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Evaluating the combined effect of a systemic phenylpyrrole fungicide and the plant growth-promoting rhizobacteria *Paraburkholderia phytofirmans* (strain PsJN::gfp2x) against the grapevine trunk pathogen *Neofusicoccum parvum*

Evaluation of a treatment combining fungicide and PGPR against GTDs

Hanxiang WU^a, Alessandro SPAGNOLO^b, Cécile MARIVINGT-MOUNIR^a,
Christophe CLÉMENT^b, Florence FONTAINE^{b,*}, Jean-François CHOLLET^{a,*}

^a Institut de Chimie des Milieux et des Matériaux de Poitiers (IC2MP), Unité Mixte de Recherche CNRS 7285, Université de Poitiers, 4 rue Michel Brunet, TSA 51106, F-86073 Poitiers cedex 9, France

^b SFR Condorcet - FR CNRS 3417, Université de Reims Champagne-Ardenne, Unité Résistance Induite et Bioprotection des Plantes (RIBP), Moulin de la Housse, Bât. 18, BP 1039, 51687 Reims cedex 2, France

*co-last authors

Corresponding authors:

Jean-François CHOLLET, jean.francois.chollet@univ-poitiers.fr, +33 5 49 45 39 65
Florence FONTAINE, florence.fontaine@univ-reims.fr, +33 3 26 91 33 18

E-mail addresses: hanxiang.wu@scau.edu.cn; alessandrospagnolo78@gmail.com;
cecile.marivingt.mounir@univ-poitiers.fr; christophe.clement@univ-reims.fr;
florence.fontaine@univ-reims.fr; jean.francois.chollet@univ-poitiers.fr

ABSTRACT

BACKGROUND: A new chemical control strategy for grapevine trunk diseases (GTDs) is to develop site-targeted fungicides to protect grapevine vascular tissues. Due to the complexity of GTDs, the effectiveness of a single method is limited. Investigation of the interactions between chemical and biological agents is an essential requirement for integrated control strategies. The effect of a phloem-mobile derivative of the fungicide fenpiclonil (SM 26) in combined use with the plant growth-promoting rhizobacteria, *Paraburkholderia phytofirmans* PsJN on the *Neofusicoccum parvum* strain Bourgogne (NpB) was evaluated.

RESULTS: SM 26 was found to be translocated to the shoot apices and roots of grapevines through both xylem and phloem after foliage application. *In vitro* studies demonstrated that SM 26 exhibited no inhibitory effect on the growth of PsJN and could be largely absorbed into the bacterial cells. *In vivo* evaluation showed that the combined use of SM 26 and PsJN was the most effective following artificial inoculation of NpB on the stems of rooted Chardonnay and Sauvignon cuttings. Finally, the expression of defence-related genes, including the genes associated with secondary metabolism (*ANTS*, *PAL*, *STS*, *VvI7.3*), defence proteins (*GLUC*, *PRI*, *PGIP*), redox status (*GTSI*) and ethylene synthesis (*ACC*), was found to be strongly upregulated in PsJN + SM 26 cotreated plants compared to non-treated plants (controls), especially for Chardonnay.

CONCLUSION: The systemic profungicide SM 26 interacts with the biocontrol agent PsJN to stimulate some plant defence responses, and their combined use may present a potential integrated control strategy against GTDs.

KEYWORDS

Biocontrol; Chemical control; Grapevine trunk diseases; Integrated pest management; Ion-trap mechanism; Plant defence response; Profungicide; Systemicity.

1 INTRODUCTION

Grapevine trunk diseases (GTDs) are a serious threat to the sustainability of vineyards worldwide.^{1,2} The three main GTDs are the Esca complex, Eutypa dieback and Botryosphaeria dieback and their respective associated pathogens are *Phaeoconiella chlamydospora*, *Phaeoacremonium minimum* and *Fomitiporia mediterranea*; commonly *Eutypa lata* but also *Eutypa sp.* and *Eutypella sp.*; and finally several species of Botryosphaeriaceae family.³ *Botryosphaeria dothidea*, *Diplodia seriata* and *Neofusicoccum parvum* are the most commonly isolated pathogens for Botryosphaeria dieback.⁴ Pathogenic fungi infect the xylem vessels of grapevines, causing wood discoloration and necrosis.⁵⁻⁷ Wood infections can also cause visible foliar symptoms because of the disruption of water and mineral transport^{8, 9} as well as phytotoxins secreted by the pathogens.¹⁰ Different control methods, including cultural, biological and chemical techniques, have been extensively studied, but none of them has been proven effective in managing GTDs.^{3, 7, 11}

Chemical treatments have been found to be insufficient for GTD management in the field, although several fungicides display good activity against GTD pathogens *in vitro*.^{3, 12, 13} Existing active ingredients with limited systemicity in plants are usually effective in controlling surface pathogens as well as protecting pruning wounds, because they do not penetrate grapevines well enough to control organisms inhabiting the vascular tissues.³ Thus, a new fungicide with properties that would allow translocation and accumulation in the plant vascular system could be considered an effective tool to manage GTDs, especially for Botryosphaeriaceae family herein.¹⁴

Almost all current systemic fungicides move predominantly in the xylem and accumulate at the sites of high transpiration. The development of phloem-mobile fungicides has long been needed to improve the efficiency of vascular disease control.^{15, 16} One approach to develop phloem-mobile fungicides is based on an ion-trap mechanism, which modifies the chemical structure by introducing a weak acidic function.¹⁷ Fenpiclonil (Fig. 1) is a non-systemic fungicide from the

phenylpyrrole family. We previously developed a fenpiclonil derivative containing a carboxylic acid group (F 30, Fig. 1), which displayed considerable *in vitro* fungicidal activity against the pathogenic fungus *Eutypa lata*,¹⁵ with pathogenic fungal growth completely inhibited at a concentration of 1 mM. When the acidic compound was used at 50 μ M, fungal development was not totally inhibited, but a large inner part of the colony around the inoculum disk became necrotic after three-weeks of culture.¹⁵ Systemicity tests using *Ricinus* seedlings showed good phloem mobility of this acidic derivative.¹⁵ Thus, the further evaluation of the *in vivo* biological activity of this molecule against GTD pathogens as well as its possible propesticide behaviour is a worthwhile endeavor.¹⁴

Due to the complexity of GTDs, it seems impossible to manage these destructive diseases by a single method of control.³ Integrated management strategies that combine both biological and chemical controls have been recommended to achieve good control performance. Recent studies using various biocontrol agents, including bacteria and fungi, have shown potential biological activities.^{11, 18, 19} *Paraburkholderia phytofirmans* PsJN is known as a plant growth-promoting rhizobacteria (PGPR) that exerts beneficial effects on plant growth by producing/modulating plant hormones, providing adequate nutrition and reducing susceptibility to diseases.²⁰ These bacteria have been found to colonize different host plants and significantly improve their tolerance to biotic and abiotic stresses.²⁰ For grapevine, *P. phytofirmans* PsJN has been reported to promote plant development, to improve tolerance to chilling and confer resistance against *Botrytis cinerea*.²¹⁻²³ The PsJN-associated tolerance to low temperature is related to enhanced expression of the stress-related genes stilbene synthase (*STS*) and phenylalanine ammonia-lyase (*PAL*), as well as the expression of genes encoding PR proteins (*Chit4c*, *Chit1b* and *Gluc*) and lipoxygenase (*LOX*).²⁴ The PsJN-induced resistance against *B. cinerea* is associated with primed expression of defence genes (*PR1*, *PR2*, *PR5*) and jasmonic acid-related genes (*JAZ*).²² Furthermore, it has been shown that *P. phytofirmans* PsJN is able to colonize the grapevine root interior 3 h after inoculation, and was detected in aerial tissues 84 h later.²⁵ This beneficial bacteria is a possible new biocontrol agent

against the pathogenic fungi that cause GTDs. The investigation of the interaction of systemic agrochemicals and biocontrol agents in colonizing woody tissues is an important prerequisite to successfully develop an integrated approach for the control of GTDs.

The purpose of the present work was to (i) study the translocation and distribution of phloem-mobile derivatives of fenpiclonil in grapevine plants and (ii) test *in vivo* the effect of the systemic fungicide and *P. phytofirmans* PsJN, separately and in combination, on the pathogenic fungus *Neofusicoccum parvum* and on the plant response by evaluating the expression of defence-related genes. Additionally, acidic compounds are usually formulated as esters to facilitate their uptake by plants and then hydrolysed to the active acidic molecules inside the plants because of their poor ability to penetrate the plant cuticle.²⁶ One carboxylic methyl ester of the acidic fenpiclonil derivative (SM 26, Fig. 1) was selected as the precursor compound. Here, we investigate the systemicity of SM 26 and its synergistic activity with PsJN in a simple grapevine model.²⁷

2 MATERIALS AND METHODS

2.1 Chemicals

The detailed synthesis of the carboxylic methyl ester SM 26 (purity > 99%) and its acidic derivative F 30 (Fig. 1) were previously described.¹⁵

2.2 *In vitro* tests

2.2.1 Antagonistic activity of SM 26 against *N. parvum* strain *Bourgogne*

Potato dextrose agar (PDA) medium was prepared and sterilized. Individual 100-fold concentrated SM 26 solutions were prepared by dissolving in absolute ethanol, and added to the medium to obtain the final concentrations ranging from 10 to 1000 μ M once it had cooled to 55 °C. Then the PDA medium was poured into petri dishes. The control consisted only PDA with 1% ethanol, namely, 1% EtOH. The plates were inoculated with agar plugs (3 mm diameter) from a 5-day-old growing

colony of plant pathogen NpB and incubated in the dark at 28 °C. The assays were performed twice with 3 plates per condition. The colony diameter was measured at 1, 2, 3, 4, 7, 15 and 28 days after confrontation by using ImageJ 1.50b software (National Institutes of Health, USA). In 7 days, NpB control covers the whole plate. Therefore, for 7, 15 and 28 days, the plate diameter was considered as the NpB control to estimate the inhibition of NpB growth in the presence of SM 26. The inhibitory effect of each isolate was calculated based on the relative mycelium growth inhibition (MI) through the formula $MI\% = ((Mfg-Mga)/Mfg) \times 100$, where Mfg corresponds to the diameter of the mycelium in the untreated group (without 1% EtOH and SM 26) and Mga to the diameter of mycelium growth in the presence of SM 26 at a given concentration.

2.2.2 Antagonistic activity of SM 26 against *P. phytofirmans* PsJN

Paraburkholderia phytofirmans PsJN was cultivated in liquid King's B medium under agitation (180 rpm at 28 °C). After 24 h, the bacterial density was measured by spectrophotometry at 600 nm and adjusted to obtain an optical density of 0.05. Five concentrations of SM 26 were tested: 10, 50, 100, 500 and 1000 µM, along with a control containing 1% of ethanol since SM 26 was solubilized in ethanol with a final concentration of 1%. The bacterial density was measured at 24 h post treatment with SM 26. The culturing of the bacteria (10^2 and 10^3 CFU) on King's B solid medium was duplicated at days 0 and 1 to verify that the cultures were free of contamination. Each concentration was tested in triplicate, and the experiment was repeated twice.

2.2.3 In vitro metabolization of SM 26 by *P. phytofirmans* PsJN

The bacteria were cultivated in liquid medium containing three concentrations of SM 26 (50, 100 and 500 µM) in three replicates. After 24 h of incubation, the bacterial cells in the culture medium were separated by centrifugation (4500 rpm for 15 min) and washed three times with 20 mL phosphate-buffered saline (PBS), which was removed each time by centrifugation for 15 min at 4 °C. The bacterial cells and culture medium were rapidly freeze-dried and stored at -80 °C until extraction. The freeze-dried bacterial cells were extracted using 5 mL acetonitrile, and the mixtures

were heated to reflux for 1 h. After evaporation under reduced pressure at 40 °C, the residue was redissolved in 0.5 mL acetonitrile and filtered through a 0.45 µm nylon membrane filter. For the culture medium, the samples were dissolved with acetonitrile/H₂O solution (50/50, V/V) and then placed in an ultrasonic bath for 8 min. The SM 26, its acid metabolite F 30 and fenpiclonil in the bacterial cells and culture medium were easily separated and quantified by HPLC with a UV/Vis photodiode-array detector at 218 nm. We employed reverse-phase chromatography using an Ascentis Express RP-amide C16 column (5 µm particle size; 4.6 × 250 mm; Supelco, France) with a mobile phase flow rate of 0.8 mL.min⁻¹. To achieve complete separation of the ester and the acidic parent compounds, a mobile phase composed of acetonitrile and 0.1% TFA aqueous solution (50/50, V/V) was used. The results were processed with PC 1000 software v.3.5 from Thermo Fisher Scientific (Courtaboeuf, France).

2.3 *In planta* assays

2.3.1 *Plant materials*

A simple grapevine model was used for plant bioassays as recently described.²⁷ This study focused on two grapevine cultivars, Chardonnay and Sauvignon, globally known for their low and high susceptibility to GTDs, respectively.^{2,28} Cuttings were taken from 15-year-old pruned woody stems collected from established vineyards in January after a cold period. The three-node-long grapevine (*Vitis vinifera* L., cv. Chardonnay and Sauvignon) cuttings were prepared and kept in a cold chamber at 4 °C for 1 month. Cuttings were surface-sterilized with 0.05% cryptonol (8-hydroxyquinoline sulfate) and rooted as previously described.²⁹ Briefly, the cuttings were placed in Gramoflor Special soil (Gramoflor GmbH & Co. KG, Vechta, Germany) using 350 mL pots in a culture chamber (25 °C day/night, 60% relative humidity, and 16 h photoperiod at 400 µmoles.m⁻².s⁻¹) and watered twice a week. Only the cuttings that developed roots were used for further experiments. The plants remained in the same growing conditions for the future experiments described below.

2.3.2 *In vivo* artificially inoculated plant assays

The experiments consisted of four conditions: non-inoculated plants (namely, control), plants treated with SM 26 only (namely, SM 26), plants inoculated with *P. phytofirmans* only (namely, PsJN) and plants treated with a combination of *P. phytofirmans* and SM 26 (namely, PsJN + SM 26). According to the method described,^{27, 30} a plug containing the phytopathogen NpB or a PDA plug (control) was artificially inoculated onto the third internode of green stems. For the PsJN treatment, the roots were inoculated twice with 30 mL of fresh *P. phytofirmans* solution at 10^7 CFU.g⁻¹ of soil at 28 and 21 days before the artificial green stem inoculation by the phytopathogen NpB (Fig. 2). For the control, the roots were inoculated with PBS solution (pH 7.5) that was used to suspend the bacteria. SM 26 was applied to the cuttings (described in § 2.3.4) 2 days before artificial inoculation with NpB (Fig. 2). Each condition included a total of 19 plant replicates, and the experiment was repeated twice.

2.3.3 *Evaluation of green stem necrosis and reisolation of N. parvum strain Bourgogne*

Sixty days after inoculation with the phytopathogen NpB, 10 plants from each treatment group were used for visual evaluation of the external necrosis of green stems and for the reisolation of NpB (Fig. 2). The external lesions of the wood cuttings were measured for their width and length from the inoculation wound, and the reisolation process was carried out from green stems at the artificial inoculation spot (necrotic tissues) and at 1 cm both above and below this area, according to the protocol reported by Larignon and Dubos (1997)³¹ and adapted by Pinto et al. (2018).¹⁹

2.3.4 *SM 26 mobility and metabolism in grapevine*

SM 26 was applied with a small brush onto the upper and lower surfaces of leaves that were above and below the site of inoculation. Two leaves (L3 and L4; Fig. 2) were treated. To facilitate the uptake of SM 26, the leaves were pretreated with the same procedure using a solution of 0.05% Agral 90 (Syngenta France SAS, 945 g. L⁻¹

nonylphenol polyethoxyle), which is a nonionic surfactant used in the agrochemical industry. After 4 h, SM 26 was applied at a final concentration of 5 mM in a solution composed of 25% ethanol and 0.05% Agral 90 (Fig. 2). Approximately 250 μL per leaf was applied. For the examination of SM 26 movement and metabolism, different parts of the grapevine cuttings (the third young leaf from the top of the green shoot, which was designated the young leaf; phloem and xylem from woody stems; and roots) were separately isolated 16 days after treatment (Fig. 2). To separate the xylem and phloem, the epidermis was first eliminated, and then under binocular magnification, the xylem and phloem were collected separately. Six plants per condition were individually collected and analysed. The samples were rapidly freeze-dried overnight and stored at $-20\text{ }^{\circ}\text{C}$ until extraction.

The freeze-dried samples were ground and extracted in 10 mL acetonitrile. The mixtures were heated to reflux for 1 h. Then, the samples were centrifuged (4000 rpm for 5 min) and washed three times with 5 mL acetonitrile. The combined supernatants were evaporated under vacuum at $40\text{ }^{\circ}\text{C}$. The residue was redissolved in 1 mL acetonitrile and filtered through a $0.45\text{ }\mu\text{m}$ nylon membrane filter prior to LC-MS/MS analysis. The analysis and quantification of SM 26 and its metabolites in plant extracts are not possible using the previous simple HPLC method described in § 2.2.3 and were performed using a Waters Acquity UPLC system equipped with a Xevo TQD Triple Quadrupole Mass Spectrometer. Chromatographic separation was performed on an ACQUITY UPLC BEH C18 column ($1.7\text{ }\mu\text{m}$ particle size, 2.1×100 mm, Waters). The mobile phase consisted of 0.1% formic acid in MilliQ water (A) and acetonitrile (B), using the following gradient in a total run time of 13 min: 0.0 to 3.0 min, holding at 80% A and 20% B; 3 to 8 min, conversion to 10% A and 90% B; 8 to 10 min, holding at 10% A and 90% B then returning to 80% A and 20% B at 10.5 min and equilibration for an additional 2.5 min. The flow rate was $0.2\text{ mL}\cdot\text{min}^{-1}$, and the injection volume was $10\text{ }\mu\text{L}$. The optimized MS parameters were as follows: the capillary voltage was set at 2.5 kV, source temperature at $600\text{ }^{\circ}\text{C}$ and desolvation gas flow at $1000\text{ L}\cdot\text{h}^{-1}$. Quantification was performed by multiple reaction monitoring (MRM). The retention time, cone voltage, MRM transition and collision energy for all

analytes are listed in Table 1.

2.4 Molecular analyses

The experiments were performed as described in § 2.3.2. At the kinetic point 4 days after NpB infection (Fig. 2), two leaf samples (L5 and L8) were collected from three plants among the nineteen under the following conditions: control (C, plants without treatment), uninfected plants treated either with SM 26 or PsJN or the combination SM 26 + PsJN, plants only infected by NpB without treatment and plants infected by NpB and treated by SM 26 or PsJN or the combination SM 26 + PsJN. Total RNA was isolated from 2 x 50 mg of leaf tissues collected 4 days after Np infection using a Plant RNA Purification Reagent (Invitrogen, Cergy Pontoise, France). The RNA pellet was resuspended in 20 µL of RNase-free water, treated with RQ1 DNase enzyme (Promega, Charbonnières les bains, France) and quantified by measuring the absorbance at 260 nm following the manufacturer's instructions. In total, 150 ng of total RNA was reverse-transcribed using the Verso SYBR 2-step QRT ROX enzyme (ABgene, Surrey, UK) according to the manufacturer's protocol. The PCR conditions were as described by Bézier et al.³² The expression of 17 targeted genes was tracked by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using the primers indicated in Table 2. The panel included genes encoding proteins related to (i) secondary metabolism, including stilbenes (*PAL*, *STS*), epicatechin (*CHI*, *ANR*), indoles (*ANTS*) and salicylic acid (*VvI7.3*) pathways, (ii) (PR)-proteins (*PR1*, *PR6*, *PR10*) and other defence proteins (*CHI1B*, *CHI4C*, *PGIP*), (iii) proteins involved in redox status (*GST1*) as well as one enzyme involved in ethylene synthesis (*ACC*) and one of the membrane lipids processes (*LOX*) as described by Dufour et al.³³ Two housekeeping genes were used as the internal standard to normalize the starting template of the cDNA (*EF1* and *60SRP*). Reactions were carried out in a real-time PCR detector Chromo 4 apparatus (Bio-Rad) using the following thermal profile: 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing/extension) for 40 cycles. Melting curve assays were performed from 65 to 95 °C at 0.5 °C.s⁻¹. Melting peaks were visualized to check the specificity of each

amplification. The results are expressed as relative expression values and are the means from three independent experiments, i.e. 3 plants. The genes analysed were considered significantly up- or downregulated when the changes in their expression were $>2\times$ or $<0.5\times$ compared to that of the 1x control (C), respectively.

2.5 Statistical analyses

The non-parametric Kruskal-Wallis test coupled with Dunn's multiple comparison test was used to compare the data from the *in vitro* study of SM 26 metabolism and the *in vivo* artificially inoculated assays, assuming a significance of $p \leq 0.05$.

3 RESULTS

3.1 SM 26 mobility and metabolism *in planta*

A systemicity test of SM 26 on grapevine cuttings was performed to investigate its uptake, translocation and distribution after foliar application. The LC-MS/MS analysis showed that SM 26 and its metabolites (F 30 and fenciclonil) could be detected in all parts of the grapevine cuttings (Fig. 3), especially the compounds were translocated towards the young leaves and root. The total amount of the compounds (SM 26, F 30 and fenciclonil) in the young leaves was approximately 12 times higher than that in the roots. The distribution of SM 26 and its metabolites above and below the site of application indicated that SM 26 was a systemic compound with both phloem and xylem mobility, which was also confirmed by the LC-MS/MS determination of the isolated phloem and xylem tissues (Fig. 3).

As a known phenomenon of ester hydrolysis, the prodrug behaviour of SM 26 was observed in grapevine. SM 26 was hydrolysed by esterase to the corresponding carboxylic acid F 30 within plants. Sixteen days after treatment with SM 26, the ratio of F 30 / SM 26 in the young leaf, phloem and xylem was higher than that in the roots (36.6%, 37.0% and 36.4% vs 7.4%). Furthermore, the active parent compound fenciclonil was observed to be slowly released in grapevine tissues over 16 days,

which was not found in the short-term experiment (5 h in *Ricinus* plants).¹⁵ The highest release rate of fenpiclonil was 41.8% of the total amount, which was obtained in phloem tissues. Under the experimental conditions used, SM 26 therefore behaved like an ambimobile profungicide.

3.2 *In vitro* SM 26 activity against the pathogen *Neofusicoccum parvum* strain Bourgogne

In vitro tests were carried out to evaluate the antifungal activity of SM 26 on colonies of the Botryosphaeria dieback agent NpB growing on solid culture medium (Fig. 4a). For untreated group (without 1% EtOH and SM 26), NpB entirely filled the Petri dish in one week (data not shown). The effect of SM 26 on the growth of NpB colonies was already perceptible after 1 day of culture, especially at the 500 and 1000 μM concentrations, where an inhibition of mycelial growth of approximately 60% was noted (Fig. 4a). Moderate inhibition of approximately 40% was noted from the second day after the beginning of the test for the 50 and 100 μM concentrations. This activity was maintained for approximately two weeks with the same intensity for the two highest concentrations tested and for one week with the 50 and 100 μM concentrations. However, NpB growth then rebounded, and after four weeks, it was comparable to that of the control 1% EtOH for the 10, 50 and 100 μM groups and was only inhibited by 20% for the 500 and 1000 μM groups (Fig. 4a). It should be noted that even the lowest concentration used for the *in vitro* tests was higher than that of SM 26 and its metabolites detected in plant systemicity assays, suggesting that the current application dose of SM 26 was not able to exert a direct effect on NpB *in vivo*.

3.3 *In vitro* SM 26 activity and metabolism in the presence of *P. phytofirmans* PsJN

Experiments were designed to investigate the possible effects of SM 26 on the PGPR *P. phytofirmans* strain PsJN::gfp2x in liquid culture (Fig. 4b). Using the same concentrations as in the *in vitro* test against NpB, no effect on the growth of *P. phytofirmans* PsJN was noted during the classical 24-hour kinetic experiment typically used for these types of bacteria (Fig. 4b). Further studies were conducted to

quantify SM 26 and its metabolites in bacterial cells and liquid culture. The results are shown in Table 3. Surprisingly, a very high proportion of SM 26 was found in the bacterial cells, which was approximately one third of the initial quantity applied 24 h after incubation. The acidic metabolite F 30 and fenpiclonil were not detected in the bacterial cells. On the other hand, both SM 26 and F 30 were detected in the culture medium, and no fenpiclonil was detected in all samples (Table 3). The proportions of F 30 varied according to the initial concentration of SM 26. When the culture medium contained lower concentrations of SM 26 (50 and 100 μM), the proportion of F 30 metabolized in the culture medium was approximately 4 to 5 times higher than that of the detected SM 26. However, the proportion of F 30 and SM 26 was found to be equal when the initial SM 26 concentration was 500 μM in the culture medium. It could be speculated that the enzymatic system that allows the methyl ester SM 26 to be cleaved into its acidic metabolite F 30 was saturated under our experimental conditions.

3.4 Artificial inoculation of rooted cuttings

The lesion sizes observed in the control group were always limited to the wound made for the inoculation with sterile PDA (Table 4). In Chardonnay (Table 4), the largest lesion sizes were found on the plants inoculated with NpB ($71.0 \pm 56.9 \text{ mm}^2$), whereas the smallest necroses were observed on the plants treated with the combination of PsJN and SM 26 ($23.9 \pm 13.1 \text{ mm}^2$). For NpB + PsJN + SM 26 treatment, the lesion sizes did not differ significantly from the uninfected control, which indicated a beneficial effect of the cotreatment in reducing pathogen-induced necroses. Unfortunately, no significant differences in necrosis sizes were found between groups for NpB and NpB + PsJN + SM 26, despite a 3-fold difference in the mean of lesion sizes existed between the two groups. This may be due to the great heterogeneity and variability of the group NpB ($71.0 \pm 56.9 \text{ mm}^2$). In Sauvignon, the lesion sizes of PsJN or SM 26 treated groups were almost the same as that of plants inoculated with NpB. As for Chardonnay, the necrosis sizes observed for the plants receiving the combined treatment of PsJN and SM 26 were not significantly different

from those in uninoculated control, confirming a positive effect of PsJN + SM 26. Moreover, the dual treatment PsJN + SM 26 induced a significant decrease of the lesion size compared to the untreated group NpB (Table 4). NpB was always reisolated from the edges of the lesions associated with its artificial inoculation (data not shown); thus, Koch's postulates were fulfilled for both Chardonnay and Sauvignon, i.e., the development of the lesions was due to the pathogen but not to the wound.²⁷

3.5 Molecular impacts of SM 26 with or without *P. phytofirmans* PsJN on grapevine cuttings infected or not by *N. parvum* strain Bourgogne

The data summarized in the Table 5 represent the leaf response four days after NpB infection. For Chardonnay, the expression of most of the targeted genes in plant leaves was slightly induced after SM 26 treatment or the bacterization by PsJN with levels of induction between 2.00 and 4.39 (*GLUC* at SM 26 condition) (Table 5a). For the combination of PsJN and SM 26, the expression of these genes was highly induced, especially for *STS* (14.03), *PAL* (8.64), *GLUC* (25.49), *PR6* (9.06) and *PGIP* (9.31). When the treated plants were infected by NpB, 9 out of 17 targeted genes were also induced with a level of gene expression higher than 2. The strongest responses were still observed in the co-treatment group with 12 out of 17 genes up-regulation (*STS*, *PAL*, *CHI*, *GST1*, all PR protein and defense protein analysed except *PGIP*). Nevertheless, increases in gene expression showed a lower intensity under experimentally infected conditions than under the conditions without NpB as for example *STS* with 5.08 in NpB + PsJN + SM 26 and 14.03 in PsJN + SM 26. For Sauvignon, the expression of genes was lower under all conditions than in Chardonnay (Table 5b). Remarkable inductions of some defence-related genes, *PAL* (6.51), *GTSI* (20.03), *PGIP* (8.46) and *VvI7.3* (15.39), were especially induced in NpB + SM 26-treated plants. No synergistic-like effect as observed for Chardonnay was reported in Sauvignon under the same experimental conditions, and the trend of a downregulation was inverse, especially for *CHIIB* (for NpB + PsJN + SM 26: 2.11 in Chardonnay, 0.55 in Sauvignon), *CHI4C* (for NpB + PsJN + SM26: 6.42 in

Chardonnay, 0.37 in Sauvignon) and *PR6* (for NpB + PsJN + SM26: 6.27 in Chardonnay, 0.27 in Sauvignon).

4 DISCUSSION

The present study showed that the ester derivative SM 26, which is the methyl ester of the F 30 acid derivative of the fungicide fenpiclonil, displayed ambimobile movement in grapevine plants, translocating from the site of application to the young leaves at the top of shoots and to the roots. However, combining the findings of the plant systemicity assays and *in vitro* tests, the dose of SM 26 and its metabolites in grapevines was not enough to achieve a direct antagonistic effect on pathogenic fungi under our experimental conditions. Further structural optimization is needed, especially for the degradable spacer groups in the profungicide.

On the other hand, SM 26 inhibited the fungal growth of NpB in the preliminary *in vitro* evaluation at different doses (from 50 to 1000 μM) but did not show any inhibitory effect on the growth of *P. phytofirmans* PsJN::gfp2x, even at the highest dose of 1000 μM . This was not surprising because the parent compound fenpiclonil is a phenylpyrrole fungicide, an analogue of pyrrolnitrin that is an antifungal antibiotic produced by *Pseudomonas* bacteria.³⁴ Phenylpyrroles were reported to inhibit a protein kinase (PK-III) that regulates the glycerol synthesis in fungi, resulting in an abnormal accumulation of glycerol in fungal mycelia.³⁵ The same glycerol synthesis signal transduction system has not been reported in bacteria. Furthermore, after 24 h incubation with SM 26, only 40-50% (SM 26 and its metabolites) of the original dose was found in the bacterial cells and culture medium. This may be due to metabolic pathway diversity in bacteria,³⁶ SM 26 is likely transformed by PsJN to other unknown metabolites. Anyway, the absence of an inhibitory effect on PsJN enabled us to carry out further experiments *in vivo* to study the effects of SM 26 and PsJN on the development of NpB after artificial inoculation as well as on plant responses as planned.

The *in vivo* activity of SM 26 was evaluated alone or in the presence of PsJN.

The results of artificial infection with NpB on rooted cuttings of Chardonnay and Sauvignon showed that the highest reduction in lesion sizes was obtained when SM 26 was applied in combination with PsJN. This is the first report of the beneficial effect of PsJN against a GTD pathogen by reducing necrosis. Hence, further studies on the mechanisms that are responsible for the synergistic effect of SM 26 and PsJN are needed. This response could be correlated with an increase in plant immunity by PsJN, as described in other plant-pathogen interactions.^{20,37} In addition, *in vitro* tests showed no antagonistic effect of PsJN on NpB growth (data not shown) that eliminates the direct effect of PsJN on NpB development. PsJN is inoculated in the soil, stays mainly in the rhizosphere but can also colonize the plant roots, especially for *in vitro* cultured plantlets, thus it is considered as an endophyte.^{21,24}

Comparative expression analysis of targeted genes was thus performed during the plant defence responses following the different treatments. It was found that a slight induction of defence-related genes occurred in Chardonnay when the sole treatment with PsJN (*GLUC*, *CHIIB*) or SM 26 (*STS*, *CHIIC*, *GLUC*, *PR6*, *PR10*) was performed. The reason for this finding could have been the features of the single treatments: PsJN may exert an already known priming effect,²² while the tenuous eliciting outcome observed in the case of SM 26 could be the result of a slight toxic effect on the plant. For example, the phenylpyrrole derivative tralopyril, which is the active metabolite of the pro-insecticide chlorfenapyr, showed significant phytotoxicity when applied to plants.³⁸ In addition, chemical stress has been found to coordinately induce the expression of β -1,3-glucanase and chitinase.³⁹ In SM 26 or PsJN plants, the slight induction of some defence responses seems not enough to result in a significant decrease of the necrosis compared to NpB infected plants. Conversely, both the significant decrease of lesion sizes and the strong activation of the most selected defence-related genes in PsJN + SM 26-treated cuttings of Chardonnay indicated a synergistic-like effect of the combined treatments, leading to the speculation that defense priming by PsJN herein was able to activate a strong defence response after the SM 26 treatment. However, this outcome was likely stronger in uninfected plants than in the NpB-infected plants. This behaviour, as well as the absence of a strong

activation of defence responses in NpB + PsJN and NpB + SM 26 plants, might be the consequence of an impairing effect of NpB toxins⁴⁰ that is stronger than the eliciting activity of PsJN and/or SM 26. The synergistic effect was only slightly visible in Sauvignon uninfected plants after transcript analysis; this result was therefore in contrast with the reduced lesion sizes observed in PsJN + SM 26-treated plants. The reason for this discrepancy may be the fact that the leaf sampling for transcript analysis was carried out four days after artificial inoculation with NpB, whereas lesion sizes were measured 60 days after fungal inoculation (dpi). Hence, defence responses in Sauvignon cuttings might be activated later than in Chardonnay cuttings, being therefore visible in terms of lesion sizes at 60 dpi, but not in terms of gene expression at 4 dpi. This hypothesis was supported by the fact that defence responses in grapevine cultivars less susceptible to GTDs are faster and stronger than those in more susceptible cultivars.^{2, 41, 42} In fact, based on observations in French^{43, 44} and European² vineyards, Sauvignon is generally considered among those cultivars more susceptible to GTDs, while Chardonnay ranks among the less susceptible cultivars, even if herein with our simple model, Chardonnay showed greater necrosis than Sauvignon. Looking at the detailed results of the transcript analysis of Sauvignon, notable gene induction was only detected in NpB + SM 26 plants. Considering this trend with the absence of the synergistic effect in Np-infected plants together leads to the hypothesis that the delayed eliciting effect of PsJN represents the underlying cause of a delayed activation of defence responses in cuttings of Sauvignon. Another hypothesis could be the non-optimal selection of the targeted genes for Sauvignon compared to Chardonnay. For example, Spagnolo et al. reported a specific protein pattern in response to *Botryosphaeria dieback* expression for Chardonnay, Gewurztraminer and Mourvèdre.⁴²

The panel of genes analysed in this study was chosen with the objective of obtaining complementary indications of the possible differential activation of the signaling pathways depending on the condition. For Chardonnay, the expression of the targeted genes associated with secondary metabolism (*ANTS*, *PAL*, *STS*, *CHI*, *ANR*, *Vv17.3*) as well as the redox status (*GTS1*), membrane lipids (*LOX*) and

ethylene synthesis (*ACC*) was strongly upregulated in a the PsJN + SM 26 groups, with or without NpB. These responses seem to be cultivar-dependent since the expression levels were lower in Sauvignon PsJN + SM 26 control plants and not detectable in the presence of NpB. The induction of such pathways by beneficial microorganisms to control GTD pathogens was recently reported by Trotel-Aziz et al¹⁸ using *Bacillus subtilis* against Np and by Pinto et al¹⁹ for *Aureobasidium pullulans* against *Diplodia seriata*, another GTD pathogen. *P. phytofirmans*, like other potentially beneficial microorganisms could efficiently attenuate Botryosphaeria dieback by enhancing some host immune responses.^{18, 19, 45}

The combined use of PGPR and agrochemicals has been reported to provide higher control efficacy and wider control spectrum than the use of either strategy alone in other studies.^{46, 47} For example, combined application of hymexazol with PGPR resulted in significantly higher protection against *Fusarium* crown and root rot on tomato than single controls alone.⁴⁷ For the first time, we report that the combined use of a systemic phenylpyrrole fungicide and PGPR can stimulate the defence responses of grapevine within cutting model. Such similar synergistic effects may provide a new possibility for integrated control of plant pathogens and would merit to further investigations with grafted-plants and under semi-controlled conditions up to a final validation in the field.

5 CONCLUSION

In conclusion, our results indicate that a potential integrated control strategy against GTDs might be achieved by the combined treatment with a systemic fenpiclonil derivative (SM 26) and *P. phytofirmans* (strain PsJN::gfp2x) in Chardonnay and Sauvignon. Notably, under our experimental conditions, this ester compound showed an ambimobile movement *in planta*. Meanwhile, its profungicide properties were evidenced by detecting the corresponding acidic derivative and the parent molecule fenpiclonil in all parts of the plant. The strong activation of host immune responses in Chardonnay, especially for defence-related genes and

phenylpropanoid pathways, was observed after PsJN + SM 26 treatment and resulted in the highest control efficiency against the GTD pathogen *N. parvum* strain Bourgogne. The interaction of systemic pesticides with endophytic microorganisms in plants has started to attract research attention in recent years, leading to new ideas for the design and development of systemic pesticides.⁴⁸ Further investigations are needed to improve our understanding of the mechanisms of interaction between PGPRs and systemic fungicides that occur in plants.

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FIGURE LEGENDS

Figure 1. Chemical structure of fenpiclonil and two derivatives bearing an acid (F 30) or ester (SM 26) function.

Figure 2. Design of experiments to study the biological activity of the systemic fungicide SM 26, with or without the plant growth-promoting rhizobacteria *P. phytofirmans* strain PsJN::*gfp2x*, on cuttings of Chardonnay and Sauvignon artificially inoculated with *N. parvum* strain Bourgogne (NpB). First, plants were inoculated with *P. phytofirmans* PsJN via a two-time soil application (T0 – 28 d and T0 – 21 d), then treated with 5 mM SM 26 solution (T0 – 2 d) via application on leaves 3 (L3) and 4 (L4) and finally artificially infected by NpB at the third internode (T0). At T0 + 4 d, two leaves (L5 and L8) were collected for molecular analysis. At T0 + 16 d, different parts of the plant (the third apical leaf, xylem and phloem from the woody stems, roots) were collected for SM 26 detection. Finally, at T0 + 60 d, the surfaces of the necrosis induced by NpB were measured. d: days.

Figure 3. SM 26 translocation and metabolism in grapevines after foliar application on L3 and L4. The different parts of the grapevine (3rd apical leaf, phloem and xylem of the woody stem, roots) were isolated 16 days after treatment. LC-MS/MS was used to quantify SM 26 and its metabolites F 30 and fenpiclonil in the samples. Mean \pm SE, n = 6.

Figure 4. Effect of SM 26 on the colony growth of *N. parvum* strain Bourgogne (NpB) over 28 days on solid culture medium **(a)** and *P. phytofirmans* strain PsJN::*gfp2x* over 24 h in liquid culture **(b)**. SM 26 concentrations ranging from 10 to 1000 μ M (dissolved in 1% ethanol, final concentration) were tested, and the control contained 1% ethanol. **(a)**: Mean \pm SE; n = 6. **(b)**: The same experiment was conducted a second time with almost identical results.

Table 1. Mass spectrometer (MS/MS) parameters for MRM of SM 26 and its metabolites.

Compound	Retention time (min)	Electrospray ionization	Cone (V)	MRM transition: parent ion > fragment ion (<i>m/z</i>)	Collision energy (V)
SM 26	8.9	+	25	323 > 228	25
				323 > 263 ^Q	20
F 30	7.3	-	25	307 > 201 ^Q	20
				307 > 227	15
Fenpiclonil	8.3	-	25	235 > 163	30
				235 > 199 ^Q	25

^Q indicates the MRM transition used for quantification of the compounds.

Table 2. Primers of genes analysed by real-time reverse-transcription polymerase chain reaction.

Genes	Primer sequences	Genbank or TC TIGR accession number
<i>EF1</i> (EF1- α elongation factor)	5'-GAACTGGGTGCTTGATAGGC-3' 5'-AACCAAAATATCCGGAGTAAAAGA-3'	GU585871
<i>60SRP</i> (60S ribosomal protein L18)	5'- ATCTACCTCAAGTCCTAGTC-3' 5'-CAATCTTGTCTCCTTTTCCT-3'	XM_002270599
<i>ANTS</i> (anthranilate synthase beta subunit)	5'-GGGGTGCTTATATCCCCAGG-3' 5'-TCCCTCCAAAAGCTTCTCCG-3'	XM_010650272.2
<i>PAL</i> (phenylalanine ammonia lyase)	5'-TCCTCCCGGAAAACAGCTG-3' 5'-TCCTCCAAAATGCCTCAAATCA-3'	X75967
<i>STS</i> (stilbene synthase)	5'-AGGAAGCAGCATTGAAGGCTC-3' 5'-TGCACCAGGCATTTCTACACC-3'	FJ851185
<i>CHI</i> (chalcone isomerase)	5'-GCAGAAGCCAAAGCCATTGA-3' 5'-GCCGATGATGGACTCCAGTAC-3'	XM_002282072
<i>ANR</i> (anthocyanidin reductase)	5'-GGTTCAGTCTCATTGCACATG-3' 5'-TTGGCAGCACAGATGTAT-3'	XM_002271336
<i>GSTI</i> (glutathion S-transferase)	5'-TGCATGGAGGAGGAGTTCGT-3' 5'-CAAGGCTATATCCCCATTTTCTC-3'	AY156048
<i>ACC</i> (1-aminocyclopropane-1-carboxylate oxidase)	5'-AAGGTCAGCAACTACCCTCC-3' 5'-CGCATCGGTGGAACATCAAT-3'	XM_002273394.3
<i>LOX</i> (lipoxygenase)	5'-CCCTTCTTGGCATCTCCCTTA-3' 5'-TGTTGTGTCCAGGGTCCATTCC-3'	AY159556
<i>AOS</i> (allene oxide synthase)	5'-CACCCAATTAGCCCAGGAGA-3' 5'-AGAGTCGTGGCTTTTCGATCA-3'	XM_002283744.3
<i>CHIIb</i> (class I basic chitinase)	5'-ATGCTGCAGCAAGTTTGTT-3' 5'-CATCCTCCTGTGATGACATT-3'	Z54234
<i>CHIIc</i> (class IV chitinase)	5'-TCGAATGCGATGGTGGAAA-3' 5'-TCCCCTGTGAAACACCAAG-3'	AY137377
<i>PR1</i> (pathogenesis-related protein)	5'-GGAGTCCATTAGCACTCCTTTG-3' 5'-CATAATTCTGGGCGTAGGCAG-3'	XM_002273752.3
<i>GLUC</i> (β -1,3 glucanase)	5'-TCAAATGGCTGCAATGGTGC-3' 5'-CGGTCGATGTTGCGAGATTTA-3'	DQ267748
<i>PR6</i> (serine-protease inhibitor 6)	5'- AGGGAACAATCGTTACCCAAG-3' 5'- CCGATGGTAGGGACACTGAT-3'	AY156047
<i>PR10</i> (pathogenesis related protein 10)	5'-CGTTAAGGGCGGCAAAGAG-3' 5'-GCATCAGGGTGTGCCAAGA-3'	XM_002274206
<i>PGIP</i> (polygalacturonase inhibiting protein)	5'-GTTTGACGTCGTTGGACCTT-3' 5'-CACCGGAATCTTACCACACA-3'	NM_001281177.1
<i>Vv 17.3</i> (unknown function, Salicylic Acid marker gene)	5'-GTACCATCAGACCACCCATAAGTAGTG-3' 3'-AGACCAACGGCAAATCAAGTG-3'	XM_002283642.1

Table 3. SM 26 metabolism *in vitro* with *Paraburkholderia phytofirmans* (strain PsJN::gfp2x). SM 26 and its metabolites F 30 and fenpiclonil detected in samples 24 h after incubation were expressed as the percentage of the initial quantity of SM 26 applied.

Sample	Initial concentration of SM 26	Percentage of SM 26 detected (%)	Percentage of F 30 detected (%)	Percentage of fenpiclonil detected (%)
Bacterial cells	50 μ M	26.5 \pm 2.8 ^a	0	0
	100 μ M	33.0 \pm 3.2 ^a	0	0
	500 μ M	34.7 \pm 3.0 ^a	0	0
Culture medium	50 μ M	3.7 \pm 2.1 ^b	18.7 \pm 0.4 ^c	0
	100 μ M	2.1 \pm 0.6 ^b	9.0 \pm 0.4 ^{cd}	0
	500 μ M	3.3 \pm 0.3 ^b	3.2 \pm 1.2 ^d	0

Values are expressed as the means \pm CI 95% for 3 independent experiments for each concentration. Differences among the means were evaluated by the Dunn's multiple comparison test after that the null hypothesis (equal means) was rejected in the Kruskal–Wallis test, assuming a significance of $p \leq 0.05$. Means with the same superscript letter within a column are not significantly different.

Table 4. Effect of different treatments after artificial inoculation with *Neofusicoccum parvum* strain Bourgogne (NpB) on the stems of rooted cuttings Sauvignon and Chardonnay. Lesion sizes were measured 60 days after the inoculation with NpB (mean \pm 95% CI). To check the effectiveness of NpB contamination, a control was performed by inoculating the cuttings with the sterile culture medium (Potato Dextrose Agar) without NpB. Three treatments were evaluated: inoculation of the roots with *Paraburkholderia phytofirmans* strain PsJN::gfp2x (NpB + PsJN), foliar application of the profungicide SM 26 (NpB + SM 26) and cotreatment with PsJN and SM 26 (NpB + PsJN + SM 26). Two comparisons were made: i/ plants infected with NpB (treated and untreated) *versus* the uninoculated control; ii/ plants infected with NpB and treated with PsJN or SM 26 or PsJN + SM 26 *versus* plants infected with NpB and untreated. Differences among the means were evaluated by the Dunn's multiple comparison test after that the null hypothesis (equal means) was rejected in the Kruskal–Wallis test, assuming a significance of $p \leq 0.05$. S: significant; NS: non-significant. Each condition included a total of 10 plant replicates.

Grapevine cultivars		Control (without NpB)	NpB	NpB + PsJN	NpB + SM 26	NpB + PsJN + SM 26
Chardonnay	Lesion size (mm ²)	6.8 \pm 2.0	71.0 \pm 56.9	42.3 \pm 19.0	41.9 \pm 18.8	23.9 \pm 13.1
	Infected sets vs Control	-	S $p = 0.0138$	S $p = 0.0125$	S $p = 0.0121$	NS $p = 0.2813$
	Treated sets vs NpB	-	-	NS $p > 0.9999$	NS $p > 0.9999$	NS $p = 0.5864$
Sauvignon	Lesion size (mm ²)	6.6 \pm 1.8	29.6 \pm 7.3	29.0 \pm 12.2	24.8 \pm 14.1	12.2 \pm 3.0
	Infected sets vs Control	-	S $p = 0.0001$	S $p = 0.001$	S $p = 0.0107$	NS $p = 0.4135$
	Treated sets vs NpB	-	-	NS $p > 0.9999$	NS $p = 0.4680$	S $p = 0.0078$

Table 5. Expression levels of selected genes in leaves (L5 and L8) of rooted cuttings of Chardonnay (a) and Sauvignon (b) with or without PsJN and/or SM 26 treatment and inoculated with sterile PDA (C + SM 26, C + PsJN, C + PsJN + SM 26) or with *N. parvum* strain Bourgogne (NpB, NpB + PsJN, NpB + SM 26, NpB + PsJN + SM 26). Expression levels were measured four days after the Np infection. Values represent the expression levels under the reported conditions relative to those under the control (C). The results correspond to the means of three plants and are from one representative replicate among two independent experiments showing the same trends. Expression of a given gene was considered up- or downregulated when the value of relative expression was < 2 or > 0.5 as compared to the control, respectively.

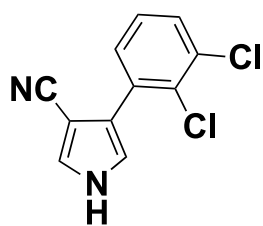
a

	Genes	SM26	PsJN	PsJN + SM26	NpB	NpB + SM26	NpB + PsJN	NpB + PsJN + SM26
Secondary metabolism	ANTS	1.24	0.96	2.77	1.27	1.66	1.35	1.46
	STS	3.00	1.84	14.03	3.96	2.34	0.82	5.08
	PAL	2.14	1.58	8.64	1.85	2.80	1.06	11.94
	CHI	2.19	1.82	3.25	1.59	6.83	1.54	4.57
	ANR	1.67	1.09	4.31	1.22	7.11	1.72	1.80
Redox status	GST1	2.46	0.84	3.69	1.04	0.52	0.40	3.91
Ethylene	ACC	1.38	1.95	2.74	2.13	1.49	1.79	1.34
Membrane Lipids	LOX	2.03	1.13	3.65	2.03	1.64	2.04	1.34
PR protein and defense protein	AOS	2.10	1.61	5.38	2.28	4.03	2.94	3.47
	CHI1B	1.59	2.77	3.58	3.62	1.67	2.50	2.11
	CHI4C	4.21	1.79	4.64	3.97	2.34	1.07	6.42
	GLUC	4.39	4.96	25.49	5.68	2.10	2.65	7.40
	PR1	0.24	0.76	2.13	0.39	0.02	0.70	4.23
	PR6	2.43	1.78	9.06	6.63	2.10	4.18	6.27
	PR10	2.64	1.00	1.88	0.67	0.77	0.78	5.34
	PGIP	1.41	1.17	9.31	1.74	1.42	1.70	1.34
	Vv17.3	1.85	3.76	3.30	2.82	1.87	3.23	2.11

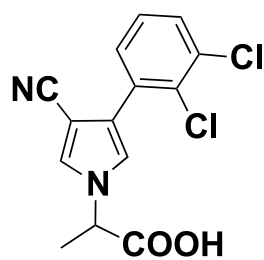
b

	Genes	SM26	PsJN	PsJN + SM26	NpB	NpB + SM26	NpB + PsJN	NpB + PsJN + SM26
Secondary metabolism	ANTS	1.57	1.44	3.41	1.17	1.28	1.18	1.31
	STS	0.76	0.57	1.85	0.73	5.32	0.56	1.28
	PAL	1.18	0.74	1.36	0.65	6.51	0.42	0.98
	CHI	1.93	1.84	1.51	0.94	1.75	0.65	1.80
	ANR	5.20	4.03	3.67	0.86	2.49	1.02	1.64
Redox status	GST1	0.46	1.03	0.36	0.74	20.03	1.02	1.34
Ethylene	ACC	1.19	0.94	2.66	0.92	0.73	1.06	0.87
Membrane Lipids	LOX	1.57	1.14	1.31	0.72	2.62	1.06	1.42
PR protein and defense protein	AOS	2.24	1.44	2.76	1.19	2.22	1.22	1.82
	CHI1B	0.64	0.78	2.22	1.06	0.91	0.80	0.55
	CHI4C	0.73	0.49	0.93	1.11	3.10	0.76	0.37
	GLUC	1.33	0.93	2.94	0.87	2.56	1.04	1.32
	PR1	1.71	1.24	1.63	1.29	1.80	1.78	1.29
	PR6	0.33	0.32	1.14	0.16	0.97	0.29	0.27
	PR10	0.75	0.57	1.01	1.76	1.43	0.98	0.76
	PGIP	3.66	1.72	2.65	1.12	8.46	1.37	1.31
	Vv17.3	1.60	1.40	6.57	1.70	15.39	1.19	1.56

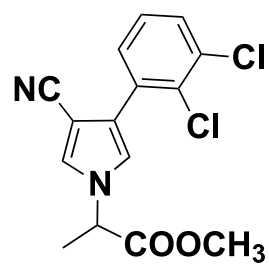




Fenpiclonil



F 30



SM 26

Figure 1. Chemical structure of fenpiclonil and two derivatives bearing an acid (F 30) or ester (SM 26) function.

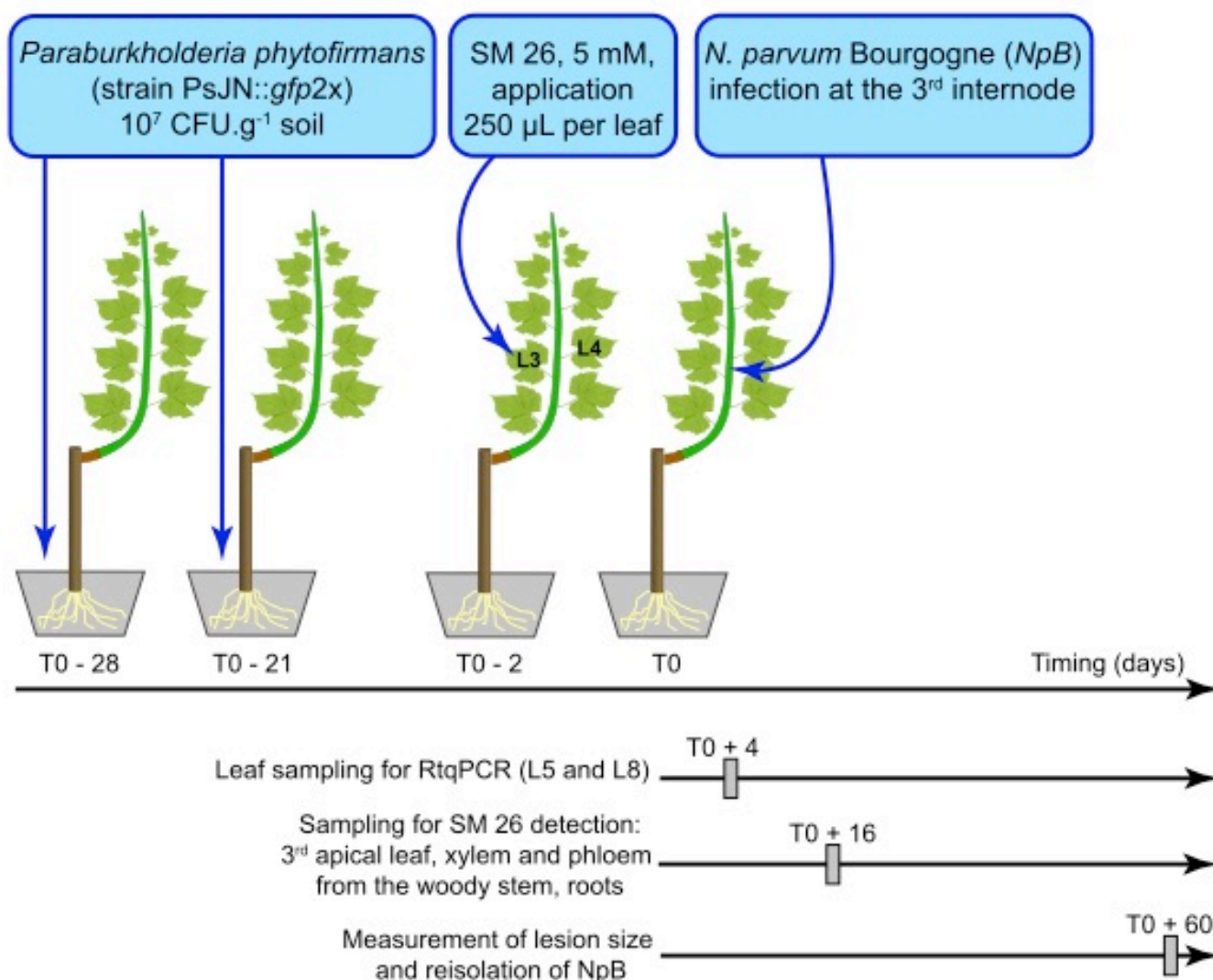


Figure 2. Design of experiments to study the biological activity of the systemic fungicide SM 26, with or without the plant growth-promoting rhizobacteria *P. phytofirmans* strain PsJN::gfp2x, on cuttings of Chardonnay and Sauvignon artificially inoculated with *N. parvum* strain Bourgogne (NpB). First, plants were inoculated with *P. phytofirmans* PsJN via a two-time soil application (T0 - 28 d and T0 - 21 d), then treated with 5 mM SM 26 solution (T0 - 2 d) via application on leaves 3 (L3) and 4 (L4) and finally artificially infected by NpB at the third internode (T0). At T0 + 4 d, two leaves (L5 and L8) were collected for molecular analysis. At T0 + 16 d, different parts of the plant (the third apical leaf, xylem and phloem from the woody stems, roots) were collected for SM 26 detection. Finally, at T0 + 60 d, the surfaces of the necrosis induced by NpB were measured. d: days.

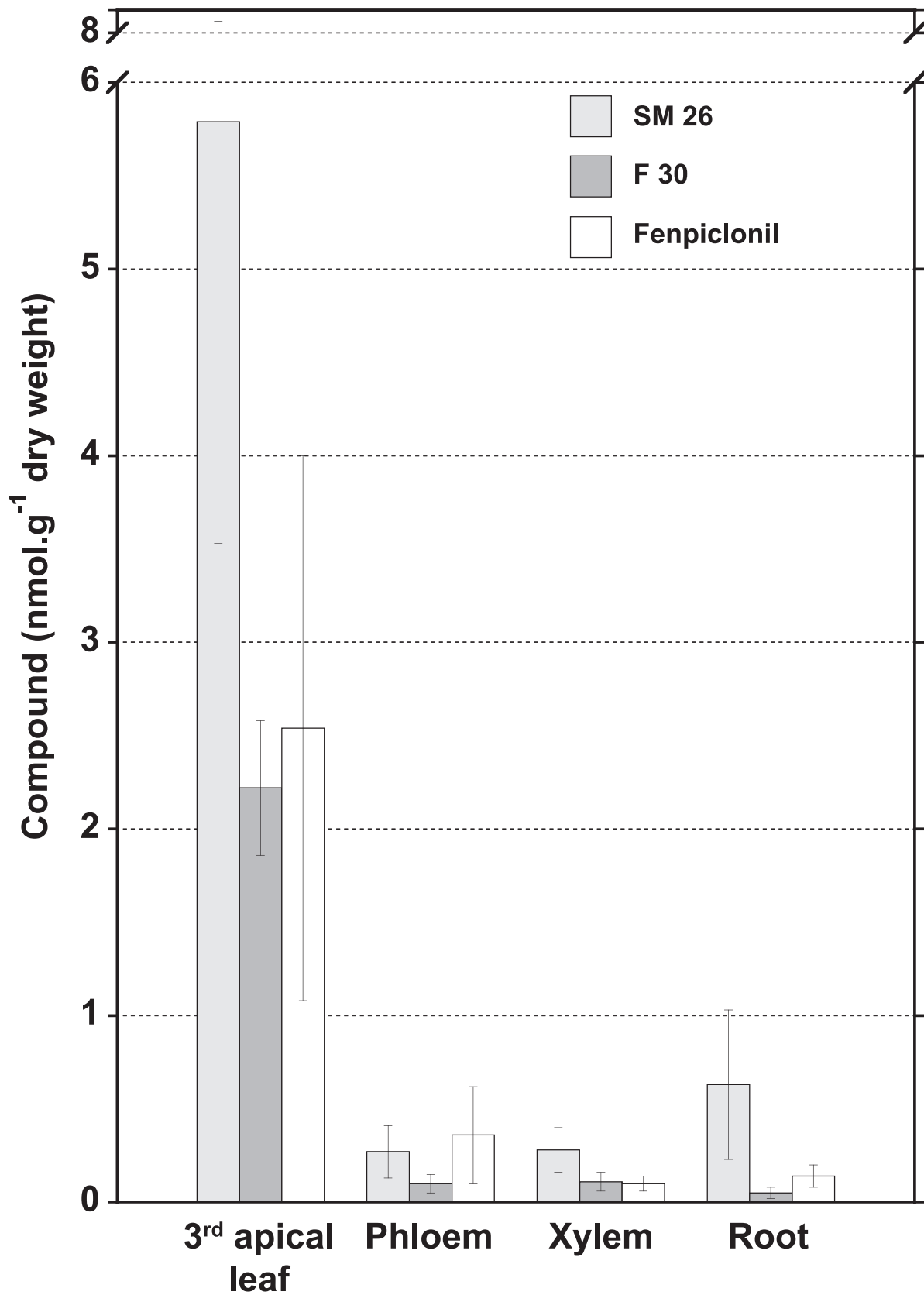


Figure 3. SM 26 translocation and metabolism in grapevines after foliar application on L3 and L4. The different parts of the grapevine (3rd apical leaf, phloem and xylem of the woody stem, roots) were isolated 16 days after treatment. LC-MS/MS was used to quantify SM 26 and its metabolites F 30 and fenpiclonil in the samples. Mean \pm SE, n = 6.

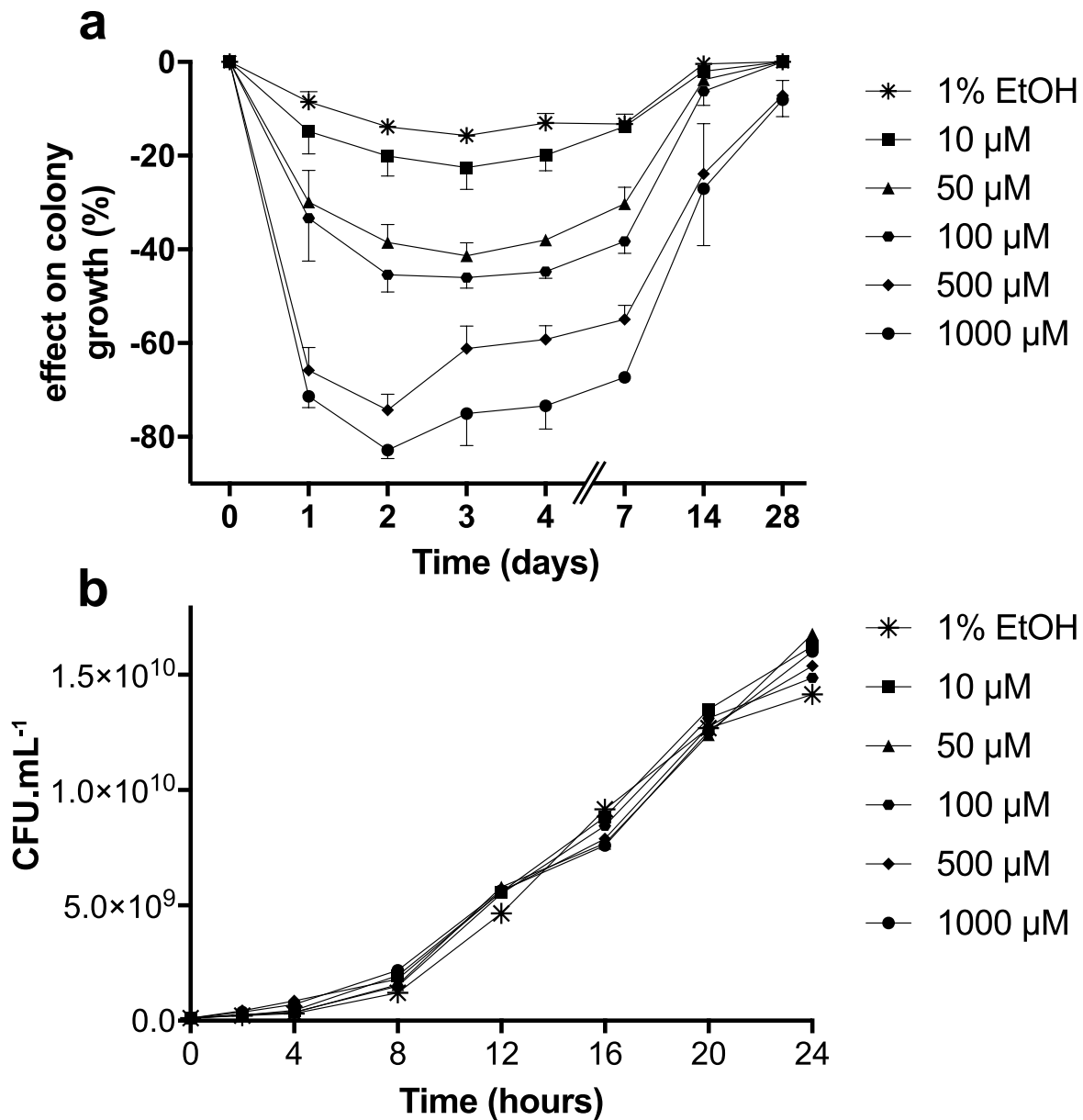


Figure 4. Effect of SM 26 on the colony growth of *N. parvum* strain Bourgogne (NpB) over 28 days on solid culture medium (a) and *P. phytofirmans* strain PsJN::gfp2x over 24 h in liquid culture (b). SM 26 concentrations ranging from 10 to 1000 μ M (dissolved in 1% ethanol, final concentration) were tested, and the control contained 1% ethanol. (a): Mean \pm SE; n = 6. (b): The same experiment was conducted a second time with almost identical results.