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ACIBENZOLAR-S-METHYL MAY PREVENT VECTOR-MEDIATED FLAVESCENCE DORÉE PHYTOPLASMA TRANSMISSION, BUT IS INEFFECTIVE IN INDUCING RECOVERY OF INFECTED GRAPEVINES

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Dimitrios E Miliordos[a], Luciana Galetto[b], Ester Ferrari[a], Mattia Pegoraro[a], Cristina Marzachì[b], Domenico Bosco[a,b*]

[a]DISAFA – Entomologia, Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy
[b]CNR – Istituto di Protezione Sostenibile delle Piante, IPSP, Strada delle Cacce 73, 10135 (TO), Italy
*Correspondence to : Domenico Bosco, DISAFA – Entomologia, Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy. Email: domenico.bosco@unito.it

Abstract

BACKGROUND: Acibenzolar-S-methyl (BTH), a functional analog of Salicylic Acid (SA), is known to elicit a systemic resistance across a broad range of plant–pathogen interactions but, so far, it was not tested against Flavescence dorée (FDP), one of the most devastating grapevine diseases. Aims of this work were to evaluate the activity of BTH in preventing FDP transmission by the insect vector and in inducing recovery of infected grapevines.

RESULTS: Repeated applications of 2 mM BTH to test grapevine cuttings (cv Barbera) exposed to adults of the infectious vector, *Scaphoideus titanus* Ball, reduced the rate of infected plants. The effect was not recorded following similar BTH applications to highly susceptible young *in vitro* propagated vines. A high natural recovery rate (more than 70%) was observed over a three-year-period in field-infected grapevines of the same cv. Under these conditions, BTH repeated applications over the whole period clearly failed to increase recovery of field-infected grapevines.

CONCLUSION: Following a three-year experiment, it can be concluded that, although high doses and repeated applications of BTH reduced vector transmission of FDP, BTH was ineffective in inducing recovery of FDP-infected grapevines *cv* Barbera under field conditions.

Key words: Resistance elicitor, Flavescence dorée, *Scaphoideus titanus*, grapevine, transmission, recovery

1 INTRODUCTION

The use of induced resistance to increase the effectiveness of plant natural defences is a promising environment-friendly tool for plant protection. Its introduction into agricultural practices can reduce the use of chemicals for pathogen control, thus contributing to the development of sustainable agriculture. The phenomenon of induced resistance has been described as Systemic Acquired Resistance (SAR) and as Induced Systemic Resistance (ISR). SAR is a long-lasting kind of induced resistance effective against a broad spectrum of pathogens[1-3]. In the vast majority of cases, it depends on the early increase of the endogenously synthesized signal salicylic acid (SA)[4] and is correlated with the immediate expression of a specific set of genes called "SAR genes", which includes genes coding for pathogenesis-related (PR) proteins[5-7]. ISR is controlled by a signaling pathway in which the phytohormones jasmonic acid (JA) and ethylene play key roles as revealed by the use of SA, JA and ethylene signaling mutant plants[8]. ISR is a resistance active against various bacterial, viral, fungal and nematode pathogens and has been found active in many plant species.

The use of SAR and ISR to cope with crop diseases and improve integrated pest management seems promising. BTH and INA (2,6-dichloroisonicotinic acid) are by far the best studied chemical elicitors available. Both are considered functional analogs of SA, and elicit a systemic form of induced resistance across a broad range of plant–pathogen interactions[9-10]. The efficacy of BTH as a resistance inducer has been demonstrated in many crops to a wide variety of pathogens also under field conditions. Its effect lasts longer in monocots, such as wheat, than in dicots crops in

which repeated applications are required to extend protection over time[11-12]. Indeed, BTH was originally marketed as a tool to control powdery mildew of wheat and barley in Europe[11].

Despite the almost 30 year long BTH application under different field conditions, studies of systemic resistance induced by BTH against phytoplasmas are in their infancy. So far, most BTH applications have been tested on phytoplasma diseases of herbaceous plants. For instance, BTH showed a strong protection activity against X-disease phytoplasma when applied to *Arabidopsis thaliana* plants[13]. Moreover, these authors reported a reduced survival of the leafhopper vector *Colladonus montanus* (Van Duzee), when it fed on 4.8 mM BTH treated plants, suggesting that SAR in this plant may have some detrimental effect on the leafhopper *C. montanus*. BTH application to daisy plants delayed symptom development and phytoplasma ('*Candidatus* Phytoplasma asteris', strain CYP) multiplication in treated plants compared with the control ones[14]. As for the application of BTH to phytoplasma-infected grapevines, field treatment with resistance inducers for the control of Bois Noir phytoplasma provided encouraging results[15]. Flavescence dorée is a phytoplasma-associated disease of grapevine that causes severe damages in large viticultural areas of Europe, namely in Northern Italy and Southern France. In some areas, e.g. North-Western Italy, it is the main constraint to viticulture. Flavescence dorée phytoplasmas, FDP, belong to the 16Sr V-C and D ribosomal groups and are transmitted by the leafhopper *Scaphoideus titanus* Ball (Hemiptera, Cicadellidae). FDP is a quarantine organism, its control is compulsory by law, and it relies mainly on insecticide applications against the vector and rouging of infected plants. In spite of all the efforts, a satisfactory control of the disease is difficult to achieve because many disease and vector reservoirs occur in the uncultivated areas and woodlands nearby vineyards[16].

Aims of this study were to investigate the activity of BTH in preventing Flavescence dorée phytoplasma (FDP) transmission by the vector *Scaphoideus titanus* Ball and in inducing recovery of FDP-infected grapevines. For the first goal, healthy grapevine cuttings were sprayed with BTH and

then exposed to FDP-infectious *S. titanus*. For the second goal, FDP-infected grapevines were identified in the vineyard, BTH was applied to a group of infected plants over a three-year period and recovery from FDP was recorded on BTH-treated and control grapevines.

2 MATERIALS AND METHODS

2.1 Vector and FD phytoplasma

Two species of FDP leafhopper vectors were used in the experiments: *S. titanus*, the natural vector[17], and *Euscelidius variegatus* Kirschbaum, a laboratory vector, routinely used to maintain FDP under laboratory conditions[18]. *S. titanus*, which has one generation per year, was used to inoculate test grapevines, while *E. variegatus*, which can be continuously reared, was used to maintain under laboratory conditions the FDP-C strain[19] on broad bean (*Vicia faba L.*). To obtain healthy *S. titanus* nymphs, two year-old branches bearing leafhopper eggs were collected in infested vineyards during the winter period, cut into 20-30 cm long pieces and kept in plastic bags in a cold room at 4°C. To allow egg hatching, branches were caged inside insect-proof screen houses (100 x 100 x 100 cm) in a glasshouse with natural light and temperature ranging from 20 to 30 °C. Potted grapevine cuttings and healthy broad bean plants from seed were introduced in the screen house to feed the newly hatched nymphs. Insect nymphs and adults were periodically collected and used for the experiments. Healthy colonies of *E. variegatus* were reared on potted oat plants inside plexiglas and nylon cages in growth chambers at 23 ° C (photoperiod L16:D8 light:dark) and periodically checked to be phytoplasma-free by nested PCR with the universal primers P1/P7[20-21], followed by F1/R1(V) primers[22].

FDP-C strain[19] was originally isolated from infected grapevines in Piemonte Region and transmitted to broad bean plants by *S. titanus*. Once the FDP was transmitted to broad beans, the

phytoplasma was maintained by insect transmission, from broad bean to broad bean, under laboratory conditions. Third and fourth instar nymph of *E. variegatus* were introduced in a plexiglas and nylon cage and fed on FDP-infected broad beans for 7 day acquisition access periods (AAP). Then, leafhoppers were transferred onto healthy oat plants for three weeks, to complete the latent period (LP), and finally transferred onto healthy broad beans for successive inoculation access periods (IAP). Inoculated broad beans were replaced twice a week and, after the IAP, they were maintained in a climatic chamber at 23-25 °C (photoperiod L16:D8 light: dark) for at least one month and observed for specific symptom development. Following the IAP, broad beans were treated with the insecticide Dichlorvos (Dedevap, Bayer Crop Science) and later served as inoculum source for healthy *S. titanus*. To obtain FDP-infectious S. titanus, nymphs were introduced in a plexiglas and nylon cage with four FDP-infected broad beans for a 7 day AAP. The infected broad beans were then replaced with healthy broad bean plants for 21 day LP, when infected insects were collected for grapevine inoculations.

2.2 Protection from FDP transmission by *Scaphoideus titanus* - grafted cuttings

Two year-old plants of *Vitis vinifera cv* Barbera grafted onto Kober 5BB rootstock were used for this experiment. Plants were grown in a screen house under field conditions in 10 L pots containing peat and pumice mixture (2:1). Inoculation by infectious *S. titanus* was performed at the beginning of July, when each plant had two shoots and each shoot had 8-12 leaves. For the experiment, four screen houses (two treated and two not treated) containing 20 potted grapevines were set, for a total of 80 plants. The experiment was repeated for three consecutive years: 2011, 2012 and 2013. To evaluate the resistance induced by BTH (Bion, Syngenta Crop Protection) against FDP, 40 grapevines were foliar sprayed with 2.4 mM BTH until run off point one week before the inoculation by infectious *S. titanus*. A total volume of 2 L was applied to the 40 plants and

therefore, about 50 ml of 0.5 g BTH was applied to each of the potted cuttings. The concentration of 2.4 mM was selected on the basis of the results obtained in a previous study on the activity of BTH in preventing the transmission of another phytoplasma (chrysanthemum yellows phytoplasma, '*Candidatus* Phytoplasma asteris') to daisy plants[14]. The application was repeated every 10 days until the end of September. Forty control grapevines were sprayed with water at the same dates.

Twenty-five FD-infectious adults obtained as described above were introduced in each of the four screen houses (on both BTH-treated and untreated grapevines), while other 20 leafhoppers were analyzed by nested PCR in order to estimate the number of FDP-positive insects. Every year, in December, all potted grapevines were pruned and transplanted in an experimental vineyard under field condition. In the following year the grapevines (BTH treated and untreated) were cultivated according to conventional practices. Moreover, BTH was applied, at the same dose, to the BTH-treated field transplanted vines, from mid-May until mid-September every 10 days.
The infection status of the test plants was checked by symptom observation and PCR two months after inoculation and in the following year.

In order to exclude any effect of BTH against the leafhopper vector, two grapevines were BTH treated as described above and two were water treated. Ten newly emerged adults of *S. titanus* were caged on each vine and the survival of the leafhoppers recorded one week later.

2.3 Protection from FDP transmission by *Scaphoideus titanus* - *in vitro* propagated grapevines

Grapevine plants (*V. vinifera* cv. Barbera) were propagated *in vitro*[23] and then transplanted in 12 cm pots and grown in a greenhouse at 20-25 °C. Vines with 7-10 leaves, one month after transplant, were used for the experiment. One week before the inoculation of FDP with infectious *S. titanus,* BTH (2.4 mM) was sprayed onto leaves of 10 plants, whilst other 10 plants were water

treated as control. BTH was applied until the run off point, and the application was repeated every 10 days until the end of the experiment (11 weeks post inoculation). FDP-infected *S. titanus* adults were obtained as described above and all twenty grapevines were singly exposed to four leafhoppers for an inoculation access period (IAP) of seven days. After the inoculation period the surviving insects were collected and stored at -20° C. Total DNA was extracted from twenty insects and analyzed by nested PCR in order to estimate the number of the FDP-positive individuals. At five and eleven weeks post inoculation five leaves were sampled from each test plant, DNA was extracted and FDP was detected by PCR. Samples that tested positive by PCR were further analyzed by q-PCR in order to estimate the phytoplasma population in BTH-treated and untreated test grapevines.

2.4 Induction of recovery in field-infected grapevines

The study was carried out in a vineyard of cv Barbera at Cocconato (Asti province), Piemonte Region, north-western Italy. The vineyard was planted in 1998 on SO4 rootstock and consisted of 76 rows for a total of about 8800 grapevines, the training system was cordon pruning and vines were submitted to conventional agronomic practices, including fungicide and insecticide applications. In particular, for the control of the FDP vector, insecticide was applied twice during the vegetation season, as enforced by the law in the areas were both FDP and its vector are present (Italian Ministry of Agriculture, 2000). The first application was done at mid-end of June, against *S. titanus* nymphs (Actara, Syngenta Crop Protection), and the second application took place one month later at mid or end of July against leafhopper adults with Chlorpyriphos- ethyl (Dursban, Dow AgroSciences).

FDP-infected grapevines were identified during sampling surveys at the end of June – beginning of July in 2011. Plants with phytoplasma symptoms were sampled and diagnosed for the

presence of FDP. According to the symptoms and the diagnostic PCR assays, 72 FDP-infected grapevines were identified for the experiment. Half of the plants, randomly distributed in the vineyard, were foliar sprayed with 2.4 mM BTH until run off point every 10 days from mid-May (mid-July in 2011) until mid-September for three consecutive years (2011, 2012 and 2013). A total volume of 15 L was applied to the 36 plants and therefore, about 400 ml of 2.4 mM BTH was applied to each plant. Control plants were sprayed with similar volumes of water. When two infected vines were next to each other, both were included in the same treatment (BTH or water), in order to avoid drifting of BTH to a neighboring control plant. Every year, at the beginning of September, each grapevine was checked for FDP symptoms and analyzed for the presence of FDP by nested PCR. In the final year, 2013, FDP positive grapevines were analyzed by quantitative real time PCR (q-PCR) in order to measure the phytoplasma load in treated and untreated test grapevines.

2.5 Total DNA extraction and FDP detection

Total DNA was extracted from 1.5 g of leaf veins according to a phytoplasma enrichment protocol followed by a CTAB-based extraction procedure and dissolved in 100 µl sterile double distilled water (SDW)[24]. Total DNA was also extracted from single leafhoppers following a procedure previously described by Marzachì *et al*.[25]. DNA concentration of extracts was quantified with a Nanodrop (ND 1000 Spectrophotometer; Nanodrop technologies, Inc.). Nested PCR was used for phytoplasma detection. Universal primers pair P1/P7 was used in direct PCRs[20-21]. Reaction products were diluted 1:40 in SDW and used as templates in nested reaction driven by primers R16(V)F1/R1 for the specific detection of 16S group V[22]. Amplicons were separated by electrophoresis in 1% agarose TBE (90 mM Tris- borate, 2 mM EDTA) gel and visualized under UV light after staining with ethidium bromide.

2.6 Quantification of Flavescence dorée phytoplasma cells

FDP titre in infected grapevines was measured by q-PCR as the number of FD phytoplasma cells per ng of grapevine DNA according to the method described in Roggia *et al*.[26], based on a double absolute quantification assay (phytoplasma and plant DNA) designed by Marzachì and Bosco[27]. To determine the number of FD phytoplasma cells, specific primers targeting a portion of the *sec*Y gene of the FD phytoplasma were used. For the quantification of grapevine DNA, specific primers targeting the 18S rDNA gene of grapevine were used. Standard curves for the absolute quantification of FD phytoplasma and grapevine DNA were obtained by dilution of plasmid p26SecYFD, containing the appropriate *sec*Y gene target sequence from FD-C phytoplasma, and of total DNA extracted from healthy grapevine grown from seed, respectively. Dilution of grapevine samples, reaction mix and cycling conditions were as described in the original paper[26]. Each sample was run in triplicate in the same plate. For each PCR, water instead of DNA was used as negative control. Threshold levels, threshold cycles and standard curves were automatically calculated by the iCycler software (BioRad), v. 3.06070. Per-well baseline cycles were determined automatically. Specificity of the reaction was tested by running a melting curve analysis of the amplicons following each quantification reaction.

2.7 Data analysis

Chi-square (or Fisher exact test) was applied to the comparison of the proportion of infected/recovered plants in the two treatments (BTH and water) in all the experiments. For the analysis of the phytoplasma population, raw data (obtained as log) were used and the concentration of FDP in each plant was expressed as the difference between the logarithm of the number of FDP cells and the logarithm of ng grapevine DNA. FDP population in treated and not

treated Barbera plants was compared with t-test. For all the analyses SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA) was used.

3 RESULTS

3.1 Protection from FDP infection by *Scaphoideus titanus*

Some plants (8 among the BTH-treated and 19 among the control plants) did not survive following the transplantation into the vineyard, and therefore the results refer to the surviving plants. Only one plant tested PCR positive for FD infection the year of the inoculation. A total of 8 out of 113 (7.0 %) BTH-treated grapevines were infected by FDP after the inoculation in the three-year experiment, whilst 18 out of 101 (17.8 %) control grapevines were infected under the same conditions (Table 1). Chi-square analysis showed a significant effect of BTH in protecting from FDP infection (Chi-square = 4,803, 1 df, P = 0,028), on pooled data collected during the three years. The analysis of the year by year results showed a significant/almost significant effect of BTH in 2012 (P = 0.020) and in 2013 (P = 0.055), while no effect was recorded in 2011 (P = 0.367).

The three batches of 20 *S. titanus* specimens analyzed every year to estimate the proportion of infected insects showed 60 to 70 % of FDP-positive leafhoppers (2011, 12/20; 2012, 14/20; 2013, 12/20).

No activity of BTH against *S. titanus* was recorded, as the survival was similar on both BTH-treated and untreated vines.

3.2 Protection from FD infection by Scaphoideus titanus – *in vitro* propagated cuttings

Eight out of ten BTH-treated and six out of ten control grapevines became infected with FDP following inoculation with four leafhoppers per plant (Table 2). Thus, no differences between the treated and untreated *in vitro* propagated vines were detected (Fisher exact test, P = 0.628). Seventeen out of 20 *S. titanus* sampled after inoculation of the test plants were FDP-positive following nested PCR assay (85%). The first FD symptoms appeared during the 5th week post inoculation, on both BTH treated and control plants.

Phytoplasma population in the FDP-infected grapevines, BTH-treated and untreated, was measured on leaf samples at 5 and 11 weeks post-inoculation (wpi), and at the former date FDP load was very low regardless of the treatment. At both times, an apparently higher number of FDP cells was estimated in BTH-treated plants, but these differences were not significant (t-test: 5 wpi P = 0.178, 11 wpi P = 0.082). A significant increase of FDP cells over time was recorded in both in BTH-treated (t-test, P = 0.004) and untreated plants (t-test, P = 0.016), demonstrating an active phytoplasma multiplication in the infected plants during the early phases of infection.

3.3 Induction of recovery in field-infected grapevines

Two FDP-infected grapevines died during the second year of the experiment and therefore were excluded from the analysis. After the three-year field experiment, most of the FDP-infected Barbera plants underwent recovery. Fifty-eight and 73.5% of the FDP-infected grapevines were recovered (symptomless and FDP negative by PCR) at the end of the study among BTH-treated and untreated plants, respectively (Table 3). The statistical analysis showed no significant differences between the treatments (Chi-square = 1.181, 1 df, P = 0.277) and therefore plants recovered from FDP regardless of the treatment.

The temporal pathways of recovery of the single vines over time are shown in Supplementary Tables 1 (BTH-treated) and 2 (control plants). Thirteen and 20 grapevines recovered

in the second year of the study in the BTH and control treatments, respectively. Nine and 7 grapevine plants recovered the third year of the study in the BTH and control treatments, respectively. Four plants, two among the BTH-treated and two among control plants, following a recovery in the second year, were newly infected in the third year (Supplementary Tables 1 and 2). The total numbers of FDP-infected grapevines during the three-years of the experiment are shown in Figure 1 (BTH-treated and control plants). In the third year, at the end of the experiment, FDP population was about 700 and 1,200 cells per ng of grapevine DNA in BTH-treated and control plants, respectively (Table 3). The difference was not statistically significant (t-test = -1,281, 19 df, P = 0.216) and thus the treatment had no effect on the phytoplasma multiplication in the infected plants.

4 DISCUSSION AND CONCLUSIONS

One of the main challenges in Northern Italy and Southern France viticulture is to control/suppress the spread of FD disease. Several measures are applied, such as compulsory insecticides against the vector, rouging of infected plants, cleaning of abandoned vineyard and of wild rootstocks that can host the phytoplasma and the vector. In spite of all the efforts, the disease is still spreading in several areas where it represents the main constraint to viticulture. Recently, induction of "recovery" of the infected grapevines is being explored as a tentative solution[28]. Also, in the frame of integrated FD management, agronomic techniques such as severe pruning or rouging of infected plants and the application of compounds or plant symbiotic microorganisms that may act as resistance elicitors, are under evaluation[29]. The present study represents the first trial of BTH application on grapevines to induce resistance against FDP.

Setting of the experimental conditions (dosage and application timing) was based on previous work measuring the activity of BTH against chrysanthemum yellows phytoplasma (CYP) in

daisy plants[14]. However, dosage and application frequency of this experiment are unlikely to be applied in commercial vineyards; indeed, they were designed to maximize the identification of any BTH effect on FD infection.

After the three-year experiment, considering all the inoculated grafted cutting test plants, FDP-infected grapevines were significantly more numerous in the control plants than among those sprayed with BTH, thus indicating an activity of BTH in preventing transmission of FDP. However, due to the low proportion of infected plants recorded for both BTH and control experiments, and the fact that repeats of the three years (2011, 2012 and 2013) provided slightly different results, BTH activity in preventing FDP transmission to grapevines could not be proven definitively. On the other hand, most of the cuttings from *in vitro* propagated vines were successfully infected by infectious *S. titanus,* irrespective of BTH applications. The reasons for the higher susceptibility of *in vitro* derived plants compared to grafted ones may be different. Herbaceous hosts of phytoplasmas are generally very susceptible and easy to infect, as it is the case of daisy for CYP[30], broad bean for FDP[31], and many other herbaceous hosts for different phytoplasmas that can be routinely and efficiently transmitted by their vectors. A high susceptibility to FDP was also reported by Jarausch *et al*.[32] for young grapevines obtained from *in vitro* multiplication following inoculation by the vector *S. titanus*. Also, a different feeding behavior of the vector on young, tender shoots/leaves cannot be excluded and possibly explain the different transmission results. Finally, it is possible that the absence of rootstock increases susceptibility of grapevine to the pathogen. The higher infectivity of leafhoppers that inoculated in vitro propagated vines, besides the higher number of insects per plant, may also explain the different transmission, despite their shorter IAP. Moreover, no effect of BTH was measured on the multiplication of FDP in infected cuttings from *in vitro* propagated vines, a further indication that this molecule is not effective in eliciting resistance on these very susceptible plants.

To our knowledge, there are only two reports on the activity of BTH in preventing the transmission of phytoplasmas by the insect vectors[13-14]. Interestingly, the results are not consistent for the two phytoplasma-plant vector associations. While Bressan and Purcell[13] reported a significant effect of BTH in reducing X-disease phytoplasma transmission to *Arabidopsis thaliana* (L.) Heynh by the leafhopper *Colladonus montanus* (Van Duzee), D´Amelio *et al.*[14] reported no differences in transmission efficiencies of CYP to *C. carinatum* by the vector *M. quadripunctulatus*. In this work, a significant effect was found in preventing transmission of FDP to grafted grapevines and no effect in preventing FDP transmission to herbaceous *in vitro*-propagated grapevines with the vector *S. titanus*. In spite of the lack of activity in preventing CYP transmission, D'Amelio *et al.*[14] reported a significantly lower phytoplasma load in the BTH-treated test plants at early stages of infection, while no differences in FDP load were found between treated and untreated grapevines under our experimental conditions. The activity of BTH in preventing phytoplasma transmission is highly dependent on the phytoplasma-plant-vector association.

In the last decade, the spontaneous remission of symptoms in previously infected plants (recovery) has attracted a lot of attention for the potential consequences on economically important woody crops. In particular, recovery from phytoplasmas was reported for apple[33-34], apricot[35], pear[36] and grapevine[37-39]. Recovery from Bois Noir (BN), another grapevine yellows of phytoplasma origin, can be promoted by the application of resistance elicitors compounds or stress practices such as partial uprooting of the plants[40-41]. In the present work, repeated BTH foliar applications to FDP-infected grapevines under field conditions for three years did not increase the number of Barbera grapevines that underwent recovery compared to water-treated control plants. Indeed, a very high spontaneous recovery rate occurred in the control plants, and even more untreated grapevines underwent recovery compared to the treated ones, though the difference was not significant. Moreover, no significant difference was recorded in the number of FDP cells in

treated and control plants that were still infected at the end of the experiment, as measured by q-PCR. These results are in line with those showing that BTH application do not affect either the apple proliferation phytoplasma infection rate or phytoplasma multiplication in treated apple trees under controlled conditions[42].

Interestingly, our results are not consistent with those published by Romanazzi et al.[15], who found a positive effect of BTH in inducing recovery of BN-infected grapevines. Several hypotheses can explain these different results: i) the resistance response elicited by BTH is active against BNP but not against FDP infection ii) different environmental conditions of the two vineyards (in northern and central Italy, characterized by a very different amount of summer rains) may influence plant response to the resistance elicitor iii) the grapevine cultivar (Barbera vs Chardonnay) is a clue element in BTH-elicited defense response. In Sardinia (Italy), the resistance elicitor Kendal (Valagro, Italy), containing glutathione and oligosaccharines, also failed to promote recovery of BN-infected Chardonnay and Vermentino grapevines[43]. It is interesting to note that BTH is active in inducing resistance of grapevines toward other pathogens, such as *Plasmopara viticola* and *Erisyphe necator* (downy and powdery mildews)[44] and *Botrytis cinerea* (gray mold)[45-46]. These data suggest that BTH induces resistance response in grapevine but this response is pathogen-specific.

Due to the above results, in spite of the great interest in natural, synthetic and biotic resistance elicitors, a lot of caution should be taken in promoting the application of these tools and specific evaluations are required for each pathogen-plant combination. Interestingly, in this work BTH reduced FDP transmission by the vector but was ineffective in inducing recovery of the infected vines. It is then likely that physiological and molecular mechanisms regulating FDP-grapevine interactions during early and late stage of plant infection are different, and BTH is only able to interfere with the former ones. The information on the mechanisms of plant response to

phytoplasmas are still very limited; only a deeper knowledge of physiological and molecular relationships between the pathogen and the host plant will possibly allow manipulation of such mechanisms, thus providing new effective and environmental friendly tools for the control of phytoplasma diseases of crops.

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Table 1: Number of FDP-infected plants over the total number of grapevines recorded every year among BTH or control treatment plots in the three-year experiment.

	Treatment		
YEAR	**BTH**	**CONTROL**	**P**
2011	4/32	1/26	0.367 (Fisher exact test)
2012	4/41	12/35	0.020 (Chi-square)
2013	0/40	5/40	0.055 (Fisher exact test)

Table 2: Number of FDP-infected plants over the total number of *in vitro* propagated grapevines and estimated amount of FDP cells per ng of vine DNA (mean ± s.e.) in BTH-treated and control grapevines at 5 and 11 weeks post inoculation (wpi). Within columns and within rows, different letters indicate significant differences.

		FDP cells/ng vine DNA	
Treatment	**Number of FD infected plants**	**5 wpi**	**11 wpi**
BTH	8/10	3.60 ± 1.47[a]	114.56 ± 56.53[b]
CONTROL	6/10	1.03± 0.47[a]	21.43 ± 7.50[b]

Table 3: Number of recovered plants over the total number of FDP-infected field grapevines at the end of the three-year experiment and estimated amount of FDP cells per ng of vine DNA (mean ± s.e.) in the grapevines still infected at the end of the experiment in BTH-treated and control grapevines.

Treatment	**Number of recovered/infected plants**	**FDP cells/ ng vine DNA**
BTH	21/36	731.12 ± 187.18
CONTROL	25/34	1256.73 ± 405.16

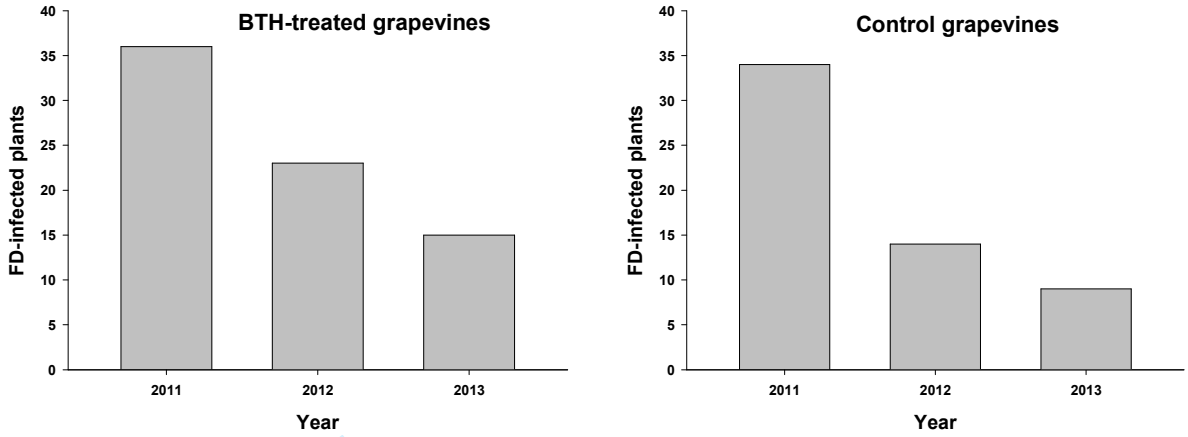

Figure 1: Number of FDP-infected grapevines in the three years of the experiment following BTH or water control treatments.

Supporting Table 1: FDP-infected or recovered grapevines in the BTH-treatment over the three-year period. Each line represents the sanitary status of an individual plant. **+**, FDP-infected grapevine; **-**, recovered grapevine.

2011	2012	2013
+	+	-
+	-	-
+	-	-
+	+	+
+	-	-
+	+	+
+	+	+
+	-	-
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+	+	-
+	+	+

Supporting Table 2: FDP-infected or recovered grapevines in the control treatment over the three-year period. Each line represents the sanitary status of an individual plant. **+**, FDP-infected grapevine; **-**, recovered grapevine.

2011	2012	2013
+	+	-
+	-	-
+	-	-
+	+	-
+	-	-
+	-	-
+	-	-
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