# FIRST REPORT OF PHYTOPLASMAS ASSOCIATED WITH YELLOW DISEASE IN CARROTS (*Daucus carota* L.) IN CIANJUR, WEST JAVA

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## ABSTRACT

Yellow disease was discovered in carrot fields in Cianjur but has never been reported. The disease is thought to be associated with phytoplasma, but the pathogen remains unidentified until now. The purpose of this study, therefore, was to characterize the suspected phytoplasmas associated with yellow disease on carrot in Cianjur and also observe the potential role of two leafhopper species in the transmission of the disease. DNA target of phytoplasma was amplified by nested-PCR using universal primers P1/P7 followed by specific primers R16F2n/R16R2. Specific DNA fragment of 1250 bp in size was successfully amplified, and this amplicon was subjected for nucleotide sequencing followed by BLAST analysis. Based on nucleotide analysis, the phytoplasma associated with yellow disease of carrots is identified as 16SrII-Cactus witches'- broom group. The transmission study indicated that 2 species of leafhoppers, *i.e*. *Balclutha incisa* and *Orosius argentatus* may play a role as vector and assist in the spread of yellow disease of carrot. This is a new report on phytoplasmas infecting carrots in Indonesia and the potential of two leafhopper species as disease vectors.

**Key words :** *Balclutha incisa*, insect transmission, nested-PCR, *Orosius argentatus*, sequence analysis

## INTRODUCTION

Phytoplasma has a wide host range among cultivated as well as weed plants. Several types of phytoplasma were reported to infect carrots in some countries, including the phytoplasma from groups 16SrII-B, 16SrII-D, 16SrIII, 16SrV, and 16SrXII-A in Asia; 16SrI-A, 16SrI-B, 16SrII-C, and 16SrXII-A groups in Europe, among others (Kumari et al. 2019). Phytoplasma infections in Indonesia were previously reported in soybean (Asniwita et al. 1999), peanuts (Nugroho et al. 2000), Bermuda grass (Mutaqin et al. 2003), ornamental peanuts (Budiyarto and Mutaqin 2012), coconut plants (Prasetyo et al. 2017), as well as cacti and bamboo (Prasetya et al. 2018). Yellow disease of carrots in Cianjur, West Java was never reported, although its incidence in the field has been found. Yield losses due to phytoplasma infection in carrots were reported from other countries. The infection of Aster yellows phytoplasma In carrots in Canada caused a 70-80% decrease in yield (Arcelin and Kushalappa 1991).

The host range of phytoplasm is highly dependent on the eating preferences of the insect vector (Weintraub and Beanland 2006). Tomato big bud phytoplasma (group 16SrII-D) which causes yellow disease on grapes in Australia is reported to be transmitted in the field through the leafhoppers *Orosius orientalis* (Constable 2010). It is becoming important to identify the potential of insects found in the field as the vector of yellow disease. This study was initiated to identify the associated phytoplasma in

carrot plants with yellow disease and to determine the potential for phytoplasma transmission through two species of leafhoppers found in the carrot field in Cianjur.

## MATERIALS AND METHODS

**Symptoms observation and phytoplasma frequency assessment.** The observation of yellow disease was conducted on a 1.5 - 3 months old carrot field in Cianjur Regency (Ciherang Village, Pacet District) from March 2016 to August 2018. Plant samples was taken using a diagonal method, with a total of 20 symptomatic plant samples which were further analyzed using the PCR method. The frequency of phytoplasma infection was calculated as the proportion of plant samples giving positive reaction to PCR detection divided by the total number of samples detected and multiplied by 100%.

**Leafhoppers trapping from the field.** Insect trapping was conducted in the same location as those of plant sampling. The insects were trapped using insect nets, *i.e*. as much as 5 swings of 2.5 m or covering 20 plants. Two leafhopper species, namely *Balclutha incisa* and *Orosius argentatus* were selected from the insect net collections and allowed to reproduce on healthy carrot plants for further use in transmission experiments. These two species of leafhoppers were considered as potential vector of phytoplasma based on previous studies, in which phytoplasma was successfully amplified from the 2 species by PCR detection. Confirmation of leafhopper species was conducted in advance before rearing, *i.e*. based on morphological characters following the key dichotomous of Wilson and Claridge (1991).

**Phytoplasmas identification based on nucleotide sequence analysis of 16S rRNA genes.** The total DNA isolation of symptomatic leaf samples was performed using DNeasy Plant Mini Kit (Qiagen, Germany) with a slight modification, *i.e*. addition of 2% CTAB buffer. Furthermore, the total DNA was used as a template in the amplification stage using P1/P7 primers in a standard PCR procedure. Then, the amplicon obtained was diluted (1:30 v/v) and used as a template in the next amplification stage using R16F2n/R16R2 primers in nested-PCR procedure (Table 1). DNA amplification was carried out using MyTaqTM HS Red Mix reaction kit (Bioline, London) on an Applied Biosystems Veriti 96 Well thermal cycler machine (Thermo Fisher Scientific, USA) according to a predetermined program. Amplification of target DNA at the first PCR begins with denaturation of DNA strands at 94 ºC for 4 min for 1 cycle, then continues with 32 cycles at several stages: denaturation at 94 ºC for 1 min, annealing at 59 ºC for 1 min and DNA synthesis at 72 ºC for 1.5 min. The DNA synthesis stage ended with 1 cycle at 72 ºC for 5 min (Deng and Hiruki, 1991; Schneider et al., 1995). The nested-PCR stage begins with the preparation of denaturation at 92 ºC for 1 min for 1 cycle, then continues with 35 cycles at several stages: denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min and DNA synthesis at 72 ºC for 1.5 min, the DNA synthesis stages ended with 1 cycle at 72 ºC for 10 min (Gundersen and Lee 1996). The amplified DNA was visualized by electrophoresis using 1% agarose gel. Cacti-infected phytoplasma that has been identified in the previous study (Prasetya et al. 2018) was used as positive control in this study.

**Table 1.** Primer sequences used for amplification of phytoplasma

| Primer | Sequence | PCR product (bp) | Reference |
|---|---|---|---|
| P1 | 5'-AAG AGT TTG ATC CTG GCT CAG GATT-3' | 1800 | Deng and Hiruki (1991) |
| P7 | 5'-CGT CCT TCA TCG GCT CTT-3' | | Schneider et al. (1995) |
| R16F2n | 5'-GAA ACG ACT GCT AAG ACT GG-3' | 1250 | Gundersen and Lee (1996) |
| R16R2 | 5'-TGA CGG GCG GTG TGT ACA AAC CCC-3' | | |

The amplified DNA fragments were sent to First Base DNA sequencing (Malaysia) for nucleotide sequencing. Nucleotide sequence analysis was performed using Bio Edit version 7.1.7, followed by the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Analysis of the relationship between phytoplasma samples and construction of phylogenetic tree was carried out using ClustalW and Molecular Evolutionery Genetic Analysis (MEGA 6) with the Neighbor-Joining algorithm method using 1000 bootstrap replications. *Spiroplasma citri* (M62054) was used as a comparison outside the group (out group). DNA fragments as the products of amplification using nested-PCR were used for DNA cloning using TA cloning method (Thermo Scientific, USA). Confirmation of insert DNA on recombinant clones was carried out through nucleotide sequence analysis.

**Phytoplasma cell morphology.** Observation of phytoplasma cells using a transmission electronic microscope (JEOL JEM-1010) was carried out at the Eijkman Institute for Molecular Biology, Jakarta. Phytoplasma cell morphology was observed in the phloem tissue of carrot leaves. Electron microscope sample preparations were carried out following the positive stain method according to the Bozzola and Rusell (1999) protocol.

**Transmission study using leafhoppers**

***Rearing of leafhoppers.*** Two month-old healthy carrot plants, confirmed by PCR detection, were used for rearing the leafhoppers. Insect rearing was carried out in carrot fields at Ciherang Village, Pacet District, Cianjur Regency. *B. incisa* and *O. argentatus*, were reared on separate carrot plants. A total of 60 leafhoppers of respective species were invested in each healthy carrot plant. Following insect infestation, each plants were contained in insect-proof cages to obtain phytoplasma-free offspring of leafhoppers. Rearing of the leafhopper was carried out for approximately 45 days.

***Propagation of inoculum source.*** Carrot plants showing yellow symptoms were obtained from the test site. These plants were confirmed positively infected by phytoplasma using nested-PCR method. The carrot plants were then isolated by placing them in an insect-proof cage until these were ready for use.

***Transmission of phytoplasma.*** Carrot seeds were sown in a greenhouse for 1.5 months. All plants used in the transmission study were confirmed phytoplasma-free by nested-PCR detection as described above. Leafhoppers were given an acquisition feeding period on infected plants for 3 days and later transferred to the test plant to be given a latent period. This was followed by an inoculation feeding period of 3 days. As a control treatment, the transmission stages were carried out using phytoplasma-free leafhoppers.

The parameters of this study consisted of disease incidence and incubation period. Disease incidence is measured based on the proportion of the number of infected plants and the total number of plants observed and multiplied by 100%; whereas the incubation period is determined based on the time when disease symptoms, on leaves and clumps of plants, first appear after inoculation.

## RESULTS AND DISCUSSION

**Symptoms of yellow disease and frequency of phytoplasma infection.** Symptom variation was observed on carrot plants in the survey location, including yellow and reddish yellow leaves; shape malformation, smaller size, and hairy root growth on tuber; and dwarf plants (Fig. 1). Infection of phytoplasma may cause chlorophyll damage in leaves, which in turn results in inhibition of chlorophyll formation process, pigment changes and chlorosis (Agrios 2005). Although the symptoms found were very severe in some samples, the frequency of phytoplasma infection was relatively low (7.47%). Many factors may influence this condition, including low leafhopper population as potential vectors and the low concentration of phytoplasma in the infected plants. Symptoms appeared seven days after infection

through the insect vector. Even in some cases, symptoms appeared 6-24 months after infection. The latency period of phytoplasma in the host plant depends on the type of phytoplasma and the plant species (Hogenhout et al. 2008).


**Fig. 1.** Symptoms of yellow disease on carrots in Cianjur, West Java. Yellow leaves (a, b and c); tuber with hairy roots (d)

**Identity of phytoplasma based on the sequence of 16S rRNA genes.** Specific fragments of DNA were visualized at 1250 bp (Fig. 2) and nucleotide sequence of 1251 to 1253 in length was obtained from this fragment. Based on the BLAST analysis, one type of phytoplasma was identified, *i.e*. 16SrII-Cactus witches'- broom group (Table 2). Similarly, phylogenetic analysis showed that the phytoplasma from the carrot plant was closely related to the 16SrII-Cactus witches'-broom group (Fig. 3). One recombinant DNA plasmid was successfully obtained, with an inserted fragment size of 1450 bp. When this plasmid was sequenced, it was shown that it has 87.7% similarity to 16SrI-Henon bamboo witches'-broom group (Table 2). This result was different from those of amplicon fragments, which may indicate a mixed infection of several types of phytoplasma in the field. A similar result was reported wherein three different subgroups of phytoplasma, *i.e.* 16SrI-A, 16SrI-B and 16SrXII-A were detected from symptomatic carrots in Serbia (Duduk et al. 2008).

**Table 2.** Nucleotide homology of phytoplasma on carrots with phytoplasm in the GenBank database based on sequence of 16Sr RNA

| Phytoplasma isolates | Sequence length (bp) | Query cover (%) | Homology (%) | 16Sr Group | Identified type of phytoplasma | Accession Number |
|---|---|---|---|---|---|---|
| Carrots CJ-1 | 1253 | 94 | 95.6 | II | Cactus witches'- broom phytoplasma | EU099572 |
| Carrots CJ-2 | 1251 | 98 | 94.7 | II | Cactus witches'- broom phytoplasma | EU099565 |
| Carrots CJ-3 | 1251 | 97 | 94.9 | II | Cactus witches'- broom phytoplasma | EU099573 |
| Carrots CJ-4 | 1251 | 98 | 94.5 | II | Cactus witches'- broom phytoplasma | EU099573 |
| Carrots CJ-5 | 1253 | 98 | 94.2 | II | Cactus witches'- broom phytoplasma | EU099565 |
| Carrots_CJ (Cloning) | 1326 | 94 | 87.7 | I | Henon bamboo witches' -broom phytoplasma | AB242433 |

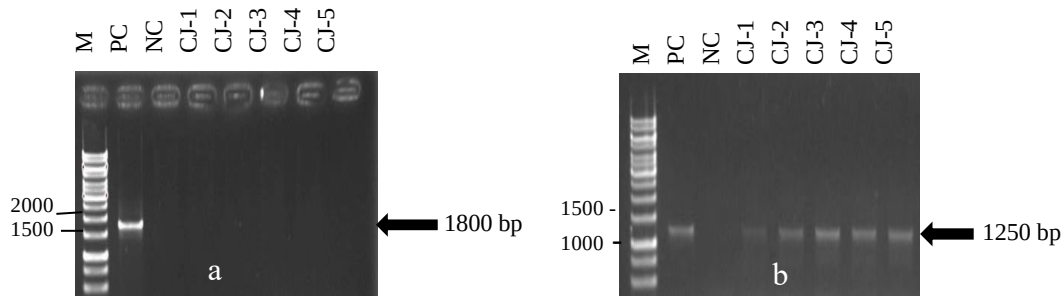


**Fig. 2.** Amplification of specific DNA fragment of phytoplasma from carrot leaves using standard PCR (a) and nested-PCR (b) methods. CJ1 to CJ5, yellow leaf samples; PC, positive control; NC, negative control (nuclease free water); M, DNA ladder (1 kb)

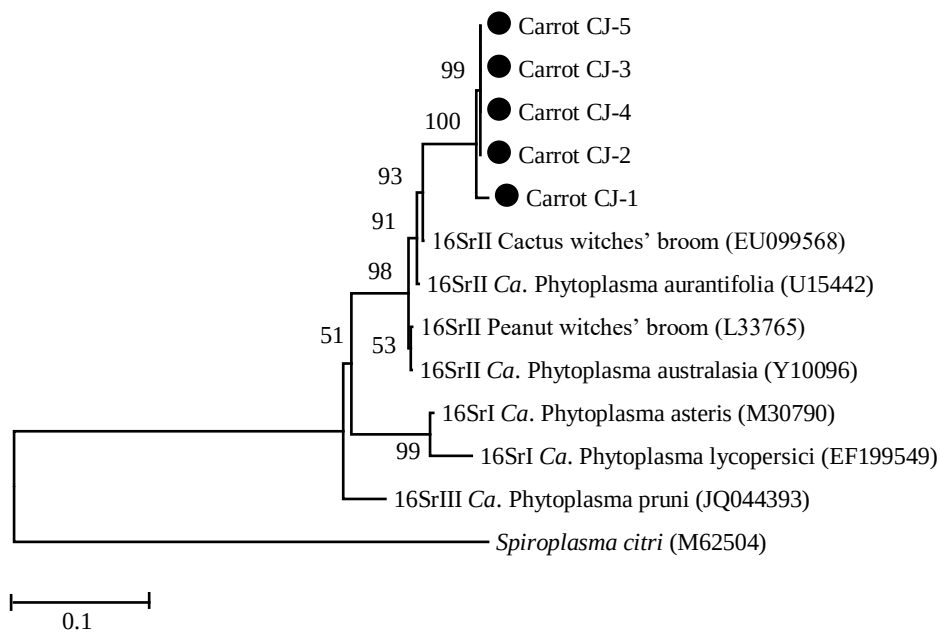


**Fig. 3.** Phylogenetic tree of phytoplasm from carrot (●) based on 16S rRNA gene sequence. The sequences of several phytoplasma reference groups were included, *Spiroplasma citri* was used as the outgroup sequence

Phytoplasma infections of group 16SrII in carrots associated with yellow disease were previously reported in Iran (Salehi et al. 2016) and Qassim province, Saudi Arabia (Omar 2017). Phytoplasma group 16SrII in Indonesia was previously reported to infect coconut plants in Derawan Island (Prasetyo et al. 2017); peanuts, soybeans, string beans and cacti (Prasetya et al. 2018). The 16SrI-*Candidatus* Phytoplasma asteris in Indonesia was previously reported to infect banana cv. mangosteen in Tasikmalaya and cv. king jackfruit in Banjar (Sibarani et al. 2019). The occurrence of phytoplasma groups 16SrII and 16SrI in the carrot planting area in Cianjur is a concern because this pathogen is known to infect many host plants. In addition, this phytoplasma can be transmitted through leafhoppers *O. argentatus*, which is known to exist in Indonesia.

**Presence of phytoplasma cells in phloem tissue.** Cells of phytoplasma in various shapes and sizes were found in phloem tissue of diseased plants but were not found in healthy plants. The phytoplasma cells are rounded (spherical) but not fixed (pleomorphic) measuring about 0.2-0.5 μm (Fig. 4). Similar results were reported in Pakistan with 16SrII group phytoplasma cells infecting carrots. In the phloem tissue of carrot leaves with yellow symptoms, phytoplasma cells were found with various shapes in diameter ranging from 200 - 600 nm, while in healthy tissue there were no phytoplasma cells (Sharif et al. 2019).

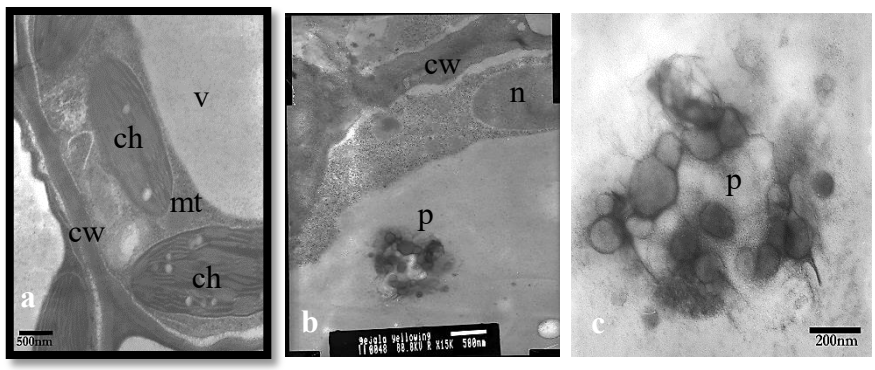


**Fig. 4.** Micrograph of carrot leaf phloem cell. Healthy tissue, no phytoplasma cells (magnification 15000x) (a); Diseased leaf tissue showing phytoplasma cells (magnification 40000x) (b and c). cw, cell wall; ch, chloroplast; mt, mitochondria*;* n, nucleus; p, phytoplasma; v, vacuole

**Leafhopper transmission of phytoplasma.** The phytoplasma will remain in the insect's body until the end of its life, but the phytoplasma cannot be passed on to the next generation of insect vector (non-transovarial). *Ca.* Phytoplasma ulmi were not found in in the egg, 1st and 5th instar of the insect vector *Amplicephalus curtulus* although it was detected at the imago stage (Arismendi et al. (2015). Furthermore, none of the eggs samples of *Diaphorina citri* (200 eggs from 10 phytoplasma-infected female) were positive for *Ca.* Phytoplasma aurantifolia (Queiroz et al.2018). Chlorosis was observed after transmission using *B. incisa* and *O. argentatus* (Fig 5). The incubation period for phytoplasma transmission using *B. incisa* and *O. argentatus* were 26 days and 28 days, respectively; while the percentage of transmission (disease incidence) was 80%, for both of them (Table 3). This shows that *O. argentatus* and *B.incisa* have the same ability to transmit phytoplasma to carrots. Detection using nested-PCR method was carried out to confirm the association of phytoplasma in these carrot plants showing chlorosis symptoms. Association of phytoplasma was evidenced except for Bi1 and Oa2 (Fig 6). This transmission study indicated the importance of monitoring the populations of *O. argentatus* and *B. incisa* on carrot to prevent further spread of the phytoplasma, so than it can reduce the incidence of yellow disease in the field.

**Table 3.** Transmission study of phytoplasma in carrots through leafhoppers

| **Leafhoppers** | **Transmission result[a]** | | | **The frequency of positive nested-PCR in the testing plants at day-** | | | |
|---|---|---|---|---|---|---|---|
| | **Symptoms (DI)** | | **IP** | **7** | **14** | **21** | **30** |
| | **Treatment** | **Control** | | | | | |
| *B. incisa* | 4/5 (80%) | Asymptomatic | 26.75 | 0/5 | 4/5 | 4/5 | 4/5 |
| *O. argentatus* | 4/5 (80%) | Asymptomatic | 28 | 0/5 | 1/5 | 3/5 | 4/5 |

[a] Transmission was carried out respectively on 5 carrots

DI = Disease Incidence

IP = The incubation period was calculated as the day after inoculation.


**Fig. 5.** Transmission of phytoplasma using leafhoppers. Control plants in transmission using *Balclutha incisa* (a) and *Orosius argentatus* (b); Infected plants in transmission using *B. incisa* (c) and *O. argentatus* (d). Symptoms of phytoplasma include chlorosis on leaves, small leaves, stunted plant growth, small petioles with many branches (c) and (d)

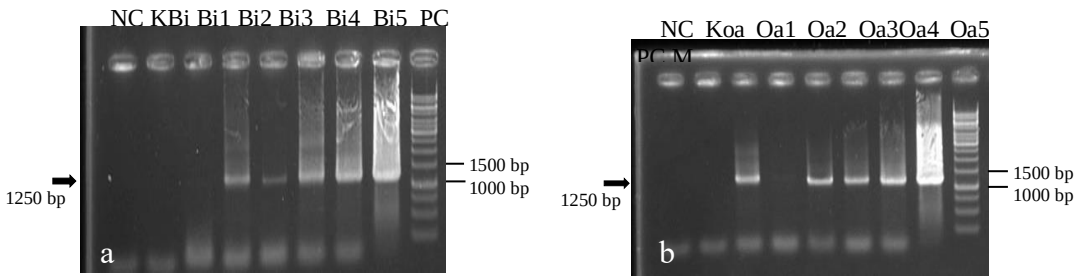


**Fig. 6.** Amplification of phytoplasmas DNA by nested-PCR from carrot plants in the transmission study using *Balclutha incisa* (a) and *Orosius argentatus* (b). KBi and Koa, control plant samples; Bi1 to Bi5, plant samples from *B. incisa* transmission study; Oa1 to Oa5, plant samples from *O. argentatus* transmission study; PC, positive control (source of inoculum); NC, negative control (nuclease free water); M, DNA ladder (1 kb)

## CONCLUSION

Cells of phytoplasma in various shapes and sizes were found in phloem tissue of carrot plants showing yellow symptoms. Two species of leafhopper, *O. argentatus* and *B. incisa*, found in carrot fields showed the same efficiency in transmitting yellow disease. Molecular identification based on 16S rRNA sequences confirmed the association of 16SrII-Cactus witches'- broom group and 16SrI-Henon bamboo witches'-broom group with the diseased plants. Further investigation should be carried out to identify the full range of phytoplasmas associated with yellow disease on carrot.

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