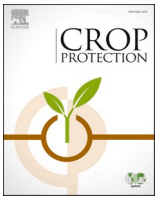
Crop Protection 176 (2024) 106472

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro



Protective and curative effect of an experimental biocomplex against Flavescence dorée phytoplasma infection and transmission by *Scaphoideus titanus* under laboratory conditions

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ARTICLE INFO

Keywords:
Phytoplasma vector
Grapevine
Systemic penetration
Antibacterial activity
Transmission efficiency

ABSTRACT

Flavescence dorée (FD) is one of the most devastating diseases threatening grapevine cultivation in Europe. The causal agents are phytoplasmas, which reside in phloem vessels of infected plants and in the body of leafhopper vectors, the most important being *Scaphoideus titanus*. Current control mainly relies on vector management, which is often insufficient to prevent from disease outbreaks. Suitable methods to interfere with phytoplasmas *in planta* are constantly sought to support insecticide sprays. We tested a biocomplex containing trace elements and soluble sugars, with potential systemic spread and antimicrobial activity, with the aim to verify its capability to penetrate plant phloem and to interfere with phytoplasma survival in vector-inoculated plants, using two FD isolates (FD-C and FD-D). The penetration into the plant phloem, as well as its acquisition by phloem-feeding vectors, were tested using an iridium-marked biocomplex. Phytoplasma infection was detected by real Time PCR and compared in plants exposed to biocomplex treatment before inoculation (short-time and mid-time preventive application), and to treatment after inoculation (curative application). We also assessed if the biocomplex interferes with the vector competence of *S. titanus,* by conducting phytoplasma transmission trials with sequential inoculation steps, where untreated and biocomplex-treated grapevine plantlets were sequentially offered to infectious leafhoppers. Foliar treatment with the biocomplex supported the penetration of phloem tissues, and the product was successfully acquired by the experimental phytoplasma vector *Euscelidius variegatus* after feeding on the phloem of treated plants. When the biocomplex was applied on grapevines, it induced a significant reduction of phytoplasma infection regardless of FD isolate, both as a preventive and curative treatment, indicating an antibacterial activity in the plant. Moreover, sequential inoculation experiments showed a decrease of *S. titanus* inoculation rates after vector exposure to a biocomplex-treated plant, suggesting a reduction of transmission efficiency that was higher using the FD-C rather than FD-D isolate. Our results indicate the tested biocomplex as a promising tool for the creation of anti-phytoplasma formulations to support vector management in FD containment.

1. Introduction

Phytoplasmas are wall-less bacteria residing in the plant phloem tissues and in the body of phloem-feeding insects, which are responsible for their transmission from plant to plant (Namba, 2019). Infected plants display a variety of symptoms related to the impairment of sieve tubes functionality, carbon consumption and the release of effector molecules that induce responses (e.g., suppression of flowering, expression of immunity factors) that result in vector attraction (Lewis et al., 2022). In many plant species, major symptoms are colour alteration, such as yellowing or reddening, causing a general decline and often the complete loss of production, or even the plant death (Bertaccini 2022). One of the plants in which phytoplasma infections determine acute damage is grapevine. The most relevant phytoplasmas in grapevine belong to the 16SrV taxonomic group, subgroups –C and -D, and cause a disease called Flavescence dorée (FD). The FD phytoplasma (FDp) is native to the

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https://doi.org/10.1016/j.cropro.2023.106472
Received 10 August 2023; Received in revised form 11 October 2023; Accepted 12 October 2023
Available online 18 October 2023

Palearctic region; however, its main vector is the Nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera, Cicadellidae), whose introduction in Europe in the first half of the 20th Century resulted in the appearance of FD outbreaks (Chuche and Thiéry, 2014; Malembic-Maher et al., 2020). Presently in Europe, FD is a major threat for grapevine, causing severe yield and quality losses (Belli et al., 2010). Infected vines show leaf rolling, yellowing or reddening, stunted growth, unripe cane wood and shrivelled berries (Oliveira et al., 2019), leading to severe economic losses due to missed production and to cost for replacement of dead plants. To date, no resistant grapevine cultivars have been found, although a range of susceptibility levels have been detected according to the plant genotype (Eveillard et al., 2016; Ripamonti et al., 2021).

To date, no curative methods are available for direct phytoplasma control, as only the use of antibiotics is resolutive in eliminating them from the plant phloem, yet resulting in serious damages of plant tissues (Bertaccini, 2021); moreover the use of antibiotics in agriculture is not allowed in several countries (Stockwell and Duffy, 2012). The use of admitted products, such as naturally-derived molecules or resistance inducers, has been proposed to reduce symptom development in crops (Oliveira et al., 2019; Bertaccini 2021; Rastegar et al., 2021). However, these systems do not allow actual pathogen reduction and have been exploited at small scale only. Therefore, the production of phytoplasma-free nursery stocks (e.g., by hot water treatment) and vector management are still the most important available tools to contain phytoplasma-borne diseases. As for FDp, which is a quarantine pathogen in Europe, compulsory measures are applied to limit the disease spread, including the rouging of infected plants and spray of insecticides against *S. titanus* (Oliveira et al., 2019). However, the recent ban of several active ingredients, and the consequent limited number of available insecticide substances, are recently resulting in decreased success of control campaigns; additionally, possible insecticide resistance is likely to further threaten the insect containment (Prazaru et al., 2023).

The search for new anti-phytoplasma molecules, less impacting than antibiotics, is regarded as a key step to improve phytoplasma control (Bertaccini, 2021). However, an efficient system to interfere with phytoplasmas *in planta* is difficult to obtain, thus hardening the actual phytoplasma inhibition of many potentially effective molecules or biotic agents. A crucial point that currently limits the capability to contain phytoplasmas inside the plant is the need to reach phloem vessels, where phytoplasmas are restricted. Seeking for effective substances capable of addressing these requirements to contain the spread of phytoplasmas, in this work we tested the biocomplex prototype formulation PRC2022 (Pireco Productie B.V., The Netherlands). The prototype is an acid formulation containing trace elements and sugars whose chemical forms potentially allows systemic delivery. Moreover, literature data regarding the biocomplex composition indicate that it may support the plant nutritional status and defence, interfering with development of pathogens (Kalia et al., 2020; Tahjib-Ul-Arif et al., 2021; Jeandet et al., 2022; Tripathi et al., 2022). Therefore, we aimed at assessing the anti-phytoplasma activity of the biocomplex in the plant phloem and in the body of phloem-feeding vectors. We set up a sequence of experiments to determine if: i) the biocomplex effectively reaches the phloem of plants; ii) the product co-occurrence with FDp in grapevine results in an inhibitory activity against phytoplasma survival, due to the antimicrobial effect displayed by its components (Kalia et al., 2020; Tahjib-Ul-Arif et al., 2021; Jeandet et al., 2022; Tripathi et al., 2022); iii) phytoplasma vectors are able to acquire the biocomplex following phloem ingestion of a treated plant; iv) the biocomplex interferes with FDp in *S. titanus*, reducing the transmission rates.

2. Materials and methods

2.1. Plant and insect source

Phytoplasma-free broad bean (*Vicia faba* L. cv. Aguadulce) seedlings and micropropagated grapevines (*Vitis vinifera* L.) were maintained under an insect-proof screen until used for experiments testing biocomplex penetration into the phloem and the effect on FDp transmission. The cultivar Barbera was selected as it is one of the grapevine varieties most susceptible to FD (Ripamonti et al., 2021). Two phytoplasma infected potted grapevines (cv. Barbera) were also used in this study. The potted vines were experimentally infected with two phytoplasma isolates, namely *map* genotypes M51 and M54 (Malembic-Maher et al., 2020), belonging to the 16SrV-C and –D taxonomic groups, respectively (hereafter FD-C and FD-D). Infections were achieved by exposing them to infectious *S. titanus* specimens, which were previously maintained for at least one month on other infected potted grapevines from the plant collection established at DISAFA (University of Torino), whose infection status was previously checked by molecular diagnosis. Infected plants were kept in separated cages for the whole experiment duration, and phytoplasma infection was periodically checked by Real Time PCR.

Two FDp vector species were used in this work, namely the experimental vector *Euscelidius variegatus* (Kirschbaum) (Hemiptera, Cicadellidae), used as a model species for FDp transmission (Salar et al., 2013), and the field vector *S. titanus*. A phytoplasma-free colony of *E. variegatus* was maintained under laboratory conditions on oat plants (*Avena sativa* L., cv. Aveny) in a climatic chamber at 25 °C, photoperiod 16 L:8 D, to be used for the experiments testing biocomplex penetration into the phloem. Healthy *S. titanus* nymphs were obtained by rearing nymphs emerging from grapevine canes containing eggs (2-year-old wood, or older), since there is no evidence of transovarial transmission for FDp in vectors (Alma et al., 1997). Grapevine canes were collected during winter from 2019 to 2021 in several Piedmontese grapevine growing areas (in the municipalities of Vaglio Serra, Asti province; Piverone, Torino province; and Clavesana, Cuneo province) where abundant *S. titanus* populations were reported during the previous years. Canes were cut in pieces of approximately 20 cm length, placed in plastic bags, periodically sprinkled to avoid desiccation of eggs, and preserved into a cool chamber (+4 °C) before use. The twigs were placed into BugDorm® (NHBS Ldt, Devon, UK) insect rearing cages (47.5 × 47.5 × 138 cm) at springtime, along with potted broad bean plants to support insect development until they reached the third nymphal instar and were used for FDp transmission trials. The cages were kept either outdoors, under a shelter against rain and sunlight, from 15 May to 1 September, or into a greenhouse from 15 March to 15 May and from 1 September to 15 November, and periodically sprinkled.

2.2. Experiment 1: phloem penetration by the biocomplex and acquisition by phloem-feeding leafhoppers

To verify if PRC2022 was capable of entering the plant phloem tubes, two separate experiments were conducted using an iridium-enriched (Ir-enriched) lot of the prototype biocomplex. The Ir-enriched lot was experimentally produced by the manufacturer (Pireco Productie B.V.) and had the same composition of the prototype, but with the addition of Ir to reach a concentration of 1.2 g/L. The use of Ir as a marker was selected because it is one of the rarest elements in nature (Pinto et al., 2021), and it is almost absent in living organisms. In addition, it can be safely absorbed by plants and animals, showing some toxicity only after long-term accumulation, and it is easily detectable with no interference (Wang et al., 2007; Kabata-Pendias and Mukherjee, 2007).

In the first experiment, batches of four two-week old broad bean plants (*Vicia faba* L.) were isolated and sprayed with one of the following compounds: a 0.5 mL/L of the Ir-enriched biocomplex (final concentration 0.6 mg Ir/L); a 0.5 mL/L of PRC2022; distilled water containing 0.6 mL/L of iridium atomic absorption standard solution 1000 μg/mL (Merck KGaA, Darmstadt, Germany); distilled water. After the foliar treatment, plant batches were separately maintained in insect-proof net cages (930 × 475 × 475 mm) placed in climatic chambers (25 ± 1 °C, L: D 16:8) for 24 h. Subsequently, plants were transferred to the laboratory,

where they were washed with distilled water. At least 20 leaves with petioles were collected for each plant batch, and submitted to phloem exudates collection according to Tetyuk et al. (2013). Briefly, leaf petioles were dipped in a 20 mM K_2-EDTA solution in a microcentrifuge tube, and incubated for 1 h. Then, they were washed in Milli-Q water and transferred in new microcentrifuge tubes containing Milli-Q water for 4 h. Finally, the leaves were removed from tubes and the resulting exudates were dried using a centrifugal evaporator (Genevac SF50, Genevac Ltd., UK). Dried samples were weighted and used for quantitative analysis of Ir. Four replicates were performed for each plant treatment.

A second experiment was also set up to measure the capability of phloem feeding insects to ingest the biocomplex from phloem sap. This experiment was conducted with the same conditions as explained above, but 24 h after the treatments the broad beans were accurately washed with distilled water and subsequently they were offered to groups of 200 adults of the phloem feeding phytoplasma vector *E. variegatus* (Caudwell et al., 1972), which were reared on these plants for 24 h. Subsequently, insects were collected and frozen at −80 °C; then they were powdered using liquid nitrogen. The obtained powder was dried at 60 °C for 48 h. Dried samples were weighted and used for Ir quantification. Two replicates were performed for each plant treatment.

Phloem exudates and insect samples were submitted to microwave Nitric-Peroxide digestion using a START D Microwave Digestion System (Milestone S.r.l., Sorisole BG Italy) and analysed to evaluate quantitative Ir amount with ICP-MS (inductively coupled plasma mass spectrometry) using a Nexion 350d (PerkinElmer, Waltham, MA, USA). The analysis was performed according to EPA 200.8 and ISO 17294-2:2016 methods.

2.3. Experiment 2: FDp transmission trials on biocomplex-treated grapevines

Two separate sets of transmission trials were set up using grapevines infected with FD-C and FD-D phytoplasmas as acquisition sources. For each pathogen isolate, groups of 50 *S. titanus* third instar nymphs were fed with the infected potted vine in an insect-proof cage made of nylon mesh and polyethylene film (40 × 40 × 30 cm), and kept outdoors under a shading coverage. An acquisition access period of 7 days was applied, followed by a latency period of 21 days on the same plants (total time on infected vines: 28 days). Afterwards, the insects were transferred into a climatic chamber (25 ± 1 °C, L:D 16:8) for FDp inoculation. Specifically, batches of 3–5 infected *S. titanus* were placed on a single micropropagated grapevine plantlet caged inside a Plexiglas cylinder (h = 20 cm; diameter = 12 cm), for an inoculation access period of 7 days. Before being used, grapevines were divided into four groups corresponding to the following treatments: i) short-time preventive biocomplex application; ii) mid-time preventive biocomplex application; iii) curative application; iv) no treatment (control). Preventive applications were applied to test if the enhanced plant nutritional and immune status provided through biocomplex application (Kalia et al., 2020; Tahjib-Ul-Arif et al., 2021; Jeandet et al., 2022; Tripathi et al., 2022) reduced the probability of phytoplasma infection. Curative application was applied to test if the biocomplex occurring in the phloem vessel after the treatment was capable of interfering with phytoplasma survival. For treatments i) to iii), a 0.5 mL/L solution of PRC2022 was used. In i), the solution was sprayed onto the grapevine plantlets, and then the plants were maintained in an insect-proof cage for 24 h before being exposed to FDp inoculation. In ii), the plants were left in the insect-proof cage for five days after biocomplex delivery, and subsequently exposed to FDp inoculation. In iii) the plants were exposed to FDp inoculation prior to be sprayed with the biocomplex. In iv), the plants were only used for FDp inoculation and never treated with PRC2022. All treatments were replicated 10 times and repeated with both FDp isolates. During the inoculation access period, plants were checked daily, and dead specimens were periodically collected. At the end of the experiments, *S. titanus* specimens were collected and preserved at −20 °C before molecular analyses. Inoculated plants were treated with an insecticide (Etofenprox, TREBON® UP, SIPCAM ITALIA S.p.A., Italy; 0.5 mL/L) to avoid insect infestations during the breeding period, and they were kept inside an insect-proof cage in a climatic chamber for three weeks from the beginning of phytoplasma inoculation. At the end of this period, leaf vein samples were collected from all plants for molecular analyses.

2.4. Experiment 3: effect of the biocomplex on the transmission efficiency of S. titanus

To test if the vector inoculation efficiency was altered after feeding on a plant treated with the biocomplex, FDp transmission trials with the FD-C and FD-D isolates were separately set up, with sequential inoculation steps. In detail, acquisition access period and latency period were conducted with the same conditions as described above, after that insects where submitted to 3 sequential inoculation periods (24 h each), named IAP1-3. For IAP1, batches of 3–5 specimens were left on a micropropagated caged plantlet for 24 h. For IAP2, the same insect batches were moved to a second grapevine plantlet, which had been treated with a 0.5 mL/L solution of PRC2022 24 h earlier, and they were maintained on this plant for further 24 h. For IAP3, the same insects were moved to a new untreated grapevine plantlet for other 24 h. The experiment was replicated 10 times, and repeated with both FDp isolates. At the end of the trials, insects and plants were treated as explained above prior to be used for molecular analyses.

2.5. DNA extraction and Real Time PCR

Total DNA was extracted both from whole, single insects used in transmission experiments and from inoculated grapevine samples. DNA extraction from *S. titanus* was carried out according to Marzachì et al. (1998). Plant DNA was extracted from leaf tissue previously ground with liquid nitrogen in a sterile mortar, according to the DNeasy Plant Pro Kit protocol instructions (Qiagen, Milan, Italy). Real-time PCR was carried out to measure the presence of 16Sr-V group phytoplasmas in insect and plant samples. Reactions were performed in a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad, United States) with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). Reactions targeting the 16S rRNA gene of group 16SrV phytoplasmas were carried out on all samples by using the fAY/rEY primer pair (Marcone et al., 1996; Marzachì et al., 2001), with the conditions described by Galetto et al. (2005). Amplicons showing a quantification cycle (Cq) > 30 were considered as negative.

2.6. Statistical analyses

Statistical analyses were performed with SPSS Statistics 27 (IBM Corp. Released, 2020; Armonk, NY, USA). Each insect batch and the corresponding inoculated plant(s) were considered as a separate replicate to calculate the FDp inoculation rate in plants and vectors infection rate corresponding to each treatment. The plant inoculation rates were expressed as the number of FDp-positive plant samples out of the total number of grapevine samples in the same experiment. As well, vector infection rates were expressed as the number of FDp-positive insects out of the total number of *S. titanus* samples in the same experiment. These rates were compared using a generalized linear model (GLM) with a binomial probability distribution—with positive samples marked as “1”, and negative samples as “0”—and a Bonferroni post hoc test ($P < 0.05$).

3. Results

3.1. Experiment 1:detection of the biocomplex in plant phloem and in insects

ICP-MS analysis performed on the phloem exudates collected from broad beans and from insect bodies showed the occurrence of Ir only in

samples treated with enriched water or biocomplex, in agreement with the almost total absence of this element in organic tissues. Despite the abundance of Ir was always higher in samples exposed to Ir-enriched biocomplex, in the phloem exudate no significant difference was found, suggesting that water can penetrate the vascular tissues of the plant via foliar spray, as well as the biocomplex. However, the abundance of Ir in insect samples exposed to Ir-enriched biocomplex was almost five times higher than in enriched water (Table 1). Being *E. variegatus* a phloem feeder (Caudwell et al., 1972; Bosco et al., 1997), the increased amount of Ir in insect bodies confirms the plant localization of the biocomplex in the phloem sap and the ingestion by the vector.

3.2. Experiment 2: effect of treatment with the biocomplex on FDp in grapevine

In total, 145 and 144 *S. titanus* specimens were used for transmission experiments using FD-C and FD-D phytoplasmas, respectively. Insects used for untreated controls showed similar infection rates for both isolates, as 27 out of 39 samples from FD-C and 23 out of 35 samples from FD-D tested positive for the plant pathogen according to Real Time PCR, with an average infection rate among batches of 70.50% and 66.33%, respectively. All replicates contained at least one infected insect, and effective inoculation was obtained for 90% and 80% of grapevine plantlets of the control batches using FD-C and FD-D isolates, respectively. For both phytoplasmas, all the applied treatments caused a dramatic drop of successfully inoculated grapevines compared to controls according to binomial GLM (Fig. 1A, Table S1), with no significant differences among groups submitted to different biocomplex applications. Two out of ten grapevine plantlets tested positive for the phytoplasma after short-time and mid-time preventive applications in experiments using both phytoplasma isolates, three positive plants were found after curative treatment in experiments with FD-C and none in those with FD-D. The phytoplasma load in inoculated plants was also reduced in all treatments and with both FD isolates, with a raise of recorded Cq from ~22 in controls to ~29 in treated samples (Table S2). In contrast, only preventive applications resulted in a significant reduction of phytoplasma infection in *S. titanus* compared to controls, according to binomial GLM (Fig. 1B and C, Table S1). As for FD-C experiments, both short-time and mid-time preventive treatments decreased insect infection, with 15 phytoplasma-positive samples out of 34 (average infection rate among batches: 42.67%) and 13 positive samples out of 35 (average infection rate among batches: 36.67%) for short-time and mid-time preventive applications, respectively. In trials with FD-D phytoplasma, only the short-time preventive treatment resulted in reduced infection in *S. titanus*, with 15 phytoplasma-positive samples out of 37 (average infection rate among batches: 40.17%), whereas after the mid-time preventive application of the biocomplex we found 16 phytoplasma-positive samples out of 33 (average infection rate among batches: 50.00%), which was not significantly divergent from the control. Curative applications, where exposure to the biocomplex occurred after insect removal, were never related to a significant reduction of phytoplasma infection. Indeed, after curative treatment, 17 phytoplasma-positive samples out of 35 (average infection rate among batches: 47.33%) and 27 positive samples out of 39 (average infection rate among batches: 69.00%) were found in experiments using FD-C and FD-D isolates, respectively. These values were not significantly different from those obtained in controls, where we found 27 phytoplasma-positive samples out of 39 (average infection rate among batches: 70.50%) in experiments with FD-C and 23 positive samples out of 35 (average infection rate among batches: 66.33%) using FD-D. The average phytoplasma load in positive insect samples seemed rather stable among treatments and FD isolate, with mean Cq values ranging from 22.87 to 24.39 (Table S2).

Table 1
Detected abundance of the iridium tracker in the phloem exudate collected from broad bean plants and from dried *E. variegatus* samples. For each sample type, different letters refer to significantly different values according to normal GLM followed by sequential Bonferroni pairwise comparisons ($P < 0.05$).

Treatment	Mean μg Ir/g of phloem exudate (*V. faba*) ± SE	μg Ir/g of insect sample (*E. variegatus*) ± SE
Ir-enriched PRC2022	44.30 ± 5.14 **a**	13.3 ± 0.61 **a**
Ir-enriched H_2O	33.41 ± 7.61 **a**	2.83 ± 0.63 **b**
PRC2022	<0.005 **b**	<0.005 **c**
H_2O	<0.005 **b**	<0.005 **c**

3.3. Experiment 3: effect of the biocomplex on the transmission efficiency of S. titanus

In total, 126 *S. titanus* specimens were employed for transmission experiments with sequential inoculation steps, equally divided between FD-C and FD-D trials. When the second plantlet (corresponding to IAP2) was treated with biocomplex before *S. titanus* inoculation, a consistent decrement of successfully inoculated grapevines was detected from IAP1 to IAP3 with both phytoplasma isolates (Fig. 2A). Conversely, the inoculation rates underwent a consistent growth from IAP1 to IAP3 with both phytoplasma isolates in controls, where all grapevine plantlets offered to *S. titanus* for inoculation were untreated (Fig. 2A). As a consequence, in IAP1 no significant differences were observed between plant groups inoculated with the same phytoplasma isolate, whereas a difference was found between treatments using FD-D in IAP2 and using FD-C in IAP3 (Table S1). The Cq recorded in Real Time PCR for inoculated plants were quite uniform among IAPS and FD isolates. Comparing these Cq values with those obtained in the previous experiment (where a longer inoculation was performed), they were generally closer to the values from biocomplex-treated plants that to controls, suggesting a moderate phytoplasma load (Table S3). The recorded opposite trends between batches with treated IAP2 plant and controls were not related to any significant divergence in the vector infection rates at the end of the experiments, according to binomial GLM (Fig. 2B, Table S1). Indeed, the average *S. titanus* infection rate among batches ranged from 40.00% (in FD-C experiments with biocomplex-treated IAP2 plant, with 12 positive samples out of 31) to 59.17% (in FD-C control experiments, with 19 out of 32 positive insects). In experiments with FD-D phytoplasma, the average *S. titanus* infection rate among batches was 58.33% in trials with biocomplex-treated IAP2 plant (18 positive insects out of 31) and 41.67% in controls (14 out of 32 positive insects). Similarly to the previous experiment, the Cq detected by Real Time PCR in positive insects was quite uniform in different treatments and FD isolates (Table S3).

4. Discussion

The search for antimicrobials that effectively limit phytoplasma infection *in planta* is crucial for the implementation of plant protection programs against FD. The first requirement for a putative anti-phytoplasmal active substance is the capability to reach phloem vessels in the plant. We demonstrated that foliar treatment with the prototype biocomplex PRC2022 supports its penetration into phloem tissue. Indeed, we found a significantly higher concentration of the enriching agent Ir in the exudates from plants treated with enriched substances than in unenriched treatments. Despite no significant differences were found between exudate samples from plants exposed to Ir-enriched biocomplex and in those treated with enriched water, Ir had a concentration more than three times higher in the former group than in the latter. Since water penetration via the leaf surface is a slow process subject to high resistance (Riederer, 2006; Guzmán-Delgado et al., 2018), only a little amount of Ir is expected to be introduced through the water itself. The systemic capability of the biocomplex, allowed by its composition (Bromilow et al., 1990), may have supported the increase of penetration in the samples treated with the enriched biocomplex. It

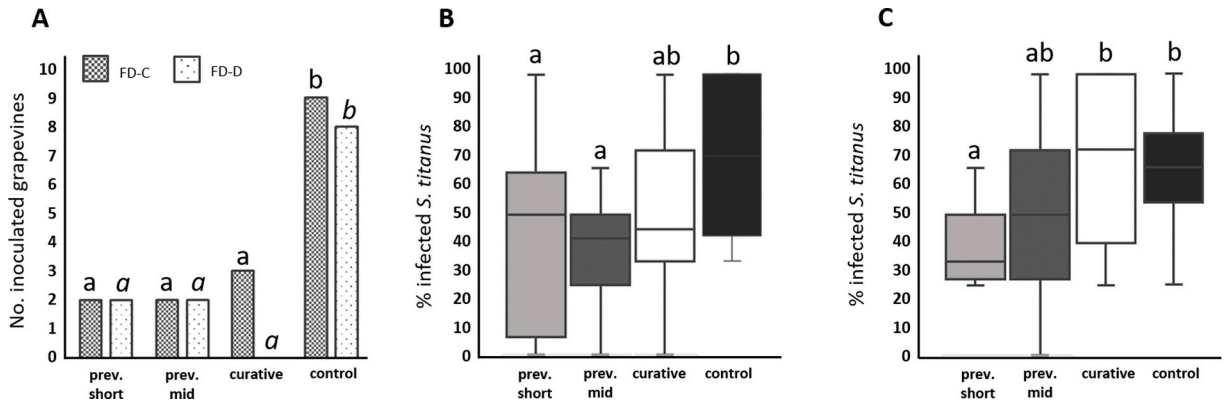

Fig. 1. Phytoplasma infection in inoculates grapevine plantlets and in *S. titanus* specimens used for transmission trials using FD-C and FD-D isolates. A) Number of grapevine plantlets inoculated with FD-C or FD-D phytoplasma (N = 10) after short-time preventive treatment with the PRC2022 biocomplex, after mid-time preventive application, after curative treatment, and in controls. B, C) Percentage of infected *S. titanus* specimens among those used in FD-C (B) or FD-D (C) transmission trials with short-time or mid-time preventive treatment with the biocomplex, with curative application, and in controls. For each phytoplasma isolate, different letters refer to significantly different values according to binomial GLM followed by sequential Bonferroni pairwise comparisons ($P < 0.05$).

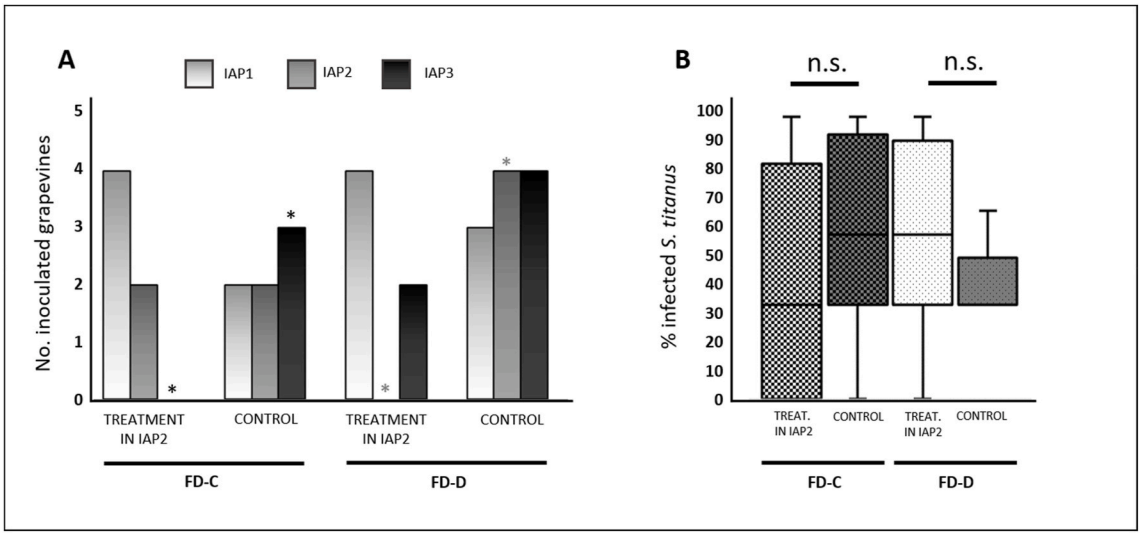

Fig. 2. Phytoplasma transmission experiments with sequential inoculation steps. A) Number of grapevine plantlets (N = 10) inoculated by *S. titanus* after three sequential inoculation access periods of 24 h (IAP1-3). B) Percentage of infected *S. titanus* specimens used in this experiment, which were collected at the end of trials. For each phytoplasma isolate, the replicates where a biocomplex-treated plant was offered to vectors for IAP2were compared to controls, where untreated grapevines were used for all IAPs. Asterisks refer to significant difference according to binomial GLM in the same IAP and with the same phytoplasma isolate; n.s. = not significant.

must be pointed out that the exudate sampling cannot totally prevent from sap contamination by fluid from surrounding cells and from xylem (Lewis et al., 2022); in contrast, the collection of sap flowing from stylets of phloem-feeding hemipterans allows specific collection of phloem sap (Will et al., 2013). We collected whole *E. variegatus* samples for iridium detection, rather than collecting phloem sap through stylectomy, in order to clarify whether the insect was capable of retaining the biocomplex in the digestive system after feeding. Using this method, we cannot exclude any partial contamination of the vector gut from other saps, as many phloem feeders are known to ingest a certain amount of xylem as well (Stafford and Walker, 2009; Chuche et al., 2017). No data are available about the amount of non-phloem tissues ingested by *E. variegatus*; however, we can assume that phloem is the main feeding source. Indeed, the percentage of time spent on phloem ingestion was shown to be correlated with phytoplasma transmission efficiency, due to the specific phloem-restricted localization of these pathogens (Ripamonti et al., 2022), and *E. variegatus* is an extremely efficient vector of phytoplasmas to broad bean (Rashidi et al., 2014; Rossi et al., 2023). Therefore, by measuring the iridium contained in vector insects, we further support the specific penetration of the biocomplex into the sieve tubes. We detected a lower Ir abundance in insect bodies compared with plant exudates, as expected due to the low sap amount ingested by insects (Lewis et al., 2022). However, we detected a significantly increased concentration in samples fed in the presence of the enriched biocomplex than with enriched water, in agreement with its efficient penetration of the vascular tissues.

Thanks to its capability to reach phloem vessels, when the biocomplex was applied on grapevine plantlets it induced a significant reduction of their phytoplasma infection regardless of the isolate, both as a preventive measure and as a curative treatment. The drop of infected plants after curative treatments suggests that the biocomplex exerts an antimicrobial effect in the phloem, where phytoplasmas reside. On the other hand, we assume that in preventive treatments with the biocomplex, the final outcome in terms of reduced phytoplasma infection in inoculated grapevines may be a combination of at least three distinct events. These are: a biostimulation that enhances plant capability to contrast the pathogen invasion, due to the enhancement of the nutritional status; a direct antibacterial effect played into the phloem by

the established biocomplex once phytoplasmas are inoculated; and a reduction of vector competence in insects that feed on the phloem of a treated plant, resulting in reduced inoculation. As for the anti-pathogen preventive and curative mechanisms occurring in the plant, it is very hard to discriminate the contribution of these factors (Monnier et al., 2020; Abdelkhalek et al., 2022; Montesinos et al., 2023). Conversely, the possible reduction of the insect capability of inoculating the phytoplasmas is suggested by the decrease of infection rates in *S. titanus* specimens used for preventive treatments. The contribution of a reduction of transmission efficiency in the decrease of successful inoculation events appeared different for FD-C and FD-D isolates. This was especially true for the mid-time preventive treatment, in which *S. titanus* infection rates were higher in the trials using FD-D than using FD-C, suggesting either a reduced persistence of the biocomplex in the phloem, or an imperfect phytoplasma suppression in insects infected with the FD-D isolate. Strikingly, FD-D was shown to be less efficient than FD-C in colonizing the plant and the insect body (Rossi et al., 2020, 2023), and the relationship between the slower spread in the hosts and the ability to escape containment action would hence merit further investigation. In agreement with the observed differential influence of the treatment on insect transmission efficiency, in experiments with sequential inoculation events, we recorded contrasting results using different phytoplasma isolates, despite in both cases we found a decrease of inoculation rates overtime, in opposition with control trials, where the percentage of inoculated plants increased overtime. In IAP2, a significant decrease of inoculated plants was observed in biocomplex-treated plants using FD-D, suggesting a curative effect. In contrast, when insects where moved to another non-treated plant for IAP3, they inoculated a significantly lower number of grapevines only using FD-C, suggesting a higher influence on vector competence with this isolate.

On the whole, our results indicate the PRC2022 biocomplex as a promising tool for limiting FDp infection in grapevine, thanks to its capability to penetrate phloem vessels, along with its antibacterial activity against phytoplasmas, which was observed both in the plant and in the vector. The search for control measures that successfully limit the phytoplasma pressure in plants is certainly a priority for many crops as an additional tool to vector control. Indeed, vector management alone, which is at present the main available tool, cannot be sufficient as the high transmission efficiency of vectors puts the economic thresholds very close to zero (Murphy et al., 2014; Chen et al., 2022). This is especially true when dealing with FD phytoplasma, since in Europe, where it is a major threat, the use of antibiotics in agriculture is not allowed, with a meaningful restriction of the curative options, since antibiotics are the most effective antimicrobials against phytoplasmas (Bertaccini, 2021). Our results on the tested biocomplex provide a starting point for developing new plant protection products against phytoplasmas.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Elena Gonella, Luca Picciau, Federico Lessio, Alberto Alma reports financial support and equipment, drugs, or supplies were provided by Pireco Productie B.V.

Data availability

Data will be made available on request.

Acknowledgements

The authors are grateful to Bianca Orrù and Chiara Arcolin for their support in molecular analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2023.106472.

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