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Study of the natural resistance towards apple proliferation disease and establishment of an *in vitro* resistance screening system in view of the development of resistant apple rootstocks

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1. INTRODUCTION 5
1.1 APPLE PROLIFERATION 5
1.2 SYMPTOMS AND ECONOMIC LOSSES 5
1.3 CANDIDATUS PHYTOPLASMA MALI 7
1.3.1 Taxonomic Position 7
1.3.2 Detection 8
1.3.3 Quantification of *Ca.* Phytoplasma mali 8
1.4 CONTROL STRATEGIES AGAINST AP 9
1.4.1 Vectors 9
1.4.2 Control of the vectors 10
1.4.3 Resistant plant material 10
1.4.4 Resistance strategy 11
1.5 APOMIXIS 12
1.6 BREEDING WITH APOMICTIC SELECTIONS 12
1.7 APPLICATION OF MOLECULAR MARKERS TO SCREEN THE PROGENIES 14
1.8 FLOW CYTOMETRY 14
1.9 IN VITRO CULTURE OF APPLE 14
1.10 IN VITRO CULTURE OF AP-INFECTED APPLE AND MICROGRAFTING 15
2. AIM OF THE WORK 17
3. RESULTS 19
3.1 Apple Proliferation Resistance in Apomictic Rootstocks and its Relationship to Phytoplasma Concentration and SSR Genotypes 20
3.2 Application of microsatellite (SSR) markers in the breeding of apple rootstocks with polyploid, apomictic Malus sieboldii used as source of resistance to apple proliferation 37
3.3 In vitro screening for resistance towards apple proliferation in Malus ssp. 60
3.4 Micropropagation of apple proliferation-resistant apomictic Malus sieboldii genotypes 81
4. DISCUSSION 95
5. CONCLUSIONS 101
6. SUMMARY 103
7. ZUSAMMENFASSUNG 106
8. REFERENCES 109
9. ACKNOWLEDGEMENTS 119

1. INTRODUCTION

1.1 APPLE PROLIFERATION

Apple proliferation (AP) represents one of the most important phytoplasmoses in Europe, detected in several major apple growing areas where it causes considerable economic losses (Kunze, 1989). The disease manifests a range of symptoms that are either specific such as witches' brooms, enlarged stipules, undersized and unmarketable fruits, either non-specific such as foliar reddening, yellowing and growth suppression.

The disease was first reported from northern Italy in 1950 (Rui *et al.* 1950). In the following decades, the occurrence of AP was reported from most European countries. Due to its transmissibility by grafting the disease initially was thought to be caused by a virus until wall-less bacteria, then named mycoplasma-like organisms, were identified in symptomatic trees in 1968 (Giannotti *et al.,*1968). Restriction fragment length polymorphisms (RFLP) and sequence analysis of ribosomal DNA revealed that AP is induced by a distinct phytoplasma that forms, together with the pear decline and European stone fruit yellows agents and a few other phytoplasmas, a distinct group in the phytoplasma phylogenetic clade (Lee *et al.* 2000; Seemüller *et al.* 1998). Unlike most or all other phytoplasmas, which are transmitted by leafhoppers, the fruit tree phytoplasmas of the AP-group are mainly spread by psyllids (Frisinghelli *et al.* 2000; Tedeschi *et al.* 2002). Recently, the AP phytoplasma was taxonomically delineated under the provisional status '*Candidatus*' for unculturable bacteria as '*Candidatus* Phytoplasma mali' (Seemüller and Schneider 2004b).

AP can be transmitted from tree to tree by grafting. Transmission by root bridges was experimentally confirmed by inserting roots from one AP-inoculated tree and one healthy apple tree into a narrow plastic hose and growing the trees for 2 years in the same pot. In addition, spontaneous anastomosis was also observed in the same experiment and other trials (Ciccotti *et al.,* 2005).

1.2 SYMPTOMS AND ECONOMIC LOSSES

AP induces specific and non-specific symptoms on shoots, leaves, fruits, and roots. First indication of disease is often foliar reddening in late summer in contrast to the normal yellow autumn coloration of healthy trees. In July or August of the following year symptomatic trees may form witches' brooms as the most conspicuous symptom through the suppression of apical dominance and by the growth of normally dormant axillary buds on the upper part of vigorous shoots. The resulting secondary shoots are steeply erect and differ from normally wide-angled growth of lateral buds. On less vigorous shoots or later in the season, leaf

rosettes on the apex may appear instead of witches' brooms. Leaves of rosettes, upper parts of brooms and current season's shoots as well as basal leaves of young shoots of diseased trees often have enlarged stipules which differ from other forms of large stipules by distinct denticulation and symmetric shape. Witches' brooms, rosettes, and enlarged stipules are the only symptoms of AP that allow a reliable diagnosis (Seemüller 1990).
Undersized and unmarketable fruits are the main economic losses due to AP. While fruit number is usually not affected, fruit weight is often reduced by 30-60%, the fruit colour is unsatisfactory, and the taste is poor, with the result that as much as 80% of the fruit is unmarketable. The small fruits have longer peduncles than fruits from healthy trees. Diseased trees leaf out earlier in spring, but flowering is slightly delayed. Leaves are often light green to chlorotic and are often smaller and more susceptible to powdery mildew than normal. The vigour of young trees is severely reduced and their root system poorly developed and characterized by dense tufts of short, knotted feeder roots. Larger roots, from which subterraneous witches' brooms may arise, are reduced in number and size. Such trees decline and have to be uprooted because they do not or only poorly recover. Trees that become infected in a later stage and having a well developed root system when infected suffer less from the disease and show a better recovery (Kunze 1989; Seemüller 1990). Like other temperate fruit tree phytoplasmoses, AP decreases frost resistance of otherwise frost tolerant cvs. such as Antonovka and Landsberger Renette (Zawadzka 1988).

The symptoms occur irregularly. Witches' brooms and undersized fruits are typical for newly diseased trees and can be observed for only one or a few years after they first appear. Then, trees often start to recover and show just foliar reddening and enlarged stipules and may eventually become no symptomatic for one or more years. During the remission period, yield, fruit size and fruit quality increase, but usually not to the level of healthy trees (Kunze 1989; Seemüller 1990). The yield of partially recovered trees reaches about 60% of that of healthy trees (Kunze 1989).

There is indication that the variation in symptom expression is affected by the seasonal fluctuation of the phytoplasma population in aerial parts of infected trees. As phytoplasmas depend on functional phloem sieve tubes and because the sieve tubes in the stem cease to function in late autumn and early winter, the pathogen is eliminated in the aerial parts during winter. It survives in the roots, where functional sieve tubes are present throughout the year. From the roots the scion may be recolonized in spring when new phloem is being formed. Recolonization appears to take place more frequently in the first years of disease, but later the

stem may not be recolonized every year or not at all. These differences in colonization explain the fluctuation in symptom expression. Trees intensively colonized in the aerial parts usually develop characteristic symptoms, whereas those only partially, weakly, or not colonized develop mild or no symptoms. The root system of infected trees remains colonized for the life of the tree (Schaper and Seemüller 1982; Schaper and Seemüller 1984; Seemüller *et al.* 1984a; Seemüller *et al.* 1984b; Carraro *et al.,* 2004; Pedrazzoli *et al.* 2007a).

The symptomatology described is typical for cultivars grown on *Malus x domestica*-based rootstocks. However, there are some differences in the response to infection in this genetic material. According to Kunze (Kunze 1989) disease incidence and severity is higher in trees on vigorous rootstocks such as M 4, M 11 and MM 104 than on dwarfing standard stock M 9. Also, the cultivars differ to some extent. Scab-resistant cvs. Florina, Prima, and Priscilla proved very susceptible to AP (Loi *et al.* 1995). In work by Zawadzka (Zawadzka 1976), vigour and yield of cvs. Starking, McIntosh, and Jonathan were more affected than that of cvs. Idared, Spartan, and Jonathan.

Symptom development also depends on the virulence of the infecting *Ca.* Phytoplasma mali strain. In a graft inoculation experiment, different sources of the pathogen reduced terminal growth by 20 to 77%. Trunk girth and fruit size were similarly affected (Kunze 1976). In a more recent study the virulence of 24 randomly collected strains was examined by graft-inoculating Golden Delicious trees on M 11 and monitoring symptom development over a 12-years period. The strains were categorized as not or mildly, moderately, or severely virulent. The three groups occurred in about the same frequency. Trees infected by the first group were virtually indistinguishable from healthy controls or showed only occasionally mild symptoms. Trees inoculated with the other groups showed pronounced to severe symptoms including decline of trees. The vigour of moderately affected trees was reduced by 26% and that of severely affected trees by 62%. Determination of phytoplasma titres by quantitative real-time PCR with DNA from roots revealed similar phytoplasma concentrations in all virulence groups (Seemüller and Schneider 2007).

1.3 C*ANDIDATUS* PHYTOPLASMA MALI

1.3.1 Taxonomic Position

Superkingdom: *Bacteria*; Phylum: *Firmicutes*; Class: *Mollicutes*; Order: *Acholeplasmatales*;

Family: *Acholeplasmataceae*; Genus: *Candidatus* Phytoplasma; Species: *Candidatus* Phytoplasma mali; Species group: 16SrX (Apple proliferation group).

Phytoplasmas are small bacteria that cause yellows and decline diseases and were, after their discovery (Doi *et al.,* 1967), assigned to the mycoplasmas or *Mollicutes* due to their morphological similarity, the lack of a firm cell wall. The phylogenetic position based on 16S rDNA revealed that the phytoplasmas are only distantly related to the cultivable mycoplasmas. A comprehensive analysis of 16S rDNA sequences showed that phytoplasmas represent a monophyletic group which is most closely related to saprophytic acholeplasmas (Family: *Acholeplasmataceae*) (Seemüller *et al.,* 2002).

1.3.2 Detection

Taxonomy and classification of phytoplasmas is mainly based on sequence and restriction fragment length polymorphism (RFLP) analysis of cloned or polymerase chain reaction (PCR) - amplified 16S rDNA (Lee *et al.,* 2000; Seemüller *et al.,* 2002). In the resulting phylogenetic taxonomy, 17 groups and more than 40 subgroups have been classified, and 24 '*Ca.* Phytoplasma' species have been proposed to date (Lee *et al.,* 2006).
In the current taxonomy '*Ca.* P. mali' is assigned, together with '*Ca.* P. pyri' (the pear decline agent), '*Ca.* P prunorum' (the cause of European stone fruit yellows), and the peach yellow leaf roll agent, in the apple proliferation or 16SrX group (Seemüller and Schneider 2004). The genome size of '*Ca.* P. mali' strains varies, ranging between 600 to 640 kb (Seemüller and Schneider 2007). The determination of '*Ca.* P. mali' subtypes by PCR-RFLP (Jarausch, *et al.* 1994) has been used to mark strains in epidemiological studies (Jarausch *et al.,* 2004; Cainelli *et al.,* 2004)
Primers based on non-ribosomal sequences are available too (Jarausch *et al.*, 1994; Lorenz, *et al.,* 1995; Smart *et al.,* 1996; Seemüller and Schneider 2007; Galetto *et al.*, 2005). Some combinations are specific for '*Ca.* P. mali' (Baric *et al.,* 2006; Jarausch *et al.* 2000a) while others cross-amplify the target from the related fruit tree phytoplasmas or do not detect all '*Ca.* P. mali' strains (Lorenz *et al.,* 1995). Nested PCR assays using a sequential amplification with a set of universal and more or less specific ribosomal primers are often employed (Lee, *et al.,* 1995; Bertaccini *et al.,* 2001).

1.3.3 Quantification of *Ca.* Phytoplasma mali

The determination of the phytoplasma titre in the inoculated material has been done in the past by semi-quantitative DAPI staining and epifluorescence microscopy (Kartte and

Seemüller, 1991b; Jarausch *et al*., 1999). Nowadays, real-time PCR using ribosomal or nonribosomal primers in combination with TaqMan™ probes or SYBR green stain is increasingly employed (Torres *et al.,* 2005; Baric and Dalla Via 2004; Jarausch *et al*., 2004b; Galetto *et al.,* 2005; Baric *et al*., 2006) Applications based on TaqMan™ probes were proposed to detect *Ca.* P. mali in roots of apomictic selections resistant to AP (Bisognin *et al.*, 2007b) and as a tool to evaluate the concentration in *in vitro* plants infected with AT2 strain (Bisognin *et al.*, 2007a).

In conventional PCR the amplified product is made visible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. Specialized thermal cyclers equipped with fluorescence detection modules are applied to monitor the fluorescence as amplification occurs.

The main advantage of q-PCR is determining the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Q-PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and contamination probability. At present, real-time assays are more expensive than conventional PCR but are simpler, faster and slightly more sensitive (Baric *et al.,* 2006).

1.4 CONTROL STRATEGIES AGAINST AP

As no curative treatments exist, preventive measures are the only means to control this phytoplasma disease. One way is to apply insecticide treatments against the insect vector(s). Another strategy is to use resistant plant material.

1.4.1 Vectors

Most important for the natural spread of *Ca.* Phytoplasma mali are insect vectors. Two psyllids, *Cacopsylla picta* (Förster) (syn. *C. costalis*) and *Cacopsylla melanoneura* (Förster) are actively spreading the disease agent (Frisinghelli *et al.,* 2000; Tedeschi *et al.,* 2002). *C. picta* has a palearctic distribution and is monophagous on *Malus* spp. The insect completes one generation per year and overwinters as an adult on shelter plants (conifers). At the end of winter/early spring *C. picta* re-immigrants from the shelter plants to apple trees for oviposition. The insects of the springtime generation feed on the primary host until the beginning of July when they leave the apple trees as adults. *C. melanoneura* has a palearctic distribution and is widely oligophagous on *Rosaceae* such as *Crataegus, Malus* and *Pyrus* spp. The life cycle is similar to that of *C. picta* but the overwintering adults appear earlier in the year on apple trees and the springtime generation abandons the primary host earlier than *C. picta* to migrate to its overwintering hosts (Mattedi *et al.,* 2007b). *C. picta* and *C.*

melanoneura transmit the pathogen in a persistent manner. The presence of phytoplasma-infected and infective psyllids among the first re-immigrants collected in apple orchards suggests winter-retention of the pathogen in both species (Jarausch *et al.,* 2004; Mattedi *et al.,* 2007b). In addition to *C. picta* and *C. melanoneura,* there are also reports on successful AP transmission by the leafhopper *Fieberiella florii* (Stål) from Germany (Krczal *et al.* 1988) and Piemonte (Tedeschi and Alma 2006). The role of *F. florii* as an AP vector remains to be further investigated.

1.4.2 Control of the vectors

Control of the vectors is the most important measure against AP. Previous work carried out before *Ca.* P. mali vectors were known showed that reductions in the standard insecticide programs drastically increase infection (Kunze 1989). Now, several vectors are known and their biology has been elucidated so that better timing of insecticide applications is possible. The aim of the control strategy must be to prevent reproduction of the vectors that are in a given area known to transmit *Ca*. P. mali. This is usually *C. picta* or *C. melanoneura* or both. The first step must be measures against the re-immigrating overwintering adults. A particular problem may arise in the control of overwintering *C. picta* adults in years when oviposition coincides with blossoming and insecticides cannot be applied. In this case the strategy has to be focused on the control of the new generation. Organophosphates as well as neonicotinoids (thiametoxan, thiacloprid) were found to be appropriate products to control the developing larvae of *C. picta* (Mattedi *et al.* 2007a). In areas where the disease is present, both the re-immigrants and the springtime generation, which is potentially infective when born on infected trees, must be controlled. In Trentino, field trials revealed that Ethofenprox is the most efficient product to control the overwintering adults of both *C. picta* and *C. melanoneura* before blooming. *C. melanoneura* has also been controlled successfully with organophosphates (Mattedi *et al.,* 2007a). Further data are needed in order to judge the role of *F. florii* in natural AP transmission before control measures against *F. florii* can be recommended.

1.4.3 Resistant plant material

Cultivars and rootstocks of the domestic apple *Malus x domestica* are naturally infected by *Ca.* P. mali and develop AP symptoms. They frequently develop symptoms, remain permanently infected, and favour the development of high phytoplasma concentrations (Seemüller *et al.,* 1992). Screening of a large number of other *Malus* taxa revealed that they

are often more susceptible to infection than *M. x domestica* genotypes (Kartte and Seemüller 1991a). However, resistance was observed in some experimental apomictic rootstock selections derived from crossings between the apomictic species *M. sieboldii* and genotypes of the non-apomictic species *M. x domestica* or *M. purpurea*. In contrast to trees on established rootstocks, trees on some experimental apomictic rootstock selections that mostly derived from crossings between *M. sieboldii* and *M. domestica* develop only mild symptoms such as foliar reddening or slight yellowing. The small fruit symptom occurred not or very seldom. Following graft inoculation the trees may show foliar reddening and enlarged stipules for one or two years and then recover. In the remission period, only light foliar reddening or yellowing may appear. Apomictic selections were developed with the aim to obtain rootstocks for easy propagation by seeds, virus free plants, and better anchorage than dwarfing *M. x domestica*-based stocks (Schmidt, 1988). Promising resistance was identified in several selections including 4551, 4608, D1131, D2212, and H0909.

1.4.4 Resistance strategy

Data on the fluctuation in the phytoplasma colonization in the stem and the persistence of the pathogen in the roots has led to the presumption that growing trees on resistant rootstocks can prevent the disease (Kartte and Seemüller 1991a; Seemüller *et al.* 1992).

There is indication that the variation in symptom expression is affected by the seasonal fluctuation of the phytoplasma population in aerial parts of infected trees. As phytoplasmas depend on functional phloem sieve tubes and because the sieve tubes in the stem cease to function in late autumn and early winter, the pathogen is eliminated in the aerial parts during winter. It survives in the roots, where functional sieve tubes are present throughout the year. From the roots the scion may be recolonized in spring when new phloem is being formed. Recolonization appears to take place more frequently in the first years of disease, but later the stem may not be recolonized every year or not at all. These differences in colonization explain the fluctuation in symptom expression. Trees intensively colonized in the aerial parts usually develop characteristic symptoms, whereas those only partially, weakly, or not colonized develop mild or no symptoms. The root system of infected trees remains colonized for the life of the tree (Carraro *et al.,* 2004; Schaper and Seemüller 1982; Schaper and Seemüller 1984; Seemüller *et al.,* 1984a; Seemüller *et al.,* 1984b; Pedrazzoli *et al.*, 2007a). Phytoplasma concentration in the roots of apomictic genotypes and compared with *M. x domestica*-based rootstocks are extremely low, indicating unsuitable host properties. Both low titre and unsuitable host properties appear to negatively affect recolonization of the stem and, thus, development of foliar symptoms (Bisognin *et al.,* 2007b; Kartte and Seemüller 1991b; Kartte and Seemüller 1991a; Seemüller *et al.,* 1992).

Phytoplasmas were also detected in the roots of most ungrafted apomicts. This shows that such genotypes are visited by *Ca.* P. mali vectors and that the pathogens are able to spread over long distances in low titre hosts. However, the titre in the roots of ungrafted trees was in most cases considerably lower than in grafted trees. This may be due to phloem interactions between root and scion.

1.5 APOMIXIS

The ability of flowering plants to propagate by seed is not necessarily linked to sexuality. Some angiosperms, belonging to the families of *Poaceae*, *Asteraceae*, *Rosaceae* and *Rutaceae* commonly reproduce asexually through seed by a process called apomixis.
Apomixis mainly occurs in two forms. In *agamogenesis*, the embryo arises from an unfertilized egg via a modified meiosis. In *agamospermy*, also called *apogamy*, a nuclear embryo is formed from the surrounding embryo sac tissue. Progenies derived from apomictic parents are genetic copies of the maternal genotypes. A unique example of male apomixis has recently been discovered in the Saharan Cypress, *Cupressum dupreziana*, where the seeds are derived entirely from the pollen with no genetic contribution from the female "parent" (Pichot *et al.,* 2000, Pichot *et al.,* 2001).Apomixis as *apospory* is common in the family of *Rosaceae* and widespread among polyploid species of the genus *Malus*. An attempt was undertaken to analyse the inheritance of apomixis in *Malus* spp. and their F_1 and F_2 hybrids (Schmidt, 1977). Cytogenetic and embryological investigations confirmed the presence of this process. Somatic cells form unreduced embryosacs, indistinguishable from normal ones. The egg is capable of developing into a seed without fertilization but, an additional fertilisation is possible when pollen supply is high, especially following artificial pollination This proves that the development of apomictic gametes is dependent on the genotype and on the environmental conditions (Schmidt, 1977, Nogler *et al.,* 1984b; Serek *et al.,* 2003).
Obligate apomixis is rare among the Rosaceous apomicts, i.e. in a seedling progeny from an apomictic there will be some hybrids too among the apomictic seedlings (Schmidt, 1964). Current hypotheses on the inheritance of apomixis accept some kind of dosis effect of single genes or whole genomes. In *Malus* apomixis is reported to be a dominant character (Sax, 1959).

1.6 BREEDING WITH APOMICTIC SELECTIONS

However, the resistant rootstocks are not fully satisfactory from the agronomical point of view. Trees on these rootstocks are mostly more vigorous and/or crop less than trees on standard stock M9. Thus, a breeding program mainly consisting of crossing resistant

apomictic selections with M9 has been initiated to ameliorate agronomic traits (Jarausch *et al.,* 2005).

In the genus Malus apomixis is, among others, present in the tetraploid asian species *M. sieboldii* and *M. sargentii*. However, apomixis is not obligate in these species. Progenies from open pollination contain a certain percentage of hybrids from unreduced and reduced maternal gametes. Taken a tetraploid apomictic female parent and a diploid nonapomictic male parent, pentaploid hybrids are obtained from unreduced female gametes by having an allele at each locus segregating from the male parent. Triploid recombinants result when both gametes are reduced (Bisognin *et al.*, 2003; Schmidt, 1988). Attempts to develop apomictic rootstocks were undertaken for easily propagation by seeds, virus free plants, better anchorage and higher resistance to some fungal and bacterial diseases than dwarfing *M. x domestica*-based stocks (Schmidt, 1988).

Resistance to AP in field conditions was recently confirmed for some *Malus sieboldii* first generation selections (4608, 4551), and also for second generation selections (C1907, D2118, D2212, Gi477, H0909, H0801) (Bisognin *et al.,* 2007b). However, due to their high vigour and alternate cropping (Webster, 2002) available rootstock breeding lines are not competitive with standard stocks like M9. Nevertheless they represent valuable and advanced material for new breeding programs oriented to provide a durable solution to AP by introducing resistance traits in commercial rootstocks.

When *M. sieboldii* (2n=4x=34) (Schmidt, 1964) and its hybrids resistant to AP, supposed to be allotetraploid (Nekrutenko and Baker, 2003), are crossed with M. x domestica genotype, ploidy level could affect the yield of seeds. Moreover, the lack of clear visual morphological features at early stage prevents the distinction of seedlings originated by apomixis from the ones actually derived by sexual reproduction (Schmidt *et al.,* 1977; Menendez *et al.,* 1986; Ur-Rahman *et al.,* 1997; De Oliveira *et al.,* 2002).

With the goal to obtain apple rootstocks resistant to AP and suitable to modern fruit-growing cross combinations using *M. sieboldii* and derived selections both as maternal parent and pollen donor should be done to transfer the traits of resistance. To select the real hybrid lines obtained from each cross and to distinguish them from apomictic lines a screening method based on the pattern of segregation of Simple Sequence Repeats (SSR) DNA markers could be applied to the seedlings at 2-3 leaves stage. Polymorphic markers were developed from the available locus specific co-dominant SSRs generated in apple for mapping projects in the recent years (Guilford *et al.,* 1997; Gianfranceschi *et al.,* 1998; Hokanson *et al.,* 2001; Liebhard *et al.,* 2002, Liebhard *et al.,* 2003b; Hemmat *et al.,* 2003; Kenis and Keulemans, 2005; Silfverberg-Dilworth *et al.,* 2006.

1.7 APPLICATION OF MOLECULAR MARKERS TO SCREEN THE PROGENIES

The development of highly informative markers, such as microsatellite markers (SSRs) resulted well-suited for genotype segregating of progenies because they have desirable features such as a codominant mode of inheritance, high abundance in plant genome, multi-allelism, low costs and the potential for running in multiplex reactions (Morgante and Olivieri, 1993; Szewc-Mc Fadden *et al.,* 1996; Lübberstedt *et al.,* 1998; Harris *et al.,* 2002). In particular, SSRs isolated from apple have been developed for cultivar identification (Guarino *et al.,* 2006), assessment of genetic diversity (Larsen *et al.,* 2006) and construction of genetic linkage maps in scion apple cultivars (Guilford *et al.,* 1997; Gianfranceschi *et al.,* 1998; Hokanson *et al.,* 2001; Liebhard *et al.,* 2002, 2003b; Hemmat *et al.,* 2003; Kenis and Keulemans, 2005; Silfverberg-Dilworth *et al.,* 2006). These markers developed from apple genome have also been used for cultivar identification in Japanese pear, for genetic differentiation of pear species (Yamamoto *et al.,* 2001) and to fingerprinting and diversity studies in apple rootstocks (Oraguzie *et al.,* 2005).

1.8 FLOW CYTOMETRY

The breeding with apomicts could be further complicated by the fact that *M. sieboldii* (Schmidt 1964) is tetraploid (2n=4x=34) and its hybrids, resistant to AP, are supposed to be allotetraploid (Nekrutenko and Baker, 2003). Determination of ploidy level is conventionally conducted by means of microscopic chromosome counting using meristematic tissues from individual plants. Mitotic cells are arrested in methaphase, followed by DNA stained squash preparation. This work needs a well equipped cytology laboratory as well as trained technicians. Moreover in *Malus spp*. chromosome counting is rather difficult. Since automated fluorescence methods have been introduced ploidy measurements have found a wide field of application also in determination of ploidy of plants. Using flow cytometry (FCM) it is possible to measure the nuclear DNA content of plant cells in a wide variety of species (Galbraith *et al.,* 1983).

1.9 *IN VITRO* CULTURE OF APPLE

Vegetative propagation is the only means to produce homogenous rootstock material deriving from apomictics as these genotypes are difficult to root by classical methods (Magnago, pers. comm.). Therefore, micropropagation is the method of choice to produce uniform planting material in a commercial scale. Tissue culture methods have been successfully applied for the propagation of *Malus* sp. (Lane, 1992). However, it has been reported that different cultivars and rootstocks do not respond in the same way during micropropagation and *in vitro* rooting (Zimmerman and Fordham, 1985; Webster and Jones, 1989; Webster and Jones, 1991).

Micropropagation of apomictic *Malus* genotypes has been attempted only once (Miller *et al.,* 1988). In this study almost exclusively *M. sargentii* hybrids have been used. Many of them were recalcitrant and difficult to micropropagate.

1.10 *IN VITRO* CULTURE OF AP-INFECTED APPLE AND MICROGRAFTING

In vitro culture was successfully employed to maintain *Ca.* P. mali in micropropagated *Malus* cultivars (Jarausch *et al.* 1996; Ciccotti *et al.* 2003).

In vitro grafting of apices, known as micrografting, was initially developed to obtain pathogen-free Citrus (Murashige *et al.*, 1972; Navarro *et al.*, 1975). Since then it has also been employed for apple (Alskieff and Villermur, 1978). In the *in vitro* system *Ca.* P. mali could be transmitted by micrografting to other *M. domestica* cultivars (Jarausch *et al.* 1999) but also to *M. sieboldii* and hybrids of *M. sieboldii* x *M. domestica* (Bisognin *et al.* 2007b). Under aseptic conditions, infected shoot tips were grafted *in vitro* on healthy material (Jarausch *et al.,* 1999). For each genotype several independent experiments could be done. After one month graft contact the success of the graft could be recorded. Only strong grafts were considered as successful grafts because only in these grafts a good phloem connection between the different genotypes is ensured. Transmission rate (number of infected plants per number of successful grafts at three months post inoculation), presence of necrosis and survival rate were evaluated.

The determination of the phytoplasma titre in the inoculated material has been done in the past by semi-quantitative DAPI staining and epifluorescence microscopy (Kartte and Seemüller, 1991; Jarausch *et al.*, 1999). Nowadays, new tools for the quantification of phytoplasmas in plants are available with the development of new techniques of quantitative real-time PCR.

2. AIM OF THE WORK

The work of this thesis was integrated in a research project on AP between the Istituto Agrario di San Michele (Trentino, Italy), AlPlanta – Institute for Plant Research (Neustadt/Weinstrasse) and Biologische Bundesanstalt, Institut für Pflanzenkrankheiten im Obstbau (Dossenheim). The major objective of this project was the development of AP-resistant rootstocks with agronomic value in order to find a long-term solution of disease control.

- The first object of the thesis was therefore concentrated on the re-evaluation of the AP resistance in apomictic rootstocks in a 12-years field trial under natural infection pressure at BBA Dossenheim in order to select the best donors for resistance in the breeding program.

- As individual trees of the resistant genotypes showed an altered behaviour, molecular analyses should be performed to verify if the seed propagated, apomictic material used for the trial was really true-to-type. For this analysis, co-dominant microsatellite (SSR) markers which derive from published work should be applied. For each genotype suitable, polymorphic markers should be selected.

- The work of the thesis should also contribute to the breeding program where a wide range of cross combinations between different donors of resistance with different donors of agronomic value should be performed. With respect to the different degrees of apomixis and polyploidy in the genotypes used as donors of resistance the best suited cross combinations for the production of a high percentage of recombinant progeny should be selected. All seedlings of the progeny should be examined by microsatellite analysis in order to distinguish recombinant from non-recombinant, apomictic progeny.

- As resistance screening of AP is long-lasting *in vivo* and necessitates several years of observation in the field a further aim was to develop an *in vitro* screening of resistance towards AP. This method should be based on *in vitro* graft-inoculation of the genotype to test and the analysis of the resistance by determining the phytoplasma concentration by quantitative real-time PCR and recording the phenotype *in vitro*.

- The *in vitro* resistance screening system had to be based on the establishment of *in vitro* cultures of all parental genotypes of the breeding program. For each genotype the optimal culture medium had to be defined in order to obtain homogenous, well growing shoot cultures of each genotype.

3. RESULTS

3.1 Apple Proliferation Resistance in Apomictic Rootstocks and its Relationship to Phytoplasma Concentration and SSR Genotypes

Phytopathology accepted

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ABSTRACT

In an effort to select and characterize apple rootstocks resistant to apple proliferation (AP) progenies from seven apomictic rootstock selections and their parental apomictic species *Malus sieboldii* and *M. sargentii* were tested in comparison to standard stocks M 9 and M 11. Seedlings derived from open pollinated mother plants were grafted with cv. Golden Delicious and grown under natural infection conditions. The progenies differed greatly in resistance to the AP agent '*Candidatus* Phytoplasma mali'. Progenies of *M. sieboldii* and its descendent rootstock selections D2212, 4608, 4551, and D1131 showed a high level of resistance whereas progenies of *M. sargentii* and its descendent selections D1111 and C1828 proved susceptible. M 9 and M11 showed and intermediate position. Phytoplasma titer in roots of the *M. sieboldii* and *M. sargentii* progeny groups was similar and significantly lower than in standard stocks. In trees on most of the resistant stocks only a minority was colonized in the scion while in trees on susceptible stocks infection rate was often higher. Also, the titer in the top of trees on resistant stocks was usually lower than in trees on susceptible stocks. Four

progenies derived from open pollinated *M. sieboldii* and *M. sieboldii* descendents were subjected to DNA typing using codominant microsatellite (SSR) markers. This study revealed that the selected groups consisted mainly of motherlike plants (apomicts) and hybrids type I (unreduced mother genotype plus one male allele at each locus). Hybrids type II (full recombinants) and autopollinated offspring were rare. In the 4608 progeny, trees grown on hybrid I rootstocks were significantly less affected than trees on motherlike stocks. In other progenies with fewer or no hybrids type I, trees on hybrids II or autopollinated offspring suffered considerably more from disease than trees on motherlike stocks.

INTRODUCTION

Phytoplasmas are wall-less bacteria of the class *Mollicutes* that cause diseases in more than thousand plant species (21). Many of these diseases are of great economic importance. In Europe, one of the most damaging phytoplasma diseases is apple proliferation (AP) that is present in several major fruit growing areas and caused by '*Candidatus* Phytoplasma mali' (25). The pathogen induces a range of symptoms that are either specific such as witches' brooms, enlarged stipules, and rosettes, or nonspecific such as foliar reddening and yellowing, growth suppression and undersized, unmarketable fruits. AP is mainly spread by the psyllids *Cacopsylla picta* and *C. melanoneura* (6,11,27). The leafhopper *Fieberiella flori* is also reported to vector the pathogen (14). Furthermore, infection via root bridges is possible (4).

AP is difficult to control. Phytosanitary measures such as the use of healthy planting material and uprooting diseased trees are often not satisfactory as new infections do occur. The results obtained with insecticide application against insect vectors are contradictory. According to Kunze (15) infection rate can be kept at low level by regular chemical treatments while reduced insecticide programs do not provide satisfactory control. More recent experiments carried out in northern Italy showed that frequent and obviously well timed treatments do not always sufficiently reduce disease spread (L. Mattedi and W. Jarausch, unpublished results).

The most promising approach to control AP appears to be the use of resistant plants. Previous work has shown that '*Ca*. P. mali' is eliminated in the stem during winter due to degeneration of phloem sieve tubes on which the pathogen is depending. Overwintering occurs in the roots where functional sieve tubes are present throughout the year. From the roots the stem may be recolonized in spring when new phloem is being formed (17,19,24). This fluctuation in the

colonization pattern has lead to the presumption that growing scion cultivars on resistant rootstocks can prevent the disease or reduce their impact.

However, extensive studies with many established and experimental rootstocks, which were mainly based on *Malus x domestica*, have shown that there is no satisfactory resistance in this group. They frequently develop symptoms, remain permanently infected and show, as estimated by DAPI (4'-6-diamidino-2-phenylindole) fluorescence staining, a high phytoplasma titer (22). Screening of a large number of other *Malus* taxa revealed that they are often more susceptible to infection than *M. x domestica* genotypes. Many of them showed a high mortality rate. Resistance was observed in some experimental rootstock selections derived from crossings between the apomictic species *M. sieboldii* and genotypes of the nonapomictic species *M. x domestica* or *M. purpurea*. In preliminary trials, resistance was also observed in progenies of similar crossings with *M. sargentii*. Resistant plants either developed never symptoms or recovered within a few years. In these genotypes the pathogen was not or only difficult to detect by fluorescence microscopy, indicating a low phytoplasma titer (12,22).

Apomixis is asexual reproduction characterized by the formation of seeds that are genetically identical to usually the female parent. It occurs in several plant families including the *Rosaceae*. In the genus *Malus* apomixis is, among others, present in the tetraploid Asian species *M. sieboldii* and *M. sargentii*. However, apomixis is not obligate in these species. Progenies from open pollination contain a variable percentage of hybrids from unreduced and reduced maternal gametes. Taken a tetraploid apomictic female parent and a diploid nonapomictic male parent, pentaploid hybrids are obtained from unreduced female gametes by having an allele at each locus segregating from the male parent. Triploid recombinants result when both gametes are reduced (3,20). Attempts to develop apomictic rootstocks were undertaken for easy propagation by seeds, virus free plants, better anchorage and higher resistance to some fungal and bacterial diseases than dwarfing *M. x domestica*-derived stocks (20).

Based on the results obtained in previous work (12,22), a field trial under standard growing conditions was established in which experimental apomictic rootstocks were compared with *M. x domestica*-type rootstocks M 9 and M 11. Whereas in previous trials the trees were graft-inoculated, one objective of this trial was to evaluate the trees under natural infection conditions in order to avoid impairment of the test by a limited strain spectrum or an

inoculum dose that is much higher than when infections occurred by insects. Also, to make sure that the infection rate is high and that there is sufficient time for disease development, the trial was planned to be observed for much longer than in previous trials. In the course of this trial, new technologies became available that allowed more sophisticated studies of phytoplasma resistance. They include quantitative real-time PCR (q-PCR) with which the phytoplasma titer can be determined much more sensitively and precisely than by using the previously employed DAPI fluorescence microscopy. Another new technique is DNA typing using codominant microsatellite (SSR) markers with which the various classes of allele combinations in apomictic progenies can be determined (3).

MATERIALS AND METHODS

Plant material and disease rating. Seedling progenies from 7 experimental apomictic rootstock selections (Table 1) and their apomictic parents *M. sieboldii* and *M. sargentii* were obtained from H. Schmidt, formerly Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Ahrensburg, Germany. Clonal standard stocks M 9 and M 11 were included in the study. The seedling progenies and standard stocks were grown in the nursery and budded with cv. Golden Delicious. In spring of 1993, fourteen to 35 trees of each rootstock-scion combination were transplanted to the field in a randomized block design consisting of 2 to 5 replicates. Maintenance including plant protection measures was similar to that of commercial orchards. From 1994 to 2005 foliar symptoms and terminal growth of the trees were recorded annually in late summer/early fall using a rating system from 0 to 3. Symptom rating categories were: slight reddening or mild yellowing = 0.5; severe reddening and yellowing, premature leaf drop, leaf roll and enlarged stipules = 1; reduced vigor = 2; witches' brooms, undersized fruits, and severe stunting = 3. Mortality was rated with 10. At the end of the observation period the highest annual ratings of each tree were added so that accumulated disease indices were obtained. In addition the occurrence of undersized fruits was recorded over 10 years. The years of appearance were accumulated to obtain the value per tree.

DNA extraction and qPCR. From each tree examined samples from three different roots and current season's shoots were collected in early fall of 2005. Phloem from healthy and infected trees was prepared as described (1) and DNA was extracted from 0.2 to 0.8 g of phloem tissue according to Doyle and Doyle (5). The nucleic acid pellets were suspended in 500 µl of sterile water. Quantitative PCR was performed with a Bio-Rad iCycler IQ. The amplification was

performed in 50-μl reactions containing 25 pmol each primer (fAT 5'-CATCATTTAGTTGGGCACTT-3', rATRT 5'-CGCTTCAGCTACTCTTTGTG-3'), 2 pmol Taqman probe (5'-CCCTTATGACCTGGGCTACA-3') with a reporter fluorescence dye (FAM) at the 5' end and a quencher dye (TAMRA) at the 3' end, 0.2 mM each dNTP, 1 U heat stable polymerase (Tempase, Amplicon), 1x polymerase buffer and 5 μl of DNA. The following parameters were used for amplification: 30 min at 95°C followed by a two step protocol consisting of 41 cycles at 95°C for 15 s and 56°C for 1 min. The samples were run in duplicates. Calculation of phytoplasma titer was by means of a cloned AP phytoplasma 16S rRNA gene standard dilution ranging from 10^6 to 10^1 copies. Sample values distinctly higher than that of control DNA from healthy trees maintained under insect-proof conditions were considered as phytoplasma positive.

Microsatellite analysis. The progenies of *M. sieboldii* and apomictic selections D2212, 4551, and 4608 were DNA typed using SSR markers in order to establish their pedigree. DNA was extracted as described above. Twelve pairs of oligonucleotide primers were used: CH01f03b, CH01g05, Ch02a08, CH02c02a, CH02c11, CH03d02, CH04e03 and CH04g07 (7,16) and GD 96, GD 142, GD147 and 02b1 (8-10). This set of microsatellites was chosen based on the high level of heterozigosity in the above *Malus* accessions. PCR was performed in a 15-μl volume containing 50-100 ng of genomic DNA, 0.2 U of AmpliGold Taq polymerase (Applied Biosystem), 1x GeneAmp PCR Buffer II, 1.5 mM $MgCl_2$ with primers "CH" or 2.2 mM with the others primers, 0.2 mM dNTPs and 0.3 μM each primer pair. Reverse primers were fluorescently labeled with Dye Phosphoramidities (HEX, 6-FAM and NED). PCR was carried out using the Gene Amp PCR System 9700 (Perkin Elmer) at three different programs. Amplification with primers "CH" was performed for 33 cycles at the following conditions: initial denaturation at 95°C for 10 min, 95°C for 30 s, 60°C for 30 s, 72°C for 60 s and final extension at 72°C for 7 min. Amplification with primers "GD" was performed for 25 cycles at the following conditions: initial denaturation at 95°C for 10 min, 95°C for 60 s, 55°C for 120 s, 72°C for 120 s and a final extension of 7 min at 72°C. Amplification with primer "02b1" was performed for 35 cycles at the following conditions: initial denaturation at 95°C for 10-min, 95°C for 40 s, 50°C for 40 s, 72°C for 20 s and final extension at 72°C for 7 min. Separation and sizing of SSR alleles were performed on a 3100 ABI Prism apparatus (Applied Biosystems) and analysis was carried out with softwares Gene Scan 2.1 and Genotyper using Gene Scan 500 ROX as size standard (Applied Biosystems). Use of three dye colors allowed automated detection of fragments arising from multiple loci within one lane simultaneously (multiplexing).

Statistical analysis. The differences between means were tested for significance using the Tukey test with a significance level of P = 0.05. No statistical comparison of data was attempted when sample numbers appeared insufficient.

RESULTS

Level of resistance. Most trees showed symptoms for the first time between the first and the fifth year of observation. Only ten out of a total of 287 trees never developed symptoms. Evaluation of the data obtained from disease rating revealed that the rootstocks examined differ considerably in resistance to AP as expressed by the cumulative disease indices and the cumulative undersized fruit values (Table 2). Progenies of *M. sieboldii* and of *M. sieboldii*-derived apomicts 4608, 4551, D1131, and D2212, (in the following referred to as "resistant *M. sieboldii*-derived progenies") responded similarly and showed a high level of resistance. In contrast, progenies from *M. sieboldii*-derived selection 20186, *M. sargentii* and *M. sargentii*-based selections D1111 and C1828 proved susceptible. Standard rootstocks M 9 and M 11 showed an intermediate position. The low level of resistance in the group of susceptible apomicts is also evident from mortality. In each of the progenies of *M. sargentii*, selections 20186, and D1111 three trees and in the C1828 progeny six trees died. Also, 2 trees on M 9 declined. There was no mortality in the resistant *M. sieboldii*-derived progenies. Moreover, witches' brooms as the most typical symptom were never observed in trees of this group, in contrast to M 11 where brooms occurred repeatedly.

In all root-scion combinations a considerable variability in resistance was observed. However, there were great quantitative differences (Table 2). On resistant *M. sieboldii*-derived progenies 80% or more of the trees were not or only slightly affected by showing disease indices between 0 and 8.0. For trees on standard stocks and, in particular, on susceptible apomicts, this percentage was considerably lower, ranging from 68% for M 11 and 25% for C1828.

Phytoplasma concentration. Six to nine trees from each kind of rootstock were selected to determine the phytoplasma titer in roots and current season's shoots. For this analysis equal or nearly equal numbers of not or little and severely affected trees were chosen for each rootstock. In the roots all but one tree on M 9 proved to be infected (Table 3). Phytoplasma concentrations in the apomictic rootstocks were, irrespective of the level of resistance, in the

same range and did not differ significantly. However, in standard stocks M 9 and M 11 the means were 100 to more than 5,000 times higher than in the apomicts. In shoots of trees grown on *M. sieboldii* and on *M. sieboldii*-derived stocks, *M. sargentii* and M 9 the infection rate in the scion was much lower than in the root. Furthermore, the phytoplasma titer in the few infected scions of resistant stocks was lower than in the roots although scion cultivar Golden Delicious is a high titer host. On the other hand, nearly all trees on *M. sargentii*-derived rootstock selections and M 11 were infected in the shoots, which showed mostly a higher phytoplasma concentration than shoots from resistant apomicts. Only a minority of the shoot-infected trees (1 out of 9) of the resistant *M. sieboldii*-derived progenies showed symptoms in the year of sampling whereas the majority of trees on the remaining stocks (13 out of 23) was symptomatic.

To examine possible correlations between disease severity and phytoplasma titer, the phytoplasma concentrations in not or slightly (disease index ≤ 8) and in moderately to severely affected trees (disease index >8) were compared. In most rootstocks the titer in moderately to severely affected trees was similar to or higher than in trees that suffered not or little from disease. However, in rootstocks 4608 and 20168 slightly affected trees showed higher values than moderately to severely affected trees. The higher mean of the latter group is, across all rootstocks, statistically significant at P = 0.05 (data not shown).

SSR genotyping and resistance. Four apomictic, *M. sieboldii*-derived progenies were subjected to SSR genotyping. Based on the allele patterns, the genetic origin of each seedling has been supposed (Table 4). Plants were assigned to four different classes: “motherlike” when the SSR profile was identical to the female parent, “hybrid I” when in addition to the unreduced mother genotype one father’s allele was observed at each locus, “hybrid II” when both parents contributed with a half of their genotype, and “autopollinated” when SSR pattern were consistent with selfing events. As expected for apomicts, motherlike plants were in all progenies predominant, comprising 65% of the plants across all progenies. The other classes occurred unevenly. In the progenies of apomicts 4551 and 4608 having substantial numbers of hybrids I, trees on this class were markedly less affected than trees on motherlike rootstocks. The differences between these genotypes in the 4608 progeny are statistically significant. The differences observed are mainly due to the fact that 4 and 5 trees on motherlike stocks of 4608 on 4551, respectively, were moderately to severely affected by showing disease indices between 8.8 to 21.5. In contrast, the highest disease index of hybrid I-grown trees was 7.0. In the progenies of D2212 and *M. sieboldii* the hybrid I category was rare or missing. In these

progenies trees on motherlike roots were considerably less affected than trees on stocks derived from hybridization or selfing. While on motherlike stocks 4 and 25%, respectively, were moderately to severely affected, the values for stocks derived from hybridization or selfing ranged between 50 to 67%. The differences in phytoplasma titer between the various genotypes of the progenies examined were relatively small and in no case statistically significant.

DISCUSSION

The rootstocks examined differ significantly in resistance to AP. In decreasing order of resistance, three major groups can be distinguished that consist of (i) progenies of *M. sieboldii* and the resistant *M. sieboldii*-derived selections, (ii) standard stocks M 9 and M 11, and (iii) progenies of *M. sargentii*, *M. sargentii*-based selections, and selection 20186. The first group has a high level of resistance as expressed by low disease rating values, a high percentage of not or little affected trees and a very rare occurrence of undersized fruits, the economically most imported impact of the disease. In all these disease categories the resistant group shows better values than the standard stocks although the differences are not always statistically significant. Of particular importance is the significantly lower occurrence of undersized fruits than of trees on M 9, the major commercial rootstock in Europe. Despite the better resistance of the members of the resistant group in comparison to standard stocks, they are not fully satisfactory from the agronomical point of view. Their major disadvantage is that they are often too vigorous, tend to alternating cropping, and mediate lower yields than M 9. Thus, further selection and/or breeding is required to obtain suitable resistant stocks for commercial apple growing. Such work has to be based on the resistance of *M. sieboldii*, the only known source of AP resistance.

Considerable differences in disease severity were observed in all rootstocks. In case of clonal stocks M 9 and M 11 this variation may be explained by differences in virulence of the infecting phytoplasma. Recent work has shown that phytoplasma strains differ strongly in this respect and that about one third of the strains is either avirulent to weakly virulent, moderately virulent, or severely virulent (26). Differences in strain virulence is with no doubt also involved in the variability of apomicts. As in the apomicts examined four different combinations of the parental genetic contribution were deduced from SSR profiles, these genetic differences seem also account for variation. It could be shown that in the progenies of the triploid selections 4608 and 4551 trees on motherlike plants are more severely affected

than trees on hybrids I. This phenomenon may be due to the unknown father's genetic contribution. On the other hand the higher variation of disease severity of trees on motherlike stocks than on hybrids I may be due to their lower level of resistance. This may result in a wider range of disease indices following infection by differently virulent phytoplasma strains than in trees on the more resistant hybrids I. The fact that trees on hybrids of type II of selection D2212 show higher susceptibility than trees on motherlike stocks indicate segregation of the resistance trait of a susceptible male parent. Also, the low level of resistance of trees on autopollinated stocks may be due to recombination at resistance loci. These observations are the first molecular data on AP resistance which will be valuable for future studies on the inheritance of AP resistance and for breeding work. Furthermore, the variation within progenies from apomicts indicates that such progenies, like seedlings from nonapomictic plants, are unsuitable for being used as rootstocks. Rather, suitable genotypes have to be carefully selected and than vegetatively propagated.

Virtually all trees examined became naturally infected during the observation period as evidenced by the presence of phytoplasmas in the roots. However, phytoplasma concentrations in the roots differed considerably. There were two categories, the high-titer *M. x domestica*-based stocks M 9 and M 11 and the low-titer progenies of apomictic rootstock selections and their apomictic parents. Within the latter group there were no significant differences although *M. sieboldii*- and *M. sargentii*-derived stocks differed strongly in resistance. The latter group proved very susceptible, much more than the *M. x domestica* stocks. This indicates that host suitability per se as expressed in phytoplasma titer is obviously not the deciding factor for resistance. Instead it appears that the pathogenic effect of phytoplasma infection is of qualitative rather than quantitative nature. This may be explained by the fact that the phloem residing phytoplasmas cause formation of pathological callose, sieve tube necrosis, and depletion of starch in the roots of susceptible genotypes of apple and pear (2,13). In previous work it has been shown that a severely affected *M. sargentii*-derived apomict showed extended sieve tube necrosis together with a low starch level and a very low phytoplasma titer. On the other hand, two slightly affected plants, a *M. x domestica*-related genotype and a 4551-derived seedling, showed little phloem necrosis and high starch contents together with high and low phytoplasma concentration, respectively (13). This indicates that the response of the phloem to infection is an important factor in resistance and that this response is not markedly influenced by phytoplasma concentration. The AP resistance of *Malus* genotypes as reported here largely agrees with previous findings obtained following graft inoculation (12,22). However, the high susceptibility of progenies of *M. sargentii*-

derived apomicts C1828 and D1111 became evident only in this work after longer observation than in previous trials.

In contrast to the roots only a minority of the trees was colonized in the top at the end of the observation period although sampling was performed at a time when phytoplasma concentration is highest. Progenies showing low infection rates include mostly *M. sieboldii*-derived rootstocks. Also, the titer in the scion was in most cases lower than in the roots. Both findings are probably related to seasonal changes in the colonization patterns of the AP phytoplasma. Previous studies based on periodic grafting and DAPI fluorescence microscopy have shown that the pathogen persists the winter in the roots from where recolonization of the scion may take place in spring when new phloem is being formed. However, because infected trees often did not develop symptoms and phytoplasmas could not be detected in the stem, it was concluded that recolonization does not occur every year, particularly when trees were older or infected for longer time (17,19,23,24). These findings obtained by examining trees on *M. x domestica* rootstocks are consistent with the results of this work in which the much more sensitive q-PCR technology was employed. With this approach we succeeded to detect the very low phytoplasma concentrations usually present in the stem of trees on apomictic rootstocks. The low infection rate and the low titer in the stem of trees on resistant apomictic stocks seem to result from low phytoplasma concentrations in the roots. The low starting concentration in and poor host suitability of apomictic rootstocks may have a negative effect on the spread of the pathogen from the roots into the scion. Thus, the low titer in the roots is likely to contribute to the resistance of *M. sieboldii*-derived stocks. It is well established that severe symptoms such as witches' brooms and undersized fruits are only developed when the phytoplasma concentration in the stem is high (18).

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TABLE 1. Parentage of apomictic rootstocks examined

Rootstock	Parentage	Ploidy
D2212	(*M. x domestica* cv. Laxton's Superb x *M. sieboldii*), open pollinated	4n
4608	*M. purpurea* cv. Eleyi x *M. sieboldii*	3n
4551	*M. domestica* cv. Laxton's Superb x *M. sieboldii*	
D1131	(*M. sieboldii* x *M. x domestica* cv. Pigeon), open pollinated	4n
20186	*M. purpurea* cv. Eleyi x *M. sieboldii*	3n
D1111	*M. sargentii* x *M. x domestica* cv. Filippa	3n
C1828	M. *sargentii* x *M. x domestica* 'Niedzwetzkyana'	3n

TABLE 2. Apple proliferation resistance of apomictic rootstocks in comparison to apomictic parents and standard rootstocks

Rootstock	No. of trees examined	Cumulative disease index/tree[a]	Slightly affected trees (%)[b]	Undersized fruits (cumulative value/tree)[c]
4608	28	4.0 a[d]	83	0.21 a[d]
D2212	35	4.3 a	80	0.25 a
4551	28	4.9 a	83	0.28 a
D1131	28	5.0 a	82	0.46 a
M. sieboldii	28	5.2 a	83	0.36 a
M 11	28	7.2 a b	68	0.57 a
M 9	28	8.3 a b	64	1.46 b c
20186	28	10.3 b c	57	1.43 b c
D1111	14	10.8 b c	57	1.50 b c
M. sargentii	21	11.0 b c	43	2.19 d
C1828	21	13.2 c d	25	1.95 c d

[a] Annual disease ratings accumulated over 12 years

[b] Trees with cumulative disease index ≤ 8.0.

[c] Occurrence of small fruit symptom accumulated over 10 years

[d] Values with the same letter are not significantly different from each other at P = 0.05.

TABLE 3. Phytoplasma concentrations in phloem preparations from roots and shoots of trees on apomictic and standard rootstocks

Rootstock	Phytoplasmas in roots		Phytoplasmas in shoots	
	Phytoplasma-positive trees[a]	Phytoplasma concentration (cells/g phloem)[b]	Phytoplasma-positive scions[a]	Phytoplasma concentration (cells/g phloem)[b]
D2212	7/7	$2.2.10^5$ a[c]	2/6	$1.3.10^5$
4608	9/9	$1.6.10^6$ a	1/6	$7.3.10^4$
4551	6/6	$1.2.10^6$ a	1/6	$2.2.10^4$
D1131	8/8	$2.8.10^6$ a	4/6	$7.6.10^4$
M. sieboldii	6/6	$1.1.10^6$ a	1/6	$2.6.10^5$
20186	6/6	$2.8.10^5$ a	2/6	$4.0.10^7$
D1111	6/6	$9.1.10^6$ a	5/6	$3.1.10^5$
M. sargentii	7/7	$4.6.10^5$ a	2/6	$7.3.10^4$
C1828	7/7	$3.3.10^5$ a	6/6	$4.9.10^6$
M 9	6/7	$8.7.10^8$ b	2/6	$1.6.10^7$
M 11	6/6	$1.4.10^9$ c	6/6	$1.3.10^9$

[a] Numerator, number of phytoplasma-positive trees; denominator, total trees tested.

[b] Average of infected trees.

[c] Values with the same letter are not significantly different from each other at P = 0.05.

TABLE 4. Disease ratings and phytoplasma concentrations in relation to SSR genotypes

Apomictic progeny	SSR genotype	Disease index		Phytoplasma titer	
		No. of trees	Cumul. value/tree [a]	No. of trees	Cells/g phloem
4608	Motherlike	10	6.6 a[b]	5	$6.0.10^5$
	Hybrid I	18	2.6 b	4	$2.6.10^6$
D2212	Motherlike	26	3.5 a	4	$2.6.10^5$
	Hybrid I	1	0.5	1	$5.2.10^4$
	Hybrid II	6	7.2 b	2	$2.2.10^5$
	Autopollinated	2	8.5	n. d.[c]	
4551	Motherlike	19	5.8 a	4	$1.2.10^6$
	Hybrid I	7	2.6 a	2	$1.0.10^6$
M. sieboldii	Motherlike	22	4.8	4	$5.2.10^5$
	Autopollinated	4	7.5	2	$2.4.10^6$

[a] Annual disease ratings accumulated over 12 years

[b] Values with the same letter within the same progeny are not significantly different from each other at P = 0.05. Other values were not tested due to too low sample numbers.

[c] Not done.

3.2 Application of microsatellite (SSR) markers in the breeding of apple rootstocks with polyploid, apomictic *Malus sieboldii* used as source of resistance to apple proliferation

Plant Breeding, submitted

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Abstract

In order to obtain apple rootstocks resistant to apple proliferation and suitable to modern fruit growing 24 cross combinations were performed over a 5-years period using *M. sieboldii* and its hybrids as donors of the resistance trait and standard apple rootstock *M. domestica* genotypes as donors of agronomic value. Breeding with these genotypes was achieved despite different degrees of apomixis and polyploidy. For identification of recombinant progeny co-dominant SSR DNA markers were applied. Sets of 5-7 polymorphic markers suitable for characterizing each progeny were developed and more than 3000 individuals derived from 17 cross combinations that produced seeds were genotyped. Apomictic genotypes in which marker profiles matched the complete maternal genotype encountered for 79% of the progeny. In 13% of all progenies a complete recombination of maternal and paternal genotype was found at all loci and in 25% the unreduced apomictic genotype was recombined with one haploid *M. domestica* genotype. The different degrees of ploidy created in the progeny could be determined by DNA markers segregation and were perfectly confirmed by flow cytometry analysis.

Keywords: polyploidy, apomixis, flow cytometry, *Malus domestica*, *Candidatus* Phytoplasma mali

Introduction

Apple proliferation (AP) is a disease widespread in central and southern Europe which causes important economic losses due to undersized fruits with poor taste (Kunze, 1989). All currently grown apple cultivars and rootstocks are susceptible to the disease and no curative treatments are applicable. A specific, non-cultured phloem-restricted phytoplasma, the *Candidatus* Phytoplasma mali, is associated with the disease and spread by psyllid vectors (Frisinghelli et al., 2000; Tedeschi et al., 2002). Furthermore, the phytoplasmas may also be transmitted through root bridges (Ciccotti et al., 2005) and the disease can be introduced into the orchards by infected planting material. Due to this different ways of disease spread a control of AP is difficult. Thus, the use of resistant plant material could be the only means to control the disease.

Since 1980, natural resistance towards AP was looked for in *Malus* genotypes. Hundreds of genotypes consisting of established, more recent and experimental rootstocks as well as wild and ornamental *Malus* genotypes were tested. Natural resistance to AP was discovered only in wild, apomictic *Malus* species, namely in *Malus sieboldii* (Kartte and Seemüller, 1988; 1991; Seemüller et al., 1992). Crossings of *M. sieboldii* with *M. domestica* were carried out in the 1950s and 1970s in order to obtain apomictic rootstocks for apple amenable to seed propagation (Schmidt, 1964; 1988). However, due to their high vigour and alternate cropping (Schmidt, 1988) these rootstock breeding lines are not competitive with standard stocks like M9. Nevertheless, resistance to AP was recently confirmed in field conditions for some *Malus sieboldii* F_1 hybrids (4608, 4551), and also for *M. sieboldii* F_2 hybrids (C1907, D2118, D2212, Gi477, H0909, H0801) (Bisognin et al. submitted). These genotypes represent valuable and advanced material for new breeding programs oriented to provide a durable solution to AP by introducing resistance traits in commercial rootstocks.

This natural resistance towards AP can be exploited in a resistance strategy because the phytoplasmas are eliminated in the upper part of the tree, the grafted cultivar, once a year during phloem re-newal in late winter/early spring (Schaper and Seemüller, 1984; Seemüller et al., 1984a; 1984b). The phytoplasmas survive in susceptible rootstocks all over the year because phloem re-newal is a constant process in the root system. Thus, resistant rootstocks could impair this survival and the re-colonisation of the stem in spring.

Apomixis occurs in several plant families (Nogler 1984) including *Rosaceae*. Especially in polyploid species of the genus *Malus* apomixis in form of apospory is widespread (Schmidt, 1977). Somatic cells form unreduced embryosacs and the egg is capable to develop into a seed without fertilization. Therefore, seedlings are genetically identical to the female parent. However, apomixis is not obligate in *Rosaceae* and additional

fertilization is possible when pollen supply is high, especially following artificial pollination. Recombinant progenies can be generated in varying amount by crossing, open pollination or selfing, as reported by Schmidt (1964) for offsprings of *M. sieboldii*. However, the lack of clear visual morphological features at early stages prevents the distinction of seedlings originated by apomixis from the ones derived by sexual reproduction (Schmidt et al., 1977).

The objective of the present study was to breed apple rootstocks resistant to AP and suitable to modern fruit growing. In order to overcome problems with apomixis and varying degree of polyploidy a wide range of cross combinations was performed using *M. sieboldii* and derived selections both as maternal parent and pollen donor. For the early selection of recombinant progeny at 2-3 leaves stage a screening method based on the pattern of segregation of simple sequence repeats (SSR) DNA markers was established. Polymorphic markers were developed from the available locus specific co-dominant SSRs generated in apple for mapping projects in the recent years (Hemmat et al., 2003; Liebhard et al., 2002; 2003; Silfverberg-Dilworth et al., 2006).

Material and Methods

Plant material

Parental lines used in the breeding program and analyzed in this study are listed in Table 1. *Malus sieboldii* and derived apomictic selections were used as donor of resistance to AP. Plants were obtained from H. Schmidt, formely Bundesanstalt fur Züchtungsforschung an Kulturpflanzen, Ahrensburg (Germany), and maintained at BBA Dossenheim. Crossings were performed for five years (2001-2005) with *Malus domestica* clonal standard stock J-TE-F, M9, M27, P22 and Supporter1, and commercial apple cultivars Gala, Golden delicious and Prima. Cross combinations are reported in Table 2. M9 was preferentially used for its appreciated traits of low vigour and high productivity. Reciprocal crosses of rootstock genotypes could only be done for M9 as no flowering trees were available at BBA Dossenheim for the other genotypes. Flowers of genotypes J-TE-F, M27 and P22 were obtained from the Bundessortenamt Wurzen, Germany.

Crossings were done as recommended by H. Schmidt (pers. comm.). Pollen was prepared from flowers which were cut from the trees when they were still closed and then maintained on humid sand in the greenhouse for further pollen ripening. As most of the *Malus*

species are not self fertile a castration of the flowers prior artificial pollination was not done. Pollinated flowers were protected by specific bags from open pollination.

The obtained seeds were germinated according to standard protocols. All progenies were grown in pots in the greenhouse under standard conditions as described in Westwood (1980).

Genotyping with SSR markers

Parents of the crosses and all individuals of progenies listed in table 2 were DNA typed using molecular markers. DNA was isolated from leaves of parents and young seedlings (at 2-3 leaves stage) using DNAeasy 96 Plant Kit (Qiagen).

SSR markers were developed testing the following primers pairs: CH01f03b, CH01g05, CH02a08, CH02c02a, CH02c11, CH03d02, CH04g07, CH04e05 (Liebhard et al., 2002), GD 12, GD 96, GD147, GD 162 (Hokanson et al., 1998), 02b1, 04h11 (Guilford et al., 1997). Sets of suitable markers were selected based on locus position in different linkage groups (Liebhard et al., 2002; Hemmat et al., 2003; Silfverberg-Dilworth et al., 2006), unambiguous banding patterns and degree of polymorphism.

PCR was performed in a 15 µl volume containing 1 µl of the DNA solution obtained by the Qiagen extraction protocol as template, 0.2 U of AmpliGold Taq polymerase and 1x GeneAmp PCR Buffer II (Applied Biosystem); 1.5 mM $MgCl_2$ with primers “CH” and 2.2 mM with remaining primers; 0.2 mM dNTPs and 0.3 µM each primer pair. Reverse primers were fluorescently labeled with Dye Phosphoramidities (HEX, 6-FAM and NED). PCRs were carried out using the Gene Amp PCR System 9700 (Perkin Elmer) at three different programs. Amplifications with primers “CH” were performed for 33 cycles at the following conditions: initial denaturation at 95°C for 10 min, 95°C for 30 s, 60°C for 30 s, 72°C for 60 s and final extension at 72°C for 7 min. Amplification with primers “GD” were performed for 25 cycles at the following conditions: initial denaturation at 95°C for 10 min, 95°C for 60 s, 55°C for 120 s, 72°C for 120 s and final extension of 7 min at 72°C. Amplifications with primer “02b1” and primer “04h11” were performed for 35 cycles at the following conditions: initial denaturation at 95°C for 10 min, 95°C for 40 s, 50°C for 40 s, 72°C for 20 s and final extension at 72°C for 7 min. PCR products were multiplexed (3 loci) and analysed on a 3100 ABI Prism capillary electrophoresis system using the Gene Scan 500 ROX size standard and the software Gene Scan 2.1 and Genotyper (Applied Biosystems).

Analysis of plant ploidy

Flow cytometric analysis was applied to the parental lines and seedlings listed in Table 6 in order to establish their ploidy. Suitable tissue material was obtained by plants cultivated *in vitro*. Establishment and maintenance of *in vitro* cultures was done according to standard protocols (Jarausch et al., 1994; 1996).

Samples were prepared following the protocol of Galbraith et al. (1983) with minor modifications. Briefly, young leaves were finely chopped with a razor blade in a Petri dish containing 500 µl of Tris buffer (10 mM tris(hydroxymethyl)aminomethane, 10 mM NaEDTA, and 100 mM NaCl, pH 7.4) + 0,1% Triton. Nuclear suspensions were filtered twice through a 15-µm nylon mesh to remove debris.

To define plant ploidy and to control instrumental and staining variation when comparing measurements referring to different samples, nuclei extracted from tetraploid *M. sieboldii* and from diploid '*Golden delicious'* were added as reference internal standards to each sample. The mixed nuclei were stained with the DNA binding fluorochrome DAPI at a final concentration of 5.5 µM. The fluorescence intensity of the nuclei was measured with an arc lamp-based flow cytometer (Bryte-HS; Bio-Rad, Hercules, CA) equipped with a 75 W xenon lamp and a 365 ± 5 excitation filter. The DAPI fluorescence emitted was selected by a band-pass 450-490 filter. A total of 20000 nuclei were measured for each cytogram. At least three different plants for each line were measured, and each sample analysis was repeated 3 times. Data were analysed with Win-Bryte software (Bio-Rad, Hercules, CA).

Results

Three thousands and thirty two seedlings belonging to 17 progenies were finally obtained from 24 different cross combinations carried out in the years 2001-2005. Combinations 4551 x M9, 4608 x M9, *M. sieboldii* x M9 and H0909 x M9 were repeated 2 or 3 times, in order to increase the number of seedlings. Apomictic selections 4551, 4608, D2212 and *M. sieboldii* used both as female parent and male parent generated a different yield of seeds. Fruit set, seed quality and population size obtained from each cross combination are reported in Table 2.

All plant material was typed with SSR markers in order to distinguish the seedlings originated by the sexual combination of each pair of parents from the seedlings originated by apomixis, selfing or out crossing. Different sets of 5-7 SSR markers were applied to the whole progenies (Table 3) based on polymorphism and reliability of fragments generated at thirty

SSR loci previously tested on parental lines. Common loci to most sets were finally: CH01g05, Ch02a08, CH02c11, CH04g05, CH04g07, GD 96 and 02b1.

Every parent used in the breeding program generated a unique profile at SSR loci (Table 4). DNA markers of different size (allele combination) at the same locus were thus considered for establishing the genetic origin of each individual progeny. Based on pattern of markers inheritance, seedlings were assigned to different classes as reported in Table 5. Individuals with a genotype at SSR loci identical to the female parent were classed as "mother like" and considered originated by apomixis. When seedlings exhibited a more complex SSR profile at each locus, composed by all the alleles of the mother (unreduced genotype) added to one allele derived from the male parent, the class of individuals was named "hybrid I". Seedlings exhibiting half of both mother and father SSR profiles were considered the real hybrids ("hybrid II"). Few cases of real hybrids were attributed to uncontrolled crossing as the male parent allele were different from expected ("open pollinated" class). Finally, seedlings showing different allele combinations of the mother SSR profile were considered originated by selfing and grouped as "autopollinated".

Crosses H0909 x M9 and H0801 x M9 were actually backcrosses due to the origin of H0909 from the cross 4556 x M9 (Table 1). In these cases, both parent profiles have at least one allele in common and the distinction of genotypes "mother like" from genotypes "hybrid II" was possible only when their M9 derived allele was different from the M9 related allele of H0909.

In the crossings with apomictics used as female parents, "mother like" SSR profiles were predominant in all progenies. Non recombinant genotypes were actually expected from apomixis and accounted for 55% of the seedlings generated by the breeding program. Other genotypic classes occurred from these crosses at variable rates. In progenies *M. sieboldii* x M27; 4551 x M9; 4608 x M9 and D2212 x M9 (Table 5) only "mother like" and "hybrid I" type of seedlings were observed together with very few cases supposed to be originated by open pollination. In addition to "mother like" and "hybrid I", a group of 'hybrid II' was detected in the rest of progenies from cross combinations with apomictics used as female. The highest rates of "hybrids II", 100% and 68%, were obtained from cross combinations Gala x *M. sieboldii* and M9 x D2212, respectively. However, the largest amount of real hybrids was produced by the three repetitions of crossing H0909 x M9 and by the combination Gala x *M. sieboldii.* Genetic origin by selfing was deduced for very few individuals spread on half of progenies with the exception of the cross H0909 x M9.

The cross H0909 x M9 showed a decrease in the degree of apomixis during 3 years of observations (61% in 2002 year, 54% in 2003 year and 44% in 2004 year) compensated by an

increased proportion of hybrids I (15%, 23%, and 16%); hybrids II (18%, 13% and 20%) and autopollinated (6%; 10%; and 20%).

Flow cytometry analysis

Flow cytometry analyses produced unambiguous information about genome copy number for all materials tested (Fig.1, Table 6). The ploidy of *M. sieboldii* was established as *4 n* while its first generation descendants, 4551 and 4608, resulted triploids. Based on segregation of DNA markers, 4551 and 4608 should be considered real hybrid as both have half alleles of the *M. sieboldii* female parent in their SSR profile at each locus.

For the second generation of apomictic parents (C1907, D2118, D2212, H0801, H0909) a tetraploid genome was determined. These parental plants were related to “hybrid I” as they show the unreduced maternal genetic contribute (i.e. 4608, *3 n*) added to the paternal allele at each locus.

Besides parental lines, ploidy was established for a set of individuals belonging to most of the progenies obtained by the breeding program. Polyploidy was deduced for all plants analyzed (Table 6) and related to the category to which each seedling was more easily assigned based on parental allele combination at SSR loci. A perfect agreement between the two sets of data was found.

Discussion

Breeding with apomictic *Malus sieboldii* and its F_1 hybrids has been done between the 1950s and 1970s for developing seed propagated apple rootstocks which are free of not-seed transmitted viruses (Schmidt, 1964; 1977; 1988). The objective was therefore to maintain apomixis in order to guarantee the production of homogenous plant material. In this respect the apomixis of this material was extensively studied and it was shown that recombinant progeny was obtained in varying amounts with the different genotypes (Schmidt, 1977). However, the material was never used in apple growing because its performance in the orchard could not compete with the dwarfing standard stock M9. Only in the 1980s it became evident that *Malus sieboldii* and its hybrids exhibit resistance towards apple proliferation disease (Kartte and Seemüller, 1988). These findings were recently confirmed by the analysis of a 12-years field trial (Bisognin, submitted). In these trials it could be shown that AP resistance was only derived from *M. sieboldii* and was maintained in some of its F_1 and F_2 hybrids.

As apple proliferation disease is a major threat for apple production in important apple growing regions in northern Italy and southwest Germany, the objective of the present study was to develop AP-resistant rootstocks with agronomic values equivalent to the standard stock M9. Therefore, the best donors of resistance were crossed with different genotypes of dwarfing apple rootstocks. The objective of these crosses was to produce a high amount of recombinant progeny. According to previous data, the degree of apomixis is varying between the different genotypes and is less complete in 4n genotypes. Thus, predominantly these genotypes (*M. sieboldii* and its F_2 hybrids) were used. As 3n F_1 hybrids 4551 and 4608 are excellent donors of resistance also crosses were performed with these genotypes. In order to find a suitable cross combination which yields a high percentage of recombinant progeny a wide range of different combinations were analysed. As controls for cross compatibility crosses with *M. domestica* cultivars were included in the study. The results demonstrated that fruit set, seed formation and seed quality differed considerably between the different cross combinations. For some combinations no vital seeds were obtained which might indicate an incompatibility of the genotypes. As expected, fruit set was good if the apomict was used as maternal genotype. Contrary, fruit set was mostly poor if a non-apomict was pollinated with pollen of an apomict. In part this can be explained by a poor pollen vitality, especially of genotypes 4551, 4608 and *M. sieboldii* (data not shown). However, exceptions are possible as demonstrated by the good fruit set of the combination Gala x *M. sieboldii*. As reciprocal crosses could only be done with M9 due to missing flowering trees for the other genotypes and fruit set was poor if pollen of apomicts was used, the majority of crosses were performed with the apomictic genotype as maternal genotype.

As the phenotype of apomictic and recombinant progeny cannot be distinguished at the early seedling stage, a new strategy was developed in order to screen for recombinant progeny as soon as possible. This strategy was based on genotyping with SSR markers. SSRs are co-dominant DNA markers and for apple a large amount of sequence information for SSR is available. They are markers of choice in apple mapping experiments and, thus, for hundreds of SSR loci map position in different genetic backgrounds of *Malus* spp is well known (Liebhard et al., 2003; Silfverberg-Dilworth et al., 2006). Thirty map-based SSRs were tested in the parental lines of the breeding program in order to select the most informative loci for characterizing each cross combination and enabling fast and effective genetic origin evaluation of progenies. Preferred SSR markers were highly polymorphic among accessions and frequently heterozygous so that DNA profiles with presence of 4 alleles were observed at almost all loci.

As expected, most of the seedlings generated from all cross combinations in which apomictic lines were used as female parent were non-recombinant individuals. They were easily identified thanks to a complete overlapping of genotypic profile with the mother's alleles at all considered SSR loci. Consequently, this breeding material was not further evaluated. On the contrary, a whole maternal allele pattern with an additional allele derived by the paternal parent at each locus was an unexpected genotype found on a large amount of individuals (743) belonging to all progenies. As these seedlings represent nevertheless new genetic combinations they were graft-inoculated with AP-infected material and maintained for further resistance and vigour evaluations. Fortunately, also a discrete number (398) of real hybrids was generated and to this group of seedlings a special evaluation effort has been dedicated.

Flow cytometry analysis confirmed the polyploid status of *M. sieboldii* and its derivatives as determined in previous studies by chromosome counts for the apomictic parents of the crosses (Schmidt 1964). Flow cytometry was preferred for the reliability of data because the nuclei of apple are small in size and, thus, incertitude remains in the chromosome counts in root tip cells. Furthermore, this technique is labour-intensive and time-consuming (Kim et al., 2003). Flow cytometry analysis was also favoured by the fact that *in vitro* cultures of the material were established for other purposes, e.g. micropropagation of promising progeny genotypes (Ciccotti et al., submitted).

SSR DNA marker segregation as well as flow cytometry confirmed the existence of four genotypic classes in the progeny, each of which with a distinct ploidy level. Mother like plants were only derived from crosses with apomicts as female parent and presumably derived from unreduced egg cells. No male apomixis was detected as it has recently been discovered in Saharan Cypress, *Cupressus dupreziana*, where seeds are derived entirely from the pollen with no genetic contribution from the female parent (Pichot et al., 2000). The mother like genotypes had the same ploidy level as the female parent, e.g. 3n for selections 4551 and 4608, 4n for all other apomictic genotypes used. Hybrids I had an increased ploidy level with respect to the female parent (4n and 5n, respectively) and resulted from unreduced egg cells plus one parental haplotype. For triploid female parentals only hybrids I were obtained as recombinant progeny indicating that reduced egg cells could not be formed in these genotypes. In the reciprocal cross, however, diploid gametes must have been developed to a low extent in the pollen as hybrid II progeny of 3n was obtained in the cross M9 x 4551. Hybrids II are classified as fully recombinants between male and female parents. As predicted by Schmidt (1977) they were almost exclusively obtained from tetraploid apomictic parents for which a higher degree of sexual reproduction can be observed. Consequently, the ploidy

level of these hybrids II was 3n. A fourth genotypic class was detected by SSR analysis in the progeny of most of the tetraploid female parentals: a recombination of the female genome without contribution of a male genome. These autopollinated genotypes show that a varying degree of self fertility exists in these tetraploid apomicts. This has already been observed by Schmidt (1964) if experiments of artifical selfing were carried out. The highest amount of autopollinated progeny was obtained with genotype H0909 which is a more recent F_2 hybrid of *M. sieboldii.* This cross is already a cross with M9 and, therefore, the distinction of hybrid II and autopollinated progeny in the cross combination H0909 x M9 needed the application of more SSR markers. Flow cytometry confirmed the 4n level of this material.

Progenies with different ploidy level may vary in germination capacity and/or seedling survival ability that may bias results based on the ploidy examination of established plants (Krahulcová and Suda, 2006). Less vital cytotypes are bound to be underestimated. However, this limitation is not relevant in the present breeding program in which only vital genotypes are interesting. For the exact determination of the different genotypic classes these genotypes could be underestimated.

The highest number of hybrid II progeny was obtained with cross combination H0909 x M9 which was for this reason repeated in several years as only one flowering tree was available for the genotype H0909. The degree of apomixis of this tree decreased in the three consecutive years. This could have been influenced by the climatic conditions during embryosac formation as reported by Schmidt (1977) or might be related to the increasing age of the tree.

The different genotypic classes have different impact on the further analysis of the breeding progeny. Whereas motherlike genotypes were excluded from further evaluation all recombinant progeny is currently being analysed for AP-resistance. This resistance screening is done by graft-inoculation of *Ca.* Phytoplasma mali and subsequent observation of symptom expression for several years in the field. Therefore, these data are not available yet. Resistant genotypes will be further analysed for their agronomic value. Whereas the most promising genetic recombination is expected in the class of hybrids II, tetraploid genotypes of hybrid I or autopollinated genotypes could be interesting material for further breeding. This is especially the case for the autopollinated progeny of H0909 in which a new recombination of *M. sieboldii* and M9 haplotypes occurred. Crosses H0909 x M9 and Gala x *M. sieboldii* were the only cross combinations in which a substantial number of hybrid II progeny could be obtained. This progeny is currently being exploited for the construction of linkage maps used to locate the genes associated to the resistance trait.

SSR markers proved to be a reliable and valuable tool to rapidly screen the breeding progeny at an early stage. For each cross combination a defined set of markers can now be proposed for future analyses and a large set of seedlings can easily be screened. Furthermore, SSR markers were also reliable in predicting the different ploidy level of the progeny, thus, avoiding in future laborious and time consuming ploidy analyses like chromosome counts or flow cytometry.

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Table 1. Apomictic species and selections crossed in this study with *M. domestica sp.*
(op.*: unknown pollen donor due to open pollination)

Apomictic genotypes		*M. domestica* genotypes
Wild species	Parentage	Rootstocks of agronomic value
M. sieboldii	ancestor	JTE-F
		M 9
1st generation selections		M 27
4551	Laxton's Superb x *M. sieboldii*	P 22
4608	M. purpurea "Eleyi" x *M. sieboldii*	Supporter 1
2nd generation selections		Apple Cultivars
C1907	4608 (*M. purpurea* "Eleyi" x *M. sieboldii*) x op.*	Gala
D2118	4556 (Laxton's Superb x *M. sieboldii*) x op.*	*Golden Delicious*
D2212	(Laxton's Superb x *M. sieboldii)* x op.*	Prima
H0801	4556 (Laxton's Superb x *M. sieboldii*) x M 9	
H0909	4556 (Laxton's Superb x *M. sieboldii*) x M 9	
Gi477/4	4608 (*M. purpurea* "Eleyi" x *M. sieboldii*) x op.*	

Table 2. Fruit set, seeds traits and number of seedlings germinated from cross combinations made over a five-years period.

Cross combination	Fruit set	Seed formation	Seed quality	N. of seedlings				
				2001	2002	2003	2004	2005
Apomictic genotypes as recipient								
M. sieboldii x JTE-F	poor	fair	no seeds			0		
M. sieboldii x M9	moderate	good	fair		82	71		
M. sieboldii x M27	moderate	fair	fair			9		
M. sieboldii x P22	poor	poor	fair			1		
M. sieboldii x Gala	moderate	fair	fair			188		
M. sieboldii x GD	poor	fair	no seeds		0			
M. sieboldii x Prima	poor	fair	no seeds		0			
4551 x M9	good	good	good	534	170			
4608 x M9	good	good	good	334	220			
C1907 x M9	good	good	good					128
C1907 x supporter 1	good	good	good					59
D2118 x M9	good	good	good					132
D2118 x supporter 1	good	good	good					123
D2212 x M9	moderate	fair	fair		67			
H0801 x M9	good	good	good					29
H0909 x M9	good	good	good		177	143	599	
H0909 x P22	moderate	fair	fair	25				
H0909 x supporter 1	poor	poor	fair					0
Gi477/4 x M9	no	no seeds	no seeds					0
Gi477/4 x supporter 1	no	no seeds	no seeds					0
Apomictic genotypes as pollen donor								
M9 x 4551	poor	poor	fair		28			
M9 x 4608	poor	poor	poor	0				
M9 x D2212	poor	fair	fair		28			
Gala x M. sieboldii	good	good	good			117		

Table 3. Sets of SSR markers applied to the genetic characterization of progenies obtained from 15 different cross combinations. SSR loci were selected from thirty publicly available sequences based on DNA fragments reliability and polymorphism in the parental lines.

Cross-combination	SSR LOCUS													
	CH01 g05	CH02 a08	CH01 f03b	CH02 c02a	CH02 c11	CH03 d02	CH04 e05	CH04 g07	GD 12	GD 96	GD 147	GD 162	02 b1	04 h11
M. sieboldii x M 9	√	√			√			√		√			√	
M. sieboldii. x Gala						√	√		√	√	√	√	√	
4551 x M 9	√	√			√					√			√	
4608 x M 9	√	√			√			√		√			√	
C1907 x M9	√						√	√	√	√		√	√	
C1907 x supporter1	√			√			√		√	√		√		
D2118 x M9				√		√	√			√	√	√		
D2118 x supporter 1	√			√			√			√		√	√	√
D2212 x M 9	√	√			√			√			√		√	
H0801 x M9	√			√			√			√	√		√	
H0909 x P22		√	√		√	√	√			√			√	
H0909 x M 9	√	√			√			√		√			√	
Gala x *M. sieboldii*						√	√		√	√	√	√	√	
M 9 x D2212	√	√			√			√			√		√	
M9 x 4551	√	√			√			√		√			√	

Table 4a. Genotype at SSR loci for each parental line used in the breeding program and analyzed in this study. Allele sizes are given in bp.

Parental line	SSR LOCUS						
	CH01g05	CH02a08	CH02c02a	CH02c11	CH03d02	CH04e05	CH04g07
M. sieboldii	153:157:163:175	133:153	130:151:156:185		188:194:200	181:201	158:166:170:190
4551	151:163:175	146:153		196:219			158:166:174
4608	157:175:187	153:161		196:237			170:190
C1907	157:175:187		156			195:219	135:158:170:190
D2118			167:191		200:219	170:181:197:201	
D2212	138:155:157:175	133:140:150:153		196:202:212:234			194:200:220
H0801	142:157:170:175		167:178			181:197:201:219	
H0909	149:171:179	133:140:146:153		196:210:213	194:200:219	181:195:197:201	137:150:166:190
M9	142:171	146:171	179:191	210:231	220	196:219	137:150
Supporter 1	131:142		193			185:219	
P22		140:172		220:231	192	220:227	
Gala					220:242	170	

Parental line	SSR LOCUS					
	GD12	GD 96	GD 147	GD 162	02b1	04h11
M. sieboldii	152:156	147:149:153	117:126:138	201:213:243	239:253	
4551		149:153			215:239:253	
4608		149:153:177			229:239	
C1907	156	149:153:179		201:213:230	221:235	
D2118	152	149:153:193	117:126:138:149	201:213:219:235	215	210: 222:228
D2212		149:153:169			212:239	
H0801		149:153:169	117:126:138		215:222:239:253	
H0909		149:153:169			215:229:239:253	
M9		161:169	138:151	210	222:229	
Supporter 1	158	168	130:151	207	221:229	205
P22		161:167			222:226	
Gala	145:188	149:177	135:149	211:232	226:237	

Table 4b. Allelic pattern at SSR loci for representative individuals of each progeny class to which seedlings generated from the 12 cross-combinations with apomictic maternal parents have been assigned. Based on observed parental allele segregation, 5 different progeny classes were established: mother like, hybrid I, hybrid II, autopollinated and open pollinated (see the results for definition). Allele sizes are given in bp. Abbreviations: *M. sie.* : *Malus sieboldii*; sup. 1: supporter 1.

Cross-combination	Progeny class	SSR LOCUS						
		CH01g05	CH02a08	CH02c02a	CH02c11	CH03d02	CH04e05	CH04g07
M. sie. x M 9	mother like	153:157:163:175	133:153		196:200:212			158:166:170:190
	hybrid I	153:157:163:171:175	133:146:153		196:200:212:231			150:158:166:170:190
	hybrid II	153: 163:171	133:153:171		196:200:231			137:158:166
M. sie. x Gala	mother like					188:194:200	181:201	
	hybrid I					188:194:200:242	170:181:201	
	hybrid II					188: 200:242	170:201	
4551 x M 9	mother like	151:163:175	146:153		196:219			158:166:174
	hybrid I	151:163:171:175	146:153		196:219:231			150:158:166:174
	hybrid I	142: 151:163:175	146:153:171		196:210:219			137:158:166:174
4608 x M 9	mother like	157:175:187	153:161		196:237			170:190
	hybrid I	157:171:175:187	153:161:171		196:210:237			137:170:190
	hybrid I	142:157:175:187	146:153:161		196:231:237			150:170:190
C1907 x M9	mother like	157:175:187					195:219	135:158:170:190
	hybrid I	157:171:175:187					195:219	135:150:158:170:190
	hybrid II	157:171:187					195:219	135:150:190
	autopollinated	157:187					195	158:190
C1907 x sup.1	mother like	157:175:187		156			195:219	
	hybrid I	142:157:175:187		156:193			195:219	
	hybrid II	142:187		156:193			185:219	
	autopollinated	157:175		156			181	
D2118 x M9	mother like			167:191		200:219	170:181:197:201	
	hybrid I			167:179:191		200:219	170:181:197:201:219	
	hybrid II			167:179		200:219:220	170:197:219	
	autopollinated			167		200:219	181:201	
D2118 x sup.1	mother like			167:191			170:181:197:201	
	hybrid I			167:191:193			170:181:197:201:219	
	hybrid II			167:193			181:201:219	
	autopollinated			167:191			181:197:201	
D2212 x M 9	mother like	138:155:157:175	133:140:150:153		196:202:212:234			194:200:220
	hybrid I	138:155:157:171:175	133:140:146:150:153		196:202:210:212:234			150:194:200:220
	hybrid I	138:142:155:157:175	133:140:150:153:171		196:202:212:231:234			194:200:220
H0909 x P22	mother like		133:140:146:153		196:210:213	194:200:219	181:195:197:201	
	hybrid I		133:140:146:153:172		196:210:213:220	192:194:200:219	181:195:197:201:220	
	hybrid II		140:146:172		210:213:220	192: 194:219	197:227	
H0801 x M9	mother like	142:157:170:175		167:178			181:197:201:219	
	hybrid I	142:157:170:175		167:178:191			181:197:201:219	
	hybrid II	142:157:170		167:191			181:195:197	
H0909 x M 9	mother like	149:171:179	133:140:146:153		196:210:213			137:150:166:190
	hybrid I	149:171:179	133:140:146:153:171		196:210:213:231			137:150:166:190
	hybrid II	149:171	140:146:171		196:210:231			150:166:190
	autopollinated	149:179	140:146:153		196:231			166:190

Cross- combination	Progeny class	SSR LOCUS					
		GD12	GD 96	GD 147	GD 162	04h11	02b1
M. sie. x M 9	mother like		147:149:153				239:253
	hybrid I		147:149:153:169				222:239:253
	hybrid II		149:153:169				229:239
M. sie. x Gala	mother like	152:156	147:149:153	117:126:138			239:253
	hybrid I	152:156:188	147:149:153:177	117:126:138:149			226:239:253
	hybrid II	145:152:188	149:153:177	117:126:135			237:239:253
4551 x M 9	mother like		149:153				215:239:253
	hybrid I		149:153:161				215:222:239:253
	hybrid I		149:153:169				215:229:239:253
4608 x M 9	mother like		149:153:177				229:239
	hybrid I		149:153:177:169				222:229:239
	hybrid I		149:153:177:169				229:239
C1907 x M9	mother like		149:153:179		201:213:230		221:235
	hybrid I		149:153: 161:179		201:210:213:230		221:229:235
	hybrid II		153:161:179		210:213:230		229:235
	autopollinated		149:179		213:230		235
C1907 x sup.1	mother like	156	149:153:179		201:213:230		
	hybrid I	156:158	149:153:168:179		201:207:213:230		
	hybrid II	156:158	153:168:179		201:207:213		
	autopollinated	156	149:179		201:213		
D2118 x M9	mother like	152	149:153:193	117:126:138:149			
	hybrid I	152:188	149:153:169:193	117:126:138:149			
	hybrid II	152:188	149:161:193	117:138			
	autopollinated	152	149:153:193	117:126			
D2118 x sup.1	mother like		149:153:193		201:213:219:235	210:222:228	215
	hybrid I		149:153:168:193		201:207:213:219:235	205:210:222:228	215:222
	hybrid II		149:153:168		201:207:235	205:222:228	215:229
	autopollinated		149:153		201:213:219	222:228	215
D2212 x M 9	mother like			117:126:138:153			212:239
	hybrid I			117:126:138:153			212:222:239
	hybrid I			117:126:138:151:153			212:222:239
H0909 x P22	mother like		149:153:169				215:229:239:253
	hybrid I		149:153:161:169				215:222:229:239:253
	hybrid II		153:167:169				215:226:229:239
H0801 x M9	mother like		149:153:169	117:126:138			215:222:239:253
	hybrid I		149:153:161	117:126:138:151			215:222:229:239:253
	hybrid II		149:161	117:126:151			215:222:239
H0909 x M 9	mother like		149:153:169				215:229:239:253
	hybrid I		149:153:169				215:229:239:253
	hybrid II		149:169				215:222:239
	autopollinated		149:153				215:229

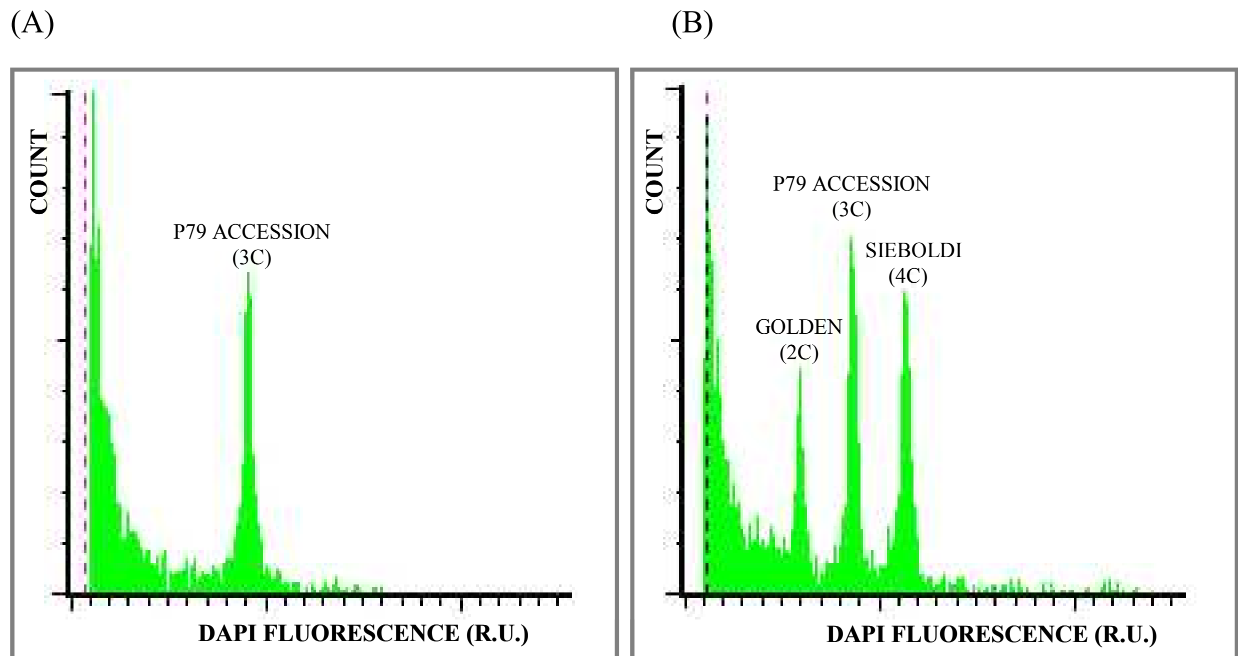
Table 5. Number of seedlings assigned to each progeny class based on allele pattern at selected SSR loci for all cross combinations made in the breeding program.

Progeny n.	Cross combination	Progeny class					
		mother like	hybrid I	hybrid II	autopollinated	open pollinated	Total
1	M. sieboldii x M9 (2002)	53	27	2			82
	M. sieboldii x M9 (2003)	52	12	6	1		71
2	M. sieboldii x M27	8	1				9
3	M. sieboldii x P22	1					1
4	M. sieboldii x Gala	151	7	18	12		188
5	4551 x M9 (2001)	309	225				534
	4551 x M9 (2002)	37	29			14	80
6	4608 x M9 (2001)	180	153			1	334
	4608 x M9 (2002)	40	38				78
7	C1907 x M9	76	34	12	6		128
8	C1907 x supporter 1	49	2	3	5		59
9	D2118 x M9	72	35	22	3		132
10	D2118 x supporter 1	102	1	17	3		123
11	D2212 x M9	50	17				67
12	H0801 x M9	22	1	6			29
13	H0909 x M9 (2002)	108	26	32	11		177
	H0909 x M9 (2003)	77	33	19	14		143
	H0909 x M9 (2004)	265	95	119	120		599
14	H0909 x P22	16	7	2			25
15	M9 x D2212			19	9		28
16	M9 x 4551 (2002)			4	24		28
17	Gala x M. sieboldii			117			117
	Total	1168	743	398	208	15	3032

Table 6. Ploidy level of parental lines and individual progenies as established by flow cytometry analysis. The category to which each seedling was assigned based on SSR markers (hybrid I, hybrid II, mother like, autopollinated) is indicated as well as the cross-combination of origin.

PLANT MATERIAL	PROGENY N.	CROSS COMBINATION	PLOIDY LEVEL (CWF analysis)
Parental lines			
M. sieboldii			4n
4551			3n
4608			3n
C1907			4n
D2218			4n
D2212			4n
M9			2n
G. delicious			2n
H0801			4n
H0909			4n
Seedlings			
hybrid I	1	(M. sieboldii * M9)	5n
hybrid I	5	(4551 * M 9)	4n
hybrid I	6	(4608 * M9)	4n
hybrid I	7	(C1907 * M9)	5n
hybrid I	8	(C1907 * supporter 1)	5n
hybrid I	9	(D2118 * M9)	3n
hybrid II	9	(D2118 * M9)	5n
hybrid II	10	(D2118 * supporter 1)	3n
hybrid I	11	(D2212 * M9)	5n
hybrid II	13	(H0909 * M 9)	3n
hybrid I	13	(H0909 * M 9)	5n
mother like	13	(H0909 * M 9)	4n
autopollinated	13	(H0909 * M 9)	4n
hybrid II	14	(H0909 * P22)	3n
hybrid II	15	(M 9 * D2212)	3n
hybrid II	16	(M9 * 4551)	3n
hybrid II	17	(Gala * M. sieboldii)	3n

Fig 1. Graphic example of FCM analysis. (A) DNA histogram of nuclei isolated from in vitro plants of accession P79. (B) DNA histogram of P79 nuclei mixed with nuclei isolated from facultative apomictic *M. sieboldii* (2n = 4x), and *Golden delicious* (2n), used as reference standards to determine the plant ploidy.



3.3 In vitro screening for resistance towards apple proliferation in *Malus ssp.*

Plant Pathology, submitted

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Abstract
A rapid screening system for apple proliferation resistance was developed which is based on *in vitro* graft-inoculation with the causal agent, *Candidatus* Phytoplasma mali. For this, *in vitro* cultures of the field-resistant apomictic genotypes *M. sieboldii,* H0909, D2212 and the susceptible *Malus domestica* genotypes Golden Delicious and rootstock M9 were established as well as *in vitro* cultures of Rubinette and Golden Delicious infected with *Ca.* P. mali strains PM4 and PM6, respectively. Healthy *in vitro* shoots were inoculated by micrografting with infected shoots used as graft tip. After 1.5 months graft contact no significant differences for graft quality were observed between healthy and infected grafts. Mortality of grafts and transmission rates were not significantly different among the different genotypes. The phytoplasma concentration in inoculated shoots was determined at different time points after inoculation by quantitative real-time PCR. The phenotype of the inoculated plants and the survival were recorded. Infected *M. sieboldii* and D2212 had lower phytoplasma concentration than the susceptible controls and their phenotype was comparable to the healthy control. Their resistance was confirmed *in vitro*. H0909 showed an intermediate behaviour exhibiting lower phytoplasma concentrations with strain PM4 but showing an affected phenotype. The dynamics of phytoplasma concentration reached a maximum at 6-8 months p.i. for all genotypes but the values for 3-5 and 10-12 months p.i. were similar. Thus, an evaluation of resistance *in vitro* can be performed already 3 months p.i. by analysing phytoplasma concentration and phenotype. Interestingly, a significant difference in phytoplasma concentration was observed between strains PM4 and PM6.

Keywords: micrografting, quantitative real-time PCR, *Candidatus* Phytoplasma mali, *Malus sieboldii*

Introduction

Apple proliferation (AP) is one of the most important phytoplasmoses in Europe, spread in several major apple growing areas where it causes considerable economic loss. The disease manifests a range of symptoms that are either specific such as witches' brooms, enlarged stipules, undersized and unmarketable fruits, either non-specific such as foliar reddening, yellowing and growth suppression. AP is associated with *Candidatus* Phytoplasma mali, an unculturable bacterium of the class *Mollicutes* (Seemüller *et al.,* 2002). The phloem-restricted phytoplasma is naturally spread by the psyllids *Cacopsylla picta* and *Cacopsylla melanoneura*

(Frisinghelli *et al.*, 2000; Tedeschi *et al.*, 2002). However, insecticide treatments proved to be either not applicable or insufficient to control the disease. One reason is that the disease spreads also by other means: naturally formed root bridges (Ciccotti *et al.*, 2005) or by the use of infected planting material. As there is no cure of the phytoplasma infection, the most promising approach to face AP appears to be the use of resistant plant material.

All currently grown cultivars and rootstocks are susceptible to the disease. In an extensive screening of *Malus* genotypes natural resistance to AP was discovered only in wild, apomictic *Malus* species, namely in *Malus sieboldii* (Kartte & Seemüller, 1988; 1991; Seemüller *et al.*, 1992). Crossings of these wild *Malus* species with *M. domestica* were carried out in the 1950s and 1970s in order to obtain apomictic rootstocks for apple amenable to seed propagation (Schmidt, 1964; 1988). Although the obtained progeny turned out to be too vigorous for modern apple culture a certain number of genotypes, like selections D2212 and H0909, remained resistant to AP disease. They developed never symptoms or recovered within few years. In these genotypes the pathogen was not or only difficult to detect by fluorescence microscopy, indicating a low phytoplasma titer in the roots. Studies on the colonisation behaviour of the phytoplasmas in apple revealed that they were eliminatied once a year in the aerial parts of the tree during phloem renewal in early spring whereas they persisted in the roots (Schaper & Seemüller, 1984). This data led to the presumption that growing trees on resistant rootstocks can prevent the disease or reduce their impact. Therefore, a new breeding program was started, based on several cross combinations between apomictic resistant accessions and *M. domestica* susceptible parents in order to find the best selection comparable to M 9, containing both features of dwarfing and resistance to AP (Jarausch *et al.*, 2005). The resistance screening of the progeny is currently done by graft inoculation of the fully developed woody plants which are then observed in the nursery for symptom expression over a period of 2-5 years.

Therefore, the objective of the present study was to speed up this resistant screening which is long-lasting, labour-intensive and costly. As an alternative, an *in vitro* resistance screening system was developed. It uses *in vitro* grafting, also known as micrografting, which was initially developed to obtain pathogen-free Citrus (Murashige *et al.*, 1972; Navarro *et al.*, 1975). Since then it has also been employed for apple (Alskieff & Villermur, 1978). The efficient transmission of phytoplasmas by micrografting was first described by Jarausch *et al.* (1999). The method requires the establishment of *in vitro* shoot cultures of the genotype to analyse and the maintenance of *Ca.* P. mali in micropropagated apple. The long-term maintenance of healthy and phytoplasma-diseased apple has been previously reported by Jarausch *et al.* (1996). The determination of the phytoplasma titre in the inoculated material

has been done in the past by semi-quantitative DAPI staining and epifluorescence microscopy (Kartte & Seemüller, 1991; Jarausch *et al.*, 1999). Nowadays, new tools for the quantification of phytoplasmas in plants are available with the development of new techniques of quantitative real-time PCR either based on SYBR Green™ technology (Jarausch *et al.*, 2004) or on TaqMan™ probes (Baric & Dalla Via, 2004). A further objective of the present study was to employ these new methods for the evaluation of the resistance in the *in vitro* system by accurately determining the phytoplasma concentrations in the different genotypes.

Different strains of *Ca.* P. mali exist, e.g. three different subtypes can be distinguished by PCR-RFLP (Jarausch *et al.*, 2000). Recently, a considerable variation in the pathogenicity was observed among different isolates of *Ca.* P. mali (Seemüller & Schneider, 2007). Thus, an other objective of the present study was to enable a rapid resistance screening using different *Ca.* P. mali strains in parallel. For this study, two different strains were selected: a subtype AP-strain which is dominant in Germany (Jarausch *et al.*, 2000; 2004) and a subtype AT2-strain, widely diffused in Trentino (Italy) (Cainelli *et al.*, 2004).

Materials and methods

In vitro culture of healthy and phytoplasma-infected plant material

In vitro cultures were established from three AP-resistant genotypes: *M. sieboldii* and it hybrids H0909 [4556 (Laxton's Superb x *M. sieboldii*) x M9] and D2212 [(Laxton's Superb x *M. sieboldii*) x open pollinated]. As susceptible controls *Malus domestica* cultivars Golden Delicious, Rubinette and M9 were used. Culture establishment was done according to standard procedures (Jarausch *et al.*, 2004) by surface sterilisation of actively growing shoots and culturing of the sterilised nodal explants on modified Murashige & Skoog (MS) medium as described by Jarausch *et al.* (1996). Plantlets developed from sprouted axillary buds were subcultured every 4-6 weeks on MS modified for iron source (Van der Salm *et al.*, 1994) containing 0.25 µM indole-3-butyric-acid, 4.44 µM 6-benzylaminopurine, 0.28 µM gibberellic acid, 88 mM sucrose and 0.7% (w/v) of Microagar (Duchefa). Vitamins were those of MS modified for thiamine at 2.96 µM (Lloyd & McCown, 1981).

Ca. Phytoplasma mali-infected shoot cultures were obtained according to the same protocols. A German AP-subtype isolate was derived from a naturally infected tree of cv. Rubinette and was subsequently named strain PM4. An Italian AT2-subtype isolate was derived from a naturally infected tree of cv. Golden Delicious and was termed PM6. The phytoplasma-infected shoot cultures were subcultured on the same medium as described above.

In vitro graft inoculation experiments

Under aseptic conditions, infected shoot tips were grafted *in vitro* on healthy material of resistant and susceptible genotypes as described by Jarausch *et al.*, 1999. For each combination several independent experiments with at least 10 repetitions were done. After 1.5 months graft contact the success of the graft was recorded. Only strong grafts were considered as successful grafts because only in these grafts a good phloem connection between the different genotypes is ensured. Percentage of mortality in the first 3 months post inoculation (m.p.i) and transmission rate (number of infected plants per number of successful grafts at 3 m.p.i.) were evaluated. Successful strong grafts (SG) were further subcultured and then analysed by PCR and quantitative PCR for *Ca.* P. mali detection and concentration. One half of the rootstock was maintained and the other half was used for PCR analysis. As control, micrografts with healthy material of the same genotype combination were performed to evaluate incompatibility of the graft or hypersensitivity reactions due to phytoplasma infection.

Inoculated shoots were subcultured every month and one half of the material was analysed by qPCR to follow the dynamics of phytoplasma concentration. This analysis was done up to 12 months p.i. At 6 and 12 months p.i. the survival of the inoculated shoots was recorded. After this period the phenotype of the stably infected genotypes was recorded and compared the healthy genotype. For this, a phenotypic index (P.I.) was developed: 0 = phenotype like healthy control, 1 = weak growth reduction, smaller leaves than healthy control, 2 = growth reduction, small leaves, 3 = stunted, proliferation, enlarged stipules, small leaves.

Nucleic acid extraction and phytoplasma detection by PCR

Total DNA was extracted according to Doyle & Doyle (1990) from 0.1 - 0.5 g of *in vitro* plant material. Both tip and rootstock were analysed. Direct PCR was carried out with 100-150 ng total DNA with *Ca.* P. mali-specific primers fAT/rAS (Smart *et al.*, 1996) or AP3/AP4 (Jarausch *et al.*, 1994) as described. Healthy samples and reagent blanks were included in each experiment as negative controls. DNA from infected plant was used as positive control. PCR amplification products (5µl) were analysed by electrophoresis on 1.5% w/v agarose gels. DNA was stained with ethidium bromide and visualized on a UV transiluminator.

Quantitation of *Ca.* P. mali in infected *in vitro* plants

A real-time PCR assay based on the method published by Baric & Dalla Via (2004) was established to quantify the *Ca.* P. mali in the inoculated plants using the automated ABI Prism 7700 apparatus (Applied Biosystem). A multiplex qPCR was performed simultaneously amplifying a fragment of the 16SrRNA gene of *Ca.* P. mali and the *Malus* chloroplast gene coding for tRNA leucine as housekeeping gene. Absolute quantity of phytoplasma DNA was determined by comparison with a standard curve based on serial dilutions of a plasmid containing a fragment of the 16SrRNA gene from *Ca.* P. mali. Primers, probes and conditions of reaction were as described by Baric & Dalla Via (2004). The absolute quantity of host DNA was determined on serial dilutions of plant DNA as described by Baric & Dalla Via (2004). The genome units of *Ca.* P. mali in the plant material were normalised with the quantitity of host DNA and were finally expressed as number of genome unit of phytoplasma per nanogram of plant DNA.

Data analysis

Means of one-way analysis of variance (ANOVA) were performed (Statigraphics Plus 4.1) with the data for mortality, percentage of strong grafts, transmission rate at 3 months p.i., survival at 6 and 12 months p.i., and quantitative real-time PCR data for the five different genotypes used (*M. sieboldii*, D2212, H0909, Golden Delicious, M 9) and subsequently compared using Fisher's least significant difference (LSD) and post hoc multirange test (Statigraphics Plus 4.1). Main factors: genotype phytoplasma concentration, strains (PM4 and PM6) and sampling dates at 3, 4, 5, 6, 7, 8, 10 and 12 months p.i. were subjected to ANOVA multivariate analysis. The differences between means were tested for significance using LDS test with a significance level of probability ($\alpha = 0.05$) followed by multirange test.

Results

Establishment of *in vitro* cultures of healthy and *Ca.* Phytoplasma mali-infected *Malus spp.*

In vitro shoot cultures of AP-resistant genotypes *Malus sieboldii*, H0909 and D2212 as well as of susceptible genotypes Golden Delicious, Rubinette and M9 were established with success following previously published protocols (Jarausch *et al.*, 1994; 1996). *Ca.* P. mali-infected *in vitro* shoot cultures were obtained in the same way: a culture of Golden Delicious infected with *Ca.* P. mali subtype AT2-strain PM6 isolated in Trentino (Italy) and a culture of Rubinette infected with subtype AP-strain PM4 isolated in Palatinate (Germany). Healthy and infected cultures of the different *Malus* genotypes could be propagated efficiently according

to the protocol published by Jarausch *et al.* (1996). Only the standard Murashige & Skoog medium was replaced by a MS medium modified for the iron source (van der Salm *et al.,* 1994). A better growth of the cultures was observed on this modified medium (data not shown). For all genotypes homogeneous cultures could be obtained if subculture intervals of 4-6 weeks were respected.

Establishment of an *in vitro* screening system for apple proliferation resistance in *Malus spp.*

The established *in vitro* screening system was based on the results of Jarausch *et al.* (1999) that *Ca.* P. mali can be efficiently transmitted *in vitro* by micrografting of infected shoot tips on healthy shoots used as rootstock. Preliminary experiments confirmed the published data that the efficiency of phytoplasma transmission does not vary between a graft contact period of one or two months (data not shown). Therefore, a graft contact period of 1.5 months was used in all experiments. As the two different phytoplasma strains which were compared in the study were isolated from two different *M. domestica* cultivars, a first experiment was carried out with healthy grafts in order to evaluate whether the compatibility of the different cultivars was comparable with all five genotypes tested. For this, the quality of the grafts was analysed. Jarausch *et al.* (1999) described that a reliable transmission of phytoplasmas occurred only in grafts which grew well together and, thus, formed a phloem connection. These grafts were difficult to separate after the graft contact period of one month and were therefore called "strong" grafts. The same approach was adopted in the present study and by this means the compatibility of the different genotypes with the two inoculum genotypes was tested. A statistical analysis of 34 independent experiments revealed no significant difference of the percentage of strong grafts for the 10 different combinations graft tip genotype x rootstock genotype.

In a next step the three resistant genotypes *M. sieboldii*, H0909 and D2212 and the two susceptible genotypes *M. domestica* cvs. Golden Delicous and M9 were graft-inoculated with the two *Ca.* P. mali strains PM4 and PM6. The quality of grafts in terms of "strong" grafts was analysed by a multifactor ANOVA for 53 independent experiments of all 10 graft combinations. The percentage of strong grafts was significantly higher ($P = 0.0221$) with strain PM6 (mean of 73% of strong grafts) than with strain PM4 (mean of 60% of strong grafts). However, regarding the percentages of strong grafts of the individual combinations genotype x strain, no significant effect could be observed (Table 1). Thus, the quality of grafts with one strain was comparable for all genotypes tested.

Whereas no mortality of the healthy grafts was observed, a varying mortality of infected grafts was recorded. The relevance of this mortality was analysed by a multifactor ANOVA for the 53 independent experiments carried out for all 10 graft combinations. Although higher percentages of mortality were observed in grafts inoculated with strain PM6 (mean of 24%) compared to PM4 (mean of 16%), these differences were not significant. For both strains the highest mortality rates were observed with cv. M9 and the lowest with cv. GD. Resistant genotypes showed an intermediate behaviour for both strains. As mortality was not correlated to resistance the reasons for the observed mortality were not studied in further detail.

The success of the *in vitro* graft inoculation was recorded in terms of transmission rates. The graft tips were separated from the rootstock after a period of 1.5 months and the inoculated rootstocks were subcultured for another period of 1.5 months in order to allow the establishment of the phytoplasma infection in the shoot. After this period, in total 3 months p.i., the shoots were divided: one half was used for PCR detection and the other half was further subcultured. PCR detection of *Ca.* P. mali was achieved using either specific ribosomal primers fAT/rAS or non-ribosomal specific primers AP3/AP4. The transmission rates were calculated as inoculated, PCR-positive shoots per number of strong grafts. If strong grafts were PCR-negative, the phytoplasma-infection of the graft tip was confirmed by PCR. A comparison of all data of the 53 independent experiments of all 10 graft combinations revealed that a significantly better transmission ($P = 0.0116$) was achieved with strain PM6 (mean of 46%) than with strain PM4 (mean of 25%). The transmission rates 3 months p.i. for the individual combinations genotype x strain are shown in Table 1. With strain PM4 sufficient transmission rates (31 – 51%) were obtained with the susceptible genotypes cvs. Golden Delicious and M9 as well as with the resistant genotype D2212. The transmission rates with the genotypes H0909 (13%) and *M. sieboldii* (8%) were significantly lower. On the contrary, strain PM6 could be readily transmitted to all resistant genotypes (45 – 50%) as well as to cv. Golden Delicious (79%). Only the transmission rates of strain PM6 to cv. M9 were significantly lower (19%).

The survival of the inoculated shoots was recorded after 6 and 12 months. Survival rates were calculated as percentage of surviving shoots per number of inoculated shoots 3 months p.i.. The data shown in Table 1 for the each genotype x strain combination demonstrate a generally good survival of all genotypes inoculated with strain PM6. There are no statistical significances among the genotypes. PM4-infected shoots of cvs. Golden Delicious and M9 and of the resistant genotype D2212 survived comparably well but all PM4-inoculated plants of genotypes H0909 and *M. sieboldii* died within a period of 12

months. However, due to the high standard errors these differences in survival are not significant. The reasons for the loss of the material were multiple and were not studied in further detail.

Evaluation of resistance towards *Ca.* Phytoplasma mali *in vitro*

The natural resistance to AP in the field is characterised by two parameters: a low concentration of phytoplasmas in the plant and the absence of AP-specific symptoms. Both parameters were also studied *in vitro*.

The concentration of *Ca.* Phytoplasma mali was measured by quantitative, real-time PCR. For this, the TaqMan™-assay published by Baric & Dalla Via (2004) was employed. With this multiplex qPCR it was possible to quantify simultaneously the concentration of pathogen DNA compared to the host DNA, overcoming eventual different yields of DNA from the extraction procedure. For data analysis a mean quantification approach was adopted using the mean values of all inoculated plants per genotype expressed in number of copies of phytoplasma per ng of DNA of *Malus spp*.. The phytoplasma concentration was assessed in the shoots 3 months p.i. and then after each further subculture in approximately monthly intervals until 12 months p.i.. The analysis of the data was done only in those shoot culture lines which survived this period. The mean phytoplasma concentration values for several culture lines are shown for the different genotype x strain combinations in Table 2. Three months p.i. the data exhibit a high variation leading to high standard errors. Due to these high standard errors only the higher phytoplasma concentration in the susceptible cv. Golden delicious is significant for both strains. Although not significant, resistant genotypes had remarkable lower phytoplasma concentrations than cv. Golden delicious. The problem of the high standard errors could be reduced when all data obtained between 3 months p.i. and 12 months p.i. for each individual culture line were analysed. In this case PM6-infected shoots of the resistant genotypes had a significantly lower phytoplasma titer than the susceptible genotypes. PM4-infected resistant genotypes maintained a lower phytoplasma concentration over the entire period of 12 months but the standard errors were still too high because of the lower number of samples analysed.

The phenotype of the *in vitro* inoculated shoots was monitored after the 12 months period in established infected cultures. It was compared to synchronously subcultured healthy shoots of each genotype. This study could not be done for genotypes *M. sieboldii* and H0909 infected with strain PM4 because no stably infected shoot culture lines could be maintained. A phenotypic index was developed to evaluate the observations. As shown in Table 3, resistant genotypes showed no or only a slight reaction to the phytoplasma infection. Whereas

the susceptible genotypes were stunted and exhibited typical AP-symptoms like proliferation and enlarged stipules, genotype H0909 showed a reduction in growth and smaller leaves than the healthy control. The growth of infected *M. sieboldii* shoots was only slightly reduced and leaves were slightly smaller. Almost no difference between healthy and infected shoots was seen with genotype D2212.

Influence of the *Ca.* Phytoplasma mali strain and dynamics of the infection

A significant difference among the two phytoplasma strains was detected when all values of the qPCR obtained for both strains were compared (Fig. 1). However, different reactions of the genotypes became evident when the data were analysed for the individual genotypes separately. Strain PM4 multiplied to significantly higher concentrations only in genotypes D2212 ($P = 0.0212$) and cv. Golden delicious ($P = 0.0004$). An invers relationship was found in genotype H0909 where strain PM6 developed to higher concentrations than strain PM4 ($P = 0.0428$). No significant differences in concentration of the two strains was observed in genotypes *M. sieboldii* and cv. M9.

A dynamics of phytoplasma colonisation in the graft-inoculated shoots could be established by measuring the phytoplasma concentration in monthly intervals. Fig. 2a shows the dynamics of PM4-infection in all genotypes where as Fig. 2b presents the data for the PM6-infected culture lines. The phytoplasma concentration reaches a maximum 6-8 months p.i. and decreases afterwards to the level of 3 months p.i.. Whereas these differences in phytoplasma concentratrion are significant for strain PM6 the same dynamics can be seen for strain PM4 as a trend.

Discussion

Natural resistance towards apple proliferation was discovered in so-called apomictic rootstocks and was classified as reduced phytoplasma titer in the roots and as absence of symptoms in the aerial part of the tree (Kartte & Seemüller, 1991; Seemüller *et al.*, 1992). In this regard the results fit to the terminology proposed by Cooper & Jones (1983): the genotypes are resistant to *Ca.* Phytoplasma mali and are tolerant to the disease. On the contrary, *M. domestica* genotypes were found to be susceptible to the phytoplasma and sensitive to the disease as they enabled high titers of the pathogen and exhibited severe symptoms of the disease. However, a wide range of plant responses were observed with different apomictic rootstock selections indicating varying degrees of resistance in this material. New data (Jarausch *et al.*, 2005; Seemüller, unpublished data) revealed that AP resistance is only found in *M. sieboldii* and its hybrids. Furthermore, the evaluation of AP

resistance *in vivo* needed several years of observation in the field after experimental graft-inoculation. Therefore, the first objective of the present study was to investigate whether the resistant phenotype could be reproduced in an *in vitro* system. On this basis a more rapid screening system for resistance could be established. As the apomictic rootstocks are not suitable for modern apple culture, a breeding program is currently ongoing aiming to combine AP resistance with the agronomic value of the standard apple rootstock M9 (Jarausch *et al.,* 2005). As individual genotypes of the breeding progeny have to be screened for AP resistance as a single plant *in vivo*, a further objective of the present study was to develop a system which enables to perform repetitions of inoculations and the simultaneous use of different *Ca.* P. mali strains.

A prerequisite for this study was the establishment of *in vitro* cultures of *M. sieboldii*-genotypes. Using standard protocols for the establishment and maintenance of *M. domestica* genotypes (Jarausch *et al.*, 1996) this objective could be achieved. The published culture medium based on MS macro and micro elements was slightly modified by replacing the iron source Fe-EDTA with Fe-EDDHA as described by Van der Salm (1994). This improved the quality of the microshoots and, thus, well growing, homogenous shoot cultures of the resistant reference genotypes *M. sieboldii*, D2212 and H0909 were obtained. These genotypes were selected for the following reasons: *M. sieboldii* is thought to be the donor of resistance, D2212 is considered as highly resistant and H0909 is already a cross with the standard apple rootstock M9.

As inoculum, *Ca.* P. mali strains representing the predominant subtypes in two highly AP-infected apple growing regions in Northern Italy (Trentino) and southwestern Germany were selected. The strains were derived from naturally infected trees showing typical AP-symptoms and were classified according to the PCR-RFLP system of Jarausch *et al.* (2000). Recent data, however, indicate that a high genetic variability and important differences in virulence can be found among different *Ca.* P. mali strains (Seemüller & Schneider, 2007).

For the establishment of the *in vitro* screening system the method of *in vitro* grafting was chosen to inoculate the genotypes to test. Jarausch *et al.* (1999) demonstrated that *Ca.* P. mali can be efficiently transmitted by this method from an infected shoot culture used as graft tip to a healthy shoot culture used as rootstock. In this study only autografts between infected and healthy material of the same genotype *M. domestica* cv. MM106 were used. Attempts to transmit *Ca.* P. mali in heterografts to *Pyrus* or *Prunus* failed because of cellular incompatibility (Jarausch *et al*., 2000b). Therefore, before using this method for the inoculation of *M. sieboldii* genotypes with phytoplasma strains maintained in different *M.*

domestica cultivars the graft compatibilities of the different genotype combinations were first tested by using healthy grafts. According to Jarausch *et al.* (1999) a graft contact period of 1.5 months was used and the quality of the grafts was evaluated as percentage of "strong" grafts after this period. No significant differences in the results were found indicating that the compatibility of the grafts was homogenous for all combinations. By performing the same graft combinations with phytoplasma infected graft tips significant differences were found between the two strains regarding the quality of grafts and the transmission rates. The success of the grafting was reduced with strain PM4 which also multiplied to significantly higher concentrations in the inoculated shoots. It can be assumed that this strain is more virulent and that the cellular connection of phloem tissue at the graft union, which is necessary to allow a migration of the phytoplasma from one genotype to the other, is negatively influenced by the infection with this strain. This influence of the strain might also explain the lower rates of successful grafts and the lower transmission rates obtained in the present study compared to the autografts performed in the reported study. As only the autograft control of cv. Golden delicious reached similar high transmission rates (e.g. 79% with strain PM6) an influence of the genotype cannot be excluded. However, as the influence of the strain on the graft quality was similar for all genotypes and no significant differences among resistant and susceptible genotypes were found, the analysis of the data was not impaired. The method was, thus, suitable for an *in vitro* screening of AP resistance.

The analysis of the data showed that the resistance trait could be analysed *in vitro* in a similar way than *in vivo*. The concentration of the phytoplasmas in the resistant genotypes was always lower than in the susceptible control and the phenotype was clearly different. As already demonstrated by Jarausch *et al.* (1996) AP-infected sensitive *M. domestica* genotypes are severely affected by the disease *in vitro* and show stunted growth with proliferation of shoots, small leaves and enlarged stipules. The same symptomatology was observed in the present study in PM4- and PM6-infected shoot cultures of the susceptible genotypes cvs. Golden delicious and M9. On the other hand, as *in vivo*, the highly resistant genotype D2212 was almost not affected by the disease *in vitro*. The data obtained for *M. sieboldii* and H0909 infected with strain PM6 indicate a variable degree of resistance in these genotypes. Whereas *M. sieboldii* was only slightly affected and could still be classified as resistant, the genotype H0909 showed an intermediate behaviour. This evaluation was supported by the higher concentration of phytoplasmas measured in this genotype. Although the statistical analysis of the phytoplasma concentrations in the inoculated genotypes was hampered by high standard errors, quantitative PCR is judged as a valuable tool to evaluate the resistance trait in the *in vitro* inoculated shoots. As the qPCR values were consistent for each sample analysed, the

observed variation has to be attributed to an inhomogenous repartition of the phytoplasma in the shoots as only a part of each shoot was analysed after each subculture. For susceptible *M. domestica* it could be shown by DAPI staining and epifluorescence microscopy that the phytoplasma concentration increases by the age of the shoot (Jarausch *et al.*, 1996). Comparable histological investigations of *in vitro* inoculated resistant genotypes are necessary to verify this hypothesis. At present, a substantial number of qPCR data for a given genotype are necessary for a reliable evaluation of the resistance trait *in vitro*.

An increased mortality was observed during field evaluation of AP resistance in apomictic rootstock genotypes (Kartte & Seemüller, 1991; Seemüller *et al.*, 1992). Whereas no mortality occurred for healthy micrografts a varying percentage of mortality was found with phytoplasma-infected grafts. The susceptible cultivar M9 exhibited the highest mortality rates but cultivar Golden Delicious showed almost no mortality. Thus, it is not clear whether this mortality is a reaction to a high inoculum pressure as it can be observed *in vivo*. Further studies are needed to elucidate the causes of the mortality *in vitro*, especially for the resistant genotypes which showed an intermediate behaviour.

Considerable variation in the pathogenicity was observed among different isolates of *Ca.* P. mali studied after experimental inoculation in the field for several years (Seemüller & Schneider, 2007). Different virulence was also evidenced between the two *Ca.* P. mali strains in the *in vitro* system. The multiplication efficiency of strain PM4 was much higher especially in the susceptible cultivar Golden delicious. Thus, the *in vitro* system is also suitable to evaluate the virulence of different *Ca.* P. mali strains and to study their host-pathogen interactions. These interactions became also evident during analysis of the dynamics of phytoplasma infection after *in vitro* graft inoculation. For both strains an increase of the phytoplasma concentration was measured for the first 6-8 months p.i.. The general differences in phytoplasma concentration between resistant and susceptible genotypes were kept stable and followed the same dynamics. These data can be interpreted by an adaptation of the phytoplasma population to the new host. After this period the plant defense reaction became active and the phytoplasma concentration decreased. After 10-12 months a steady-state level was reached which is similar to the phytoplasma concentration after 3 months p.i.. An evaluation of the phytoplasma concentration in a genotype to test can therefore be done already after 3 months p.i..

The results obtained in this study indicate that the resistance to *Ca.* P. mali is based on a specific host-pathogen interaction which is also expressed in the *in vitro* system. It is not based on solely physical properties which might distinguish fully developed woody plants of

resistant and susceptible genotypes. The *in vitro* system can therefore be used for the screening of resistance towards AP. Due to the high inoculum pressure applied in the *in vitro* system only a high level of resistance - as expressed by the genotype D2212 – can be detected with statistical significance. However, an advantage of the system is its usefulness to evaluate a genotype with a substantial number of repetitions and with different phytoplasma strains simultaneously. This avoids the risk to screen for a resistance which might be only strain-specific. The results indicate that the resistance of D2212 is directed against both strains studied. The results obtained for genotype H0909 can be interpreted as a strain-specific resistance directed more against strain PM4 than against PM6. Provided that qPCR data can be obtained without high variation an evaluation of the resistance *in vitro* can already be done 3 months p.i.. The developed system is particularly useful to screen the progeny of a breeding program. In this case each single genotype can be screened in a short time and the established *in vitro* cultures can be used to multiply the genotype for further agronomic evaluation studies.

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Table 1. Strong grafts, transmission rate and survival rate of resistant genotypes *M. sieboldii*, D2212, H0909 in comparison to susceptible genotypes cvs. Golden delicious and M9 after *in vitro* graft-inoculation with *Ca.* Phytoplasma mali strains PM4 and PM6.

genotype	strain	nb. of indipendent experiments	total nb. of successful grafts	total nb.of strong grafts 1.5 m.p.i.[a]	strong grafts % ±S.E.	total nb.of PCR+ plants 3 m.p.i.	transmission rate % ±S.E. 3 m.p.i.	nb. of independent experiments with transmission	total nb.of PCR+ plants 6 m.p.i.	survival rate % ±S.E. 6 m.p.i.	total nb.of PCR+ plants 12 m.p.i.	survival rate % ±S.E. 12 m.p.i.
M. sieboldii	PM4	8	125	56	54.7±8.0 a [b]	6	7.6±7.4 c [b]	3	3	55.7±24.9 a [b]	0	0.0±24.1 a [b]
D2212	PM4	6	88	46	55.6±9.3 a	16	31.4±8.5 ab	5	12	71.4±19.3 a	9	45.2±18.6 a
H0909	PM4	7	102	50	55.0±8.6 a	4	13.2±7.9 bc	4	1	25.0±21.6 a	0	0.0±24.1 a
GD	PM4	5	37	32	79.6±10.2 a	15	38.9±9.34 a	3	9	91.6±24.9 a	6	66.7±24.1 a
M 9	PM4	4	47	28	53.5±11.4 a	16	50.9±10.4 a	4	10	54.9±21.6 a	9	41.7±20.9 a
M. sieboldii	PM6	3	58	35	66.9±10.9 a	17	45.0±17.8 ab	3	17	100±19.6 a	12	81.0±22.9a
D2212	PM6	6	75	54	71.9±7.7 a	26	50.4±12.6 ab	6	21	83.3±13.8 a	16	48.2±16.2 a
H0909	PM6	6	97	74	74.6±7.7 a	45	46.8±12.6 ab	5	30	68.2±15.2 a	22	38.0±17.7 a
GD	PM6	3	33	27	82.5±10.9 a	20	79.2±17.8 a	3	15	72.7±19.6 a	13	66.0±22.9 a
M 9	PM6	5	57	40	66.7±8.4 a	7	18.6±13.8 b	3	3	46.7±19.6 a	3	46.7±22.9 a
TOTAL		53						39				

[a] m.p.i.: month post inoculation

[b] Data are presented in each column for combined analysis of different replicated experiments. Means within a column followed by the same letter are not significantly different (α = 0.05) based on Fisher's protected Least Significant Difference (LSD) and post hoc multirange test. Statistical analysis was made separately when used PM4 and PM6 strains as sources of inoculum.

Table 2. Comparison of *Ca.* Phytoplasma mali concentrations (nb of copies of phytoplasma/ng DNA of apple) of strains PM4 and PM6 in *in vitro* inoculated resistant genotypes *M. sieboldii*, D2212, H0909 in comparison to susceptible genotypes cvs. Golden delicious and M9 at 3 m.p.i. and in established cultures (mean of all monthly data from 3 to 12 months p.i.)

Genotype	3months p.i.				mean of all monthly data from 3 to 12 months p.i.			
	nb. of samples	PM4 strain	nb. of samples	PM6 strain	nb. of samples	PM4 strain	nb. of samples	PM6 strain
M. sieboldii	6	15689±14531 a [a]	5	11289±4905 ab [a]	6	20978±14768 a [b]	38	13682±2060 a [b]
D2212	6	11082±14532 a	6	6955±4477 a	26	26151±6450 a	48	14493±1831 a
H0909	4	7727±17798 a	5	16481±4905 ab	5	14132±15343 a	28	29426±2430 b
GD	6	72714±14532 b	3	24702±6332 b	35	65166±5461 b	31	34801±2270 bc
M 9	7	36564±13454 ab	2	18162±7755 ab	23	43837±7392 a	14	40279±3386 c

[a]Means (deriving from one way ANOVA) within a column followed by the same letter are not significantly different ($\alpha = 0.05$) based on Fisher's protected Least Significant Difference (LSD) and post hoc multirange test.

[b] Means (deriving from multifactor ANOVA) within a column followed by the same letter are not significantly different ($\alpha = 0.05$) based on Fisher's protected Least Significant Difference (LSD) and post hoc multirange test.

Table 3. Phenotype of *Ca.* Phytoplasma mali-infected genotypes compared to the healthy genotype.

genotype	phytoplasma strain	nb. of grafts	Phenotype Index [a]
M. Sieboldii	PM4 (AP subtype)	nt	nt
D2212	PM4 (AP subtype)	10	0
H0909	PM4 (AP subtype)	nt	nt
GD	PM4 (AP subtype)	10	3
M 9	PM4 (AP subtype)	10	3
M. Sieboldii	PM6 (AT2 subtype)	10	1
D2212	PM6 (AT2 subtype)	10	0
H0909	PM6 (AT2 subtype)	10	2
GD	PM6 (AT2 subtype)	10	3
M 9	PM6 (AT2 subtype)	10	2,5

[a] phenotype index: 0 = like healthy control; 1 = weak growth reduction, smaller leaves than healthy control, 2 = growth reduction, small leaves; 3 = stunted, proliferation, enlarged stipules, small leaves; nt = not tested.

Fig 1. Comparison of *Ca.* Phytoplasma mali concentrations (nb of copies of phytoplasma/ng DNA of apple) of strains PM4 and PM6 in all *in vitro* inoculated shoots at 8 time points in an ANOVA multifactor analysis

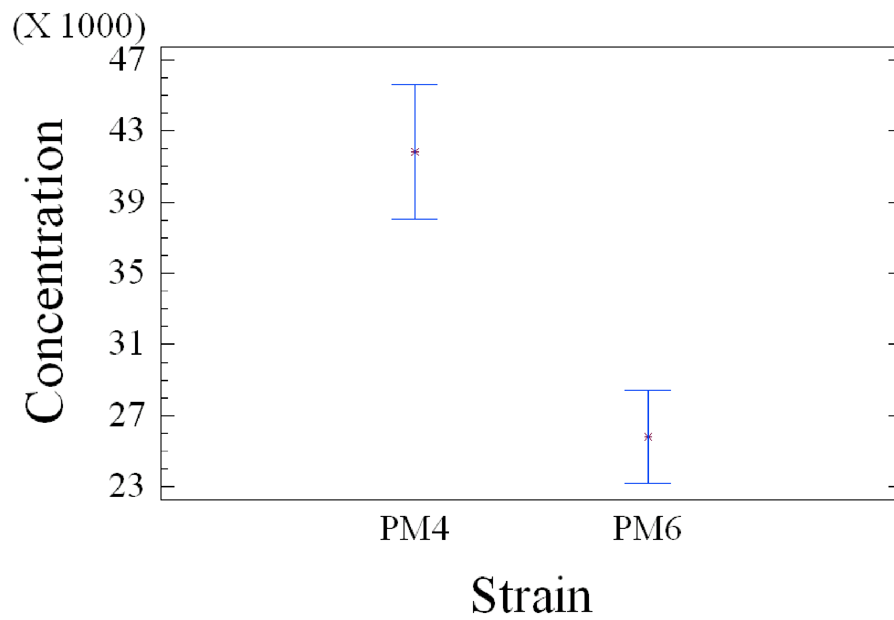


Fig 2a. Dynamics of *Ca.* Phytoplasma mali strain PM4 infection in all *in vitro* inoculated shoots at 8 time points as expressed as means of phytoplasma concentration (nb of copies of phytoplasma/ng DNA of apple) in an ANOVA multifactor analysis

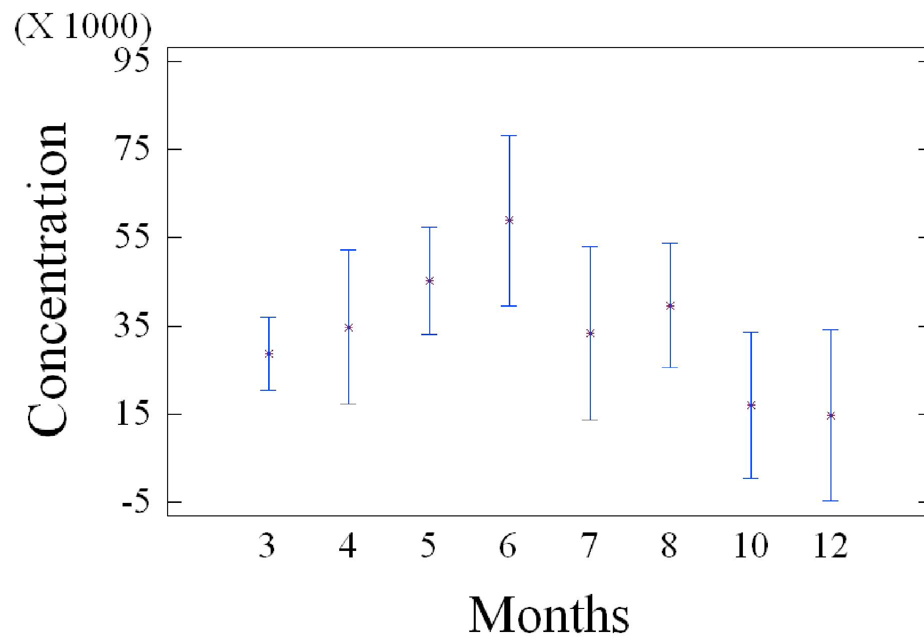
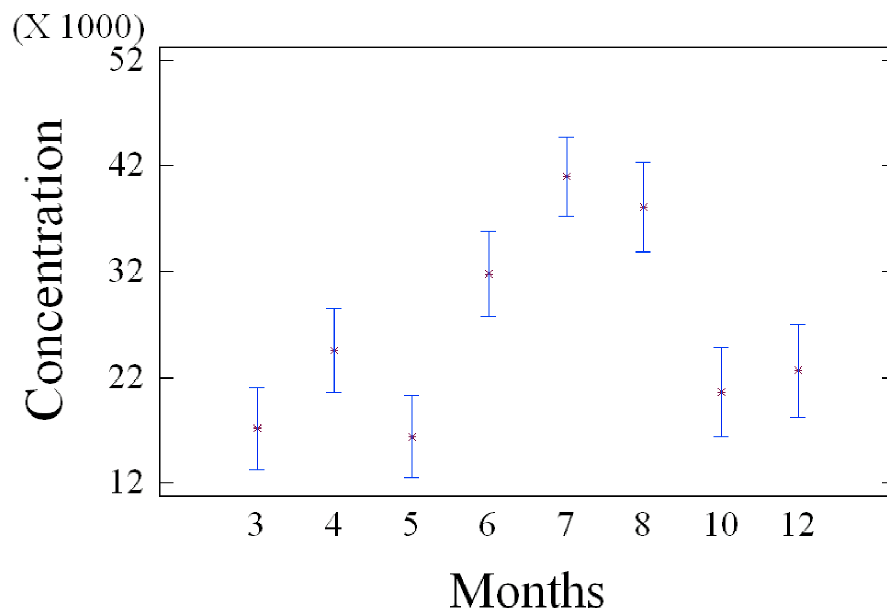


Fig 2b. Dynamics of *Ca.* Phytoplasma mali strain PM6 infection in all *in vitro* inoculated shoots at 8 time points as expressed as means of phytoplasma concentration (nb of copies of phytoplasma/ng DNA of apple) in an ANOVA multifactor analysis



3.4 Micropropagation of apple proliferation-resistant apomictic *Malus sieboldii* genotypes

Agronomy Research submitted

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Abstract. Apple proliferation (AP) is a serious disease of apple in Europe. Natural resistance was found in apomictic *Malus sieboldii*-derived genotypes which can be used as rootstocks and whose agronomic value is actually improved in ongoing breeding programs. As these genotypes are difficult to propagate by standard procedures micropropagation was established and validated in this study to multiply the material in larger scale. A propagation protocol was developed for *in vitro* establishment, multiplication and rooting of eleven interesting AP-resistant genotypes. For the optimisation of the multiplication medium, four different macro and micro element formulations were tested: MS, QL, WPM and DKW. Phytohormones (0.25 µM IBA, 4.44 µM BAP and 0.28 µM GA_3) and vitamins (MS modified for thiamine at 2.96 µM), established for the propagation of *M. domestica*, were also suitable for the propagation of *M. sieboldii*-genotypes. The MS medium yielded in general the highest proliferation rates and the best shoot growth. The significant better growth with the MS medium was also favoured by replacing Fe-EDTA by Fe-EDDHA as iron source. By comparing four different rooting treatments a significantly higher percentage of rooting was observed when the induction was carried out in the dark with 25 µM IBA either in liquid or agarised medium. The time required for root formation on hormone-free medium varied among the different genotypes and three classes of low, medium and high rooting efficiency could be defined. The acclimatisation method adopted for the *ex vitro* plants in the greenhouse yielded survival rates between 90-100% for most of the genotypes.

Key words: apple rootstocks, culture initiation, *Candidatus* Phytoplasma mali, *in vitro* rooting, plant tissue culture, culture media

Abbreviations: BAP - 6-benzylaminopurine; DKW - Driver and Kuniyuki (1984); GA_3 - gibberellic acid; IBA - indole-3-butyric-acid; IAA – 3-indoleacetic-acid; MS - Murashige & Skoog (1962); QL - Quorin & Lepoivre (1977); WPM - woody plant medium (Lloyd & McCown, 1981); EDTA - ethylendiaminetetracetic acid; EDDHA – ethylenediamine-di-(o-hydroxyphenyl)-acetic acid.

INTRODUCTION

Apple proliferation (AP) caused by *Candidatus* Phytoplasma mali is a serious disease in Central and Southern Europe. The disease threatens the apple production in Trentino (Northern Italy) and in South-western Germany. As no curative treatments exist, preventive measures are the only means to control this phytoplasma disease. One way is to apply insecticide treatments against the insect vector(s) (Frisinghelli et al., 2000; Tedeschi et al., 2002). Another strategy is to use resistant plant material. All commercial apple rootstocks – as well as all apple cultivars - are susceptible to the disease. However, a great variability on response towards AP was observed in several wild and ornamental *Malus* species and hybrids when experimentally inoculated with *Candidatus* Phytoplasma mali (Kartte & Seemüller, 1988; Kartte & Seemüller, 1991). In several apomictic apple rootstock selections derived from crosses between *M. domestica* and the apomictic species *M. sieboldii* and *M. sargentii* (Schmidt, 1964; Schmidt, 1988) natural resistance towards AP was observed. Therefore, a promising way to control AP disease is the use of resistant rootstocks (Seemüller et al., 1992). The resistance strategy is based on the fact that the phytoplasmas are eliminated from the aerial part of the tree, the cultivar, once a year during phloem inactivation and renewal in winter/early spring

(Seemüller et al., 1984) and that re-colonisation from the roots in spring and summer is impaired by the resistance of the rootstock genotype.

Several breeding generations exist for these apomictic rootstocks (Jarausch et al., 2007). As apomixis in these genotypes is not complete, seed propagation leads not to uniform material as originally thought (Schmidt, 1988). Therefore, vegetative propagation is the only means to produce homogenous rootstock material. However, these genotypes are difficult to root by classical methods (Magnago, pers. comm.). Therefore, micropropagation is the method of choice to produce uniform planting material in a commercial scale. Tissue culture methods have been successfully applied for the propagation of *Malus* sp. (Lane, 1992). However, it has been reported that different cultivars and rootstocks do not respond in the same way during micropropagation and *in vitro* rooting (Zimmerman & Fordham, 1985; Webster & Jones, 1989; Webster & Jones, 1991). Micropropagation of apomictic *Malus* genotypes has been attempted only once (Miller et al., 1988). In this study almost exclusively *M. sargentii* hybrids have been used. Many of them were recalcitrant and difficult to micropropagate.

The objective of the present study was to develop an efficient micropropagation protocol for *in vitro* establishment, multiplication and rooting of the most interesting AP-resistant apomictic genotypes derived from crosses with *M. sieboldii*, the presumed donor of the resistance. The aim was to quickly obtain enough material for an *in vitro* screening method for AP resistance (Jarausch et al., 1999), field evaluation studies and for commercial use as rootstock.

MATERIALS AND METHODS

Plant material

Eleven AP phytoplasma-resistant apomictic genotypes were used, selected by BBA at Dossenheim (Germany) and cultivated at IASMA (San Michele all'Adige, Italy) and AlPlanta-IPR (Neustadt, Germany) in the screen house. These were *Malus sieboldii* ; 4551 (Laxton's Superb x *M. sieboldii*); 4556 (Laxton's Superb x *M. sieboldii*); D2118 (Laxton's Superb x *M. sieboldii* open pollinated); D2212 (Laxton's Superb x *M. sieboldii* open pollinated); H0801 [4556 (Laxton's Superb x *M. sieboldii*) x M9]; H0901 [4556 (Laxton's Superb x *M. sieboldii*) x M9]; H0909 [4556 (Laxton's Superb x *M. sieboldii*) x M9]; 4608 (*M. purpurea* cv.Eleyi x *M. sieboldii*); Gi477/4 (4608 open pollinated); C1907 (4608 open pollinated). The *Malus domestica* cv. Golden delicious was included in this study as control.

In vitro culture establishment

Actively growing shoots of the year were collected in spring (May) from plants in pots in the greenhouse. For sterilisation two methods were utilised: a) at IASMA segments with one or two buds were rinsed in a stream of tap water for 1 h, dipped in 70 % (v/v) ethanol and surface sterilized for 20 min by manual agitation in 1% sodium hypochlorite containing 0.1% Tween80 (surfactant); they were then rinsed three times in sterile distilled water; b) at AlPlanta-IPR segments with one or two buds were incubated in tap water over night, washed with commercial detergent, incubated for 15 min in 1% sterilium (a commercial disinfectant), surface sterilized for 1-2 min in 70% (v/v) ethanol and 5 – 10 min in 2.5% freshly prepared solution of $Ca(Cl_2O)_2$. Then the segments were washed under sterile conditions two times with 0.25% $CaCl_2$ and three times with sterile water. Sterilised nodal explants were placed in 25x150 mm glass tubes with micropropagation medium based on macro and micro elements of MS (Murashige & Skoog, 1962), vitamins MS, with thiamine at 1.18µM and the following additions: 4.44 µM BAP, 0.28 µM GA_3, 0.25 µM IAA, 57 µM ascorbic acid, 88 mM sucrose and 0.7% (w/v) agar (Ciccotti, 1987) or on the same medium, but with 0.25 µM IBA (Jarausch et al., 1996). Media were adjusted to a pH 5.6–5.7 with 0.1 N KOH and autoclaved at 120°C for 20 min. Cultures were placed in usual growth chamber at 23/18 °C ± 1°C day/night and 16h photoperiod under cool-white fluorescent lights (60 µE m^{-2} s^{-1}). Plantlets developed from sprouted axillary buds were subcultured every 4-6 weeks.

Shoot multiplication

The cultures of the different genotypes were initiated and first cultured on media as described above. After three months, four different macro and micro salt formulations were tested. Shoot tips (2-2.5 cm long) were transferred into test tubes containing ten ml of the following media: MS modified for iron source (Van der Salm et al., 1994), DKW (Driver & Kuniyuki, 1984), WPM (Lloyd & McCown, 1981) and QL (Quorin & Lepoivre, 1977). In all basic media growth regulators and organic compounds were constant: 0.25 µM IBA, 4.44 µM BAP, 0.28 µM GA_3, sucrose (88 mM) and 0.7% (w/v) of Microagar (Duchefa). Vitamins were those of MS modified for thiamine at 2.96 µM (Lloyd & McCown, 1981). Proliferation rate (PR = mean of new axillary shoots produced per microshoot) and mean length of shoots were recorded after 30 days of culture. Explants were also visually evaluated for leaf necrosis, hyperhydricity and chlorosis. Six single shoots for each genotype were cultured in different media and the experiment was repeated twice (12 shoots in total/ genotype/medium).

Rooting and acclimatisation

Four treatments and different auxin concentrations were compared in rooting experiments as follows: a) half strength MS modified salts (Van der Salm et al., 1994), MS vitamins with 2.96 µM thiamine, 58.6 mM sucrose, 0.6% (w/v) agar, and 10 µM IAA (treatment IAA2), pH adjusted to 5.6 before autoclaving; b) the same as a) but with 10 µM IBA (treatment IBA2); c) water solution with 25 µM IBA and 88 mM sucrose, without vitamins, pH adjusted to 6.59-6.60 (treatment IBA5-LIQ); d) water-agar solution (0,6% agar) supplemented with 25 µM IBA and 88 mM sucrose, pH 6.59-6.6 (treatment IBA5-AG). To support *in vitro* shoots, saturated cylindrical Sorbarods plugs (Baumgartner Papiers, Switzerland) have been used in liquid medium. Magenta GA7 jars were used for all treatments. Homogeneous shoots, longer than 2 cm, were excised from proliferating cultures: 10-30 explants per treatment were incubated in darkness at 24 ± 1°C for 7 days (medium IAA2 and medium IBA2) or for 4 days (medium IBA5-LIQ and IBA5-AG). After dark induction, explants were transferred to the light on agarised (0.5%) auxin-free medium with half strength MS salts, without vitamins and with 29.3 mM sucrose. For root development ventilated Microbox ECO2 jars (Micropoli, Italy) were used. The percentage of rooted shoots was recorded at different time intervals: 10, 15, 18 and 25 days. After 25 days mean number and length of primary roots developed per explants were determined. Root number and length were calculated on 5 replicate explants. After rooting, plantlets were removed, rinsed in tap water and transplanted in sterilised potting mixture of peat and agriperlite (15%) in greenhouse benches under closed environments ("woven non woven mini green-house") under natural daylight conditions. Humidity (RH), maintained near saturation by intermittent mist system (Defensor 505), was reduced over a period of 4 weeks by gradually opening the vent. A weak solution of fungicide was sprayed on the plants to prevent fungal contamination. Acclimatized plantlets were then transplanted to plastic square pots and survival in the greenhouse was recorded.

Data analysis

A completely randomised design was applied. Mean number of shoots and roots per microshoot were analysed by a two-factor (genotype and medium) ANOVA procedure. Separation of treatment means was done by Duncan's multiple range test ($P < 0.05$). Proportions of rooted shoots, noted at different days per genotype and medium, were analysed by multifactor ANOVA (Statgraphics 4.1)

RESULTS

***In vitro* culture establishment**

Two methods of sterilizing were equally effective. Contamination rate (data not shown) during *in vitro* establishment was very low (between 1% and 5%) for the genotypes *M. sieboldii*, 4608, 4551, 4556, D2212 and D2118, but higher (between 18% and 20%) for C1907, Gi477/4, H0909 and H0801. Browning exudation occurred only with *M. sieboldii* and H0909. Initial development and growth was very genotype dependent. After three months, shoot development stopped and decreased in the initial culture medium for some genotypes like 4608, H0901 and Gi477/4 (Tab.1).

Shoots multiplication

To optimise micropropagation of these apomictic genotypes four macro and micro salt formulations commonly used in woody plant propagation were tested. Shoot proliferation rate and shoot length for the single genotypes are reported in Table 2. Significant differences were observed for each genotype when the shoot proliferation rate or the mean shoot height was compared for the four different media. Nevertheless, for each genotype a best suited medium could be defined which enabled a good shoot proliferation rate in combination with a sufficient shoot height. For the majority of genotypes modified MS salts were the best propagation medium reaching proliferation rates ranging from 3.3 (*M. sieboldii*) to 5.7 (C1907). However, QL salts led to a significantly better proliferation rate with D2212 (3.3) and H0801 (3.9). For H0909 equivalent growth was observed with QL (4.4) and MS (3.9). Using WPM salts the best proliferation rates were recorded for *M. sieboldii* (2.3), 4551 (3.3) and D2212 (2.8), but the shoots exhibited a stunted growth (1.6 cm and 1.9 cm). This was also observed for WPM medium with 4556 (3.1 and 1.8 cm) and Gi477/4 (2.7 and 1.6 cm). Furthermore, chloroses were seen with genotypes 4551 and 4556 when grown on WPM salts. In genotypes C1907, 4608 and D2118 WPM salts induced the formation of big basal calli. For these reasons WPM medium was judged to be the least suitable medium for the propagation of the apomictic genotypes. The use of DKW medium yielded in some cases good results (4608, D2118) but failed for other genotypes. The *Malus domestica* cv. Golden Delicious used as control showed good multiplication on all media tested. However, the proliferation rate was significantly higher on MS salts (4.8 shoots/explant and 2.4 cm height).

The statistical analysis (ANOVA) of all data revealed that under the same condition for growth regulators and vitamins, the salt composition significantly ($P < 0.0001$) affected shoot production and elongation. MS medium was significantly ($P > 0.05$) better for propagation (3.6 shoots/explant) than QL (3.2), DKW (2.9) and WPM (2.8) media. The mean height of the shoots was best with the media DKW and MS (2.5 and 2.4 cm, respectively). Shoots grown on QL (2.3 cm) or WPM (1.7 cm) had a significantly reduced height. Furthermore, the statistical analysis showed a highly significant effect ($P < 0.0001$) of the factor "genotype" and for the interaction of "genotype" and "medium", both for the proliferation rate and the shoot height (data not shown).

Problems with hyperhydricity were not observed with the different culture media except for the genotype 4608 grown on DKW. The culture of 4608 is therefore best of QL medium which combines a good proliferation (3.5) with a good development of the shoot (height 2.0 cm) without exhibiting hyperhydricity problems.

Rooting and acclimatisation

Different rooting treatments were tested for root induction. After an initial induction phase in the dark shoots were maintained on hormone-free medium. Table 3 shows rooting percentage, number and length of roots 25 days after root induction. For all genotypes the percentage of rooting was significantly higher when the induction was carried out with 25 µM IBA either in liquid or agaraised medium ($P < 0.05$). Only with the genotype D2118 no significant differences in rooting with either treatment were observed. For most of the genotypes the average root length was also higher with the treatment of 25 µM IBA. The absolute length of the roots, however, was not dependent on the treatment but on the genotype ($P < 0.0001$). The longest roots were formed by the cv. Golden Delicious control and by genotype 4556. Independent of the treatment a medium formation of basal callus was observed with the genotypes 4608, Gi477/4 and C1907.

The dynamics of root formation was monitored after cultivation for 10, 15, 18 and 25 days on hormone-free medium after induction with 25 µM IBA in liquid medium (IBA5 LIQ). As shown in Fig 1 a-c the speed of root formation is different for the various genotypes. After 10 days genotypes D2212, D2118, H0901 and H0909 showed already a percentage of rooting of 60% while this value was reached for the genotypes 4551, 4608, C1907, H0801 and *M. sieboldii* only after 15 days. Except for the genotypes C1907 and Gi477/7 the percentage of rooting increased only slightly after the period of 15 days. According to their rooting ability three classes of genotypes could be defined by statistical analysis (Fig. 2): genotypes with low rooting efficiency (rooting % < 60%) were 4551, 4556, Gi477/4 and H0801, genotypes with medium rooting efficiency (rooting % between 60 and 70%) were 4608 and C1907 and genotypes with high rooting efficiency (rooting % > 70% to 100%) were D2212, D2118, H0909, H0901, *M. sieboldii* and cv. Golden Delicious.

Regarding the acclimatisation of the *ex vitro* plants in the greenhouse the adopted method showed a satisfying result for most of the genotypes with survival rates between 90-100%. Only the genotypes 4556, D2118 and D2212 had a reduced survival rate of 75-77%.

DISCUSSION

As a consequence of the recent epidemics of apple proliferation disease apomictic genotypes resistant to *Candidatus* Phytoplasma mali have gained major interest. Therefore, an efficient and commercially sustainable method of propagation had to be developed. For the production of homogenous planting material vegetative propagation is fundamental. However, most of the apomictic genotypes are difficult to root by traditional nursery methods. To overcome this obstacle *in vitro* culture has been employed. Micropropagation of apomictic *Malus* genotypes has been attempted only once in the past and almost exclusively *M. sargentii* hybrids have been used (Miller et al., 1988). In this study attention was given to *M. sieboldii* hybrids which have been shown to exhibit a better AP resistance than *M. sargentii* hybrids (Kartte & Seemüller, 1991). The objective of the present study was therefore to develop an efficient propagation system for these promising rootstock genotypes. In total 11 genotypes were analysed which have a different genetic background. As this work was integrated in a breeding program to increase the agronomic value of these rootstock genotypes (Jarausch et al., 2007) it was important to define the best protocols for culture initiation, propagation and *in vitro* rooting of the different genotypes. The results of this work will also be useful for the propagation of the progeny of the breeding program.

For the success of *in vitro* culture initiation of woody plants it is most important to avoid contamination by bacteria and fungi as well as phenolic oxidation of the tissue. Satisfying results were obtained with the studied genotypes when actively growing shoots were used and explants were soaked in tap water for at least 1h before sterilisation, as suggested by Cresswell & Nitsch (1975) and Vieitez & Vieitez (1980). Furthermore, the use of ascorbic acid helped to reduce oxidation in the establishment stage. The best culture initiation results were obtained with a standard procedure based on disinfection with ethanol and $Ca(Cl_2O)_2$ as previously employed (Jarausch et al., 1994; 1996). As first culture medium a MS derived medium with vitamins and phytophormones as published by Jarausch et al. (1996) was used. Whereas most of the genotypes developed well-growing shoots on this medium some genotypes like 4608, H0901 and Gi477/4 showed in the first months after culture initiation a weak growth which led to a decline of the culture. Because the multiplication rate and growth is the major economic parameter for successful large-scale plant production, further investigations were required to achieve optimal propagation of these genotypes. The classical growth regulators BA, IBA and GA_3 are commonly used in similar concentrations for *in vitro* propagation of apple cultivars and rootstocks (Quorin et al, 1977a; Zimmerman, 1984; Webster and Jones, 1991). Therefore, the phytohormone concentrations successfully employed in previous work (Jarausch et al., 1996) were kept constant. However, the concentration of micro and macro element salts may play an important role in micropropagation of woody plants (Andreu & Marìn, 2005). Therefore, four standard formulations of macro and micro elements were tested to optimise the quality of shoots for large scale micropropagation and *in vitro* rooting. The media differed mainly in nitrogen content: higher in MS (60 mM) and DKW (44 mM), lower in QL (35 mM) and WPM (12 mM).

With WPM medium a reduced shoot height was observed for most of the genotypes which could be due to the low nitrogen content. On the contrary, the MS modification with the highest content of nitrogen yielded for most of the genotypes the highest proliferation rates and the best shoot growth. The MS medium - commonly used for micropropagation of *M. domestica* cultivars - was also suitable for the propagation of *M. sieboldii*. However, the interaction between the different *M. sieboldii* hybrid genotypes and the different media was significantly genotype-dependent which can be explained by the different ploidy levels and the different parentages of the *M. sieboldii* hybrids. Particularly, two out of three genotypes with *M. purpurea* cv. Eleyi as mother parental behaved differently. For the propagation of the genotypes 4608 and Gi477/4 the MS medium was the least efficient whereas the media with lower nitrogen content (QL, DKW) were better suited.

The significant better growth with the MS medium could also be favoured by the modification of the iron source which was not used with the other media. The Fe-EDDHA in the MS Van der Salm modification resulted in a longer and higher availability of Fe to the shoots of *Rosa* and *Prunus* (Van der Salm et al, 1994; Molassiotis et al., 2003). Preliminary experiments to the present study showed that the replacement of Fe-EDTA by Fe-EDDHA in the MS medium used for *M. domestica*-propagation increased the quality of the microshoots also in *Malus* (Jarausch, unpublished data).

Inefficient *in vitro* rooting has usually been the major obstacle when establishing micropropagation protocols for new apple rootstock genotypes and has therefore been extensively studied. IBA was found to be the most suitable auxin for *in vitro* rooting of fruit trees (Welander, 1983; Zimmerman, 1984). Our results show that IBA at 25 µM, both in liquid or agarized medium, is very adequate for root induction on all genotypes including *M. domestica* cv Golden delicious. This is in agreement with results reported by Pua et al. (1983) with Ottawa 3, a rootstock selection difficult to propagate and root. These authors obtained 100% rooting with IBA a 6.25 mg/l, but without dark induction. However, a high concentration of auxins may lead to the formation of callus which is negatively influencing the subsequent acclimatisation phase (Welander, 1983). Prolonged exposure to auxin is not suitable and dark treatment during root induction and transfer of the cutting to auxin-free medium improved rooting percentage (Zimmerman, 1984; Karhu & Zimmerman, 1993). In this work dark treatment was used for different time periods dependent from the auxin concentration in the medium: 4 days with the highest concentration, 7 days with the lowest concentration. No important callus formation was observed with the apomictic genotypes when high concentrations of IBA were used for a short period of induction and the material was then cultured on hormone-free medium.

Thus, rooting percentage obtained in this study was better than results obtained in previous work with other apomictic *Malus* genotypes (Miller et al., 1988) and most of the genotypes rooted at sufficiently high percentages. However, the individual rooting efficiencies were genotype-dependent. Whereas *M. sieboldii* rooted well some of the hybrids, especially those with a contribution of the genome of cv. M9, exhibited the lowest rooting percentages. Furthermore, all hybrids of *M. purpurea* cv. Eleyi showed a slow development of roots with callus formation and the efficiency of rooting was only medium.

In conclusion the obtained results demonstrate that apomictic genotypes can be easily propagated and rooted *in vitro.* Therefore, micropropagation represents an efficient way of multiplication for this interesting material. For each genotype the best suited medium for propagation was defined according to the following criteria: good shoot proliferation combined with good shoot growth, absence of important callus formation, vitrification and chlorosis. Efficient *in vitro* rooting could be achieved for all the genotypes with one protocol: induction of root formation with high auxin concentrations in a 4 days dark phase followed by culture on hormone-free medium.

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Table 1. Percentage of shoots developed 1, 2 and 3 months after culture initiation using two different methods for disinfection

	method A		method B			
Genotype	nb.initial explants	% shoots 1 month	nb.initial explants	% shoots 1 month	% shoots 2 months	% shoots 3 months
M. sieboldii	30	67	17	47	41	47
4551	30	86	51	59	63	63
4556	24	79	17	18	12	41
D 2118	20	60	n.t.	n.t.	n.t.	n.t.
D 2212	30	80	49	47	27	39
H 0801	24	42	29	72	162	252
H 0901	n.t.	n.t.	37	43	35	35
H 0909	32	56	26	50	96	219
4608	11	36	11	9	91	36
Gi 477/4	24	25	18	33	50	33
C1907	24	29	24	25	29	38
Golden del.	30	83	n.t.	n.t.	n.t.	n.t.

n.t. = no tested

Table 2. Multiplication rate and average height of shoots from different apomictic genotypes grown on different media. Data are given as means +/- S.E. Means followed by the same letter are not significantly different (P< 0.05).

Genotype and Parentage	Medium	nb. shoots *				height (cm) *			
Malus sieboldii	MS	3.3	+/-	0,4	a	2.8	+/-	0.1	a
	WPM	2.3	+/-	0.3	ba	1.6	+/-	0.1	b
	DKW	1.8	+/-	0.3	b	1.7	+/-	0.1	b
	QL	1.3	+/-	0.3	b	1.9	+/-	0.2	b
4551	MS	3.7	+/-	0.4	a	3.3	+/-	0.2	a
(Laxton's Superb x M.Sieboldii)	WPM	3.3	+/-	0.4	a	2.3	+/-	0.1	b
	DKW	2.8	+/-	0.3	a	2.9	+/-	0.1	a
	QL	1.6	+/-	0.2	b	2.1	+/-	0.1	b
4556	MS	4.6	+/-	0.4	a	2.4	+/-	0.2	b
(Laxton's Superb x M.Sieboldii)	QL	3.3	+/-	0.2	b	2.7	+/-	0.2	b
	WPM	3.1	+/-	0.3	b	1.8	+/-	0.1	c
	DKW	2.4	+/-	0.1	b	3.3	+/-	0.1	a
D2118	MS	3.5	+/-	0.4	a	2.2	+/-	0.1	ba
(4556 o.p.)	DKW	2.9	+/-	0.3	a	2.0	+/-	0.1	b
	WPM	2.8	+/-	0.3	a	1.5	+/-	0.1	c
	QL	1.8	+/-	0.2	b	2.5	+/-	0.1	a
D2212	QL	3.3	+/-	0.1	a	2.3	+/-	0.2	ba
(Laxton's Superb x M.Sieboldii o.p.)	WPM	2.8	+/-	0.2	ba	1.9	+/-	0.2	bc
	MS	2.3	+/-	0.3	b	2.5	+/-	0.2	a
	DKW	1.6	+/-	0.2	c	1.8	+/-	0.1	c
H0801	QL	3.9	+/-	0.4	a	2.6	+/-	0.3	b
(4556 x M9)	MS	2.7	+/-	0.2	b	2.1	+/-	0.1	b
	WPM	2.7	+/-	0.3	b	2.3	+/-	0.3	b
	DKW	2.2	+/-	0.3	b	3.7	+/-	0.3	a
H0901	MS	4.9	+/-	0.4	a	2.2	+/-	0.1	b
(4556 x M9)	QL	4.6	+/-	0.4	ba	2.6	+/-	0.2	b
	DKW	3.8	+/-	0.3	b	3.3	+/-	0.2	a
	WPM	2.5	+/-	0.3	c	1.5	+/-	0.1	c
H0909	QL	4.4	+/-	0.4	a	2.7	+/-	0.1	a
(4556 x M9)	MS	3.9	+/-	0.2	a	2.9	+/-	0.1	a
	DKW	2.8	+/-	0.2	b	2.3	+/-	0.1	b
	WPM	2.1	+/-	0.3	b	1.5	+/-	0.1	c
4608	DKW	4.3	+/-	0.4	a	2.3	+/-	0.1	a
(M.purpurea cv Eleyi x M.Sieb.)	QL	3.5	+/-	0.3	a	2.0	+/-	0.1	a
	WPM	3.3	+/-	0.3	a	1.6	+/-	0.1	b
	MS	2.2	+/-	0.3	b	2.0	+/-	0.1	a
Gi 477/4	QL	3.4	+/-	0.1	a	1.3	+/-	0.1	c
(4608 o.p.)	DKW	3.0	+/-	0.2	ba	1.9	+/-	0.1	ba
	WPM	2.7	+/-	0.2	b	1.6	+/-	0.1	bc
	MS	1.8	+/-	0.2	c	2.3	+/-	0.2	a
C1907	MS	5.7	+/-	0.3	a	2.0	+/-	0.1	b
(4608 o.p.)	DKW	4.3	+/-	0.4	b	2.7	+/-	0.1	a
	QL	3.8	+/-	0.2	bc	2.1	+/-	0.2	b
	WPM	3.1	+/-	0.1	c	0.8	+/-	0.1	c
Golden delicious	MS	4.8	+/-	0.6	a	2.4	+/-	0.2	ba
	WPM	3.1	+/-	0.3	b	2.0	+/-	0.2	b
	QL	3.1	+/-	0.3	b	2.9	+/-	0.2	a
	DKW	2.5	+/-	0.2	b	2.3	+/-	0.2	b

* n = 12

Table 3. Rooting percentage, number and length of roots of apomictic genotypes produced with different treatments and measured after 25 days. Data are given as means +/- S.E. Means followed by the same letter are not different significantly (P< 0,05)

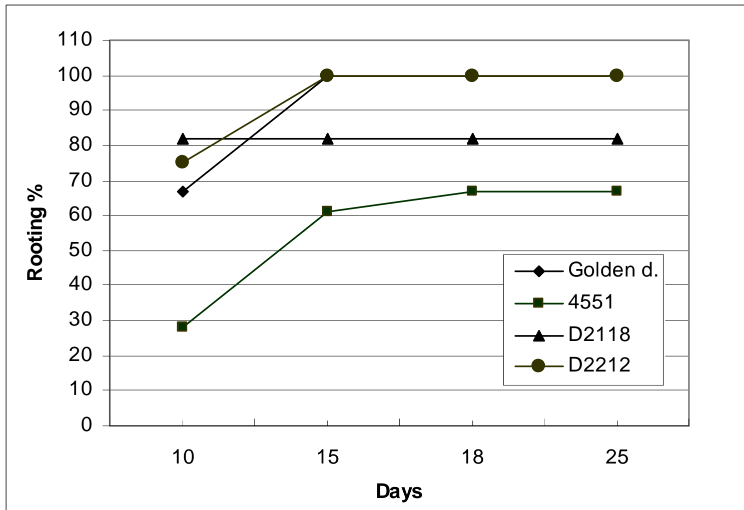
Genotype and Parentage	Rooting method	No. rooted microplants / total expl.	%	No. of roots per microshoot *				Length (cm) of roots *			
Malus sieboldii	IBA5 LIQ	26/26	100	5.8	+/-	0.4	ab	2.0	+/-	0.3	a
	IBA5 AG	18/19	95	6.8	+/-	0.4	a	1.4	+/-	0.1	b
	IBA2	17/20	85	5.4	+/-	0.2	bc	2.2	+/-	0.1	a
	IAA2	15/20	75	4.6	+/-	0.2	c	2.5	+/-	0.0	a
4551	IBA5 LIQ	12/18	67	6.4	+/-	1.3	a	2.9	+/-	0.3	a
(Laxton's Superb x	IBA5 AG	9/19	47	2.8	+/-	0.8	b	2.8	+/-	0.3	a
M. sieboldii)	IBA2	5/20	25	4.6	+/-	0.6	ba	2.6	+/-	0.2	a
	IAA2	6/17	35	2.6	+/-	0.4	b	3.5	+/-	0.8	a
4556	IBA5 LIQ	12/22	55	3.4	+/-	0.5	a	6.6	+/-	0.3	a
(Laxton's Superb x	IBA5 AG	12/35	34	2.8	+/-	0.4	ba	5.5	+/-	0.4	ab
M. sieboldii)	IBA2	5/30	17	2.8	+/-	0.8	ba	4.8	+/-	0.6	bc
	IAA2	6/23	26	1.4	+/-	0.3	b	3.2	+/-	0.5	c
D2118	IBA5 LIQ	9/11	82	7.6	+/-	0.8	a	1.6	+/-	0.3	b
(4556 o.p.)	IBA5 AG	8/10	80	8.4	+/-	2.1	a	1.5	+/-	0.2	b
	IBA2	8/10	80	5.4	+/-	1.5	a	1.9	+/-	0.3	ab
	IAA2	10/20	50	5.8	+/-	0.8	a	2.7	+/-	0.3	a
D2212	IBA5 LIQ	12/12	100	5.6	+/-	0.6	ab	3.3	+/-	0.2	a
(Laxton's Superb x	IBA5 AG	11/12	92	7.0	+/-	0.6	a	3.4	+/-	0.2	a
M. sieboldii o.p.)	IBA2	9/10	90	5.6	+/-	0.7	ab	2.9	+/-	0.1	a
	IAA2	9/10	90	3.2	+/-	0.8	a	4.4	+/-	0.9	a
H0801	IBA5 LIQ	12/20	60	4.4	+/-	0.7	ab	4.3	+/-	0.7	a
(4556 x M9)	IBA5 AG	12/20	60	5.2	+/-	1.1	a	4.0	+/-	0.7	a
	IBA2	7/20	35	2.5	+/-	0.6	bc	2.8	+/-	0.4	a
	IAA2	3/20	15	1.3	+/-	0.3	c	4.3	+/-	0.3	a
H0901	IBA5 LIQ	19/20	95	7.0	+/-	0.6	b	2.6	+/-	0.2	b
(4556 x M9)	IBA5 AG	17/19	89	11.4	+/-	1.2	a	3.8	+/-	0.3	ab
	IBA2	10/15	66	6.6	+/-	1.3	b	5.1	+/-	0.8	a
	IAA2	13/24	54	4.4	+/-	1	b	5.1	+/-	0.7	a
H0909	IBA5 LIQ	18/18	100	6.2	+/-	0.6	ab	3.0	+/-	0.2	a
(4556 x M9)	IBA5 AG	14/20	70	7.2	+/-	0.3	a	2.6	+/-	0.2	a
	IBA2	19/20	95	6.4	+/-	0.7	ab	3.2	+/-	0.2	a
	IAA2	17/20	85	5.4	+/-	0	b	2.6	+/-	0.1	a
4608	IBA5 LIQ	15/20	75	5.6	+/-	0.4	a	1.8	+/-	0.2	b
(M. purpurea cv	IBA5 AG	10/19	53	5.0	+/-	0.6	ba	2.3	+/-	0.1	b
Eleyi x M. sieboldii)	IBA2	10/20	50	3.6	+/-	0.5	b	3.9	+/-	0.3	a
	IAA2	10/20	50	2.0	+/-	0.3	c	3.6	+/-	0.3	a
Gi 477/4	IBA5 LIQ	8/11	73	7.0	+/-	0.4	a	3.4	+/-	0.2	a
(4608 o.p.)	IBA5 AG	5/11	45	4.0	+/-	1.2	b	2.8	+/-	0.3	a
	IBA2	5/24	21	2.0	+/-	0.0	b	1.8	+/-	0.1	b
	IAA2	4/24	17	2.2	+/-	0.2	b	1.4	+/-	0.2	b
C1907	IBA5 LIQ	17/20	85	7.6	+/-	1.2	b	3.9	+/-	0.3	b
(4608 o.p.)	IBA5 AG	20/20	100	10.4	+/-	1.4	a	5.6	+/-	0.2	a
	IBA2	16/20	80	6.6	+/-	0.7	b	2.7	+/-	0.2	c
	IAA2	19/20	95	7.0	+/-	0.6	b	3.0	+/-	0.2	c
Golden delicious	IBA5 LIQ	15/15	100	8.6	+/-	0.9	a	7.0	+/-	0.2	ab
	IBA5 AG	12/15	80	4.8	+/-	0.4	b	6.6	+/-	0.2	ab
	IBA2	16/20	80	5.6	+/-	0.6	b	6.2	+/-	0.2	b
	IAA2	17/20	85	5.2	+/-	0.7	b	7.4	+/-	0.3	a

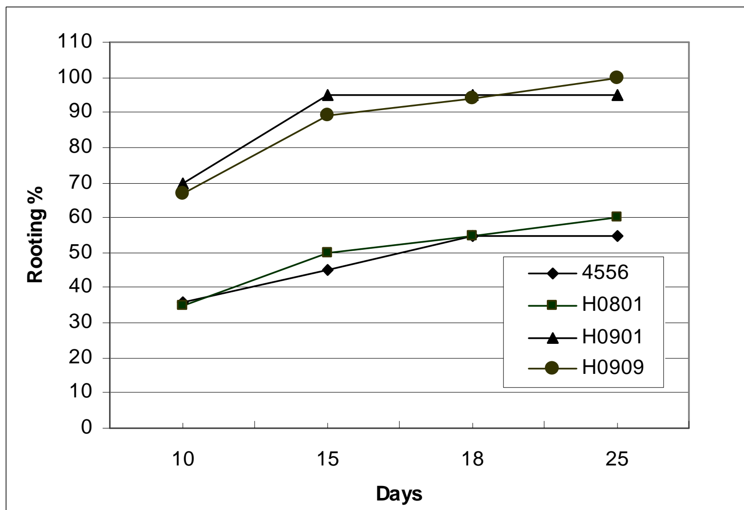
* n = 5

Legends to figures

Fig. 1. - Increase in rooting percentage of different genotypes, as a function of time, after rooting induction on IBA 5 Liq.

Fig. 2. - Results of the multifactor ANOVA: rooting percentage referred to the different genotypes after induction on IBA 5 Liq. medium





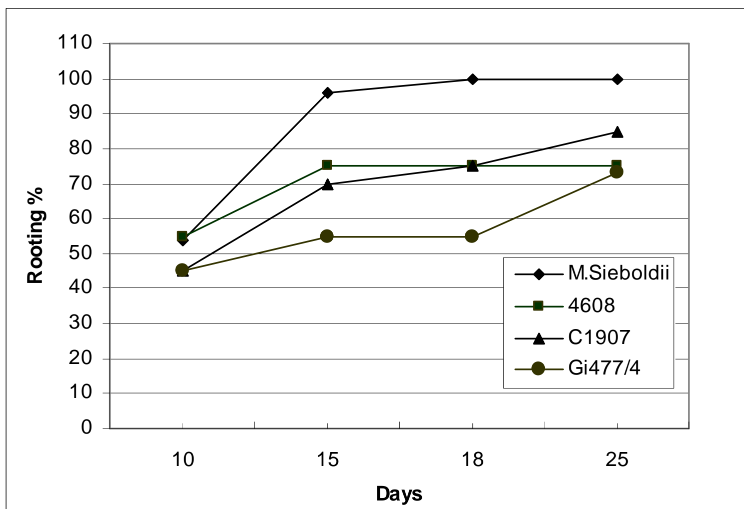


Fig. 1

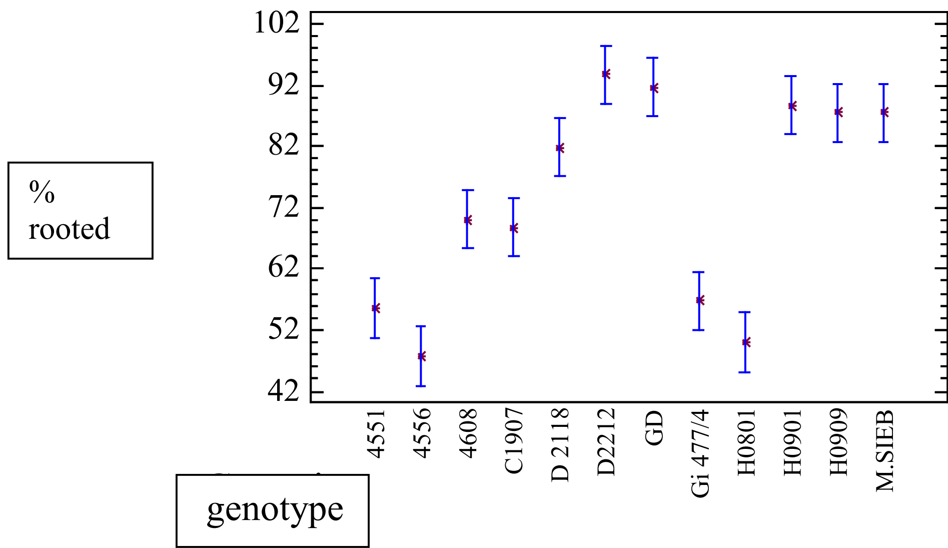


Fig. 2

4. DISCUSSION

Apomictic rootstocks examined in the field after graft inoculation differ significantly in resistance to AP. Progenies of *M. sieboldii* and the resistant *M. sieboldii*-derived selections have a high level of resistance as expressed by low disease rating values, in particular a very rare occurrence of undersized fruits. Results reported in this work largely agree with previous findings obtained following graft inoculation (Kartte and Seemüller, 1991; Seemüller *et al.,* 1992). However, the high susceptibility of progenies of *M. sargentii*-derived apomicts C1828 and D1111 became evident only in this work after longer observation than in previous trials.

Considerable differences in disease severity were observed in all rootstocks. Recent work has shown that phytoplasma strains differ strongly in this respect and that about one third of the strains is either avirulent to weakly virulent, moderately virulent, or severely virulent (Seemüller and Schneider, 2007). Differences in strain virulence are with no doubt also involved in the variability of apomicts. As in the apomicts examined four different combinations of the parental genetic contribution were deduced from SSR profiles, these genetic differences seem also account for variation. The fact that trees on hybrids of type II of selection D2212 show higher susceptibility than trees on motherlike stocks indicate segregation of the resistance trait of a susceptible male parent. Also, the low level of resistance of trees on autopollinated stocks may be due to recombination at resistance loci. These observations are the first molecular data on AP resistance which will be valuable for future studies on the inheritance of AP resistance and for breeding work.

Despite the better resistance of the members of the resistant group in comparison to standard stocks, they are not fully satisfactory from the agronomical point of view. Their major disadvantage is that they are often too vigorous, tend to alternating cropping, and mediate lower yields than M 9. Thus, further selection and/or breeding are required to obtain suitable resistant stocks for commercial apple growing. Such work has to be based on the resistance of *M. sieboldii* and derivatives, the only source of AP resistance confirmed in this study. Therefore, the best donors of resistance were crossed with different genotypes of dwarfing apple rootstocks. In order to find a suitable cross combination which yields a high percentage of recombinant progeny a wide range of different combinations were carried out. According to previous data, the degree of apomixis is varying between the different genotypes and is less complete in 4n genotypes. Thus, predominantly these genotypes (*M. sieboldii* and its F_2 hybrids) were used. As 3n F_1 hybrids 4551 and 4608 are excellent donors of resistance also

crosses were performed with these genotypes. Furthermore, crosses with *M. domestica* cultivars were included in the study as controls for cross compatibility. The results demonstrated that fruit set, seed formation and seed quality differed considerably between the different cross combinations. For some combinations no vital seeds were obtained which might indicate an incompatibility of the genotypes. As expected, fruit set was good if the apomict was used as maternal genotype.

As the phenotype of apomictic and recombinant progeny cannot be distinguished at the early seedling stage, a new strategy was developed in order to screen for recombinant progeny as soon as possible. This strategy was based on genotyping with SSR markers. SSRs are co-dominant DNA markers and for apple a large amount of sequence information for them is available. Preferred SSR markers were highly polymorphic among accessions and frequently heterozygous so that DNA profiles with presence of 4 alleles were observed at almost all loci. As expected, most of the seedlings generated from all cross combinations in which apomictic lines were used as female parent, resulted non-recombinant individuals. They were easily identified thanks to a complete overlapping of genotypic profile with the mother's alleles at all considered SSR loci. Consequently, this breeding material was not further evaluated. SSR DNA marker segregation as well as flow cytometry confirmed the existence of other tree genotypic classes in the progeny, each of which with a distinct ploidy level. The mother like genotypes had the same ploidy level as the female parent, e.g. 3n for selections 4551 and 4608, 4n for all other apomictic genotypes used. Hybrids I had an increased ploidy level with respect to the female parent (4n and 5n, respectively) and resulted from unreduced egg cells plus one parental haplotype. For triploid female parentals only hybrids I were obtained as recombinant progeny indicating that reduced egg cells could not be formed in these genotypes. Hybrid II progeny represent real recombinants deriving from segregating of alleles both parents. A fourth genotypic class was detected by SSR analysis in the progeny of most of the tetraploid female parentals: a recombination of the female genome without contribution of a male genome. These autopollinated genotypes show that a varying degree of self fertility exists in these tetraploid apomicts. This has already been observed by Schmidt (1964) if experiments of artificial selfing were carried out. The highest amount of autopollinated progeny was obtained with genotype H0909 which is a more recent F_2 hybrid of *M. sieboldii.* The highest number of hybrid II progeny was obtained with cross combination H0909 x M9 which was for this reason repeated in several years as only one flowering tree was available for the genotype H0909.

SSR markers proved to be a reliable and valuable tool to rapidly screen the breeding progeny at an early stage. For each cross combination a defined set of markers can now be proposed for future analyses and a large set of seedlings can easily be screened. Furthermore, SSR markers were also reliable in predicting the different ploidy level of the progeny, thus, avoiding in future laborious and time consuming ploidy analyses like chromosome counts or flow cytometry.

The different genotypic classes have different impact on the further analysis of the breeding progeny. Whereas motherlike genotypes were excluded from further evaluation all recombinant progeny is currently being analysed for AP-resistance. This resistance screening is done by graft-inoculation of *Ca.* Phytoplasma mali and subsequent observation of symptom expression for several years in the field. Therefore, these data are not yet available. Resistant genotypes will be further analysed for their agronomic value. The most promising genetic recombination is expected in the class of hybrids II, this is especially the case for the progeny of H0909 in which recombination of *M. sieboldii* and M9 haplotypes occurred. This progeny is currently being exploited for the construction of linkage maps used to locate the genes associated to the resistance trait.

As the evaluation of AP resistance *in vivo* needed several years of observation in the field after experimental graft-inoculation a study was done to investigate whether the resistant phenotype could be reproduced in an *in vitro* system. On this basis a more rapid screening system for resistance could be established. As individual genotypes of the breeding progeny have to be screened for AP resistance as a single plant *in vivo*, a further objective of the study was to develop a system which enables to perform repetitions of inoculations and the simultaneous use of different *Ca.* P. mali strains. A prerequisite for this study was the establishment of *in vitro* cultures of *M. sieboldii*-genotypes. Using standard protocols for the establishment and maintenance of *M. domestica* genotypes (Jarausch *et al.*, 1996) this objective could be achieved. *M. sieboldii*, D2212, H0909 were selected among apomictic for the following reasons: *M. sieboldii* is thought to be the donor of resistance, D2212 is considered as highly resistant and H0909 is already a cross with the standard apple rootstock M9. As inoculum, *Ca.* P. mali strains representing the predominant subtypes in two highly AP-infected apple growing regions in Northern Italy (Trentino) and south-western Germany were selected. The strains were derived from naturally infected trees showing typical AP-

symptoms and were classified according to the PCR-RFLP system of Jarausch *et al.* (2000). Recent data, however, indicate that a high genetic variability and important differences in virulence can be found among different *Ca.* P. mali strains (Seemüller and Schneider, 2007). Micrografting was applied to inoculate the genotypes to test. Jarausch *et al.* (1999) demonstrated that *Ca.* P. mali can be efficiently transmitted by this method from an infected shoot culture used as graft tip to a healthy shoot culture used as rootstock. In this study only autografts between infected and healthy material of the same genotype *M. domestica* cv. MM106 were used. Therefore, before using this method for the inoculation of *M. sieboldii* genotypes with phytoplasma strains maintained in different *M. domestica* cultivars the graft compatibilities of the different genotype combinations were first tested by using healthy grafts. No significant differences in the results were found indicating that the compatibility of the grafts was homogenous for all combinations. By performing the same graft combinations with phytoplasma infected graft tips significant differences were found between the two strains regarding the quality of grafts and the transmission rates. The success of the grafting was reduced with strain PM4 which also multiplied to significantly higher concentrations in the inoculated shoots. It can be assumed that this strain is more virulent and that the cellular connection of phloem tissue at the graft union, which is necessary to allow a migration of the phytoplasma from one genotype to the other, is negatively influenced by the infection with this strain. This influence of the strain might also explain the lower rates of successful grafts and the lower transmission rates obtained in the present study compared to the autografts performed in the reported study. As only the autograft control of cv. Golden delicious reached similar high transmission rates (e.g. 79% with strain PM6) an influence of the genotype cannot be excluded. However, as the influence of the strain on the graft quality was similar for all genotypes and no significant differences among resistant and susceptible genotypes were found, the analysis of the data was not impaired. The method was, thus, suitable for an *in vitro* screening of AP resistance.

The analysis of the data showed that the resistance trait could be analysed *in vitro* in a similar way than *in vivo*. The concentration of the phytoplasmas in the resistant genotypes was always lower than in the susceptible control and the phenotype was clearly different. The low infection rate and the low titre in the stem of trees on resistant apomictic stocks seem to result from low phytoplasma concentrations in the roots. Thus, the low titre in the roots is likely to contribute to the resistance of *M. sieboldii*-derived stocks. It is well established that severe symptoms such as witches' brooms and undersized fruits are only developed when the phytoplasma concentration in the stem is high and they appears only in susceptible ones

(Schaper and Seemüller, 1984). Also in the *in vitro* system stunted growth with proliferation of shoots, small leaves and enlarged stipules were observed in AP-infected sensitive *M. domestica* genotypes. On the other hand, as *in vivo*, the highly resistant genotype D2212 was almost not affected by the disease *in vitro*. The data obtained for *M. sieboldii* and H0909 infected with strain PM6 indicate a variable degree of resistance in these genotypes. Whereas *M. sieboldii* was only slightly affected and could still be classified as resistant, the genotype H0909 showed an intermediate behaviour.

This evaluation was supported by the higher concentration of phytoplasmas measured in this genotype. Although the statistical analysis of the phytoplasma concentrations in the inoculated genotypes was hampered by high standard errors, quantitative PCR is judged as a valuable tool to evaluate the resistance trait in the *in vitro* inoculated shoots. As the qPCR values were consistent for each sample analysed, the observed variation has to be attributed to an inhomogeneous repartition of the phytoplasma in the shoots as only a part of each shoot was analysed after each subculture. Comparable histological investigations of *in vitro* inoculated resistant genotypes are necessary to verify this hypothesis.

Considerable variation in the pathogenicity was observed among different isolates of *Ca.* P. mali studied after experimental inoculation in the field for several years (Seemüller and Schneider, 2007). Different virulence was also evidenced between the two *Ca.* P. mali strains in the *in vitro* system. The multiplication efficiency of strain PM4 was much higher especially in the susceptible cultivar Golden delicious. Thus, the *in vitro* system is also suitable to evaluate the virulence of different *Ca.* P. mali strains and to study their host-pathogen interactions. These interactions became also evident during analysis of the dynamics of phytoplasma infection after *in vitro* graft inoculation. For both strains an increase of the phytoplasma concentration was measured for the first 6-8 months p.i.. The general differences in phytoplasma concentration between resistant and susceptible genotypes were kept stable and followed the same dynamics. These data can be interpreted by an adaptation of the phytoplasma population to the new host. After this period the plant defence reaction became active and the phytoplasma concentration decreased. After 10-12 months a steady-state level was reached which is similar to the phytoplasma concentration after 3 months p.i.. An evaluation of the phytoplasma concentration in a genotype to test can therefore be done already after 3 months p.i..

The results obtained in this study indicate that the resistance to *Ca.* P. mali is based on a specific host-pathogen interaction which is also expressed in the *in vitro* system. It is not

based on solely physical properties such as the formation of pathological callose, sieve tube necrosis, and depletion of starch in the roots of susceptible genotypes (Kartte and Seemüller, 1991). The *in vitro* system can therefore be used for the screening of resistance towards AP. Due to the high inoculum pressure applied in the *in vitro* system only a high level of resistance - as expressed by the genotype D2212 – can be detected with statistical significance. However, an advantage of the system is its usefulness to evaluate a genotype with a substantial number of repetitions and with different phytoplasma strains simultaneously. This avoids the risk to screen for a resistance which might be only strain-specific.

The results indicate that the resistance of D2212 is directed against both strains studied. The results obtained for genotype H0909 can be interpreted as a strain-specific resistance directed more against strain PM4 than against PM6. Provided that qPCR data can be obtained without high variation an evaluation of the resistance *in vitro* can already be done 3 months p.i.. The developed system is particularly useful to screen the progeny of a breeding program. In this case each single genotype can be screened in a short time.

The development of efficient micropropagation and *in vitro* rooting protocols for *M. sieboldii*-genotypes is of particular importance for the further analysis of the progeny of the breeding program. Resistant genotypes can rapidly be multiplied to enable further agronomic evaluation studies as well as to continue resistance screening *in vivo* with different *Ca.* P. mali strains. As apomictic rootstocks are difficult to root by standard nursery procedures, the established micropropagation protocols can also favour the propagation of promising genotypes in a commercial scale.

The strategy proposed to face AP is a long term solution. The resistance in the breeding progeny has to be completed by an agronomic evaluation in the field. Thus, we are still far to obtain a resistant rootstock that could substitute M9 in the field. It is recommended in the meantime to apply short time strategies like the control of the vectors and the uprooting of diseased trees.

Nevertheless the results of thesis allowed a better understanding of the resistance towards AP. A high variation in plant response and in host pathogen interaction became evident in resistant and susceptible genotypes after infection with AP. Comparable results were obtained *in vivo* as well as *in vitro*. AP resistance can therefore be defined as low

concentration of *Ca.* Phytoplasma mali in the infected plant and absence of symptoms. In this respect, apomictic genotypes originating from *M. sargentii*, which also show only low concentration of the phytoplasma in the roots but which express severe symptoms, can no longer be classified as resistant. An important finding is also that the resistance has to be evaluated for different *Ca.* P. mali strains. For the screening of different virulence of the strains and of putative strain-specific resistance the developed *in vitro* system seems to be particular useful.

5. CONCLUSIONS

- The re-evaluation of the AP resistance in apomictic rootstocks in a 12-years field trial under natural infection pressure yielded a better understanding of AP resistance *in vivo.* Phenotypic evaluation and low titre of phytoplasma were confirmed in *M. sieboldii* and some of its derivatives which are, thus, resistant to AP and are candidate donors for resistance in the breeding program. Instead, *M. sargentii* was found to be susceptible in contrast to previous studies.

- A wide range of cross combinations between different donors of resistance (*M. sieboldii* and derivatives) and standard stocks were performed in order to combine AP resistance with reduced vigour. Despite different degrees of apomixis and polyploidy recombinant progeny could be obtained. This progeny is actually screened for AP resistance in the field

- Molecular markers (SSR) were applied to verify if apomictic material used in the 12-year old trial was true-to type as individual trees of the resistant genotypes showed an altered behaviour. Furthermore, all seedlings of the progenies were examined in order to distinguish recombinant from non-recombinant, apomictic progeny. For each genotype or cross combination analysed, suitable polymorphic markers were selected. Flow cytometry analysis as an independent technique confirmed SSR findings.

- As resistance screening of AP is long-lasting *in vivo* and necessitates several years of observation in the field an *in vitro* screening of resistance towards AP was developed.

This method was based on *in vitro* graft-inoculation of the genotype to test and the analysis of the resistance determining the phytoplasma concentration by quantitative real-time PCR and recording the phenotype *in vitro*. AP resistance could also be found in the *in vitro* system. The parameters to evaluate the resistance were the same as in the field: resistant genotypes developed slight or no symptoms and had lower titre of phytoplasmas in comparison with susceptible genotypes. A different virulence of *Ca.* Phytoplasma mali strains could be evidenced *in vitro* and the response of some the genotypes differed with respect to the inoculated strain.

- The *in vitro* resistance screening system has been preceded by the establishment of *in vitro* cultures of all parental genotypes of the breeding program. For each genotype the optimal culture medium was defined in order to obtain homogenous, well growing shoot cultures of each genotype. Efficient micropropagation and *in vitro* rooting protocols were established which enable a large scale propagation of the resistant genotypes in a commercial scale.

The results of the thesis are the basis for further studies in the direction of understanding the genetic inheritance of the resistance trait. A single progeny is still being under evaluation for the construction of linkage maps used to locate the genes associated to the resistance trait.

The developed *in vitro* system is particularly useful to investigate under homogenous and standardised conditions the differential gene expression between infected and healthy state of the material in order to find genes or metabolic pathways involved in resistance.

6. SUMMARY

Apple proliferation (AP) is an economically important disease of apple which occurs in all countries of central and southern Europe. Typical symptoms are witches' brooms and enlarged stipules but the main economic loss is due to undersized and unmarketable fruits. All currently grown cultivars and rootstocks are susceptible to the disease and no curative treatments are applicable. AP is caused by a phytoplasma, *Candidatus* Phytoplasma mali, which is restricted to the phloematic tissue of the plant. *Ca.* P. mali is naturally spread by psyllid vectors and by root bridges as well as by man through infected planting material. An efficient control of the disease is hampered by these different ways of transmission. The objective of the thesis was therefore to evaluate a strategy for a long-term solution to AP based on natural resistance. This resistance has been detected in the wild apomictic species *Malus sieboldii*. First and second generation hybrids of *M. sieboldii* have been obtained in the 1950s and 1970s in order to develop apomicitic rootstocks for apple amenable to seed propagation. Although the obtained progeny turned out to be too vigorous for modern apple culture, a certain number of genotypes remained resistant to AP. While phytoplasmas colonise constantly the roots of infected trees, infections in the susceptible cultivar are eliminated each year during the renewal of the phloem in early spring. A resistance strategy towards AP can therefore be solely based on AP-resistant rootstocks in order to prevent the re-colonisation of the canopy in spring.

The work of this thesis was integrated in a research project on AP between the Istituto Agrario di San Michele (Trentino, Italy), AlPlanta – Institute for Plant Research (Neustadt/Weinstrasse) and Biologische Bundesanstalt, Institut für Pflanzenkrankheiten im Obstbau (Dossenheim). The first part of the thesis was concentrated on the re-evaluation of the AP resistance in *M. sieboldii* and its hybrids in a 12-years field trial under natural infection pressure at BBA Dossenheim. The annual data for symptom recording and fruit size were analysed as cumulative disease index and cumulative undersized fruit index, respectively. By the end of the trial the phytoplasma concentration in roots and shoots was analysed by quantitative real-time PCR. The results confirmed previous data that *M. sieboldii* and its hybrids 4608, D2212, 4551 and D1131 exhibit resistance towards AP. Infected trees of these genotypes had low concentrations of phytoplasmas in the roots and phytoplasmas were found only rarely in low concentrations in the aerial parts. These infected trees showed almost no symptoms and no undersized fruits. Contrary, previously as resistant classified genotypes derived from *M. sargentii* reacted highly susceptible. As individual trees of the resistant

genotypes showed an altered behaviour, molecular analyses were performed to verify if the seed propagated, apomictic material used for the trial was really true-to-type. For this analysis, co-dominant microsatellite (SSR) markers were used which were derived from published work. However, for each *M. sieboldii* genotype suitable, polymorphic markers had to be selected. This analysis revealed that apomixis was not complete and that a varying percentage of progeny of the different genotypes was recombinant due to open pollination. However, no clear relationship was found between resistance at the phenotypic level, phytoplasma titre and the genotypic classification.

As the resistant *M. sieboldii* genotypes were too vigorous for modern apple culture, new breedings were carried out with these genotypes in combination with dwarfing rootstock genotypes such as M9. More than 3.000 seedlings have been produced in 17 cross combinations. All seedlings were examined by microsatellite analysis in order to distinguish recombinant from non-recombinant, apomictic progeny. SSR markers were also useful in determining the ploidy level of the parents and their progenies. These results were confirmed by flow cytometric analysis. The recombinant progeny is currently being evaluated in the field for its AP resistance.

As the screening of resistance towards AP in the field necessitates several years of observation, an alternative *in vitro* method was developed. This method is based on *in vitro* graft-inoculation of the genotype to test and the analysis of the resistance by determining the phytoplasma concentration and recording the phenotype *in vitro*. As a prerequisite *in vitro* cultures of all parental genotypes of the breeding program were established. For each genotype the optimal culture medium was defined. Thus, efficient micropropagation and *in vitro* rooting protocols were established for the *M. sieboldii* genotypes which are difficult to root under normal nursery conditions. The established protocols enable a large scale production of these genotypes on a commercial scale. The *in vitro* resistance screening method allows the evaluation of a given genotype under standardized conditions by using repetitions of micro-grafts. The quality of the grafts, mortality and the transmission rates of the phytoplasmas 3 months p.i. were analysed. The concentration of the phytoplasmas in the inoculated genotypes was determined by qPCR 3 – 12 months p.i.. The results showed that the phytoplasma concentration in resistant genotypes was significantly lower than in susceptible ones. Whereas susceptible genotypes exhibited stunted growth and proliferation symptoms *in vitro* the resistant genotypes had a phenotype almost comparable to the healthy control. On the other hand, the quality of the grafts and the transmission rates were not correlated to the resistance trait. Interestingly, significant differences in phytoplasma

concentration could be found between two different *Ca.* P. mali subtypes used in the experiments. The results demonstrated that the method enables a reliable result 3 months p.i. and can be used to evaluate the virulence of different *Ca.* P. mali strains.

In this work, natural resistance towards AP could be better defined *in vivo* as well as *in vitro*. It was only confirmed in *M. sieboldii* and its hybrids. In breeding for the development of AP resistant rootstocks of agronomic value this resistance could be inheritated. The evaluation of the breeding progeny was optimized by the application of SSR markers and the development of a rapid *in vitro* resistance screening system.

7. ZUSAMMENFASSUNG

Apfeltriebsucht (AT) ist eine ökonomisch bedeutende Krankheit bei Apfel, die in allen Ländern Zentral- und Südeuropas vorkommt. Typische Symptome sind Hexenbesen und vergrößerte Nebenblätter; der wirtschaftliche Schaden entsteht jedoch durch die kleinen, nicht vermarktungsfähigen Früchte. Alle gegenwärtig angebauten Sorten und Unterlagen sind anfällig gegenüber der Krankheit und eine Gesundung ist nicht möglich.

AT wird durch ein Phytoplasma, *Candidatus* Phytoplasma mali, verursacht, welches nur im Phloem der Pflanzen überlebt. *Ca*. Phytoplasma mali wird auf natürliche Weise durch Insektenvektoren aus der Familie Psyllidae und Wurzelverwachsungen, als auch durch den Menschen bei der Verwendung infizierten Pflanzmaterials verbreitet. Durch diese verschiedenen Übertragungswege wird eine effiziente Bekämpfung der Krankheit erschwert. Ziel dieser Arbeit war es daher, eine Strategie für eine langfristige Lösung der AT basierend auf natürlicher Resistenz zu entwickeln.

Resistenz ist in der wilden apomiktischen Art *Malus sieboldii* entdeckt worden. Hybride der ersten und zweiten Generation von *M. sieboldii* wurden in den 1950iger und 1970iger Jahren gezüchtet, um apomiktische, Samen-vermehrbare Apfel-Unterlagen zu erhalten. Obwohl sich die so gewonnene Nachkommenschaft als zu starkwüchsig für den modernen Apfelanbau erwies, blieb eine bestimmte Anzahl an Genotypen resistent gegenüber AT. Während Phytoplasmen die Wurzeln infizierter Bäume konstant besiedeln, werden Infektionen in den anfälligen Sorten jedes Jahr mit der Erneuerung des Phloems im zeitigen Frühjahr eliminiert. Eine Resistenzstrategie gegenüber AT kann daher auf die Entwicklung AT-resistenter Unterlagen beschränkt werden, um eine Wiederbesiedlung der Baumkrone im Frühjahr zu verhindern.

Diese Dissertation war integriert in ein Forschungsprojekt zwischen dem Istituto Agrario di San Michele (Trento, Italien), AlPlanta – Institute for Plant Research (Neustadt, Weinstrasse) und der Biologischen Bundesanstalt, Institut für Pflanzenkrankheiten im Obstbau (Dossenheim). Der erste Teil dieser Doktorarbeit konzentrierte sich auf die Überprüfung der AT-Resistenz in *M. sieboldii* und seiner Hybride im Rahmen eines 12-jährigen Feldversuchs unter natürlichem Infektionsdruck an der BBA Dossenheim. Die jährlichen Daten zu Symtombeobachtung und Fruchtgröße wurden als kumulativer Krankheitsindex bzw. als kumulativer Index der Kleinfrüchtigkeit analysiert. Zu Versuchsende wurde die Phytoplasmakonzentration in Wurzeln und Sproß mithilfe der quantitativen real-time PCR ermittelt. Die Ergebnisse bestätigten frühere Daten, dass *M.*

sieboldii und seine Hybride 4551, 4608, D1131 und D2212 eine Resistenz gegenüber AT aufweisen. Infizierte Bäume dieser Genotypen hatten niedrige Phytoplasmakonzentrationen in den Wurzeln und nur selten wurden geringe Phytoplasmakonzentrationen in den oberirdischen Teilen gefunden. Solcherart infizierte Bäume zeigten kaum Symptome und keine Kleinfrüchtigkeit. Im Gegensatz dazu verhielten sich ursprünglich als resistent klassifizierte Genotypen der Elterngeneration von *M. sargentii* als anfällig. Da einzelne Bäume der resistenten Genotypen ein abweichendes Verhalten zeigten, wurden molekulare Untersuchungen durchgeführt, um zu überprüfen, ob das für dieses Experiment verwendete Samen-vermehrte apomiktische Material wirklich typenecht war. Für diese Analyse wurden aus der Literatur entnommene ko-dominante Mikrosatelliten Marker (SSR) verwendet. Für jeden *M. sieboldii*-Gentoyp mussten jedoch noch passende, polymorphe Marker selektiert werden. Diese Untersuchung ergab, dass die Apomixis nicht vollständig ist und dass ein variabler Prozentsatz der Nachkommenschaft der verschiedenen Genotypen auf Grund offener Bestäubung rekombinant ist. Es konnte jedoch kein klarer Zusammenhang zwischen Resistenz in Bezug auf Phänotyp, Phytoplasmakonzentration und Klassifizierung des Genotyps gefunden werden.

Da die resistenten *M. sieboldii*-Gentoypen zu starkwüchsig für den modernen Apfelanbau waren, wurden neue Kreuzungen dieser Gentoypen in Kombination mit schwachwüchsigen Unterlagen-Genotypen wie M9 durchgeführt. Es wurden mehr als 3000 Sämlinge in 20 Kreuzungskombinationen produziert. Alle Sämlinge wurden mithilfe der Mikrosatellitenanalyse untersucht, um rekombinante von nicht-rekombinanten, apomiktischen Nachkommen zu unterscheiden. SSR Marker erwiesen sich auch als geeignet, um den Ploidiegrad der Eltern und ihrer Nachkommen zu bestimmen. Diese Ergebnisse wurden durch flowzytrometrische Analysen bestätigt. Die rekombinante Nachkommenschaft wird gegenwärtig im Feldversuch bezüglich ihrer AT-Resistenz bewertet.

Da die Beurteilung der Resistenz gegenüber AT im Feldversuch mehrjährige Beobachtungen erfordert, wurde eine alternative *in vitro* Methode entwickelt. Diese Methode basiert auf der *in vitro*-Pfropfinokulation des zu untersuchenden Genotyps und der Untersuchung der Resistenz durch Messung der Phytoplasmakonzentration und Beschreibung des Phänotyps *in vitro*. Als Voraussetzung wurden *in vitro* Kulturen von allen Elterngenotypen des Kreuzungsprogramms etabliert. Für jeden Genotyp wurde das optimale Kulturmedium bestimmt. Auf diese Weise wurden effiziente Protokolle für die Mikropropagation und die *in vitro*-Bewurzelung von solchen *M. sieboldii*-Genotypen erstellt, die unter normalen Baumschulbedingungen schwer zu bewurzeln sind. Die entwickelten

Protokolle ermöglichen eine Massenproduktion dieser Genotypen in wirtschaftlichem Maßstab. Die Verwendung von Wiederholungen der Mikropropfungen bei der *in vitro* screening-Methode erlaubt die Beurteilung eines bestimmten Genotyps unter standardisierten Bedingungen. Bewertet wurden die Qualität der Pfropfungen, die Mortalität und die Übertragungsrate der Phytoplasmen 3 Monate nach Inokulation. Die Phytoplasmakonzentration in den inokulierten Genotypen wurde 3-12 Monate nach der Inokulation mittels qPCR bestimmt. Die Messungen ergaben, dass die Phytoplasmakonzentration in den resistenten Genotypen signifikant niedriger war als in anfälligen. Während anfällige Genotypen *in vitro* Kümmerwuchs und Symptome von Proliferation zeigten, war der Phänotyp der resistenten Genotypen nahezu vergleichbar mit dem der Gesundkontrolle. Die Qualität der Propfung und die Übertragungsraten waren nicht mit dem Resistenzmerkmal korreliert. Interessanterweise wurden zwischen den beiden in diesen Versuchen verwendeten *Ca*. P. mali-Subtypen signifikante Unterschiede in der Phytoplasmakonzentration gefunden. Die Daten zeigten, dass es mit dieser Methode möglich ist, 3 Monate nach der Inokulation zuverlässige Ergebnisse zu erzielen und dass diese Methode dazu geeignet ist, die Virulenz verschiedener *Ca*. P. mali-Stämme zu beurteilen.

Im Rahmen dieser Arbeit ist es gelungen, die natürliche Resistenz gegenüber AT sowohl *in vivo* als auch *in vitro* besser zu beschreiben. Diese wurde nur für *M. sieboldii* und seine Hybride bestätigt. In Kreuzungen zur Entwicklung AT-resistenter Unterlagen konnte diese Resistenz weitervererbt werden. Die Beurteilung der Kreuzungsnachkommenschaft wurde durch die Anwendung von SSR Markern und die Entwicklung eines schnellen *in vitro* Resistenz screening-Verfahrens optimiert.

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Erklärung:
Ich versichere, dass ich die vorliegende Dissertation selbständig und ohne fremde Hilfe angefertigt, nur die angegebenen Hilfsmittel verwendet und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Curriculum vitae

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