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Resistance Inducers and Plant Growth Regulators Show only Limited and Transient Effects on Infection Rates, Growth Rates and Symptom Expression of Apple Trees Infected with 'Candidatus Phytoplasma mali'

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Resistance inducers and plant growth regulators show only limited and transient effects on infection rates, growth rates and symptom expression of apple trees infected with '*Candidatus* Phytoplasma mali'

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Abstract

The effects of four commercially available bio-active compounds on the infection rates, symptom expression and growth rates of apple trees (*Malus* × *domestica* Borkh.) cv. Golden Delicious infected with '*Candidatus* Phytoplasma mali' (the so-called Apple Proliferation phytoplasma or AP) were tested over a three-year period under controlled conditions. Post-infection treatments using Bion® (active ingredient: Acibenzolar-S-Methyl), Messenger® (Harpin protein), Regalis® (Prohexadione-Ca) and Dormex® (Cyanamide) had no significant effect on infection rates. Terminal growth of apple trees (grown as one-shoot pruned trees) was increased significantly by AP infection; Prohexadione-Ca was the only compound which had a significant (inhibiting) effect on the growth of both infected and non-infected apple trees. Acibenzolar-S-Methyl and Harpin had no significant effects on symptom expression. AP symptoms were masked during summer by Prohexadione-Ca, which caused severe growth abnormalities. Cyanamide changed the seasonal appearance of AP symptoms: while symptoms were delayed compared to the untreated control the first two years (2008 and 2009), symptoms appeared earlier the third year (2010). Differences in symptom expression levelled off later in the vegetative season, and no significant difference was found in October.

Key words: apple proliferation, *Malus domestica*, phytoplasma quantification, recovery, witches' broom

Introduction

Apple proliferation (AP), caused by '*Candidatus* Phytoplasma mali' (also known as AP phytoplasma) is an economically important disease of apple trees in Central and Southern Europe. Infected plants produce small and tasteless fruit which are not suitable for consumption. Other typical disease symptoms are enlarged stipulae, premature bud opening and leaf reddening and growth abnormalities that reflect perturbations in the plant hormonal balance ('witches' brooms'). The phytoplasmas colonize the sieve tubes of the phloem tissue and are transmitted mainly by the sap-sucking psyllids *Cacopsylla picta* and *C. melanoneura* (Tedeschi & Alma 2004). Field control of AP consists of the removal of symptomatic plants and insecticide treatments against the insect vectors.

Phytoplasma densities in AP-infected apple trees and symptom expression can vary significantly among seasons, plant tissue and individual plants (Rekab et al. 2010). Latent non-symptomatic infections were reported (Baric et al. 2007, Carraro et al. 2004), but it is not well established if they play an important role for disease spread. Symptom remission (or recovery) can occur naturally either transiently or permanently (Carraro et al. 2004, Seemüller et al. 1984a). The underlying mechanism is poorly understood, but several studies indicate that recovered plants have reduced levels of reactive oxygen species (ROS) and scavenging enzymes, particularly ascorbate peroxidases (APX) and catalases (CAT). Several studies have reported that recovered trees were colonized by the phytoplasma in the roots at a similar level as symptomatic plants, whereas the shoots were either phytoplasma-free (Carraro et al. 2004) or colonized by lower phytoplasma titres (Baric et al. 2011).

Infected but asymptomatic plants produce apples which are equal in size and quality to fruits of healthy plants (Bianchedi et al. 2008). Therefore, the control of apple proliferation using bioactive compounds which induce the remission of symptoms might be a successful management strategy for perennial crops in areas where the disease is already endemic. A number of commercial resistance inducers and plant regulators are available for fruit production, and preliminary field tests indicate that some of them might actually induce recovery from phytoplasma infection in annual and woody plants. Resistance inducers were used to control Bois noir, a major disease of grapevine caused by Stolbur phytoplasma, and numbers of symptomatic plants decreased significantly compared to the untreated control (Romanazzi et al. 2009, 2011). Treatments with the plant growth regulators indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) induced the recovery of the periwinkle *Catharanthus roseus* (Apocynaceae) infected with '*Ca.* P. mali' (Curković Perica 2008), but to our knowledge no experiments with apple proliferation diseased apple plants were carried out to-date.

The aim of this study was to assess if resistance inducers and plant regulators could induce recovery and modify the i) infection rates and '*Ca.* P. mali' titres, ii) symptom expression and iii) growth rates of potted apple plants artificially infected with '*Ca.* P. mali'.

Materials and methods

Plant material and inoculation

Symptomatic and healthy plant material was collected from a field orchard heavily infected with AP in Tramin (South Tyrol, Italy) in November 2007, when phytoplasmas are still present in aerial shoots before sieve tubes degenerate in late winter (Seemüller et al. 1984b). Roots and branches of the selected donor apple trees cv. Golden Delicious on M7 rootstocks were analyzed using a qualitative real-time PCR procedure (Baric & Dalla Via 2004) to confirm the presence of '*Ca.* P. mali'. Branches collected from 13 infected and 9 healthy donor trees were used for the inoculation experiment. The collected branches were stored at 4°C with relative humidity (RH) > 60% until February 2008 to prevent desiccation and death of phytoplasmas.

The inoculation was performed in February 2008 via scion grafting from collected material on healthy M9 rootstocks. Six hundred rootstocks were grafted with scions from infected donor trees and 400 with scions from healthy donors; each scion contained three buds.

Plants were potted in container substrate (Gramoflor MTL, Manna Italia) and kept in a glass house under controlled temperature and RH > 60%. On April 15th 2008, test plants were transferred to an insect-proof field tunnel and arranged in blocks of 90 plants, each consisting of 60 inoculated and 30 healthy plants. In order to start the experiments with similar sized and shaped plants and facilitate symptom evaluation, the main stems of the apple plantlets were pruned and side shoots removed, resulting in a 'one-shoot-tree'.

Resistance inducers and plant growth regulators

The commercial products tested were Bion® 50 WG (Syngenta Crop Protection, Switzerland), Messenger® (Eden Bioscience Corporation, USA), Regalis® (BASF, Germany) and Dormex® (SKW Trostberg AG, Germany). Active ingredients, application rates and treatment schedules are listed in Table 1. Plants were treated over a three year period (2008–2010).

The products, except cyanamide, were dissolved in water according to manufacturer's instructions and sprayed on the canopy of the apple trees. Each 90-plant-block was treated with a different chemical. Cyanamide was dissolved and applied to the pots of the plants. As cyanamide contains nitrogen, the plants of the other blocks were supplemented with corresponding amounts of nitrogen in form of urea during the treatment period (2008: 0.38 g N plant^{-1}; 2009 and 2010: 0.7 g N plant^{-1}). One 90-plant- block was kept as untreated control.

Plant growth rate and symptom expression

The vertical growth of the main shoot of healthy and infected plants was measured in October 2008. The development of

Table 1: Treatment schedule of apple trees with four commercial resistance inducers and plant growth regulators

Product name	Active ingredient	Recommended dosage	Year	Application dosage	Application interval (days)	N Applications	First treatment	Last treatment
Bion	Acibenzolar-S-Methyl (50%)	0.15 g l^{-1}	2008	0.15 g l^{-1}	14	4	May 7	June 18
			2009	0.15 g l^{-1}	10–14	6	April 9	June 18
			2010	0.15 g l^{-1}	10–14	6	April 14	June 18
Messenger	Harpin protein (3%)	0.9 g l^{-1}	2008	0.9 g l^{-1}	14	4	May 7	June 18
			2009	0.9 g l^{-1}	10–14	6	April 9	June 18
			2010	0.9 g l^{-1}	10–14	6	April 14	June 18
Regalis	Prohexadion-Ca (10%)	0.5–0.8 g l^{-1}	2008	1 g l^{-1}	14	4	May 7	June 18
			2009	0.8 g l^{-1} + 0.2 gl^{-1} citric acid	10–14	4	April 9	May 22
			2010	0.8 g l^{-1} + 0.2 g l^{-1} citric acid	10–14	6	April 14	June 23
Dormex	Cyanamide (49%)	n.d.	2008	0.2 g 300 ml^{-1} plant^{-1}	14	4	May 7	June 18
			2009	0.2 g 300 ml^{-1} plant^{-1}	10–14	8	March 24	July 2
			2010	0.2 – 0.3 g 500 ml^{-1} plant^{-1}	10–14	8	March 31	July 9

typical AP symptoms (witches' brooms and/or enlarged stipulae at the top of the shoots) was recorded two times per season: one week after termination of the treatment period, in July, and at the end of the growing season, in October (see Table 2). In the year 2009 symptom expression was recorded two times after termination of the treatment period (four days and four weeks after the last treatment, respectively), in order to determine the long-lasting symptom repression effect of the treatments. Dormex treated trees were recorded in 2009 after 7 treatments and after 8 treatments, respectively.

Biomass of symptomatic vs. non-infected apple plantlets

The fresh weight of symptomatic and non-infected (control) plants was determined in July and October 2009 (n = 5 for each variant). The symptomatic plants had been infected in February 2008; all plants were pruned to form a 'single-stem'-plant in the winter of 2008/2009. The biomass of aerial plant parts and roots were measured individually.

Qualitative and quantitative real-time PCR analyses of '*Ca.* P. mali'

In order to determine the presence of '*Ca.* P. mali' in inoculated apple trees that did not show visible disease symptoms until October 2009, root samples were collected for DNA extraction. DNA was isolated from 100 mg dissected phloem tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) (Baric et al. 2006) and tested with a highly sensitive real-time PCR method (Baric & Dalla Via 2004).

Infected trees treated with Dormex and the corresponding infected control trees were sampled in July and October 2009 as well as in July and October 2010 in order to be tested for an effect of cyanamide treatment on the phytoplasma titre. Dormex-treated plants were selected for quantitative real-time PCR analysis, as this test group was the only one that differed in symptom expression compared to the untreated infected control in the first two years of the study. Depending on the size of the rootstock, 2–3 root samples were taken from each tree. In addition, 3 leaves from the top part of the main shoot (leaving out the first three leaves) of each tree were collected. DNA from roots was isolated from phloem dissections using the same protocol as mentioned above. The midribs of each of the three leaf samples were cut out and finely chopped. Following proper mixing, 100 mg were used for DNA isolation (DNeasy Plant Mini Kit, Qiagen). DNA isolates were normalized to 10 ng μl^{-1} and a standard curve-based real-time PCR procedure was used for quantitative analysis of '*Ca.* P. mali' as described in Baric et al. (2011).

Briefly, in addition to the 16S rDNA gene of '*Ca.* P. mali', the ACO gene (1-aminocyclopropene-1-carboxylate oxidase) of the host plant was analyzed in parallel in a single-tube reaction. Since two gene copies of the ACO gene are contained in the diploid apple genome and the 16S rRNA gene is present in two copies in the '*Ca.* P. mali' genome, it was possible to calculate the absolute number of phytoplasma genomic units or cells per diploid host plant genome or cell (Baric et al. 2011). The discrepant number of root and leaf

Table 2: Effect of resistance inducers and plant growth regulators (in % of symptomatic plants) on the expression of typical AP symptoms (witches' broom and/or enlarged stipulae). n = total number of trees assessed for symptom expression.

Time period		Control	Bion	Messenger	Regalis	Dormex	Crosstab Chi Square
2008	July	67.4	71.8	48.9	40.4	51.1	12.3; df = 4; p=0.02
	n	*46*	*39*	*45*	*47*	*45*	*222*
	October	84.8	89.7	82.2	78.7	86.7	2.5; df = 4; p=0.6
	n	*45*	*39*	*45*	*47*	*45*	*221*
2009	June 22nd	56.5	41.0	40.9	32.6	31.8	7.5; df = 4; p=0.11
	n	*46*	*39*	*44*	*46*	*44*	*219*
	July 6th	78.3				52.3	6.7; df = 1; p=0.01
	n	*46*				*44*	*90*
	July 15th	86.7	76.9	81.8	76.6		1.9; df = 3; p=0.6
	n	*45*	*39*	*44*	*47*		*175*
	October	97.8	84.6	84.1	87.0	81.8	6.2; df = 4; p=0.18
	n	*45*	*39*	*44*	*46*	*44*	*218*
2010	July	43.9	48.6	41.7	5.7	70.5	33.8;df = 4; p< 0.001
	n	*41*	*37*	*36*	*35*	44	*193*
	October	89.5	84.8	88.2	65.7	88.6	10.5; df = 4; p=0.03
	n	*38*	*33*	*34*	*35*	44	*184*

samples per sampling point is due to the fact that in some of the leaf samples *'Ca*. P. mali' could not be detected or the titre was below the limit of quantification. These samples were thus not considered for the quantitative real-time PCR analysis.

Statistical analyses

Data were analyzed separately for each monitoring date and bioactive compound. Prior to statistical analyses, the data distribution was tested; for the effects of AP-infection on the plant growth t-tests were applied for data following normal distribution and Mann-Whitney U tests for non-normal distributions. Non-parametric one-way analysis of variance (Kruskal-Wallis test) was used to compare the effect of the bioactive compound on plant growth. Chi-square tests were utilized to compare the effect of bioactive compounds on infection rates and symptom expression.

Quantitative real-time PCR data obtained for symptomatic Dormex-treated and symptomatic untreated control trees were grouped per season (July and October) and per plant organ (roots and leaves). Univariate analysis of variance (UNIANOVA) by applying the pairwise Least Significant Differences (LSD) test and an alpha value of 0.05 was performed separately for the quantitative real-time PCR data of roots and leaves. A logarithmic transformation was used in order to normalize the data distribution. All Statistical analyses were performed with software IBM SPSS Statistics 20 for Windows.

Results

Infection rates

The infection rates of apple trees infected early 2008 with *'Ca*. P. mali' were evaluated in October 2009 by a combination of symptom evaluation and qualitative real-time PCR analysis of asymptomatic trees. The average infection rate calculated over all inoculated plants was 82.1%. Plants treated with Bion showed the lowest infection rate of 75.0%, while the plants treated with Regalis showed the highest infection rate of 87.0%. The corresponding rates for plants treated with Dormex and Messenger were 83.3% and 84.9%, respectively; and for untreated control plants 80.4%. The effect of the bioactive compounds on the infection rate was not statistically significant (Chi square = 0.45, p=0.5). Plants tested negative in the real-time PCR test were not considered in the symptom expression evaluation and discarded.

Evaluation of plant growth rates

AP infection showed an effect on the vertical growth of the main shoot of apple trees: infected plants grew about 50 cm higher than healthy ones (infected: 136 ± 16 cm; healthy: 87 ± 11 cm). The effect of the disease on the growth was highly significant (Mann-Whitney test; p< 0.001). The effect of treatment with the bioactive compounds was analyzed for infected and non-infected plants separately. The effect of treatments on growth was more pronounced in infected plants than in healthy ones (Kruskal-Wallis test: p< 0.001 and p=0.05), and in both cases only the plants treated with Regalis showed significant differences in the growth compared to the untreated plants (Mann Whitney test: infected: p< 0.001; healthy: p=0.001).

In July of 2009, control plants had slightly higher total fresh weight than symptomatic plants (infected: 131 ± 30 g; healthy: 161 ± 37 g), however the differences were not statistically significant (t-test, p=0.190). The same pattern was observed when aerial parts (infected: 61 ± 9 g; healthy: 71 ± 15 g; t-test, p=0.243) and roots (infected: 70 ± 25 g; healthy: 90 ± 24 g; t-test, p=0.231) were analyzed separately. In October of 2009, aerial parts of symptomatic plants showed a tendency to increased growth (infected: 121 ± 28 g; healthy: 100 ± 16 g); however the difference was not statistically significant; (t-test, p=0.195); whereas the root growth was significantly reduced compared to that of the control plants (infected: 77 ± 22 g; healthy: 121 ± 9 g; t-test, p=0.03). The total fresh weight of infected plants did not differ significantly from healthy ones (infected: 197 ± 34 g; healthy: 220 ± 17 g; t-test, p=0.205).

Symptom expression

Typical symptoms of AP infection (witches' broom and/or enlarged stipulae) were recorded twice yearly (July and October) over a three year period. Symptom expression followed a clear seasonal pattern: 40.6–55.9% of all infected plants showed symptoms in July, whereas these rates rose to 83.4–87.1% in October. Treatment of infected plants with bioactive compounds changed the dynamics of symptom expression compared to the untreated (but infected) control plants (Table 2). In July 2008, plants treated with Messenger, Regalis and Dormex showed reduced symptom expression (18.5%, 27.0% and 16.3% less symptomatic plants, respectively) compared to the untreated control group (Chi Square, p=0.015). The difference was highly significant for Regalis (Chi Square, p=0.01). In October 2008, no differences in the symptom expression were recorded between treated and untreated plants (Chi Square, p=0.6).

On June 22nd 2009, all treatment groups showed a reduced number of symptomatic plants compared to the control, but the difference was not statistically significant (Chi Square, p=0.11). On July 6th, one week after the last treatment, the Dormex group showed 26% less symptomatic plants compared to the untreated control group (Chi Square, p=0.01). In October 2009, again no statistically significant difference was found (Chi Square, p=0.18); even though the Dormex group contained 16% less symptomatic plants compared to the control block. Plants treated with Messenger, Bion and Regalis were evaluated for a second time on July 15th, about one month after the last application, and the decrease of the symptom-delaying effect was already visible (Table 2).

In July 2010, differences in symptom expression were recorded for the Regalis and Dormex treated groups compared to the control. The first product caused heavy growth inhibition in shoot elongation with increased secondary growth; therefore masking the typical symptoms of apple proliferation which were not recorded in July. After the treatment period, plants started to grow again and the apex of the main shoot could be evaluated for symptom expression. In October 2010, the Regalis treated group displayed 23% less symptomatic plants compared to the control (Chi Square, p=0.014). In contrast to the previous years, the Dormex treated group contained a higher number of symptomatic plants in July (Chi Square, p=0.013) compared to the untreated control. In October with the exception of Regalis treated plants no difference among treated and untreated groups could be recorded. A relatively constant percentage of infected plants did not express any typical AP symptoms when monitored in October (2008: 23.7%; 2009: 15.8%; 2010: 18.0%). In total, 7.3% of infected plants were symptomatic in 2008 and asymptomatic in 2009; 6.8% of asymptomatic plants showed again symptoms in 2010. Plants showing a stable recovery were not detected during the analysis period.

Quantitative real-time PCR analysis of '*Ca.* P. mali'

The results of the quantitative real-time PCR analyses of Dormex-treated trees and untreated control trees are shown in Fig. 1. The pairwise UNIANOVA test pointed to differences in the phytoplasma load among the groups of data, both for root and for leaf samples. In the root samples of Dormex-treated trees, no significant difference in the number of phytoplasma cells per host plant cell was found between samples collected from symptomatic trees in July and October. In the untreated control trees, however, significantly less phytoplasma cells were found in July compared to October for symptomatic trees. In the leaf samples, significantly higher phytoplasma loads were found in October for both groups. When comparing Dormex-treated and untreated control trees, a significant difference in the phytoplasma titre was only found in July in the roots, while in October no difference was revealed as was the case for all the leaf samples.

In July 2009 and October 2010, asymptomatic trees were present in both treated and untreated groups. The phytoplasma load in root and leaf samples of these trees was generally similar to the values observed for the symptomatic counterpart groups, except for the root samples from Dormex-treated trees collected in July and leaf samples from control trees collected in October (Fig. 1). As the number of asymptomatic trees available for quantitative real-time PCR analysis was very low, these data, however, need to be treated with caution.

Discussion

Apple proliferation, caused by '*Ca*. P. mali', is one of the most destructive diseases of apple trees in Europe. Current management strategies focus on chemical control of the vectors and eradication of infected trees. Hundreds of thousands of infected trees were felled in the South Tyrol (Italy), the largest apple–producing area in the European Union, during the last decade, resulting in considerable economic damage. Apple rootstocks with increased resistance to AP were developed (Bisognin et al. 2008), but due to unfavourable vigorous growth they are not suited for modern orchard production. Several recent studies indicate that application

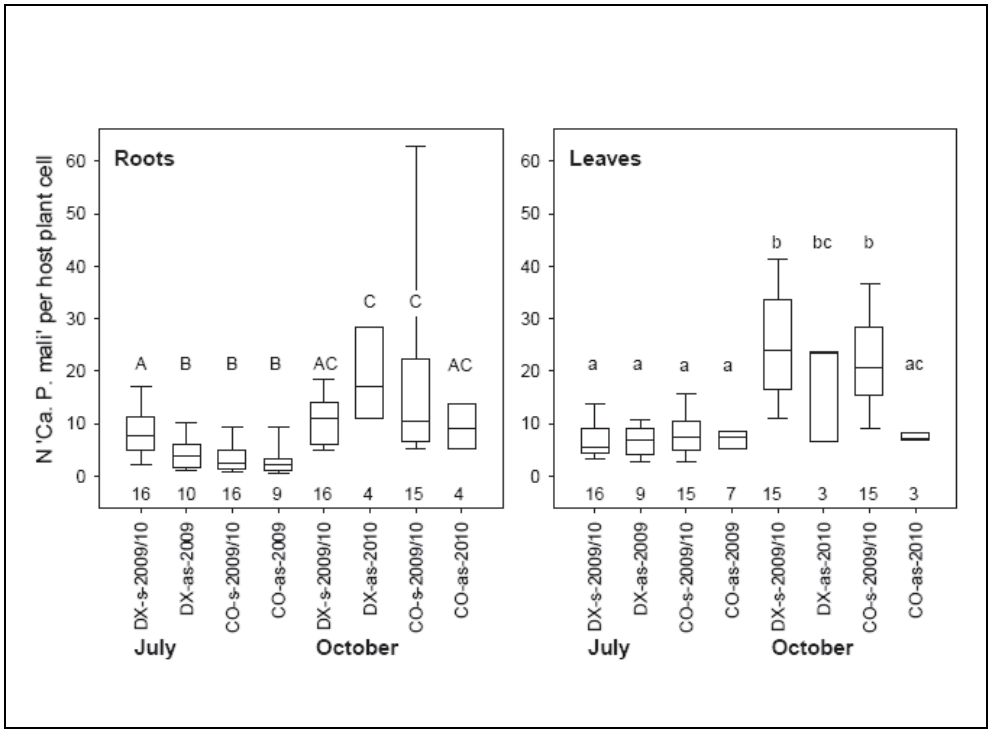

Fig. 1: Quantification of '*Ca*. P. mali' in roots and leaves of Dormex-treated apple trees and untreated control trees. The plants were infected in spring of 2008, treated with Dormex in spring/early summer of 2008, 2009 and 2010. The phytoplasma titres were determined in July and October of 2009 and 2010, respectively. The numbers below the box plots represent the sample size available for quantitative real-time PCR analysis, while the letters above the box plots refer to differences in means based on UNIANOVA testing for the root samples (uppercase letters) and leave samples (lowercase letters). Means sharing a letter are not significantly different. DX, Dormex-treated; CO, untreated control; s, symptomatic; as, asymptomatic

of resistance inducers can trigger recovery events in phytoplasma-infected herbaceous and woody hosts (Romanazzi et al. 2009, 2011). Recovery is generally defined as a spontaneous remission of symptoms associated with the disappearance of the phytoplasmas from aerial plant parts while roots remain infected (Carraro et al. 2004). Field-grown apple trees infected with '*Ca.* P. mali' show low rates of recovery (Carraro et al. 2004); 'recovered' apple trees could be used to produce commercially valuable fruits.

The aim of this study was to evaluate the effects of commercially available resistance inducers and plant growth regulators on phytoplasma colonization and symptom expression of artificially infected apple trees. The four products were selected because they induce different physiological plant responses. Bion and Messenger are known resistance inducers, respectively activating SAR and hypersensitive reaction (HR) (Vallad & Goodman 2004, Wei & Betz 2007). Bion was used in Italy to induce recovery in grapevines infected with '*Candidatus* Phytoplasma solani', causal agent of the Bois noir disease (Romanazzi et al. 2009). Weekly treatments of the canopy in spring-summer increased the number of recovered plants compared to control plants in a field experiment. Regalis is a plant growth regulator commonly used in apple orchards to regulate shoot elongation. It limits vegetative growth by inhibiting gibberellins synthesis, interferes with the metabolism of flavonoids (Spinelli et al. 2005) and decreases host susceptibility in a number of host-pathogen systems (Bazzi et al. 2003). Dormex is used for breaking bud dormancy in several deciduous fruit crops (e.g. apple) in areas with warm winters and insufficient chill hours. Hydrogen cyanamide, the active ingredient of Dormex, increases IAA and cytokinin (CK) levels (Guevara et al. 2008) and inhibits catalases (Amberger 1961). Catalases degrade hydrogen peroxide, a crucial anti-microbial compound produced by infected plants to attack plant pathogens.

The present study confirmed that scion grafting of field-infected shoots on rootstocks is an efficient method to infect apple trees with the AP phytoplasma. Infection rates were assessed the year after inoculation in October before leaf fall, when typical symptoms are expressed most unambiguously. The extended time frame between inoculation and symptom evaluation was chosen to allow for phytoplasma proliferation and movement to different plant tissues and symptom expression. Because apple trees infected with '*Ca.* P. mali' do not always develop symptoms (Baric et al. 2011), the roots of asymptomatic trees were tested using qualitative PCR and trees tested positive were included in the calculation of infection (or transmission) rates. The transmission rates from 75 to 87% are in a similar range as those reported by Jarausch et al. (1999) for *in vivo* inoculation, whereas the same authors found higher transmission success for '*Ca.* P. mali' inoculation *in vitro* (80 – 97%), using cultured infected tissue for grafting. The bioactive compounds had no significant effect on the infection rate. Based on the known chemical properties of the active ingredients, no direct bactericidal effect was expected. Indirect effects triggered by the bioactive compounds, like a possible increase of H_2O_2 levels due to catalases suppression by Dormex or growth abnormalities induced by Regalis, also had no effect on infection rates.

Infection with '*Ca.* P. mali' increased the terminal growth of apple trees significantly. The total bio-mass of infected and symptomatic potted apple plantlets was not significantly different from non-infected control plants in the year post-infection; however, the root system was visibly less well developed and significantly lighter than that of the control plants, whereas the biomass of the aerial parts of symptomatic plants in October (with elongated shoots) was slightly greater than that of the control plants. The effect of the bio-active compounds on the bio-mass was not measured because plant numbers were limited.

Regalis was the only bioactive compound which had a significant and inhibiting effect on the growth of both healthy and infected apple trees. The control of vegetative growth of healthy apple trees through Prohexadione-Ca is well documented (Unrath 1999). The effect on infected apple trees could be explained by interference with plant phytohormonal levels, which are imbalanced in phytoplasma-infected plants (Marcone 2010).

Phytoplasma concentrations in leaves varied among season, with higher levels found in fall than in summer. A similar but more subtle trend was also observed for the root samples. Interestingly, phytoplasma titres in leaves and roots of symptomatic trees were in general similar to that of asymptomatic trees. In contrast, shoot samples from symptomatic apple trees from a commercial orchard had phytoplasma titres 14 times higher compared to the ones from asymptomatic trees (Baric et al. 2011) and it was speculated that higher phytoplasma titres might trigger a series of physiological reactions eventually leading to symptom expression. Different age of the tested apple plants, infection timing in plants life history and different growth conditions (orchard vs. potted plants in a field tunnel) but also different plant organs (shoots vs. leaves) might be responsible for differences in phytoplasma titres and plant reactions. Stable recovery was not observed in our infected plants, regardless if treated with bio-active compounds or untreated. Although a significant percentage of infected plants was asymptomatic each year (23.7% in 2008 and on average 17% in 2009 and 2010), no plant which showed symptoms in 2008 was symptom-free throughout the following two years. The asymptomatic plants which tested PCR positive were probably sampled during the latency period. This result suggests that infected plants, which still show no symptoms, can potentially serve as inoculum for insect vectors, as they have aerial parts already colonized by the phytoplasma.

Treatment with bioactive compounds did not suppress AP symptoms on a long-term basis, but did change the dynamics of symptom development. Infected plants treated with Messenger, Regalis and Dormex showed significantly reduced symptoms in early summer of the first two years (2008–2009) compared to the untreated control, but no differences were found in fall of these years. Bion showed no effect the summer of the first year (2008), but did repress symptoms in the following year (2009). In 2010, Bion and Messenger did not alter symptom expression (summer and fall). Regalis induced severe growth abnormalities, like

stunted growth, which masked AP symptoms in summer, but this effect was less pronounced in fall. In contrast to its effect during the two previous years, in 2010 treatment with Dormex enhanced symptom expression in summer, but again the effect was not long-lasting and no significant differences were measured in fall. In general, all four tested bioactive compounds only changed the symptom expression throughout the treatment period, but did not induce a persistent post-treatment effect. In our experiments the timing of the treatments followed the recommendations of the commercial producers; therefore a possible effect of continuous treatment throughout the whole growing seasons was not tested. During the three years of observation, no apple plant showed a stable remission of symptoms (or recovery). Carraro et al. (2004), in their pioneering work on the recovery phenomena in '*Ca.* P. mali'-infected apple trees, describe recovery from field-grown trees, but also did not observe it on infected, potted test plants. The previously described induction of recovery of grapevines from phytoplasma-induced BN disease (Romanazzi et al. 2009) with bioactive compounds (including Bion) also occurred on field-grown plants. A number of abiotic effects typical for field conditions (like fluctuation in soil moisture levels and temperatures) as well as plant age might play a role for symptom remission; and therefore different growth conditions in the field-tunnel used for our experiments might be responsible for the lack of recovery. Based on our results with '*Ca.* P. mali'-infected potted apple trees in a controlled environment, we cannot recommend the use of resistance inducers and plant growth regulators as a management option to control apple proliferation in the field.

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