Phytology

Brief overview of Phytoplasma associated with pigeon pea cultivated in India

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Received 20 Nov 2021; Accepted 2 Jan 2022; Published 19 Jan 2022

Abstract

Pigeon pea (*Cajanus cajan* L.) is an important food legume crop predominantly cultivated in tropical and subtropical regions of Asia and Africa and also plays an important role in food and nutritional security due to rich in protein, minerals and vitamins. Pigeon pea has a unique place in Indian farming and India accounts for about 90% of the global production. Pigeon peas have been reported of phytoplasma diseases worldwide associated with the visual symptoms such as witches'-broom, little leaf, floral malformation, stunting of whole plant. Phytoplasma strains belonging to the Pigeon Pea Witches'-Broom (PPWB;16SrIX group), subgroup IX-A, IX-C, little leaf disease associated with '*Candidatus* Phytoplasma asteris' (16SrI); phytoplasma '*Candidatus* phytoplasma aurantifolia" (16SrII group) have been reported on pigeon pea worldwide. This review article focused brief current status of phytoplasma disease associated with pigeon pea of India as well as abroad.

Keywords: *Cajanus cajan*, witches'-broom, little leaf, *Candidatus* phytoplasma group

Introduction

Pigeon pea [*Cajanus cajan* (L.) Millspaugh] is an important food legume predominantly cultivated in tropical and subtropical regions of Asia and Africa and also plays an important role in food and nutritional security due to rich in protein (23-27%), minerals and vitamins (esp. vitamin B). In India, *C. cajan* grows at altitudes ranging from 150 to 2000 m. above sea level (Van der Maesen 1990) [1], and even in moderately cold climates.

The major areas of its cultivation and variability are in the states of Uttar Pradesh, Madhya Pradesh, Maharashtra, Karnataka, Gujarat, Andhra Pradesh, Telangana and Bihar. States of Arunachal Pradesh, Chhattisgarh, Kerala, Odisha, Rajasthan, Tamil Nadu and Uttarakhand have lesser cultivated areas of pigeon pea in the country. Madhya Pradesh occupies an area of about 5.79 lakh ha with production of 6.44 lakh tonnes with an average productivity of 1105 kg/ha (Anonymous 2015-16) [2].

Phytoplasmas are intracellular obligate prokaryotes which lack cell wall, have small genome (680-1,600 kb) and are mainly transmitted by leafhoppers, they are associated with typical yellowing, stunting of whole plant, virescence, phyllody, proliferation of axillary buds, witches'-broom and die back symptoms (Al-Saady and Khan, 2006; Bertaccini 2007; Harrison *et al*. 2008) [3, 4, 5]. Phytoplasma are also associated with severe yield losses in a variety of plant species of horticultural, agricultural and ornamental importance (Chaturvedi *et al.* 2010) [6].

In India recent evidence showed that phytoplasma cause diseases in several plant species including vegetable crops, fruits trees, ornamental, sugarcane, grasses & weeds and resulted in serious threat as a source of alternative natural host for the spread of phytoplasma pathogen to other economically important plants and thereby chances of causing severe losses. Efforts have been made for detection, identification and possible management of phytoplasma diseases naturally occurring in various plant species in India so that their growth and yield may be improved.

The important diseases of Pigeon pea are Wilt, Sterility mosaic disease, phytophthora blight, alternaria blight, powdery mildew and pigeon pea witches'-broom (PPWB) caused by phytoplasma.

Phytoplasma strains belonging to the Pigeon Pea Witches'-Broom (PPWB) group (16S rDNA gene RFLP group IX) has a broad host range which includes herbaceous plants, fruit trees and conifers. Harrison *et al.* (1991) [7] described for the first time PPWB phytoplasma, subgroup IX-A on symptomatic pigeon pea plants (*C. cajan*). Later Khan *et al.* (2007) [8] reported the presence of phytoplasmas within the same group, classified in the subgroup IX-C, affecting herbaceous plants in the field such as bristly oxtongue (*Pichris echioides* L.) and field scabious (*Knautia arvensis* L.).

The second phytoplasma-related disease reported in Puerto Rico was pigeon pea witches'-broom (PPWB) (Rodríguez *et al.* 1979) [9]. Witches' broom disease of pigeon pea was noted for the first time in 1980 in several plantings in southern Florida (McCoy *et al.* 1983) [10]. Harrison *et al.* (1991) [7] described for the first time PPWB phytoplasma, subgroup IX-A on symptomatic pigeon pea plants (*C. cajan*). Breeder's plots of pigeon pea were affected by a phyllody disease in February 2012 growing at a single trial site at Urrbrae, South Australia, were all shown to belong to the 16SrII phytoplasma taxonomic group, but each host species was found to be infected with a different genotype (Yang *et al.*, 2013) [11]. The best known

phytoplasma disease of pigeon peas is associated with pigeon pea witches' broom phytoplasma classified in the 16SrIX group. This phytoplasma has been recorded from China, Mexico, Myanmar, India and Puerto Rico (Caicedo *et al.* 2015) [12].

Only two groups have been reported on pigeon pea and more than ten groups of phytoplasma have been identified on other economically important plants and most of them have been reported from North-southern parts of the India (Mall *et al.* 2011) [13]. There are few reports are available in literature from Eastern, Western and Central India regarding phytoplasma infection.

Worldwide production of pigeon pea

Pigeon pea commonly known as Arhar, red gram or tur in India. The world acreage of pigeon pea is 6.2 Mha with an annual production of 4.7 M ton. Since 1976, the area under pigeon pea has increased by seven percent. Currently pigeon pea is grown on 5.2 million ha in the rain-fed areas of Asia, eastern and southern Africa, Latin American and Caribbean countries. It is a very old crop and second most important pulse crop in the country. Seeds of arhar are also rich in protein (22.3%), fat (1.7%) iron, iodine, essential amino acids like lycine, threonine, cystine and arginine etc. India is the largest producer and consumer of pigeon pea with an annual production of 2.86 M ton, followed by Myanmar (0.60 Mt), Malawi (0.16 Mt) and Kenya (0.10 Mt) (FAO 2011) [14].

Pigeon pea has a unique place in Indian farming and India accounts for about 90% of the global production. It is the second most important pulse crop next to chickpea, covering an area of around 4.42 m ha (occupying about 14.5% of area under pulses) and production of 2.86 mt (contributing to 16% of total pulse production) and productivity of about 707 kg/ha (FAOSTAT, 2011, Singh *et al*. 2013). Madhya Pradesh ranks IInd in production (15.87%). Recommended Varieties for cultivation in Madhya Pradsh state like JKM-189, TJT-501, JKM-7, TT-401, BSMR-175, ICPL-87119, BSMR-736.

Symptomatology of phytoplasma on pigeon pea

Phytoplasmas are intracellular obligate prokaryotes which lack cell wall, have small genome (680-1,600 kb) and are mainly transmitted by leafhoppers, they are associated with typical yellowing, stunting of whole plant, virescence, phyllody, proliferation of axillary buds, witches' broom and die back symptoms (Fig. 1).

Fig 1(a): A filed view of pigeon pea showing Witches'- broom disease (in circle) compared with healthy plants

Fig 1(b): Natural symptoms of little leaf disease on pigeon pea (a) healthy (b) and infected plant in field (c)

Taxonomy and genome of phytoplasma

These phytopathogenic mollicutes (mycoplasma-like organism-MLO) were named as phytoplasmas in the subcommittee on taxonomy of Mollicutes during 1992 (Lee *et al.* 2000; IRPCM 2004) [15, 16]. Molecular tools such as PCR/RFLP and nested-PCR on 16S rDNA were developed and established to ascertain a standard and reliable system of identification and classification of phytoplasmas in ribosomal groups and subgroups obtained by RFLP and/or virtual RFLP analyses of the 16S rRNA gene amplicon or sequence with a number of restriction enzymes (Lee *et al.* 1998; Zhao *et al.* 2009) [17, 18]. Since they were only recently cultured (Contaldo *et al.* 2012 and 2016) [19, 20], biological i.e., classical methods for classification are not available as yet. Currently, phytoplasmas are categorized into 33 ribosomal groups comprising a number of subgroups each (Bertaccini and Lee 2018) [21]. A provisional classification was also established to the taxon '*Candidatus* Phytoplasma' species based on a unique 16S rRNA gene sequence (>1200 bp) and a novel 'Ca. Phytoplasma' species can be named only if its 16S rRNA gene sequence has <97.5% similarity to that of any of the previously described species or if there are sufficient biological and genetic characteristics to warrant the designation of the new taxon (IRPCM, 2004) [16].

Transmission of Phytoplasma

Phytoplasmas can be spread from plant to plant by vegetative propagation through cuttings, storage tubers, rhizomes or bulbs (Lee and Davis, 1992). Phytoplasmas can also be spread via cuscuta dodder (Carraro *et al.* 1988) [22] and through grafts but unlike viruses they cannot be transmitted mechanically by inoculation with phytoplasma-containing sap from infected plants. Phytoplasmas can also be spread or transmitted from plants to plants through sap-sucking insect vectors belonging to the different families of insect vectors such as Cicadellidea (leaf-hoppers), Fulgoridea(plant-hoppers) and Psyllidea (jumping plant lice) (Grylls 1979; Tsai 1979; Ploaie 1981) [23, 24, 25].

Molecular diagnosis of Phytoplasma

For detection of Phytoplasma, total DNA was extracted from symptomatic samples (approximately 100 mg leaf tissues), employing a phytoplasma enrichment procedure (Ahrens and Seemüller 1992) [26]. Several published reports have been indicated that the Polymerase chain reaction (PCR) was performed using the total DNA and P1/P6 universal primers designed earlier (Deng and Hiruki 1999) [27], specific to 16S rRNA gene of phytoplasma. The PCR was set up in a 50µl reaction mixture containing 5µl DNA (20ng), 5µl *Taq* buffer (10X), 1µl dNTPs (10mM each) 1µl each primers (25pM/µl each), 1µl *Taq* DNA polymerase (3U/µl), and 37µl double distilled water to make up the reaction volume. The PCR were performed using an incubation regime of 94^0C for 5min once, followed by 30 cycles of 94^0C for 50 s, 60^0C for 45 s and 72^0C for 90 s, then a final incubation of 7min at 72^0C.

Further the nested PCR will be performed using 1:10 diluted first stage (P1/P6) products and R16F2n/R16R2 primers (Gundersen and Lee 1996) [28] with the standardized PCR conditions: denaturation at 94^0C for 5min, followed by 30 cycles of 94^0C for 50 s, 55^0C for 45 s and 72^0C for 90 s and a final extension for 7min at 72^0C. The resulting products of direct PCR and nested PCR will be electrophorezed on 1.0% agarose gel with DNA marker for comparison and assessing size of amplicons. The amplified PCR amplicons (size ~1.3kb) from nested PCR (R16F2n/ R16R2) will be purified from the gel using the gel PCR purification Kit and purified product were cloned in suitable cloning vector and the positive clone will be checked by restriction digestion. The selected clones were sequenced and consensus sequence data obtained will be deposited in NCBI GenBank database.

The sequence data obtained through sequencing results was analyzed for consensus data remaining no ambiguities and submitted in National Centre for Biotechnology Information GenBank database (NCBI, http://www.ncbi.nlm.nih.gov/Bankit). To observe the nucleotide identity within and with other reported strains of phytoplasma, basic local alignment search tool (BLAST) searches were performed with all available databases using the NCBI-BLAST server (www.ncbi.nlm.nih.gov). The data were compared within and with other reported strains of virus sequences obtained with the Entrez program using the BLAST (NCBI, Bethesda, USA, http://www.ncbi.nlm.nih.gov/blast). Multiple nucleotide and amino acid sequence alignments of selected strains reported from India and abroad were performed using *Genomatix DiAlign* program (www.genomatix.de/cgi-bin/dialign/ dialign.pl).

Phylogenetic and molecular evolutionary analyses of identified phytoplasma isolates using 16S rRNA gene sequences was performed with Molecular Evolutionary Genetics Analysis (MEGA version 7.0) program with 100o replicates bootstrapping and phylogram were generated with Neighbour-joining method. Dendrograms were viewed by the NJ plot program.

Virtual *in silico* restriction fragment length polymorphism (RFLP) analysis

Virtual *in silico* RFLP analysis for group/sub group level characterization was performed using genome of (~1.25 kb) phytoplasma 16S rRNA gene sequence obtained with a representative phytoplasma of '*Ca*. P. species group/subgroup using selected restriction enzymes by pDRAW32 program (http://www.acaclone.com) and iPhyClassifer online tools (iPhyClassifer.cgi). Each 16S rRNA gene sequences will be digested manually and virtually *in silico* with restriction enzymes like: *Alu*I, *Bam*HI, *Bfa*I, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Kpn*I, *Rsa*I and *Taq*I and a gel/virtual gel electrophoresis image generated from proper identification of phytoplasma species.

Identification and characterization of phytoplasma Internationally on pigeon pea

Phytoplasmas are associated with over 600 diverse plant diseases worldwide, mainly transmitted by phloem-feeding insects, especially leafhoppers and plant hoppers (Bertaccini *et al.*, 2014) [29]. For several decades the lack of effective methods to identify and characterize phytoplasmas made it not possible to know if the same bacterium was involved in diseases showing similar symptoms on the same or different host plants at various locations. The advent of molecular tools enabled the classification of phytoplasmas into groups and subgroups, depending in particular on the analysis of 16S rRNA gene sequence (Lee *et al.* 1998; IRPCM 2004) [17, 16].

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Identification and characterization of phytoplasma on pigeon pea in India

Phytoplasma cause diseases in several plant species in India and resulted in serious threat to the affected crop as a source of alternative host for the spread of phytoplasmas to other economically important plants and thereby chances of causing severe losses to a maximum level. So far, 34 phytoplasmas belonging to 7 groups have been identified on different plant species in India. Nucleotide sequence studies of 16S rDNA have shown that the '*Ca.* Phytoplasma asteris' (16SrI), '*Ca*. P. aurantifolia (16SrII), '*Ca*. P. ulmi (16SrV), '*Ca.* P. trifolii' (16SrVI), '*Ca.* P. phoenicium' (16SrIX), '*Ca.* P. oryzae' (16SrXI) and '*Ca*. P. cynodontis' (16SrXIV) are the major groups associated with various plant species from India (Rao *et al.* 2011; Kumar *et al.* 2017) [30, 31].

There are number of reports are available in literature associated with phytoplasma disease from India (Specially from North and South India) from economically important plant species but there are limited reports have been published on Pegion pea crop from India. A witches' broom disease of pigeon pea such as associated with MLO has also been reported from Hyderabad, India (Singh *et al.* 1976) [32]. Reddy (1987) [33] reported phyllody and witches' broom associated with phytoplasmas in several cultivars of pigeon pea at ICRISAT, Patencheru, Hyderabad.

Raj *et al*. (2006) [34] identified causal agent of little leaf disease of pigeon pea as '*Candidatus* Phytoplasma asteris' (16SrI) based on 16S rDNA sequence data and 16SrII group phytoplasma '*Candidatus* phytoplasma aurantifolia" associated with little leaf disease of pigeon pea First time reported from India. (Vijay Kumar Naik *et al.* 2018) [35].

Conclusion

Recent evidence showed that phytoplasma cause diseases in several plant species including vegetable crops, fruits trees, ornamental, sugarcane, grasses & weeds and resulted in serious threat as a source of alternative natural host for the spread of phytoplasma pathogen to other economically important plants and thereby chances of causing severe losses. Efforts have been made for detection, identification and possible management of phytoplasma diseases naturally occurring in various plant species in India so that their growth and yield may be improved. The best known phytoplasma disease of pigeon peas (Fabaceae) is associated with pigeon pea witches' broom phytoplasma classified in the 16SrIX group. This phytoplasma has been recorded from China, Mexico, Myanmar, India and Puerto Rico (Caicedo *et al.* 2015) [12].

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