# SYMPTOMATOLOGY, ETIOLOGY AND TRANSMISSION OF CHICKPEA PHYLLODY DISEASE IN PAKISTAN

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## SUMMARY

Chickpea (*Cicer arietinum*) plants of two types, kabuli and desi, showing phytoplasma disease-like symptoms were observed for the first time in Pakistan during 2005. The major symptoms were floral virescence, phyllody and extensive proliferation of the branches. Light microscopy of hand-cut sections of symptomatic stems treated with Dienes' stain showed blue areas in the phloem region of diseased plants but not in apparently normal ones. Phytoplasma-like pleomorphic bodies were observed in the sieve cells of symptomatic chickpea stems by transmission electron microscopy, but not in the sieve tubes of healthy plants. Amplification from diseased plants of a 16S rDNA fragment 1800 bp in size with phytoplasma primers P1 and P7, confirmed that they hosted a phytoplasma. RFLP profiles and complete sequencing of the R16F2n/R16R2 region showed that the associated phytoplasma had 100% sequence identity in this rDNA region to 16SrII-D subgroup phytoplasmas. Chickpea phyllody disease was transmitted from diseased to healthy chickpea plants by grafting and through the leafhopper *Orosius orientalis.* Sap inoculation trials were unsuccessful and transmission by *Aphis craccivora*, *Myzus persicae*, *Empoasca devastans* and an unidentified brown leafhopper failed to produce disease symptoms.

*Key words: Cicer arietinum*, phyllody, 16SrII phytoplasma*, Orosius orientalis*, epidemiology.

## INTRODUCTION

Chickpea (*Cicer arietinum*) is an important, cool-season grain legume of exceptionally high nutritive value and versatile food use. It is mostly grown under rain-fed conditions in arid and semi-arid areas around the world (Millan *et al.*, 2006). The major producers, India, Pakistan and Turkey contribute 65%, 9.5% and 6.7% respectively of the world harvest (FAOSTAT, 2005). Despite growing demand and high yield potential, chickpea yield is unstable and productivity is stagnant at unacceptably low levels with a world average of *ca.* 0.8 t/ha (FAOSTAT, 2005; Millan *et al.*, 2006). Reasons for only marginal recent improvements in productivity are a series of biotic and abiotic stresses that reduce yield and yield stability (Millan *et al.*, 2006). More than 50 diseases and 54 insect pests have been reported to date on chickpea in different parts of the world (Nene, 1980; van Rheenen, 1991; Singh and Weigand, 1994). Recently Akhtar *et al.* (2008) reported a phytoplasma associated with chickpea phyllody disease in Pakistan for the first time. A similar phyllody disease of chickpea was previously reported in 1959 from Coimbatore (Tamil Nadu, India) (Ghanekar *et al.*, 1988) and from Ethiopia, Myanmar (Ghanekar *et al.*, 1988), Australia (Saqib *et al.*, 2005) and Oman (Al-Saady *et al.*, 2006).

Phytoplasmas are a group of plant pathogenic wall-less, phloem inhabiting bacteria in the class Mollicutes that cause devastating damage to plants by loss in biomass and quality of products including flowers. These pathogens are known to affect approximately 1000 plant species worldwide including fruits, vegetables, cereals, trees and legumes (Seemüller *et al.*, 1998). New disease reports are published frequently, and the growing list of hosts includes economically important food, fibre, forage, fruit and ornamental plants (Hogenhout *et al.*, 2008). Despite their economic importance and unique biological features, phytoplasmas remain still poorly characterized plant pathogens.

Chickpea has been grown in Pakistan for many years without any reported incidence of phyllody disease, thus the presence of a phytoplasma-associated disease presents a new threat to cultivation of this crop. Diseases possibly due to phytoplasma in Pakistan have not been investigated in detail in terms of symptomatology, etiology and transmission. In this study, investigations were undertaken to study these aspects of chickpea phyllody from naturally infected plants.

## MATERIALS AND METHODS

The investigations presented here were carried out

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during 2005-2008 at NIAB, Faisalabad (Pakistan), whilst electron microscopy for the in situ observation of the phytoplasma were carried out at NIBGE (Pakistan) and studies to characterize the phytoplasma using molecular techniques were conducted at the School of Biosciences, University of Nottingham, UK.

**Symptomatology**. Observations for chickpea phyllody disease were started one week after germination. Both symptomatic and symptomless plants were tagged in naturally infected fields at different growth stages. These plants were compared for their main and distinguishing features.

**Light microscopy.** Sections 1 to 2 mm long were cut with razor blades from healthy and infected tissues collected from the field and fixed at pH 7.4 for two days at 4°C as described by Nienhaus *et al.* (1982). Free hands-cut transverse sections were made and stained for 10 min in a 0.2% solution of Dienes stain at 30°C according to Deenley *et al.* (1979).

**Electron microscopy.** Water agar-embedded healthy and phyllody infected chickpea stem samples were pre-fixed in 5% glutaraldehyde overnight, washed with 0.2 M Pipes buffer and post-fixed in 1% osmium tetraoxide for 18 h at room temperature. The samples were washed with distilled water, treated with 5% uranyl acetate for 16-18 h and washed again with distilled water. They were then dehydrated with absolute ethanol and embedded in Spurr resin at 70°C for 48 h. Sections 120 nm thick were cut on an RMC MT 7000 ultra-microtome, picked on copper grids and stained with 5% uranyl acetate for 30 min and lead citrate for 10 min. Observations were made with a JEOL JEM1010 transmission electron microscope operating at 80 KV.

**Molecular characterization.** DNA was extracted from six symptomatic chickpea plants and analysed by PCR as described by Hodgetts *et al.* (2007). Amplifications of 16S rRNA were performed in 25 µl reactions using "Ready To Go PCR beads™" (Amersham, UK) containing 15 ng template DNA and 100 ng of each primer in a MJ Research PTC200 thermocycler as described by Hodgetts *et al.* (2007). The phytoplasma universal primers P1/P7 (Smart *et al.*, 1996) were used for first-round PCR; the reaction conditions were 95°C for 3 min followed by 30 cycles of 94°C for 30 sec, 53°C for 90 sec, 72°C for 90 sec, with a final extension step of 72°C for 10 min. Following PCR, 10 µl of PCR products were digested with 0.5 U of either *Alu*I or *Hpa*II restriction enzymes (New England Biolabs, UK) overnight at 37°C in a 20 µl reaction vol. Digests were separated on 1.4% agarose gels in 1x TBE buffer containing ethidium bromide, and visualised under UV light. For sequencing of the 16S rRNA gene, the PCR product from one sample was cleaned using a Qiaquick® PCR Purification Kit (Qiagen, USA) before sequencing using primers P1/P7 and a range of internal primers, so that the complete sequence between primers R16F2n and R16R2 could be read in both orientations. Sequences were processed using Beckman Quickstart kit technology and WellRed Dye chemistry (infra-red dyes), with a CEQ 8000 Genetic Analysis System (Beckman Coulter, USA). For phylogenetic analysis, BLAST searches (Altschul *et al.*, 1990) were performed at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/).

**Sap inoculation.** Chickpea plant tissues with typical phyllody disease were collected from the field, ground in a mortar with pestle in 0.02 M phosphate buffer, pH 7.4 (1g/ml) and squeezed through very fine muslin. Young leaves of healthy chickpea plants were dusted with 500-mesh carborundum powder and mechanically inoculated with the freshly extracted sap using cotton pads. Plants were rinsed just after inoculation and kept under insect-free covers for symptom development.

**Graft inoculation.** Ten pots were sown in a glasshouse with 4-5 seeds each of chickpea kabuli genotype Pb-1. Plants were thinned to one per pot 2 weeks after germination. These plants were graft inoculated at 5-6 week age with chickpea shoots showing phyllody disease, collected from naturally infected plants. For graft inoculation, one slice was made on the stem, 1-2 inch below the tip when the test plants attained the height of 22 cm. A 12 cm long infected branch was detached from diseased plants. A similar cut was made on this branch and corresponding cut surfaces were brought together and tied with parafilm. The base of the detached stem was then placed in a 2x16 cm test tube, containing distilled water. The water was changed daily and after seven days the tubes were removed. Conventional agronomic practices and normal plant protection measures were followed to keep the crop in good condition. Plants were observed daily for symptom development.

**Insect transmission.** In a search for the vector, five different insects namely *Aphis craccivora*, *Mysus persicae*, *Orosius orientalis, Empoasca* sp. and an unidentified brown leafhopper were collected from chickpea fields using yellow sticky traps and by sweep netting chickpea plants in fields with high incidence of phyllody disease during 2005-2007. A batch of twenty insects per plant for each aphid and leafhopper species was then transferred immediately after collection to 10 caged, healthy chickpea seedlings (4-week-old) for an inoculation access period of 5 days. Insects were killed after the inoculation feeding period with Confidor (0.8 ml/l $H_2O$). Test plants were monitored daily for symptom

expression. In addition, a similar set of chickpea plants was inoculated using the same insects after 5 days acquisition access period on phyllody infected chickpea plants.

**Seed transmission.** One hundred seeds of chickpea genotype kabuli NCS 2001 were harvested at maturity from chickpea plants partially infected with phyllody disease. These seeds were planted in pots under insect free conditions in the glasshouse. Plants were observed for symptom development until maturity.

## RESULTS

**Symptomatology.** Chickpea phyllody disease can produce different types of symptoms, but the main symptoms observed were phyllody, virescence and extensive branch proliferation. The most characteristic symptom is transformation of floral parts into green leaf-like structures (phyllody). The ovary is replaced by elongated structures, almost resembling a shoot and the calyx becomes polysepalar. The sepals become leaf like and remained smaller in size (Fig. 1). The phylloid flowers become actinomorphic in symmetry and the corolla becomes polyseptalous and deep green. The stamens retain their shape but flatten, showing a tendency to become leaf-like. The anthers become green and contain abnormal pollen grains. The pistils transform into a pseudosyncarpous ovary by their fusion at the margins. This false ovary becomes very enlarged and flattened. Inside the ovary, instead of ovules there are small petiole-like outgrowths, which later grow and burst through the walls of the false ovary providing small shoots. These shoots continue to grow and produce more phylloid flowers at the axillae. Production of new shoots from closely placed axils, due to possible stimulation of axillary buds, result in crowding of shoots at the apical portion, giving a bushy appearance to the plants. The leaves may become epinastic and leathery. The main stems and axillary branches become very thin compared to healthy ones. The stalk of the phylloid flowers is generally elongated, where as the normal flower has very short pedicels (Fig. 1). Phyllody-affected plants become sterile, resulting in total loss of yield.

**Fig. 1.** Healthy and normal flowers (left upper kabuli type and lower desi type) and abnormal green structure (phyllody, virescence and proliferation) on the right.

Severity of phyllody was found to be associated with the time of infection. Plants infected during early stages of development showed severe symptoms on whole plants while plants infected later showed severe symptoms on the upper part only. Sometimes normal flowers were produced on symptomless parts of a plant, occasionally followed by some rudimentary flowers that yielded very small, sickle-shaped, upright and leathery pods with degenerate seeds. However, seeds developed on partially infected plants had a bitter taste and sometimes they germinated in the pods.

Infected plants scattered in the field were more easily spotted at flowering and podding time. When the healthy plants were drying, diseased ones remained green.

**Light microscopy.** Light microscopy of Dienes stained sections from stems of infected plants showed regularly distributed dark blue areas in the phloem region (not shown). No such areas were found in sections from healthy tissues or in diseased tissues other than phloem.

**Electron microscopy**. Infected chickpea tissues showed typically pleomorphic (phytoplasma) bodies 200-600 nm in diameter, confined to the sieve elements (not shown). Such bodies were absent from healthy samples.

**Molecular characterization.** DNA was extracted from six infected plants and amplified using the universal phytoplasma PCR primers P1/P7. PCR products were characterized by RFLP analysis and sequencing of one isolate between the R16F2n and R16R2 primers. All samples from symptomatic plants gave the expected 1800 bp PCR product and the RFLP profiles using *Alu*I and *Hpa*II were identical for all six, and the same as that associated with 16SrII-D phytoplasmas, including others from our collection at the University of Nottingham (Hodgetts *et al.*, 2007, 2008) (results not shown). Sequencing confirmed that the phytoplasma had 100% sequence identity with the 16Sr-II-D tomato big bud phytoplasma over the entire 1247 bp of the R16F2n/R16R2 region. This sequence has been deposited at GenBank with accession No. FJ870549.

**Table 1.** Results of graft and insect transmission trials.

| Method of inoculation | | Plants used for transmission trials (No.) | Plants with disease symptoms (No.) | Disease transmission (%) |
|---|---|---|---|---|
| Grafting | | 10[a] | 7 | 100 |
| Plants exposed to insects after a 5-day AAP on phyllody infected plants | *A. craccivora* | 10 | 0 | 0 |
| | *M. persicae* | 10 | 0 | 0 |
| | *O. orientalis* | 10 | 7 | 70 |
| | *E. devastans* | 10 | 0 | 0 |
| | Unidentified brown leafhopper | 10 | 0 | 0 |
| Plants inoculated with insects immediately after capture from diseased field | *A. craccivora* | 10 | 0 | 0 |
| | *M. persicae* | 10 | 0 | 0 |
| | *O. orientalis* | 10 | 2 | 20 |
| | *E. devastans* | 10 | 0 | 0 |
| | Unidentified brown leafhopper | 10 | 0 | 0 |

[a]Success of grafting was 70% as only 7 grafts survived.

**Transmission**. Under glasshouse conditions, sap and seed transmission of the infectious agent could not be achieved, which indicates that chickpea phyllody is not mechanically or seed transmissible. The phyllody disease was transmitted from infected to healthy chickpea plants by grafting. Of the 10 grafts seven survived, showing disease symptoms 20-25 days after grafting (Table 1).

Transmission trials with *O. orientalis* were successful, while *A. craccivora*, *M. persicae*, *E. devastans* and the unidentified brown leaf hopper failed to transmit the disease. The rate of transmission with *O. orientalis* was higher (70%) when chickpea plants were inoculated with leafhoppers which had been reared on infected chickpea. It was less (20%) when leafhoppers captured in the field were directly released onto healthy chickpea seedlings (Table 1).

## DISCUSSION

Since the first report of chickpea phyllody from Coimbatore in Tamil Nadu (India) in 1959 (Ghanekar *et al*. 1988), this syndrome has been observed in Ethiopia, Myanmar, (Ghanekar *et al*., 1988), Australia (Saqib *et al*., 2005) and Oman (Al-Saady *et al*., 2006). In Pakistan this disease was observed for the first time in 2005 (Akhtar *et al*., 2008). On the basis of major disease symptoms, graft inoculation, *O. orientalis* transmission, reaction with Dienes stain, direct observation of pleomorphic bodies in sieve cells and amplification of a characteristic 1800 bp 16S rDNA fragment, it was confirmed that chickpea plants are infected by a phytoplasma**.** Dienes staining showed regularly distributed areas in the phloem region similar to those observed for other phytoplasmas (Salehi and Izadpanah, 1992). TEM revealed the presence of pleomorphic bodies similar to previously reported phytoplasmas (Credi, 1994; Samad *et al*., 2002; Ajayakumar *et al*., 2007). A similar etiology for chickpea phyllody has been reported in India (Ghanekar *et al*., 1988), Australia (Saqib *et al*., 2005) and Oman (Al-Saady *et al*., 2006).

The major symptoms of chickpea phyllody in Pakistan, such like virescence, phyllody and branch proliferation are similar to those described in other parts of the world. Furthermore, the phytoplasma isolate is in the same 16SrII-D alfalfa witches' broom group as the isolates from Australia and Oman, although the isolate in Oman was mistakenly referred to as 16SrII-B by Al-Saady *et al*. (2006). In Pakistan, germination of seeds in pods of phyllody-affected plants was also observed and this has not been reported elsewhere. However germination of seeds in nature has been found in capsules of phytoplasma-infected sesame in Iran (Salehi and Izadpanah, 1992). The yellowing symptoms reported from Oman and the leaf deformity reported from Australia were not commonly observed in Pakistan.

Our present results indicate that seed and sap transmission from phyllody-infected chickpea plants to susceptible chickpea does not occur. However, the disease was transmitted from diseased to healthy chickpea by grafting and an insect vector. These results conform to previous reports for phytoplasmas. *O. orientalis* was the only major leafhopper species found in chickpea fields and this is the first time that this species is reported as a natural and experimental vector of chickpea phyllody in Pakistan. Our results are in agreement with those from India (Ghanekar *et al*., 1988) where chickpea phyllody was reported to be vectored by *O. orientalis* (Matsumura [=*albicinctus* (Distant)]. *O. orientalis* is a natural vector of 16SrII group phytoplasmas and it was also report-

ed to transmit 16SrII phytoplasma to sesame in India, Thailand, Upper Volta (Schneider *et al*., 1995), Iran (Esmailzadeh-Hosseini *et al*., 2007), 16SrVI phytoplasma to sesame in Turkey (Sertkaya *et al*., 2007) and the associated phytoplasma in Thailand belonging to the peanut witches' broom group (16SrII-D) (Schneider *et al*. 1995). This group has frequently been found in Asia and Australasia.

As the Pakistan chickpea phyllody phytoplasma is very closely related to the tomato big bud (TBB) and sweet potato little leaf (SPLL) phytoplasmas detected elsewhere, a search was conducted for phyllody disease in tomato plants grown adjacent to infected chickpea field, but no symptomatic plants were observed. This apparent lack of cross infection may be due to host resistance or vector specificity (Davis *et al*., 1997). Further experimentation is now required to determine whether similar phytoplasmas are found in other plant species in Pakistan and whether they are associated with disease in other crops.

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