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

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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. Significantly better growth with the MS medium was also favoured by replacing Fe-EDTA by Fe-EDDHA as the 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, 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, threatening 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 phytoplasmic 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 in 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 does not lead not 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 on 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 were 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 overnight, 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 the 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 the 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 a 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 an intermittent mist system (Defensor 505), was reduced over a 4-week period 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. The 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. The 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 (Table 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.

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

Genotype	method A		method B			
	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

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 good results in some cases (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 conditions 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 media DKW and MS (2.5 and 2.4 cm, respectively).

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 ($P < 0.05$); * n=12.

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

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 ($P < 0.05$); *n = 5.

Genotype and Parentage	Rooting method	No. rooted microplants	%	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

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 the 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 agarised 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 a 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 varies with genotype. After 10 days genotypes D2212, D2118, H0901 and H0909 already showed a 60% percentage of rooting 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%.

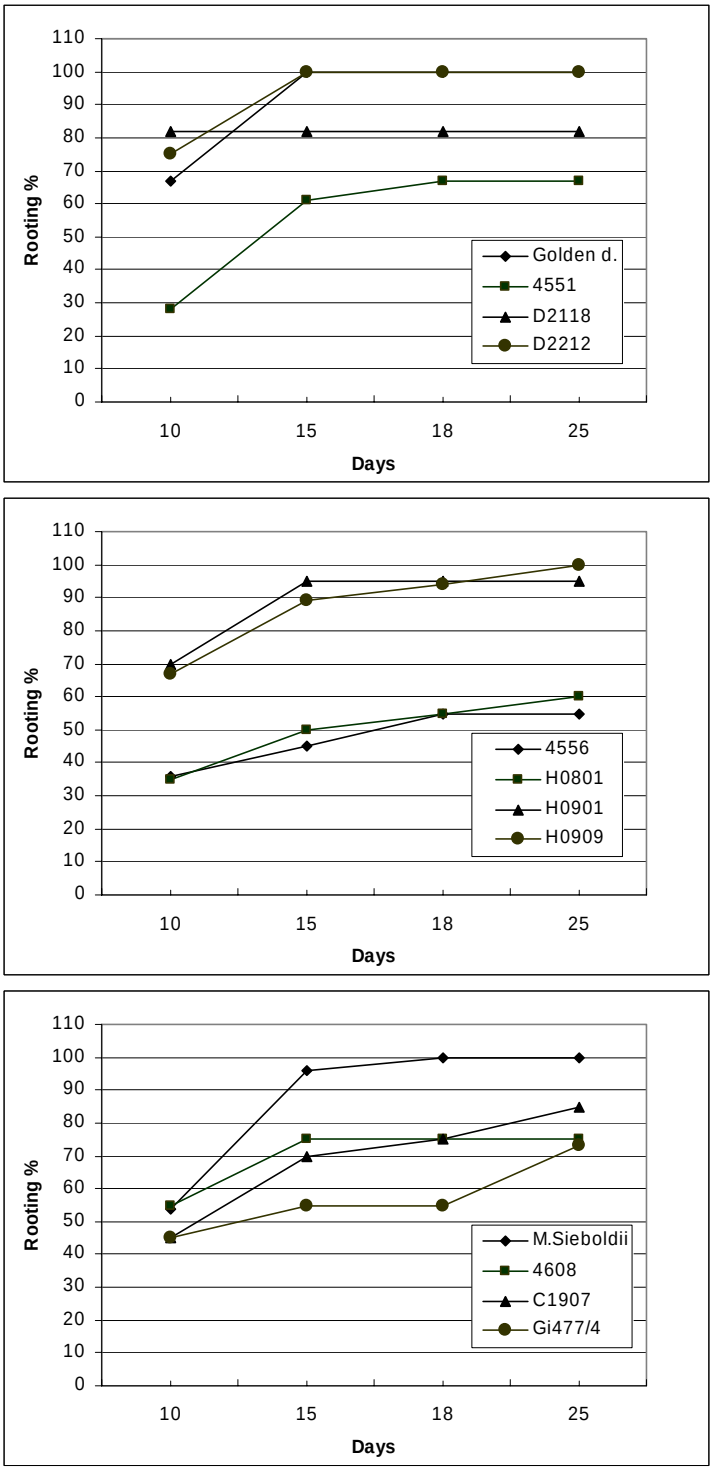

Fig. 1. Increase in rooting percentage of different genotypes, as a function of time, after rooting induction on IBA 5 Liq.

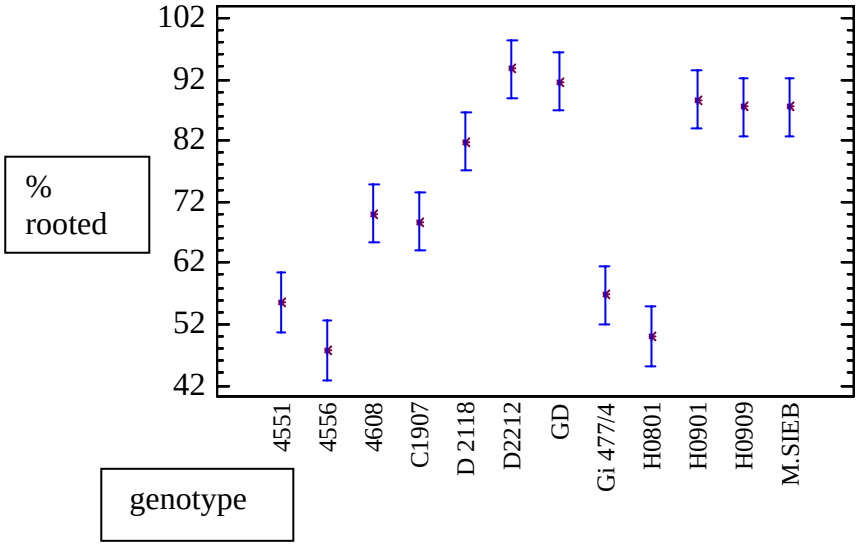

Fig. 2. Results of the multifactor ANOVA: rooting percentage referred to the different genotypes after induction on IBA 5 Liq. Medium.

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, using *M. sargentii* hybrids almost exclusively (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 which have a different genetic background were analysed. 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 1 h 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 the first culture medium an 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 weak growth in the first months after culture initiation, leading 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, possibly due to the low nitrogen content. On the contrary, the MS modification with the highest content of nitrogen yielded the highest proliferation rates and the best shoot growth for most of the genotypes. 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 more effective.

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 has a negative influence on 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 depending on 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.

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-day dark phase followed by culture on hormone-free medium.

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