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Distribution and natural incidence of Onion Yellow Dwarf virus (OYDV) on garlic and its related *Allium* species in India

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ABSTRACT

A study was done in 2010 and 2011 to determine the presence of Onion Yellow Dwarf virus (OYDV) on garlic and related *Allium* spp. in India. Garlic leaf samples were collected from 12 garlic growing states of India. Virus identification was based on DAS-ELISA, RT-PCR and qRT-PCR. Out of total 493 samples analyzed, 308 samples from all states were found positive for OYDV in variable proportions. The highest percentage of OYDV positives were recorded from Maharashtra (96%) followed by Gujarat (75%) and Madhya Pradesh (75%). The lowest percentage of OYDV positives were recorded from Rajasthan (25%) followed by Delhi (40%). To know the status of OYDV on commercial cultivars, six leading garlic cultivars were tested. Relative quantification of OYDV concentration in these garlic cultivars was determined through qRT-PCR. No correlation was observed between bulb skin colour and virus concentration. Total 13 accessions of wild and related edible Alliums tested through RT-PCR and qRT-PCR for natural incidence of OYDV, 10 species were found positive for the virus. Three species, *viz*., *A. senescens*, *A. cepa* var. *aggregatum* (Multiplier onion) and *A. fistulosum* were found free from OYDV.

Key words: Potyvirus, garlic, onion yellow dwarf virus (OYDV), qRT-PCR, wild alliums.

INTRODUCTION

Garlic (*Allium sativum* L.) is spice crop of prime importance, grown in India as well as other parts of the world. Garlic is known to be infected by multiple species of viruses, known as “garlic virus complex”. Sixteen major viruses of genus Potyvirus, Allexivirus, Carlavirus and Potexvirus (Van Dijk, 12) were reported to infect garlic. Recently, Tospovirus (Iris yellow spot virus) was also reported on garlic from India (Gawande *et al*., 5). Up to 88% reduction of bulb weight was reported by potyviruses only (Lot *et al.*, 7; Walkey and Antill, 13). Among the potyviruses affecting *Allium* species, Onion yellow dwarf virus (OYDV) is the major viral pathogen of garlic crop and is reported to occur worldwide (Van Dijk , 11, 12; Lunello *et al.*, 8; Dovas *et al.*, 4). OYDV is a member of family Potyviridae, genus potyvirus. OYDV transmitted by aphids in a non-persistent manner, produces symptoms of mild chlorotic stripes to bright yellow stripes depending on the virus isolate and cultivar.

Occurrence of OYDV is well documented on onion and garlic in India (Ghosh and Ahlawat, 6; Majumder *et al.*, 9). In recent the years, OYDV has emerged as an important virus infecting economically important Garlic crop in India (Arya *et al.*, 1). Therefore, the study was conducted with the aim to provide complete information about OYDV in garlic and related Alliums. In addition, this research paper also constitutes a first report of OYDV in *A. ampeloprasum*, *A. alticum*, *A. roylei*, *A. tuberosum*, *A. longicuspis* and *A. chinense* from India.

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MATERIALS AND METHODS

Surveys were carried out during 2010 and 2011. Garlic leaf samples were collected from farmers’ fields. For sampling, each field was walked in a ‘W’ pattern and 40-50 leaves were collected randomly irrespective of symptoms. A total of 493 symptomatic leaf samples of garlic and wild *Allium* spp. were collected from major garlic growing parts of India. The survey was carried out at several places, *viz*. Delhi, Pune (Maharashtra), Nashik (Maharashtra), Rahuri (Maharashtra), Srinagar (Jammu and Kashmir), Coimbatore (Tamil Nadu), and Ooty (Tamil Nadu) by DOGR, Pune. The rest of samples were procured under All India Network Research Project on Onion and Garlic (AINRPOG) network centres, *viz*. Kanpur (Uttar Pradesh), Chiplima (Odisha), Junagarh (Gujarat), Dharwad (Karnataka), Jabalpur (Madhya Pradesh), Durgapura, Jaipur (Rajasthan), Karnool (Andhra Pradesh) and Solan (Himachal Pradesh). The instructions to collect samples were communicated to these centres.

The samples of major garlic varieties were collected from Demonstration Farm of Directorate of Onion and Garlic Research, Rajgurunagar, Pune. These varieties were planted in 1 square metre plot in two replicates. The visual symptoms of OYDV were recorded on each accession. For ELISA testing

total ten leaves (ten plant selected diagonally) from each cultivars were composited and subjected to ELISA. Leaf samples of wild *Allium* species, *viz.*, *A. tuberosum*, *A. alticum*, *A. ampeloprasum*, *A. longicuspis*, *A.chinense*, *A. cepa* var. *aggregatum* (Multiplier onion), *A. roylei*, *A. fistulosum* and *A. senescens* were collected from the Hybridization Garden of DOGR, Rajgurunagar, Pune. Seeds of some these wild *Allium* accessions (numbers mentioned in Table 3) were originally received from CGN Plant Genetic Resources, Netherlands and HRI, UK.

All samples were tested for the presence of viruses using the Double Antibody Sandwich ELISA (DAS-ELISA). DAS-ELISA was performed by using commercial ELISA kit (Agdia, Indiana, USA) and the procedure followed as per manufacturer instructions. Plant tissue samples were extracted in 1:10 (w/v) extraction buffer (Agdia). After incubation with 4-nitrophenyl phosphate at room temperature in dark, absorbance was measured with an ELISA reader (ELX 808IU, Biotek Inc.) at 405 nm. Samples were considered positive if the absorbance value was double of the absorbance value healthy control. Virus free mericlones of garlic were used as healthy control throughout the experiment.

Total RNA was extracted from 100 mg fresh sample taken from the tip of the youngest fully developed leaf of the infected garlic and wild *Allium* plants using Qiagen RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) was used as per the manufacturer's instructions. A healthy garlic sample was used as a negative control. The quality and quantity of RNA was estimated spectrophotometrically. Reverse transcription was performed on about 10 ng of the total RNA with 1 µl specific primer (100 µm), 4 µl reaction buffer (5X), 1 µl Ribolock RNase inhibitor (20 U/µl), 2µl dNTP Mix (10 mM), 200 U/µl RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas Life Sciences) and reaction volume was raised to 20 µl by adding DEPC treated water. cDNA synthesis was carried out at 42°C for 60 min. and reaction stopped by heating to 70°C for 5 min. The cDNA synthesized has been used as template DNA in RT-PCR as well as real-time PCR.

PCR was performed by using 3 µl of the RT mix as template, 2.5 µl of 10X reaction buffer, 1.5 µl dNTPs mix (10 mM), 2 µl each upstream OYDV1 5-GAAGCACAYATGCAAATGAAGG-3 and downstream primers OYDV2 5-GCCACAACTAGTGGTACACCAC-3 (10 pmole/µl), 1.25U *Red-Taq* DNA polymerase (Bangalore Genei, Bengaluru) and distilled water to final volume of reaction mixture of 25 µl. PCR reaction was optimized to 35 cycles of denaturation at 94°C for 3 min. that included an initial denaturation at 94°C for 60 s, annealing period of 60 s at 62°C, synthesis for 60 s at 72°C and final extension at 72°C for 7 min. Amplified products were analysed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transilluminator.

Real-time PCR assays were performed on a LightCycler 480 II (Roche Applied Science GmbH, Sandhoferstr, Germany) using SYBR Green I Master (Roche Applied Science, GmbH, Sandhoferstr, Germany) and a program of one initial pre-incubation hold (hotstart) at 10 min. at 95°C followed by 45 amplification cycles of 10 s at 95°C, 15 s at 62°C, and 15 s at 72°C. Real-time PCR was performed in a total volume of 20 µl which consisted of 10 µl SYBR Green I Master Mix, 10 pM each OYDV1 and OYDV2 primers, 5 µl of template cDNA and water PCR grade. Plots of fluorescence versus time, was used to identify Crossing Point (CP) value of amplified products using Light Cycler 480 software 1.1. ROX was used as a reference dye. The data obtained were presented as fold change in amplicon level with reference to calibrator (*A. roylei*). Samples were taken in triplicates and analysed by using CRD with IRRISTAT software.

RESULTS AND DISCUSSION

Our results indicate that, the virus was found to be widespread in garlic. Total 493 samples from 12 major garlic growing states of India (Table 1) were analyzed through DAS-ELISA. OYDV was detected in variable proportion from all the states surveyed (Table 1). Out of a total 493 samples, 308 samples were found positive for OYDV. The highest percentage of OYDV positives were recorded from Maharashtra (96%) followed by Gujarat (75%) and Madhya Pradesh (75%). Whereas, the least percentage of OYDV positives were recorded from Rajasthan (25%) followed by Delhi (40%). Among 6 leading garlic cultivars (two varieties each of white, creamy white and purple) tested through qRT-PCR, the incidence of OYDV was observed in all cultivars. qRT-PCR CP value ranged from 18.64 to 19.94 (Table 2). As evident from its qRT-PCR CP value of 18.64 (Table 2), the highest titre of OYDV was observed in variety Bhima Omkar. Low titre of virus *vis-a-vis* highest CP value (CP value: 19.94) was observed in garlic cultivar Yamuna Safed-3. No correlation was observed between bulb skin colour and virus concentration.

OYDV was detected as prevalent virus on wild *Alliums* in this investigation. Out of a total of 13 wild alliums that were tested through RT-PCR and qRT-PCR (Fig.1) for natural incidence of OYDV, 10 species were found positive for OYDV (Table 3). Three species, *viz.*, *A. senescens*, *A. cepa* var. *aggregatum* (Multiplier

Table 1. Incidence of OYDV in garlic samples collected from various locations in India as determined by DAS-ELISA.

Place	Total samples	Positive samples	Percent positive
Uttar Pradesh (Kanpur)	25	13	52.0
Orissa (Chiplima)	30	20	66.6
Gujarat (Junagarh)	40	30	75.0
Maharashtra (Nashik, Rajgurunagar, Rahuri)	120	114	95.0
Delhi	25	10	40.0
Tamil Nadu (Ooty)	58	26	44.8
Karnataka (Dharwad)	30	15	50.0
Madhya Pradesh (Jabalpur)	20	15	75.0
Rajasthan (Durgapura)	40	10	25.0
Jammu and Kashmir (Srinagar)	40	30	75.0
Andhra Pradesh (Hyderabad)	35	20	57.1
Himachal Pradesh (Solan)	30	10	33.3

Table 2. OYDV incidence on leading garlic cultivars.

Cultivar	Skin colour	qRT-PCR CP value
Bhima Omkar	White	18.65
Agrifound White (G-41)	White	19.42
Phule Baswant	Purple	18.68
Godavari	Purple	18.99
Yamuna Safed-3 (G-282)	Creamy white	19.94
Yamuna Safed-5 (G-189)	Creamy white	19.23

Table 3. Incidence of OYDV on wild *Allium* spp.

Species	Source	Relative conc.
A. tuberosum	India	+
A. ampeloprasum (Leek)	India	++
A. tuberosum cv. Bawang Kucai	CGN15749	+
A. alticum	CGN 16417	+++
A. ampeloprasum cv. Balady	CGN18724	++
A. longicuspuspis	ALL146	+++
A. cepa var. *aggregatum* (Multiplier onion)	India	-
A. ampeloprasum (Winterreuzen 2-Group 1)	CGN16402	+++
A. ampeloprasum L. Zwitserse Reuzen Group I	CGN16398	++
A. chinense	NGB14574	+++
A. roylei	CGN20520	+
A. senescens	CGN 15758	–
A. fistulosum	ALL750	-

+ sign indicates the intensity of virus concentration

onion) and *A. fistulosum* were found free from OYDV. *A. roylei* was tested negative for OYDV through RT-PCR but, was tested positive when tested through qRT-PCR. Among OYDV positive wild spp. the distinct symptoms of OYDV were observed on *A. alticum*, *A. chinense*, *A. ampeloprasum*, *A. ampeloprasum* (Winterrezen Group), *A. ampeloprasum* (Zwitserse Reuzen Group I), *A. ampeloprasum* (Balady) and *A. longicuspis*. However, on *A. tuberosum* (India), *A. tuberosum* (Bawang Kucai) and *A. senescens* the distinct symptoms were not visible. As evident from relative quantification (Fig. 1), the lowest concentration

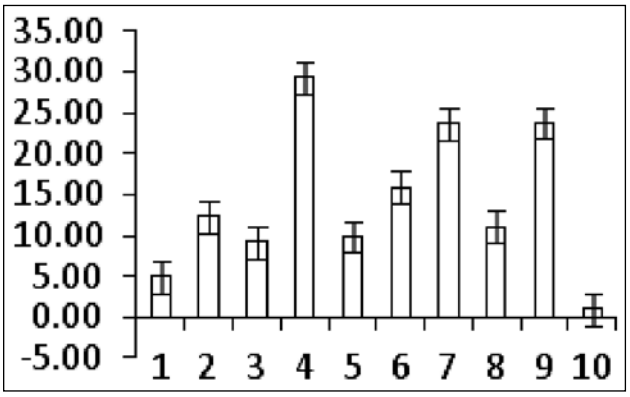

Fig. 1. Fold change in viral concentration among different *Allium* spp. with calibrator (*A. roylei*) determined through qRT-PCR: (Numbers in X axis indicates 1: *A. tuberosum*, 2: *A. ampeloprasum* (Leek), 3: *A. tuberosum* cv. Bawang Kucai, 4: *A. alticum*, 5: *A. ampeloprasum* cv. Balady, 6: *A. longicuspuspis*, 7: *A. ampeloprasum*, 8: *A. ampeloprasum* L. Zwitserse Reuzen Group I, 9: *A. chinense*, 10: *A. roylie*). Error bar indicates SE value.

of OYDV was observed in *A. roylei* followed by *A. tuberosum* (India). Moderate concentration of OYDV was observed in *A. ampeloprasum* (India), *A. tuberosum* (Bawang Kucai), *A. ampeloprasum* (Balady), *A. ampeloprasum* (Zwitserse Reuzen Group I). Whereas, OYDV was found at higher concentration in *A. longicuspis*, *A. alticum*, *A. ampeloprasum* (Winterreuzen 2-Group 1) and *A. chinense*.

Among garlic viruses, OYDV is one the major distinct symptom producing virus that is known to occur on garlic. In India, although the virus was reported way back during 90s by Ghosh and Ahlawat (6) but, systematic data about the quantum and magnitude of OYDV infection was unavailable. Present survey was carried out in order to verify the incidence of OYDV on garlic. Although, reports of incidence of OYDV and viruses on garlic are available from other countries, this is the first such from report India where the attempt has been made to provide complete information of OYDV on garlic and its related wild and other edible *Allium* spp.

Our results showed that OYDV is prevalent and widespread on garlic in India. The widespread distribution of OYDV had also been reported by Dovas *et al.* (4) and Conci *et al.* (3) wherein they reported up to 100% incidence of OYDV in Greece and Argentina respectively. Significant variation regarding the frequency and distribution of OYDV was observed in the regions surveyed which may due to variation in agro-climatic conditions of these regions. The lower percentage (25) of OYDV infection in Rajasthan may be due to large diurnal variation and strong winds, which may had hindered the development and movement of aphids and other vectors, which transmits this virus. Similar results were reported by Conci *et al.* (3) wherein they observed comparatively less incidence of LYSV (34%) in the Santa Cruz province of Argentina probably due to low temperature and strong winds. Another reason for variation in frequency and distribution of OYDV could be as in the every state, the farmers are predominantly using their own seed material or local land-races, lack of homogeneity in the planting material within and across the regions studied.

Up to 100% incidence of OYDV observed on improved cultivars. This result demonstrates the magnitude of OYDV incidence in garlic. Further, Conci *et al.* (3) recorded variation in concentration of LYSV in red and white type of garlic. In the present study, to know precisely the effect of skin colour on virus concentration, two varieties each of white, creamy white and purple colour were tested through qRT-PCR. Our result suggests that there is no correlation between garlic skin colour and OYDV concentration and incidence.

In Netherlands, Van Dijk (11) carried out extensive survey of potyviruses infecting wild and cultivated *Alliums*. He reported OYDV on wild species, *viz. A. ampeloprasum* var. *holmense*, *A. scorodoprasum*, *A. longicuspis* besides onion and garlic. Similarly, Dovas *et al.* (4) described the incidence of potyviruses, *viz.* LYSV and TuMV on wild *Allium* hosts. Recently, Ward *et al.* (14) gave extensive accounts of viruses infecting wild and ornamental alliums in New Zealand. Similarly, OYDV was reported on *A. ampeloprasum* and its varieties by Shahraeen *et al.* (10). We tested 13 wild alliums for incidence of OYDV using RT-PCR as well as qRT-PCR. These two highly sensitive methods were employed to eliminate false positives, if any. Previously, qRT-PCR has been used for the detection of OYDV and LYSV by Lunnelo *et al.* (13). In the present study, natural incidence of OYDV was observed on *A. ampeloprasum*, *A. longicuspis* and *A. chinense*. These results are in conformity with the earlier reports from several countries (Van Dijk, 11; Shahraeen *et al.*, 10). The other important species of *Allium* we tested was *A. tuberosum*, popularly known as garlic chives. It is consumed as a vegetable mostly in Asia. Garlic chives are found in the Himalayas, NE India and China. In the present investigation, both the varieties of *A. tuberosum*, *viz*. Bawang Kucai and India were found positive for the virus. We could not find any reports of OYDV on *A. tuberosum* in literature; therefore this is the first report of OYDV on *A. tuberosum*. Similarly, in case *A. alticum* and *A. roylei* we could not find any information in literature about OYDV. Although, Van Dijk (11) reported incidence of OYDV on multipler onion (*A. cepa* var. *aggregatum*) but, in our study we could not detect OYDV in Indian multiplier onion. Further, *A. fistulosum* was found negative for OYDV in the present study, but Brunt *et al.* (2) reported it to be susceptible to OYDV. Similarly, A. senescens was also found to be free from OYDV. There is no previous report of OYDV on *A. senescens* and obvious reason behind such results. A strategy for the production of virus free garlic planting material is underway in India. Therefore, the information related to virus incidence has immense role in prioritizing viruses for indexing for virus free planting material production. In addition, the data on OYDV incidence on wild alliums is also useful to formulate management strategy through host plant resistance against OYDV in garlic.

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