological detection of grapevine leafroll-associated virus 4 in grapevine growing areas of India using polyclonal antiserum raised against the recombinant coat protein. Crop Prot 109:128–135
Raikhy G, Hallan V, Kulshrestha S, Zaidi AA (2007) Polyclonal antibodies to the coat protein of carnation etched ring virus expressed in bacterial system: production and use in immuno diagnosis. J Phytopathol 155:616–622
Rana T, Chandel V, Hallan V, Zaidi AA (2011) Expression of recombinant apple chlorotic leaf spot virus coat protein in heterologous system: production and use in immuno diagnosis. J Plant Biochem Biotechnol 20:138–141
Rani P, Pant RP, Jain RK (2010) Serological detection of cymbidium mosaic and odonto glossum rings pot viruses in orchids with polyclonal antibodies developed against their recombinant coat proteins. J Phytopathol 158:542–545
Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York
Sevik MA, Akcura C (2013) Viruses occurring in onion crop in Amasya province, the major onion producing region in Turkey. Indian J Virol 24(1):78–81
Takaichi M, Yamamoto M, Nagakubo T, Oeda K (1998) Four garlic viruses identified by reverse transcription-polymerase chain reaction and their regional distribution in northern Japan. Plant Dis 82(6):694–698
Takaki F, Sanom T, Yamashita Y (2006) The complete nucleotide sequence of attenuated onion yellow dwarf virus: a natural potyvirus deletion mutant lacking the N-terminal 92 amino acids of HC-Pro. Arch Virol 151:1439–1445
Tiberini A, Mangano R, Micali G, Leo G, Manglli A, Tomassoli L, Albanese G (2018) Onion yellow dwarf virusΔΔ Ct-based relative quantification obtained by using real-time polymerase chain reaction in 'Rossa di Tropea'onion. Eur J Plant Pathol 153(1):251–264
Tiberini A, Tomlinson J, Micali G, Fontana A, Albanese G, Tomassoli L (2019) Development of a reverse transcription-loop-mediated isothermal amplification (LAMP) assay for the rapid detection of onion yellow dwarf virus. J Virol Methods. https://doi.org/10.1016/j.jviromet.2019.113680
Van Dijk P (1993) Virus diseases of Allium species and prospects for their control. In International Symposium on *Alliums* for the Tropics. 358, pp 299–306
Verma RK, Mishra R, Petrov NM, Stoyanova M, Stoev A, Bakardjieva NV, Gaur RK (2015) Molecular characterization and recombination analysis of an Indian isolate of onion yellow dwarf virus. Eur J Plant Pathol 143:437–445
Walkey DG, Antill DN (1989) Agronomic evaluation of virusfree and virus-infected garlic (*Allium sativum* L.). J Hortic Sci 64(1):53–60