REPORT on a litterature study on

’Status quo of the knowledge on phytoplasma with the focus on *Euphorbia pulcherrima* and other ornamental plants’

Euphorbia fulgens with and without phytoplasma

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This report has been made in order to give an up-to-date review on the knowledge on the interaction between *Euphorbia* spp. and phytoplasma.
Phytoplasmas, which are microorganisms, are present in probably all commercial poinsettias in which they improve the ability of poinsettia to branch. The increased branching and thus the phytoplasma is a desirable trait in poinsettia.
This report is based on a litterature study on the scientific litterature on the subject, but interviews with experts in the field (poinsettia breeders and scientists) have also been carried out either by written correspondence or by visits.
The first part of the report describes what is generally known about the phytoplasma/ plant interaction, whereas the second part describes the specific phytoplasma/ poinsettia interaction. The third part includes recommendations for future research.
Appendices includes reports from meetings with experts and written replies from the questionaires which were sent to poinsettia breeders that were not visited.

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PART 1

Description of phytoplasma

The phytoplasmas are very small prokaryotes which are related to bacteria, but in contrast to bacteria they do not have a cellwall. The phytoplasmas are found in the phloem cells of host plants, and are normally considered pathogens of plants. The phytoplasmas cannot be cultured on artificial media, and can only be maintained in their plant host. The fact that phytoplasmas cannot be cultured on artificial media has made the study of phytoplasmas very laborious and difficult.

Phytoplasmas as plant pathogens

Phytoplasmas as harmful plant pathogens.

Phytoplasmas infect numerous plant species, also including many ornamental plants, for a comprehensive list see McCoy *et al.* (1989). Examples of ornamental plants in which phytoplasmas have been reported to cause disease include: Aster (yellowing), Gladiolus (virescence and other symptoms), Lily (virescence and stunting) and Hydrangea (virescence). As a plant pathogen, it is without the scope of this report to fully review the diseases caused by phytoplasmas in ornamentals.

Plant diseases associated with the presence of phytoplasmas typically exhibit a number of symptoms that are suggestive of disturbances in the normal balance of plant hormones (Lee and Davis, 1992). These symptoms include virescence (loss of normal flower colour, green flowers), phyllody (development of floral parts into leaf-like structures), proliferation of auxilary shoots (side shoots) resulting in ’witches broom’, sterility of flowers, compact growth at the end of stems, yellowing, phloem necrosis and dieback of branches in woody plants (McCoy *et al.,* 1989).

Phytoplasmas as desirable plant pathogens

As a ’desirable’ plant pathogen, for instance as a branching factor, only few reports exist with reports on the poinsettia – phytoplasma interaction, and recently the *Euphorbia fulgens* – phytoplasma interaction dominating scientific litterature.

Fragaria multicipita was for years thought to be a diverse strawberry genotype but Jomantiene *et al.* (1998) showed that it is a natural population of *F. virginiana* only that they

are infected with phytoplasma. The morphology of this *F. multicipita* is characterized as being dwarfed, with short petioles, small leaves and flowers, and a multi-branched crown.

A test of ornamentals without typical phytoplasma disease symptoms for the presence of phytoplasma has, to my knowledge, never been carried out. Therefore, phytoplasmas may be present in other vegetatively propagated ornamentals that show increased branching, but these must then have been introduced after traditional plant breeding as phytoplasmas are not seed transmitted.

Transmission of phytoplasmas

The plant to plant spread of phytoplasmas in nature is mediated by phloem-feeding leafhoppers and psyllids (a group of insects related to aphids and leafhoppers) (McCoy, 1979). Therefore, control of phytoplasma in agriculture or horticulture is highly dependent on the control of vectors, that is of leafhoppers and psyllids.
In the laboratory, phytoplasmas may also be transmitted from plant to plant by grafting. The phytoplasmas will move from infected plants into healthy plant. Another possibility when grafting is not possible (for example between two incompatible plant species) is the use of *Cuscuta* (dodder) which is a plant that is itself a plant parasite. This plant can form an ’infection bridge’ between plants, and the phytoplasma can move through dodder into healthy plants. Phytoplasmas are not considered to be seed-transmissible, and are lost during for instance conventional breeding (McCoy, 1979; Lee *et al.*, 2000).

Elimination of phytoplasmas

Phytoplasmas can be eliminated from their plant hosts, as they are generally heat labile and are not present in the shoot meristem (Lee and Davis, 1992). Furthermore they are sensitive to some antibiotics such as tetracycline (Heintz, 1989). Several metods have been applied to clean plant material for phytoplasmas, these include *in vitro* tissue culture such as shoot tip (Dale and Cheyne, 1993) or micropropagation (Davies and Clark, 1994) sometimes in combination with heat or antibiotic treatment.

Genome structure of phytoplasmas

The genome of phytoplasmas is very small (600.000 basepairs (bp) to 1.200.000 bp) compared to bacterial genomes (Seemüller *et al.*, 1998) (the genome of *Escherischia coli* is 4.000.000 bp). The phytoplasma genome has a very low G + C content in the range of 25-30% (the average for other organisms is 50%). Studies indicate that the gene structure is

similar to that of other prokaryotes (for example *Escherichia coli*) (e.g. Berg and Seemüller, 1999).

Is phytoplasma DNA part of plant genome?

Considering the below mentioned, **the phytoplasma genome cannot be considered part of the plant genome**:

-the phytoplasma is limited to the phloem, where no other plant DNA is present (physical separation of plant and phytoplasma DNA).

-the phytoplasma can be eliminated from the plant (plant is not dependent of phytoplasma DNA)

-the phytoplasma ribosomal DNA (rDNA) is very distinct from plant rDNA (rDNA is widely used to classify organisms), this indicates that phytoplasma DNA is very distinct from plant DNA.

-the overall chemical composition of phytoplasma DNA (low G + C content) is very different from that of plant DNA.

-the gene structure of phytoplasma DNA is like the general DNA structure for prokaryotes and is thus very different from plant DNA

Taxonomic classification of phytoplasmas

Only within the last decade, with the development of molecular techniques, it has been possible to resolve the classification of phytoplasma. Since then, molecular data derived from serological studies, DNA hybridization, or most recently, amplification of DNA by the PCR reaction have provided insight into the diversity and interrelationships between individual phytoplasmas. The analysis of particularly ribosomal DNA (rDNA) by the sequence or their restriction fragment length polymorphism has provided the basis for phytoplasma taxonomy. These studies have revealed that phytoplasmas form one discrete group within the class *Mollicutes* (Lim and Sears, 1989; Gundersen *et al.*, 1994). The phytoplasmas have been further subdivided into 14 major group and up to 46 subgroups, recently updated by (Lee *et al.*, 1998) who states that ’each group and some subgroups can be assigned to the species level’ meaning that several species of phytoplasma exists.

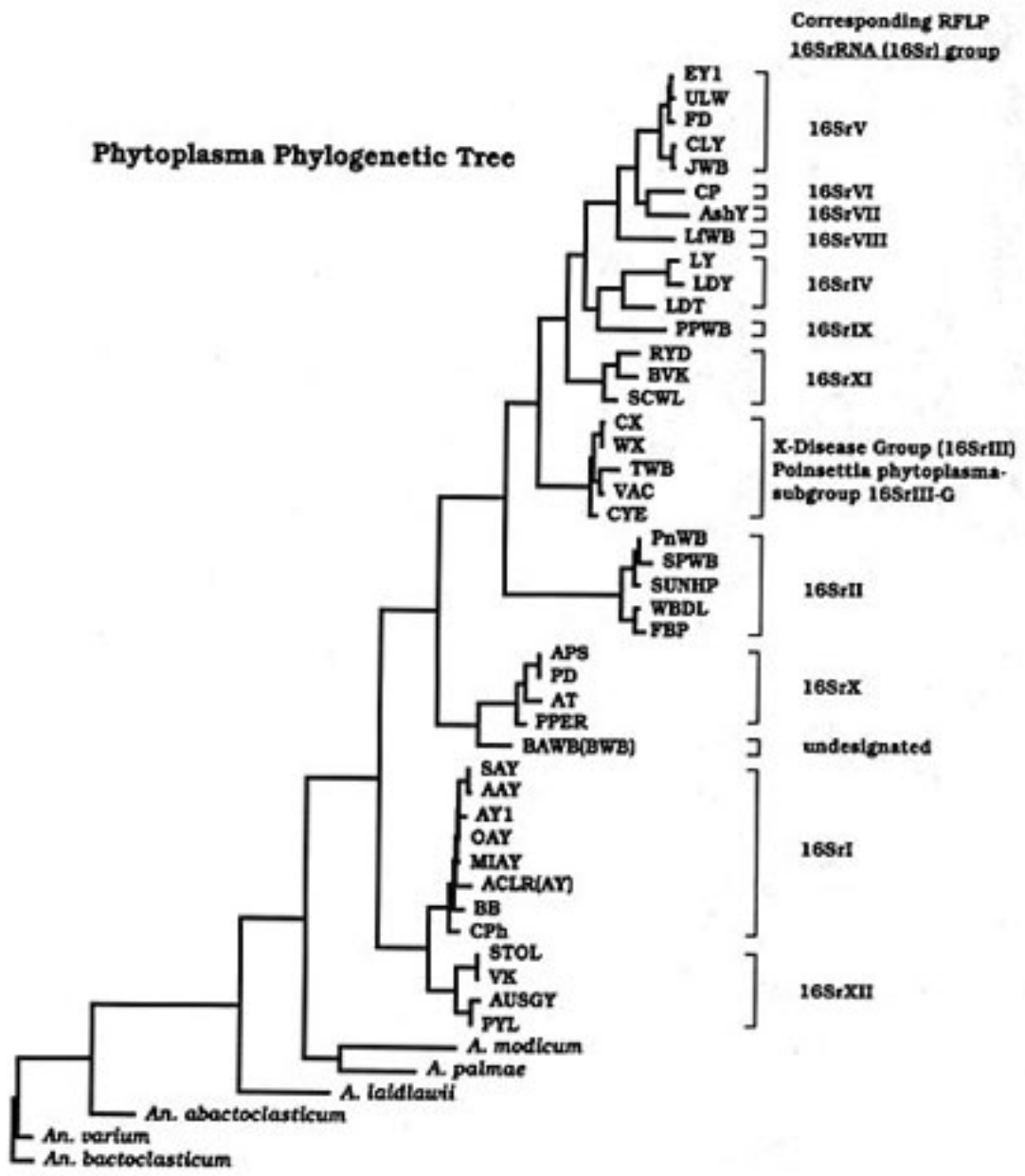

Figure 1. Taxonomic classification of phytoplasmas based on ’restriction fragment length polymorphism (RFLP)’. The dominating phytoplasma population in poinsettia belongs to the X-disease group (16SrIII). Figure from http://www.uniud.it/phytoplasma/.

Detection methods of phytoplasma.

Phytoplasmas have been classified into several groups as described in the previous chapter on the taxonomy of phytoplasmas. For the development of a detection method it is therefore important to define the target organisms that should be detected. Should all phytoplasmas be

detected or only phytoplasmas within one group. As it is not completely known which type of phytoplasma is causing phenotypic changes in poinsettia, it must be nescessary to use an assay that detects all known types of phytoplasma, or at least the 4 types found in free-branching poinsettias until now, if the aim is to determine whether a plant sample is infected with phytoplasma or not. In this report, only universal methods that are aimed at detecting all known phytoplasmas, are described.
The methods described should be able to distinguish plant material from phytoplasmas. For instance, it should be possible to distinguish phytoplasma DNA from plant DNA in PCR detection methods (see later).
Several methods for detection have been applied in the work with phytoplasmas, these include, apart from symptoms, microscopic techniques, immunological techniques, and techniques based on phytoplasma DNA.
For the reliable detection of phytoplasmas with a relatively high throughput a very sensitive method is needed as phytoplasmas can occur in very low titers, and furthermore the technique should not be too labour intensive. Finally the test should, as already stated, be universal.

Microscopic techniques

Light microscopy

Microscopic examination of plant tissue treated with different stains such as DAPI or Dienes stain that are specific of DNA has been used for the detection of phytoplasmas in phloem tissue. No plant DNA is present in the phloem that can interfere with the test (e.g. (Schaper and Seemüller, 1982), but other bacteria present in the phloem can interfere. It can be diffecult to identify true phloem cells in the microscope.
The sensitivity of these methods has not been directly examined for the detection of phytoplasma in poinsettia, but as the methods were unsuccessful in identifying phytoplasma as the cause of free-branching in poinsettia (see later), it is assumed that the methods are not sufficiently sensitive for detection of phytoplasma in poinsettia. Furthermore the methods are laborious and requires experienced workers as plant material (present in cells other than the phloem) can be very similar to phytoplasmas in the microscope (Chen *et al.*, 1989). The method is thus not easily applied for large scale testing.

Electron microscopy (EM)

Phytoplasmas can be detected by using ultrathin-section EM (Chen *et al.*, 1989). The technique is sensitive, but phytoplasmas are not always easily distinguishable from plant material or from other plant-pathogenic bacteria inhabitating the plant phloem. The method is very laborious, and only applicable in specialized laboratories with a very expensive electron microscope. To perform the analysis, highly trained personel is needed. It is therefore not suitable for high throughput analysis.

As also this electron microscopic method was unsuccessful in the previous attempts to identify the 'branching factor' in poinsettia before it was known as a phytoplasma, it is not likely that the method will be suitable for routine detection of phytoplasmas in poinsettia.

Techniques based on immunology.

Enzyme linked immunosorbent assay (ELISA)

ELISA has been used for the detection of specific phytoplasmas but not always with convincing results (Chen *et al.*, 1989).

For the production of antisera, a relatively pure preparation of phytoplasma is needed. As phytoplasmas can only by propagated in plant hosts, the complete purification of phytoplasmas from plant material is almost impossible. This plant material will interfere in the development of an ELISA test and can result in false positive reactions in phytoplasma free samples.

The use of monoclonal antibodies has recently improved the specificity and sensitivity of ELISA (Lee and Davis, 1992) but it is still difficult to develop new assays, for example for the detection of poinsettia phytoplasmas.

ELISA has not been developed for universal assay, that is for the detection of all phytoplasma groups in one test, and specifically it has not been developed for poinsettia phytoplasmas.

Polymerase chain reaction (PCR)

General steps in PCR detection

PCR detection consists of the following steps:

1) Extraction of DNA (total or phytoplasma enriched)

The choice of a suitable DNA extraction method does not depend significantly on the amplification used subsequently, but is dependent on the plant species and the concentration of phytoplasma in each case. For phytoplasma detection in *Euphorbia* at least 2 methods have been used with success. The method used in professor Jim Moyers lab at North Carolina State University (pers. comm.) is a combination of a phytoplasma enrichment procedure (Ahrens

and Seemüller, 1992) and a DNA extraction procedure (Zhu *et al.*, 1993). This method has been used in the authors lab with success. The method includes many steps and is quite laborious.
IngMing Lee describes a method for the extraction of DNA that can be used directly for PCR (Lee *et al.*, 1997a). This method is also quite laborious.
As both methods are laboriuos, it may be possible to develop a faster method.

2) Amplification of specific DNA

The crucial step of the development of a PCR test method is the design of primers of which two are needed for the priming of the amplification, a forward and a reverse primer. These are small pieces of DNA that are specific for the target sequence, therefore these must be designed so that they are identical to phytoplasma DNA and not to any irrelevant DNA (e.g. DNA from the plant host or contaminating bacteria). The amplification is most efficient when a small fragment of DNA is amplified (approx. 150 – 400 basepairs (bp)).

3) Visualization of amplified DNA

The amplification products are most often visualized by size fractionation in a gel and subsequent staining of DNA with ethidium bromide. More time-saving methods exist for visualization of amplification products (avoiding sizefractionation in gel) but these have not been implemented for the detection of phytoplasma PCR, to the authors knowledge.

PCR for the detection of phytoplasmas

PCR has been used during the last ten years for the detection of a large number of microorganisms, also including phytoplasmas. Several methods have been developed for the universal detection of phytoplasmas, these differ in principle only in the choice of primers which are normally designed so that they will amplify a region of the well characterized and highly conserved ribosomal (rDNA) genes.
The below mentioned tests are all developed for the universal test of phytoplasma on the basis of conserved 16S rDNA sequences. Most of the tests have also been developed for the discrimination of phytoplasma strains – this means that the PCR products are relatively large, which decreases sensitivity. Some of the tests have only been used in a few studies whereas others are widely used (table 1).

Table 1. List of most frequently used PCR methods. The examples in which the methods has been used is not complete, the references should be regarded as examples.

Primers	**PCR product size**	**Method developed by:**	**Used in the following studies (list not complete)**
SN910601/ SN910502	1370 bp	(Namba *et al.*, 1993)	(Sawayanagi *et al.*, 1999)
'Ahrens'	558 bp	(Ahrens and Seemüller, 1992)	(Marcone and Ragozzino, 1995)
P1/P6	1500 bp	(Deng and Hiruki, 1991)	(Khadhair *et al.*, 1999; Mpunami *et al.*, 1999)
P1/P7	1813 bp	(Deng and Hiruki, 1991; Schneider *et al.*, 1995)	(Marcone *et al.*, 1999; Cousin *et al.*, 1998; Polloni *et al.*, 1997; Jarausch *et al.*, 1999; Schneider *et al.*, 1999b; Green *et al.*, 1999; Guthrie *et al.*, 1998; Schneider *et al.*, 1997; White *et al.*, 1998; Staniulis *et al.*, 2000)
NGF/ NGR	506 bp	(Andersen *et al.*, 1998b)	(Andersen *et al.*, 1998a; Andersen *et al.*, 2001)
fU5/ rU3	865 bp	(Lorenz *et al.*, 1995)	(Daire *et al.*, 1997; Davies, 2000; Del Serrone *et al.*, 2001; Davies and Adams, 2000)
Nested PCR with R16mF2/mR1 as external primers and R16F2n/ R16R2	1248 bp	(Gundersen and Lee, 1996)	(Lee *et al.*, 1997b; Martini *et al.*, 1999; Bertaccini *et al.*, 1996b; Waterworth and Mock, 1999; Staniulis *et al.*, 2000)

As previously stated, a demand for phytoplasma PCR should be that it detects all known phytoplasma strains, it is universal, especially because several strains of phytoplasma are reported to infect poinsettia. At the same time, the PCR should not amplify DNA from other bacteria or healthy plants. Furthermore the method should be sufficiently sensitive to detect also low concentrations of phytoplasma. It should be amenable to high throughput analysis and ideally not be sensitive to contamination.

Below is discussed some of the more promising primer combinations:

The internal primers NGF/NGR in combination with external R16F2/R2 in nested PCR.
Although these primers have not been tested on a large variety of phytoplasma strains it is believed that these primers will detect all phytoplasma strains. The relatively small PCR product will probably result in increased sensitivity although the most optimal would be an amplification product in the range of 150 to 250 bp.

Nested PCR with R16mF2/mR1 as external primers and R16F2n/ R16R2 as internal primers
This assay has been widely used for the detection of phytoplasma and is probably the most thoroughly investigated. It detects all strains of phytoplasmas whereas healthy plants do not react. The large size of PCR product 1248 bp probably decreases sensitivity.

P1/P6 or 7
This assay has been widely used for the detection of phytoplasma. It detects all strains of phytoplasmas whereas healthy plants do not react. There is a risk that other prokaryotic DNA will interfere especially when using P1/P6. The large size of PCR product (1500/ 1800 bp) probably decreases sensitivity markedly.

The main drawback of the above mentioned methods is the use of ’nested’ PCR. This is a more laborious method than standard PCR methods (as it includes two PCR reactions), and in the nested method reactions are more likely to be cross-contaminated, and thus is more likely to generate false positives.

Recommended test method.

Based on the above mentioned facts (see also table 2.) it is recommended that PCR is used for the detection of phytoplasma in poinsettia. PCR can be easily developed, it has a potential for high throughput analysis, it is highly sensitive and the specificity can be designed so that it detects all known types of phytoplasma. Finally, PCR can be relatively easily implemented in most laboratories. The method easily distinguishes plant and phytoplasma so that a screening of varieties for the presence of phytoplasma could take place.

Table 2. Comparison of different phytoplasma detection methods. The cost of equipment is based on a rough estimate.

	Microscope DAPI/ Dienes	**electron microscope**	**ELISA**	**PCR**
Specificity	universal	universal	universal method not developed	can be both universal or directed towards e.g. one group of phytoplasmas
Possibility of distinguishing phytoplasma from plant material	can be difficult	can be difficult	possible with a good antiserum	easy with good primer combinations
Sensitivity	moderate	moderate	moderate	very high
Robustness	high	moderately high	very high	moderately high
Suitable for high throughput	labour intensive	labour intensive	not labour intensive	not labour intensive
Equipment costs (main equipment in brackets)	~25.000 EURO (flourescence microscope)	~250.000 EURO (electron microscope, ultramicrotome)	~15.000 EURO (ELISA reader)	~15.000 EURO (PCR cycler, electrophoresis)
Comments	Demands very skilled technicians.	Demands very skilled technicians.	Difficult to develop new test, for example for poinsettia.	Danger of cross contamination

Discrimination methods of phytoplasmas.

Discrimination between individual phytoplasmas can only be performed at present by investigating the DNA of the phytoplasma. This has mainly been done by analyzing the ribosomal genes in phytoplasmas as described in the chapter above on classification of phytoplasmas. The analysis can be performed either as determining the DNA sequence of the gene or by using restriction enzyme fragment length polymorphism, see for example (Lee *et al.*, 1998). This method has been used to discriminate phytoplasmas present in poinsettia (Abad *et al.* 1997).

PART 2

The poinsettia/ phytoplasma interaction.

It is now well established that phytoplasma is a desirable microorganism in poinsettia as it causes poinsettia to branch more willingly. All free-branching poinsettias investigated contain a phytoplasma. This part of the report will specifically describe the poinsettia / phytoplasma interaction with information from the scientific litterature supplemented with information obtained from interviews with experts.

History of the ’branching factor’ of poinsettia

(partly from Preil and Ebbinghaus (1997b) and from interview with Walter Preil)

Previously, before 1970, branching in poinsettias was very poor. In 1967 a cultivar ’Annette Hegg’ was introduced into the market by the norwegian poinsettia breeder Thormod Hegg with a dramatically increased branching habit. This cultivar arose from one seedling with improved branching in a delivery of seedlings which came from the breeder Paul Mikkelsen (Walter Preil, personal communication).

During the following years several cultivars with increased branching were introduced into the market, among these the cultivar ’Annemie’ from the breeder Gregor Gutbier.

During these years it was not known what causes the improved branching.

In 1982, Bundesforschungsanstalt für gartenbauliche Pflanzenschutz, Ahrenburg, showed that the strong branching can be lost together with viruses using *in vitro* culture techniques.

In 1983 Dr. D.P. Stimart published that the ’branching factor’ can be transmitted to new plants by grafting (Stimart, 1983).

During the following years, many attempts to identify the ’branching agent’ were made (Dole and Wilkins, 1992; Dole and Wilkins, 1991; Dole *et al.*, 1993; Ruiz-Sifre *et al.*, 1995; Ruizsifre *et al.*, 1997; Dole and Wilkins, 1988).

In 1990, Bundesforschungsanstalt für gartenbauliche Pflanzenschutz, Ahrenburg, showed that the cultivar ’Annemie’ is identical to ’Christiane Zieger’ but with the ’branching factor’.

In 1996, Walter Preil at Bundesforschungsanstalt für gartenbauliche Pflanzenschutz, Ahrenburg, transmitted the branching factor to *Euphorbia fulgens* to dramatically improve branching in this plant which has been used for cut flower production (Preil and Ebbinghaus, 1997a; Preil and Ebbinghaus, 1999; Preil and Ebbinghaus, 1998).

In 1997, Ing-Ming Lee identified a phytoplasma as the ’branching agent’ (Lee *et al.*, 1997a). The ’branching agent’ was transmitted to *Catharantus roseus* which is a host for phytoplasma but not for viruses known also to infect poinsettia. From *C. roseus* it was transmitted back into restricted-braching poinsettias and subsequently causing branching. The presence of phytoplasma was confirmed by PCR.

The phytoplasmas associated with poinsettia were molecularly characterized. Is was shown that the phytoplasmas are present as a complex of several groups of phytoplasmas (Abad *et al.*, 1997).

Bradel *et al.* (2000) showed that the free-branching pattern of poinsettia can be suppressed by the addition of tetracycline antibiotics (phytoplasmas are sensitive to antibiotics) thus supporting the finding that free-branching is caused by a phytoplasma.

Strains of phytoplasma in poinsettia

As described in a previous chapter, phytoplasmas have been grouped according to differences in their ribosomal DNA. By investigating this rDNA from phytoplasmas from 20 poinsettia cultivars, Lee (Lee *et al.*, 1997a) found that the predominant type of phytoplasma was ’a new member of the 16SrDNA group III, and is most closely related to x-disease and spirea stunt’. Two poinsettia cultivars contained a phytoplasma belonging to group 16SrI. Schneider *et al* (1999a) also found 16SrDNA group III phytoplasma in poinsettia.
Abad *et al*. (1997) found that the infection in poinsettia is a complex of different strains, the dominant one being the 16SrDNA group III as also Lee and Schneider found. Another dominant type also belonged to the 16SrDNA group III but was slightly different. The third group was similar to the 16SrDNA group I (aster yellow phytoplasma) and the fourth group did not show a clear similarity with any group. Differences in phytoplasma populations between individual poinsettia rootstocks have not been thoroughly investigated, although Lee found some variation as stated above. The work by Abad (Abad *et al.*, 1997) has not been

extended to reveal differences in the composition of phytoplasma populations between cultivars (Moyer, pers. comm.).
Based on these informations it seems likely that the phytoplasmas in poinsettia constitute a population of individual phytoplasmas with different genotypes. It is not known whether all types of phytoplasma are able to induce branching or only phytoplasmas from one group or even only a small fraction of phytoplasmas within one group are branch-inducing. Also, it has not been thoroughly investigated whether all groups of phytoplasmas are present in all available poinsettia cultivars.

Effect of different strains of phytoplasma in poinsettia

It has been speculated whether different populations of phytoplasmas originating from different poinsettia cultivars could induce different phenotypes in the same poinsettia genotype. That is, can phytoplasma populations be used as a ’treatment’ on the same poinsettia cultivar to obtain several ’cultivars’?
Several distinct phytoplasma types exist in poinsettia as described above, and these are probably not present in the same amount or in the same combination in all cultivars. Furthermore, mutation breeding (chemical or radiation) has most likely mutated not only the plant but also the phytoplasmas during the years of poinsettia breeding (Sander, Selecta Klemm, pers. comm.). Therefore, it is likely that several different populations of phytoplasma exist in different poinsettia cultivars, probably with most distinct differences in cultivars belonging to different breeders or ’breeding lines’.
Will these different populations be able to induce different phenotypes of the same poinsettia genotype?
No scientific litterature is available on this subject. According to some breeders, only minor changes of no practical importance occurs (Sander (Selecta Klemm), Dümmen (appendix)), unfortubately, it has not been possible to obtain details on what kind of minor changes they observed. Dümmen finds that differences disappear after a while. Kobayashi (Paul Ecke Ranch) did not observe any differences in plant morphology when using two different rootstocks (Annette Hegg and Angelika) with a range of cultivars, however she noticed that Annette Hegg produced more successful graftunions than Angelika. This phenomenon could be caused by either the phytoplasma, the poinsettia genotype or the physiological state of the rootstock. The experiments were not repeated. Furthermore, the number of rootstock (Annette Hegg and Angelika) is probably too low to cover the natural variation in rootstock/ phytoplasma types. However, Oglevee (see appendix) did observe differences, but the source of phytoplasma, as well as the effect, is not stated.

Unfortunately, all these observations are not based on scientifically conducted experiments, but rather ’on the breeders eyes’. Furthermore, the number of rootstock infector plants has probably only been limited, which means that it is possible that an extended investigation with rootstocks from several breeders (to obtain the largest possible variation in phytoplasma populations) would reveal more distinct morphological differences between plants infected with different rootstocks/ phytoplasma populations.

’Sideeffects’ of phytoplasma infection in poinsettia.

The well known effect of phytoplasma infection on poinsettia is termed free-branching and results from a weak apical dominance to release axilary buds giving a highly branched plant. The phytoplasma infection also results in ’sideeffects’ such as a thinner stem, shorter internodes, leaves become less deeply lobed, bract and leaf colour becomes less intense, cyathia tend to abort earlier, overall keeping quality is reduced. The characteristics mentioned in table 3 were in overall confirmed by breeders. However, (Dole and Wilkins, 1991) did not find changes in for example bract colour between free- and restricted branching poinsettias.

Table 3. Comparison of morphology of poinsettias with and without phytoplasma (partly from (Preil, 1994)).

Trait	**+ phytoplasma**	**- phytoplasma**
Growth habit	free branching	restricted branching
Internodes	short	long
Leaves	less deeply lobed	deeply lobed
Stems	thin	thick
Bract colour	less intense	intense
Anthesis	earlier	later
Cyathia abortion	earlier	later
Fertility	low	high

Procedures to infect poinsettia with phytoplasma

Phytoplasma infection of poinsettia can be performed by grafting on infected rootstocks, either as grafting on top of the rootstock or as ’approach grafting’ in which the infector and recipient plants are brought together and a branch from each is grafted (Dole and Wilkins, 1991). Nescessary time before infection takes place has been reported to be at least 10 days with a 100 % infection after 30 days (Dole and Wilkins, 1992). Poinsettia can also be infected by phytoplasma by use of the parasitic plant *Cuscuta* spp. (dodder) (Lee *et al.*, 1997a), but only with difficulty (Preil, pers. comm.).

Elimination of phytoplasma from poinsettia

Only few reports concerning the elimination of phytoplasma from poinsettia exist, as it is normally the intention to infect poinsettia with phytoplasma. The existing reports have been produced during the attempts to identify the 'branching factor' or during attempts to free poinsettias from virus. There are some examples of commercial poinsettias that lost their branching ability especialy during hot periods (Preil, pers. comm.). Whether these plant have truly lost their ability to branch, and thus their phytoplasma, or the concentration was just too low to induce branching for a period is not known.

Bradel *et al.* (2000) reports the generation of one phytoplasma freed plant after tetracycline treatment, the plant did not contain any detectable phytoplasma after 36 weeks. Lee *et al.* (1997a), Preil (1994) and Geier *et al.* (1992) used suspension culture followed by the induction of somatic embryos for the production of phytoplasma (and virus) free poinsettias. The genetic stability of plants regenerated through this method has been questioned (Ron Cramer, Paul Ecke Ranch, pers. comm.). Bech and Rasmussen (1996) freed poinsettias from phytoplasma by using meristem-tip culture, however Walter Preil did not believe that meristem culture would be an efficient method for the elimination of phytoplasma, although he did not carry out extensive research on this subject.

Other hosts for poinsettia phytoplasma?

It has been attempted to transmit the phytoplasma from poinsettia into other plant species, mainly other *Euphorbias*. Lee *et al.* (1997a) was able to transmit the phytoplasma (by the use of dodder) into periwinkle (*Catharanthus roseus)* which is not an *Euphorbia* but a widely used plant for maintaining phytoplasmas in general. This experiment has shown to be very difficult to repeat in other laboratories (Sander, Selecta Klemm, pers. comm.). Phytoplasma has been transmitted into *Euphorbia fulgens* by use of grafting with poinsettia as the rootstock (Preil and Ebbinghaus, 1999; Preil and Ebbinghaus, 1997a; Preil and Ebbinghaus, 1998; Ludolph *et al.*, 1998, Nicolaisen, unpublished results) resulting in free-branching plants which could be manufactured as pot plants, whereas the restricted-branching type can only be used as cut flowers. Attempts to transmit the phytoplasma to *E. milii* have been unsuccessful until now, although the results were initially successful, the phytoplasma disappeared from *E. milii* after few months (Nicolaisen, unpublished results). Preil has attempted to transmit the phytoplasma to other ornamentals (*Tribouchina urvilleana, Clerodendrum ugandense, Solanum rantonnettii*) by the use of dodder or grafting, but was unsuccessful (Preil, pers. comm.).

Detection of phytoplasma in poinsettia

Before the ’branching factor’ was identified as a phytoplasma, several attempts to identify phytoplasmas in poinsettia were performed by microscopy, but with no success (Preil, pers. comm.). Only when PCR was applied it was possible to detect phytoplasma using primers designed by Lee (nested PCR with R16mF2/mR1 as external primers and R16F2n/ R16R2 as internal primers) (Bertaccini *et al.*, 1996a; Lee *et al.*, 1997a) or by using specific primers designed for the detection of ’western X phytoplasmas’ (Abad *et al.*, 1997a; Abad *et al.*, 1997b). As previously discussed, these methods amplify a large PCR product and are not optimal for high sensitivity and throughput. More efficient methods could probably be developed.

Handling of the phytoplasma in breeding programmes.

As phytoplasma is lost during seed propagation it will be lost during conventional breeding, and so will the free-branching characters of the cultivar. In order to reintroduce the phytoplasma new cultivars are grafted onto phytoplasma infected poinsettia rootstocks (Sander, Selecta Klemm and Kobayashi, Paul Ecke Ranch, pers. comm.). This method is probably exploited by all poinsettia breeders. After a few weeks phytoplasma has infected the new cultivar which subsequently starts to branch more efficiently. The transmission of phytoplasma is confirmed by visual inspection. At this point the newly infected cultivar can be vegetatively propagated, and the free-branching character will remain in the new cultivar. The normal procedure is to perform an initial selection in seedlings before transmission by grafting as it would be a tremendous task to graft all seedlings in a normal sized breeding program.

The rootstock used for transmitting the phytoplasma is not important according to breeders (see appendix) and it is more important to select a poinsettia cultivar with a rootstock that produces viable grafts (Sander, Selcta Klemm, pers. comm.). Some breeders only use one cultivar as rootstock (e.g. Selecta Klemm (Sander, pers. comm.), FLOREMA (appendix)), which means one population of phytoplasma , and some breeders use several cultivars as rootstocks (e.g. Paul Ecke Ranch (Kobayashi, pers. comm.)), which might mean the use of several different populations of phytoplasmas.

PART 3
Final Conclusions and recommendations for future research

Final conclusions

In the original proposal for this litterature review several questions were mentioned:

1) Is the phytoplasma part of the host plant genome
2) Are there different strains of phytoplasma (in Euphorbia)
3) Effect of different strains of phytoplasma or mixtures of strains on the phenotype (of *Euphorbia*)
4) Detection methods of phytoplasma
5) Is it possible to clean a plant from phytoplasma
6) Is there literature on the infection of other (ornamental) plant species with phytoplasma

1)

Is the phytoplasma part of the host plant genome

As already stated in the introduction the phytoplasma cannot be regarded as being part of the plant genome as the phytoplasma DNA is physically separated from plant DNA, phytoplasma can be removed and reintroduced into plants, the DNA structure of phytoplasmas is very different from that of plants. Specifically for the poinsettia/ phytoplasma interaction is the fact that it has been observed that phytoplasma can apparantly disappear from the plant or parts of it during hot periods.

2)

Are there different strains of phytoplasma (in Euphorbia)

Several strains of phytoplasma have been detected in poinsettia. These occur together in individual plants and probably to some extent in different combinations from cultivar to cultivar. The dominant strain belongs to the 16SrDNA group III. Whether different subpopulations within this group exist has not been investigated.

3)

Effect of different strains of phytoplasma or mixtures of strains on the phenotype of *Euphorbia*.

No scientific litterature exist on this subject to my knowledge. Interviews indicated that most breeders do not see a significant effect on the phenotype when using different sources of phytoplasma, although minor effects have been observed. Breeders do not fully agree on this subject, as Oglevee (appendix) did observe differences. These investigations have not been carried out for scientific purposes, and it is not known whether sufficient number of plants have been tested for statistical analysis. It is also not known what measures were taken to ensure maximum variation in phytoplasma between rootstocks and thus obtain the largest possible phenotypic variation of test plants.

4)

Detection methods of phytoplasma

For detection of phytoplasma it is highly recommended to use PCR, as this method is less labour intensive than other known methods, and is routinely used in most phytoplasma-research laboratories, including the authors laboratory.

For discrimination of phytoplasma strains several PCR methods exist, but these have not been developed for routine detection of phytoplasma in a large number of samples.

5)

Is it possible to clean a plant from phytoplasma

Phytoplasma can be eliminated from poinsettia by *in vitro* somatic embryogenesis. The genetic stability of the plant after this procedure has been questioned (Ron Cramer, Paul Ecke Ranch).

6)

Is there literature on the infection of other (ornamental) plant species with phytoplasma

It has not been possible to find any litterature on other ornamentals infected with phytoplasmas to give a benificial trait, except *E. fulgens.*

As phytoplasmas are not seed transmissible it is not very likely that phytoplasma infections as benificials are widespread in commercial production. The phytoplasma would be lost in conventional plant breeding which of course involves the production of seed.

Recommendations by the researcher for future research

In the original proposal for this litterature review, several suggestions for laboratory experiments were mentioned:

A) Characterisation of the different strains of phytoplasma present and implementation of methods to discriminate them (PCR)

B) Effect of different strains of phytoplasma or mixtures of strains on the phenotype (To infect uninfected plants with different strains to see the outcome)

C) To check other species for phytoplasma (like *Euphorbia millii*)

D) To check the method(s) for phytoplasma detection to come to standardisation

E) To check a method for removing of phytoplasma and re-infection

A + B + E) Effect on phenotype of different phytoplasma populations

Based on the rather limited information on the effect of different phytoplasma populations on the same poinsettia cultivar, it is recommended that controlled experiments is set up to evaluate this. The experiments must be carried out with a sufficient number of individual phytoplasma-free plants to compensate for statistical variation. These experiments will also automatically include a test of methods for removing and reinfection of poinsettia with phytoplasma.

Setup of experiment

Plant and phytoplasma sources:

To investigate the effect of different phytoplasma populations on poinsettia cultivars several sorts of plant material is needed:

Phytoplasma-free clones of the same cultivars. These will serve as recipients of phytoplasma and these plants will be evaluated for their phenotype. After cleaning of a cultivar for

phytoplasma by somatic embryogenesis, this individual plant will be vegetatively propagated so that all material within one cultivar has arisen from one clone, and thus genetic variance will be eliminated. Alternatively, cultivars exist that have already been freed from phytoplasma (Cvs. Freedom and Lilo in the authors lab)

Rootstocks with different populations of phytoplasma. These will be collected so that they represent the broadest variation in phytoplasma populations. This includes collection of rootstocks from different breeders and from different 'breeding lines'. For example 10 rootstocks are selected from different breeders and with differents populations of phytoplasma (see below).

Characterization of phytoplasma populations in the poinsettia rootstocks used for infection

In order to use different rootstocks with different populations of phytoplasma it is nescessary to characterize the populations in order to discriminate between them and to select the ones with the most different populations. This should be done by comparing ribosomal genes from individual phytoplasma organisms. For example 10 individual phytoplasmas are investigated from each rootstock, this can be done by molecular methods such as cloning parts of the ribosomal DNA. The nucleotide sequence of these clones will be determined and compared to known strains of phytoplasma.

Infection of non-branching poinsettias with different populations of phytoplasma

Several non-branching poinsettias of e.g. 2 – 3 cultivars produced during this research project will be grafted onto e.g. 10 rootstocks. After transmission of the phytoplasma, plants will be detached from the grafting and rooted. The plants will be grown at identical conditions until flowering.

Evaluation of the outcome of the phytoplasma infection

The infected plants will be evaluated with respect to important morphological traits. Any observable difference in each cultivar between different rootstocks will be recorded.

The experiments with infection and evaluation may have to be repeated in order to ensure statistically significant results.

Deliverables

After the experiments have been performed and the results have been analyzed the following questions should be answered:

- it will be confirmed whether it is possible to remove and reinfect phytoplasma from poinsettia.
- the existence of diverse phytoplasma populations in poinsettia has been investigated.
- the effect of different phytoplasma populations on the phenotype of poinsettia has been investigated.

C + D) Detection method of phytoplasma and check of other *Euphorbia* species for phytoplasma infection

Depending on the decisions made by CPVO, a detection method for phytoplasma in poinsettia and perhaps also *E. fulgens* may be needed. This detection method can also be used for testing other *Euphorbia* species for phytoplasma infection.

To obtain a sensitive, robust and non-laborious method for the detection of phytoplasma in poinsettia it is recommended that

A) Published DNA extraction procedures be compared, eventually together with commercial DNA extraction kits, in order to identify an efficient and easy method for the extraction of poinsettia/phytoplasma DNA that can be used in PCR analysis.
B) A more sensitive PCR method than the above mentioned methods based on the amplification of a smal PCR product should be developed. This method could be evaluated in order to avoid the use of the laborious nested PCR step.
C) Alternatively, but probably less optimal, already published methods (described above) should be compared for the highest sensitivity and robustness.

The developed method can subsequently be used for testing other *Euphorbia* species for phytoplasma infection.

Setup of experiment

A) DNA extraction methods (e.g. 5 different methods) will be compared on phytoplasma infected poinsettia (e.g. 3 different cultivars). The DNA will be evaluated with respect to amount and its ability to act as a template for PCR analysis in an assay developed in B or C).
B) A sensitive PCR method will be developed to assay for phytoplasma infection. This includes the design of new primers, test of these on a number of poinsettia cultivars - and the selection of the best. The most suitable primer pair will be used in a large scale test of commercial poinsettia cultivars to evaluate the primers. An experiment using different parts of the plant and at different timepoints during the year for the assay will be performed to determine the best sampling practice.

C) Like B) but with already developed test methods.

A selection of other *Euphorbia* species will be tested using the above developed method for the presence of phytoplasma.

Deliverables

A detailed protocol, including sampling technique, DNA extraction, and PCR, will be delivered.

Results from a small scale survey of phytoplasma infections in other *Euphorbias* will be delivered.

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