RESEARCH ARTICLE

Identification and Characterization of 16SrIX and 16SrXI groups of phytoplasmas associated with leaf yellows and declining diseases of garlic and onion in India

SONIA GOEL¹, MADHUPRIYA¹, VIPOOL THORAT², AMIT YADAV² and G.P. RAO^{1*}

¹Division of Plant Pathology, Indian Agricultural Research Institute, Pusa Campus, New Delhi 110 012, India

²National Centre for Microbial Research (formerly, Microbial Culture Collection), National Centre for Cell Science, Pashan, Pune 411 021, Maharashtra, India

Received: 27 April 2017/ Accepted: 29 June 2017/ Published online: 24 July 2017

© Indian Phytopathological Society 2017

ABSTRACT: Leaf yellows and declining symptoms were observed on garlic and onion plants at Pune and Nasik region of Maharashtra, and Gorakhpur region of Uttar Pradesh in March-April 2016. The disease incidence varied from 12% to 30% in different surveyed regions. After nucleic acid extraction and nested PCR analysis using phytoplasma specific primer pairs (P1/P7 followed by 3F/3R) of a 16S ribosomal gene an expected DNA fragments of 1.3 kb were amplified in two symptomatic onion samples (from Gorakhpur and Narayangaon) and one symptomatic garlic sample (from Pune). Pair wise sequence comparison of 16S rRNA gene of the onion leaf yellows phytoplasma (GKP strain) showed 100% sequence identity with a strain of phytoplasma associated with sugarcane grassy shoot disease (KP746932) and 99% sequence identity with phytoplasma strain associated with *Jasminum sambac* little leaf disease (KF728950) and *Ca. P. oryzae* (AB052873) belonging to rice yellow dwarf (16SrXI) group. However, another strain of onion leaf yellows phytoplasma (Narayangaon strain) and garlic leaf yellows and declining phytoplasma (Pune strain) showed 99% sequence identity with phytoplasma strains of grapevine yellows (KX011516), eggplant big bud (JX464672) and sesame phyllody (JX464672) belonging to *Ca. P. phoenicium* (16SrIX) group. Phylogenetic analysis of 16S rRNA gene sequences of phytoplasma strains from onion and garlic in the present study confirmed their close relationships with phytoplasma strains of 16SrXI and 16SrIX groups. This is the first report of association of 16SrXI and -IX groups of phytoplasma causing leaf yellows and declining disease of onion and garlic in India.

Keywords: 16SrIX, 16SrXI, garlic, onion, phytoplasma, India

Onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) are the most important crops used as vegetables and culinary commodities in India. India is the second largest producer of both onion and garlic in the world after China. Onion is grown in 1.1 million hectares with the production of 18.9 million tons and productivity of 16.13 ton/ha, whereas garlic is grown in 0.2 million hectares with production of 1.4 million tons and productivity of 5.44 ton/ha. Maharashtra is the leading state in onion production after Uttar Pradesh and Orissa, whereas Madhya Pradesh is the major garlic producing state followed by Gujarat and Uttar Pradesh in India (Anonymous, 2015).

The onion and garlic crops are attacked by many diseases caused by fungi, bacteria, viruses and nematodes, which vary from region to region, season to season and variety to variety (Schwartz and Mohan, 2007). These diseases can affect the production and marketing with low quality and reduce the yield (Chand and Kumar, 2016). Phytoplasmas were first discovered in 1967 and were named mycoplasma-like organisms or

MLOs (Doi *et al.*, 1967). They are not-yet-cultivated prokaryotic microorganisms that cause several hundred diseases of various plants (Bertaccini *et al.*, 2014) and are transmitted by phloem-feeding leafhoppers and plant hoppers (Weintraub and Beanland, 2006). Phytoplasma diseases are increasingly important worldwide, with a high economic impact on crop production and quality (Bertaccini *et al.*, 2014). In India, both the onion and garlic crops are severely infected by *Allexivirus* causing serious economic losses in India (Kumar *et al.*, 2010). Till date, no report is available on phytoplasma incidence on onion and garlic in India. However, Phytoplasma infection in onion causing yellow dwarf disease have been reported worldwide and two phytoplasma groups, 16SrI (Lee *et al.*, 2003; Namba *et al.*, 1993; Vibio *et al.*, 1995; Jung *et al.*, 2012; Gungoosingh- Bunwaree *et al.*, 2010) and 16SrXII (Gungoosingh-Bunwaree *et al.*, 2010) have been reported. Besides, 16Sr III group was reported to be associated with garlic decline disease in Argentina (Galdeano *et al.*, 2009) and 16SrI group in the USA (Mollov *et al.*, 2014). In the present study, attempts were made to identify phytoplasma etiology associated with leaf yellows and declining disease of onion and garlic in two states (Uttar Pradesh and Maharashtra) of India.

*Corresponding author: gpao_gor@rediffmail.com

MATERIALS AND METHODS

Symptomatic samples (5 each) of onion and garlic plants were collected from farmer's fields of Narayangaon (Nasik, Maharashtra), Gorakhpur (U.P.) and experimental fields of Directorate of Onion and Garlic Research, DOGR (Pune, Maharashtra) India. Total genomic DNA from asymptomatic and symptomatic leaves of the onion and garlic was extracted by CTAB method (Ahrens and Seemuller, 1992) and amplification of phytoplasma ribosomal DNA (rDNA) was performed with the universal phytoplasma primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by nested primer pair 3F/3R (Manimekalai *et al.*, 2010). The DNA from periwinkle infected with toria phyllody phytoplasma (group 16SrIX) (Azadvar *et al.*, 2009) maintained in the greenhouse was used as positive control and DNA extracted from asymptomatic onion and garlic samples and distilled water were used as negative controls.

PCR reactions were carried out in a Mastercycler (Eppendorf, Germany) and the cycling protocol used for the first round PCR was with initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min and extension at 72°C for 2 min, with a final extension for 10 min. Two µl of the product of the first round of PCR were used in nested PCR assays with internal primer pairs (3F/3R). The reaction mixture and conditions of nested PCR were similar to those applied in the first PCR round except for the annealing temperature which was done at 63°C. The PCR products were subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with good view dye and observed under UV transilluminator.

An amplified product was purified using the Nucleospin Gel and PCR Clean-up kit (Macherey Nagel, Germany) and sequenced directly in both directions using the same primers as for the PCR amplification.

The sequences were aligned using CLUSTAL W method of Bio-Edit software. The consensus sequences were submitted to GenBank and used in the BLAST search analysis. The 16Sr DNA sequence generated from the present study and the reference phytoplasma strains sequences retrieved from GenBank were used to construct phylogeny through MEGA 6.0 version software (Tamura *et al.*, 2013) employing the neighbor joining method with default values and 1,000 replications for bootstrap analysis. *Acholeplasma laidlawii* was used as the out-group to root the tree. The similarity coefficient was calculated for each pair of phytoplasma strains through BioEdit online software.

RESULTS AND DISCUSSION

Phytoplasma suspected symptoms of leaf yellows, tip necrosis, drying and declining were observed on garlic and onion plants grown in experimental plots of Directorate of Onion and Garlic Research, DOGR (30 percent incidence, Pune, Maharashtra) and farmers' fields of Gorakhpur (Uttar Pradesh) (12 per cent incidence); and Narayangaon (Nasik, Maharashtra) (15 per cent incidence) (Fig. 1a-e). The symptomatic onion and garlic plants were stunted with the reduced size of bulbs.

The universal primer pair P1/P7 did not amplify the expected DNA products of 1.8 kb in the first round of PCR assays. In a nested PCR assay, amplicons of 1.3 kb were amplified with 3F/3R primer pair in symptomatic samples of garlic and onion from all the three locations (Fig. 2). No amplifications were observed in any of the asymptomatic samples of onion and garlic collected from healthy fields from the three locations and in water controls.

Amplified DNA products from two onion (Gorakhpur and Narayangaon) and one garlic (Pune) samples were

Fig. 1. Phytoplasma symptoms showed (a) leaf yellows and declining in garlic (Pune), (b & c) leaf yellows in onion var. Phursungi (Narayangaon), (d & e) leaf yellows in onion (Gorakhpur)

Fig. 2. Amplification of a 16S rDNA product using primer pair 3F/3R (1.3kb) from onion and garlic samples. Lane M: 100bp DNA marker; Lanes 1: Asymptomatic onion sample; Lane 2 and 3: Symptomatic onion (Gorakhpur); Lane 4 and 5: Symptomatic onion var. phursungi (Narayangaon); Lane 6: Asymptomatic garlic sample; Lane 7: Symptomatic garlic (Pune); Lane 8: Positive control (toria phyllody phytoplasma)

sequenced and the consensus sequences were submitted in GenBank (Acc. No. KX641074 (onion, GKP), KX641075 (onion, Narayangaon) and KX641076 (garlic, Pune) (Table 1). Pair wise sequence comparison of 16S rDNA sequence analysis of the onion leaf yellows phytoplasma (GKP strain) showed 100 per cent sequence identity with the strain of sugarcane grassy shoot phytoplasma (KP746932) and 99 per cent sequence identity with strains of *Jasminum sambac* little leaf phytoplasma (KF728950) and *Ca. P. oryzae* (AB052873) belonging to rice yellow dwarf (16SrXI) group. However, 16S rDNA sequences of another onion leaf yellows var. phursungi phytoplasma strain from Narayangaon and the garlic leaf yellows and declining phytoplasma Pune strain showed 99 per cent sequence identity with phytoplasma strains of grapevine yellows (KX011516), eggplant big bud (JX464672) and sesame phyllody (JX464672) belonging to pigeon pea witches' broom (16SrIX group) (Table 2). Phylogenetic analysis based on 16S rDNA sequences of the onion and garlic phytoplasma strains in the present study again revealed their close relationships with members' strains of 16SrIX and 16SrXI phytoplasma groups (Fig. 3). The similarity coefficient calculation was performed using the 16SrRNA sequences of all the three strains of onion and garlic phytoplasma in the present study and the reference strains of 16SrIX and 16SrXI group. The results showed that the onion yellows phytoplasma-Gorakhpur strain shared < 97% similarity coefficient with the representative strains of 16SrXI groups (Table 2), indicating the association of *Ca. P. oryzae*. However, the onion leaf yellows phytoplasma - Narayangaon strain and garlic leaf yellows and decline phytoplasma- Pune strain shared

<97% similarity coefficient with the representative strains of 16SrIX groups, hence identified as the strain of *Ca. P. phoenicium* (Table 2).

Several phytoplasma diseases causing various types of symptoms and damage to a variety of economically important vegetable and spice crops have been reported in India (Rao *et al.*, 2017). '*Ca. P. asteris*' (16SrI-A subgroup) was reported infecting onion and garlic crops in Canada (Khadhair *et al.*, 2002), 16SrI-A and -L subgroup phytoplasma association with onion in Lithuania (Jomantiene *et al.*, 2010) and 16SrI-B subgroup and 16SrXII group in onion from Mauritius (Gungoosingh-Bunwaree *et al.*, 2010). Besides, 16Sr III group was reported to be associated with garlic decline disease in Argentina (Galdeano *et al.*, 2009) and 16SrI group in USA (Mollov *et al.*, 2014). This indicates wider genetic diversity of phytoplasma associated with garlic and onion crops all over the world. The association of many groups and subgroups of phytoplasmas also indicate the susceptibility of these crops to many phytoplasma strains of different groups, which may be possible by natural spread of phytoplasma strains through efficient leafhopper vectors (Bertaccini *et al.*, 2014). Till date, no report is available on phytoplasma incidence on onion and garlic from any region of India. In the present study, it has been confirmed the association of two groups (16SrXI and -IX) of phytoplasmas with onion and garlic from two major onion/garlic growing states (Maharashtra and Uttar Pradesh) of India. This is the first report of association of 16SrXI and IX groups of phytoplasma causing leaf yellows and declining disease of onion and garlic in India.

Table 1. Symptoms, location, disease incidence, phytoplasma identification of onion and garlic samples

Plants	Time of survey	Location (Place/State)	Symptoms	Disease incidence (%)	PCR results with GenBank Acc No. identified	Phytoplasma group
Onion	April 2016	Gorakhpur (Uttar Pradesh)	Leaf yellows	12	+ve (KX641074)	16SrXI
Onion (var. Phursungi)	March 2016	Narayangaon (Nasik, Maharashtra)	Leaf yellows	15	+ve (KX641075)	16SrIX
Garlic	March 2016	DOGR (Pune, Maharashtra)	Leaf yellows and declining	30	+ve (KX641076)	16SrIX

Table 2. Sequence similarities based on 16S rRNA gene sequences of the onion leaf yellows phytoplasma (GKP strain, KX641074; Narayangaon strain, KX641075) and garlic leaf yellows and declining phytoplasma (Pune strain, KX641076) with representatives of 16Sr IX and –XI phytoplasma groups

Acc. No./group	1	2	3	4	5	6	7	8	9
KX641074 (ACLY)	1.00								
KX641075 (ACLY)	0.941	1.00							
KX641076 (ASLY)	0.942	0.999	1.00						
KX011516 (GYP)	0.942	0.997	0.998	1.00					
JX464669 (EBBP)	0.942	0.997	0.998	0.998	1.00				
JX857827 (<i>Ca. P. Phoenecium</i>)	0.944	0.997	0.998	0.998	0.998	1.00			
AF248957 (PPWB)	0.945	0.992	0.993	0.993	0.993	0.995	1.00		
AB052873 (<i>Ca. P. oryzae</i>)	0.984	0.938	0.939	0.939	0.939	0.941	0.943	1.00	
KP746932 (SCGS)	1.00	0.941	0.942	0.942	0.942	0.944	0.945	0.984	1.00

For each pair wise alignment, the similarity (relative to the maximum similarity) and the number of identical nucleic acids (in % of shorter sequence) is given. ACLY (*Allium cepa* leaf yellows), ASLY (*Allium sativum* leaf yellows), GYP (Grapevine yellows phytoplasma), EBBP (Eggplant Big bud phytoplasma), *Ca. P. Phoenecium* (*Candidatus* phytoplasma phoenicium), PPWB (Pigeon pea Witches'broom), *Ca. P. oryzae* (*Candidatus* phytoplasma oryzae), SCGS (Sugarcane grassy shoot phytoplasma).

Fig. 3. Phylogenetic tree constructed by neighbor-joining method showing the relationships between onion and garlic phytoplasma strains (black highlight) and reference phytoplasma strains based on the nucleotide sequences of 16S rRNA gene sequence fragment. *A. laidlawii* was used as an out group

Ca. P. oryzae (16Sr XI) has been reported associated with important phytoplasma diseases of sugarcane, rice, areca nut and coconut, however, the *Ca. P. phoenicium* was reported to infect pigeon pea, toria and ornamentals in India (Rao *et al.*, 2017). Leafhopper vectors are already known to transmit *Ca. P. oryzae* in nature in India (Rao *et al.*, 2014; Tiwari *et al.*, 2017). Hence it would be quite possible that both the reported phytoplasma strains may spread in nature by efficient leafhopper species to other important crop and thus has great potential in causing serious economic losses to many crops in India.

ACKNOWLEDGEMENTS

The authors are thankful to ICAR, New Delhi for providing ICAR Platform Research funding and Head, Division of

Plants Pathology, IARI, New Delhi for providing laboratory facilities. Authors are also acknowledged this funding support by SERB, New Delhi through the Project No. SERB/F/9182.

REFERENCES

- Ahrens U and Seemuller E (1992). Detection of DNA of plant pathogenic mycoplasma like organisms by a polymerase chain reaction which amplifies a sequence of the 16S rRNA gene. *J. Phytopathol.* 82: 828-832.
- Anonymous (2015). DOGR Annual Report 2014-2015. ICAR-Directorate of Onion and Garlic Research Rajguru nagar, Pune, Maharashtra, India.
- Azadvar M, Baranwal V K and Yadava D K (2009). First report of a 16SrIX (Pigeon pea witches' broom) phytoplasma

- associated with toria (*Brassica rapa* cv. toria) phyllody disease in India. *New Dis. Rep.* 20: 27.
- Bertaccini A, Duduk B, Paltrinieri S and Contaldo N (2014). Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. *Am. J. Plant. Sci.* 5: 46-62.
- Chand G and Kumar S (Eds.) (2016). Crop Diseases and Their Management: Integrated Approaches. Apple Academic Press, Canada. pp. 188-190.
- Deng S and Hiruki C (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *J. Microbiol. Methods* 14: 53-61.
- Doi Y, Teranaka M, Yora K and Asuyama H (1967). Mycoplasma- or PLT group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or Paulownia witches' broom. *Ann. Phytopathol. Soc. Japan* 33: 259-266.
- Galdeano E, Conci L R, González O, Paradell S, Di Rienzo J A, Nome C. and Conci V C (2009). Epidemiological aspects of garlic decline disease caused by a phytoplasma in Asiatic and Argentinean garlic cultivars. *Aust. Pl. Pathol.* 38: 437-443.
- Gungoosingh-Bunwaree A, Contaldo N, Vally V, Benimadhu SP, Duduk B and Bertaccini A (2010). Detection of phytoplasmas in watercress and onion plants from Mauritius. *Plant Health Prog.* 10: 1094.
- Jomantiene R, Davis RE, Lee IM, Zhao Y, Bottner-Parker K, Valiuna, D and Petkauskaitė R (2010). Onion is host for two phytoplasma lineages, subgroups 16Srl-A and 16Srl (-B/L) L, in Lithuania: A *HinfI* site revealed a SNP marking divergent branches of evolution. *J. Plant Pathol.* 92: 461-470.
- Jung H Y, Win N K K and Kim Y H (2012). Current status of phytoplasmas and their related diseases in Korea. *Plant Pathol. J.* 28: 239-247.
- Khadhair A H, Evans I R and Choban B (2002). Identification of aster yellows phytoplasma in garlic and green onion by PCR-based methods. *Microbiol. Res.* 157: 161-167.
- Kumar S, Baranwal VK, Joshi S, Arya M and 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: 64-68.
- Lee IM, Martini M, Bottner KD, Dane RA, Black MC and Troxclair N (2003). Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. *Phytopathology* 93: 1368-1377.
- Manimekalai R, Soumya VP, Kumar RS, Selvarajan R, Reddy K, Thomas GV, Sasikala M, Rajeev G and Baranwal VK (2010). Molecular detection of 16Srl group phytoplasma associated with root (wilt) disease of coconut (*Cocos nucifera*) in India. *Plant Dis.* 94: 636.
- Mollov D, Lockhart B, Saalau-Rojas E and Rosen C (2014). First report of a 16Srl (Aster yellows) group phytoplasma on garlic (*Allium sativum*) in the United States. *Plant Dis.* 98: 419-419.
- Namba S, Kato S, Iwanami S, Oyaizu H, Shiozawa H and Tsuchizaki T (1993). Detection and differentiation of plant-pathogenic mycoplasma like organisms using polymerase chain reaction. *Phytopathology* 83: 786-791.
- Rao GP, Madhupriya, Thorat, V, Manimekalai R, Tiwari AK and Yadav A (2017). A century progress of research on phytoplasma diseases in India. *Phytopathogenic Mollicutes*, 7(1): 1-38.
- Rao GP, Madhupriya, Tiwari AK, Kumar S and Baranwal VK (2014). Identification of sugarcane grassy shoot-associated phytoplasma and one of its putative vectors in India. *Phytoparasitica* 42: 349-354.
- Schneider B, Seemueller E, Smart CD and Kirkpatrick BC (1995). Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: S. Razin, and J. G. Tully (eds.), *Molecular and Diagnostic Procedures in Mycoplasmaology*. Academic Press, San Diego, California, USA. Vol. 1: 369-380.
- Schwartz HF and Mohan SK (2007). Compendium of onion and garlic diseases and pests. (Ed. 2). APS Press, ISBN 978-0-89054-357-3, USA. pp. 127.
- Tamura K., Stecher G, Peterson D, Filipski A and Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Bio. Evol.* 30: 2725-2729.
- Tiwari AK, Kumar S, Mall S, Jadon V and Rao GP (2017). New efficient natural leafhopper vectors of sugarcane grassy shoot phytoplasma in India. *Sugar Tech.* 19: 191-197.
- Vibio M, Camele I, Bertaccini A, Rana GL, D'Aloisio V and Benni A (1995). Metodi diagnostici molecolari per l'individuazione di infezioni fitoplasmatiche in cipolla. *L'Inf. tore Agrario* 35: 75-77.
- Weintraub PG and Beanland L (2006). Insect vectors of phytoplasmas. *Ann. Rev. Entomol.* 51: 91-111.