JURNAL FITOPATOLOGI INDONESIA

RESEARCH ARTICLE

Maret, 2026. Vol. 22, No. 1, Hlm. 1-6
DOI: https://doi.org/10.14692/jfi.22.1.1-6

First Report of Phytoplasmas Associated with Strawberry Proliferation and Tall Fleabane Witches' broom Diseases in West Java, Indonesia

Laporan Pertama Fitoplasma Berasosiasi dengan Penyakit Proliferasi Stroberi dan Sapu Jalantir di Jawa Barat, Indonesia

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Article history:
Received: 21 July 2025
Rivised: 22 January 2026
Accepted: 12 March 2026

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ABSTRACT

Phytoplasmas are bacteria from the class Mollicutes that have been widely reported to be associated with diseases in hundreds of plant species across various countries. In Indonesia, infection of phytoplasmas have been reported from more than eight different plant families. This study aimed to confirm the association of phytoplasmas with strawberry plants showing proliferation symptoms and a wild plant exhibiting witches' broom symptoms in West Java, using nested-PCR with two primer pairs designed from the 16S–23S rRNA gene region of phytoplasmas. Wild plants were identified both morphologically and molecularly based on the ITS region of the ribosomal RNA gene. Symptoms in strawberry (*Fragaria ananassa*) include shoot proliferation, phyllody, and dwarf fruits, while the wild plant exhibited witches' brooms symptoms. The wild plant, commonly known as tall fleabane (Jalantir, in Bahasa Indonesia), was successfully identified as *Conyza sumatrensis* based on morphological characteristics and nucleotide sequence analysis of PCR amplicons using universal primers ITS1/ITS4. Initial PCR amplification using a primer pair P1/P7 for phytoplasmas from both strawberries and tall fleabane produced a positive DNA band of approximately 1800 bp with inconsistent band intensities. Further amplification using nested-PCR with a primer pair R16F2n/R16R2 yielded a stronger DNA band of approximately 1250 bp. The nested-PCR results confirmed that phytoplasmas are associated with both plants. This study represents the first report of phytoplasma association with strawberries (*Rosaceae*) and tall fleabane (*Asteraceae*) in Indonesia.

Keywords: 16S-23S rRNA gene, *Conyza sumatrensis*, *Fragaria ananassa*, ITS1/ITS4, nested-PCR

ABSTRAK

Fitoplasma adalah kelompok bakteri dari kelas Mollicute yang telah banyak dilaporkan berasosiasi dengan penyakit pada ratusan spesies tanaman di berbagai negara. Di Indonesia, fitoplasma baru diketahui berasosiasi dengan tanaman-tanaman inang lebih dari delapan famili berbeda. Penelitian ini bertujuan membuktikan asosiasi fitoplasma pada tanaman stroberi bergejala filodi dan suatu tanaman liar bergejala daun kecil di Jawa Barat dengan teknik *nested-polymerase chain reaction* (*nested*-PCR) menggunakan dua pasangan primer yang dirancang dari wilayah gen 16S-23S rRNA fitoplasma. Tumbuhan liar juga diidentifikasi secara morfologi dan molekuler berdasarkan wilayah ITS gen ribosomal RNA. Gejala penyakit pada stroberi (*Fragaria ananassa*) meliputi proliferasi tunas, filodi dan buah kerdil, sedangkan pada tumbuhan liar berupa sapu. Tumbuhan liar yang dikenal masyarakat sebagai jalantir berhasil diidentifikasi sebagai *Conyza sumatrensis* berdasarkan morfologi dan analisis nukleotida amplikon hasil PCR menggunakan pasangan primer universal ITS1/ITS4. Amplifikasi PCR awal dengan pasangan primer P1/P7 untuk fitoplasma dari stroberi dan jalantir menghasilkan pita DNA positif berukuran ±1800 pb dengan ketebalannya yang inkonsisten. Amplifikasi lanjut dengan nested-PCR dengan pasangan primer R16F2n/R16R2 menghasilkan pita DNA berukuran ±1250 pb yang lebih tebal. Hasil *nested*-PCR mengonfirmasi bahwa fitoplasma berasosiasi dengan dua tanaman tersebut. Penelitian ini merupakan laporan pertama fitoplasma berasosiasi dengan tanaman stroberi (Rosaceae) dan jalantir (Asteraceae).

Kata kunci: 16S-23S rRNA gene, *Conyza sumatrensis*, *Fragaria ananassa*, ITS1/ITS4, nested-PCR

INTRODUCTION

Phytoplasma, formerly known as a mycoplasma-like organism (MLO), is a cell wall-less bacterium belonging to the class Mollicutes. It has been reported to cause diseases with a wide range of characteristic symptoms in plants, primarily due to its disruptive effects on plant hormones and nutrient transport. Common symptoms include leaf yellowing, stunted growth, and abnormal shoot proliferation (witches' broom), often accompanied by leaf shrinkage (small leaves). Other symptoms include floral abnormalities, such as the transformation of flowers into leaf-like structures (phyllody) and the development of green-colored floral parts (virescence). Phytoplasmas have been associated with diseases in 945 plant species worldwide (Wei *et al.* 2024), indicating their potential to pose a serious threat to global food security (Kumar *et al.* 2024).

Phytoplasmas have been reported to cause significant diseases in strawberries, including strawberry green petal (SbGP) and phyllody, in various strawberry-producing countries. Strawberry plants infected with phytoplasmas exhibit symptoms such as virescence, floral and fruit phyllody, dwarfism, multi-branched crowns, hypertrophy, chlorosis, and reddish leaves (Ferna´ndez *et al.* 2015; Cui *et al.* 2019; Brochu *et al.* 2021). Leafhoppers are the primary vectors responsible for the spread of SbGP disease through the acquisition and transmission of phytoplasmas (Santos *et al*. 2025).

Wild plants found around cultivated land can serve as alternative hosts for plant pathogens or their insect vectors, including phytoplasma species. Phytoplasmas associated with wild plants such as fleabane, which exhibit witches' broom symptoms, have been reported in Argentina and Brazil (Meneguzzi *et al.* 2008; Montano *et al.* 2014). Phytoplasmas from the 16SrI and 16SrVII-B groups have been reported to infect fleabane (Fernández *et al.* 2020).

Disease symptoms such as shoot proliferation in strawberries and witches' broom in tall fleabane (so-called Jalantir, in local language) have been observed in West Java; however, the causal agent has not yet been identified or reported. This study aimed to confirm phytoplasma as the pathogen associated with strawberry plants exhibiting proliferation symptoms and wild tall fleabane plants showing witches' broom symptoms in West Java, using the nested-PCR method. In addition, this study aimed to identify the species of tall fleabane based on morphological characteristics and molecular analysis.

MATERIALS AND METHODS

Plants Sampling

Sampling of fresh and intact plants was conducted in August 2024 at two locations in West Java. Strawberry plants showing proliferation symptoms were collected from Lembang, West Bandung (Geographical coordinates: S 6°43'44.5", E 107°4'31"), and wild plants exhibiting witches' broom symptoms or asymptomatic individuals were collected from Sukaresmi, Cianjur (S 6°48'8.8", E 107°36'22.9"). Bamboo plant samples with small leaf symptoms and cactus plants with broom blight symptoms, previously confirmed to be positively associated with phytoplasma, were used as positive reference plants.

DNA Extraction

Total DNA extraction from both symptomatic and healthy plants was performed using fresh, non-necrotic plant tissues, specifically veins and leaf stalks. The extraction followed the protocol of the genomic DNA mini kit (Geneaid, Taiwan). The extracted DNA was then used as templates for subsequent PCR amplification.

Identification of Wild Plant Species of Tall Fleabane

DNA extracted from healthy wild plants was amplified by standard PCR using the universal eukaryotic primer pair ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.* 1990). The PCR program was conducted according to Dogan *et al.* (2022) and included the following steps: Initial denaturation at 97 °C for 3 minutes; 35 cycles of denaturation at 97 °C for 15 seconds, annealing at 60 °C for 45 seconds, and extension at 72 °C for 2 minutes; followed by a final extension at 72 °C for 5 minutes. The amplification yielded products of approximately 700 bp. The PCR products were separated by electrophoresis on a 1% agarose gel at 100 V for 30 minutes. DNA bands were visualized under UV light using a transilluminator (Thermo Scientific, USA).

The PCR products were sent to First BASE Laboratories (Malaysia) for nucleotide sequencing. The resulting sequences were analyzed using the basic local alignment search tool (BLAST) on the national center for biotechnology information (NCBI) website (www.ncbi.nlm.nih.gov) to identify wild plant species. Phylogenetic analysis of the tall fleabane species was performed using molecular evolutionary genetics analysis (MEGA) software version 12.0.11. Phylogenetic trees were constructed using the maximum likelihood method with 1000 bootstrap replicates.

Phytoplasma Detection and Identification

The nested-PCR method targets the 16S–23S rRNA gene region (Figure 1) and involves two stages of amplification: (1) Primary PCR amplification of the extracted template DNA using the forward primer P1 (5'-AAG AGT TTG ATC CTG GCT CAG GATT-3')

(Deng and Hiruki 1991) and the reverse primer P7 (5'-CGT CCT TCA TCG GCT CTT-3') (Schneider *et al.* 1995), producing an amplicon of approximately 1800 bp; (2) Nested PCR amplification of the first-round PCR product, diluted 1:30 (v/v), using the forward primer R16F2n (5'-GAA ACG ACT GCT AAG ACT GG-3') and the reverse primer R16R2 (5'-TGA CGG GCG GTG TGT ACA AAC CCC-3') (Gundersen and Lee 1996).

Both PCR reactions were carried out using MyTaq™ HS Red Mix (Meridian Bioscience, USA) on a Thermal Cycler T100™ (Bio-Rad, USA) under the following cycling conditions: Initial denaturation at 92 °C for 1 minute; followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1.5 minutes; and a final extension at 72 °C for 10 minutes. Separation and visualization of PCR amplicons were performed as previously described.

RESULTS

Symptoms of Disease in Strawberries and Tall Fleabane

Strawberry plants in the field exhibited various symptoms affecting multiple parts of the plant, including the development of leaf-like structures on flowers (phyllody). When fruits were produced, they appeared stunted (Figure 2a, b). The plants also showed shoot proliferation, characterized by excessive shoot growth that inhibited optimal fruit development (Figure 2c).

Wild plants in the field exhibited leaf malformation and reduced size. Leaves emerged from shortened stem internodes, resulting in a typical witches' broom. Flower developing from the shortened stems were also smaller in size (Figure 2d).

Identification of Wild Plant Species as *Conyza sumatrensis*

Tall fleabane is a wild plant from the Asteraceae family and is considered an invasive weed in Indonesia. This species has serrated leaf margins and pointed tips (Figure 3a). Mature plants in the generative phase produce densely clustered, bud-shaped flowers (Figures 3b, c).

Tall fleabane was successfully identified as *C. sumatrensis* (synonym: *Erigeron sumatrensis*) based on the nucleotide sequence analysis of PCR amplicons generated using the ITS1/ITS4 primer pair, which targets the ITS region commonly used as a species marker for *Conyza* spp. PCR amplification with the ITS1/ITS4 primers successfully yielded a DNA fragment of approximately 700 bp from tall fleabane (Figure 4a). Based on BLAST analysis against sequences in the GenBank database, the wild plant showed 98.88% similarity to *C. sumatrensis* from China (Table 1).

Phytoplasma Detection by PCR Method

Amplification of phytoplasma DNA from strawberry plants using standard PCR revealed the presence of DNA fragments of approximately 1800 bp in sample St2; however, the fragment was too

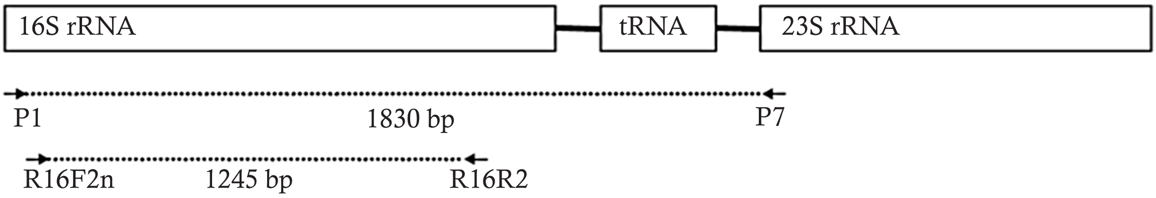

Figure 1 The primary primer pairs P1/P7 and R16F2n/R16R2 are located in the 16S–23S rRNA gene region (Hodgetts *et al.* 2008).

Figure 2 Strawberry plants from Lembang (a – c) and a wild plant from Sukaresmi (d) showing symptoms. a, Phyllody; b, Shoot proliferation; c, Stunted fruit; and d, Witches' broom symptoms.

Figure 3 Morphology of wild tall fleabane plants. a, Vegetative phase; b, Generative phase; and c, Inflorescence consisting of flowers, fruits, and compound seeds.

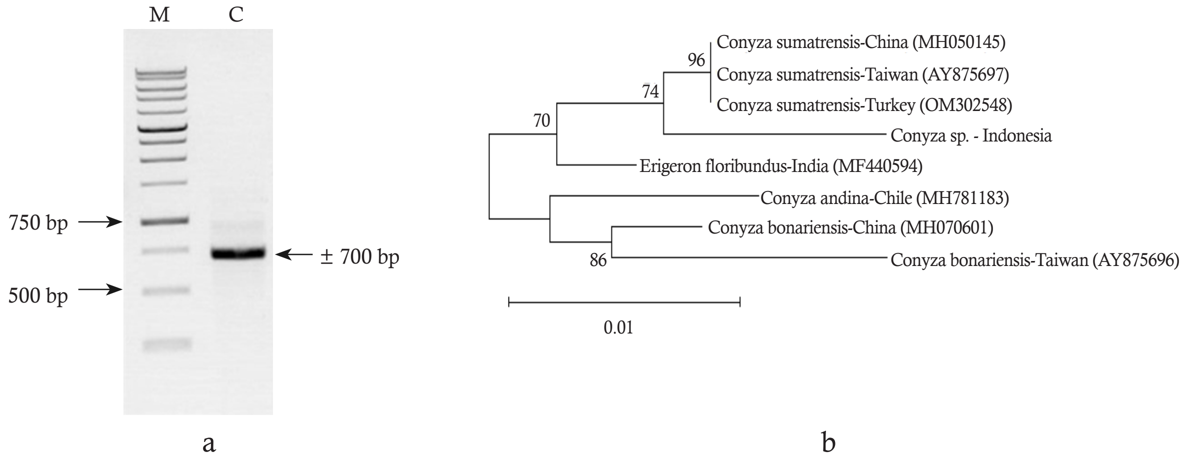

Figure 4 a, DNA fragments of *Conyza sumatrensis*; and b, Phylogenetic tree showing the relationship of *Conyza sumatrensis* from Indonesia with other *Conyza* species.

Table 1 Percentage of nucleotide identity of wild plant samples compared to available nucleotide accessions in GenBank based on BLAST analysis

Species/ Country of origin	Size (bp)	Percentage (%)		Accession number
		Query cover	Identity	
Conyza sumatrensis / China	702	100	98.88	MH050145
Conyza sumatrensis / Taiwan	625	100	98.88	AY875697
Conyza sumatrensis / Turkey	649	100	98.88	OM302548
Erigeron floribundus/ India	600	100	98.31	MF440594
Conyza andina / Chile	706	100	97.94	MH781183
Conyza bonariensis/ China	698	100	97.75	MH070601
Conyza bonariensis/ Taiwan	625	100	97.19	AY875696

faint to be reliably considered positive (Figure 5a). In contrast, nested-PCR successfully amplified DNA fragments of approximately 1250 bp in all samples (St1–St4), with clearer and thicker bands (Figure 5b). In *Conyza sumatrensis*, standard PCR also produced DNA fragments of approximately 1800 bp in all samples (Fb1–Fb4), although with varying band intensities. Weak or faint bands may lead to false-negative results. Nested-PCR yielded stronger and more distinct fragments of approximately 1250 bp in samples Fb1, Fb2, and Fb4.

DISCUSSION

Strawberry plants from Lembang exhibited symptoms commonly associated with phytoplasma infection, specifically phyllody and proliferation. Similar phyllody symptoms have been reported in strawberries in Mexico infected with Mexican periwinkle virescence (MPV) phytoplasma, which belongs to group 16SrXIII (Avendaño-Benequen *et al.* 2017). Phytoplasma infection in strawberries was also

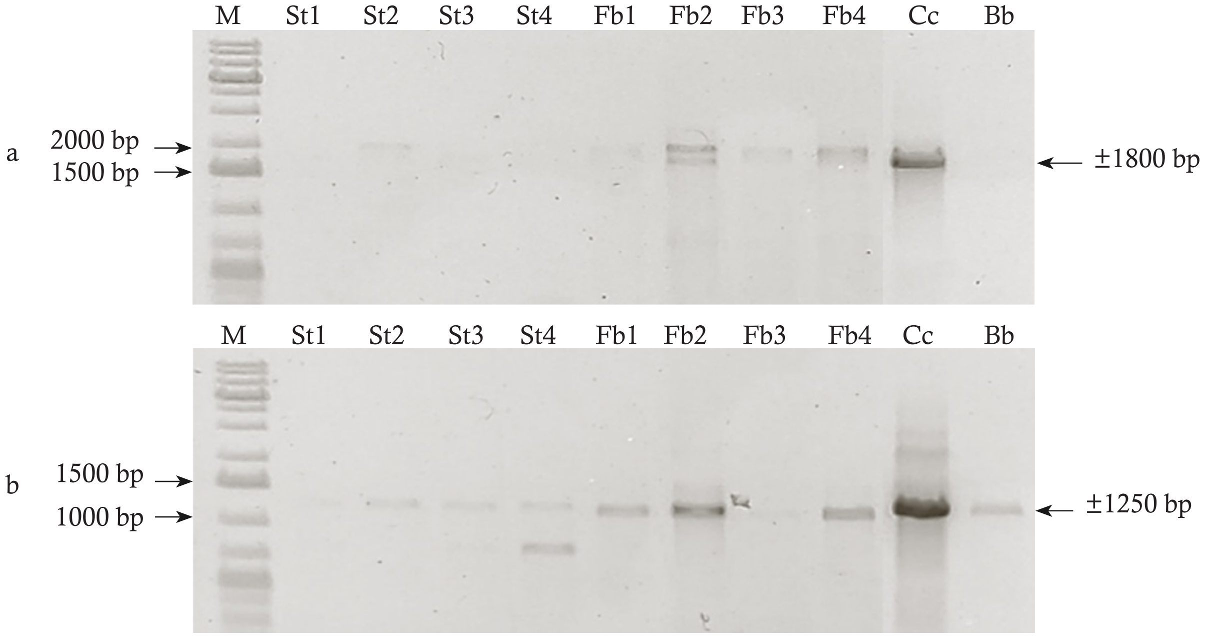

Figure 5 Phytoplasma DNA fragments amplified by standard PCR using primer pairs a, P1/P7; and b, R16F2n/R16R2. M, 1 kb Plus DNA Ladder (Thermo Scientific, USA); St, Strawberry; Fb, Fleabane; Cc, Cactus; and Bb, Bamboo.

reported by Cui *et al.* (2019), identifying 16SrXIII and 16SrXIII-F phytoplasmas as being associated with phyllody symptoms. Furthermore, the association of phytoplasmas with strawberries is not limited to group 16SrXIII, as phytoplasmas from group 16SrI, such as *'Candidatus* Phytoplasma asteris', have also been reported as causal agents (Brochu *et al.* 2021).

Detection was carried out through amplification using the nested-PCR method, which revealed the presence of DNA fragments of approximately 1250 bp visualized on agarose gel. Strawberry plants (*F. ananassa*) exhibiting symptoms of phyllody and virescence tested positive for phytoplasma using nested-PCR targeting the 16S rRNA group (Cui *et al.* 2022). Similarly, Strawberry multicipa (SM) has also been detected with phytoplasma and phylogenetically classified within the 16SrVI-B group, with symptoms such as phyllodic fruit, dwarfism, and crown proliferation (Jomantiene *et al.* 2002).

Phyllody is the most relevant symptom associated with phytoplasma presence. It involves the transformation of various plant organs into leaf-like structures. However, this type of malformation may also be induced by exposure to hormone-based pesticides (Bertaccini 2022). Strawberry plants infected with phytoplasmas typically exhibit a range of symptoms resulting from disruptions in the plant's hormonal balance (Mokbel and Kheder 2020).

Detecting phytoplasmas in weeds surrounding cultivated crops is crucial to prevent the potential role of these weeds as phytoplasma reservoirs (Meneguzzi *et al.* 2008). *Conyza sumatrensis* is an invasive weed known for its ability to rapidly adapt to new environments (Ruiz *et al.* 2021). Phytoplasma associations in *Conyza* species have been reported to cause witches' broom symptoms. These symptoms are characterized by abnormal growth patterns, particularly excessive shoot proliferation forming broom-like structures.

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