Review
On Some Significant Phytoplasma Diseases of Forest Trees: An Update

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Abstract: This paper provides an updating of information of a selected number of major phytoplasma diseases of forest trees, with a focus on the associated phytoplasma taxa. Phytoplasma diseases of forest trees have been less extensively studied than those affecting fruit trees. Research on the role of phytoplasmas as the cause of diseases of forest trees has only in the last few years been intensified, after sensitive and specific detection methods greatly based on PCR technology became available. Various phytoplasma taxa have been identified in naturally infected elm, ash, conifer, sandal, and eucalyptus trees, whereas only one phytoplasma taxon has been recorded in naturally infected alder trees. However, for almost all of the reviewed diseases, there is still sparse information about insect vectors, plant host range, strain virulence, pathogenicity, and host tolerance and resistance. Knowledge of these aspects is the basis for appropriate disease management. In particular, further research is required to clarify the role of phytoplasmas in asymptomatic trees. In addition, the etiological role of various “non-specific” phytoplasma taxa, which have been recorded in forest trees, while no data from pathological studies are available, needs to be further investigated.

Keywords: ‘Candidatus Phytoplasma’ species; 16Sr group/subgroups; PCR; yellows diseases; witches’ broom; phloem discoloration; die-back; phytoplasma strains; etiology; eucalyptus little-leaf; disease incidence

1. Introduction

Phytoplasmas are a very large group of wall-less, obligate bacteria associated with diseases of more than a thousand plant species worldwide, including economically and ecologically important forest trees. In the plant host, they colonize the phloem elements and are naturally transmitted by phloem-feeding homopteran insects [1]. Phytoplasmas cause either specific symptoms (virescence, phyllody, witches’ brooms) or non-specific symptoms (yellowing, reddening, reduced growth, die-back, and decline). However, symptom expression may be highly variable for a given plant–host combination.

Phytoplasmas are members of the class Mollicutes, which are closely related to acholeplasmas, and they are currently assigned to the provisional genus ‘Candidatus Phytoplasma’ mainly through 16S rDNA sequence analysis [2,3]. Approximately 34 phytoplasma groups or 16Sr groups (= taxonomic groups) were established by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified rDNA sequences within the mentioned genus [3,4]. In addition, multi-locus sequence typing (MLST) using less-conserved genes proved a useful tool for the identification of genetically closely related but pathologically or epidemiologically distinct strains. Identification of these strains is essential for epidemiological studies [5].

The aim of this paper is to update information of some phytoplasma diseases of forest trees, with a focus on the molecular and taxonomic aspects of the associated phytoplasmas.
2. Phytoplasma Diseases of Forest Trees

2.1. Elm

Various Ulmus (elm) species and hybrids are affected by elm yellows (EY), which is a disease common in North America and Europe [6]. This disease was formerly named elm phloem necrosis because the discoloration and death (= necrosis) of phloem tissue are the most characteristic symptoms in highly susceptible hosts, e.g., Ulmus americana (American or white elm) [7]. EY was first reported in Ohio in 1938 [7]. However, there is evidence that it was present there as well as in neighboring states long before this time [8,9]. Once established in the midwestern states, EY spread into eastern states and southeastern Ontario [10]. It is known for causing destructive epidemics in North America, which killed thousands of elm trees. Until the 1980s, EY was considered a typical North American disease. Conti et al. [11] first reported the occurrence of this disease in Italy, although it was observed in Italy since at least 1918 (for a review, see [6]). Following this finding, phytoplasma diseases of elm have also been recorded in several other European countries [6,12,13]. The detection of EY in Europe was first made on the basis of symptoms [11,14]. Later, molecular studies using mainly RFLP and sequence analyses of PCR-amplified rDNA showed that the phytoplasma diseases of elm in Europe and North America are caused by the same organism, the EY agent [15–18]. This pathogen was taxonomically delineated as ‘Candidatus (Ca.) Phytoplasma ulmi’ [2,19].

Taxonomically, the EY agent ‘Ca. Phytoplasma ulmi’ is assigned to the EY phytoplasma group or 16SrV group, subgroup 16SrV-A [19]. Other members of this group are phytoplasmas causing mainly diseases of trees and shrubs in the northern hemisphere [6]. An updated picture of the phylogenetic relationships among phytoplasmas associated with diseases of forest trees reviewed in this paper is shown in Figure 1. In contrast to 16S rRNA gene, genetic diversity within the EY pathogen have been observed in rplV, rpsC, secY, map, groEL, and imp gene sequences [19–24].
Figure 1. Phylogenetic dendrogram generated by using the neighbor-joining method software MEGA, version X [25] with 16S rDNA sequences from phytoplasmas detected in naturally infected elm, alder, ash, conifer, sandal and eucalyptus trees (in bold type), and a number of reference phytoplasmas. Acholeplasma laidlawii was used as the outgroup. Bar represents a phylogenetic distance of 2%. GenBank accession number is given for each phytoplasma. Bootstrap values are shown on branches of the dendrogram.
A phytoplasma of the clover proliferation group (=16SrVI group), subgroup 16SrVI-C, the Illinois elm yellows (ILEY) phytoplasma, was detected in American elm trees showing EY symptoms in Illinois, USA [26]. However, some trees proved to be doubly infected with the ILEY phytoplasma and a phytoplasma of the aster yellows (AY) group (16SrI group) [26]. Diseased elm trees doubly or multiply infected with the EY agent and a phytoplasma of the 16SrI and/or the stolbur group (16SrXII group) were also recorded in Italy [27]. However, in these trees, the EY agent was predominant, while the other phytoplasmas were present at low titer. A few diseased elm trees showing yellowing symptoms, singly infected with 16SrI and 16SrXII-A phytoplasmas, were found in Croatia [23], whereas diseased elm trees in China harbored 16SrV-B and 16SrI-B phytoplasmas [28]. A ‘Ca. Phytoplasma fragariae’-related strain (16SrXII-E subgroup) was detected in five symptomless elm trees in Belgium, whereas 16SrV-C phytoplasmas were detected in two elm trees in Germany with the absence of yellows disease symptoms [13,29].

EY symptoms may differ among elm species. In Northern American species, e.g., *U. americana*, *U. rubra* (red or slippery elm), *U. alata* (winged elm), *U. serotina* (September elm), and *U. crassifolia* (cedar elm), the main symptoms are yellowing, premature casting of the leaves, early bud break, brown discoloration of the phloem tissue, and tree death. Red elm often shows witches’ brooms. Discolored phloem tissue of American, winged, September and cedar elms usually have a characteristic odor of oil of wintergreen (methyl salicylate). In *U. minor* (syn.: *U. carpinifolia*, European field elm), witches’ brooms are predominant. For this reason, the disease occurring in Europe is often called elm witches’ broom. Other symptoms are yellowing, stunting, and small leaves (Figure 2).

**Figure 2.** Small leaves, yellowing, and witches’ broom on European field elm (*Ulmus minor*) shoot affected by elm yellows disease. Left, healthy shoot.

Brooming and stunting are also typical symptoms of EY-affected *U. glabra* (Scots or wych elm) and *U. parvifolia* (Chinese elm) [14,15,30]. Yellowing or reddening, reduced vigor, witches’ brooms, and die-back but not phloem discoloration are known to occur in several other European and Asian elm genotypes, including *U. pumila* (Siberian elm), *U. chenmoui* (Chenmou elm), *U. villosa* (cherry bark elm), *U. laevis* (European white elm), *U. wallichiana* (Himalayan elm), *U. wilsoniana* (Wilson elm), *U. japonica* (Japanese elm), and *U. × hollandica* (Dutch elm) [11,21,22,27,31,32]. In diseased American elm trees, an abnormal callose deposition on the sieve plates is usually followed by the collapse of sieve tube elements. Thereby, phloem transport is severely compromised. Starch accumulates in the aerial parts of affected trees while roots starve and die. The sieve tubes of affected *U. americana* and *U. rubra* trees are so sensitive to ‘Ca. Phytoplasma ulmi’ that they suddenly collapse without allowing the pathogen to reach a high titer [14,33,34]. In contrast, sieve tubes of *U. minor* and *U. parvifolia* trees as well as of other European and Asian elm genotypes being rather tolerant to the EY pathogen are only slightly damaged and allow it to reach a quite high titer [14,17,35,36]. ‘Ca. Phytoplasma ulmi’ also causes stomatal closure and elevated diffusive resistance, which is associated with high water potential,
in leaves of *U. americana* and *U. rubra* trees [10]. There are also several reports on the presence of ‘*Ca. Phytoplasma ulmi*’ in non-symptomatic trees belonging to some European and Asian elm genotypes [13,23,27,29–32,37]. In China, elm trees of unspecified species showing yellowing and witches’ brooms were found to be infected by 16SrV-B and 16SrI-B phytoplasmas, respectively [28].

EY is common in eastern North American states where several severe epidemics spread at rates of 5 to 8 km per year in some areas and destroyed a large number of native elm tree [30]. In some states, EY epidemics occurred together with the Dutch elm disease and exacerbated the latter by providing additional breeding material for elm bark beetles, which are the insects responsible for the spread of the Dutch elm disease [30]. In Italy, significant EY epidemics have been observed in some experimental fields established during the 1980s to test the adaptability of a number of elm species and various hybrid clones to local environmental conditions [31]. *U. americana*, *U. villosa*, *U. parvifolia*, *U. japonica*, *U. parvifolia × U. wallichiana (= clone P628), (U. wallichiana × (U. × hollandica ‘Vegeta’ × U. minor) (= clone 793), [(U. wallichiana × U. minor) × U. laciniata] × U. laciniata ‘Nikkoisens’ open-pollinated (= clone 1094), and (U. wallichiana × U. minor) × U. laciniata open-pollinated (= clone 1098) were severely affected [31]. In one of the experimental field, 30% of trees were infected five years after the first evidence of the disease, reaching nearly 80% within fourteen years. EY epidemics observed in southern and northern Italy on European field elm and Siberian elm showed a disease incidence greater than 80% [6,11,31,37]. A detection rate of 75% of EY phytoplasma in European white elm trees has been recorded in Croatia using nested PCR assays. More than half of the infected trees were symptomless [25]. In Croatia, 75% of the European white elm trees that were examined by nested PCR assays proved to be infected by ‘*Ca. Phytoplasma ulmi*’. Most of the examined trees were non-symptomatic [25]. In a recent survey performed in Germany on a nationwide scale by using real-time PCR, ‘*Ca. Phytoplasma ulmi*’ was detected in almost 28% of elm trees examined. Among the elm species examined, the highest detection rate was recorded in European white elm trees [23]. EY epidemics have been observed in some experimental fields established during the 1980s to test the adaptability of a number of elm species and various hybrid clones to local environmental conditions [31]. *U. americana*, *U. villosa*, *U. parvifolia*, *U. japonica*, *U. parvifolia × U. wallichiana (= clone P628), (U. wallichiana × (U. × hollandica ‘Vegeta’ × U. minor) (= clone 793), [(U. wallichiana × U. minor) × U. laciniata] × U. laciniata ‘Nikkoisens’ open-pollinated (= clone 1094), and (U. wallichiana × U. minor) × U. laciniata open-pollinated (= clone 1098) were severely affected [31]. In one of the experimental field, 30% of trees were infected five years after the first evidence of the disease, reaching nearly 80% within fourteen years. EY epidemics observed in southern and northern Italy on European field elm and Siberian elm showed a disease incidence greater than 80% [6,11,31,37]. A detection rate of 75% of EY phytoplasma in European white elm trees has been recorded in Croatia using nested PCR assays. More than half of the infected trees were symptomless [25]. In Croatia, 75% of the European white elm trees that were examined by nested PCR assays proved to be infected by ‘*Ca. Phytoplasma ulmi*’. Most of the examined trees were non-symptomatic [25]. In a recent survey performed in Germany on a nationwide scale by using real-time PCR, ‘*Ca. Phytoplasma ulmi*’ was detected in almost 28% of elm trees examined. Among the elm species examined, the highest detection rate was recorded in European white elm trees, followed by the Scots elm and European field elm. EY-specific symptoms were rarely observed only in infected Scots and European field elm trees [13]. In Illinois, a severe EY outbreak associated to ILEY phytoplasma occurred in the 1990s and caused the death of more than one thousand American elms [26]. No epidemics are known to occur in China where single disease occurrences were recorded [28].

The leafhopper *Scaphoideus luteolus* is known to transmit ‘*Ca. Phytoplasma ulmi*’ in North America. However, other vectors are most probably involved in the transmission process since numerous homopteran insects were captured on elm, whereas *S. luteolus* is not present in some EY-infested areas (for references see [6,30]). In New York State, of the various homopteran insects collected in EY-infested areas and examined for their ability to transmit ‘*Ca. Phytoplasma ulmi*’, single transmissions were obtained for the leafhopper *Allygus atomarius* and the spittlebug *Philaenus spumarius* [10]. In addition, ‘*Ca. Phytoplasma ulmi*’ was detected by real-time PCR in several leafhoppers belonging to *Allygus, Colladonus, Empoasca, Erythronneura, Graphocephala, Homalodisca, Orientus, Scaphoideus*, and *Typhlocyba*, which were collected in the University Park Campus of Pennsylvania State University, USA [38]. However, it has not been shown whether these leafhoppers transmit the pathogen. Work by Rosa et al. [39] revealed that three out of 30 American elm seedlings exposed to individuals of *Lepyro尼亚 quadrangularis, P. spumarius*, and *Latalus sp.*, captured from an EY-infected red elm tree in the Pennsylvania State University campus, were infected by ‘*Ca. Phytoplasma ulmi*’. *S. luteolus* is not present in Europe, whereas Carraro et al. [37] provided evidence that *Macropsis mendax* is vectoring ‘*Ca. Phytoplasma ulmi*’ in northern Italy. There are no reports on the role of *M. mendax* in the natural transmission of ‘*Ca. Phytoplasma ulmi*’ in other European countries as well as on its transmission efficiency. In Germany, the finding of naturally infected elm trees at elevations beyond 750 m suggests the presence of a vector different from *M. mendax*, since this insect occurs only to an altitude of 400 m [13]. ‘*Ca. Phytoplasma ulmi*’ was also detected by PCR in individuals of *Hyaleseths luteipes*, and *Iassus scutellaris, Allygidius furcatus*, and *Cixus sp.*, captured from EY-affected
Forests 2021, 12, 408

Several elm genotypes that are resistant to the Dutch elm disease have been examined for EY resistance or tolerance by graft-inoculation experiments. The inoculated trees of these genotypes greatly differed in their response to \( \textit{Ca. Phytoplasma ulmi} \) [44]. Diseased trees of ‘Frontier’, ‘Pathfinder’, and ‘Patriot’ showed foliar yellowing and reddening, witches’ brooms, reduced terminal growth, and stunting. Since phloem necrosis and death were not observed, these elms may be rated as tolerant. Trees of ‘Pioneer’ were considerably more affected; most of them became infected and died. Only two out of 20 inoculated ‘Prospector’ trees became infected, one of which died, whereas none of the inoculated ‘Homestead’ trees was infected. The latter trees showed localized phloem necrosis as a defense reaction that prevented spreading of the pathogen, suggesting thus resistance of ‘Homestead’ [44]. Trees of ‘American Liberty’ proved to be highly susceptible to \( \textit{Ca. Phytoplasma ulmi} \), which is a finding that is confirmed also under natural infection conditions [30]. The clonal cultivars Independence, New Harmony, Valley Forge, and the triploid putative hybrid cultivar Jefferson were susceptible as well [45]. Since EY is lethal to North American species, while some Eurasian genotypes are tolerant or resistant, it seems that the disease is of European origin [30]. Tolerance or resistance is usually expected in regions where the pathogen and its natural hosts co-evolved.

Currently, PCR assays are most widely used for the detection of ‘\( \textit{Ca. Phytoplasma ulmi} \)’. Primers differing in specificity have been developed and directed to either ribosomal or non-ribosomal DNA sequences. The sensitivity of detection can be increased by the use of nested PCR. For a review on primer sequences and primer combinations for the detection of \( \textit{Ca. Phytoplasma ulmi} \) and other phytoplasmas infecting elm, see [6,46,47]. A real-time PCR (qPCR) assay using nonribosomal primers in combination with TaqMan minor-groove-binder (MGB) probe chemistry was developed for the specific detection of \( \textit{Ca. Phytoplasma ulmi} \). This assay allowed detection of the pathogen in affected trees and several leafhoppers, but it did not show cross reactivity with the DNA of closely related phytoplasmas belonging to other 16SrV subgroups [38]. In Germany, ‘\( \textit{Ca. Phytoplasma ulmi} \)’ was nationwide surveyed by using a universal 16Sr DNA-based qPCR assay and a newly developed ‘\( \textit{Ca. Phytoplasma ulmi} \)’-specific qPCR Taq-Man assay directed to 16S–23S spacer region sequences. Both assays enabled the detection of ‘\( \textit{Ca. Phytoplasma ulmi} \)’ in the same number of elm trees (1801 trees out of 6486 trees examined), although the pathogen specific assay showed a slightly lower sensitivity as shown by different cycle threshold (Ct) values. In addition, the universal phytoplasma assay enabled the identification of two elm trees each infected by a different 16SrV-C phytoplasma strain [13]. Scots elm trees showed a higher phytoplasma titer in comparison to the other two elm species examined. However, the phytoplasma titer was not correlated to the presence of EY symptoms [13]. In most trees, a high titer of ‘\( \textit{Ca. Phytoplasma ulmi} \)’ was identified, as evidenced by a Ct value ≤28, which corresponds to a titer of \( 10^6 \) per gram of phloem tissue at least. In addition, all parts of an infected Scots elm tree such as roots, trunk at both ground and aerial levels, branches, leaves, and buds from the top and bottom of the tree were heavily colonized by ‘\( \textit{Ca. Phytoplasma ulmi} \)’ throughout the year. The survey also revealed that the pathogen was not homogeneously distributed throughout Germany. Sites with a high number of infected trees occurred in east, south, and central Germany, whereas only a few sites with low infection rates were found in the remaining parts of the country. Infection rates did not vary considerably among sites located at different altitude levels [13]. On the basis of \( \text{groEL} \) and \( \text{imp} \) gene sequences, 29 and 74 genotypes, respectively, have been identified among ‘\( \textit{Ca. Phytoplasma ulmi} \)’ strains infecting elms in Germany [24]. In phylogenetic
analysis, genotypes obtained from Scots elm trees clustered together, while those from European white elm and European field elm trees did not. A regional distribution pattern was evident only for a few genotypes [24].

2.2. Alder

Alder yellows (ALY) is a phytoplasma disease affecting several *Alnus* (alder) species. This disease has been described in several European countries such as Germany, Italy, Austria, France, Switzerland, Hungary, Serbia, Slovenia, Lithuania, Serbia, Montenegro, Macedonia, and Poland (for reviews, see [48,49]). ALY has also been recorded in the Washington State (USA) and Ontario, Canada, on *A. rubra* (red alder) [16,50,51]. However, molecular identification and classification of the phytoplasma(s) infecting *A. rubra* in the Washington State have not yet been reported.

Symptoms of ALY are yellowing, premature autumn coloration, sparse foliage, small leaves, die-back, and decline. Shoot proliferation may occur at the base of trunk of affected trees (Figure 3). Latent infections have been observed as well [50,52–54]. Lederer and Seemüller [50] examined 500 *A. glutinosa* (European alder) trees for phytoplasma infections using DAPI (4′-6-diamidino-2-phenylindole) fluorescence methods. All trees older than 5 years tested positive for phytoplasma infections. However, the majority of infected trees were non-symptomatic. Usually, non-symptomatic trees revealed a higher colonization density than symptomatic ones. The colonization density was always higher in leaves and young twigs than in other parts of the trees. The phytoplasma distribution within the affected phloem tissue was uneven, with some sieve tubes filled by phytoplasmas, while others completely empty or containing only a few phytoplasmas. Similar findings were obtained by examining *A. incana* trees [50]. Under natural infection conditions, high detection rates ranging from 85 to 100% of ALY phytoplasma infections in *A. glutinosa* were also recorded by PCR [53,55–58]. No phytoplasma infections have been recorded in *A. viridis* either by DAPI fluorescence methods or PCR assays [50,57]. This lack of infections may be ascribed to the host preference or unfavorable conditions of the insect vector(s).

![](image)

**Figure 3.** Pronounced shoot proliferation at the base of trunk of a several-year-old alder (*Alnus glutinosa*) tree affected by alder yellows disease.

The ALY phytoplasma is taxonomically assigned to subgroup 16SrV-C within the EY group [19]. It has a 99.7%–100% 16S rDNA sequence similarity with other members of the mentioned subgroup such as flavescence dorée (FD), Palatinate grapevine yellows (PGY), and spartium witches’ broom (SpaWB) agents and phytoplasmas infecting *Clematis vitalba* in Italy and the Balkans, and hemp dogbane (*Apocynum cannabinum*) in New York state, respectively [19,59,60]. ALY phytoplasma strains originating from different parts of Europe
are identical at the 16S rDNA and 16S–23S rDNA spacer region sequence level. However, studies through ribosomal protein gene and nonribosomal loci analyses showed a considerable molecular variability among them. Some strains proved to be identical or near identical to either FD phytoplasma strains or PGY phytoplasma strains [19,20,49,53,55,56,61,62]. Many different strains of the ALY phytoplasma have been identified through map-based gene analysis in singly and multiply infected alder trees throughout Europe. These strains, phylogenetically, do not form a homogeneous group but are scattered in various clusters that comprise FD, Palatinate grapevine yellows (PGY), and SpaWB phytoplasma strains. Some ALY phytoplasma strains were more closely related to FD and/or PGY phytoplasma strains than to other ALY phytoplasma strains. In addition, clustering was not linked to the geographic origin of the strains [20,53,55,61]. Phylogenetic analyses of the concatenated tuf, rplV-rpsC, rplF-rpfR, map, and worB-degV gene sequences showed that strains infecting alder trees in Canada (two strains) form together with strain HD1 of hemp dogbane (Apocynum cannabinum) phytoplasma a monophyletic discrete genetic cluster clearly separated from the cluster comprising ALY phytoplasma strains from Europe [51].

The ALY phytoplasma is naturally spread by the leafhopper Oncopsis alni [52], which is very common on alder trees and is characterized by an oligophagous feeding behavior. The distribution of this leafhopper in Europe corresponds to that of ALY [52]. ALY phytoplasma infections were also recorded in individuals of the psyllid Psylla alni and leafhoppers Orientus ishidae, Allygus mixtus, and Allygus modestus, which were all collected from ALY-infected alder trees. However, all attempts to transmit the ALY phytoplasma to healthy alder plants using ALY phytoplasma-infested P. alni psyllids failed, whereas successful transmission was obtained with ALY phytoplasma-infested O. ishidae, A. mixtus, and A. modestus leafhoppers [52,58,63]. It is well known that ALY phytoplasma strains are also responsible of the Palatinate grapevine yellows (PGY) disease in Germany. Spreading of these strains from alder to grapevine occurs through O. alni, which feeds occasionally on grapevine [64–66]. Thus, infected alder trees growing in proximity of vineyards may act as pathogen reservoirs. In addition, due to the close phylogenetic relationship of ALY phytoplasma with the FD agent, diseased alder trees may also function as pathogen reservoirs in areas where FD disease occurs. In these cases, some ALY phytoplasma strains may be transmitted to grapevine by occasional grapevine-feeding vectors leading to FD disease, which is then spread from grapevine to grapevine by Scaphoideus titanus, the strictly ampelophagous vector of FD phytoplasma strains. S. titanus is not known to occur in the Palatinate [20,55,58,61]. A recent study by Jurga and Zwolinska [49] has shown that diseased common mugwort (Artemisia vulgaris) plants showing witches’-broom symptoms, grown next to an ALY-affected alder (A. glutinosa) tree in Southern Poland, were infected by a 16SrV-C subgroup phytoplasma that had the same 16S rDNA sequences of the phytoplasma present in ALY-affected alder trees. Therefore, it is possible that common mugwort serves as alternative host of the ALY phytoplasma in eastern Europe [49].

As mentioned above, in nature, a high percentage of alder trees infected by ALY agent do not develop obvious symptoms. The reasons were elucidated by Berges and Seemüller [67] through graft-inoculation of healthy alder plants with icoculum from both differently affected and symptomless alder trees and observation of symptom development for five years. It resulted that the severity of symptoms shown by inoculated plants corresponded to that of plants from which icoculum was taken. Thus, it was concluded that ALY phytoplasma may affect alder by inducing severe symptoms, but avirulent strains occurring within this phytoplasma are responsible for the latent infections that are widespread in Europe. It is also possible that avirulent strains mediate cross-protection and suppress severe strains, since infected alder trees remain symptomless over time in spite of the presence of an abundant and efficient insect vector such as O. alni. Differences in strain virulence are also known for other phytoplasmas, including those causing apple proliferation, European stone fruit yellows, ash yellows and aster yellows diseases, whereas antagonistic interactions among different strains of the same pathogen have been observed in many instances [68–70]. On the other hand, it is also possible that symbiotic microorganisms
of alder such as *Frankia alni* may compensate for the detrimental effects of phytoplasma infections [54]. However, it is unknown whether these microorganisms are able to colonize systemically the phloem tissue of alder trees.

2.3. *Ash*

Ash yellows (AshY) is a prominent phytoplasma disease of *Fraxinus* (ash) species known to occur widely in North America [71]. This disease is caused by the AshY agent ‘Ca. Phytoplasma fraxini’, which is a pathogen of the AshY group or 16SrVII group, subgroup 16SrVII-A [72]. Although AshY may have occurred in the northeastern USA states since the 1930s, it was not considered as a major forest disease until the early 1980s. This late recognition was due to the difficulties met in identifying phytoplasma diseases and to the inconsistency with which infected trees show specific symptoms [71]. In North America, AshY occurs in twelve ash species and numerous intraspecific and interspecific ash genotypes [71]. Most of the affected species are native to North America. A 16SrI phytoplasma has been identified in yellows-diseased trees of *F. excelsior* (European ash) in Poland and *F. uhdei* (Urpan ash) in Colombia [73,74]. In the latter country, other studies showed that *F. uhdei* trees showing typical AshY symptoms were infected by ‘Ca. Phytoplasma fraxini’ [75,76]. In France, ash trees showing die-back symptoms were infected with ‘Ca. Phytoplasma fragariae’ (subgroup 16SrXII-E) [77].

AshY causes reduced growth, loss of apical dominance, suppressed root development, premature flowering, shoot proliferation, and witches' brooms (Figure 4). Light green and deformed leaves are also common. Highly susceptible taxa show mainly die-back, shoot proliferation, loss of apical dominance, and premature death. Short roots and necrosis of rootlets that precedes wilting and death are predominant in diseased *F. americana* (white ash) trees. Infected ash trees are highly sensitive to frost damages, which appear as cracks of the bark at the base of the trunk [10]. Histological symptoms which are more readily observed in the roots than in the aerial parts of affected plants, include autofluorescent sieve tubes and pathological sclerenchyma. Autofluorescent sieve tubes are sieve tubes that fluoresce without the addition of any reagent when exposed to UV radiation. Such sieve tubes are presumed to be non-functional and often collapse. Pathological sclerenchyma is a distinct parenchyma tissue adjacent to infected sieve tubes, which is characterized by prominently thickened and lignified cell walls, which greatly differs from the sclerotic parenchyma that is often seen in healthy phloem. Stomatal closure and high diffusive resistance are also known to occur in the leaves of affected white ash trees [10]. Witches' brooms are regarded as specific symptoms of the disease, whereas other symptoms are non-specific, since they can be induced by several other biotic and abiotic factors. A disease incidence of ≥50% has been observed in some *F. americana* stands in northern USA states and in a *F. velutina* (velvet ash) stand in Utah. Incidence ranging from of 3 to 27% was recorded in *F. pennsylvanica* (green ash) in Iowa and Wisconsin (for review, see [71]). ‘Ca. Phytoplasma fraxini’ was also detected in non-symptomatic ash trees [71,78]. In Colombia, more than 53,000 *F. uhdei* trees showing typical AshY symptoms were recorded in several areas of the Andes; many of them died. Phytoplasma infections were detected in all symptomatic trees examined (100 trees) either through DAPI fluorescence methods or PCR assays. Phytoplasma infections were strictly related to the presence of symptoms [76]. The phytoplasma identified in diseased *F. uhdei* trees, the AshY agent ‘Ca. Phytoplasma fraxini’, was also transmitted to *Catharanthus roseus* (periwinkle) through dodder (*Cuscuta* sp.) [76]. In a study conducted in France to test the presence of the ascomycete fungus *Hymenoscyphus fraxineus* in ash trees displaying symptoms of die-back, a relatively large proportion of symptomatic trees (27%) proved to be not infected by such fungal pathogens [79]. This finding prompted a further study aimed at verifying the presence of phytoplasma infections in affected trees [77]. ‘Ca. Phytoplasma fragariae’ was identified in 5% of the examined ash trees (13 out of 260 trees). Four phytoplasma-positive trees were also infected by the fungus *H. fraxineus*. However, further data are necessary to validate the role of phytoplasma infections in the die-back disease affecting ash in France.
Sinclair and Griffiths [68] investigated the virulence of 12 ‘Ca. Phytoplasma fraxini’ strains, originating from different areas of North America, by graft-inoculating F. pennsylvanica seedlings and periwinkle plants and monitoring symptom development under greenhouse conditions. In graft-inoculated plants, the examined strains caused symptoms that ranged considerably from imperceptible to severe. The same study [68] revealed the occurrence of interference among strains of the same taxon. Strains of ‘Ca. Phytoplasma fraxini’ differing in virulence have also been recorded under natural infection conditions [80]. These strains greatly differed in aggressiveness as indicated by the different rates of growth suppression, reduction of foliar greenness, and frequency of witches’ brooms that they caused. However, specific interactions between strain and cultivar were not discovered [80]. Several investigations have shown that F. pennsylvanica and F. velutina are more tolerant than F. americana to ‘Ca. Phytoplasma fraxini’ and that heritable intraspecific variation in tolerance occurs in F. pennsylvanica and is supposed to be present also in other ash species [81,82]. In graft-inoculation trials, the shoot growth of F. americana and F. pennsylvanica was suppressed earlier than that of F. velutina. In addition, the highest growth losses in height, stem diameter, and root volume were recorded for F. americana followed in decreasing order by F. pennsylvanica and F. velutina. The growth of diseased F. velutina scions on F. americana rootstocks was severely suppressed in comparison with that of diseased own-rooted F. velutina. On the other hand, diseased F. americana scions grafted on F. velutina rootstocks registered a significantly lower growth suppression than diseased own-rooted F. americana. When witches’ brooms arisen from F. americana trees were grafted on F. velutina rootstocks, they continued to maintain their original form but did not proliferate new shoots. These findings imply that although tolerant rootstocks may alleviate the impact of ‘Ca. Phytoplasma fraxini’ on scions, the management of AshY through tolerant genotypes requires tolerance in both scions and rootstocks [82]. Among the ash cultivars tested by graft inoculation to evaluate their responses to ‘Ca. Phytoplasma fraxini’, F. pennsylvanica cvs. Bergeson, Dakota Centennial, and Patmore and F. americana cv. Autumn Applause were least affected, i.e., they sustained less growth and foliar color depression [80].

There are no reports concerning insect vectors involved in the natural spread of AshY. A previous work showed that field-captured Paraphlepsius irroratus and P. spumarius individuals transmitted acquired phytoplasmas to ash seedlings [10]. However, the molecular identities of these phytoplasmas were not determined, and further experiments using pro-
Numerous genuses of these insects failed to transmit ‘Ca. Phytoplasma fraxini’. In the work by Hill and Sinclair [83], 33 taxa of leafhoppers were collected in two locations where AshY incidence was high, in New York State, and tested by PCR assays for phytoplasma infections. The most abundant genus was Scaphoideus. ‘Ca. Phytoplasma fraxini’ infections were recorded in some individuals of Scaphoideus spp. Therefore, these insects should be further examined to prove their transmission ability [83].

2.4. Conifers

After a few findings that were not confirmed by other studies on the presence of phytoplasma infections in various gymnosperms using electron microscope observations, phytoplasmas were detected and molecularly identified in several conifers over the last few years. Schneider et al. [84] reported on the presence of a previously undescribed taxon, ‘Ca. Phytoplasma pini’, in Pinus sylvestris (Scots pine) and P. halepensis (Aleppo pine) trees in Germany and Spain. Affected trees showed symptoms of yellowing, stunting, needle dwarfing, and shoot proliferation. In addition, affected P. sylvestris trees were characterized by the presence of ball-like structures in the canopy, resulting in the combination of proliferation of shoots with dwarfed needles. ‘Ca. Phytoplasma pini’ was detected in symptomatic and non-symptomatic parts of P. sylvestris and P. halepensis trees as well as in some neighboring non-symptomatic trees of both species.

Following the finding of Schneider et al. [84], ‘Ca. Phytoplasma pini’ and related strains were identified in several other Pinus species as well as in Abies procera (noble fir), Tsuga canadensis (Canadian hemlock), and Picea pungens (Colorado blue spruce) in Poland, the Czech Republic, Croatia, Lithuania, and Maryland (USA). In all affected species, the symptoms were similar to those observed in Germany and Spain, whereas ball-like structures were recorded other than in P. sylvestris also on P. mugo (mountain pine) in Lithuania (Figure 5). Abnormal shoot branching resulting in typical witches’ brooms were shown by pine trees in Maryland [85–90]. Furthermore, ‘Ca. Phytoplasma pini’ was identified in China [91] in Taxodium distichum var. imbricarium (pond cypress) showing symptoms of shoot proliferation, little leaf, and leaf necrosis.

Figure 5. Phytoplasma-infected Scots pine (Pinus sylvestris) (left) and mountain pine (P. mugo) (right) trees showing ball-like structures.

‘Ca. Phytoplasma pini’ is a member of the pine shoot proliferation group or 16SrXXI group, subgroup 16SrXXI-A. A ‘Ca. Phytoplasma pini’-related strain detected in a witches’-
broom affected pine tree in Maryland, strain MDPP, was assigned to a new subgroup, designated as 16SrXXI-B within the group 16SrXXI, on the basis of virtual RFLP pattern similarity coefficient values [90]. A phytoplasma of the X-disease group (16SrIII group) was detected in Cupressus sp. (cypress) trees showing witches’-brooms, stunting, and fasciation in Italy [92] and in Picea abies (Norway spruce) and P. glauca (white spruce) trees with symptoms similar to those associated with ‘Ca. Phytoplasma pini’ in Poland [86]. 16SrI phytoplasmas were identified in diseased P. pungens and Larix sp. (larch) trees in Poland and Ukraine, respectively [86,93]. The main symptoms observed in diseased larch trees were dwarfing and the proliferation of needles. Therefore, the name ‘larch dwarfed needle proliferation (LDNP)’ was given to the pathogen identified in this species [93]. Valiunas et al. [89] examined 300 P. sylvestris and P. mugo trees in Lithuania for phytoplasma infections using PCR assays. Of the examined trees, 80% proved to be phytoplasma-positive, of which 98% were infected by Ca. Phytoplasma pini-related strains, whereas the remaining trees harbored a subgroup 16SrI-A phytoplasma. A ‘Ca. Phytoplasma phoenicium’-related strain, subgroup 16SrIX-F, was found in diseased Juniperus occidentalis (western juniper) plants in Oregon (USA) showing symptoms of shoot proliferation, little leaves, shortened internodes, and ball-like structures. The percentage of affected plants was about 1% [94]. In India, yellows-diseased trees of Araucaria heterophylla (Norfolk Island pine) were infected with a ‘Ca. Phytoplasma trifolii’-related strain, which is a member of the clover proliferation group or 16SrVI group. The incidence of the disease was about 15% [95].

Phytoplasma titer in gymnosperms is usually so low that infections could be detected only through nested PCR assays. In addition, the mechanism by which phytoplasmas colonize systemically the affected gymnosperm plants is unknown, since pore sizes in the sieve cells of these plants being too small may hinder phytoplasma movement. Valiunas et al. [96] developed direct PCR methods based on primer pairs directed to 16S rDNA and tuf gene sequences which allowed detecting ‘Ca. Phytoplasma pini’-related strains infecting pine trees in Lithuania with great sensitivity and specificity. The amplified sequences contain molecular markers which are suitable for distinguishing closely related strains of this pathogen. The mentioned PCR methods, which did not allow the amplification of DNA from the pine-infecting phytoplasma strain MDPP recorded in Maryland, are useful tools for detecting and identifying ‘Ca. Phytoplasma pini’ on large-scale studies. A draft genome sequence of the ‘Ca. Phytoplasma pini’-related strain MDPP has recently been reported [97]. This sequence, which consists of 474,136 bases, will facilitate comparative genomic studies of phytoplasmas infecting conifer plants.

2.5. Sandal

Sandal spike (SSD) is a serious disease of Santalum album (sandal) that is widespread in India, especially in Karnataka, Tamil Nadu, and Kerala states [98–101]. The disease was first described in the late 1890s and was supposed to be of viral origin. Later, further investigations using fluorescence and electron microscopy and symptom remission achieved by tetracycline treatments proved its phytoplasmal etiology (for references, see [48]). SSD has spread gradually during the last decades, devastating large forest areas and threatening the sandal industry of southern India. The most characteristic symptom of SSD is small and extremely narrow leaves, which stand out stiffly from the shoots, giving them a spike-like appearance. Other symptoms include yellowing and decline. Severely affected trees die within a two- or three-year period (Figure 6). Disease incidences reaching up to 33 and 55% were recorded in southern Karnataka [100,102]. SSD is caused by ‘Ca. Phytoplasma asteris’, subgroup 16SrI-B [98,103]. In SSD-affected sandal trees in the Marayoor sandalwood reserve in Kerala, a new strain of the 16SrI group, which on the basis of the virtual RFLP pattern similarity coefficient values differed from the phytoplasmas of all 16SrI subgroups, has been identified. This strain represents a new 16SrI subgroup [104]. In addition, a survey conducted in the same reserve has shown the presence in SSD-affected sandal trees of a phytoplasma of the rice yellow dwarf group or 16SrXI group, subgroup 16SrXI-B (Figure 1).
This phytoplasma was recorded either in singly infected trees or in doubly infected trees with ‘Ca. Phytoplasma asteris’ [105]. SSD phytoplasma was transmitted to periwinkle, which induced witches’ brooms, and from this host back to sandal through dodder (Cuscuta subinclusa) [106]. However, attempts to transmit SSD phytoplasma from diseased sandal trees to Stachytarpheta indica, a weed common in sandal plantations, through dodder (C. subinclusa) failed [100]. SSD is naturally spread by the leafhopper Coelidia indica, which was formerly known as Jassus indicus [1].

Figure 6. Decline, small and narrow leaves in sandal spike-infected Santalum album (sandal). Right, healthy plant.

2.6. Eucalyptus

Eucalyptus little-leaf (ELL) is a phytoplasma disease affecting several eucalyptus (Eucalyptus spp.) species that was first observed in India in 1971 and was thought to be caused by the virus [107]. Later, ELL was observed by other Indian researchers, who discovered phytoplasma infections in diseased eucalyptus trees by using electron and light microscope observations [48]. The disease has also been recorded in other countries including Italy, China, Sudan, Iran, and Brazil [48,108–112]. The symptoms described from the various geographic areas and different Eucalyptus spp. are similar. They include small leaves, yellowing, and proliferation from axillary buds that confers a broom-like appearance. Diseased trees or their symptomatic parts do not set fruits, are stunted, and die-back. Severely affected trees decline (Figure 7). A 16SrV phytoplasma was identified in ELL-affected trees in Italy [108]. However, in the affected trees, the phytoplasma concentration was so low that infections could be identified only by nested PCR assays. In addition, 16SrI-B and 16SrI-C phytoplasmas were identified in diseased eucalyptus trees in Italy, whereas a 16SrI-B phytoplasma was recorded in diseased eucalyptus (E. camaldulensis) trees in Iran [48,110]. In the latter country, phytoplasmas of other taxonomic groups were recorded as well. Azimi et al. [111] reported on the detection of a ‘Ca. Phytoplasma aurantifolia’-related strain, subgroup 16SrII-D in yellows-diseased E. camaldulensis trees in Ahvaz, southwestern Iran. The percentage of affected trees was approximately 5%. Baghaee-Ravari et al. [112] reported on the detection of a ‘Ca. Phytoplasma solani’-related strain, subgroup 16SrXII-A in yellows-diseased E. camaldulensis trees in Fars and Khozestan provinces of Iran. This strain was detected through nested PCR assays in 14 out of 22 (approximately 64%) symptomatic trees examined. A ‘Ca. Phytoplasma aurantifolia’-related strain, subgroup 16SrII-C, was detected in diseased E. urophylla trees in Brazil. Fourteen out of 22 (approximately 64%) symptomatic eucalyptus trees examined using nested PCR assays tested positive [109]. The identity of phytoplasmas infecting eucalyptus in India, China, and Sudan has never been determined by molecular methods.
3. Discussion

With few exceptions, phytoplasma diseases of forest trees have been less extensively studied than those affecting fruit trees. One reason may be that until 10–15 years ago, the majority of the research carried out in forest pathology was mainly focused on fungal diseases and insect damages. A second reason is the technical difficulty in detecting and identifying phytoplasmas in forest trees due to the low phytoplasma titer and the presence of non-specific symptoms in affected plants. Research on the role of phytoplasmas as cause of diseases of forest trees has only in the last few years been intensified, after sensitive and specific detection methods, greatly based on PCR technology, became available. By using these methods, phytoplasma infections have been detected for the first time in several forest trees, highlighting that phytoplasma diseases may play in forest trees the same role as in fruit trees. Therefore, the number of phytoplasma diseases of forest trees and associated phytoplasmas has recently been increased considerably [48]. Many phytoplasma diseases of forest trees are each associated with taxonomically distinct phytoplasmas, which induce identical symptoms in a given plant and are present in the same areas or different countries. In addition, a given plant host may be singly or multiply infected with various phytoplasmas. This seems common in woody plants, which have more opportunities to be visited by insect vectors over time. Phytoplasmas belonging to various taxonomic groups and subgroups have been identified in naturally infected elm, ash, conifer, sandal, and eucalyptus trees (Figure 1). In elm, in addition to the specific agent ‘Ca. Phytoplasma ulmi’ (16SrV group, subgroup 16SrV-A), phytoplasmas of the 16SrI group, subgroup 16SrI-B, 16SrVI group, subgroup 16SrVI-C, and 16SrXII group, subgroups 16SrXII-A and 16SrXII-E were identified. Subgroups 16SrV-B and 16SrV-C phytoplasmas were also detected in elm. While the pathological role of the EY agent ‘Ca. Phytoplasma ulmi’ has been well established by numerous graft-inoculation experiments, the pathogenicity of other phytoplasma taxa present in elm has to be verified. In addition, the current knowledge on the latter phytoplasmas are insufficient to draw conclusions that EY can be considered as a disease induced by various phytoplasmas. Only one phytoplasma taxon, the ALY agent (16SrV group, subgroup 16SrV-C), has been recorded in naturally infected alder trees. Strains of this pathogen are highly homogeneous at the rDNA sequence level, but they greatly differ on the basis of less-conserved gene sequences. In particular, many different strains of the ALY phytoplasma have been identified through map-based gene analysis in singly and multiply infected alder trees. These strains phylogenetically do not form a homogeneous group but are scattered among various clusters, which comprise strains of agriculturally important phytoplasmas such as FD and PGY agents. In addition, the presence of avirulent strains occurring within this taxon may be responsible for the latent infections as has been shown by graft-inoculation experiments [67]. AshY-affected ash trees
were found to harbor only ‘Ca. Phytoplasma fraxini’ (16SrVII group, subgroup 16SrVII-A), whereas ash trees with symptoms of decline and die-back were infected with phytoplasmas of the 16SrI group and 16SrXII group (subgroup 16SrXII-E), respectively. The etiological role of the AshY agent ‘Ca. Phytoplasma fraxini’ has been clearly demonstrated by numerous graft-transmission experiments and field observations. However, more data are needed to clearly elucidate the role of 16SrI and 16SrII-E phytoplasmas in the decline and die-back diseases of ash. ‘Ca. Phytoplasma fraxini’-infected ash trees have been recorded only in North America and Colombia. Phytoplasmas belonging to several taxonomic groups such as 16SrXXI (subgroups 16SrXXI-A and 16SrXXI-B), 16SrI (subgroups 16SrI-A and 16SrI-B), 16SrIII, 16SrVI, and 16SrIX (subgroup 16SrIX-F) have been detected in several yellows-diseased conifer species. Among these, the most frequently detected agent is ‘Ca. Phytoplasma pini’ (16SrXXI group, subgroup 16SrXXI-A). This pathogen seems particularly widespread in Europe. In sandal trees affected by SSD, in addition to the AY agent ‘Ca. Phytoplasma asteris’ (16SrI group, subgroup 16SrI-B), a new strain of the 16SrI group, which on the basis of virtual RFLP pattern similarity coefficient values differed from phytoplasmas of all 16SrI subgroups, as well as a 16SrXI phytoplasma have been identified [104,105]. Taxonomic groups of phytoplasmas infecting eucalyptus trees include 16SrV, 16SrI (subgroups 16SrI-B and 16SrI-C), 16SrII (subgroups 16SrII-C and 16SrII-D), and 16SrXII (subgroup 16SrXII-A).

4. Conclusions
Phytoplasma diseases of forest trees are of considerable economic and ecological significance throughout the world. Over the last few years, several phytoplasma taxa associated with various yellows and decline diseases of forest trees have been identified in singly or doubly or multiply infected trees. However, for almost all of these diseases, including EY and AshY, which are the most studied phytoplasma diseases of forest trees, there is still sparse information about insect vectors, plant host range, strain virulence, pathogenicity, and host tolerance and resistance. Knowledge of these aspects is the basis for appropriate disease management. In particular, further research is required to elucidate the role of phytoplasmas in asymptomatic trees and its relationship to the lack of symptom expression. In addition, the etiological role of various ‘non-specific’ phytoplasma taxa, which have been recorded in forest trees while no data from pathological studies are available, needs to be further investigated.

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