In vitro screening of interspecific hybrids (*Malus* spp.) for resistance to apple proliferation

Bisognin, C.[1]; Ciccotti, A.M.[1]; Salvadori, A.[1]; Jarausch, W.[2]; Grando, M.S.[1]
[1] Fondazione Edmund Mach, IASMA Research Centre - Via Mach 1, 38010 San Michele all'Adige (TN), Italy
[2] AlPlanta – Institute for Plant Research, RLP AgroScience, Breitenweg 71, 65435 Neustadt/W., Germany

Abstract

A breeding programme was set up six years ago in Trentino as part of the Project "Scopazzi del Melo - Apple Proliferation" (SMAP) in order to obtain AP resistant apple rootstocks.

Twenty-six hybrids generated from the crossings (*Malus sieboldii*, second generation, x Malus domestica) were micropropagated and studied in standardised conditions. An *in vitro* screening system for AP resistance previously set for the parents of the crosses was adopted and modified. Specific symptoms of the disease, as well as height and basal proliferation of the shoot and size of the leaves, were recorded *in vitro* at 3 months post-inoculation. At the same time, phytoplasma concentration was determined in the whole shoot by quantitative RT-PCR. An *in vitro* disease index taking into account all the above-mentioned parameters was developed.

Each healthy genotype was graft-inoculated in triplicate with two different phytoplasma strains after plant rooting and acclimatisation. Phenotype and phytoplasma titre were evaluated in the roots the year after infection.

Preliminary results indicated that the resistance trait segregates in the progenies. The resistant genotypes had lower phytoplasma concentrations than the susceptible controls, did not show AP-specific symptoms and their growth was not affected by infection. By comparing the resistant behaviour of the same genotypes, the *in vitro* screening allows for a quick selection of genotypes that are worth evaluating in the field for agronomic traits.

Keywords: Apple Proliferation, *Malus sieboldii*, resistance screening, quantitative real-time PCR, disease index.

Introduction

Apple proliferation (AP) is one of the most serious phytoplasmoses in Europe, causing considerable economic loss (Kunze 1989). As there is no cure for phytoplasma infection, the most promising approach to combating AP appears to be the use of resistant plant material. Natural resistance to AP has been discovered only in wild, apomictic *Malus* species, namely *M. sieboldii*. Crosses of these wild *Malus* species with *M. domestica* have been carried out in the past and attempts have been made to exploit this resistance in apple rootstocks (Seemüller et al., 1992). Studies on the colonisation behaviour of phytoplasmas in apple have revealed that they are eliminated once a year in the aerial parts of the tree during phloem renewal in late winter/early spring. Field trials over one year have further demonstrated that the use of resistant rootstocks can prevent the disease and is sufficient to establish field resistance in the whole tree (Bisognin et al., 2008a). Resistance to AP in the field has been classified as reduced phytoplasma titre in the roots and absence of symptoms in the aerial parts of the tree. In the terminology proposed by Cooper & Jones (1983) the tested genotypes were resistant to 'Candidatus *Phytoplasma mali*' and tolerant to the disease. Unfortunately, all the *M. sieboldii* hybrids examined turned out to be too vigorous to be used as rootstocks in modern apple culture.

A breeding programme was set up six years ago in Trentino as part of the Project "Scopazzi del Melo - Apple Proliferation" (SMAP) aimed at developing a rootstock comparable to the standard stock, cv. M9, and having features of both dwarfing and resistance to AP. Several different cross combinations have been made between apomictic-resistant accessions and susceptible *M.* x *domestica* parents in order to find the best selection (Bisognin et al., 2009). Resistance screening of the progeny is currently carried out by graft inoculation of the seedlings which are then observed in the nursery for symptom expression over a period of 2-5 years. As an alternative to this time-consuming and labour-intensive screening, an *in vitro* resistance screening system based on micrografting has been developed (Bisognin et al., 2008b). It allows response to infection in resistant selections to be analysed and compared with infection in susceptible controls under standardised conditions.

The objective of the present study was to find an *in vitro* disease index to apply to the progenies obtained from the breeding programme in order to select the resistant genotypes, thus speeding up the procedure in the field. Twenty-six hybrids were infected *in vitro* by micrografting. Phenotypic data and phytoplasma titre were compared with the resistant parent *M. sieboldii*. Three hybrids were identified as resistant and were reinoculated in controlled conditions to verify resistance to different strains of the pathogen.

Materials and methods

The *in vitro* resistance screening system: The screening system, previously set up in the parents of the crosses (Bisognin et al., 2008b), was adopted to test twenty-six hybrids generated from the crossings *Malus sieboldii*, second generation, x Malus domestica. The system requires the establishment of *in vitro* shoot cultures of the genotype and the maintenance of 'Ca. *P. mali*' in micropropagated apple. Healthy *in vitro* shoot cultures of the progenies were successfully established and propagated in order to test susceptible (Golden Delicious, M9) and resistant genotypes (*M. sieboldii*, D2212, H0909), as well as infected cultures of cv. Golden Delicious (Ciccotti et al., 2008). Hybrids were inoculated by micrografting with infected shoots of cv. Golden Delicious used as graft tips. Micrografting was carried out in 10 repetitions for each genotype. Graft contact was maintained for 1.5 months. Only grafts where a good phloem connection between the two scions was established were considered successful grafts, and these were then subcultured and analysed for the presence of phytoplasma. The twenty-six hybrids were also studied in controlled conditions. *Ex vitro* healthy genotypes were graft-inoculated in triplicate with two different phytoplasma strains after plant rooting and acclimatisation. PM6 was the strain used for the *in vitro* screening, while PM11 is a local 'Ca. *P. mali*' strain found in commercial orchards in Trentino. Phenotype and phytoplasma titre of the roots were evaluated the year after infection.

In vitro phenotype index: Specific symptoms, i.e. enlarged stipules and/or witches' brooms, in the *in vitro* shoots were recorded at three months post-inoculation (m.p.i.). The incidence of these symptoms in all repetitions allowed three classes of varying intensity to be determined (Table 1).

The height of *in vitro* shoots was measured at three m.p.i and at five m.p.i. Each genotype was evaluated by comparing the height of the taller shoot of the healthy vs. the infected *in vitro* plant. Significant differences were found with analysis of variance (ANOVA) and these were subsequently compared using Fisher's least significant difference (LSD). Two intensity classes were thus determined. At the same time, basal shoot proliferation was measured in healthy and infected genotypes maintained under equivalent cultivation conditions. Significant differences were found with analysis of variance (ANOVA) and these were subsequently compared using Fisher's least significant difference (LSD). A phenotype index for each genotype was determined as the sum of the number of intensity classes found for each of the above-mentioned three parameters, as shown in Table 1 and Table 2.

Tab. 1 Specific symptoms considered in determining the *in vitro* phenotype index.

Parameter	Incidence %	Intensity classes
SYMPTOMS		
no symptoms	0 %	0
stipules -	< 50 %	0
stipules	= 50 %	1
stipules +	> 50%	2
witches' brooms	< 50 %	1
witches' brooms +	> 50%	2

Tab. 2 Growing parameters considered in determining the *in vitro* phenotype index.

Parameter	*p* value*	Intensity classes
Height		
infected vs healthy	$p > 0.05$	0
infected vs healthy	$p < 0.05$	1
Basal shoot proliferation		
infected vs healthy	$p > 0.05$	0
infected vs healthy	$p < 0.05$	1

* *p* value determined by analysis of variance (ANOVA).

Concentration index: Total DNA was extracted according to Doyle and Doyle (1990) from 0.1 - 0.5 g of *in vitro* plant material or from *ex vitro* root material. Direct PCR was carried out with specific primers fAT - rAS (Smart et al., 1996) to detect infected plants.

A real-time PCR assay based on the method published by Baric and Dalla Via (2004) was applied at three m.p.i. to quantify 'Ca. *P. mali*' in the inoculated and infected plants using the automated LightCycler® 480 apparatus (Roche). A multiplex qPCR was performed simultaneously by amplifying a fragment of the 16SrRNA gene of 'Ca. *P. mali*' with 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*'. The relative presence of 'Ca. *P. mali*' in the plant material was finally expressed as Genome Unit (GU) of phytoplasma per nanogram of plant DNA. Phytoplasma concentration of *M. sieboldii* was taken

as the resistance reference. The concentration index of each hybrid was obtained by estimating its phytoplasma titre in relation to *Malus sieboldii* phytoplasma concentration.

Results and discussion

As in the field, infected *M.* x *domestica* genotypes were severely affected by the disease *in vitro*,showing stunted growth with proliferation of shoots and enlarged stipules and/or witches' brooms. A phenotype index based on parameters of symptoms, height and basal shoot proliferation was developed in the parents (Table 3) and applied to the hybrids in this study (tab. 4). Leaf area was found to have no correlation with AP susceptibility. The highly resistant parents of the crosses were almost unaffected by the disease both *in vitro* and *in vivo*. In the *in vitro* system two threshold values of resistance were found. The absence or slight presence of symptoms corresponding to a phenotype index ≤ 2 and a concurrent concentration index of ≤ 1.5 in the parents allowed a given genotype to be considered resistant (table 3). In this study eleven out of twenty-six genotypes showed an *in vitro* phenotype index ≤ 2, while eight out of the twenty-six hybrids analysed had a concentration index of ≤ 1.5. Only three genotypes attained both resistance threshold values. Screening in the field confirmed the *in vitro* results (data for one of these are still being processed). As in the field, a clear correlation between a higher concentration of phytoplasma and a presence of witches' brooms was found (Bisognin et al., 2008a).

Tab. 3 *In vitro* evaluation of resistance to AP in parents inoculated with PM6 strain. Phenotype index is the sum of intensity classes of the three parameters considered in tab.1 – 2 for each genotype. Concentration index is the ratio of mean phytoplasma concentration of genotype per ng of host DNA to the mean concentration of strain PM6 per ng of *M.sieboldii* DNA

Genotype	Phenotype index	Concentration index
M. sieboldii	1	1.00
D2212	0	0.60
H0909	2	1.45
Golden delicious	4	2.80
M9	3	1.60

All genotypes were also tested in controlled conditions and their resistant behaviour was confirmed. Evaluation of visual symptoms alone was not enough to classify a genotype. Many hybrids showed only mild symptoms *in vivo* but investigation of the root system showed a wide variation in phytoplasma concentration. Quantification in the roots became essential in order to screen all genotypes displaying an uninfected phenotype after 2 years.

Comparing the behaviour of genotypes in the two systems (*in vitro* and in controlled conditions) showed that the *in vitro* procedure gave an advantage of at least one year in the breeding programme timetable. Susceptible genotypes can be promptly eliminated from the screening and only resistant ones can then be submitted to further assessment, such as agronomic evaluation and resistance to a wide spectrum of pathogens.

Conclusions

A reduced phytoplasma concentration and the absence or slight expression of disease symptoms are necessary and sufficient for assessing resistance to AP in the plants. The results obtained indicate that resistance to 'Candidatus *Phytoplasma mali*' can be observed *in vitro* in a similar way to *in vivo*, thus speeding up the resistance screening procedure. The *in vitro* disease index developed here should help to reduce to one year the time of screening it takes to select susceptible genotypes and carry out experimental trials.

The method is currently employed for testing several individual genotypes of different breeding progenies. Moreover, the *in vitro* system offers the possibility of studying specific host-pathogen interactions in standardised conditions and of simultaneously evaluating the virulence of different strains.

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