# Hot water treatment, detection methods and molecular diversity of Candidatus Phytoplasma mali

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# HOT WATER TREATMENT, DETECTION METHODS AND MOLECULAR DIVERSITY OF *CANDIDATUS PHYTOPLASMA MALI*.

**ARNAUD WARGNIES**

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## Encadrement du travail de fin d’études

Ce travail de fin d’études a été réalisé à Gembloux, au Centre Wallon de recherche agronomique (CRA-W) dans le département des sciences du vivant dans l’unité santé des plantes et forêts au sein du laboratoire de virologie.

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# Abstract

**[FR]** Cette étude porte sur la faisabilité du traitement à l'eau chaude des greffons de pommier pour éliminer *Candidatus Phytoplasma mali,* ainsi que sur l'évaluation de deux méthodes de détection en PCR imbriquée pour détecter les phytoplasmes et la caractérisation moléculaire du phytoplasme du pommier des pommiers infectés dans les vergers du CRA-W. *Candidatus Phytoplasma mali* est le phytoplasme qui infecte de façon pérenne les pommiers et provoque la prolifération du pommier. Il n'existe aucun traitement curatif public pour éliminer *C. P. mali*, et il est recommandé de détruire l'individu malade pour éviter sa propagation dans le verger. Au Centre Wallon de Recherche Agronomique, les vergers conservatoires présentent des individus infectés. La dispersion de variétés, parfois uniques, mais contaminées par la bactérie ne peut avoir lieu, et leur destruction entraînerait une diminution des ressources génétiques des pommiers en Wallonie. Une méthode curative a déjà montré son efficacité contre d'autres espèces de phytoplasmes infectant des arbres fruitiers : le traitement à l'eau chaude des greffons. Dès lors, il serait utile de s'assurer de la présence du phytoplasme dans le pommier ainsi que de connaître la souche, cette dernière pouvant caractériser la virulence de la maladie.

Quatre modalités de température-temps ont été testées pour le traitement à l'eau chaude : trois modalités à 45°C pendant 60, 90 et 180 minutes, et une modalité à 50°C pendant 15 minutes. Deux expérimentations ont eu lieu : la première a été réalisée en novembre 2023 en utilisant le greffage en écusson, et la seconde en mars 2024 en utilisant le greffage en fente. La validation des deux méthodes de détection a été réalisée en évaluant la sensibilité, la spécificité, la sélectivité, la reproductibilité et la répétabilité de celles-ci. Une méthode se concentre sur la séquence du gène ribosomal 16S, recommandée par l'EPPO, et l'autre sur la séquence du gène *secA*, qui est complémentaire aux méthodes mettant en évidence le gène ribosomal 16S. La caractérisation de la diversité moléculaire de *C. P. mali* a été réalisée en séquençant trois séquences de gènes : 16S, *secA* et *hflB*.

Les résultats des traitements à l'eau chaude n'ont pas permis de déterminer l'efficacité de la méthode durant la durée de ce travail, et la présence de *C. P. mali* devra être réévaluée dans le futur. Les méthodes évaluées ont montré que la sensibilité de la PCR imbriquée en 16S est intéressante lorsque la concentration attendue en phytoplasme dans les tissus est faible, mais ne présente pas toujours des résultats fiables et doit donc être confirmée soit par séquençage, soit par une autre PCR imbriquée. La PCR imbriquée en *secA* a une sensibilité faible mais, une fois séquencée, permet de bien différencier l'espèce du phytoplasme dans le groupe 16SrX. Enfin, la diversité moléculaire des gènes analysés a révélé que trois individus symptomatiques des vergers du CRA-W ont une virulence moyenne et douze une virulence élevée, donnant ainsi une direction pour la gestion future des pommiers infectés.

**[EN]** This study focuses on the feasibility of hot water treatment for grafts to eliminate apple tree phytoplasma, as well as the evaluation of two nested PCR detection methods to identify phytoplasmas and the molecular characterization of infected apple trees in CRA-W's orchards. *Candidatus Phytoplasma mali* is the phytoplasma that persistently infects apple trees and causes apple proliferation. There is no public curative treatment available to eliminate *C. P. mali*, and it is recommended to destroy infected individuals to prevent their spread within the orchard. At the Walloon Agricultural Research Centre, the conservation orchards contain infected individuals. The spread of sometimes unique but contaminated varieties cannot occur, and their destruction would lead to a reduction in the genetic resources of apple trees in Wallonia. A curative method has already shown effectiveness against other species of

phytoplasmas infecting fruit trees: hot water treatment of grafts. Therefore, it would be useful to confirm the presence of the phytoplasma in the apple trees and to identify the strain, as this can characterize the disease's virulence.

Four temperature-time conditions were tested for hot water treatment: three conditions at 45°C for 60, 90, and 180 minutes, and one condition at 50°C for 15 minutes. Two experiments were conducted: the first in November 2023 using chip budding, and the second in March 2024 using cleft grafting. The validation of the two detection methods was performed by assessing their sensitivity, specificity, selectivity, reproducibility, and repeatability. One method focuses on the 16S ribosomal gene sequence, recommended by EPPO, and the other on the *secA* gene sequence, which complements methods targeting the 16S ribosomal gene. Molecular diversity of *C. P. mali* was characterized by sequencing three gene sequences: 16S, *secA*, and *hflB*.

The results from the hot water treatments did not allow us to determine the effectiveness of the method within the scope of this work, and the presence of *C. P. mali* will need to be reassessed in the future. The evaluated methods demonstrated that nested PCR for 16S is useful when the expected concentration of phytoplasma in tissues is low, but it does not always provide reliable results and should be confirmed either by sequencing or through another nested PCR. The nested PCR for *secA* has low sensitivity but, once sequenced, effectively differentiates the phytoplasma species within the 16SrX group. Finally, the molecular diversity of the analysed genes revealed that three symptomatic individuals from CRA-W's orchards have moderate virulence and twelve have high virulence, providing guidance for the future management of infected apple trees.

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# 1. Introduction

## *1.1. Candidatus Phytoplasma*

Before 1967, many diseases associated with phytoplasmas were believed to be the consequence of viruses until it was found that the microorganism causing those diseases is a bacterium and has been named mycoplasma-like organisms (MLOs). (Doi et al., 1967).

In 2004, with the improvement of detection methods such as DNA-specific amplification, MLOs were redesignated as *Candidatus* Phytoplasma (The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group, 2004). "*Candidatus*" refers to the inability to cultivate the microorganism *in vitro* (Murray et al., 1994). Indeed, phytoplasmas are bacteria with no cell wall that depend on their hosts to survive (CABI, 2021).

*Candidatus* Phytoplasma is a genus belonging to the Mollicutes class in the Mycoplasmatota phylum in the Bacteria kingdom. Classification of phytoplasmas is based on the 16S ribosomal gene sequence (Lee et al., 1998) and currently counts 49 species (Bertaccini et al., 2022) spread through 34 groups according to the revision guidelines proposed in 2022 by Wei et al.. Within a species, phytoplasmas share 98.65% similarity for the 16S ribosomal gene sequence while the initial classification proposed in 2004 had a threshold of 97.5% (The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group, 2004). Restriction fragment length polymorphism (RFLP) is used to distinguish between *C. Phytoplasma* and divide them into groups and subgroups (Wei et al., 2022).

The complete list of all known phytoplasmas can be found on Annex 1.

## 1.2. Transmission

*Candidatus Phytoplasma sp.* are transmitted by one or more phloem-feeding insects, mainly leafhoppers (Cicadellidae), planthoppers (Fulgoromorpha) and psyllids (Psyllidae) depending on the phytoplasma species (Weintraub et al., 2006). The transmission of the disease comes with an acquisition access period, latency period and inoculation access period (Bertaccini et al., 2019).

The acquisition access period mainly occurs via the nymph (Alma et al., 2015). The length of this period varies from one to seven days (Alma et al., 2000; Chuche et al., 2014).

The latency period (LP) length, varies from 12-14 days to more than 21 days (Chiykowski et al., 1988; Alma et al., 2018; Roddee et al., 2019). Another study also pointed out that the LP may depend on the temperature and carbon dioxide (Galetto et al., 2011).

Finally, the inoculation is mainly done by adults for two reasons. First, nymphs cannot fly and therefore cannot infect healthy plants. Secondly, usually, the length of the latency period is longer than the time needed for nymphs to become adults (Bertaccini et al., 2019). For the moment of inoculation, it occurs at different times in the season depending on the vector (Hoy et al., 1999; Chuche et al., 2014).

## *1.3. Candidatus Phytoplasma mali*

*Candidatus Phytoplasma mali* is responsible for the apple proliferation (AP) disease (Seemüller et al., 2004). According to the European and Mediterranean Plant Protection Organization (EPPO) A2 list, it is a regulated pest and recommended as a regulated non-quarantine pest in the European Union (Picard et al., 2018; "EPPO A2 List," March-13-2024). *C. P. mali* belongs to the 16SrX group (or Apple proliferation group) amongst *Candidatus Phytoplasma pyri* responsible for pear decline (PD*), Candidatus Phytoplasma prunorum* responsible for European stone fruit yellows (ESFY) and *Candidatus Phytoplasma spartii* responsible for Spartium witches'-broom (SpaWB). Respectively, their subgroup is 16SrX-A, 16SrX-B, 16SrX-C and 16SrX-D (Marcone, Gibb, et al., 2004; Seemüller et al., 2004; Wei et al., 2022).

To distinguish *Candidatus Phytoplasma mali, pyri, prunorum* and *spartii*, the 16S rDNA sequence is not enough as nucleotides position only differs between 1.0% and 1.5% for AP, PD and ESFY (Seemüller et al., 2004) and between 2.8% and 2.9% for SpaWB (Marcone, Gibb, et al., 2004). Seemüller et al. (2004) and Marcone et al. (2004) analysed other regions of the rDNA and DNA (the 16S–23S rDNA spacer region, protein-encoding genes and randomly cloned DNA fragments) to underscore the differences between these species making possible the differentiation within the 16SrX group.

### 1.3.1. Host plants

The main host for *C. P. mali* is *Malus spp.* (Seemüller, Carraro, et al., 2011). This is mostly a consequence of the insect vector, *Cacopsylla picta* which is monophagous on *Malus* spp. (Jarausch et al., 2003) (see 1.3.6). However, other plants have been reported as naturally infected with the bacterium : *Crataegus* spp. (Tedeschi et al., 2009), *Corylus avellana* (Marcone et al., 1996), *Pyrus communis, Pyrus pyrifolia, Prunus salicina* (Lee et al., 1995), *Carpinus betulus, Convolvulus arvensis* (Seemüller, 2002 as cited in Janik et al., 2020), *Prunus avium, Quercus robur and Quercus rubra* (Seemüller, Carraro, et al., 2011).

### 1.3.2. Geographical distribution

Europe is the main continent with apple proliferation. It is widespread in Czech Republic, Belgium, Germany, Hungary, Italy, Slovakia, Slovenia, and Switzerland. Other countries highlighted in Figure 1 have restricted distributions or few occurrences. Outside Europe, the disease has been reported in Tunisia, the province of Nova Scotia in Canada and Syria ("'Candidatus Phytoplasma mali' (PHYPMA)[World distribution]| EPPO Global Database," March-21-2024).

It is worth mentioning that in the United Kingdom, a sole case of apple proliferation has been detected in 1978 and eliminated in 1986. Since then, no records exist on its presence and the disease is still considered as eliminated (Davies et al., 1986).

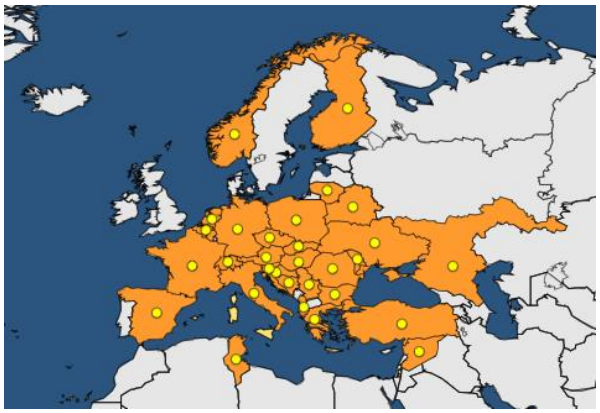


*Figure 1. European distribution of Candidatus Phytoplasma mali (“‘Candidatus Phytoplasma mali’ (PHYPMA)[World distribution]| EPPO Global Database,” March-21-2024).*

In Belgium, *C. P. mali* has been recorded for the first time in 1981 in Namur province (Maroquin et al., 1981). And in 2010, it was identified for the first time by molecular identification (Olivier et al., 2010). Even though no prevalence of the bacteria have been made in Belgium, it is commonly found (Hellin, personal communication, June 2024).

### 1.3.3. Symptoms

Apple proliferation leads to a variety of symptoms that can either be specific or not specific (Janik et al., 2020). Specific symptoms are unambiguously related to AP, and correspond to the formation of witches’ brooms (Figure 2A) and larger and dentate stipules (Figure 2B) (Seemüller, 1990; Seemüller, Carraro, et al., 2011).


*Figure 2. Symptoms of Apple proliferation. A: witches’ broom (photo taken on 26/10/2023). B: larger and dentate stipules (photo taken on 22/11/2023). C: rosette formation of apical leaves and early leaves reddening (photo taken on 26/10/2023).*

Non-specific symptoms appear on infected apple trees but cannot be directly linked to AP (Janik et al., 2020). One of the most visible non-specific symptom is early leaf reddening

(Figure 2C) (Bovey, 1960) even though this symptom only appears on some cultivars and at different period of the year (Janik et al., 2020). However, recent breakthrough documented by Jarausch et al. (2024) demonstrated that partially or entirely premature leaf reddening is a good indicator to detect AP. The disease also induces undersized branches and rosette formation of apical leaves (Figure 2C) (Zawadska, 1976 as cited in Janik et al., 2020). Infected trees tend to be more sensitive to powdery mildew (*Podosphaera leucotricha* (Ellis & Everh.) E.S.Salmon) (Bovey, 1963; Zawadska, 1976 ; Maszkiewicz et al., 1979 as cited in Janik et al., 2020). Concerning the fruit, non-specific symptoms include smaller size, lack of flavour, reduced colour, and a longer peduncle (Blattny et al., 1963; Seemüller et al., 2011; Seidl, 1980 ; Schmidt et al., 2009 ; Zawadzka, 1976 as cited in Janik et al., 2020). Those symptoms are the ones responsible for economic losses by inducing up to 80% of the fruits' unmarketability (Seemüller, 1990). A combination of two or more non-specific symptom may be linked to apple proliferation (Thomann and Tumler, 2000; Mattedi et al., 2008 as cited in Janik et al., 2020).

It is also important to note that because of the delay between infection and the appearance of the first symptoms, apple trees can be symptomless but infectious. Indeed, the latency period has been evaluated around one and a half and two years after infection (Bovey, 1963; Unterthurner and Baric, 2011 as cited in Janik et al., 2020). This means that symptoms are not always dependable for the detection of AP and further molecular investigation should be made to assert AP infection.

### 1.3.4. Economic impact

Apple proliferation can lead to 80% of productivity losses (Seemüller, Carraro, et al., 2011; Rao et al., 2018). An AP outbreak in 2001 caused economic losses of 25 million euros in Germany and 100 million euros in Italy. In 2006 and 2013, the South Tyrol region alone experienced estimated losses of around 50 million euros each year. (Strauss, 2009; Janik et al., 2020).

### 1.3.5. Distribution in apple trees

The distribution of *C. P. mali* in apple trees varies seasonally. During winter, the presence of phytoplasma is higher in the roots. In the shoot, it is almost undetectable in late winter/beginning of spring (Seemüller et al., 1984; Pedrazzoli et al., 2008 as cited in Baric et al., 2011).

When there are symptoms such as witches' brooms and undersize fruits, the concentration of phytoplasmas is higher (Schaper et al., 1984; Seemüller, Kunze, et al., 1984; Seemüller, Schaper, et al., 1984; Bisognin et al., 2008; Baric et al., 2011).

### 1.3.6. Insect vectors

Two main insects are responsible for the transmission of AP, *Cacopsylla picta* Förster (1848) and *Cacospylla melanoneura* Förster (1848) (Frisinghelli et al., 2000; Tedeschi et al., 2002, 2004; Jarausch et al., 2003). They both belong to the Psyllidae family within the Psylloidea superfamily (MNHN & OFB, March-15-2024a, March-15-2024b).

#### 1.3.6.1. Cacopsylla picta

It was confirmed that *Cacopsylla picta* is a vector of apple proliferation for the first time in Trentino, Italy (Frisinghelli et al., 2000). Later, this was confirmed in Germany (Jarausch et al., 2003). The psyllid distribution is palearctic and it is monophagous on *Malus spp.* (Lauterer, 1999 as cited in Jarausch et al., 2011).

*C. picta* adults overwinter on conifers (Cermak et al., 2008; Mayer et al., 2011). In spring, they migrate to apple orchards for two to three months, during which mating, oviposition and juvenile development occur. (Mayer et al., 2008). To ensure the spread of *Candidatus Phytoplasma mali*, the infected vector will not lay its eggs on an infected tree as it results in a higher mortality rate for the offsprings. Instead, *C. picta* will preferentially lay its eggs on an uninfected tree as well as feed on it and passing the bacteria by doing so (Mayer et al., 2011).

It is also worth mentioning that vertical transmission of AP to the progeny of *C. picta* is possible (Mittelberger et al., 2017).

#### 1.3.6.2. Cacopsylla melanoneura

*Cacopsylla melanoneura* (hawthorn psyllid) has been reported as a vector of apple proliferation for the first time in northwestern Italy in 2002 (Tedeschi et al., 2002). In Germany, Switzerland and France, however, *C. melanoneura* is not a vector of C. *P. mali* (Mayer et al., 2009). It is oligophagous on *Malus* spp., *Pyrus communis* spp. and *Crataegus* spp. (hawthorn) (Conci et al., 1993 ; Ossiannilson, 1992 as cited in Mayer et al., 2009).

The hawthorn psyllid overwinters on conifers in high-altitude regions and gradually descends toward orchards and hawthorns, with a preference for the latter, as winter ends. By late March, they mate and lay their eggs, and the juveniles develop until the end of June, at which point they begin to migrate back toward the higher mountain conifers. (Mayer et al., 2007).

#### 1.3.6.3. Other insect vectors

Another specie of insect was proved to be vector of *C. P. mali*: *Fieberiella florri Stål* (1864). However, the transmission of AP has only been observed experimentally and could not be proved on the field (Tedeschi et al., 2006).

Other studies suggest the possible implication of other vectors as the bacteria have been found in multiple other species : *Cacopsylla mali* (Baric et al., 2010; Miñarro et al., 2016), *Cacopsylla peregrina* (Tedeschi et al., 2009), *Cacopsylla crataegi* (Miñarro et al., 2016), *Cacospylla spatula* and *Cacopsylla eucalypti* (Rosa García et al., 2014; Miñarro et al., 2016).

It is important to note that experiments have been conducted on aphids, which were found carrying C. *P. mali,* to evaluate their role as vectors. However, the research concluded that aphids did not contribute to the transmission of AP (Cainelli et al., 2007 as cited in Janik et al., 2020).

### 1.3.7. Other means of spreading

The transmission of *C. P. mali* also occurs by natural root bridges (anastomosis) (Ciccotti et al., 2007; Vindimian et al., 2002 as cited in Janik et al., 2020), dodder (*Cuscuta sp*.) (Marwitz et al., 1974 as cited in Janik et al., 2020) and grafting (Seemüller, Kunze, et al., 1984; Seemüller, Schaper, et al., 1984).

Because grafting is the standard way of commercial production for apple trees, it is possibly an important way for AP to spread (Seemüller, Kunze, et al., 1984; Seemüller, Schaper, et al., 1984). Depending on the method and period of grafting, *C. P. mali* will spread efficiently. Chip budding, for example, has a low transmission rate between March and May but high between June and August. This means that grafting during the dormancy period lowers the transmission of AP (Pedrazzoli et al., 2008).

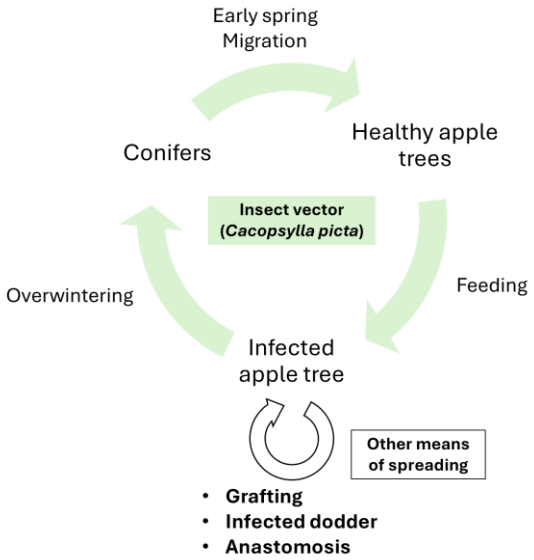



*Figure 3. Life cycle of Candidatus phytoplasma mali with the insect vector Cacopsylla picta and other means of spreading.*

### 1.3.8. Control measures

#### 1.3.8.1. Preventive methods

To date, no curative treatments exist to get rid of AP in infected trees in orchards. Therefore, a combination of preventive methods is recommended.

As the phytoplasma has a biphasic cycle, one of the preventive methods is population reduction of *C. picta* and *C. melanoneura* using pesticide. Since the discovery of those vectors, this method proved to be efficient as their population decreased in the following years. However, an outbreak of AP occurred in South Germany in 2013 (Janik et al., 2020). Alternatively to pesticide, kaolin had also been proved to be efficient on *Cacopsylla pyri* and could be used against AP vectors (Pasqualini et al., 2002; Daniel et al., 2005; Erler et al., 2007). *Pandora* sp., a entomopathogenic fungus, have been found efficient in lab condition to biologically control *C. picta* (Görg et al., 2019).

Another preventive method is the use of certified non-infected planting material based on EPPO guidelines (“PM 7/133 Generic detection of phytoplasmas,” 2018). In addition, some cultivars are tolerant to AP (mostly based on symptoms) such as “Lord Lambourne” (Friedrich,

1993 as cited in Janik et al., 2020), “Clivia”, “Herma” (Friedrich and Rode, 1996 as cited in Janik et al., 2020), “Roja de Benejama” (Janik et al., 2020), “Antonovka”, “Cortland”, “Spartan”, “Yellow Transparent”, “Wealthy” (Németh, 1986 as cited in Janik et al., 2020; Thakur et al., 1999), “Melrose” (Richter, 2003 as cited in Janik et al., 2020), “Goldstar”, “Rubinola”, “Lotos” and “Rosana” (Korte et al., 2005 as cited in Klaehre, 2008). In 2008, Seemüller et al. discovered a resistant *Malus* specie that could be used for grafting : *Malus sieboldii* (Seemüller et al., 2008). Even though it has great potential, the rootstock is not suitable for commercial apple growing compared to normally used M9 rootstock. Therefore, an effort has been made to genetically improved *M. sieboldii* with M9 rootstock, but are still under evaluation (Seemüller et al., 2018; Janik et al., 2020).

To stop the spread of the disease, the elimination of the infected trees is also recommended (Janik et al., 2020).

#### 1.3.8.2. Curative treatments against phytoplasmas

**a) Chemotherapy**

In 1967, Ishiie et al. discovered that tetracycline was efficient against phytoplasmas but symptoms reemerged when the treatment stopped (Ishiie et al., 1967). Now, antibiotics are banned from European Union (Sundin et al., 2018). Other methods exist and have been assessed experimentally such as plasma activated water (PAW) which proved to reduce symptoms in grapevines (Laurita et al., 2015; Zambon et al., 2017 as cited in Laimer et al., 2019)

**b) Thermotherapy by hot water treatment**

First efficient use of thermotherapy by hot water treatment (HWT) against a phytoplasma was achieved by Kunkel (1936) on peach tree attacked by peach yellow (as cited in Laimer et al., 2019). After that, HWT was successfully used against multiple phytoplasmas (Thung, 1952; Liu, 1963; Adams et al., 1992; Caudwell et al., 1997; Viswanathan et al., 2011).

Depending on the plant material and the phytoplasma species, the time duration and temperature modalities efficient against them differ from 10 minutes to 3 days and from 32°C to 50°C (Caudwell, 1966 ; Kunkel, 1936 as cited in Laimer et al., 2019). As an example of a closely related phytoplasma eliminated by HWT, Adams et al. (1992) successfully eliminated *Candidatus phytoplasma pyri* treating scion at 47.5°C for half an hour, 45°C for an hour and 42.5°C for 240 minutes. One of the main inconvenient in HWT would be the survival of the plant material after the treatment. Indeed, if the temperature is too high during a too long period, it can diminish the plants’ recovery rate (Brans et al., 2023).

No information could be found concerning a treatment against *Candidatus Phytoplasma mali* using HWT but only on a survival rate at different time-temperature modalities of scion. Indeed, as a treatment against infected *Malus Domestica* (MM106 cultivar) and *Pyrus Communis* (Kirchensaller cultivar) by fire blight (*Erwinia amylovora* (Burrill 1882) Winslox et al. 1920), the Thermofruit project focused on non-lethal time-temperature modalities for plant material. Temperature above 50°C during 15 minutes does not allow grafting recovery compared to every tested time modalities at 45°C which got similar recovery rates than the non-treated controls (Brans et al., 2023).

## 1.4. Polymerase chain reaction detection for phytoplasmas

As previously mentioned, differentiation between phytoplasmas is based on the 16S ribosomal gene sequence (Lee et al., 1998). To this end, universal primers pairs have been developed through the years for polymerase chain reaction (PCR).

After DNA amplification of the 16S ribosomal gene sequence, samples can be processed using RFLP which proved to be important to distinguish phytoplasmas within the same group (Lee et al., 1991; Lee, Davis, et al., 1992; Lee, Gundersen, et al., 1992; Marcone, Gibb, et al., 2004; Seemüller et al., 2004). As a complementary method, sequencing/barcoding is also used to identify subtypes as described by Wei et al. (2022).

However, direct PCR of the 16S gene is less sensitive and less specific because it also amplifies non-phytoplasma sequences (Gundersen et al., 1996).

Nowadays, one of the recommended method by EPPO for phytoplasma's detection is a nested PCR using P1/P7 (Deng et al., 1991; Schneider et al., 1995) and R16F2n/R16R2 (Lee et al., 1993; Gundersen et al., 1996) primers pairs. P1/P7 primers target the entire 16S rRNA gene as well as the 16S/23S rRNA spacer region ("PM 7/133 Generic detection of phytoplasmas," 2018). A nested PCR is a twostep PCR where the first PCR product is use as the DNA template for the second PCR amplification. This technique increased both the sensitivity and specificity of the reaction (Bonin et al., 2011).

Because nested PCR involves a higher risk of contamination, real-time PCR is also recommended to reduce that risk. Furthermore both qPCR and nested PCR for the detection of phytoplasmas were designed to be universal, false positive may occurred and EPPO recommended more specific PCR tests or sequencing when the outcome of the test result is critical ("PM 7/133 Generic detection of phytoplasmas," 2018). Moreover, genes other than 16S rRNA have been targeted to detect phytoplasmas : ribosomal protein (*rp*) (Martini et al., 2013), *Tuf, secY* (Foissac et al., 2013), *imp*, *aceF* (Danet et al., 2011), *hflB* (Schneider et al., 2009), *secA* (Dickinson et al., 2013).

## 1.5. Objectif of the study

Walloon Agricultural Research Centre's (CRA-W) apple orchards purpose is to preserve the existing biodiversity among cultivars in Belgium. The only way to propagate those cultivars is by grafting which is a problem when permanent pathogens (such as phytoplasmas) are in the plant material.

Preventive methods like the destruction of the infected tree are not an option especially if there are only few individual representatives of a cultivar. The same goes for reduction of psyllids vectors population using pesticide as orchards are purposely not treated. Using certified non-infected planting material remain the only possibility to constrain the spread of the disease outside and within CRA-W's orchards but it is not always possible as some cultivars are infected with AP .

Therefore, curative treatments could be a great solution to ensure the conservation of apple tree cultivar biodiversity. In this study, hot water treatment was evaluated to eliminate phytoplasma from scions before grafting. This method should allow the CRA-W to disseminate cultivars without spreading AP outside of their conservatives' orchards.

Furthermore, detection and identification of *Candidatus Phytoplasma mali* is crucial to know the extent on which apple trees in the conservative orchard are infected. It is especially true

for those who are symptomless, in which case, PCR method is the most efficient way to detect AP. From the seven methods initially selected to detect *Candidatus Phytoplasma Mali*, two nested PCR were assessed for validation: one recommended by EPPO guidelines on phytoplasma detection ("PM 7/133 Generic detection of phytoplasmas," 2018) which focus on the 16S ribosomal gene sequence. And another one focusing on *secA* gene sequence (Dickinson et al., 2013).For this, both methods have been following the EPPO guidelines for accreditation of a plant pest diagnostic as a way to validate those methods for the detection of *C. P. mali* ("PM 7/98 (5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity," 2021) .

Finally, a molecular diversity of symptomatic apple trees from CRA-W's orchards and elsewhere have been made to define and characterise strains of AP within it focusing on 16S ribosomal gene sequence, *secA* gene sequence and *hflB* gene sequence.

# 2. Material and methods

## 2.1. Samples harvesting

Apple trees samples collected during this study mainly came from two different orchards located in Gembloux, Namur province in Belgium. One of them is located at rue du Liroux around Emile Marchal CRA-W's building (20 + 2.5 ha)(50°33'45.7"N 4°43'33.5"E) and the other one is located at chaussée de Charleroi around Jean-Baptiste de La Quintinie CRA-W's building (5 ha)( 50°33'15.8"N 4°39'37.1"E). Three other samples came from Ormeignies, Blanmont and Hévillers.


*Figure 4. Localisation of the two orchards where the majority of the samples were harvested ("Google Earth," May-2-2024).*

Samples have been harvested from infested apple trees which were previously identified based on specific and non-specific symptoms. They were harvested using shears or a telescopic shear. Between each use, tools were disinfected using 70% ethanol ignited by fire. Harvested branches were labelled, humidified, and sealed in plastic film for storage in a fridge at 4°C until use (between one and five weeks depending on the tests). The positive extraction controls came from potato tubers infected with *Candidatus Phytoplasma solani* that were already prepared before the beginning of this work. Potato tuber samples infected by *C. P. solani* came from north of France and were harvested in 2023.

*Table 1. Harvested samples from apple trees used in this work. A35 and A65 were harvested first for the first water treatment, for the second one and for the evaluation of the detection methods (in this order).*

| ID | Cultivar | Origins | Harvested date |
|---|---|---|---|
| | Dello | Ormeignies, Belgium | 2023 |
| PH23 | Malus spp. ornamental | Blanmont, Belgium | 08/11/2023 |
| PH24 | Belle de Boskoop | Hévillers, Belgium | 08/11/2023 |
| A35 | Santana (no symptoms) | Quintinie | 08/11/2023, 13/02/2024 and 17/04/024 |
| A65 | Santana (symptomatic) | Quintinie | 08/11/2023, 13/02/2024 and 17/04/24 |
| A17 | Blenheim Orange | Orchard 1 Marchal | 16/11/2023 |
| A40 | Chailleux | Orchard 1 Marchal | 16/11/2023 |
| A7 | Devonshire Quarrenden | Orchard 1 Marchal | 16/11/2023 |
| B19 | Gris Braibant | Orchard 1 Marchal | 16/11/2023 |
| C41 | Gris Braibant | Orchard 1 Marchal | 16/11/2023 |
| A20 | Jacques Lebel | Orchard 1 Marchal | 16/11/2023 |
| D51 | Président Henry Van Dievot, cabaret | Orchard 1 Marchal | 16/11/2023 |
| A23 | Reinette Blanche du Canada | Orchard 1 Marchal | 16/11/2023 |
| A25 | Reinette de chênée | Orchard 1 Marchal | 16/11/2023 |
| C25 | Reinette de Grez-Doiceau | Orchard 1 Marchal | 16/11/2023 |
| B30 | Reinette Descardre | Orchard 1 Marchal | 16/11/2023 |
| B20 | Reinette Grise d'automne | Orchard 1 Marchal | 16/11/2023 |
| L45 | Calville Rouge d'automne | Orchard 3 Marchal | 16/11/2023 |
| L40 | Reinette Verbraeck | Orchard 3 Marchal | 16/11/2023 |
| P44 | Semis Louette | Orchard 3 Marchal | 16/11/2023 |
| K74 | Blenheim Orange | Orchard 4 Marchal | 16/11/2023 |
| L95 | Boskoop ? | Orchard 4 Marchal | 16/11/2023 |
| R108 | Coop 29 | Orchard 4 Marchal | 16/11/2023 |
| P97 | Jonagold Jonica | Orchard 4 Marchal | 16/11/2023 |
| R105 | Primadela | Orchard 4 Marchal | 16/11/2023 |
| P78 | Reinette Massart | Orchard 4 Marchal | 16/11/2023 |

## 2.2. PCR detection for phytoplasmas

Phytoplasma detection was mainly conducted using the accredited protocol of the CRA-W. This procedure includes DNA extraction using CTAB 3%, DNA amplification via conventional PCR and/or nested PCR and electrophoresis migration of the PCR products visualized under UV light.

All DNA extraction, DNA amplification and electrophoresis migration were performed in the accredited ISO17025 virology laboratory in Emile Marchal CRA-W's building, Gembloux. DNA amplification was performed on a Biorad T100 Thermal cycler or an Eppendorf Mastercyler epgradient thermocycler. For nested PCR, because the DNA used for the second round PCR come from the first round PCR (therefore highly concentrated), incorporation into individual PCR tubes were done under a hood.

### 2.2.1. DNA extraction

Depending on the plant material, 0.3 g (main leaves veins, petioles, phloem chips) or 0.15 g (washed root hair) were weighed and placed on a 2 ml tube with a grinding ball for grinding in a Retsch MM400. When the plant material was not properly grinded during the first try, 2 or 3 drops of CTAB ($C_{19}H_{42}BrN$) 3% were added to ease the process.

Two ml of CTAB 3% was added in each sample which was transferred to a dry water bath at 65°C for about 30 minutes. After centrifugation, 800 µl of the supernatant was transferred to a new 2 ml tube. In this last, 800 µl of chloroform ($CHCl_3$) was added under a fume hood before being centrifuged. Next, 400 µl of the supernatant was transferred to a 1.5 ml tubes with 400 µl of isopropanol to be centrifuged.

The supernatant was discarded, and the pellet was cleaned with 100 µl of ethanol 70%. After one last centrifugation, the supernatant was discarded, and the tubes were placed on a Savant SVC-100H SpeedVac machine until all traces of ethanol were eliminated (5 to 10 minutes). Finally, the pellet was suspended with 100 µl of biomolecular water.

Extracted DNA was stored at -20°C.

*Table 2. Target sequences and primers used in this work.*

| Target sequence | Diagnostic method | Primer name | Primer sequence | Amplicon expected size (bp) | References |
|---|---|---|---|---|---|
| *secA* | Nested PCR[1] | SecAFor1 | GARATGAAAACTGGRGAAGG | 840 | (Dickinson et al., 2013) |
| | | SecARev3 | GTTTTRGCAGTTCCTGTCATNCC | | |
| | | SecAFor5-u | ASTCGTGAAGCTGAAGG | 600 | |
| | | SecAFor5-1 | AGCTAAAAGAGAATTTGAAGG | | |
| | | SecAFor5-LY | CTGATAGAGAAGCTAATGG | | |
| | | SecARev2 | CCNTCRCTAAATTGNCGTCC | | |
| *hflB* | PCR | fHflB3-1-Schneider | TTCTAGCTATTCATCGTGAA | 530 | (Schneider et al., 2009) |
| | | rHflB3-Schneider | CGGCGCGATTAGTAGCTCC | | |
| 16S | Nested PCR[1] | P1 | AAGAGTTTGATCCTGGCTCAGGAT T | 1,850 | (Deng et al., 1991) |
| | | P7 | CGTCCTTCATCGGCTCTT | | (Schneider et al., 1995) |
| | | R16F2n | GAAACGACTGCTAAGACTGG | 1,250 | (Lee et al., 1993) |
| | | R16R2 | TGACGGGCGGTGTGTACAAACCCCG | | (Gundersen et al., 1996) |
| | PCR[2] | fU5L | TTCGGCAATGGAGGAAACTCTGACC | 1,000 | (Lorenz et al., 1995) |
| | | R16R2 | TGACGGGCGGTGTGTACAAACCCCG | | (Gundersen et al., 1996) |
| | Nested PCR | P1 | AAGAGTTTGATCCTGGCTCAGGAT T | 1,850 | (Deng et al., 1991) |
| | | P7 | CGTCCTTCATCGGCTCTT | | (Schneider et al., 1995) |
| | | f01 | CGGAAACTTTTAGTTTCAGT | 1,100 | (Lorenz et al., 1995) |
| | | r01 | AAGTGCCCAACTAAATGAT | | |

[1] Validation
[2] Under accreditation in CRA-W

### 2.2.2. Direct PCR targeting 16S rRNA gene (accredited)

This method is under accreditation at the virology lab (CRA-W). A full validation following the EPPO PM 7/98 had therefore already been performed. DNA was amplified using GoTaq® G2 Flexi DNA Polymerase kit (Promega) with fU5L (modified version of fU5 primer from Lorenz et al., 1995) and R16R2 (Gundersen et al., 1996) primers. This primer pair give an expected amplicon size around 1000bp.

*Table 3. Volume of PCR reagents per reaction used for direct PCR targeting 16S rDNA gene to detect phytoplasmas used by CRA-W.*

| Reagents | Volume (µl) |
|---|---|
| **Water** | 9.5 |
| **Buffer** | 4.0 |
| **Primer fU5L (20 µM)** | 0.8 |
| **Primer R16R2 (20 µM)** | 0.8 |
| **dNTPs 2,5mM** | 1.6 |
| **$MgCl_2$** | 1.6 |
| **Taq polymerase** | 0.1 |
| **DNA** | 1.6 |
| **Total** | 20 |

PCR conditions were as followed: 1 cycle at 95°C for 2 minutes; 35 cycles of 94°C for 1 minute, 68°C for 45 seconds and 72°C for 2 minutes; then a final extension of 72°C for 10 minutes and held at 15°C forever.

### 2.2.3. Direct PCR targeting *hflB* gene

This method is used to assess the virulence of different strain of *C. P. mali*. Sequencing of this gene sequence can highlight two motif linked to mild virulence strain: TTA184 and T-C227 (Seemüller, Kampmann, et al., 2011).

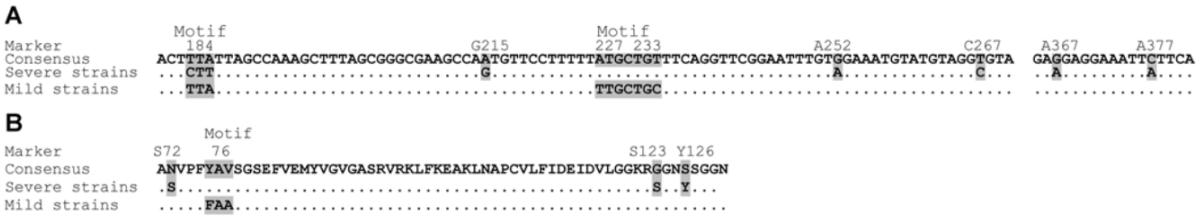



**Fig. 4.** Virulence-related markers in the **A,** nucleotide and **B,** deduced amino acid sequence of *hflB* gene fragments of '*Candidatus* Phytoplasma mali' accessions. The markers occur singly or in combination. The nucleotide markers G215, A367, A377, and motif T-C227 correspond to the amino acid markers S72, S123, S126, and motif F-A76, respectively.

*Figure 5. Virulence-related markers in hflB gene sequence (Seemüller, Kampmann, et al., 2011)*

DNA was amplified using Q5 Hot Start High Fidelity DNA polymerase (NEB) kit with fHflB3-1-Schneider/rHflB3-Schneider primers. The expected amplicon size from this primer pair is 530bp.

*Table 4. Volume of PCR reagents per reaction used for direct PCR targeting hflB gene.*

| Reagents | Volume (µl) |
|---|---|
| **Water** | 14.5 |
| **Buffer** | 5.00 |
| **Primer fHflB3-1-Schneider (20 µM)** | 0.625 |
| **Primer rHflB3-Schneider (20 µM)** | 0.625 |
| **dNTPs (2,5 mM)** | 2.00 |
| **Taq polymerase** | 0.250 |
| **DNA** | 2.00 |
| **Total** | 25.0 |

PCR conditions were as followed: 1 cycle at 98°C for 30 seconds; 39 cycles of 98°C for 10 seconds, 58°C for 30 seconds and 72°C for 30 seconds; then a final extension of 72°C for 5 minutes and held at 10°C forever.

### 2.2.4. Nested PCR targeting 16S rRNA gene

#### 2.2.4.1. P1/P7 followed by R16F2n/R16R2

This method is the preferred method for generic identification of phytoplasma by the EURL-virology (European reference laboratory). Indeed, database of 16S ribosomal gene sequence is the most furnished and therefore the most reliable to identify phytoplasma species. DNA was amplified using GoTaq® G2 Flexi DNA Polymerase kit (Promega) with P1/P7 primers for the first round PCR and R16F2n/R16R2 primers for second round PCR (based on “PM 7/133 Generic detection of phytoplasmas,” 2018). The first PCR had an expected amplicon size around 1850bp and the second one 1245bp.

*Table 5. Volume of PCR reagents par reaction used for nested PCR targeting 16S rDNA gene (P1/P7; R16F2n/R16R2).*

| Reagents (First PCR) | Volume (µl) | Reagents (Second PCR) | Volume (µl) |
|---|---|---|---|
| **Water** | 9.5 | **Water** | 9.5 |
| **Buffer** | 4.0 | **Buffer** | 4.0 |
| **Primer P1 (20 µM)** | 0.80 | **Primer R16F2n (20 µM)** | 0.80 |
| **Primer P7 (20 µM)** | 0.80 | **Primer R16R2 (20 µM)** | 0.80 |
| **dNTPs 2,5mM** | 1.6 | **dNTPs 2,5mM** | 1.6 |
| **$MgCl_2$** | 1.6 | **$MgCl_2$** | 1.6 |
| **Taq polymerase** | 0.10 | **Taq polymerase** | 0.10 |
| **DNA** | 1.6 | **DNA** | 1.6 |
| **Total** | 20 | **Total** | 20 |

PCR conditions for first round PCR were as follow: 1 cycle at 95°C for 2 minutes; 35 cycles at 95°C for 1 minute, 53°C for 1 minute, 72°C for 2 minutes; then a final extension at 72°C for 10 minutes and held at 17°C forever.

PCR conditions for second round PCR were as follow: 1 cycle at 95°C for 2 minutes; 35 cycles at 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; then a final extension at 72°C for 10 minutes and held at 15°C forever.

#### 2.2.4.2. P1/P7 followed by f01/r01

This PCR is specific for the detection and identification of the 16SrX group phytoplasmas and is recommended by EPPO guidelines after a generic phytoplasma detection method or direct detection of AP group. DNA was amplified using GoTaq® G2 Flexi DNA Polymerase kit (Promega) with P1/P7 primers for the first round PCR and f01/r01 primers for second round PCR ("PM 7/62 (3) 'Candidatus Phytoplasma mali', 'Ca. P. pyri' and 'Ca. P. prunorum,'" 2020). The first PCR had an expected amplicon size around 1,850bp and the second one 1,100bp.

*Table 6. Volume of PCR reagents par reaction used for 16S nested PCR (P1/P7; f01/r01).*

| Reagents (First PCR) | Volume (µl) | Reagents (Second PCR) | Volume (µl) |
|---|---|---|---|
| **Water** | 9.5 | **Water** | 13.9 |
| **Buffer** | 4.0 | **Buffer** | 5.00 |
| **Primer P1 (20 µM)** | 0.80 | **Primer f01 (20 µM)** | 0.500 |
| **Primer P7 (20 µM)** | 0.80 | **Primer r01 (20 µM)** | 0.500 |
| **dNTPs 2,5mM** | 1.6 | **dNTPs 2,5mM** | 2.00 |
| **$MgCl_2$** | 1.6 | **$MgCl_2$** | 2.00 |
| **Taq polymerase** | 0.10 | **Taq polymerase** | 0.125 |
| **DNA** | 1.6 | **DNA** | 1.00 |
| **Total** | 20 | **Total** | 25.0 |

First round PCR condition was the same as the one from previous PCR descripted for P1/P7 pair of primers.

PCR conditions for second round PCR were as follow: 1 cycle at 95°C for 2 minutes; 37 cycles at 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; then a final extension at 72°C for 8 minutes and held at 17°C forever.

### 2.2.5. Nested PCR targeting *secA* gene

This method is not recommended by neither EPPO guidelines nor EURL but according to Bertaccini et al. (2022) it is a great complementary method to 16S targeting PCRs as it is a universal method for phytoplasma. However, database of this gene sequence is rather scarce and cannot be reliable to identify some phytoplasma species. DNA was amplified using GoTaq® Flexi DNA Polymerase kit (Promega) with SecAFor1/SecARev3 primers for the first round PCR and SecAFor5-u; SecAFor5-1; SecAFor5-LY/SecARev2 primers for second round PCR (based on Dickinson et al., 2013). The first PCR have an expected amplicon size around 840bp and the second one 600bp.

*Table 7. Volume of PCR reagents par reaction used for secA nested PCR (secAfor1/secARev3; secAFor5-u, secAFor5-1, secAFor5-LY/secARev2).*

| Reagents (First PCR) | Volume (µl) | Reagents (Second PCR) | Volume (µl) |
|---|---|---|---|
| **Water** | 10.3 | **Water** | 10.3 |
| **Buffer** | 4.00 | **Buffer** | 4.0 |
| **Primer SecAFor1 (20 µM)** | 0.400 | **Primer SecAFor5-u (20 µM)** | 0.133 |
| **Primer SecARev3 (20 µM)** | 0.400 | **Primer SecAFor5-1 (20 µM)** | 0.133 |
| **dNTPs 2,5mM** | 1.60 | **Primer SecAFor5-LY (20 µM)** | 0.133 |
| **$MgCl_2$** | 1.60 | **Primer SecARev2 (20 µM)** | 0.40 |
| **Taq polymerase** | 0.10 | **dNTPs 2,5mM** | 1.6 |
| **DNA** | 1.6 | **$MgCl_2$** | 1.6 |
| **Total** | 20 | **Taq polymerase** | 0.1 |
| | | **DNA** | 1.6 |
| | | **Total** | 20 |

PCR conditions for first round PCR were as follow: 1 cycle at 94°C for 2 minutes; 30 cycles at 95°C for 30 seconds, 53°C for 1 minute, 72°C for 1 minute; then a final extension at 72°C for 10 minutes and held at 15°C forever.

PCR conditions for second PCR were the same as the first round.

### 2.2.6. Electrophoresis migration for visualisation

Agarose gel was made using 1,2% agarose NEEO ultra-quality (Roth) in 1X TAE Buffer (Tris base 40mM, acetic acid, 20mM and 1mM EDTA). The solution was microwaved until dissolved and cooled down with tap water until it was cool enough to be safe to handle without a potholder. One percent volume of GelRed (Biotium) was added and mixed under a hood. The agarose was then poured into tray with a comb already inserted and allowed to harden.

Once it had hardened, the comb was removed, and the tray was placed into an electrophoresis chamber filled with 1X TAE solution. 10µl of PCR product were loaded into the wells and for each row, a 6µl of GeneRuler 1kb Plus DNA Ladder (Thermo Scientific) was added (Annex 2). The power output and duration of the migration were adjusted according to the volume of the gel.

*Table 8. Gel preparation and migration parameters use in this work.*

| Gel volume (ml) | Agarose quantity (g) | GelRed volume (µl) | Maximum electric current (A) | Migration duration (minutes) |
|---|---|---|---|---|
| 30.0 | 0.36 | 3.0 | 120 | ~60 |
| 100 | 1.2 | 10 | 200 | ~120 |
| 150 | 1.8 | 15 | 250 | ~120 |
| 250 | 3.0 | 25 | 200 | ~180 |

The gel was visualised using a transilluminator under UV light and saved on photographic film. Pictures were digitalized and labelled for interpretation.

## 2.3. Hot water treatment

Hot water treatment (HWT) has been applied twice in separate experiments. Symptomatic and symptomless samples were gathered from Santana cultivar (Table 1: A65 and A35) and from the same apple trees for both experiments. Ten scions from each tree have been harvested for each HWT. Scions from the symptomatic tree were identified as SX while ASX identification was used for the asymptomatic tree (with X the number of the sample).

Hot water treatment 1 (HWT1) was conducted on the 16th of November 2023 with plant material gathered the 8th of November 2023. Hot water treatment 2 (HWT2) was completed the 22nd of March 2024 with plant material gathered the 13th of February 2024.

The device use for both HWT were a Hoake W26 and a Memmert W200. For each experimentation, infected and uninfected plant material were treated separately in one of the devices. Because branches were sometimes too long to be placed into the device, they were shortened. The Hoake W26 device applied a constant stream of water, so the scions were attached to a metal track with strings.

*Figure 6. Hot water treatment using Hoake W26 device (photo taken on the 16th of November 2023).*

Hot water treatment modalities are based on Brans et al. (2023). In this last paper, various combination of time-temperature couple have been evaluated to assess the survival rate from different cultivar of apple tree (Reinette Clochard, Discovery, Golden Delicious, Durello di Forli and Dulmener Rosenappel).

Therefore, four different modalities have been applied:

- 45°C for 60 minutes (45-60)
- 45°C for 90 minutes (45-90)
- 45°C for 180 minutes (45-180)
- 50°C for 15 minutes (50-15)

Before and directly after each HWT (before grafting), scions were tested to assess if they were infected or not using PCR methods. For HWT1, only the accredited PCR method was performed. For HWT2, the accredited PCR method was done as well, but because this method is less sensitive than a nested PCR, it was decided to conduct two nested PCR methods: one on the 16S ribosomal gene sequence using P1/P7 follow by R16F2n/R16R2 pairs of primers and the other on *secA* gene sequence).

Based on the PCR results, scions were comparatively ordered according to the intensity of their migration bands observed in the nested PCR using P1/P7 followed by R16F2n/R16R2 primers. The infected scions were categorized based on these band intensities to reflect varying degrees of positivity across different modalities.

For each modality, infected and uninfected plant material have been treated. In addition, infected and uninfected plant material were not treated (NT). Plant material was stored at 4°C until grafting.

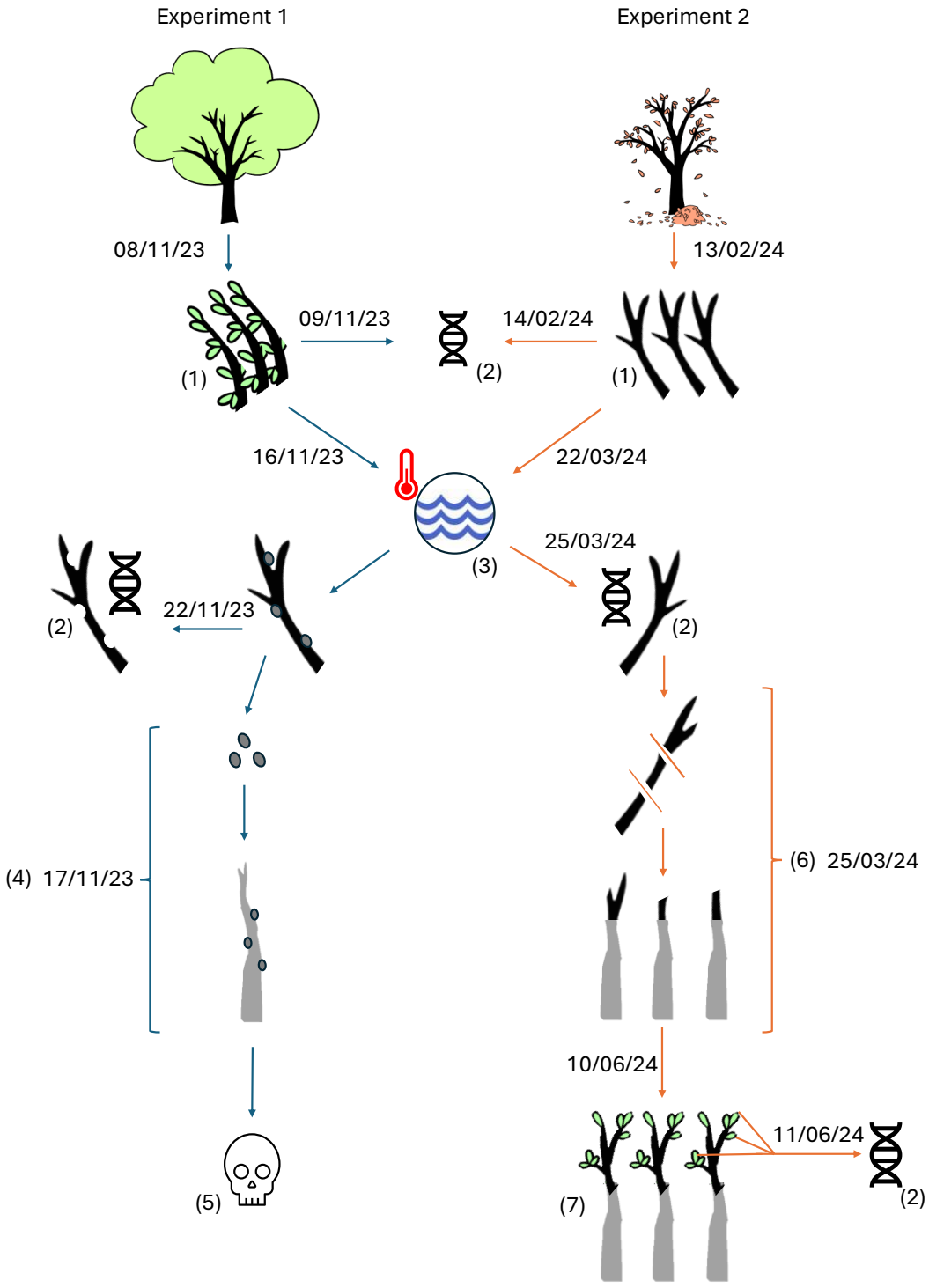



*Figure 7. Timeline and illustration of the two hot water treatments experiments. (1) Scions harvesting; (2) DNA extraction; (3) Hot water treatment; (4) Chipped budding grafting; (5) Death of the grafted apple trees; (6) Cleft grafting; (7) Leaves harvesting of the recovered grafted apple trees.*

### 2.3.1. Grafting

Depending on the HWT, two grafting techniques were utilized. For HWT1, scions were grafted using chip budding method. For HWT2, they were grafted using cleft grafting method. For both grafts, M9 rootstock was used.

The first grafting was made on the 17th of Novembre 2023. The next day, the grafted apple trees were left to grow on an outside tree nursery next to the Jean-Baptiste de La Quintinie building in Gembloux. For HWT1, each replica from Table 9 referred to 2 or 3 grafted buds (depending on the amount available on each scion) on one M9 rootstock.

After the HWT2, cuttings were divided to maximise the amount of scion with at least three buds. Therefore, each replica for HWT2 from Table 9 represents one scion with at least three buds on it. The second grafting was performed on the 25th of March 2024. The next day, the grafted trees were potted with compost and left to grow on a greenhouse at the Emile Marchal building. Grafted apple trees from the HWT2 were regularly watered.

*Table 9. Number of replicas by modality for hot water treatments. Modalities: 45-60 = 45°C for 60 minutes; 45-90 = 45°C for 90 minutes; 45-180 = 45°C for 180 minutes; 50-15 = 50°C for 15 minutes.*

| Modalities | | 45-60 | 45-90 | 45-180 | 50-15 | NT | Total |
|---|---|---|---|---|---|---|---|
| HWT1 | Uninfected | 2 | 2 | 2 | 2 | 2 | 10 |
| | Infected | 6 | 6 | 5 | 7 | 6 | 30 |
| | Total | 8 | 8 | 7 | 9 | 8 | 40 |
| HWT2 | Uninfected | 6 | 6 | 6 | 4 | 4 | 26 |
| | Infected | 8 | 6 | 8 | 9 | 9 | 40 |
| | Total | 14 | 12 | 14 | 13 | 13 | 66 |

Only the second batch of grafted apple trees' leaves were harvested for PCR detection. It was done on the 10th of June (78 days after grafting). When the graft did not recover but the buds' rootstock grown a branch, leaves from them were harvested for analyses. The PCR used were all the one that focused on the 16S ribosomal gene sequence (Table 2) as well as a P1/P7 primer pair followed by fU5l/R16R2 primer pair.

*Figure 8. (Left) Grafted trees from the first hot water treatment (photo taken on the 13th of February 2024); (right) grafted trees from the second hot water treatment (photo taken on the 10th of June 2024)*

## 2.4. Detection methods for validation

On a separate experiment, detection methods for validation focusing on the 16S ribosomal gene sequence and *secA* gene sequence were assessed following the same protocol and based on the EPPO methodology for accreditation of a plant pest diagnostic ("PM 7/98 (5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity," 2021). Accreditation of a plant pest diagnostic involves multiple factors to consider: sensitivity, specificity, selectivity, reproducibility, repeatability, and robustness[3].

### 2.4.1. Sensitivity

Sensitivity was assessed by diluting a positive sample into a negative sample to a concentration of $10^{-8}$ the initial concentration with ten times serial dilution. Because the concentration of phytoplasma was not known, the goal was to determine the maximum dilution giving a positive result. For this, the CTAB 3% DNA extraction protocol was modified before adding the chloroform as represented in Figure 9.

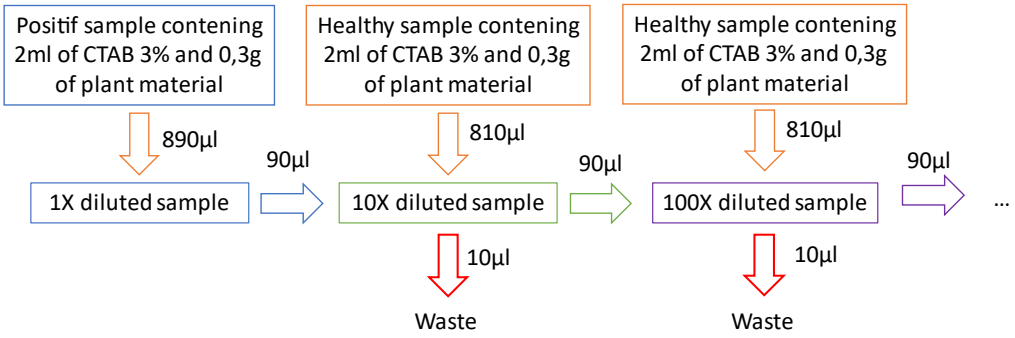



*Figure 9. Modification made in the DNA extraction protocol using CTAB 3% to dilute positive plant material in healthy plant material.*

### 2.4.2. Specificity

#### 2.4.2.1. Inclusivity

Both detection methods were performed on a various diversity of phytoplasmas from different groups already owned by CRA-W's virology laboratory.

[3] No evaluated

*Table 10. Phytoplasma species tested to assess inclusivity of detection methods. Samples come from two proficiency test performed by CRA-W in 2019 (detection of Grapevine flavescence dorée phytoplasma (GFDP)) and in 2022 (detection and identification of phytoplasmas).*

| Species | Reference/origin |
|---|---|
| *C. P. solani* (bois noir) 1 | Proficiency test for the detection and identification of phytoplasmas (D1263/21) |
| *C. P. solani* (bois noir) 2 | Proficiency test for detection of GFDP (DNA-E) |
| *C. P. solani* (bois noir) 3 | Proficiency test for detection of GFDP (DNA-F) |
| *C. P. solani* (stolbur) | 22/0274/Vi-28 (positive DNA extraction control) |
| *C. P. aurentifolia* 1 | Proficiency test for the detection and identification of phytoplasmas (NIB F 122) |
| *C. P. aurentifolia* 2 | Proficiency test for the detection and identification of phytoplasmas (NIB F 123) |
| *C. P. phoenicium* | Proficiency test for the detection and identification of phytoplasmas (NIB F 121) |
| *C. P. fraxini* | Proficiency test for the detection and identification of phytoplasmas (NIB F 119) |
| *C. P. australiense* | Proficiency test for the detection and identification of phytoplasmas (NIB F 120) |
| *C. P. vitis* 1 | Proficiency test for detection of GFDP (DNA-A) |
| *C. P. vitis* 2 | Proficiency test for detection of GFDP (DNA-G) |
| *C. P. asteris* | Proficiency test for detection of GFDP (DNA-C) |
| *C. P. mali* | 23/0370/Vi-2 (cfr. Table 1; A17) |

#### 2.4.2.2. Exclusivity

During the sensitivity and reproducibility tests if non-specific bands appeared during the electrophoresis, 20 µl of the PCR product was sent for purification and sequencing at Eurofins Genomics along with 15µl of the last pair of primer used (R16F2n/R16R2) at a concentration of 10µM.

### 2.4.3. Selectivity

For selectivity, three different plant materials were planned for analysing: potato tubers and potato leaves infected with *Candidatus phytoplasma solani* and apple tree petioles infected with *Candidatus phytoplasma mali* (Table 1; A65)

To assess the detection methods with potato leaves, potato tubers identified with Candidatus phytoplasma solani were grown in a non-quarantine greenhouse at the Emile Marchal building.

### 2.4.4. Repeatability

After finding the limit of detection by serially diluting each plant material and for each method, three to five repetitions (if the results from the sensitivity test were not consistent, more repetition have been made) of the entire process were performed at the last detected dilution level.

### 2.4.5. Reproducibility

For every method and plant material that gave results from the sensitivity test, three repetitions of the last detected dilution were performed by another person from the virology lab of the CRA-W on a different date.

## 2.5. Sequencing/bar-coding

In addition to validate the two nested PCR methods, various apple trees identified exhibiting specific and/or multiple non-specific symptoms from the previously mentioned orchards (Table 1) were sampled. These samples were analysed using the CRA-W's accredited PCR method (direct PCR focusing on 16S ribosomal gene sequence), *secA* nested PCR and *hflB* direct PCR. Moreover, positive control of *C. P. mali* (Table 11) were analysed using *secA* nested PCR and *hflB* direct PCR.

*Table 11. Origins of the positive control own by CRA-W used to build phylogenetic trees.*

| Sample ID | Cultivar | Origins | Harvested date |
|---|---|---|---|
| TP1 | Gueule de mouton D17 | Gembloux (CRA-W) | 2015 |
| TP2 | Sainte-Catherine 97 | Mussy (CRA-W) | 2016 |
| TP3 | Reinette struel K39 | Gembloux (CRA-W) | 2015 |
| TP5 | Président | Ormeignies (CEHW) | 2009 |
| TP6 | Golden | Bovesse | 2014 |
| TP7 | Pomme Bleue T4 | Gembloux (CRA-W) | 2020 |
| TP8 | Belle fleure de France R | Gembloux (CRA-W) | 2020 |

PCR products of positive samples were sent to Eurofins Genomics for purification and sequencing to assess the genetic diversity of *Candidatus Phytoplasma mali* within the orchards. For the nested PCR targeting *secA* gene sequence (SecAFor1/SecARev3 followed by SecAFor5-u;SecAFor5-1;SecAFor5-LY/SecARev2), because multiple forward primers were used in the second round PCR and only one pair of primer can be send for analyse, a pre-test was made to determine which one of them was used during the amplification of *secA* gene sequence. It was secAFor5-u primer.

Sequences were aligned, trimmed, and corrected using the software Unipro UGENE. The mapping was made using a reference sequence of the focused genes from the entire sequenced genome of *C. P. mali* strain AT (CU469464.1) ("Candidatus Phytoplasma mali strain AT complete chromosome," 2015; "PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests," 2021).

All partial sequences were individually gathered by to create one phylogenetic tree for each of the genes sequences (16S, *secA* and *hflB*) along with reference sequences of their respective gene sequence from *C. P. pyri* and *C. P. prunorum* and other *C. P. mali*. Phylogenetic trees were made using IQ-TREE website (Trifinopoulos et al., 2016) using align sequences files from each gene sequence. Then, consensus trees in newick format were use in Interactive tree of life website (Letunic et al., 2024). Parameters used for all step of the process can be found in annexes 3 to 6.

For these phylogenetic trees, when they existed, new strains from NCBI were added for clades or individuals that did not have an identical one already integrated to the tree. This was done

by blasting the sequences on NCBI website (parameters used in annexe 7). The one with the lowest E value and highest percent identity was added to the tree.

In addition, a concatenation of all three gene sequences were made to create one phylogenetic tree, as it provide better accuracy (Gadagkar et al., 2005; Paul, 2023) For this, trimmed gene sequences have been put in the continuity of one and other in this order: 16S, *secA*, *hflB*. Aligned sequences were integrate into phylogeny.fr website (Dereeper et al., 2008) to create a newick format of the phylogenetic tree for it to be used in Interactive tree of life website. Parameters used in phylogeny.fr can be found in annex 8.

## 2.6. Statistical analysis

Statistical analyses were conducted using RStudio (Posit team, 2023). Hot water treatment was analysed using ANOVA to determine whether damaged or dried post-treatment grafts resulted in higher mortality rates. Following that, a Kruskal-Wallis's test was conducted to assess if there were significant differences in survival rates across treatment groups overall. The test was separately applied to samples pre-identified as positive and negative to investigate survival differences within these subsets specifically. Moreover, Kruskal-Wallis's test was also used to compare pre-identified as positive and negative to highlight survival differences between them.

# 3. Results

## 3.1. Hot water treatments (HWT)

### 3.1.1. *Candidatus Phytoplasma mali* detection before and after hot water treatments

The analyses of the samples collected for HWT1, using fU5I/R16R2 pair of primers, confirmed that all samples coming from the symptomatic tree were positive. However, among the asymptomatic samples, one was detected positive and therefore was dismissed for the hot water treatment. Moreover, Table 12 only shows the results from the scions treated. Indeed, four scions were discarded for HWT1 because of their small width, making them unusable for chip grafting.

Six days after HWT1, samples deprived from buds and stocked at 4°C in plastic film after chipped budding graft were tested again using the same pair of primers. Two samples treated at 45°C for 60 minutes and one out of two samples treated at 45°C for 180 minutes were still positive. Other samples previously detected as positive were found to be negative after the hot water treatment.

*Table 12. PCR results before and six days after hot water treatment 1 (HWT1) targeting the 16S ribosomal gene sequence. Modalities: 45-60 = 45°C for 60 minutes; 45-90 = 45°C for 90 minutes; 45-180 = 45°C for 180 minutes; 50-15 = 50°C for 15 minutes. Sx are the scions coming from the symptomatic tree and ASx are the scions coming from the asymptomatic tree used for HWT1.*

| Modality | Sample name | Pair of primer | |
|---|---|---|---|
| | | fU5I/R16R2 | |
| | | Before HWT | After HWT |
| 45-60 | S1 | + | + |
| | S8 | + | + |
| | AS6 | - | - |
| | AS8 | - | - |
| 45-90 | S8 | + | - |
| | S7 | + | - |
| | AS4 | - | - |
| | AS5 | - | - |
| 45-180 | S7 | + | + |
| | S4 | + | - |
| | AS7 | - | - |
| | AS10 | - | - |
| 55-15 | S4 | + | - |
| | S10 | + | - |
| | AS9 | - | - |
| | AS10 | - | - |

For HWT2, four out of ten samples coming from the symptomatic tree were tested positive using fU5l/R16R2 pair of primer. To increase the sensitivity of the detection method, it was decided to use the nested PCR recommended by EPPO for the detection of phytoplasma (P1/P7; R16F2n/R16R2). With this method, all the samples coming from the symptomatic tree were detected positive and none from the asymptomatic tree.

Three days after HWT2, direct PCR targeting the 16S ribosomal gene sequence (fU5l/R16R2) successfully amplified nine out of ten positive samples, including some that had previously tested negative with the same method. The nested PCR focusing on the 16S ribosomal gene sequence (P1/P7; R16F2n/R16R2) showed the same results as before HWT2, using the same primer pairs. However, nested PCR amplification for the secA gene sequence identified only eight out of the ten previously detected positive samples as positive after HWT2.

*Table 13. PCR results before and six days after HWT2 targeting the 16S ribosomal gene sequence and secA gene sequence. Modalities: 45-60 = 45°C for 60 minutes; 45-90 = 45°C for 90 minutes; 45-180 = 45°C for 180 minutes; 50-15 = 50°C for 15 minutes. Sx are scions coming from the symptomatic tree and ASx are scions coming from the asymptomatic tree used for HWT2.*

| Modality | Sample name | Detection methods | | | | |
|---|---|---|---|---|---|---|
| | | fU5l/R16R2 | P1/P7 follow by R16F2n/ R16R2 | fU5l/R16R2 | P1/P7 follow by R16F2n/ R16R2 | Nested PCR *secA* |
| | | Before HWT | | After HWT | | |
| 45-60 | S1 | - | + | + | + | + |
| | S3 | + | + | + | + | + |
| | AS1 | - | - | - | - | - |
| | AS2 | - | - | - | - | - |
| 45-90 | S2 | - | + | + | + | - |
| | S4 | + | + | + | + | + |
| | S6 | - | + | + | + | + |
| | AS3 | - | - | - | - | - |
| | AS4 | - | - | - | - | - |
| 45-180 | S5 | - | + | - | - | - |
| | S7 | - | + | + | + | + |
| | S10 | - | + | + | + | + |
| | AS5 | - | - | - | - | - |
| | AS6 | - | - | - | - | - |
| 55-15 | S8 | + | + | + | + | + |
| | S9 | + | + | + | + | + |
| | S+1 | - | + | + | + | + |
| | AS7 | - | - | - | - | - |
| | AS8 | - | - | - | - | - |

### 3.1.2. *Candidatus Phytoplasma mali* detection after grafting

After 72 days, DNA was extracted from the petioles of the grafted trees from HWT2. Four detection methods targeting the 16S gene sequence were applied to determine the presence of *C. P. mali*. The direct PCR using the fU5I/R16R2 primers and the nested PCR with the P1/P7; f01/r01 primers yielded no results. Positive results obtained with the P1/P7; R16F2n/R16R2 primer pairs were deemed unreliable, as multiple bands appeared in most samples tested. Previous sequencing of a non-specific band indicated that these nonspecific bands were due to the amplification of another bacterium.

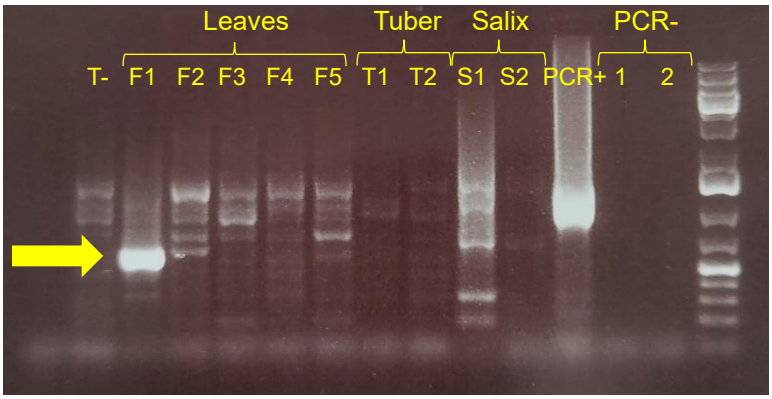



*Figure 10. Example of a nested PCR targeting the 16S ribosomal gene sequence (P1/P7; R16F2n/R16R2) migration presenting an isolated non-specific band send for sequencing (these partial results came from a fine-tuning of this method on potato leaves). The bacterium was identified as Candidatus Ovatusbacter abovo. T- = negative DNA extraction control; PCR+ = positive control for PCR reaction; PCR- = negative control for PCR reaction.*

This led to the use of a combination of the primers from first round PCR of nested PCRs targeting 16S ribosomal gene sequence (P1/P7) followed by the pair of primer from the direct PCR fU5I/R16R2. No consistent results were observed, and it was decided to sequence the PCR products from PCR products were a band appeared at the expected amplicon size from P1/P7; fU5I/R16R2 pairs of primer. It was found that all of them resulted from the amplification of the 16S ribosomal gene sequence of an unidentified bacterium or *Streptococcus* sp., *Gemmobacter fulvus*, *Fusobacterium vincentii* and *Legionella* sp. for AS12-2, AS13-3, S8-2 and S1-4, respectively.

*Table 14. PCR results 72 days after grafting from HWT2 using PCR detection targeting the 16S gene sequence. Modalities: 45-60 = 45°C for 60 minutes; 45-90 = 45°C for 90 minutes; 45-180 = 45°C for 180 minutes; 50-15 = 50°C for 15 minutes. NA = not applicable (did not recovered); NS = nonspecific band(s); (+) = bands with the expected amplicon size but were unreliable.*

| Modality | Sample | Detection method | | | |
|---|---|---|---|---|---|
| | | FU5L/R16R2 | P1/P7 follow by F01/R01 | P1/P7 follow by R16Fn2/R16R2 | P1/P7 follow by FU5L/R16R2 |
| 45-60 | S1-1 | - | - | NS | (+) |
| | S1-2 | - | - | NS | - |
| | S1-3 | - | - | - | - |
| | S3-1 | - | - | - | (+) & NS |
| | S3-2 | - | - | (+) & NS | NS |
| | S3-3 | NA | NA | NA | NA |
| | AS11-1 | - | - | NS | NS |
| | AS11-2 | - | - | NS | NS |
| | AS11-3 | - | - | (+) & NS | NS |
| | AS12-1 | - | - | NS | (+) & NS |
| | AS12-2 | - | - | (+) & NS | (+) & NS |
| | AS12-3 | - | - | (+) & NS | NS |
| 45-90 | S2-1 | - | - | (+) & NS | NS |
| | S2-2 | - | - | - | - |
| | S2-3 | - | - | - | - |
| | S4-1 | - | - | - | - |
| | S4-2 | - | - | - | - |
| | S6-1 | - | - | - | - |
| | S6-2 | - | - | (+) & NS | - |
| | S6-3 | NA | NA | NA | NA |
| | AS13-1 | NA | NA | NA | NA |
| | AS13-2 | - | - | - | - |
| | AS13-3 | - | - | NS | (+) |
| | AS14-1 | - | - | NS | (+) |
| | AS14-2 | NA | NA | NA | NA |
| | AS14-3 | - | - | (+) | (+) & NS |
| 45-180 | S5-1 | - | - | (+) | - |
| | S5-2 | - | - | (+) & NS | (+) & NS |
| | S5-3 | - | - | - | NS |
| | S7-1 | - | - | (+) & NS | - |
| | S7-2 | - | - | (+) & NS | - |
| | S7-3 | - | - | NS | - |
| | S10-1 | NA | NA | NA | NA |
| | S10-2 | - | - | - | (+) |
| | S10-3 | NA | NA | NA | NA |
| | AS15-1 | - | - | NS | - |
| | AS15-2 | - | - | NS | - |
| | AS16-1 | NA | NA | NA | NA |
| | AS16-2 | - | - | NS | - |
| 50-15 | S8-1 | - | - | NS | - |
| | S8-2 | - | - | - | (+) |
| | S8-3 | - | - | NS | - |
| | S9-1 | - | - | - | - |
| | S9-2 | - | - | - | - |
| | S9-3 | - | - | NS | (+) |
| | S+1-1 | - | - | (+) & NS | (+) & NS |
| | S+1-2 | NA | NA | NA | NA |
| | S+1-3 | NA | NA | NA | NA |
| | AS17-1 | - | - | (+) & NS | - |
| | AS17-2 | - | - | (+) & NS | - |
| | AS18-1 | - | - | NS | - |
| | AS18-2 | - | - | (+) & NS | (+) |
| NT | S1-4 | - | - | NS | - |
| | S1-5 | - | - | NS | (+) |
| | S4-1 | - | - | NS | NS |
| | S4-2 | - | - | NS | NS |
| | S5-4 | - | - | NS | NS |
| | S5-5 | - | - | NS | (+) |
| | S9-4 | - | - | - | - |
| | S9-5 | - | - | - | - |
| | AS19-1 | NA | NA | NA | NA |
| | AS19-2 | - | - | NS | (+) |
| | AS19-3 | - | - | - | NS |
| | AS20-1 | - | - | NS | - |
| | AS20-2 | - | - | - | - |
| | AS20-3 | - | - | NS | (+) |

### 3.1.3. Grafting survival rate

None of the grafted apple tree from the HWT1 survived, implying no further results.

However, the grafted apple trees from HWT2 presented various survival rate depending on the modality as shown in Figure 11 and Figure 12. Overall this results did not consider other factors influencing the survival rate of a grafted apple tree. Indeed, during the process of phloem DNA extraction, multiple grafts have been damaged and lead to a highly significantly increased of the mortality rate (p-value = $8.51*10^{-7}$).

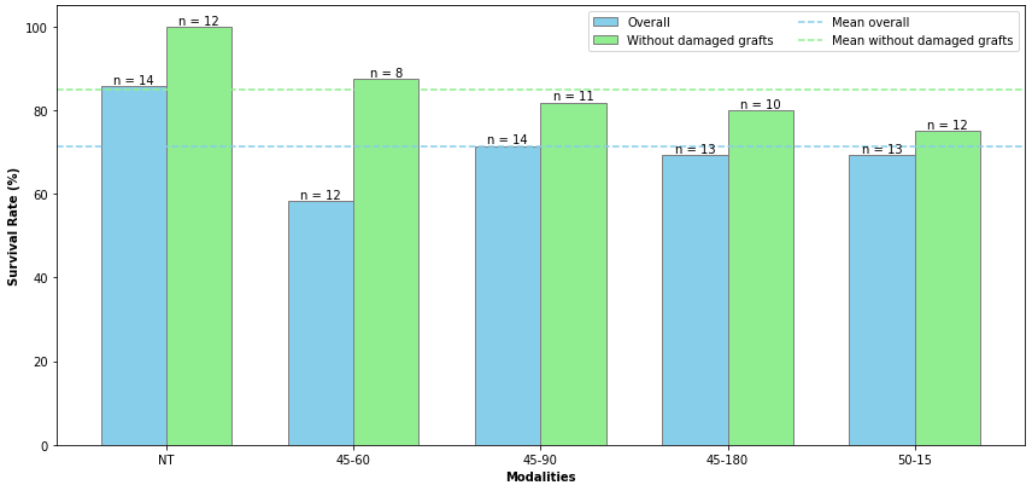



*Figure 11. Survival rate by modality with and without accounting for damaged grafts for HWT2. Modalities: 45-60 = 45°C for 60 minutes; 45-90 = 45°C for 90 minutes; 45-180 = 45°C for 180 minutes; 50-15 = 50°C for 15 minutes.*

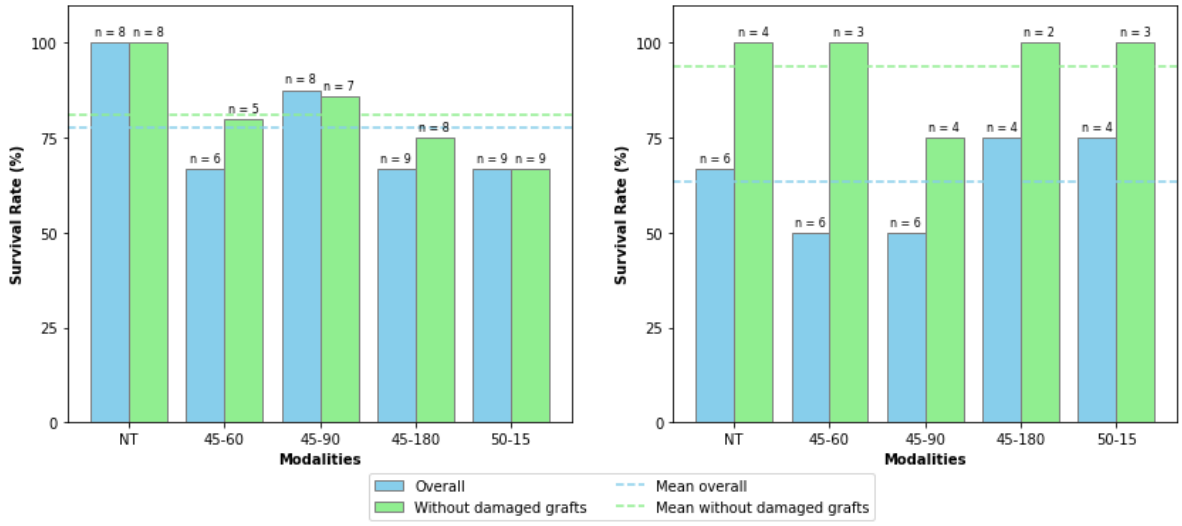



*Figure 12. Survival rate by modality with and without accounting for damaged grafts for HWT2. (Left) Detected positive before HWT2. (Right) Detected negative before HWT2. Modalities: 45-60 = 45°C for 60 minutes; 45-90 = 45°C for 90 minutes; 45-180 = 45°C for 180 minutes; 50-15 = 50°C for 15 minutes.*

Kruskal-Wallis's statistical test was performed to assess significant differences between modalities for the survival rates without accounting damaged grafts. They were not significantly different overall (p-value = 0.509) and for detected positive (p-value = 0.511) or negative (p-value = 0.558) before HWT2. When previously detected positive and negative samples are

compared (using Kruskal-Wallis' statistical test), no significant differences exist (p-value = 0.241).

## 3.2. Detection methods for validation

### 3.2.1. Sensitivity, repeatability, reproducibility, and selectivity

Among the tested protocols, the sensitivity was higher for the nested PCR targeting the 16S ribosomal gene sequence (P1/P7 follow by R16F2n/R16R2 ) than the nested PCR focusing on *secA* gene sequence (SecAFor1/SecARev3 follow by SecAFor5-u; SecAFor5-1; SecAFor5-LY/SecARev2) for potato tuber. However, during the validation on potato tubers infected with *C. P. solani*, results from the nested PCR targeting 16S shown a multitude of nonspecific bands for all dilutions and the observation of bands at the expected amplicon size of phytoplasmas for more diluted samples. Adjustment in the protocol have been made to try eliminated nonspecific bands (change in annealing temperatures, with or without dilution of the first round PCR product for the second round, and new primers) but result from this nested PCR remained similar and inconsistent.

*C. P. solani* was not detected from potato leaves by both detection methods, not allowing further investigation to assess the repeatability and reproducibility.

Detection methods used on infected apple tree petioles shown a detection only for the non-diluted samples, two out of three times for P1/P7 followed by R16F2n/R16R2 pairs of primer and one out of three times for the nested PCR targeting on *secA* gene sequence. The inconsistency of those results eliminated the need for further investigation on reproducibility. Therefore, selectivity was only achieved for one out of three plant material tested.

*Table 15. Detection methods' results for sensitivity, repeatability, and reproducibility of the nested PCRs targeting the 16S ribosomal gene sequence using P1/P7 follow by R16F2n/R16R2 pairs of primer (16S) and secA gene sequence using SecAFor1/SecARev3 follow by SecAFor5-u; SecAFor5-1; SecAFor5-LY/SecARev2 pair of primer (secA). Sensitivity is the last reliable dilution giving positive results.*

| Plant material | Sensitivity | | Repeatability | | Reproducibility | |
|---|---|---|---|---|---|---|
| | 16S | *secA* | 16S | *secA* | 16S | *secA* |
| Potato tuber | 0.001X | 0.1X | 5/5 | 5/5 | 3/3 | 3/3 |
| Potato leaves | Not detected | Not detected | | | | |
| Apple tree petioles | 1X | 1X | 2/3 | 1/3 | | |

### 3.2.2. Specificity

Both methods were able to detect all the nine phytoplasmas tested except the nested PCR targeting the 16S ribosomal gene sequence for which one of the two isolates of *C. P. aurentifolia* was not detected. During specificity test, nonspecific bands only appeared for potato tuber sample contaminated with *C. P. solani*.

Concerning exclusivity of those methods, P1/P7; R16F2n/R16R2 was found to produce various non-specific bands during the migration of the PCR products even for negative DNA extraction controls (Figure 10). The origin of these bands was not investigated as sequencing was not possible due to too many nonspecific bands.

However, *secA* never shown nonspecific bands after electrophoresis migration and further sequencing of PCR products from this detection method always proved to amplified Candidatus Phytoplasma sp..

*Table 16. Results from nested PCR detection to assess the specificity (inclusivity) of the nested PCRs targeting the* 16S *ribosomal gene sequence using P1/P7 follow by R16F2n/R16R2 pairs of primer (*16S*) and secA gene sequence using SecAFor1/SecARev3 follow by SecAFor5-u; SecAFor5-1; SecAFor5-LY/SecARev2 pair of primer (secA). Nine different species of Candidatus Phytoplasma sp. have been used.*

| Species | 16S | *SecA* |
|---|---|---|
| *C. P. solani* (bois noir) 1 | + | + |
| *C. P. solani* (bois noir) 2 | + | + |
| *C. P. solani* (bois noir) 3 | + | + |
| *C. P. solani* (stolbur) | + | + |
| *C. P. aurentifolia* 1 | - | + |
| *C. P. aurentifolia* 2 | + | + |
| *C. P. phoenicium* | + | + |
| *C. P. fraxini* | + | + |
| *C. P. australiense* | + | + |
| *C. P. vitis* 1 | + | + |
| *C. P. vitis* 2 | + | + |
| *C. P. asteris* | + | + |
| *C. P. mali* | + | + |
| Specificity (inclusivity) | 92.31% | 100.0% |

### 3.3. Molecular diversity

Detection methods used to assess phytoplasmas contamination of the symptomatic apple tree found that all samples were PCR positive by at least one method, except PH24 (Belle de Boskoop, Hévillers). Only 9 out of 22 positive samples were detected positive by all three methods. 5 out of 22, 6 out of 22 and 3 out of 22 were false negative for 16S, *secA* and *hflB* gene sequence, respectively.

Sequencing of the positive samples amplified using the fU5l/R16R2 primers for the 16S ribosomal gene sequence was the PCR method with the highest failure rate in producing usable sequences, with 3 out of 17 samples yielding no results (NR). This method also generated the most partial results from sequencing, with 2 out of 17 samples yielding incomplete sequences (P). For the sample D51 (Président Henry Van Dievot, cabaret, orchard 1), which was only detected by the 16S ribosomal gene sequence, sequencing failed to produce any results, making it impossible to identify the infecting phytoplasma species.

After BLASTing the sequences in NCBI website, all of the sample proved to be *C. P. mali* (between 99,15% and 100% similarities with *C. P. mali* from NCBI (accession number : CU469464.1)).

*Table 17. Partial DNA sequences assessed for molecular diversity of Candidatus Phytoplasma mali within CRA-W's orchards and elsewhere using three PCR detection methods focusing on three genes sequences. Sample ID referred to Table 1. R = result from sequencing; P = partial result from sequencing (one out of two primers worked); NR = no result from sequencing; empty = negative result from PCR.*

| Sample ID | 16S ribosomal gene sequence (fU5l/R16R2) | *secA* gene sequence | *hflB* gene sequence |
|---|---|---|---|
| A7 | R | P | |
| A17 | P | R | R |
| A20 | | R | R |
| A23 | R | R | R |
| A25 | R | R | R |
| A40 | P | R | R |
| B20 | | | R |
| C25 | | | R |
| C41 | NR | R | R |
| D51 | NR | | |
| B19 | R | R | R |
| B30 | R | R | R |
| L95 | R | R | R |
| P78 | R | R | R |
| P97 | R | | R |
| R105 | R | | R |
| R108 | NR | | R |
| K74 | R | R | |
| L40 | | R | R |
| L45 | R | R | R |
| P44 | | NR | R |
| PH23 | R | R | R |
| PH24 | | | |
| TP1 | / | R | R |
| TP2 | / | | R |
| TP3 | / | R | R |
| TP5 | / | R | R |
| TP6 | / | R | R |
| TP7 | / | R | R |
| TP8 | / | R | R |

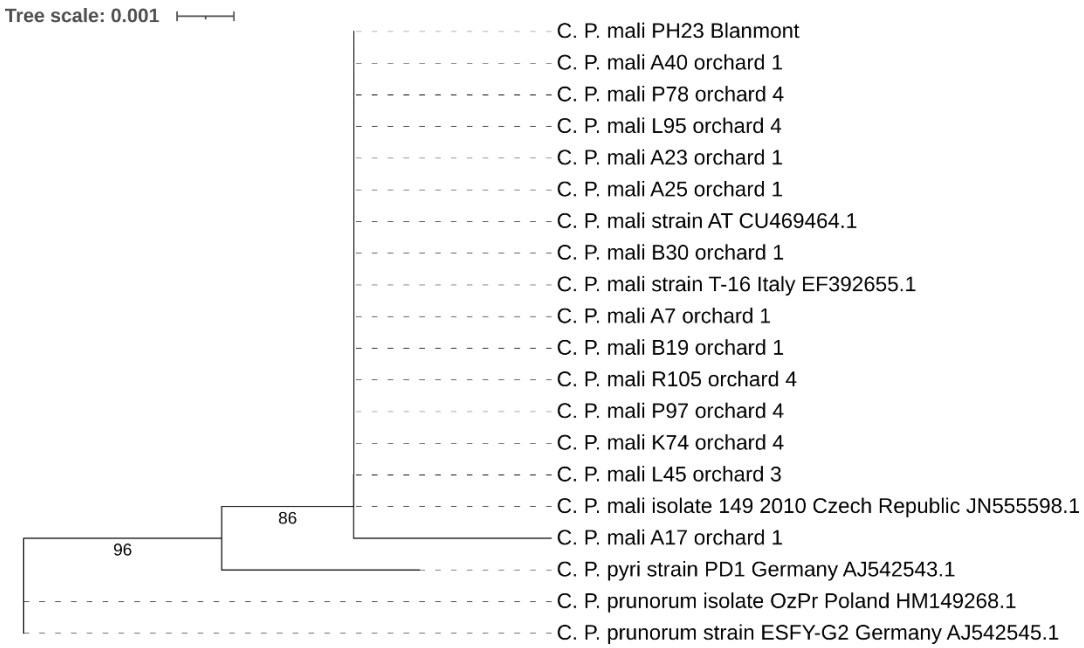



*Figure 13. Phylogenetic tree of the* 16S *ribosomal gene sequence amplified using fU5I/R16R2 primers of samples from CRA-W's orchards and elsewhere compared to the same gene sequence of different strains or isolates of C. P. mali, pyri and prunorum found in NCBI database. Internal gene sequences: [Phytoplasma] [sample ID] [origin]; Exterior gene sequences: [Phytoplasma] [strain/isolate] [origin] [accession number].*

The phylogenetic tree of the 16S ribosomal gene sequence revealed two clades (define here as a genetic cluster) for *C. P. mali*. One clade contained only the isolate from tree A17, while the other clade included 16 isolates that were 100% identical. However, as indicated in Table 17, the sequencing of A17 was partial, as one of the two primers failed to work, suggesting that the sequence may be less reliable. All *C. P. mali* strains and isolates found in the NCBI database belong to the same clade. Sequences from *C. P. prunorum* were clearly distinguished from *C. P. mali* and *C. P. pyri*, with a bootstrap value of 96%. *C. P. pyri* and *C. P. mali* were somewhat less distinct, with a bootstrap value of 86%.

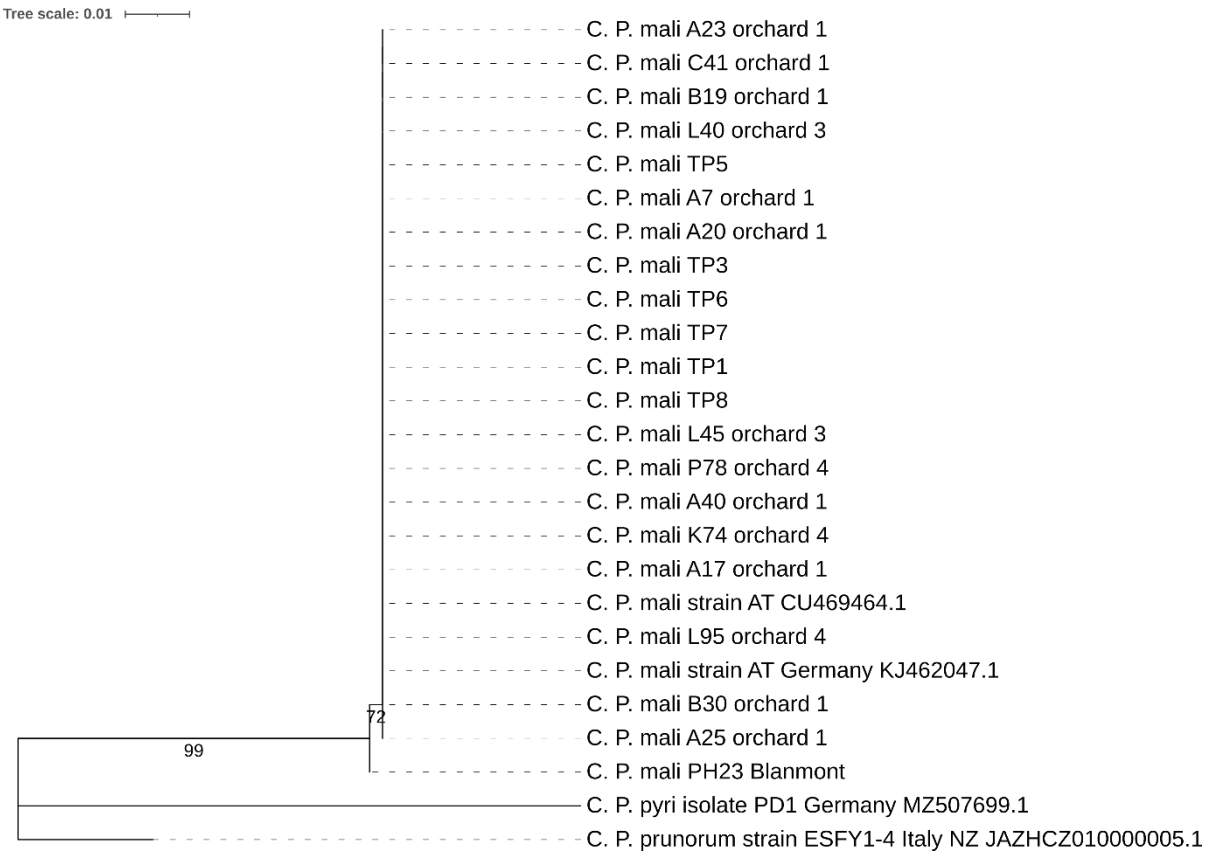



*Figure 14. Phylogenetic tree of the secA gene sequence amplified using the nested PCR focusing on that gene (Table 2) of samples from CRA-W's orchards and elsewhere compared to the same gene sequence of different strains or isolates of C. P. mali, pyri and prunorum found in NCBI database. Internal gene sequences: [Phytoplasma] [sample ID] [origin]; Exterior gene sequences: [Phytoplasma] [strain/isolate] [origin] [accession number].*

The phylogenetic tree from *secA* gene sequence revealed that two clades existed for *C. P. mali*, one containing all the samples from CRA-W's orchards and positive controls own by CRA-W (100% identical) and the other one only with the sample coming from Blanmont (PH23). Every strain and isolates of *C. P. mali* found on NCBI database belonged to the same clade. *C. P. pyri* and *prunorum* are well distinguished with *C. P. mali* with a bootstrap value of 99%.

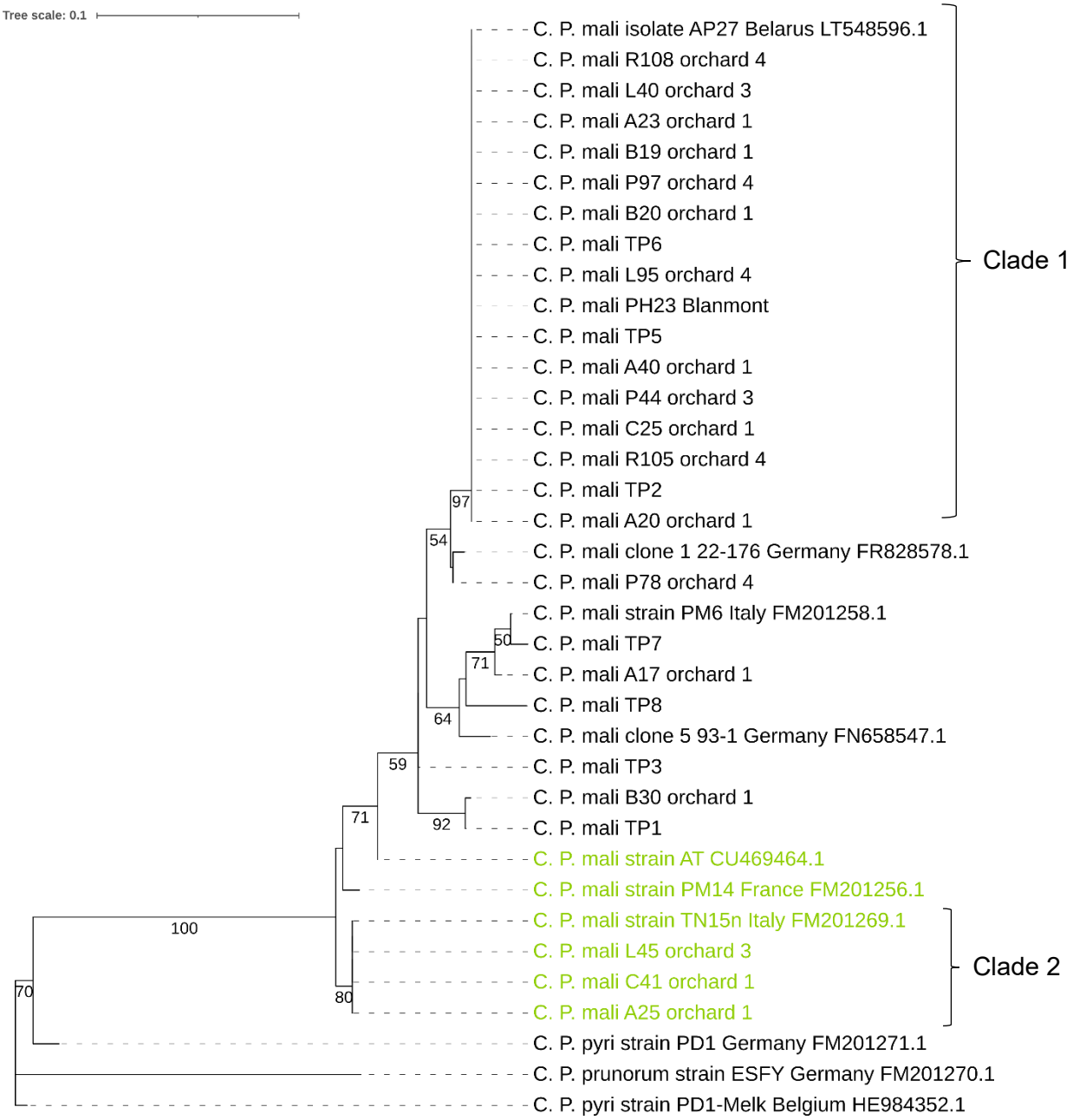



*Figure 15. Phylogenetic tree of the hflB gene sequence amplified using the direct PCR focusing on that gene (Table 2) of samples from CRA-W's orchards and elsewhere compared to the same gene sequence of different strains or isolates of C. P. mali, pyri and prunorum found in NCBI database. Light green = sequences containing motif TTA184 and T-C227. Internal gene sequences: [Phytoplasma] [sample ID] [origin]; Exterior gene sequences: [Phytoplasma] [strain/isolate] [origin] [accession number].*

The phylogenetic tree from *hflB* gene sequence showed great genetic diversity: two clades containing at least two internal gene sequences existed and the rest are genetically separated individuals. Clade 1 containing 16 (including 12 internal samples) are molecularly identical to AP17 isolate from Belarus (LT548596.1) (100% identical). Clade 2 containing L45, C41 and A25 are molecularly identical to TN15n strain from Italy (FM201269.1) (100% identical).

Analyses of the partial sequences revealed that L45, C41 and A25, as well as the strain TN15n, PM14 and AT have the motif TTA184 and T-C227. This imply that the virulence of those strain is mild. All the other samples, strains and clones have a severe virulence.

*C. P. pyri* and *prunorum* are less distinguishable from *C. P. mali* compared to the 16S ribosomal gene sequence and *secA* gene sequence. Indeed, the two strain of *C. P. pyri* are not regrouped, one is closer to *C. P. mali* (PD1 strain) than the other (PD1-Melk).

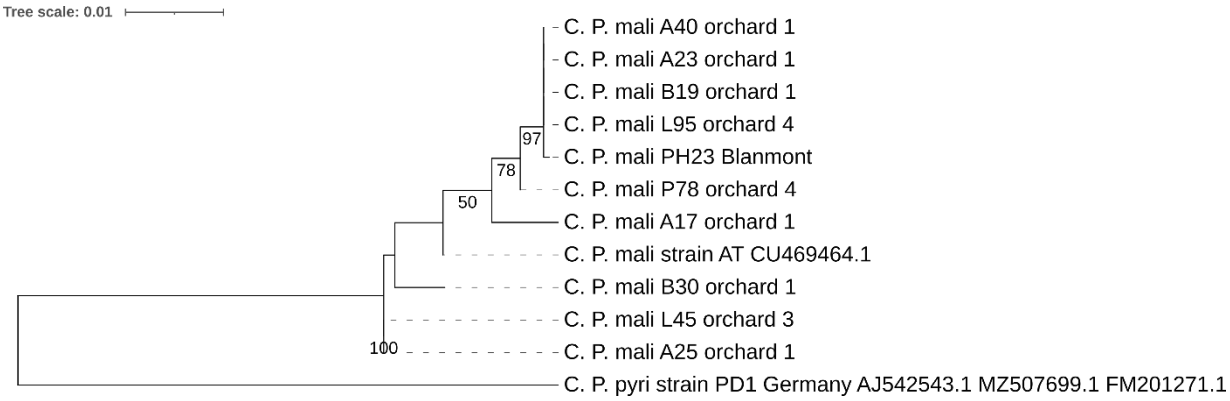



*Figure 16. Phylogenetic tree of the combined* 16S*, secA and hflB gene sequences of samples from CRA-W's orchards and elsewhere compared to the combined gene sequences of different strains of C. P. mali and pyri found in NCBI database. Internal gene sequences: [Phytoplasma] [sample ID] [origin]; Exterior gene sequences: [Phytoplasma] [strain/isolate] [origin] [accession number(s)].*

Because the phylogenetic tree of *hflB* gene sequence is the one that show the greatest genetic diversity, the combined phylogenetic tree reflects it and show a similar phylogenetic tree.

# 4. Discussion

*Candidatus Phytoplasma mali* is a wall-less bacterium that perennially infects *Malus* species and causes a disease called apple proliferation (Seemüller et al., 2004; CABI, 2021). No curative treatment exists to eradicate it on field, except the complete elimination of the infected apple tree (Janik et al., 2020). The objective of this study was to find an alternative to tree removal, especially since the apple orchards involved aim to conserve the cultivar biodiversity of apple trees in Belgium.

Before any treatment can be applied, a reliable detection method to identify infected apple trees is essential. Thus, one of the objectives of this study was to validate two nested polymerase chain reaction (PCR) techniques, focusing on the 16S ribosomal gene sequence and the *secA* gene sequence for detecting phytoplasmas. The first technique is recommended by EPPO guidelines as a universal detection method for phytoplasmas, while the second is recommended by Bertaccini et al. (2022) as a complementary detection method to PCRs targeting the 16S ribosomal gene sequence ("PM 7/133 Generic detection of phytoplasmas," 2018).

Characterizing the molecular diversity of *C. P. mali* is also essential, as Seemüller, Kampmann, et al. (2011) determined that gene sequences such as *hflB* indicate the virulence of the bacterium, which may be a key indicator in prioritizing treatments for the disease.

Prior to this study, the elimination of *Candidatus Phytoplasma mali* from apple trees for grafting had never been attempted. Therefore, another objective of this study was to evaluate the efficacy of thermotherapy via hot water treatment (HWT) in reducing or eliminating *C. P. mali* from infected plant material. Building on historical successes with HWT against various phytoplasmas and works on the elimination of fire blight from apple trees (Thung, 1952; Liu, 1963; Adams et al., 1992; Caudwell et al., 1997; Viswanathan et al., 2011; Brans et al., 2023), this study explored different time-temperature modalities to identify optimal conditions that balance pathogen eradication with plant survival.

During the validation of phytoplasma detection methods, concerns arose about the nested PCR targeting the 16S ribosomal gene sequence recommended by the EPPO (P1/P7 followed by R16F2n/R16R2). Validation on potato tubers infected with *C. P. solani* showed numerous nonspecific bands and re-emergence of a band at the expected amplicon size for phytoplasmas in more diluted samples. Sequencing one nonspecific band revealed amplification of another bacterium, suggesting other nonspecific bands might also result from amplifying other bacterial gene sequences and should be investigated via cloning for example. However, these nonspecific bands did not appear during the method's inclusivity evaluation, indicating the issue might stem from the plant material or DNA extraction process. The main concern was bands similar in size to the expected phytoplasma 16S ribosomal gene sequence amplified, leading to false positives. This is worrying as detected positive samples may have not been from phytoplasma but another organism. About inclusivity, one strain out of two of *C. P. aurantifolia* was not detected which indicates that this nested PCR may have a chance to result in false negatives as it has been detected positive with the nested PCR targeting *secA* gene sequence.

Finally, even though this method is recommended by EPPO guidelines to detect phytoplasmas, no validation data exists at the moment, but they are currently being validated (Hellin, personal communication, July 2024). It is also important to note that, in EPPO guidelines on the detection of phytoplasma, the kit used for the nested PCR targeting the 16S gene sequence using P1/P7 follow by R16F2n/R16R2 may not be the same as the one used during this study as the buffer used in EPPO protocol is not mentioned ("PM 7/133 Generic detection of

phytoplasmas," 2018). Even though this method has been following pre-tuning tests, nonspecific bands appeared nonetheless but may be a consequence of the kit used.

The nested PCR targeting the *secA* gene sequence proved more reliable, despite its lower sensitivity. It detected non-diluted and 10-fold diluted samples from potato tubers infected with stolbur, compared to up to 1000-fold dilution detection for the nested PCR targeting the 16S ribosomal gene sequence. Apart from occasional nonspecific bands, PCR products migration was clear and at the expected amplicon size. This method detected all phytoplasmas in inclusivity tests, is a good complementary method to the 16S PCR, and could be useful for distinguishing phytoplasma species within the 16SrX group when sequenced.

Overall, validation of these detection methods was challenging due to the plant material used not being suitable for phytoplasma detection at the time. Potato leaves from infected tubers had insufficient bacterial load for detection by either method. Subsequent independent tests on the same plants yielded positive results, indicating later tests could have been informative. Apple tree petioles from previously positive trees only gave results for non-diluted samples two out of three times for 16S nested PCR and one out of three times for *secA* nested PCR. This might be due to the seasonal behaviour of *C. P. mali* within apple trees, becoming almost undetectable by late winter/early spring (Seemüller et al., 1984; Pedrazzoli et al., 2008 as cited in Baric et al., 2011). The samples used were harvested on the 17$^{th}$ of April 2024, potentially explaining the insufficient bacterial load for consistent detection by both methods when not diluted. Time constraints prevented further tests with samples theoretically having higher bacterial loads.

During this study, the method of detection under accreditation in CRA-W was used many times to pre-assess the presence of phytoplasmas on most samples. This method produced false negatives multiple times. Possibly due to a lower sensitivity of direct PCR as mentioned in the EPPO guidelines for phytoplasma detection ("PM 7/133 Generic detection of phytoplasmas," 2018) but did not produce nonspecific bands. During sequencing of this PCR products, it was also the method that produced the most partial sequencing or no sequencing at all for positive samples. This may indicate that the primers used for this method are not always dependable for sequencing.

Both the validated methods and the accredited method have their advantages and disadvantages. The nested PCR targeting the 16S ribosomal sequence using P1/P7 followed by R16F2n/R16R2 is the most sensitive and should be employed when the bacterial concentration in the plant material is expected to be low. However, the results from this method must be carefully analysed, as nonspecific bands and/or false positives frequently occurred, as observed in this study.

The nested PCR targeting the *secA* gene sequence using SecAFor1/SecARev3 followed by SecAFor5-u; SecAFor5-1; SecAFor5-LY/SecARev2 is less sensitive and is better suited for situations where the concentration of phytoplasma is higher. The same applies to the direct PCR accredited by CRA-W, which targets the 16S ribosomal gene sequence with fU5l/R16R2.

Overall, nested PCR takes longer than direct PCR, especially the nested PCR targeting the 16S sequence, which requires over three hours per thermocycler cycle. Additionally, nested PCR demands careful and time-consuming handling under a hood when transferring the first PCR product to the second PCR master mix, which can also lead to cross-contamination between samples.

Despite these considerations, the direct PCR accredited by CRA-W is the most effective method for phytoplasma detection in most cases. The nested PCR targeting the 16S gene sequence should be used selectively when the expected concentration of phytoplasmas is low,

while the nested PCR targeting the *secA* gene sequence has limited value, as its performance is similar to that of direct PCR.

The analysis of molecular diversity revealed that the 16S ribosomal gene sequence of the infected apple trees from CRA-W's orchard and elsewhere was identical across all samples except for A17. This discrepancy is likely due to a lack of consensus from mapping, resulting from partial sequencing. Another hypothesis is that A17 represents a unique and undocumented strain, as no matching strain was found in the NCBI database. However, this is improbable given the well-maintained and comprehensive database for this gene sequence, which is primarily used to identify phytoplasmas. A17 should have been reassessed but time constrain prevented it.

Samples from CRA-W's apple orchard and other locations were analysed using nested PCR targeting the *secA* gene sequence, sometimes showing negative results when other methods were positive. This may be explained by the low sensitivity of the *secA* method, as assessed during its validation. The phylogenetic tree from the *secA* gene sequence presented results comparable to those from the 16S ribosomal gene sequence, except that PH23, not A17, was excluded from the main clade sharing the same nucleotide sequence. Interestingly, PH23 was the only detected positive sample from outside CRA-W's orchards and came from an ornamental apple tree. The limited *secA* gene sequence database on the NCBI website prevented comparison with a similar strain, suggesting that the *secA* gene sequence from PH23 might belong to an undocumented strain.

The *hflB* gene sequence highlighted significant molecular diversity, with multiple samples diverging from each other, though sometimes closely related to strains in the NCBI database. On the phylogenetic tree, three internal samples containing motifs TTA184 and T-C227 (associated with mild virulence) were molecularly identical. *C. P. mali* strains with mild virulence might require less aggressive management strategies for apple proliferation, while those with high virulence might necessitate stricter control measures. These highly virulent strains should be prioritised if hot water treatment for grafting against *C. P. mali* proves efficient in the future to prevent further spread of AP within the orchards. However, apple trees can be carriers of infection without showing symptoms, and no assessment of AP prevalence has been conducted in CRA-W's orchards (only symptomatic apple trees have been tested). To evaluate mild and severe virulence within the orchards, the prevalence of the bacteria should be determined, and the *hflB* gene sequence of the positive samples should be sequenced.

Overall, the phylogenetic tree based on *secA* gene sequences for the 16SrX group shows the greatest distinction between species, particularly between *C. P. mali*, *C. P. pyri*, and *C. P. prunorum*. This makes the *secA* gene sequence more effective for determining the specific species of phytoplasma compared to phylogenetic trees based on the other genes sequences.

The *hflB* gene sequence may exhibit a higher rate of evolution compared to the 16S ribosomal gene sequence and *secA* gene sequence, as these latter sequences are nearly identical across all tested individuals. Consequently, the virulence of the strains could evolve over time, potentially impacting the epidemiology of apple proliferation (AP) in the future.

The failure of grafts to recover from the first hot water treatment might be attributed to the severe climatic conditions to which they were exposed. Additionally, chip budding is typically performed from mid-summer to early autumn ("Chip budding / RHS Gardening," July-23-2024), but this experiment was conducted on November 17, 2023. Due to time constraints, grafting could not be performed earlier. To enhance survival chances, the grafted apple trees could have been cultivated in CRA-W's greenhouse. Also, It might be interesting to assess the

rootstock from this grafting to determine the presence of phytoplasma and therefore, if the buds from infected plant material inoculated them even if they did not survive.

The survival rate of grafted trees from the second hot water treatment indicated that different treatments similarly affected the recovery rate of the grafts overall, aligning with Brans et al. (2023). The prior presence of *C. P. mali* did not impact the survival rate, suggesting that pre-existing *C. P. mali* did not influence graft survival. The grafts mortality was significantly higher for damaged scions from phloem DNA extraction. Moreover, only one cultivar was evaluated, not representing the existing diversity and its response to hot water treatment.

Despite the initial grafting experiment's failure, which yielded no significant results on the efficiency of the treatment post-grafting, phytoplasma detection six days post the hot water treatment one (HWT1) suggests that prolonged treatment at 45°C (90 and 180 minutes) or at higher temperatures (50°C for 15 minutes) might reduce bacterial load. But this may be interpretated in another way: the degradation of the DNA of dead cell may have been more important for prolonged times and higher temperature. As a result, it can only be said that even though DNA was detected for shorter times treatment at 45°C, it does not indicate that the phytoplasma are still alive, only that the DNA was not degraded enough to be detected.

However, this result was not observed in the second hot water treatment, where phytoplasma detection increased after HWT2 using the fU5l/R16R2 method targeting the 16S ribosomal gene sequence. Comparable results were obtained using nested PCR targeting the same gene sequence (P1/P7 followed by R16F2n/R16R2). Direct PCR focusing on the 16S ribosomal gene sequence is less sensitive than nested PCR, potentially explaining the consistent detection of phytoplasma before and after the second hot water treatment using the nested PCR. This, however, does not clarify why direct PCR detected more post-treatment positive samples.

Furthermore, comparing nested PCR targeting 16S and *secA* gene sequences post-treatment revealed that a previously positive sample was not detected by *secA* nested PCR. Validation tests indicated lower sensitivity for *secA* nested PCR, suggesting that the undetected sample might have had a lower bacterial load.

During the evaluation of hot water treatments for grafting, a more specific PCR, such as P1/P7 followed by f01/r01 targeting the 16S ribosomal gene sequence, could have been employed to directly confirm the presence of *C. P. mali* in scions without sequencing. This method has shown to avoid producing nonspecific and has high sensitivity. Besides direct and nested PCR, specific qPCR should also be investigated as it can reduce the time needed to process samples, limits contamination and provide quantification of the bacterium.

In the second hot water treatment, phytoplasmas were undetectable 72 days after grafting, even in untreated samples that were previously positive. This could be due to an insufficient bacterial load in the leaf petioles, making detection by any PCR method difficult. The validation of two nested PCR methods on apple tree petioles yielded poor results, suggesting that this plant material may not be ideal for detecting apple proliferation. However, fine-tuning in November 2023 showed no significant differences between DNA extraction from petioles and phloem. This might be related to the seasonal variation in phytoplasma distribution within apple trees (Seemüller et al., 1984; Pedrazzoli et al., 2008 as cited in Baric et al., 2011). Moreover, no specific symptoms nor multiple non-specific symptoms have been detected during the harvesting of the leaves. Inoculation experiments of *C. P. mali* on healthy apple trees indicate that symptoms may not occurred within three months (Aldaghi et al., 2007). Periodic testing of petioles and phloem until detection would have been valuable, but time constraints hindered this.

In the work of Brans et al. (2023), *Candidatus Phytoplasma prunorum* infecting *Prunus armeniaca* and *Prunus salicina* were treated by hot water treatment. Even though the tree species was not comparable to *Malus domestica* concerning the tolerance against hot water treatment, it is interesting to note that the phytoplasma belong to the same group as *C. P. mali*. Result from this study where plant material was tested after one and two vegetative cycle show that 45°C during 90 minutes eliminated the phytoplasma. This might indicate that this temperature-time couple is also efficient against *C. P. mali* as it is a closely related phytoplasma.

In summary, since every treatment had the same impact on the survival rate and no phytoplasma was detected, it is crucial to regularly reassess *C. P. mali* presence in the future to identify the most effective treatment for eliminating it from apple tree scions. The plant material and period of harvesting are crucial to detected *Candidatus Phytoplasma mali* and should be considered. Nested PCR targeting the 16S ribosomal gene sequence such as P1/P7 followed by f01/r01 which is specific to AP and is more sensitive due to its nature, could be use during late winter and early spring and other method such as the one accredited by CRA-W (fU5l/R16R2) during the rest of the year. Considering the survival rates of grafted trees, the treatment requiring the least time and energy (in this case 45°C during 60 minutes) is important for large-scale decontamination of apple proliferation in apple tree propagation. Furthermore, only one of the two hot water treatment experiments will continue which will only allow to validate the method for only one kind of grafting and cultivar. This is why new experiments should be conducted in the future testing new grafting techniques and cultivars.

# 5. Conclusions

To conclude, the validation of the detection method targeting the 16S ribosomal gene sequence (P1/P7 followed by R16F2n/R16R2) for phytoplasmas yielded inconsistent results, with numerous nonspecific bands and occasional bands at the expected amplicon size in highly diluted samples, raising concerns about potential false positives. Although it is the most sensitive method (detecting up to a 1000-fold dilution), it may require sequencing for confirmation. The other validated detection method, targeting the secA gene sequence of phytoplasma (SecAFor1/SecARev3 followed by SecAFor5-u; SecAFor5-1; SecAFor5-LY/SecARev2), consistently provided clear results but had a lower detection limit (up to a 10-fold dilution). This method complements the 16S PCR by distinguishing phytoplasma species within the 16SrX group when sequenced. However, the selectivity of these methods was fully assessed using only one type of plant material, limiting conclusions about their efficacy in other materials. These findings underscore the importance of considering the seasonal behaviour of phytoplasma in apple trees when conducting assessments.

For routine detection, a method that is quick and reliable is essential, such as the direct PCR accredited by CRA-W (fU5l/R16R2). However, this method's sensitivity may sometimes be inadequate for detecting phytoplasma, particularly when the concentration in the plant material is low. In such cases, nested PCR targeting the 16S ribosomal gene sequence (P1/P7 followed by R16F2n/R16R2) may be more suitable but should be confirmed by sequencing or a more specific and sensitive nested PCR, such as the one targeting the 16S ribosomal gene sequence of the 16SrX group (P1/P7 followed by F01/R01), when applicable.

Currently, no comprehensive assessment of AP prevalence has been conducted in CRA-W's orchards. Sequencing the hflB gene could be a valuable tool for identifying other potentially infected apple trees, as it would offer better insights into the management and molecular

diversity of apple proliferation within the orchards, particularly because the hflB gene sequence evolves more rapidly than the 16S ribosomal gene sequence and the secA gene sequence.

Finally, the effectiveness of hot water treatment (HWT) for apple tree scions against apple proliferation remains inconclusive. Further assessment of AP presence is needed, as the bacterial load of Candidatus Phytoplasma mali was too low for reliable detection during this study. Employing a specific PCR method for the 16SrX group, such as P1/P7 followed by f01/r01, could have provided a more direct and reliable confirmation of C. P. mali presence in scions and should be used in the future, even when phytoplasma concentration is expected to be low. Additionally, exploring qPCR could enhance sample processing efficiency and enable quantification of the bacterium. Although two experiments with different grafting techniques and timings were conducted, only one method can be pursued further. To fully validate this treatment approach, future experiments should explore a variety of grafting methods and apple cultivars. However, evaluating this method will require several years due to the latency period and seasonal behaviour of Candidatus Phytoplasma mali, which may pose challenges for future research on this topic.

# 6. Student personal contribution

I carried out all the steps and analyses mentioned in this study, except for those that required the involvement of another person (such as the reproducibility of the validation of detection methods) and the harvest of apple tree leaves gathered for validation of detection method. Additionally, I assisted a technician from CRA-W during the grafting process.

# 7. Annexes

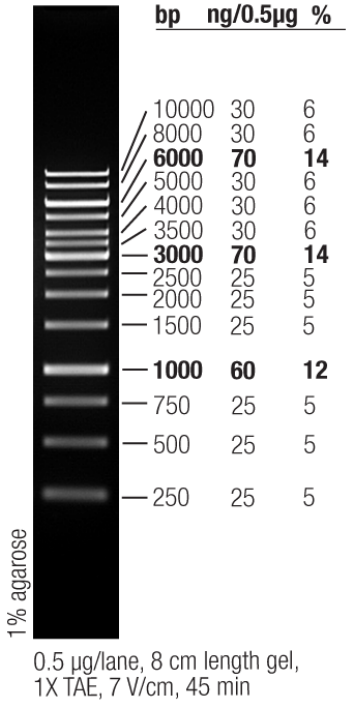
*Annex 1. Complete list of known phytoplasmas from Wei et al. (2022) (modified).*

| Group | Number of 'C. Phytoplasma' Species | | Accession Number of Reference Strain | Subgroup | References |
|---|---|---|---|---|---|
| 16SrI: Aster yellows group | 3 | 'C. Phytoplasma asteris' | M30790 | 16SI-B | (Lee, Gundersen-Rindal, et al., 2004) |
| | | 'C. Phytoplasma lycopersici' | EF199549 | 16SrI-Y | (Arocha et al., 2007) |
| | | 'C. Phytoplasma tritici' | NZ AVAO01000003 | 16SrI-C | (Zhao et al., 2020) |
| 16SrII: Peanut witches' broom group | 1 | 'C. Phytoplasma aurantifolia' | U15442 | 16SrII-B | (Zreik et al., 1995) |
| | § | 'C. Phytoplasma australasia' | Y10096 | 16SrII-D | (Davis et al., 2013) |
| 16SrIII: X-disease group | 1 | 'C. Phytoplasma pruni' | JQ044393 | 16SrIII-A | (Davis et al., 2013) |
| 16SrIV: Coconut lethal yellows group | 2 | 'C. Phytoplasma palmae' | U18747 | 16SrIV-A | (The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group, 2004; Bertaccini et al., 2022) |
| | | 'C. Phytoplasma cocostanzaniae' | X80117 | 16SrIV-C | |
| 16SrV: Elm yellows group | 4 | 'C. Phytoplasma ulmi' | AY197655 | 16SrV-A | (Lee, Martini, et al., 2004) |
| | | 'C. Phytoplasma ziziphi' | AB052876 | 16SrV-B | (Jung, Sawayanagi, Kakizawa, et al., 2003) |
| | | 'C. Phytoplasma rubi' | AY197648 | 16SrV-E | (Malembic-Maher et al., 2011) |
| | | 'C. Phytoplasma balanitae' | AB689678 | 16SrV-new subgroup | (Win et al., 2013) |
| 16SrVI: Clover proliferation group | 2 | 'C. Phytoplasma trifolii' | AY390261 | 16SrVI-A | (Hiruki et al., 2004) |
| | | 'C. Phytoplasma sudamericanum' | GU292081 | 16SrVI-I | (Davis et al., 2012) |
| 16SrVII: Ash yellows group | 1 | 'C. Phytoplasma fraxini' | AF092209 | 16SrVII-A | (Griffiths et al., 1999) |
| 16SrVIII: Loofah witches' broom group | 1 | 'C. Phytoplasma luffae' | AF248956 | 16SrVIII-A | (Davis et al., 2017) |
| 16SrIX: Pigeon pea witches' broom group | 1 | 'C. Phytoplasma phoenicium' | AF248956 | 16SrIX-D | (Verdin et al., 2003) |
| 16SrX: Apple proliferation group | 4 | 'C. Phytoplasma mali' | AJ542541 | 16SrX-A | (Seemüller et al., 2004) |
| | | 'C. Phytoplasma pyri' | AJ542543 | 16SrX-C | |
| | | 'C. Phytoplasma prunorum' | AJ542544 | 16SrX-F | |
| | | 'C. Phytoplasma spartii' | X92869 | 16SrX-D | (Marcone, Gibb, et al., 2004) |
| 16SrXI: Rice yellow dwarf group | 3 | 'C. Phytoplasma oryzae' | AB052873 | 16SrXI-A | (Jung, Sawayanagi, Wongkaew, et al., 2003) |
| | | 'C. Phytoplasma cirsii' | KR869146 | 16SrXI-D | (Šafárŏvá et al., 2016) |
| | | 'C. Phytoplasma sacchari' | VWXM00000000 | 16SrXI-B | (Kirdat et al., 2020) |
| 16SrXII: Stolbur group | 5 | 'C. Phytoplasma australiense' | L76865 | 16SrXII-B | (Davis et al., 1997) |
| | | 'C. Phytoplasma japonicum' | AB010425 | 16SrXII-D | (Sawayanagi et al., 1999) |
| | | 'C. Phytoplasma fragariae' | DQ086423 | 16SrXII-E | (Valiunas et al., 2006) |
| | | 'C. Phytoplasma solani' | AF248959 | 16SrXII-A | (Quaglino et al., 2013) |

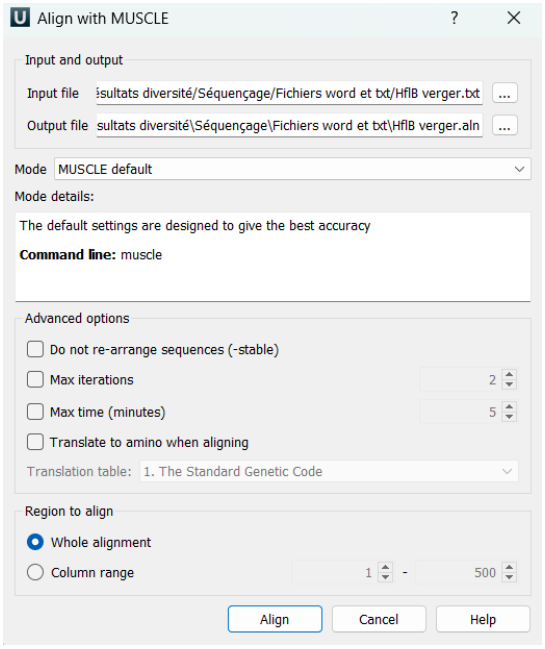
§ abolished

| | | | | | |
|---|---|---|---|---|---|
| | | ‘C. Phytoplasma convolvuli’ | JN833705 | 16SrXII-H | (Martini et al., 2012) |
| 16SrXIII: Mexican periwinkle virescence group | 2 | ‘C. Phytoplasma hispanicum’ | AF248960 | 16SrXIII-A | (Davis et al., 2016) |
| | | ‘C. Phytoplasma meliae | KU850940 | 16SrXIII-G | (Fernández et al., 2016) |
| 16SrXIV: Bermudagrass white leaf group | 1 | ‘C. Phytoplasma cynodontis’ | AJ550984 | 16SrXIV-A | (Marcone, Schneider, et al., 2004) |
| 16SrXV: Hibiscus witches’ broom group | 1 | ‘C. Phytoplasma brasiliense’ | AF147708 | 16SrXV-A | (Montano et al., 2001) |
| 16SrXVI: Sugar cane yellow leaf syndrome group | 1 | ‘C. Phytoplasma graminis’ | AY725228 | 16SrXVI-A | (Arocha et al., 2005) |
| 16SrXVII: Papaya bunchy top group | 1 | ‘C. Phytoplasma caricae’ | AY725234 | 16SrXVII-A | |
| 16SrXVIII: American potato purple top wilt group | 1 | ‘C. Phytoplasma americanum’ | DQ174122 | 16SrXVIII-A | (Davis et al., 2012) |
| 16SrXIX: Japanese chestnut witches’ broom group | 1 | ‘C. Phytoplasma castaneae’ | AB054986 | 16SrXIX-A | (Jung et al., 2002) |
| 16SrXX: Buckthorn witches’ broom group | 1 | ‘C. Phytoplasma rhamni’ | X76431 | 16SrXX-A | (Marcone, Gibb, et al., 2004) |
| 16SrXXI: Pine shoot proliferation group | 1 | ‘C. Phytoplasma pini’ | AJ632155 | 16SrXXI-A | (Schneider et al., 2005) |
| 16SrXXII: Nigerian coconut lethal decline group | 1 | ‘C. Phytoplasma palmicola’ | KF751387 | 16SrXXII-A | (Harrison et al., 2014) |
| 16SrXXIII: Buckland Valley grapevine yellows group | | 1 unnamed species identified | AY083605 | 16SrXXIII-A | (Wei et al., 2007) |
| 16SrXXIV: Sorghum bunchy shoot group | | 1 unnamed new species identified | AF509322 | 16SrXXIV-A | |
| 16SrXXV: Weeping tea tree witches’ broom group | | 1 unnamed new species identified | AF521672 | 16SrXXV-A | |
| 16SrXXVI: Mauritius sugar cane yellows D3T1 group | | 1 unnamed new species identified | AJ539179 | 16SrXXVI-A | |
| 16SrXXVII: Mauritius sugar cane yellows D3T2 group | | 1 unnamed new species identified | AJ539180 | 16SrXXVII-A | |
| 16SrXXVIII: Havana derbid group | | 1 unnamed new species identified | AY744945 | 16SrXXVII-A | |
| 16SrXXIX: Cassia witches’ broom group | 1 | ‘C. Phytoplasma omanense’ | EF666051 | 16SrXXIX-A | (Al-Saady et al., 2008) |
| 16SrXXX: Salt cedar witches’ broom group | 1 | ‘C. Phytoplasma tamaricis’ | FJ432664 | 16SrXXX-A | (Zhao et al., 2009) |
| 16SrXXXI: Soybean stunt phytoplasma group | 1 | ‘C. Phytoplasma costaricanum’ | HQ225630 | 16SrXXXI-A | (Lee et al., 2011) |
| 16SrXXXII: Malaysian periwinkle virescence group | 1 | ‘C. Phytoplasma malaysianum’ | EU371934 | 16SrXXXII-A | (Nejat et al., 2013) |
| 16SrXXXIII: Allocasuarina group | 1 | ‘C. Phytoplasma allocasuarinae’ | AY135523 | 16SrXXXIII-A | (The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group, 2004) |
| 16SrXXXIV: grapevine yellows | | No new species identified, abolished | DQ232752 | | |
| 16SrXXXV: Pepper witches’-broom | | No new species identified, abolished | EU125184 | | |
| 16SrXXXVI: foxtail palm yellow decline group | 1 | ‘C. Phytoplasma wodyetiae’ | KC844879 | 16SrXXXVI-A | (Naderali et al., 2017) |
| 16SrXXXVII: Stylosanthes little leaf group | 1 | ‘C. Phytoplasma stylosanthis’ | MT431550 | 16SrXXXVII-A | (Rodrigues Jardim et al., 2021) |
| 16SrXXXVIII: Bogia coconut syndrome group | 1 | ‘C. Phytoplasma noviguineense’ | LC228755 | 16SrXXXVIII-A | (Miyazaki et al., 2018) |
| 16SrXXXIX: Palm lethal wilt group | 1 | ‘C. Phytoplasma dypsidis’ | MT536195 | 16SrXXXIX-A | (Jones et al., 2021) |

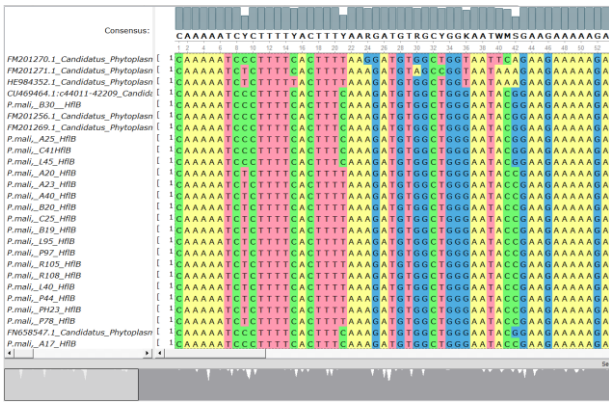
*Annex 2. GeneRuler 1kb DNA ladder (Thermo Scientific)*



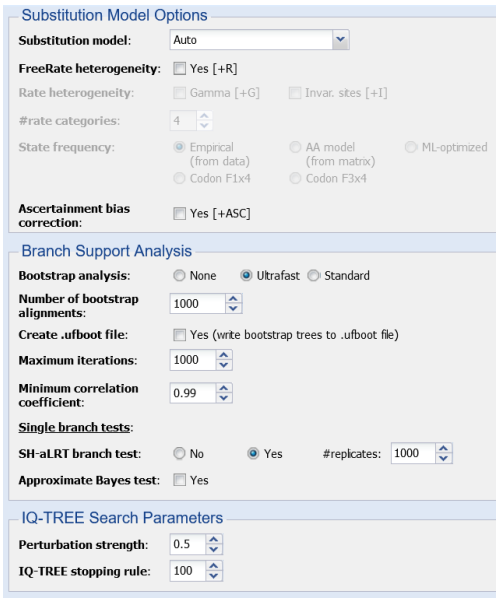

*Annex 3. Parameters used in UGENE to align gene sequences.*



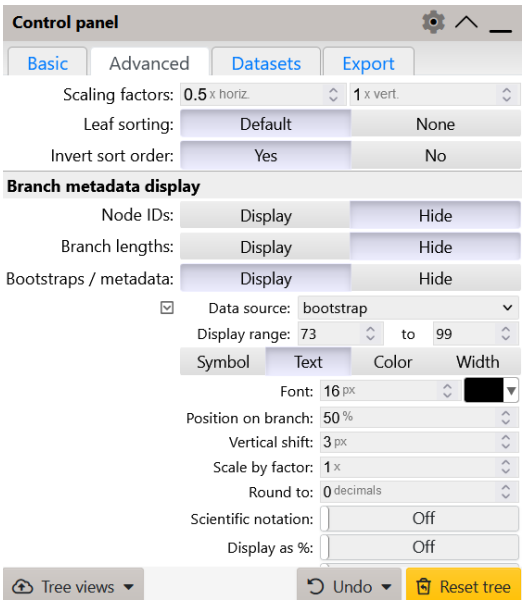

*Annex 4. Example of gene sequences aligned using UGENE.*



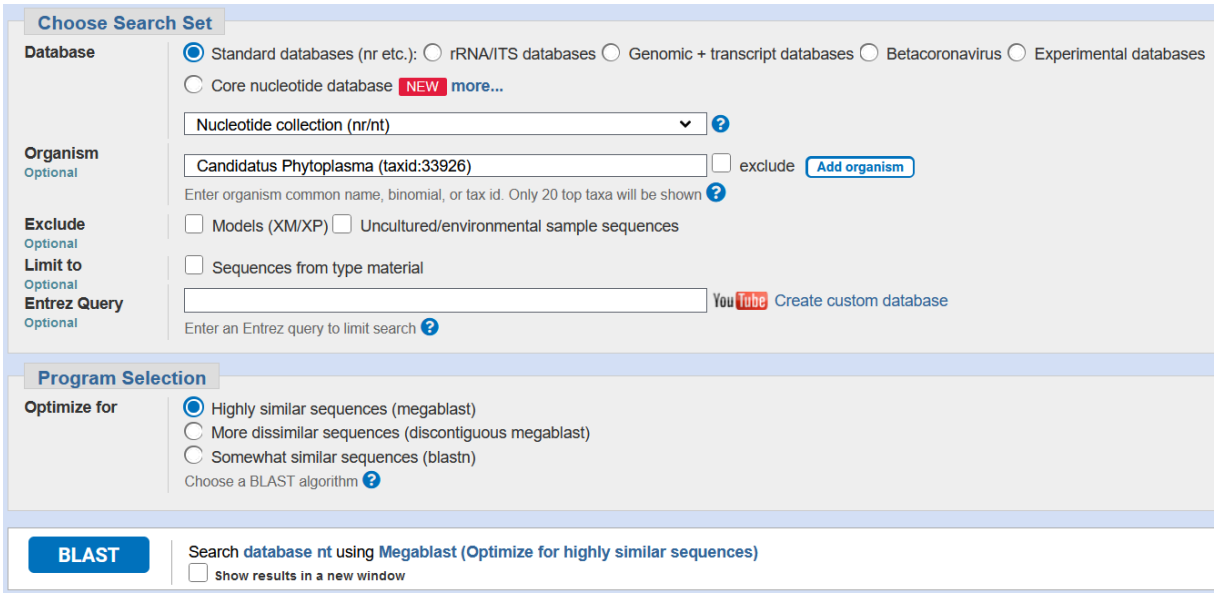
*Annex 5. Parameters used to create a phylogenetic tree using IQ-TREE website.*



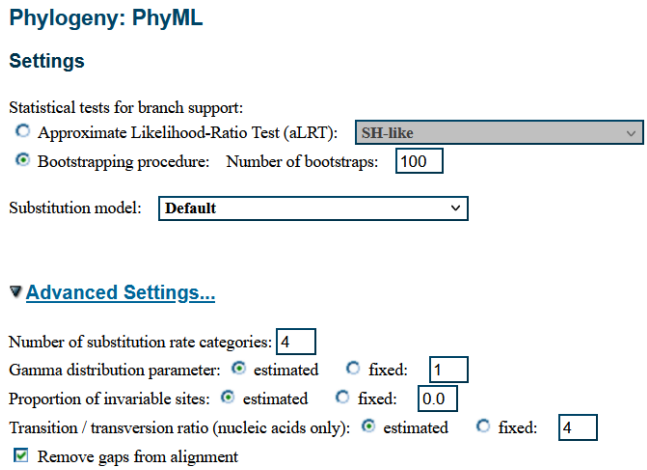
*Annex 6. Advanced control panel with the parameters used to format phylogenetic trees in iTOL.*




*Annex 7. Parameters used in NCBI website to BLAST nucleotide sequences.*




*Annex 8. Parameters used in Phylogeny.fr to create a newick file of the combined sequences.*



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