Food and Agriculture Organization of the United Nations

FOURTH INTERNATIONAL SYMPOSIUM ON BIOLOGICAL CONTROL OF BACTERIAL PLANT DISEASES

Abstracts

DAFNE
Dipartimento di Scienze Agrarie e Forestali

Università degli Studi della Tuscia

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Preface

Welcome message from FAO

Dear Colleagues,

It is my great pleasure to welcome you to the 4th International Symposium on Biological Control of Bacterial Plant Diseases, here in beautiful Viterbo, Italy. After a year of planning and organization with Dipartimento di Scienze Agrarie e Forestali (DAFNE), Università degli Studi della Tuscia to finish and approve the program.

Since many years Food and Agriculture Organization of the United Nations (FAO) dedicates much attention to the use of environmental friendly methods in agriculture production and protection. Biological control is a key element of a sustainable agriculture but also the main feature to protect the biodiversity and to reduce the pollution in the world.

Protect the planet and save the environment has always been at the center of our efforts, mainly focused on technical support to the countries and to small scale farmers. The need to promote biological control of plant pests and disease becomes a necessity especially for those hard to control by using the conventional methods; bacterial diseases are in the top list of them.

I would like to draw the attention to the amount of transboundary pest and diseases moving around the world, in particular bacterial diseases most of them are difficult to control; here we need the science to find a tangible solutions and innovative control measures.

I would like to give my particular welcome and special thanks to all the scientists and invited speakers coming from all the world who will give us information about the state of the art, the research and possible developments of biological control of bacterial disease.

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Welcome Message from DAFNE, Unitus

Dear All,

It is my honor and pleasure to synthesize about the 4th International Symposium on Biological Control of Bacterial Plant Diseases (BIOCONTROL2019) held in Viterbo, Italy, from 9 – 11 July 2019, organized in collaboration with FAO.

Since 2016, when I received the witness from the Scientific Committee of BIOCONTROL 2016, in Belgrade (Serbia), inside our Dept. DAFNE – University of Tuscia, we worked a lot to maintain as well as to improve the interest about the biological control of bacterial plant diseases worldwide.

The stimulating opportunity to meet and discuss among scientists, researchers, farmers associations, private companies, politicians, students, and people interested to move on together by sustainable approaches to control these plant pathogens, has become reality.

The latest research results and developments in the biocontrol of bacterial plant diseases, have been successfully discussed by an increasing interest inside the different sessions thanks to more than one hundred original scientific contributes.

The Italian Ministry of Agricultural, Food, Forestry and Tourism Policies within the Italian Scientific Societies of Plant Pathology, Plant Protection (AIPP, MPU, SIPaV) and ISPP, gave a relevant contribute for the success of the event.
Several representatives of the industrial world, agronomists, technicians and freelancers pointed out topic subjects that need to be followed as other to be implemented.

A lot of young researchers and students attend to BIOCONTROL2019; they are the future, they are the concrete hope to continue as to increase the common efforts to contrast several phytosanitary emergencies caused by phytobacteria around the world, by eco–sustainable strategies.

The whole sector of organic agriculture increases exponentially; People request much more respect for the environment, food, and health.

The mission of science, industry and politics is to give answers, shared and sustainable.

I believe that BIOCONTROL2019 has made a contribution in this sense and, more motivated than before, we will continue.

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Extended Abstracts

Species interactions within the microbiome mediate potential for disease suppression

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Summary
Microbes in soil exist within complex networks of interacting plant and microbial species. In addition to the significant role for antibiotics in mediating microbial fitness, antibiotic–producing microbes can also significantly enhance plant productivity via their capacities to suppress diverse soil-borne plant pathogens. Consequently, there is substantial interest in the use of antibiotic–producing microbes for inoculative disease biological control in agriculture. However, all soils harbor substantial populations of indigenous disease–suppressive populations. As an alternative to inoculation, can we impose selection for antibiotic inhibitory phenotypes within indigenous soil communities to achieve consistent disease suppression? Our work explores the roles of plant host, plant community diversity, soil nutrient characteristics, and microbial species interactions in determining the pathogen–suppressive potential and composition of soil microbiomes, and the consequences for plant productivity. Unraveling the complex coevolutionary interactions among plants and soil microbiomes suggests novel insights for active management of soil microbes to support plant productivity.

Our studies began in a naturally–occurring potato scab suppressive soil. Potatoes were grown every year in this plot for over 30 years, by which time potato scab and *Verticillium* wilt disease had been eliminated from the field: the field had become suppressive to disease. In–depth studies of the soil microbial communities in the suppressive soil and an adjacent disease–conducive soil showed that the suppressive soil supported greater densities of *Streptomyces*; that a greater proportion of *Streptomyces* in the suppressive soil could inhibit any one of a collection of 21 different pathogenic bacteria; the mean zone of inhibition against plant pathogens was significantly greater among a collection of *Streptomyces* from the suppressive vs. from the conducive soil; and the diversity of inhibitory phenotypes was greater for *Streptomyces* from the suppressive soil (Kinkel *et al.*, 2012). In total, these data suggest that long–term potato production induced density–dependent selection for inhibitory *Streptomyces* populations, and, subsequently, frequency–dependent selection for novel antibiotics (rare fitness advantage conferred on isolates that produce novel antibiotics). Collectively, this suggests a 'recipe' for creating a disease suppressive soil in any field. Specifically, if one can effectively 'feed' the soil microbiome to increase microbial densities, will density– and frequency–dependent selection result?
In the past decade, we have explored multiple strategies for feeding soil microbiome to induce suppression. However, we recognize that a model that suggests a significant role for density and frequency–dependent selection for enriching antibiotic inhibitory phenotypes in the soil microbiome makes a critical (and untested) assumption. Specifically, this assumes that antibiotics are important to microbial fitness. Thus, we set out to determine whether there is evidence of selection for antibiotic inhibitory phenotypes in soil Streptomyces and thus evidence that antibiotics are important to fitness. To accomplish this goal, we randomly collected *Streptomyces* from diverse locations on 5 continents. We quantified the capacities of these individual *Streptomyces* to inhibit sympatric (from the same location) vs. allopatric *Streptomyces* isolates. We found that isolates were significantly better at inhibiting populations that they lived with then populations from other locations. This suggests that there is positive selection for antibiotic inhibitory phenotypes that are effective within local habitats. We found further that, among sympatric populations, *Streptomyces* populations were better at killing other isolates with which they had strong niche overlap (similar nutrient use preferences) vs. isolates that preferred different nutrients (Kinkel *et al.*, 2014). Collectively, these data provide strong

evidence that there is positive selection for inhibitory phenotypes, and that nutrient use preferences among coexisting populations mediate selection for inhibition.

What factors are likely to mediate selection for inhibitory phenotypes? Over the past decade we have explored the soil bacterial and fungal microbiomes associated with prairie plant species growing across a range of plant species richness (one, four, eight, or 16 plant species) to enhance understanding of the conditions under which inhibitory phenotypes are most likely. We found that *Streptomyces* associated with the same plant host (*Andropogon gerardii, Schizachyrium scoparium, Lespedeza capitate,* or *Lupinus perenne*) are significantly more pathogen–suppressive when the host grew in monoculture vs. within a 16 species, high–diversity plant community (Bakker *et al*., 2013). In contrast, populations of *Streptomyces* in the rhizosphere of plant hosts growing in high–diversity communities are more niche–differentiated than populations associated with the same host in monoculture (Bakker *et al*., 2013, 2014). These data suggest that plant community diversity plays a critical role in determining the likelihood of antagonistic arms race coevolution vs. niche differentiation among sympatric soil populations, with significant implications for plant disease suppression. This may help to explain the association of naturally–suppressive soils with long–term crop monocultures.

In more recent work, we have studied interactions between *Streptomyces* and another soilborne saprophyte who has been associated with disease suppressive soil: *Fusarium*. Similar to interactions among *Streptomyces* populations, we found that *Streptomyces* were significantly better at killing sympatric vs. allopatric populations of *Fusarium*, and that *Fusarium* were better at killing sympatric vs. allopatric populations of *Streptomyces* (Essarioui *et al*., 2016, 2017, in press). This suggests that antibiotic inhibitory phenotypes play important roles in mediating interactions between these two abundant soil genera. Moreover, we have little evidence that pathogen populations themselves the target of our quest to increase the densities and inhibitory capacities of soil saprophytes play any role in this antagonistic arms race. Instead, pathogens appear to be collateral damage in the saprophytic arms race.

If resource competition incites an antagonistic arms race, can we alter microbial nutrient availability in soils to enhance selection for inhibitory phenotypes? We have been testing soil amendments to impose selection for pathogen–inhibitory *Streptomyces* (Schlatter *et al*., 2009). We have shown the capacity of amendments to significantly increase niche overlap among coexisting *Streptomyces*, and to select for more–inhibitory *Streptomyces* in soil (Dundore–Arias *et al*., in press). This suggests the potential for active management of indigenous soil populations to suppress plant diseases, including potato scab, Verticillium wilt, and other bacterial and fungal plant pathogens.

In more recent research, we have been exploring the roles of competitive species interactions, and especially antibiotic inhibitory phenotypes, in assembly of foliar endophytic communities (Kinkel *et al*., 2018). Though we have strong evidence that resource competition and antagonistic species interactions mediate community assembly in the soil, are antibiotics important to fitness inside plants? Understanding the potential roles of inhibition to microbial species interactions and fitness within plants offers the potential for mediating endophytic assembly to control bacterial and fungi aboveground.

Our work sheds important light on the ways in which networks of species interactions within soil and endophytic microbiomes contribute to determining the pathogen–inhibitory potential of indigenous microbes, and suggests the potential for specific crop management approaches targeting species interactions to offer for sustainable disease control.

Acknowledgements

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Role of Cruciferous weeds in the epidemiology and biological control of seedborne *Xanthomonas campestris*

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Summary
Cruciferous weeds can serve as a major reservoir of inoculum of *Xanthomonas campestris* pv. *campestris* (Xcc). Surveys of Cruciferous weeds have shown black rot (BR) to be common among cruciferous weeds in central coastal non–cultivated areas of California. In California atypical leaf spotting strains of BR were found along the central coast in several weedy species including: *B. campestris, B. nigra, B. geniculata, R. sativus, Sisymbrium officinale, S. irio*, and *Cardara pubescens*. Plants with typical symptoms consisting of yellow V–shaped lesions were found but species with atypical lesions consisting of black spots without any yellowing were widespread. We propose controlling BR by releasing weakly virulent leaf spotting strains of *Xanthomonas*. Potentially, weakly virulent pathogenic strains could be deployed as biological control agents in order to activate defense mechanisms, particularly at the physiological level, to increase persistence of bacteriophages specific to the target bacterium on leaf surfaces, and to be a pathogen competitor on host plant leaf surface. Cruciferous weeds make up a large part of the ecosystem along the central coast of California and make a good target for biological control.

Keywords*: Brassica black rot, biological control*

Black rot infections of cruciferous crops continue to threaten the growers profits worldwide (Cook 1952). The pathogen, *Xanthomonas campestris* pv. *campestris* (Xcc) is a seed–transmitted Gram–negative phytopathogenic bacterium with an annual threat in temperate climates with high humidity. *Xanthomonas campestris* includes six pathovars distinct in host virulence and symptom development among cruciferous plants: *Xcc, X. campestris* pv. *aberrans, X. campestris* pv. *barbareae*, *X. campestris* pv. *incanae*, *X. campestris* pv. *armoraciae,* and *X. campestris* pv. *raphani* (Vauterin *et al*. 1995). *X. campestris* pv. *armoraciae* and *X. campestris* pv. *raphani* are probably synonymous pathovars (Vicente *et al*. 2006). *Xcc* enters the plant through wounds, stomata, and hydathodes (Staub and Williams 1972). The typical black rot yellow V–shaped lesions are caused by bacteria that enter through the hydathodes and move through the plant xylem causing wilt, chlorosis and death of infected tissues. The presence of *Xcc* may increase infection by soft rot pathogens by 10–fold and cause death of plants after a winter season or heavy losses during storage. Seeds, plant debris, and cruciferous weeds have been implicated as inoculum sources for black rot infections (Schaad and Dianese 1981). In a study of cruciferous weeds in California, Ignatov *et al*. (2007) concluded that bacterial populations isolated from weeds had different genetic properties than those associated with seed infections in cultivated Brassica crops. The leaf spoting pathovars are usually more numerous on weedy plants and can cause leaf damage, but they do not result in a systemic infection comparing to *Xcc* (Alvarez *et al*. 1994; Vicente *et al*. 2006). The leaf spotting pathovars infect leaves though stomata under cool and wet conditions (Shaw and Kado 1988). The objectives of this research were to (i) understand the role of leaf spotting strains on weedy brassica populations for black rot outbreaks, (ii) evaluate reaction of cultivated Brassica species to the leaf spotting strains, and (iii) determine the usefulness of the leaf spotting strains for biological control of black rot through plant immunity stimulation or bacteriophage maintenance. The data presented by several authors demonstrate that the systemic *Xcc* population is more uniform comparing to leaf spotting pathovars (Alvarez *et al*., 1994; Ignatov *et al*., 2007), and new seed–transmitted haplotypes are a major source of infection on cultivated brassicas (Lange *et al*., 2016).

A collection of 24 weakly pathogenic leaf spotting strains of *X. campestris* was studied in this work. They were isolated from different geographical regions, mainly in Russia, in 2006–2012. A number of *Xcc* strains typical of different races (NCPPB 528, PHW231, HRI1279a, B100, and LMG 8004) were included for plant

inoculation. Cabbage F_1 “Express” (*B. oleracea,* Plant Breeding St. by N.N. Timofeev, Moscow) was used as susceptible host. All plant seeds were sown in 8–cm pots and plants were grown in a growth chamber under standard conditions (24/20oC, 16 hours light day). Plants were treated by weakly pathogenic strains (sprayed by hundred million CFU/ml) and inoculated by *Xcc* (by mouse–tooth tweezers soaked in bacterial suspension million CFU/ml). The development of the disease was assessed 16 days after inoculation.

The genetic diversity of the leaf spotting was determined by sequencing fragments of three house–keeping genes (*ahyR, nrdB,* and *purA*), which showed from 97 to 100 percent identity to type *Xcc* strains, except the 12 bp deletion in the gene *ahyR* (quorum sencing LuxRI homologs *XCC2818* of *Xcc* ATCC 33913 (=NCPPB 528) genome), highly important for bacteria phenotype expression (Whitehead *et al*., 2001).

The data indicated that plants treated with weakly pathogenic strains suppressed black rot development after inoculation in interval from 40 hours to 10 days after pre–inoculation with an average rate from 50 to 75 percent, when compared to control treated with water or non–pathogenic bacteria. According to previously published results, inoculation of *Brassica rapa* leaves with the incompatible *X. campestris* pv. *Vitians* induced β–1,3–glucanase and chitinase/lysozyme activity. The induction of chitinase/lysozyme one was associated with the hypersensitive reaction caused by *X. c. vitians* (Newman, *et al*. 1994).

Alternatively, weakly pathogenic strains were used as host for bacteriophages specific to the target strains of *Xcc*. Treatment of cabbage seeds of cv. “Moscow late 15” with 25,6 percent seeds naturally contaminated by the pathogen with the cocktail of 5 bacteriophages resulted a significant decrease in the black rot infection of seedlings. Estimated biological efficacy of the bacteriophage cocktail has reached 90.6 percent (Orynbaev *et al*., 2019).

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Exploring rain–isolated bacteria as potential biopesticides to control fire blight

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Summary
Fire blight, caused by *Erwinia amylovora*, is a devastating disease affecting apple and pear production worldwide. Due to the emergence of antibiotic–resistance, biological control has long been explored as an alternative control option. Unfortunately, the efficacy of biological products heavily depends on environmental conditions. Therefore, we tested the hypothesis that bacteria locally isolated from rain in Virginia, USA, could be particularly well adapted to local conditions and provide effective control. *In vitro* assays with rain–isolated bacteria identified two isolates of the genus *Pantoea* with strong inhibitory effect against *E. amylovora*. The isolates were then tested for survival on apple branches during the winter and as pre–treatment to protect against fire blight on detached apple blossoms and on whole trees in the field in the same area of Virginia where they had been originally isolated. Survival was similar to *E. amylovora* and fire blight control was similar to a commercial product but not as effective as streptomycin. Our results show the potential of rain as a source of locally adapted biocontrol stains to control fire blight on apple. However, more field testing will be needed to compare the control provided by the identified isolates with already available commercial products.

Keywords: *fire blight; biocontrol; precipitation; mutagenesis; genome sequencing*

Fire blight is a major bacterial disease of apples and pears. Epidemics are both highly destructive and sporadic in nature, able to develop rapidly in orchards with no history of the disease. For example, in the USA, estimates of annual losses to fire blight exceed $100 million (Norelli *et al*. 2003). To date, none of the popular commercial apple cultivars are resistant to fire blight. Current management practices rely on phytosanitary measures to reduce overwintering bacterial inoculum within the orchard and preventative copper and/or antibiotic sprays to protect susceptible plant tissue during an infection period. Although pesticide application timing has been significantly improved by Maryblyt™, a fire blight forecasting program (Lightner and Steiner 1992), control remains difficult. Long periods of rainy weather can prevent orchard access and timely sprays through a long bloom period and streptomycin–resistant strains of *E. amylovora* have developed in major apple producing regions in Washington (Loper *et al*. 1991), Michigan (McManus and Jones 1994), and New York (Russo *et al*. 2008).
In Virginia, non–antibiotic control of fire blight has been challenging. Idared trees sprayed with treatments not involving streptomycin had substantial blossom blight and were not significantly different from non–treated control (Yoder *et al*. 2014b). Spray programs that combined streptomycin with a non–antibiotic product or alternated streptomycin with a non–antibiotic pesticide were significantly less effective at suppressing blossom blight than spray programs that relied on streptomycin alone (Yoder *et al*. 2013). While this is good news for now, there is the risk that streptomycin–resistant strains of *E. amylovora* could develop in Virginia as well.
Because of the existing challenges in the control of fire blight and the desire to lower the chemical input in agriculture, several microorganisms antagonistic to *E. amylovora* including bacterial strains of *Bacillus subtilis*, *Pantoea agglomerans*, and *Pseudomonas fluorescens* (Bonaterra *et al*. 2012), and the yeast *Aureobasidium pullulans* (Kunz 2004) have been developed into commercial biopesticides. Unfortunately, field trials in the eastern U.S. have documented substantial variability, and none of the biological products evaluated were as effective as streptomycin, the standard treatment option (Sundin *et al*. 2009). Likely, this inconsistency is partly due to the dynamic microbial community within the apple flower (Shade *et al*. 2013) and the ability of virulent *E. amylovora* to modify the floral environment to its own benefit (Johnson *et al*. 2009). Also, *A. pullulans* causes russetting on some cultivars and is inhibited by fungicide applications (Yoder

et al. 2014a). Furthermore, *E. amylovora* can be spread later in the season by wind–driven rain from tropical storms and remain pathogenic on mature fruit (Ordax *et al*. 2009).

We estimate that fire blight costs the Virginia apple industry $1.5 million annually, including costs of management, loss of young trees and bearing surface of bearing trees, and increase of secondary fungal fruit rots following buildup of inoculum on blighted wood. A serious epidemic at some location in Virginia occurs almost every year. Therefore, biopesticides that are effective under the local environmental conditions in Virginia would be highly desirable.

One yet unexplored source of biopesticides is precipitation. We recently isolated and identified over 1200 bacteria from rain and snowfall (Failor *et al*. 2017). Over 90 percent of these bacteria belong to the genera *Pantoea*, *Bacillus*, and *Pseudomonas*, *i.e.*, the same genera currently used in commercial biopesticides for fire blight. Also, using rain as inoculum, we found that *Pantoea* and related bacteria are very efficient colonizers of tomato leaves (unpublished results). Therefore, we hypothesized that the bacteria we isolated from rain in Virginia may include potential biocontrol strains for fire blight that could be more efficient than current commercial products because they were isolated in Virginia and, therefore, may be better adapted to the regional climatic conditions allowing them to persist longer in the orchard.

To test the hypothesis, we chose 200 precipitation–borne strains and performed *in vitro* inhibition assays on Petri dishes by inoculating the strains on Petri dishes on which we had distributed *E. amylovora*. The diameter of the inhibition zones forming around potential biocontrol strains was used as first indication that strains interfered with the growth of the fire blight pathogen *in vitro*. Performing this assay with high concentrations of rain–borne strains, nine strains were identified to have at least some inhibitory effect on *E. amylovora*. The assay was then repeated with lower concentrations of strains. Two strains remained highly effective even at these lower doses and were chosen for further characterization.

Since one of the main issues with biocontrol products is their limited survival on plants in the field, we wanted to test our two strains for survival on plants. Ideally, a biocontrol strain would even get established in an orchard and remain viable and function in suppressing disease from one growing season to the next. Therefore, we compared the survival of our two best rain–borne strains on detached apple branches during the winter. We used *E. amylovora* and *Escherichia coli* for comparison. Interestingly, the two rain–borne strains survived as well as *E. amylovora* while *E. coli* could not be found anymore after one week.

To start testing the two strains *in planta* for biocontrol of fire blight, we used a detached blossom assay. We found that a repeatable measure of control was the length of necrosis visible on the peduncle and the population size of the pathogen in the peduncle seven days after pathogen inoculation. In repeated assays, we found the two rain–borne strains to significantly reduce the length of necrosis as well as the *E. amylovora* population size compared to *E. coli* and compared to a water control.

After the encouraging results obtained with detached blossoms, we performed field assays. The field assays consisted in inoculating blossom clusters with at least one open blossom with the two rain–borne bacteria, a commercial biocontrol product, streptomycin, and a negative control that consisted only in water and surfactant. The pathogen was inoculated approximately one hour later. Clusters were then evaluated twice for symptoms. Clusters were rated as healthy, mildly diseased, or severely diseased. Based on these results, only streptomycin provided almost complete control. The two rain–borne bacteria provided similar control levels to the commercial product.

To start characterizing the molecular basis of the biocontrol activity of one of the rain–borne strains, the whole genome of the strain was sequenced and annotated. A UV–mutagenesis screen was also performed. Five mutants with complete loss of inhibitory activity in plate assays were obtained. DNA of the five mutants was extracted, sequenced and compared to the wild–type strain. Non–synonymous single nucleotide polymorphisms were identified in several genes. Comparison of the predicted function of mutated genes with the frequency of mutations in these genes allowed to identify genes with putative roles in the strain's biocontrol activity.

Finally, the strains were precisely identified using the Life Identification Number (LIN) approach (Vinatzer *et al*. 2017) at the LINbase website (www.linbase.org). One strain is a member of the species *P. agglomerans* and over 98.5 percent identical to the most similar *P. agglomerans* strains in LINbase. The other strain was identified as *P. ananatis*.

In conclusion, our results showed that precipitation is a promising source of bacterial strains for fire blight control. However, additional field tests need to be performed to determine if locally isolated precipitation–borne strains can outperform commercial products. Also, additional precipitation–borne strains will

need to be tested on additional crops for control of additional disease to evaluate if rain could be considered as an isolation source to use in large screens to develop new commercial biocontrol products.

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Biocontrol of black rot pathogen clonal group predominant in Russia

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Summary
Xanthomonas campestris pv. *campestris* (*Xcc*) is one of devastating diseases of *Brassica* vegetables. We have collected *Xcc* isolates in the main areas of brassicas cultivation in Russia from 2006 to 2018. Multilocus sequence typing based on the partial sequence of loci *dnaK, fuyA*, *rpoD, gyrB, cytP450* and *avrXcc2109* was applied to over 100 strains representative for 22 collection sites. Comparison of *Xcc* strains collected at the same regions of Russia showed considerable genetic changes occurred after 2012. The phylogenetic reconstruction using a data set of gene *rpoD*, placed the strains into three distinct genetic groups. Group one (35 strains collected before 2012 and only two – at 2018) was similar to the strain NCPPB5268^T, LMG8004, and HRI1279a. Small Group two (four strains) was similar to B100 and HRI 3811. Most of strains obtained after 2012 were nearly identical in *rpoD* sequence and similar to *Xcc* 0656 and 0657 from USA. The race structure of *Xcc* strains showed that the pathogen strains collected after 2012 had significant race shift. The strains reaction with 25 bacteriophages confirmed the distinct grouping of *Xcc* strains obtained since 2012. Application of the bacteriophage cocktails was efficient against black rot caused by homogeneous *Xcc* population on seedlings of brassicas.

Keywords*: Brassica black rot, biological control, bacteriophages, genetic diversity.*

Brassicas (family Brassicaceae) plants are affected by many pathogens, including *Xanthomonas campestris* pv. *campestris* Pam. (Dow.) (*X. campestris*) causing black rot. *X. campestris* strains are divided into physiological races, serotypes, and genotypes (Kamoun *et al*., 1992, Ignatov, 2006). In 2006–2012, the collection of new strains of the pathogen was carried out in Russian Federation, and they were studied in this work. The collection included 86 strains isolated from different geographical regions, mainly in Russia, in 2006–2012. A number of strains typical of different races, pathovars, genotypes, and species of xanthomonads (inc. *X. campestris* pv. *campestris* NCPPB 528, PHW231, HRI1279a, 2D520, B100, LMG8004, *X. campestris* pv. *aberrans* NCPPB 2986, X. *campestris pv. raphani* NCPPB 1946*, X. campestris* pv. *armoraciae* NCPPB 347, *X. vesicatoria* NCPPB 422, and *X. gardneri* NCPPB 881) from international collections were included for comparison.
Only pathogenic strains for at least one plant species of the *Brassica* spp. plants were used in the experiments. For inoculum, bacteria were grown on King's B medium at 28^{o}C for 36 hours. The differential series described by Kamoun *et al* (1992) and Vicente *et al*. (2001) were used for race identification, including: "Seven Top Green", F_1 "Tokyo Cross", F_1 "Just Right" (all *B. rapa*), "Florida Broad Leaf" (*B. juncea*), F_1 "Miracle" (*B. oleracea*). In addition, *B. carinata* PI 199947/V12 and *B. napus* "Cobra" R4 inbred lines were included. Cabbage F_1 "Express" (*B. oleracea,* Plant Breeding St. by N.N. Timofeev, Moscow) was used as susceptible control. All plant seeds were sown in 8–cm pots and plants were grown in a climatic chamber under standard conditions (24/20oC, 16 h light day). Plants were inoculated six weeks after emergence (stage of four–five true leaves), three leaves per plant were inoculated by mouse–tooth tweezers soaked in bacterial suspension (million CFU/ ml) with 10 inoculation points made on each leaf. From three to four plants per strain were inoculated. The development of the disease was assessed in 16 days after inoculation. Strains tested for virulence were assayed with specific PCR analysis for the *X. campestris* pv. *campestris* and *X. campestris* pv. *raphani* (Berg *et al*. 2005; Park *et al*. 2004; Vo Thi Ngoc Ha 2015; Tsygankova *et al*. 2004), targeted on several variable genes, including *cytP450, hrpF*, *xopAD, tonB*, and by sequencing fragments of virulence gene *avrXcc2109* (Castaneda *et al*. 2005), *cytP450,* and six house–keeping genes (MLST) (Young *et al*. 2008; Ignatov *et al*. 2007).

For genetic analysis, bacterial strains were grown on LB agar at 28°C for 48 hours. Genomic DNA was obtained using modified CTAB–SDS method (Vo Thi Ngoc Ha 2015). Amplification of the gene fragments was done on Mastercycler Eppendorf AG using PCR kit "Encyclo" (Evrogen, Russia) according to the recommendations of the manufacturer. Amplification was carried out according to the following profile: initial denaturation 95°C (60 s), 30 cycles: denaturation 95°C (30 s), annealing 55–65°C (30 s), elongation 72°C (30 s). Primer pairs were used according to Young *et al*. (2008), Ignatov *et al*. (2007) and Vo Thi Ngoc Ha (2015). Isolation and purification of PCR fragments was done by "CleanUp Standard" kit (Evrogen) according to the manufacturer's recommendations. Sequencing of purified PCR fragments was done with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The nucleotide sequence was obtained by automatic sequencer 3730 DNA Analyzer (Applied Biosystems, USA). The obtained de novo nucleotide sequences were aligned using the Clustal W algorithm. Manual alignment checking was performed in the program BioEdit V. 7.2.5. Dendrograms of phylogenetic relationships of strains were constructed in the MEGA 7.0 program (Kumar *et al*. 2016).

Based on the data obtained, the Russian strains of the pathogen belong mainly to races one, three and four. Bacteria isolated in 2000–2007 were more variable compared to the isolates obtained after 2012 and international strains. The most significant changes occurred in the frequency of virulence reaction to plants with the resistance gene in *Brassica rapa* and *B. napus* (18.6 percent before 2012 and 56 percent–after), virulence reaction to plants with the resistance gene in *Brassica juncea* and *B. carinata* (c 4.7 percent to 2012 and 16 percent – after). This demonstrated that the race–specific reaction of *X. campestris* population in Russia changed at 2012.

Analysis of specific PCR results showed that the majority of 86 of the strains gave a positive reaction with the primers for *Xcc007–tonB* intergenic region, genes *hrpF*, *xopAD, wxcO,* and *cyt186* (*cytP450*). Only 52 strain (60 percent) had positive reaction with all specific primers for *X. campestris* pv. *campestris* and 11 strains (12.8 percent) – positive reaction with specific primers for *X. campestris* pv. *raphani*. However, 21 (24.4 percent) strains were positive with primers for the *xopAD* gene linked to the same genetic locus as *X. campestris* pv. *raphani* – specific PCR marker.

Multilocus gene analysis has revealed from 5 (*cytP450*) to 20 (*rpoD*) allelic variants. Atypical for *X. campestris* variants of the gene *rpoD* were found in isolates obtained after 2012. All the strains of *X. campestris* of this group have *rpoD* alleles most close to the strains of *Xcc* 0656 and 0657 from USA (Lange *et al*. 2016). At the same time, dendrograms based on other 5 genes placed the isolates of post–2012 together with type strains of *X. campestris* species and strains from Russia obtained at 2000–2007. This may indicate the *X. campestris* population in Russia had change in genotype, virulence, and in phage specificity (as found later) probably, due to pathogens introduced with imported brassicas seeds.

21 isolates of bacteriophages specific to 11 target strains of *X. campestris* pv. *campestris*, were isolated from soil samples collected under black rot–infected cabbage plants at 2014–2017. Electron microscopic study of bacteriophage morphology allowed to classify three isolates as members of Siphoviridae family, and the remaining 18 isolates – as Myoviridae family. After analysis of phagotyping for 73 phytopathogen strains against newly isolated isolates and four collection strains of bacteriophages, it was proposed to create a phage cocktail of isolates BT2, Ph30–1, Ph44, DB1 and Tir2, which together can infect 88 percent of the strains of the *X. campestris* pv *campestris* collection, representative for the Russian strains of the pathogen isolated after 2012, and phage SM10 was specific for the pathogen isolates of pre–2012 type. Treatment of cabbage seeds of cv. “Moscow late 15” with 25,6 percent seeds naturally contaminated by the pathogen with the cocktail of 5 bacteriophages resulted a significant decrease in the black rot infection of seedlings. Estimated biological efficacy of the bacteriophage cocktail has reached 90.6 percent.

From a practical point of bacteriophage application, it was important to test the compatibility of phages with pesticides and agrochemicals used for brassicas protection. Our test results for 20 agents of different chemical composition incubated with bacteriophages showed that only copper–based preparations (Coside 2000) and strong sterilizers (peroxyacetic acid) affected survival of phages.

The designed bacteriophage cocktail with the addition of 0.75 percent skimmed milk as protector against UV light was applied during two years on young cabbage plants in greenhouse conditions. The spraying by the bacteriophage cocktail three times with 10–days interval during seedlings growing period over naturally infected plants reduced the spread of black rot on susceptible cultivars by 71.7 percent, when standard treatment by Coside 2000 had average efficiency about 59 percent.

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Epidemiology and forecasting systems in biological control of diseases caused by plant pathogenic bacteria

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Summary
Biological control of bacterial diseases of plants is the result of complex relationships between pathogen, host and biological control agent, which interact with the environment across time and space. Disease progression and pathogen spread are influenced by pathogen aggressiveness. and among a given plant pathogenic bacteria there is generally a wide range of aggressiveness within the population. The efficiency of biocontrol depends greatly on pathogen aggressiveness as has been demonstrated by analysing the quantitative relationships between the dose of biological control agent, the dose of pathogen and disease intensity. The environment and host influence the fitness of biological control agents affecting colonization, survival, spread and decay. However, there are methods to improve fitness of biocontrol agents to counteract adverse conditions. The knowledge of the biological cycle of the pathogen and biocontrol agent in relation to the host and forecasting models have provide tools for guided application and timing of biocontrol agents. Climate change is expected to have a strong influence on efficacy of biological control. There are different scenarios possible, but it can be expected a need for new biocontrol strains due to the arrival of unfavourable environmental conditions.

***Keywords**: pathogen aggressiveness, dose-response relationships, biocontrol agent fitness, treatment scheduling, climate change.*

Plant disease is influenced by the pathogen, host, biocontrol agent, environment and time-space factors, which conform the five corners of a "disease tetrahedron" with interactions between the three living organisms implicated. Biological disease control is the result of relationships between pathogen, host and biological control agent, that interact with the environment across time and space.
I will focus on some key points for a successful management of disease, such as the disease-biocontrol agent and pathogen-dose relationships, and the influence of the environmental conditions and host in fitness of the biological control agents. The use of disease forecasting systems as tools to schedule biocontrol treatments, and the scenario of climate change and biological control of bacterial diseases will be also discussed. Most examples will be chosen from the area of biological control of fire blight.
The efficiency of biological control depends primarily on disease intensity. This has been illustrated by experiments on biological control of fire blight with the bacterial antagonists *Pseudomonas fluorescens* A506, *Pantoea agglomerans* C9-1, *Pantoea agglomerans* E325 and *Bacillus subtilis* QST713 that were evaluated for efficacy in Michigan, New York, and Virginia in USA, in experiments conducted between 2001 and 2007 (Sundin *et al.* 2009). In a more detailed analysis of the data from the authors, the main effects on the efficacy of biocontrol were found to be disease intensity, followed by year and location, but it was not significantly affected by the antagonist used (Montesinos, E. data not shown).
Disease intensity is affected by several conditions mainly environment and host, but also by pathogen aggressiveness. Among a given plant pathogenic bacteria there is generally a wide range of aggressiveness within the population. Ercolani, who was a pioneer in the characterization of aggressiveness among bacterial plant pathogens, reported in several *Pseudomonas* spp. affecting different hosts, a strong variability in their median infective dose, ED_{50} (Ercolani, G.L. 1973). But pathogen aggressiveness can be calculated as well as from the ED_{50}, also by the rate of disease progression (r_g) and the time delayed to start the disease progression curve (t_0). The aggressiveness of 49 strains of *Erwinia amylovora* from several world outbreaks, mainly from Europe, have been studied by means of infectivity titration and time-course infection

experiments on pear (Cabrefiga and Montesinos 2005). It was found a wide range of the ED_{50}, r_g and t_0 among the strains of *E. amylovora*. A lower ED_{50} within a strain was related to short t_0 and high r_g.

The efficiency of biocontrol depends on pathogen aggressiveness. This has been demonstrated by analysing the quantitative relationships between the dose of biological control agent and pathogen, and disease intensity. Fitting experimental data to mathematical models, permitted the estimation of parameters describing the efficiency of the biological control agent (Montesinos and Bonaterra 1996). One of these parameters is the ratio between the biological control agent and the pathogen doses to obtain a 50% disease control (*Kz/Kx*). Immature fruits from two pear cultivars were treated with different doses of the antagonist *Pseudomonas fluorescens* EPS62e and inoculated with different doses of *E. amylovora*, and the disease incidence was recorded. An inverse relationship was found between *Kz/Kx* and the pathogen aggressiveness (Montesinos and Cabrefiga, unpublished results). Thus, high doses of the biological control agent are required to control highly aggressive strains of a pathogen.

The environment and host, influence fitness of biological control agents, such as colonization, survival, spread and decay of the biological control agent. However, field studies are complicated by the fact that in plant leaves and other aerial plant parts, frequencies of epiphytic bacteria follow a log-normal distribution (Hirano *et al.* 1982). The consequence is a strong variability in population levels (e.g. from leaf to leaf) complicating sampling and data analysis (need of bulked samples) and affecting the distribution of the biological control agents.

Two of the main environmental factors affecting the epiphytic fitness of the pathogen and biocontrol agent in the aerial plant part are wetness (W) and temperature (T) during wetness periods (W). The daily dynamics of W and T is generally complex, but upon establishing threshold values of T and W for a period of time, suitable periods of activity of the pathogen/biocontrol agents can be determined. Also, from long-term observations in a given area, the favourable periods for infection under field conditions can be identified. Colonization and survival of the antagonist *P. fluorescens* EPS62e upon inoculation on pear trees during bloom, was monitored by a strain specific quantitative PCR (total population) and by plate counting (culturable population) (Pujol *et al.* 2006, 2007). Active growth and colonization were observed during bloom, survival was reported on immature fruit, but very low survival or even death was observed on leaves upon inoculation. Similar results have been reported for other biocontrol agents such as *Bacillus amyloliquefaciens* EPS2017, *Pantoea agglomerans* EPS125 and *Lactobacillus plantarum* PM411 (Daranas *et al.*, 2018).

It is therefore evident that a key issue in the success of biological disease control in the phylosphere is the improvement of the antagonist fitness. Ways to improve fitness of biocontrol agents have been achieved to counteract adverse conditions by means of different approaches that include physiological or genetic improvement of the strains. We have reported successful improvement of the efficacy using physiological adaptation approaches such as osmoadaptation during production or nutritional enhancement of the formulation in *P. agglomerans* EPS125, *P. fluorescens* EPS62e and *L. plantarum* PM411 (Cabrefiga, J. *et al.* 2011, Daranas N. *et al.* 2018). Interestingly, not only survival under field conditions was improved by a better adaptation to drought onto the leaf surface, but also higher efficiency of biological control was observed.

The knowledge of the biological cycle of the pathogen and biocontrol agent in relation to the host have provide tools for guided application and timing of biocontrol agents, and several examples have been reported, mainly in fire blight biological control. For example, stigmata imprinting to verify the presence and incidence of *E. amylovora* colonizing flowers has been used to estimate the risk of infection and then to schedule biocontrol treatments (Thomson, S.V. 1992). Also, this technique has been used to confirm colonization of the plant host by the biological control agent after spraying it to the orchard (Sundin *et al.* 2009).

Forecasting models of several bacterial diseases have been used for risk mapping or treatment scheduling, as well as to analyse disease dynamics in space and time. Forecasting models are available for diseases caused by *P. syringae* pathovars on bean, tomato and pear, *E. amylovora* on apple and pear, and *Xanthomonas* pathovars on rice, *Prunus* and walnut (Steiner P.W., 1988, Llorente I. *et al.* 2017, Morales *et al.* 2018). Interestingly, these models also have an important role as tools to schedule treatments of the biological control agents. An example has been reported for the timing of *Aureobasidium pullulans* strains treatments for fireblight control using the Maryblyt system (Kunz, S. 2004).

Finally, it can be speculated about the influence of climate change in biological control of bacterial diseases. The possible scenarios suggest a need for new BCA strains better adapted to higher temperatures and drought. It is expected that current commercial biocontrol agents may not work properly due to the arrival of

unfavourable environmental conditions that compromise their efficacy and ecological fitness. In addition, there will be also a need to establish new threshold actions and possibly to re-evaluate the existing disease forecasting models under the new conditions expected due to global climate change.

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The interactions among biocontrol agents, pathogens and the environment: the concept of environmental niches

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Summary
The use of biological control agents (BCAs) is supported by Directive 128/2009/EC on the Sustainable Use of Pesticides, which gives priority to non-chemical methods for disease control. Although the intensive research of the last decades, the practical use of BCAs is still challenging. Biocontrol of plant bacterial diseases involves complex interactions among the target pathogen, the host plant, and the BCA population in a changing environment. The colonization rate and the efficacy of the BCA, as well as the bacterial growth and infectivity, are all influenced by weather conditions, such as temperature and moisture. To achieve an effective integration of BCAs in a disease management program is then relevant to know how the target pathogen and the BCA are influenced by the environment. The concept of "environmental niches" is proposed to understand how the environment influence the interactions of BCAs and plant pathogenic bacteria by using *Erwinia amylovora* as a case study. Environmental niches are defined as the environmental conditions necessary for the presence of a species and the maintenance of its population.

Keywords: *Biological control, Erwinia amylovora, fire blight disease*

Fire blight, caused by *Erwinia amylovora* (Burrill 1882) Winslow *et al.*, is a destructive bacterial disease for apple and pears, which can affect other plants in the Rosaceae family (Vanneste 1996). This pathogen causes considerable yield losses and constitutes a market access barrier between countries (Vanneste 1996). The search for biological control agents against *E. amylovora* has been increased as an alternative to chemical control for many reasons (Johnson and Stockwell 2000, Vanneste 1996), including the public concern about the effects of chemicals on human health and the environment (Alavanja *et al.* 2004, Epstein 2014). In Europe, the use of antibiotics against phytopathogenic bacteria is forbidden and the chemical control of fire blight is based on copper products, which will be strongly limited in next years (La Torre *et al.* 2018). Therefore, there is an increasing interest in explore alternatives to chemical control, which include the use of microorganisms like yeasts, fungi, and bacteria, that may suppress *E. amylovora* via antibiosis, cell-to-cell interaction, nutrient competition, and competitive exclusion by colonization of the entry sites on the plant host (Cabrefiga *et al.* 2007, Johnson and Stockwell 1998, 2000, Vanneste 1996, 2010). These microorganisms, known as biological control agents (BCAs), have the potential to complement and/or replace chemicals. Unfortunately, only few products are commercially available today for controlling fire blight (Vanneste 2010). Although a high number of BCAs have been tested under laboratory conditions, only a few ensure good control in the field, even though divergences among locations and years have been observed in many cases (Johnson and Stockwell 1998). A possible reason for poor and variable field efficacy of BCAs is that they are living organisms that dynamically interact with the target pathogen, the host plant, and the microbial communities on the host surfaces, in a changing physical environment. Indeed, the timing and the weather conditions strongly affect the BCA establishment and growth on the host surfaces, and, finally, its efficacy in controlling fire blight (Johnson *et al.* 2000, Pusey and Curry 2004). Also, the ability of *E. amylovora* to cause infection is strictly related to the environmental conditions (Shwartz *et al.* 2003). For these reasons, a successful integration of BCAs in a disease management strategy requires an understanding of their ecological requirements, so that they can be used when environmental conditions are favorable for their establishment, growth and efficacy.
In this work, we propose an "environmental niche" approach to study the effect of the environment in the BCA-pathogen relationships and to improve the selection of BCAs in the practical management of plant bacterial diseases. Environmental niches are defined as the environmental conditions necessary for the

presence of a species and the maintenance of its population (Chesson *et al.* 2001). This concept is broadly used in ecology, but not in phytopathology. In this work, the environmental niches were defined for two microorganisms commonly used in the biocontrol of fire blight, *Aureobasidium pullulans* and *Pantoea agglomerans* (Vanneste 2011). For these BCAs, the environmental niches were defined considering temperature and humidity intervals in which their growth was null (no growth), minimal (<=20% of maximum growth), marginal (>20-50%), considerable (>50-80%), and maximal (>80%). A score was assigned to the above growth rates as follows: no growth=0; minimal=1; marginal=2; considerable=3; and maximal=4. This score was used to determine the temperature (T) and humidity (RH) combinations at which the growth rate of the two BCAs (*A. pullulans* or *P. agglomerans*) was higher than, equal to, or lower than the growth rate of *E. amylovora*. A second analysis was conducted to determine the extent of environmental niche sharing by each BCA and *E. amylovora*. First, the growth scores (from 0 to 4) of each BCA and of *E. amylovora* were multiplied. In a second step, a matrix was developed in which rows were the above products for humidity, and columns were the products for temperature; in this matrix, cells were the products of the values in rows and columns. The frequency of cells in which the product was >0 showed the extent of environmental combinations in which the two microorganisms interact; the sum of the values in the cells provided information on the intensity of such an interaction.

Erwinia amylovora grows between 4 and 35°C, with relative humidity >40%, and shows optimal growth at 15-30°C and RH>80% (Billing 1974, Pusey 2000, Shreshta *et al.* 2005). *Aureobasidium pullulans* grows at 2-34°C and RH>52%, with optimal growth at 15-29°C and RH>77% (Vero *et al.* 2009, *not published data*). *Pantoea agglomerans* grows at 8-38°C and RH>94.5%, with optimal growth at 17-36°C and RH>96% (Costa *et al.* 2002). Based on this information, the environmental niche approach shows that *E. amylovora* prevails over *A. pullulans* when T>30°C and RH <67%; under these conditions, *E. amylovora* is expected to be more competitive than *A. pullulans* in colonizing the host's surfaces. When temperature is in the range of 12-30°C and RH>68%, the two microorganisms grow at similar rate; finally, *A. pullulans* prevails over *E. amylovora* at temperatures of 3-12°C and RH>50%. Under the latter conditions, the BCA is expected to be more competitive in occupying the niches where it interacts with *E. amylovora* and, as a consequence, to be more effective in disease control. For the second BCA, the environmental niche approach shows that *E. amylovora* prevails over *P. agglomerans* under most temperature and RH conditions, due to the strong limiting effect of RH on *P. agglomerans* growth; *P. agglomerans* is expected to prevail over *E. amylovora* only at temperatures >30°C and RH>96%. The comparison between the two BCA shows that the pathogen shares a wider ecological niche with *A. pullulans* than with *P. agglomerans*. The frequency of niche sharing is 85 and 13% for *A. pullulans* and *P. agglomerans*, respectively, and the intensity of interaction is approximately 5.5 times higher for *A. pullulans* than for *P. agglomerans*. These results suggest that *A. pullulans* has the potential to compete with the target pathogen under a wider range of environmental conditions than *P. agglomerans*, and that *A. pullulans* would be able to grow under a wider range of environmental field conditions.

The environmental niches described in this work were built by using the results of the experiments developed by Billing (1974), Pusey (2000), and Shrestha *et al.* (2005) for *E. amylovora*; Vero *et al.* (2009) and our experiments (*data not published*) for *A. pullulans*; and Costa *et al.* (2002) for *P. agglomerans*. These niches could probably be improved by considering additional literature or by conducting additional research. The intention here is not to give definitive recommendations based on the environmental knowledge of these microorganisms (the pathogen and the BCAs), but to propose the concept of environmental niche as a tool for improving the biological control of fire blight, as well as for other pathogens. This approach may help researchers identifying those BCAs that occupy (or partially occupy) the same niche as the target pathogen. Those BCAs may therefore have higher probability of growing under the same environmental conditions than the pathogen; this may lead to greater interaction between the microorganisms and the pathogen, and potentially to higher BCAs efficacy. Furthermore, when different BCAs are available for a specific pathogen, the selection of the BCA to be used in a specific field application should include considerations regarding the environmental conditions at the time of application and in the following days, to increase the probability that the selected BCA will occupy the target environmental niche. Environmental niches could also be a starting point for the development of dynamic, weather-driven models for BCA–pathogen systems. Development of these models will require a deeper knowledge of the biology and epidemiology of the BCAs and how the environment affects their fitness and efficacy against the target pathogen. Since obtaining the needed information could be challenging, priority should be given to the most-studied species of BCAs and for those already included in commercial products. Once new information becomes available, models accounting for

both biocontrol mechanisms and environmental conditions could be developed, which may help enhance the biocontrol of bacterial diseases.

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EPPO activities in efficacy testing and safety assessment in biological control

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Summary
Since 1996, EPPO has worked on biological control agent (BCAs) with the Joint EPPO/IOBC[1] Panel on Biological Control Agents. Within this Panel, the focus is on safety of BCAs. EPPO has published a 'List of biological control agents widely used in the EPPO region` (PM 6/3) which represents a 'positive list' for which EPPO recommends its member countries to use a simplified procedure for import and releases. These are BCAs which are either indigenous and widespread in the EPPO region, or non-indigenous but established and widespread in the EPPO region or used by at least 5 EPPO countries for at least 5 years without records of any negative non-target effects. This positive list was first adopted in 2001 and has been annually amended (except between 2002 and 2008).

The assessment of the potential benefits and impact of a biological control agents are essential requirements when evaluating the risks of releasing organisms into an area. The requirements and procedures for the approval process will vary depending on the type of organism to be released (macro-organisms: arthropods, compared to micro-organisms: fungi and nematodes). Additionally, the application or mode of release (whether it is formulated of released as such), the target pest (whether it is a plant pest or an invasive alien plant) and the type of biological control being applied. For the latter, for example, biological control can be defined into classical biological control (the utilisation of natural enemies from the pest`s origin aiming at their establishment) and augmentative biological control (the mass production and periodic release of natural enemies) which can further be divided into inoculative augmentative biocontrol, the release of small numbers, or inundative augmentative biocontrol where a large number of natural enemies are released to control a pest population). For completeness, conservation biological control involves the manipulation of the habitat or environment to increase the abundance and effectiveness of a naturally occurring natural enemy.
Requirements and procedures for the approval of import and releases of biological control agents (BCAs) such as micro-organisms and invertebrates vary widely between EPPO countries. Implementation at the national level varies, even within the EU. At a global scale, the International Plant Protection Convention (IPPC) provides guidance on the assessment of the use of BCAs. The Organisation for Economic Co-operation and Development (OECD) provides guidance on the use of biopesticides. At the EU level, the EU Regulation on Invasive Alien Species (1143/2014), the EU Regulation on Placing of Plant Protection Products on the Market (1107/2009) and the EU Habitats Directive all have relevance for BCAs (EMPHASIS, 2016).
Following a Joint EPPO/COST-SMARTER Workshop on the Evaluation and Regulation of the Use of Biological Control Agents in the EPPO Region, in 2015 (Budapest), recommendations were presented that could be put into practice to improve the regulatory framework for BCAs. These included:

- Guidance is needed on which regulations should be applied in which cases (e.g. the scenarios presented at this workshop). EPPO/IOBC and EU could have a role in this
- Common definitions would be useful (e.g. 'indigenous')
- National authorities should be encouraged to establish effective co-ordinating mechanisms to ensure a coherent respond to requests to use and release BCAs (e.g. between authorities responsible for environment, agriculture and health regulation)
- Proposed releases of BCAs should be discussed early on with the national authorities in order to agree host test lists etc. in advance

[1] http://www.iobc-global.org/

- More harmonisation should be achieved through recognition and use of existing EPPO guidance, additional guidance where needed, sharing of information on applicable regulations and on specific applications for releases between regulators in neighbouring countries, and development of a form of "mutual recognition" between countries with similar conditions
- An independent expert review group for applications at European level should be explored again, building on EFSA's experience at reviewing the evidence on release of a non-native BCA against an invasive acacia in Portugal
- A distinction should be made between BCAs expected to establish (normally introduced with the intention of classical biological control) and BCAs not expected to establish (normally introduced on a commercial basis as augmentative biocontrol)
- Decisions on import and release of BCAs should be made in the context of a background level of introductions of new organisms into the EPPO region and their spread within the region. Not all spread of organisms can be avoided – particularly within the EPPO region across land borders
- Potential use of biological control should be included in contingency planning for arrival of new pests in the EPPO region, so that some of the information needs and regulatory hurdles can be addressed in advance
- Some of this contingency planning should take place at a European level
- Fast track procedures should be considered for emergency situations
- The Euphresco research funders and managers network2 offers one way in which research on biological control options might be co-ordinated between countries to which a pest is native (or where it is well established) and countries to which that pest is likely to spread,
- Analysis of a proposed release should include the environmental, economic and social benefits as well as risks including:
    - benefits from reduced environmental damage by the target pest
    - benefits from reduced use of other control options, which have a negative impact on the environment
    - other benefits e.g. human health benefits from control of allergenic plants and pests
- Benefits and risks should be quantified where possible, even though there may be a large level of uncertainty e.g. about the efficacy of a classical BCA
- Inclusion of benefits in the analysis requires some evidence of efficacy
- Information should be exchanged between national authorities on the spread and impacts of BCAs which have been released (with or without authorisation)

Efficacy. Within the remit of plant protection products, EPPO`s work focuses on efficacy of plant protection products which includes biopesticides. EPPO Standard PP 1/296 *Principles of efficacy evaluation for low-risk plant protection products* describes the requirements for an efficacy evaluation of low-risk plant protection products in a registration procedure. More specifically PP1/276 *Principles of efficacy evaluation for microbial plant protection products* describes the principles for determining the requirements for an efficacy evaluation of plant protection products containing micro-organisms. For microbial products, a similar approach should be used as for chemical products. However, by their nature, products based on micro-organisms may be highly specific in the pests that they affect or require specific environmental conditions to reach optimal effectiveness (EPPO, 2012).
For classical BCAs, the expectations of the efficacy level often rely on their efficacy in the areas of their origin and especially in the areas where they have already been used for classical biocontrol (e.g. low damage caused by *Agrilus planipennis* in China and efficacy of the pest parasitoids used for biocontrol in the USA support the expectation of good efficacy of those parasitoids if established in Europe). Extrapolation from laboratory tests is also possible, but these should be interpreted with caution.

Safety. As previously mentioned, until now, EPPO activities in biological control have focused mainly on the safety aspects of introduction of BCAs. The Joint Panel has to-date worked on macro-BCAs (though they have also considered nematodes). Evaluating the safety aspects of biological control agents is one of the most important factors to ensure non-target damage in the area of introduction. Biocontrol practitioners collect a large amount of data during the evaluation phase of the BCA. A large part of the evaluation process

[2] https://www.euphresco.net/

includes host range testing of the BCA to ensure no non-target effects. But equally, information on the ecology and biology of the agent is collected, along with data on climatic suitability and other factors that may influence establishment and spread. Coupled with this, information on the potential benefits of a BCA is important information that can be included in a dossier to inform decision-makers.

To facilitate this, EPPO has recently published the Standard PM 6/4 *Decision-support scheme for import and release of biological control agents of plant pests* which provides guidance on impact assessment for a BCA. Although the Decision-support scheme (DSS) was developed for invertebrates as BCA, the elements of the DSS could also be applied to micro-BCAs, but questions may have to be amended to reflect this. The Standard provides detailed instructions for the following elements of environmental impact assessment (EIA) for biological control agents (BCAs) of plant pests (including pathogens): initiation, probability of BCA establishment and spread in the impact assessment area (IAA), and assessment of potential positive and negative environmental consequences. PM 6/4 consists of two parts, the first is an express assessment, that evaluates if the BCA can be assessed using the express assessment or if the user should proceed to the second part, the full assessment. PM 6/4 stipulate that the full scheme should always be used in the case of a classical BCA introduced into a new area against weeds or invasive plants. Using expert judgement, an overall conclusion on the benefit and associated consequences of the introduction of a BCA is drawn.

PM 6/4 supports decision-making on introduction and aims to harmonize the assessment procedure within the region. This Standard is based on ISPM 11 (FAO, 2013) Pest risk analysis for quarantine pests, ISPM 3 (FAO, 2005) Guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms, EPPO Standard PM 5/3 (EPPO, 2011) Decision-support scheme for quarantine pests and EPPO Standard PM 6/2 (EPPO, 2014) Import and release of non-indigenous biological control agents.

In conclusion, the regulatory framework for the release and use of biological control agents within the EPPO region requires further harmonisation between member countries. This can help to ensure the utilisation of safe biological control agents in an era where the availability of chemical control methods is decreasing. To facilitate this, EPPO has worked to bring together biocontrol practitioners and decision-makers to evaluate the current regulations and make recommendations for further harmonisation within the region. EPPO has published a number of Standards that can be utilised by the biological control community to evaluate the efficacy of microbial formulations and the safety of macro-BCAs.

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Evaluation of tannins respect to their biostimulant and antibacterial activity on tomato plants

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Summary
The phytopathogenic bacterium *Pseudomonas syringae* pv. tomato (Pst) is the causal agent of the tomato bacterial speck disease. Its control is mainly related to the balanced agronomic practices and the preventive use of commercial formulations based on cupric salts. The EU restrictions about the copper use and the needs to reduce the chemicals in agriculture requires to find sustainable alternative that can replace/reduce of cupric salts usually employed to control Pst. In this study, the *in vitro* and *in vivo* biostimulant and antibacterial activity of different tannins were verified respect to Pst. *In vitro* experiments were carried out testing different concentrations (0.5-5% W/V), measuring the colonies units formed. *In vivo* experiments were conducted foliar-spraying tannins at 1% before bacterial inoculation. Results underlined that, two of the tested tannins (UTUST 1, UTUST 3) expressed a remarkable biostimulant and antibacterial activity both.

***Key words**: Pseudomonas syringae pv. tomato, bacterial speck, tomato, natural substances, copper, tannins, biostimulants, biocontrol.*

Bacterial diseases control is mainly based on the preventive and constant use of copper products (Balestra, 2003). The environmental problems related to the accumulation of copper ions in the soil have focused the pesticides research to develop products with lower environmental impact (De Waard *et al.*, 1993). These new control methods are based on the use of natural antagonists and natural substances active against phytopathogenic bacteria (Balestra *et al.*, 2017; Fortunati *et al*., 2016). Tannins represent a very interesting 'group' of natural compounds, since they have antimicrobial properties against fungi, bacteria and yeasts (Brownlee *et al.,* 1990).
The aim of this work was to evaluate *in planta*, the possible use of some tannins in the biological control of *Pseudomonas syringae* pv. *tomato* (Pst), the causal agent of tomato bacterial spot, and to evaluate also a possible biostimulant activity of the selected tannins.
Pseudomonas syringae pv. *tomato* (Pst) CFBP 1323 was the known strain used.
Previously, tannins have been tested in *vitro* at different concentrations 0,5-5% (W/V), incorporating them into synthetic medium, in order to evaluate their possible antimicrobial activity against Pst inoculated in petri dishes at $1x10^6$ concentration, very closing concentration of bacteria attack in nature.
Since tannins resulted to have *in vitro* antimicrobial activity at each concentration tested, *in vivo* experiments were conducted using tannins at 1% (W/V).
By the *planta* tests, Table 1 shows the different treatment considered and their respective concentrations.

Table 1. List of tannins used in in planta tests and relative concentration

Tannins used	Concentration % (W/V)
UTUST 1	1%
UTUST 2	1%
UTUST 3	1%
UTUST 4	1%
UTUST 1 + c. hydroxide ½ F.D.	1% + 0,045%
UTUST 2 + c. hydroxide ½ F.D.	1% + 0,045%
UTUST 3 + c. hydroxide ½ F.D.	1% + 0,045%
UTUST 4 + c. hydroxide ½ F.D.	1% + 0,045%

The *in planta* tests were carried out in a greenhouse under controlled environmental conditions. For each thesis (Tab. 1), 30 San Marzano's tomato plants were used and all the tests were repeated 3 times. Tests were carried out on small tomato plants (1^{st} true leaf stage) and on developed plants (4^{th} true leaf). On both phenological stages, foliar area (cm^2) and shoot and root dry weight (g) were calculated to evaluate a possible biostimulant activity. Besides, NBI index (calculated as ratio chlorophyll/flavonoids) of the tomato

culture was calculated by an optical sensor (Dualex). While, only for the plants at 1st true leaf stage, shoot and root dry weight (g) were also calculated to evaluate a possible biostimulant activity. Moreover, Pst epiphytic survival (cfu/cm^2) was considered to evaluate tannins antibacterial activity. At 1st true leaf stage, plants were treated three times and then, after 24 hours, spray-inoculated with Pst at 10^6 cfu/ml. Instead of, at 4th true leaf stage, plants were treated once before the pathogen inoculation. Leaves were sampled after 1, 7, 14- and 21-days post inoculation and tomato leaf area was calculated using APS Asses software. Moreover, leaves were washed out with sterile distilled water (10mL), in order to calculate the epiphytic survival on Petri dishes (cfu/mL) and, relating these values to the measured leaf areas. All data were analyzed by ANOVA (Analysis of Variance) and Tukey test.

By *in vitro* tests, all tannins tested against Pst did not show colonies formed, confirming their antimicrobial activity (Fig. 1). UTUST 2 mixed with a half dose of copper hydroxide showed colonies formed as much as an half dose of copper used alone.

Leaf development (1st true leaf stage), related to treatments with different tannins, showed smaller areas respect to the water control at second and third weeks (Fig. 1). Moreover, the effect of all different substances used was the same in these two weeks. At the last week, FITOLTAN U1 showed the best result, and its value resulted to be significantly higher than controls (P < 0,01).

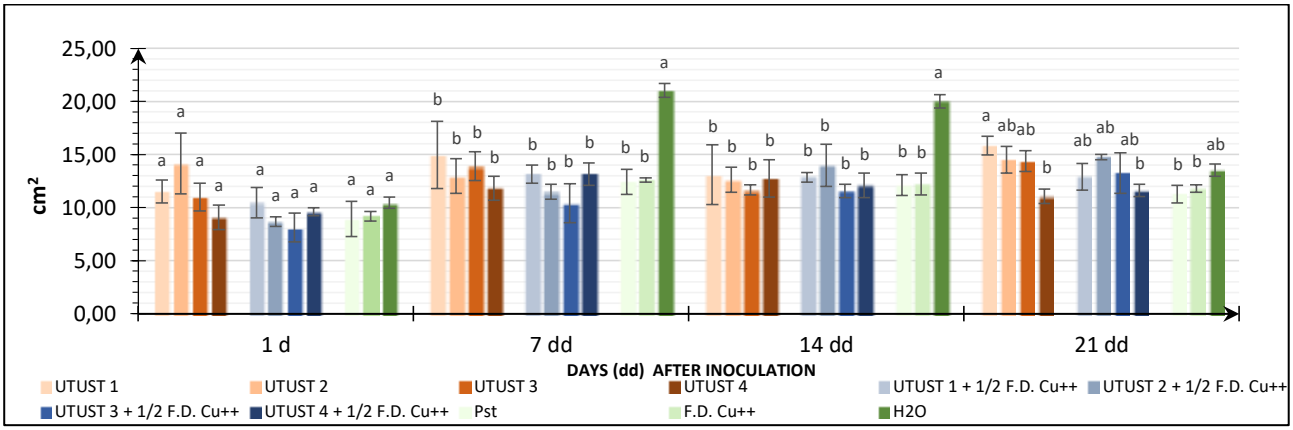

Figure 1 Development area on tomato plants at first true leaf stage

After three foliar treatments, the nitrogen condition in all thesis, showed a significant increase (P < 0,01), except in the control plants inoculated with Pst (Fig. 2).

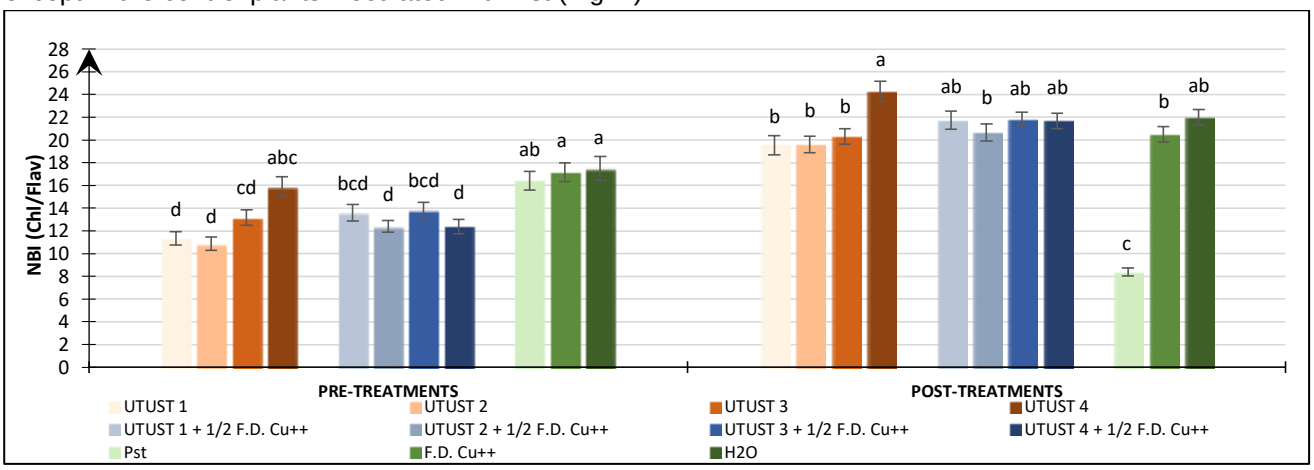

Figure 2. Nitrogen Balance Index (NBI) on tomato plants at first true leaf stage.

Apparently, plants treated with tannins resulted to have an epigeous dry biomass greater than plants treated with copper hydroxide, but not significant differences were showed by ANOVA analysis (n.s.). Shoot root ratio resulted to be not affected by the foliar tannins treatments (Data not showed).

Pst epiphytic survival evaluation showed that tannins have antibacterial activity as much as copper hydroxide. In particular, on 21th day post inoculation, Pst survival resulted to be much more affected by all tannins used alone and by tannins mixed with copper hydroxide (except for UTUST 2 and UTUST 4) than by

copper hydroxide as control (Fig. 3). Moreover, UTUST 3 mixed with copper hydroxide has shown the best significant statistically result (P < 0,01).

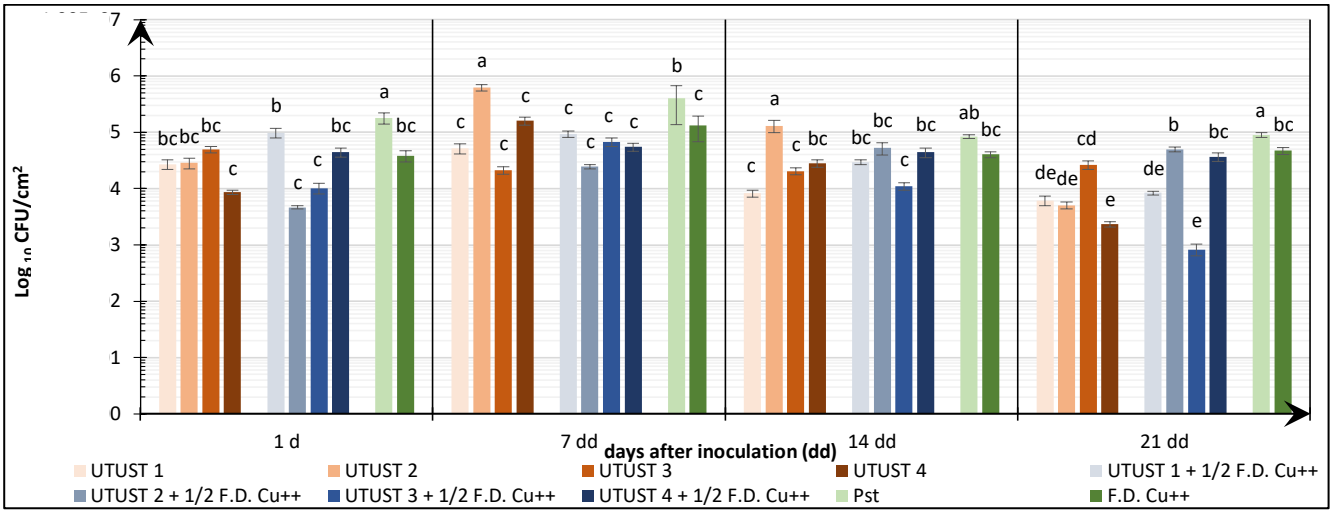

Figure 3. Epiphytic survival of Pst (cfu/cm2) on tomato plants at 1st true leaf stage.

Leaf development (4th true leaf stage) related to treatments with different tannins, was not subjected to significant differences during all the experimental trial (data not showed).
The nitrogen condition of the developed plants did not show significant differences after only one treatment (data not showed).
Epiphytic survival evaluation (Fig. 4) showed that tannins reduced Pst survival for all the infection trial. Importantly, at 14th day, all thesis with tannins mixed with copper hydroxide showed reduced epiphytic survival as much as the control. At the last washing sample, tannins still had an equal antibacterial activity as much as the control and their values were significant by ANOVA analysis (P < 0,01).

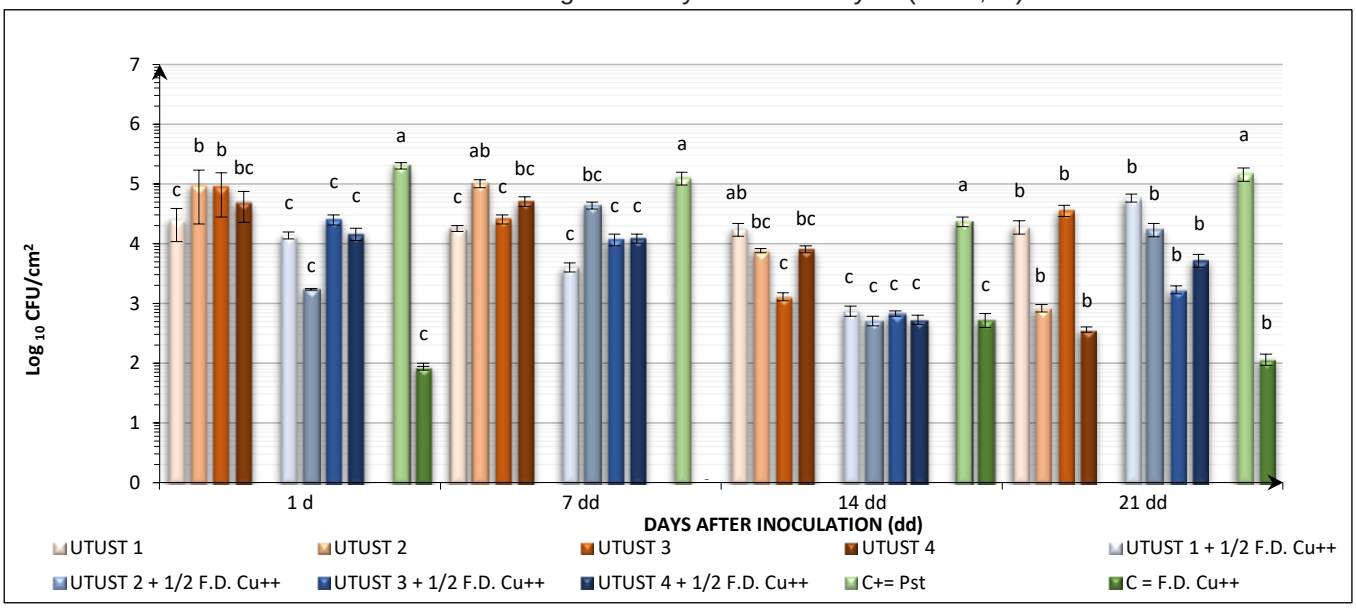

Figure 4. Epiphytic survival of Pst (cfu/cm2) on tomato plants at 4th true leaf stage.

The present study highlights that tannins are not phytotoxic when used at the above concentration. They have been shown to have biostimulant characteristics expressed by the increase of nitrogen content after three preventive foliar treatments with tannins. Moreover, tannins showed a notable antibacterial activity against Pst, and, notably, a long-term application resulted to be more effective than copper hydroxide.

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Biological control of Pierce's disease of grape by an endophytic bacterium

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Summary
The potential for *Paraburkholderia phytofirmans* strain PsJN to reduce Pierce's disease of grape caused by *Xylella fastidiosa* was explored. Strain PsJN achieved population sizes as large as 10^6 cells/g and moved 1 meter or more within 4 weeks after inoculation into vines. While *X. fastidiosa* grew and moved extensively in grape when inoculated alone, few viable cells were recovered when it was co-inoculated with strain PsJN and disease severity was always greatly reduced. Large populations of strain PsJN could be established in both leaf lamina and petioles by topical application of cell suspensions in 0.2% of an organo-silicon surfactant conferring low surface tension; such treatments were as effective as direct puncture inoculations of this biocontrol strain in reducing disease severity. Inoculation of strain PsJN into plants at the same time as or 4 weeks after the pathogen resulted in large reductions in disease severity; much less disease control was conferred by inoculation 4 weeks prior to that of the pathogen. The expression of grapevine PR1 and ETR1 was substantially higher in plants inoculated with both *X. fastidiosa* and strain PsJN compared to that in plants inoculated only with the pathogen or strain PsJN, suggesting that this biological control agent primes innate disease resistance pathways in plants that otherwise would have exhibited minimal responses to the pathogen.

***Keywords**: Xylem, Xylella fastidiosa, Paraburkholderia phytofirmans, surfactants*

Xylella fastidiosa is a highly successful pathogen of a wide variety of plants including grape, olive, citrus, almond and other crops because of its ability to move readily through the xylem vessels of plants after inoculation by xylem sucking insects. The success of the pathogen seems to be due primarily to its ability to move without restriction of the plant. Much of the previous work on *Xylella fastidiosa* and the control of Pierce's disease in the Lindow lab at the University of California has dealt with a cell density-dependent gene expression system mediated by a family of small signal molecules called diffusible signal factor (DSF) which includes 2-Z-tetradecenoic acid (C14-cis), and 2-Z-hexadecenoic acid (C16-cis)(Chatterjee *et al.* 2008) This work revealed that cell density signaling modulated the adhesiveness of cells in the plant, and that movement of the pathogen is essential for its virulence and that artificially increasing DSF levels in transgenic plants greatly increased the resistance of these plants in both greenhouse and field studies to Pierce's disease by limiting the spread of the pathogen after infection (Lindow *et al.* 2014). Limiting spread of the pathogen in various ways is important in controlling disease because not only can the pathogen itself block the flow of xylem fluids in the xylem vessels, but the plant appears to perceive *X. fastidiosa* and attempt to limit its distribution by the production of tyloses, which themselves can block the flow of xylem fluids and thereby make disease symptoms worse (Chatterjee *et al.* 2008). While endophytic bacteria might be exploited to produce DSF in plants, until recently, no strains capable of growth or movement in grape had been found. We found however that *Burkholderia phytofirmans* strain PsJN (Sessitsch *et al.* 2005) was capable of extensive growth and movement within grape. *Burkholderia phytofirmans* stain PsJN has recently renamed *Paraburkholderia phytofirmans* due to the recognition that it is genetically unrelated to other *Burkholderia* strains which are potentially human or plant pathogens, and is thus genetically similar to a variety of environmental strains known not to be plant pathogens. Our intention therefore was to use such a strain as a surrogate host for the *rpfF* gene from *X. fastidiosa* that encodes DSF synthase. We found however that this *Paraburkholderia* strain itself was capable of mediating very high levels of control of Pierce's disease (Baccari *et al.* 2018).

The extent of biological control of Pierce's disease by *P. phytofirmans* was remarkable in several ways. The effect of biological control of plant diseases by the inoculation of beneficial bacteria normally is optimum when the biological control agent is inoculated into plants before that of the pathogen. In such a process, the

biological control agent is able to colonize the plants before the onset of inoculation with the pathogen, thereby achieving sufficiently high population sizes that optimum competitive exclusion or perhaps induction of host defenses can occur, thereby limiting the ability of the pathogen to multiply in the plant and cause disease. Remarkably, while inoculation of grape with *P. phytofirmans* up to three weeks before that of inoculation with *X. fastidiosa* led to a reduction in disease incidence and severity, much greater reductions in disease severity were achieved when the two species were inoculated at the same time (Baccari *et al.* 2018). It was noteworthy that the two strains is not need to be co-inoculated together at the same site in the plant for biological control to occur when inoculated at the same time. Even more remarkable however was the observation that the biological control of Pierce's disease of grape was usually greatest when *P. phytofirmans* was inoculated up to three weeks or more after that of *X. fastidiosa* (Baccari *et al.* 2018). Unlike the control of Pierce's disease achieved by increasing the abundance of pathogen signal molecules in the plant which reduced the rate at which the pathogen moved in the plant, the population size of *X. fastidiosa* was often undetectably low in plants that had also been inoculated with *P. phytofirmans* either at the same time as or even up to three weeks after that of the pathogen (Baccari *et al.* 2018). The apparent death of *X. fastidiosa* even at some distance away from the apparent presence of *P. phytofirmans* suggested that disease control was mediated by the resistance reaction by the plant itself. Indeed, whereas the expression of plant disease response genes such as PR1 were not induced in plants inoculated with the pathogen itself, or by *P. phytofirmans* itself, high levels of induction of such genes occurred in plants inoculated with both strains (Baccari *et al.* 2018). Such a phenomenon suggests that *P. phytofirmans* in some way primed the innate resistance of grape.

X. fastidiosa is restricted to the xylem vessels within plants, interactions with *P. phytofirmans* and any host response apparently must occur within xylem vessels. Inoculation of plants with either the pathogen or *P. phytofirmans* can be achieved by a so-called droplet puncture method wherein small droplets of bacterial suspensions are placed on stems or petioles and then punctured with a needle, allowing the negative pressure within xylem vessels to draw the cell-containing fluid into the plant. While this is a highly efficient method of inoculation of the biological control agent, such a method may not be practical on large-scale under field conditions. Other more high-throughput and rapid methods of inoculation were therefore explored. Topical application of cell suspensions to which organosilicon surfactants having very low surface tension enabled the penetration of the fluid, and thereby the cells, into the plant. While many cells apparently would remain within the apoplast of the plant, substantial populations of *P. phytofirmans* were found apparently within the xylem tissue after such topical application (Baccari *et al.* 2018). The associated watersoaking is rapid and extensive after such treatments. (Figure 1). High levels of disease control were achieved by topical application of *P. phytofirmans* by this method (Baccari *et al.* 2018). This method therefore appears to be a very practical one for application to grape, perhaps two other plants infected by *X. fastidiosa.*

Figure 1.
Appearance of Pinot Noir grape leaves approximately two minutes after spray application of *Paraburkholderia phytofirmans* in a solution of 0.2% Breakthru. Note the water-soaked areas on the leaf indicating the spontaneous infiltration of the bacterial suspension into the leaf.

Our continuing results from greenhouse and field studies show remarkable ability of this biological control agent to move within plants and to inhibit the movement of *X. fastidiosa*, thus achieving very high levels of disease control. The current work is providing a better understanding of the ways in which this biological control agent can be used for disease control, and extensive field evaluations to exploit the information learn from greenhouse studies are underway. Preliminary results suggest that the biological control agent will be highly efficacious, and that it could be used in conjunction with other disease control strategies such as DSF-mediated pathogen confusion in transgenic plants or by topical application of signaling molecules, as well as with other resistant plants that are being developed in other laboratories.

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Successful control of fire blight: Can bacteriophages do the job?

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Summary

The use of lytic bacteriophages for the control of the fire blight pathogen, *Erwinia amylovora*, exploits the ability of this biological to kill the pathogen on the blossom surface. In our system, an epiphytic bacterium, named the carrier, is infected by phages. The carrier acts as a delivery and propagation system and a biological control agent. Upon application and on the pistil surface, the carrier-phage populations increase prior to the arrival of the pathogen. During the development of this biopesticide, multiple developmental processes were employed in order to improve efficacy at field level. This includes the selection, isolation and characterization of broad host range lytic phages, selection of *Pantoea agglomerans* isolates as carrier, *in planta* bioassays and multi-year field trials. In addition, the study examined the effect of host EPS on phage infection and the presence of lysogeny in field collected pathogen and carrier. Currently, the focus is on the development of a stable formulated carrier-phage preparation and the continued of field-based trials.

***Keywords**: biological control agent, biopesticide, agriculture, phage therapy, Erwinia herbicola*

Bacteriophages were independently discovered by Frederick W. Twort and Félix d'Herelle at the beginning of the 20th century (Summers, 2005). d'Herelle was the first to coin the term bacteriophage, 'the eater of bacteria' and recognise the potential of using bacteriophages as therapeutic agents for the treatment of bacterial pathogens (d'Herelle,1917; Summers, 2005). Recent review articles have examined the use of bacteriophages for the control of agricultural pathogens (Buttimer *et al*., 2017; Jones *et al.,* 2018; Nagy *et al*., 2012; Svircev *et al*., 2018). The introduction of the fire blight pathogen from North America to Britain resulted in definitive work on *E. amylovora* and its bacteriophages by Eve Billing in the 1960s (Billing, 1960; Billing *et al*., 1961). Light microscopy and India ink study revealed that the majority of the 150 *E. amylovora* isolates collected from infected orchards were capsulated (Billing *et a*l., 1961). Billing noted that the capsulated and non-encapsulated *E. amylovora* isolates were infected by different bacteriophages and produced distinctly dissimilar plaques (Billing, 1960). The bacterial capsule of *E. amylovora* would later be shown to play a critical role in pathogenicity and phage infection (Roach *et al*., 2013). Erskine (1973) was the first to demonstrate reduced or no disease symptoms when pear slices were co-inoculated with a yellow saprophyte lysogen (*Erwinia herbicola,* currently *Pantoea agglomerans*) and white colonies of the fire blight pathogen. Significantly, it was recognised that the yellow saprophyte individually was antagonistic to the fire blight bacteria and the phages released from the lysogen had the combined ability to act as biological control agents. The saprophyte also protected the lysogenized phages from sunlight and high temperatures (Erskine, 1973). Ritchie (Ritchie and Klos, 1977) isolated, enriched, purified and fully characterised a number of *E. amylovora* bacteriophages recognizing their potential for the control of fire blight pathogen (Ritchie and Klos, 1979). In the 1980s and 1990s *Erwinia* bacteriophage research remained dormant mainly due to the high efficacy of streptomycin. The advent of streptomycin resistance, popularity of organic fruit production and the public aversion to the use of antibiotics in agriculture led to a phage renaissance (Born *et al*., 2011; Boulé *et al*., 2011; Müller *et al*., 2011; Nagy *et al*., 2012; Nagy *et al*.,2015). The following short paper describes our work on the development of the phage-carrier biopesticide for the control of fire blight in the orchard. This development is based on phage characterization, description of interactions with *E. amylovora* and *P. agglomerans* including host range and mechanisms by which phage resistance may develop, and production and testing of formulations in field trials.

The initial phage isolation and enrichment using six wild type isolates of *E. amylovora* yielded 42 bacteriophages that were characterization by PCR (limited to a single phage), restriction endonuclease patterns and electron microscopy (Gill *et al*., 2003). Later characterization has been supplemented with genomic sequencing and, in particular, high throughput DNA analyses. The first *Erwinia* phage to be fully sequenced was phage φ21-4 isolated in southern Ontario, Canada (Lehman *et al*., 2009). Full genomic sequences of the 40 bacteriophages in the collection have shown that the phages in the collection fall into four distinct taxonomic groups. The *Myoviridae* are represented by ϕ21-4 (Lehman *et al.,* 2009) and ϕ35-70 (Yagubi *et al.,* 2014) and the *Podoviridae* by ϕ31-1(unpublished data) and ϕ9-2 (Wittmann *et al.* 2015).

Phage host range was determined by the traditional double agar overlay method, where plaques were visually counted and phages were recognised simply by plaque appearance. Based on plaque assays, *Erwinia* phages isolated from orchard soil in southern Ontario had a wide host range. Interestingly, some phages showed limited ability to infect bacterial isolates from west coast of Canada (Gill *et al.,* 2003). This host range study depended on the visual appearance of the plaques; however, Roach *et al.,* (2013) showed later that the plaque appearance 'hazy vs clear' was very much influenced by the quantity of exopolysaccharides (EPS) produced by the host. Using bacterial EPS mutants and phage production as estimated with quantitative PCR (qPCR), we demonstrated that the *Erwinia Podoviridae* phages had an absolute requirement for amylovoran (Roach *et al*., 2013). Recently, the host range work was expanded to test the ten phages used as biopesticides against a global collection of *E. amylovora* wild type isolates (Steven Gayder, unpublished data). The host range was determined using qPCR and a standardised plasmid protocol. In this system primers were designed for four distinct phage types and host allowing quantification of each of the populations following 8 h of culture. The data revealed that broad host range phages have the ability to infect and produce high titres on isolates from eastern North America, Europe and the Middle East (Steven Gayder unpublished data). Additionally, the phage primers and qPCR were used to determine if lysogens are present in the wild type population of *E. amylovora* and *P. agglomerans* (Roach *et al*., 2015). Lysogens were not detected in 162 global isolates of *E. amylovora* and 82 isolates of *P. agglomerans* from southern Ontario. Induction of stable lysogens in the laboratory proved difficult and only a single stable lysogen was recovered. The work showed that lysogeny can occur in *E. amylovora* but is likely to be far too infrequent in natural conditions to be a concern in the implementation of a phage-based biocontrol. Lastly, the primers can be used to track and quantify phages, pathogen and carrier populations under field conditions (Lehman, 2007).

Phage therapy commonly uses phage mixtures or cocktails to avoid the development of host resistance (Jones *et al*., 2018; Svircev *et al.,* 2018). The production of a phage-carrier that contains a mixture of phages has its own inherent challenges including finding an optimised quantitative protocol that allows the study of phages and their respective host (in this case the carrier). One of the greatest challenges faced in the phage-mediated biocontrol in *E. amylovora* is understanding why one phage is more effective at controlling *E. amylovora* than another. Much of this shortness of understanding stems from a lack of reproducibility from traditional phage assays and protocols. For this reason, much of our focus has been the development of molecular techniques to quantify and characterize *Erwinia* phage. Our most recent progress in this regard was the adaption of the phage one-step growth curve into a new molecular technique (Michael Parcey, unpublished data). This has allowed us to begin exploration of different infection parameters, such as adsorption and burst size, which were previously poorly described for *Erwinia* phages.

"Which phages should be incorporated into the mixtures?". Data from plaque and qPCR based host range and the forced flower bioassays (Lehman, 2007) has been used to determine combinations of phage-carrier that should be used in the field. Laboratory based assays while helpful are often poor predictors of performance in the field. In proof of concept field trials from 2005-2017, non-formulated phage-carrier combinations were tested and efficacies in controlling the pathogen varied from 54-63% compared to the water control. Using the qPCR-based detection technology we can focus on the trials where combinations of phage-carrier carrier succeeded or failed to provide control of the pathogen to better understand the infection dynamics.

Encapsulation of the biopesticide will allow for the survival of the phage-carrier system during processing, storage and field application. Phage-carrier encapsulation will protect phages against hostile conditions such as sunlight and dryness (Ma *et al.,* 2008; Tang *et al.,* 2015). In our current research, spray drying approach was chosen to encapsulate and stabilize phage-carrier system. *P. agglomerans* Pa39-4 (Pa 39-4). Phage □21-4 were selected to study the effect of different formulation protocols on the viability of the bacterial cells and lytic activity of phage after spray drying. Various polymers were screened for formulation prior to spray

drying (unpublished results). Talc/CMC (2 percent) + Trehalose (20 percent) formula was chosen since it did not have a significant effect on the phage infectivity. Subsequently, this material was selected to formulate the phage-carrier system prior to spray drying. Spray drying of a formulated bacterial cells alone resulted in a significant reduction in Pa39-4 by 3 log CFU/ml. However, using osmotically stressed Pa39-4 cells resulted in less than 0.5 log CFU/ml reduction in the bacterial count after the spray drying. While, only one log reduction in bacterial and phage counts was observed after reconstitution of the dried powder produced by spray drying the phage-carrier mixture. The dried phage and its host powder was stable for up to 8 weeks at 4□C. The formulated biopesticide will be field tested in the spring of 2019 using artificially applied *E. amylovora* on three-year-old Gala trees on M9 rootstock.

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Light quality modulates infection and defense response to fire blight in pear trees

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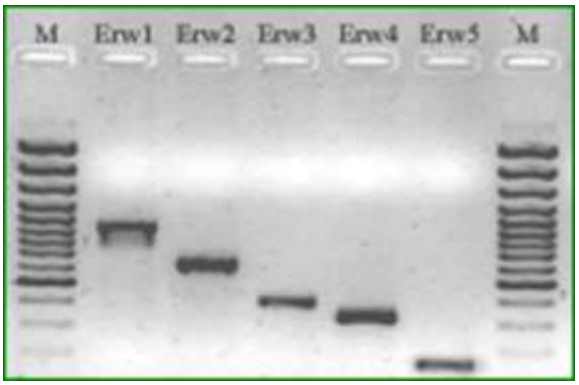
Summary
Erwinia amylovora causes fire blight disease. Pathogenesis-related (PR) proteins are part of the systemic signaling network that perceives pathogens and activates defenses in plant. Eukaryotic and bacterial species have a 24 hour 'body-clock' known as circadian rhythm. This rhythm regulates organisms' lives, as the activity and/or mRNA accumulation of phytochromes (*phys*) and cryptochromes (*crys*) genes, which in turn synchronize the internal clock, working as zeitgeber molecules. Salicylic acid accumulation is under light control and upregulates *PR* genes expression. Moreover, cherry plants overexpressing *phyA* showed increase resistance to *Pseudomonas syringae*. In this work, five bacterial transcripts (*erw1-5*), expressed in asymptomatic *E. amylovora*-infected plants, have been isolated. This research studied how the circadian clock, light quality and related photoreceptors regulate *PR* and *erw* genes expression, in vitro-cultured Pear plantlets of three lines of cv DarGazi. Plantlets were exposed to different circadian conditions, and continuous Blue-, Red- and Far-Red- light. Results showed that *PR10* and *erw* are under circadian control while *PR1* is expressed without clear evidence of circadian regulation. In this regulation framework the active form of phytochrome enhances the expression of *PR1* five to 15 times more. An ultra-dian rhythm has been observed according with the zeitgeber role played by *CRY1*.

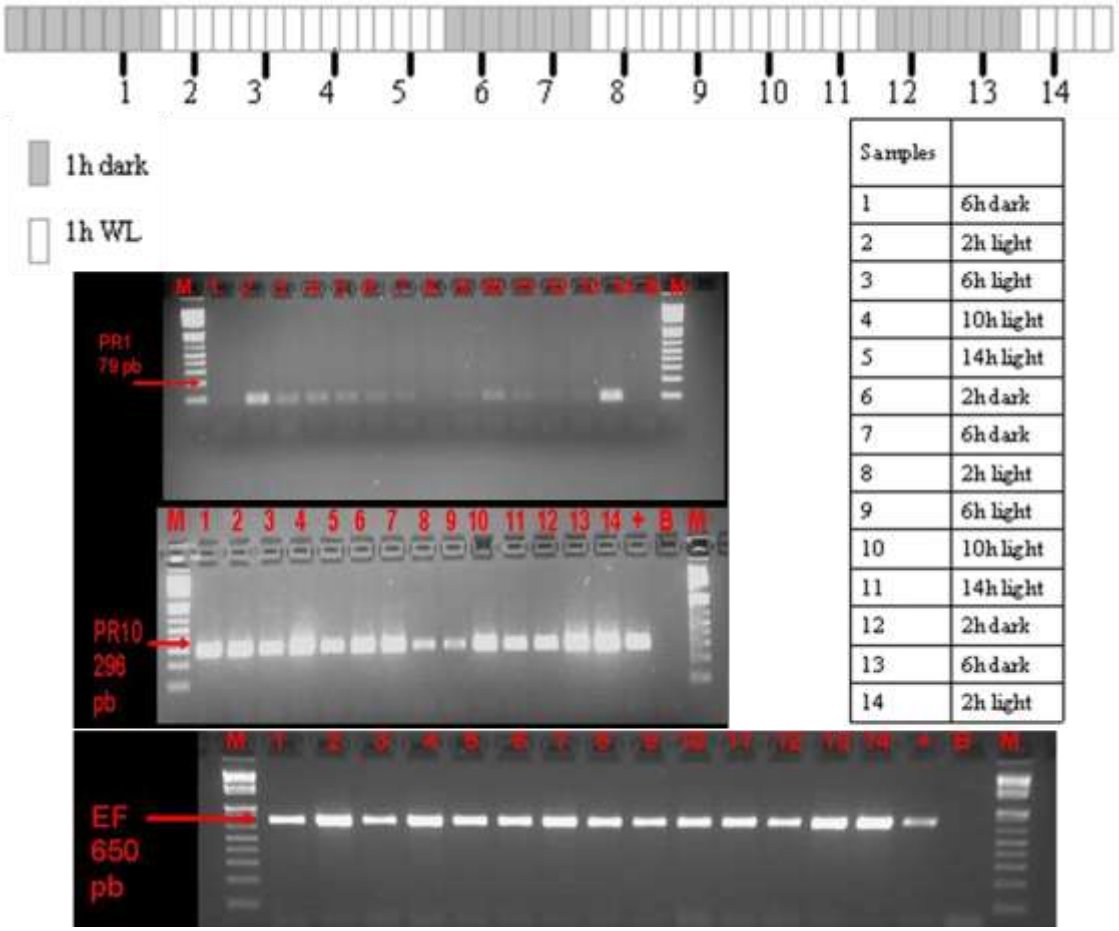
During plant-pathogen interactions a dialogue occurs between the two organisms: plant synthesizes molecules for signalling system and defense, pathogen synthesizes molecules suppress the host defense to break down the barriers of the host and mimic plant hormones. Fire blight, caused by *Erwinia amylovora*, is a disease of agronomic and economic importance that attacks many Rosaceae species, *in primis* pear and apple trees. Pathogenesis-related (PR) proteins are part of the articulated systemic signaling network active in plants to perceive the presence of the pathogen and activate defenses.
Plants, fungi, animals and some bacteria have a 24 hour 'body-clock' known as circadian rhythm. The circadian rhythms regulate organisms' lives in many ways. Tòth *et al*., (2001) demonstrated that the circadian clock regulates the promoter activity and/or mRNA accumulation of *Phytochromes* (*PHYs*) and C*ryptochromes* (*CRYs*) genes, which in turn play also an important role in the synchronization of internal clock, working as zeitgeber, or time-keeper, molecules. Furthermore, Karpiński and collaborators (2003; 2013) have reported that the accumulation of salicylic acid (SA), which upregulates *PR* genes expression, is under control of light. Genaud *et al*., (2002), using *Arabidopsis thaliana phys* nil mutants, proved that *PR1* gene is under the direct control of photoreceptors. More recently, this regulation has been confirmed by Yanovsky group (Faigón-Soverna *et al.,* 2006). Cherry plants overexpressing *phyA* showed an increased resistance to *Pseudomonas syringae* pv. *mors-prunorum*, suggesting a putative role of PHYA in the regulation of *PRs* genes (Cirvilleri *et al*., 2007). In this work, we isolated five bacterial genes (*erw1-5*, Figure 1), which are expressed in asymptomatic *E. amylovora*-infected plants and that could play an important role in the initial phase of the infection. Transcritional changes in host and pathogen genes expression during early *E. amylovora* infection indicated that both plant *PR* and bacterial *erw* genes were temporarily expressed and differentially regulated.

Figure 1.
Exclusivity tests have shown that *erw* primers amplify exclusively specific *E. amylovora* sequences from RNA extracted from infected pear tree plants.



The aim of this research was to understand how the circadian clock, the light quality and the related photoreceptors, affect the regulative system of *PR* and *erw* genes expression. To investigate if the internal clock autonomously regulates the abundance of *PR1* and *PR10* transcripts, an *in vitro*-cultured plantlets system of *Pyrus communis* L. cv Dar Gazi was used. Plantlets of three different lines: Dar Gazi-wt, Dar Gazi-*phyB* (transgenic plant overexpressing *phyB*) and Dar Gazi-*cryI* (transgenic plant overexpressing *cryI*) were exposed to different circadian experimental conditions, and to continuous Blue-Red- and Far-Red- light conditions. Fluorescent white light lamps were used as a control.
Results showed that *PR10* is under circadian control, being highly expressed during dark period, while *PR1* was expressed at low level ratio irrespective of dark/light period without clear evidence of circadian regulation (Figure 2). Results also showed that *erw* genes were regulated by circadian rhythms, suggesting that light quality plays a role in the host-parasite interaction. A complex regulatory system has emerged in which each photoreceptor plays a specific role, highlight the importance of photoperception during systemic rather than local resistance induction (Griebel T. and Zeier, 2008). In this regulation framework the active form of phytochrome seems to play a clear role in enhancing the expression of *PR1* five to 15 times more. An ultra-dian rhythm has been observed under light-dark cycles indicating CRYI as a possible crucial zeitgeber. The overexpression of the photoreceptors showed a drastically reduction of the expression ratio of the *PR10* gene.

Figure 2.
Gene expression of Pathogen Related proteins (PR1 and PR10) as affected by the circadian clock, whose activity is kept synchronous by the activity of the photoreceptors



Samples	
1	6h dark
2	2h light
3	6h light
4	10h light
5	14h light
6	2h dark
7	6h dark
8	2h light
9	6h light
10	10h light
11	14h light
12	2h dark
13	6h dark
14	2h light

Results will be discussed in relation to the expression of photoreceptors during photoperiodic conditions and pathogen attack.

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Microbial groups and biocontrol agents associated with different crop succession in relation to Potato Brown Rot suppression

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Summary
Four reasonable naturally infested areas in Egypt (0.12 ha each) were selected to follow three years of crop succession. Two areas were sandy (Wardan, Giza governorate and Ganoub El-Tahrir, Behera governorate) and two areas were silty clay soils (Talia, Minufyia governorate and Sids, Bani Suef governorate). The applied cropping succession system included, corn, potato intercropped with cabbage and onion (first year), cowpea, and wheat (second year), corn again and ended by potato (third year). The pathogen was undetectable after onion, wheat and second corn in the four fields. The pathogen was undetectable at the end of the crop succession program in all fields under investigations except in Sids. The failure of the eradication of the pathogen in this field correlated to the high ratio of NO_3^- and Na^+ in this area as compared to the other three fields. Disease suppression at the end of crop rotation associated with clear shift in cultural soil biodiversity as indicated by enhancing oligotrophism, increased ratio of fluorescent pseudomonads, endospores bacteria and actinomycetes ratios. Also, the suppressed potato soil and rhizosphere supported high ratio of a diverse of *R. solanacearum* antagonists, similar to *Pseudomonads* spp, *S. maltophilia, Citrobacter freundii, Acinetobacter sp, Delftia* sp and *Serratia marcescens* as identified by16S rRNA gene sequencing.

***Keywords**: R. solanacearum, corn, cabbage, wheat, onion and 16S rRNA.*

Wardan area was characterized with low density of the pathogen, high alkalinity (pH8.8), high phosphorus and sulfur contents and poor nutrients. Negative correlation between pH and disease severity was addressed by Messiha *et al*., (2007). Phosphorous and sulfur have had an inhibitory effect against *R. solanacearum* (Norman *et al*., 2006, Williams and Cooper, 2003). Ganoub El-Tahrir soil was characterized with higher density of the pathogen. Although the soil had supported high ratio of fluorescent pseudomonads and actinomycetes, the volunteer infected potato and the secondary hosts decreased this suppressiveness effect. The third area (Talia, silt clay soil) was characterized with high pathogen level, and low C:N ratio which may explain the soil conduciveness as postulated by Grunwald, (1997). The greater iron content in that area had decreased potential of siderophores producing biocontrol agents. The fourth area (Sids) supported moderate pathogen level. C:N ratio was high in this area which is expected to slow the OM decomposition by decreasing the microbial activity (Shunfeng *et al*., 2013). The high incidence of the disease may be attributed to the extremely high ratios of $N\text{-}NO_3$ and Na^+ in soil of that area as compared to the other three areas. The positive correlation between the soil nitrate and sodium from one side and bacterial wilt incidence on the other side was addressed by Messiha *et al.,* (2007).
The pathogen was below the detection level in corn soil and rhizosphere in Wardan and Sids areas (Figure 1), accompanied by an increase in endospores, fluorescent pseudomonads and actinomycetes densities. The soil in both areas supported reasonable ratio of antagonistic *Streptomyces* spp. (Table 1) which can be considered as indicators of soil suppressiveness (van Bruggen and Semenov, 2000). Planting corn had negative effect at Talia was due to much iron suppressing the efficiency of siderophore producing agents.
The pathogen was significantly suppressed in potato rhizosphere intercropped with cabbage as compared to potato rhizosphere grown solely which prove the suppressive effect of cabbage against *R. solanacearum*. This was most clear at Talia area where significant increase in actinomycetes, and endospores in cabbage

rhizosphere along with high ratio of antagonists in rhizosphere of both cabbage and potato. Onion soil and rhizosphere supported the highest ratio of antagonists as compared to other crops with increasing oligotrophic bacteria (oligotrophism) and fluorescent pseudomonads along with significant increase of *S. maltophilia* and *Leclercia adecarboxylata*. Oligotrophism as a result of ecological succession as well as fluorescent pseudomonas are indicators of soil health and disease suppressiveness as postulated by van Bruggen and Semenov, (2000). The pathogen was detectable again in cowpea soil with even higher ratio in cowpea rhizosphere as compared to its soil. This was correlated with a decrease in oligotrophic ratio in cowpea rhizosphere as well as a reduced fluorescent pseudomonas ratio. No antagonists were detected either in cowpea soil or rhizosphere. These conditions may reflect the increase of soil conduciveness for the pathogen except at Talia where cowpea rhizosphere supported significant ratio of antagonistic bacteria similar to *P. moorei, P. aeruginosa* and *S. maltophilia*. Cowpea and corn supported the growth of *S. maltophilia* which was accompanied by decrease in survival of *R. solanacearum* as addressed by Elhalag *et al*., (2015). The pathogen was undetectable in wheat soil and rhizosphere. This decline in pathogen persistence was accompanied by a drastic decrease in endospores along with a significant increase in fluorescent pseudomonads ratio along with considerably great antagonistic *Streptomyces* spp. The suppressive effect of wheat soil and rhizosphere may be related to the long growing period (6 months). The pathogen was not detectable in second corn and final cropping potato soil and rhizosphere. This soil suppressiveness accompanied by an increase in endospore, fluorescent pseudomonads and actinomycetes ratios as compared to the beginning of the experiment. Unlike the three other areas, Sids area was characterized with dominant copiotrophic while the ratio of actinomycetes, fungi and fluorescent pseudomonads were neglected in final potato soil and rhizosphere which reflected the failure of eradication of the pathogen after the crop succession in this area. Also, the high nitrates and sodium contents may partially explain the disease incidence in that area as compared others.

In conclusion, Wheat and onion followed by corn are highly recommended to be used as crop successions before cropping potato but should be used spontaneously according to the suitable planting season. Through this crop succession a shift in soil microbial biodiversity to the suppressive direction in a sustainable natural way is expected. Other edaphic factors, such as C:N ratio, pH, OM and soil minerals should be taken into consideration. Meanwhile, secondary hosts and volunteer infected tubers would make crop rotation ineffective.

Figure 1.

Inoculum density of the pathogen in different soil and rhizosphere (Log CFU+1) after different crop successions in 4 areas. Wardan and Ganoub El-Tahrir (sandy soil). Talia and Sids (clay soil)

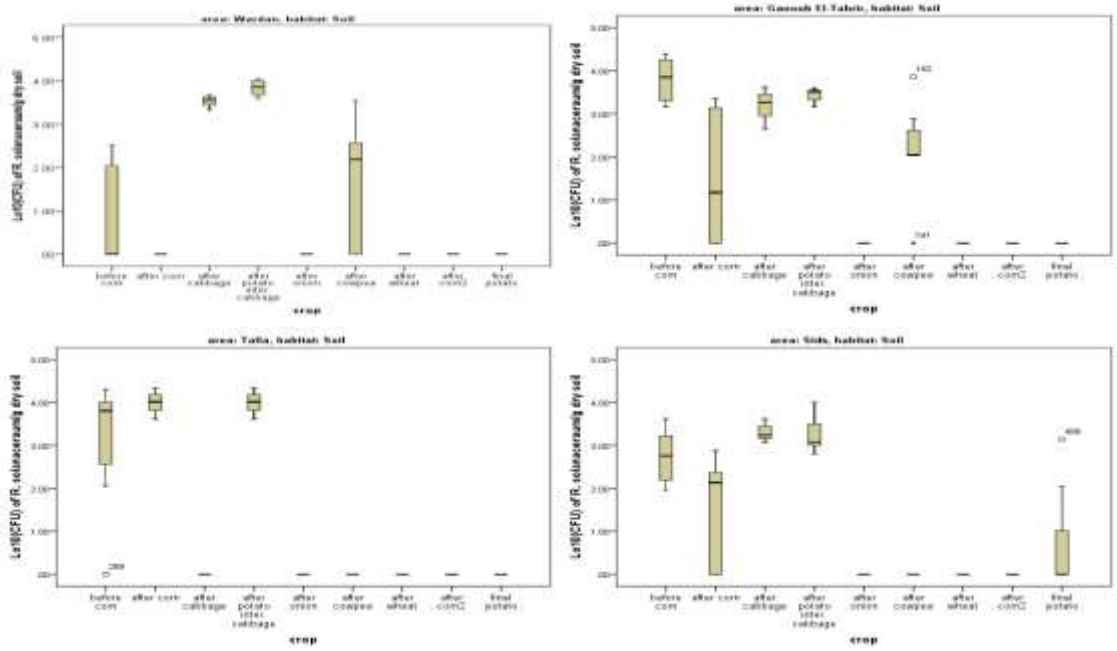

Table 1. List of isolated bacterial antagonists following different cropping system

	Estimated ratio of identified *R. solanacearum* antagonists to the total cultural community							
	Wardan		**Ganoub El-Tahrir**		**Talia**		**Sids**	
Crop								
Corn	*Streptomyces intermedius*	1.0%					*S. erythrogriseus* (99%)	1.0%
							S. coeruleorubidus group (99	1.0%
							Streptomyces spp (100%)	1.0%
Potato/cabl	*P. monteilii* (96%)	0.6%	*S. albidoflavus* group	0.8%	*Stenotrophomonas maltophilia*	1.0%	*Bacillus marcorestinctum*	2.0%
			P. putida group (99%	0.7%	*Citrobacter murliniae* (99%)	1.0%	*Serratia quinivorans* (99%)	2.0%
					Serratia quinivorans (99%)	2.0%	*P. putida group (99%)* [1]	0.2%
					P. protegens (92%)[1]	3.0%		
Cabbage			*Streptomyces caerule*	1.4%	*Pseudomonas sp* (98%)[1]	1.0%	*B. cereus/B. thuringiensis (99*	1.0%
					P. rhizosphaerae (97%)	1.0%	*P. brassicacearum/ P. corrug*	1.0%
					P. vranovensis (98%)	1.0%	*P. vranovensis/ P. reidholzens*	1.0%
					S. proteamaculans (86%)	1.0%	*P. putida* group (99%)	1.0%
					Perlucidibaca aquatic (89%)	1.0%		
Onion	*Leclercia adecarboxylata*	0.7%	*P. putida* group	8.2%	*P. aeruginosa* (99%)	0.4%	*P. mendocina* (97%)[1]	3.0%
	P. putida group (99%)[1]	7.1%	*S. maltophilia*	3.2%			*S. maltophilia strain SA21-01*	1.0%
	P. fluorescens group	0.7%	*Bacillus* spp	1.2%			*P. plecoglossicida* (99%)	1.0%
	P. fulva (99%)	5.0%	*P. entomophila/*	2.4%			*P. putida group (99%)1*	1.0%
	S. maltophilia (99%)	0.6%	*P. monteilii* (99%)[1]				*B. cereus/ B. thuringiensis*	1.0%
							Ochrobactrum	1.0%
Cowpea					*P. moorei (99%)*	2.0%		
					S. maltophilia	4.0%		
					P. aeruginosa (99%)	4.0%		
Wheat	*S. erythrogriseus* group (9	1.0%	*S. maltophilia*	1.0%		1.0%		
			D.	1.0%		1.0%		
Second cor	*Pseudomonas putida* grou	1.0%	*Beijerinckia fluminen*	1.0%	*Enterobacter asburiae (99%)*	1.0%	*Pseudomonas fluorescens*	1.0%
			P. fluorescens group	1.0%	*Providencia sneebia* (99%)	1.0%		
			Burkholderia	1.0%	*S. maltophilia* (99%)	1.0%		
			S. maltophilia	1.0%				
			P. prosekii (99%)	1.0%				
Final Potato			*P. putida* group	2.0%	*P. seleniipraecipitans* (98%)	1.0%		
			S. maltophilia	1.0%	*P. putida* (99%)	1.0%		
			P. aeruginosa (99%)	2.0%	*S. maltophilia* (99%)	3.0%		
					P. rhizosphaerae (99%)	1.0%		
					Citrobacter freundii (97%)	1.0%		
					Acinetobacter sp (99%)	1.0%		
					D. tsuruhatensis (99%)	1.0%		
					Serratia marcescens (99%)	1.0%		
					P. aeruginosa (99%)	1.0%		

Identification was made by DNA sequencing either for the V6 to V8 region of the 16S rRNA gene as described in Hiddink et al. (2005) or by MicroSeq®500 (16S rDNA microbial identification kit) provided by *Applied Biosystem* using 8-capillary Genetic Analyzer (*Applied Biosystem*).

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Control of tomato bacterial wilt (*Ralstonia solanacearum*) by grafting and bacteriophage application

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Summary
Tomato bacterial wilt disease caused by *Ralstonia solanacearum* is difficult to control. Grafting with H-7996 and local rootstocks suppressed bacterial wilt into some degree of protection. This paper reported the use of grafted plant and application of bacteriophage to control tomato bacterial wilt. Local rootstocks (Amelia, Mawar) were sown two days before scion (Servo) while H-7996 was sown at the same day with scion. The plants were grafted by tube grafting method at three weeks after sowing the scion. Bacteriophages were isolated from several region of tomato fields and tested in vitro for their infection activity against *R. solanacearum*. The phage that had widest spectra against several isolates of *R. solanacearum* then was chosen for the next experiment. Soil in polybags were infested with 20 ml of water suspension of *R. solanacearum* at 10^8 cfu/ml then added with 10 ml bacteriophage solution. A bacteriophage from a region in Central Java showed the widest host range compare with the others. Clear phages were produced in the lawn of *R. solanacearum* in CPG medium. Combination of grafting and bacteriophage application enhanced protection against bacterial wilt of tomato caused by *R. solanacearum*.

***Keywords**: Tomato, Ralstonia solanacearum, bacterial wilt, grafting, bacteriophage*

Bacterial wilt disease caused by *Ralstonia solanacearum* is one of the most important diseases of tomato. Depending on crop season, disease incidence of tomato bacterial wilt range from 10 percent - 80 percent in China (Jiang *et al*., 2017), and sometime reach 100 percent in Indonesia. *R. solanacearum* is one of the most important plant pathogens, especially in the tropics and subtropics area (Mansfield *et al.*, 2010). The pathogen and the disease it caused is difficult to control due to high genetic variability of the pathogen (Prior and Fegan, 2005), wide host range (Hayward, 1994), and its complex of entry points to the host plants (Goto, 1992). *R. solanacearum* is a soilborne plant pathogen and survived long time in the soil even without the presence of its host plant (Hayward, 1991). Therefore, pathogen-free soil is ideal place to grow tomato without bacterial wilt threat, but it is almost impossible to find such soil except clearing new land. However, this problem has been solved by growing tomato in a hydroponic system. While the vast majority of tomato growing in the tropic is conducted outdoor, a reliable control method should be found to minimize disease incidence. Grafting has been practiced in many countries to enhance production and to control plant diseases including bacterial wilt (Lee, 1994; Kubota *et al*., 2008). Grafting with tomato H-7996 and eggplant Eg-203 suppressed tomato bacterial wilt and increase yield (Arwiyanto *et al*., 2015), however the control effect was not consistent (unpublished data). Indonesian local tomato varieties have been used as rootstoks and suppressed bacterial wilt and root knot nemathodes into some degree (Arwiyanto *et al*., 2018). To enhance the degree of protection it is necessary to find other method of control that compatible with grafting. Bacteriophage has been used to control plant pathogens (Jones *et al*., 2012) including to control *R. solanacearum* (Fujiwara *et al*., 2011). Here, we report the use of grafting combined with bacteriophage to control tomato bacterial wilt.

Bacterial strain. *R. solanacearum* race 1, biovar 3, phyllotype1 was isolated from local area in Yogyakarta, Indonesia and used as challenge strain. Working culture was grown on CPG (Casaminoacid peptone glucose) agar media while stock culture was preserved in 50 percent glycerol and kept at – 80 °C.

Tomato cultivars and sowing condition. Tomato var. H-7996, Amelia, and Mawar were used as rootstocks and var. Servo as scion. H-7996 was obtained from AVRDC Taiwan (now WDC); Amelia, Mawar, and Servo were purchased from a local agriculture shop. Amelia and Mawar were sown two days before H-7996 and Servo in cocopeat:rice hull charcoal media (50 percent / 50 percent, vol/vol). The media was irrigated with water until cotyledons emerge then by half strength of Hoagland solution until ready for grafting.

Grafting procedure. The tomato plants were grafted according the method of Arwiyanto *et al*., 2015.

Bacteriophage isolation. Bacteriophage was isolated from the rhizosphere soil of tomato plants collected from different places. The phage was isolated according the method of Brunchoth *et al*., 2015.

Inoculation. Grafted plants were transplanted into vinyl pots containing sterilized field soil. Holes were made in the soil to position plants. Inoculum of *R. solanacearum* in a water suspension at 10^8 cfu/ml was poured into the holes (20 ml per hole) then followed by pouring a water suspension of bacteriophage into the same holes (10 ml per hole).

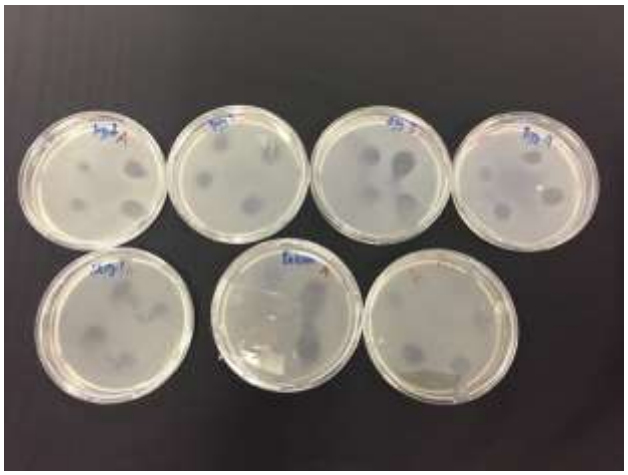
Five bacteriophages isolated from five different tomato field produced clear zone in the lawn of *Ralstonia solanacearum* (Figure 1). Bacteriophage from Kopeng area had widest activities against isolates of *R. solanacearum* in vitro and then was used for the next experiments along with *R. solanacearum* isolate number 4 which was the most sensitive isolate (Table 1).

Table 1. Activity of bacteriophages against *Ralstonia solanacearum*

Isolates of *R. solanacearum*	Bacteriophage isolated from				
	Kopeng	Bojong	Sawangan	Ketep	Muntilan
1	+	-	-	-	-
2	+	-	-	-	+
3	+	-	-	-	-
4	+	+	+	+	+
5	+	+	-	-	+
6	+	-	-	-	-
7	+	-	-	-	+

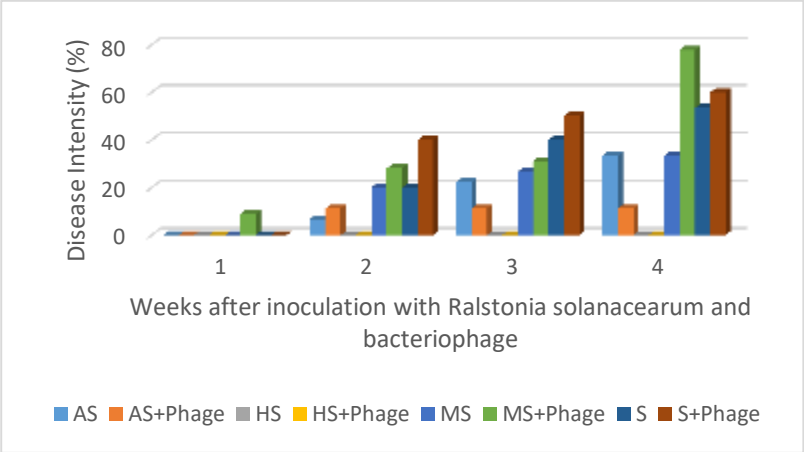
Note : + there was clear zone in the lawn of *R. solanacearum*, - no clear zone

Figure 1. Plaque of bacteriophage isolated from the rizosphere of tomato at the lawn of *Ralstonia solanacearum*



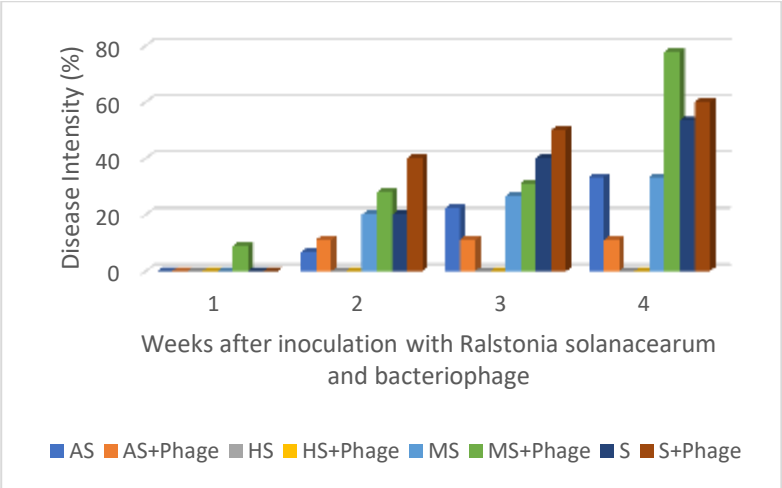
Grafted plants inoculated with *Ralstonia solanacearum* showed the disease development with various degrees of disease intensity (Figure 2).

Figure 2. Bacterial wilt development on grafted tomato plants



At the first weeks after inoculation there was no symptom of bacterial wilt at any grafted plants as well as at non grafted plants. Tomato var Servo grafted with H-7996 (HS) showed no any symptom until 4 weeks after inoculation while non grafted plants (S) the disease intensity reached 53.3 percent. Tomato plants grafted with two local rootstocks (AS and MS) exhibited higher disease intensity compared with those grafted with H-7996 but still have lower disease intensity compared with those non grafted plants (S). The local rootstocks was less superior compared with H-7996 in the bacterial wilt suppression, and this result agree with previous report (Arwiyanto *et al.*, 2018).
When grafting combined with application of bacteriophage, the degree of protection against bacterial wilt varied depended on rootstocks (Figure 3).

Figure 3.
Bacterial wilt development on grafted tomato plants added with bacteriophage



While application of bacteriophage in the tomato plants grafted with H-7996 did not exhibit any bacterial wilt symptom, it is difficult to justify whether addition of bacteriophage played a role in bacterial wilt suppression. However, two local tomato rootstocks reacted differently when combined with bacteriophage. Application of bacteriophage on tomato grafted with Mawar (MS) did not enhanced the degree of suppression, instead, the disease intensity was higher in the plot added with bacteriophage. When tomato grafted with another local rootstock (AS) then added with bacteriophage, there was significant difference in the suppression of bacterial wilt compare with those non added with bacteriophage. The results indicated that grafting with resistant rootstock combined with application of bacteriophage suppressed the development of bacterial wilt of tomato. The degree of protection depended on rootstocks and its combination with bacteriophage.

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Recent advances on the control of *Xylella fastidiosa* and its vectors in olive groves: state of the art from the ongoing Europe's Horizon 2020 Research Program

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Summary
Search for therapeutic solutions to suppress the development of diseases caused by strains of *Xylella fastidiosa* in infected plants has been, since the last century, one of the most challenging objective in several applied research programs developed in the Americas, and more recently this topic is of high priority in the European research programs. A wide range of sustainable approaches are included in the multidisciplinary research workplan funded by the European Commission: (i) search for olive varietal resistance or tolerance; (ii) applications of antimicrobial formulations and use of antagonists are also at an advanced stage (i.e. *in planta* and under field conditions); (iii) control of the vector populations, a complementary research task, aiming on one side to elucidate the feeding behaviour toward setting strategies for disrupting the transmission events, on the other side by testing organic or inert compound to suppress juveniles and adults. The implications of having strategies for mitigating *Xylella*-disease are particularly relevant for those European territories (demarcated areas) where the bacterium has established and containment strategies are in place

***Keywords**: Xylella fastidiosa,* vector control, genetic resistance

Olive resistance to *Xylella fastidiosa*.
Among the numerous plant species listed in the database of the European susceptible plant species of *Xylella fastidiosa*, olive is one of the major affected crop species, with trees that upon infections by a specific strain of *X. fastidiosa* succumb to the deadly disease termed Olive Quick Decline Syndrome (OQDS). This is the situation occurring in southern Italy (Apulia region) where infections, associated with *X. fastidiosa* subsp. *pauca*, ST53, are causing a severe epidemic (Saponari *et al*. 2019). However, periodic surveys and monitoring in the epidemic area provided consistent element on the phenotypic resistance (mild or no symptoms) in infected trees of some cultivars, and specifically of Leccino and in the selection FS17®. Likewise, infection rates in the olive groves of these cultivars were consistently lower. Because the use of genetic resistance is considered one of the most promising long-term management strategy for OQDS, an intense screening program for resistance was started for more than 100 selections, combining exposure of the plants to the natural pressure of inoculum in the field, and by artificially inoculating the Apulian strain on potted plants maintained under controlled conditions. Although, these ongoing experiments require long-term evaluation, preliminary data confirm that differential susceptibility to *X. fastidiosa* exists in the olive germplasm. Briefly, based on the molecular analysis and visual inspections, the cultivars are categorized in: (i) genotypes with low propensity to sustain systemic infections, i.e. vector and needle-inoculations result in a low percentage of plants infected; (ii) genotypes that upon infections support low bacterial populations levels; (iii) genotypes that upon infections harbour high bacterial population sizes but do not show symptoms; (iv) genotypes that upon infections harbour high bacterial population sizes and develop within a shorter time than other cultivars symptoms of desiccation. Indeed, a set of genes with altered expression in resistance *vs* susceptible cultivars (Giampetruzzi *et al*., 2016) is under validation on a large panel of infected cultivars. These genes are mainly related to the drought stress imposed by the pathogen, particularly controlling abscissic acid (ABA) and to the pathogen perception by receptor kinases (RLKs). Interestingly, similar findings have been described in grapevine (Rapicavoli *et al*. 2018; Zaini *et al*. 2018) in which the pathogen is perceived, in the early stage of the infection.

Field testing of N-acetylcysteine applications to reduce symptoms of *X. fastidiosa* in olives.

Field tests for the assessment of the therapeutic effects of several compounds (Fosetyl alluminium; Protein of Harpin; COS-OGA; Acibenzolar S-methyl) against *X. fastidiosa*, proved their inability to reduce the occurrence and the severity of the desiccation phenomena induced by *X. fastidiosa* on the susceptible olive cultivars, except for some applications of N-acetylcysteine (NAC), known to disrupt the biofilm matrix and cell aggregates. NAC was tested in field under in four different trials under different experimental conditions (trees of different age, with different initial incidence of symptoms and infections) and mode of applications (fertirrigation, soil application mixed with organic fertilizers, trunk injections). Trials started on 2015 and plants were periodically checked for the presence *X. fastidiosa* and for the presence of symptoms. Diagnostic tests were performed by qPCR and symptoms severity was scored on a 0 (asymptomatic plant) to 5 (plant completely desiccated) scale. Endotherapic applications (one application/year) in new plantations (low incidence of infections and symptoms) or in olive groves with only limited initial incidence of the infections, were the only conditions that yielded some reductions in the occurrence of dieback and branch desiccation. Even if, quantitative PCR on the trees did not show any significant reductions (treated vs non-treated controls) in the bacterial population size. Regardless the mode of application, the uptake of NAC was confirmed in all cases by HPLC analysis and by the phytotoxicity effects (leaf drop) recorded when the highest doses were used. Along with further observation and tests, the competence of the bacterium to colonize the new growth and its vector-transmissibility from the NAC-treated trees will be assessed.

Use of *Paraburkholderia phytorfirmans* PsJN as potential biocontrol agent against *Xylella fastidiosa* in olives. Among the numerous attempts to use endophytic bacteria to control diseases caused by *X. fastidiosa*, a new encouraging possibility came from the observation that the bacterium *Paraburkholderia phytofirmans* PsJN was able to reduce the symptoms caused by *X. fastidiosa* in grapes affected by Pierce's Disease (Baccari *et al*., 2018). Our study aimed at testing the ability of PsJN to colonize olive xylem vessels and consequently act as biocontrol agent. Although *in vitro* tests showed the absence of competitive inhibitory effects on *Xylella*-growth or biofilm formation, several trials were started in the field on naturally infected trees (curative treatments) or on newly planted trees exposed to the natural inoculum pressure (preventive treatments). Only upon needle inoculation of 1- to 2-year- old shoots, PsJN proved to remain viable for a period of time >500 days, and time course diagnostic tests clearly showed that it moves slowly away from the point of inoculation. The results of the visual inspections upon one single application and one season of observations did not revealed significant differences in the reduction of OQDS symptoms nor the population size of *X. fastidiosa* or reduction of the new infections upon preventive applications. Applications will be continued in the upcoming years and preliminary results of PsJN impact, on the resident microbiome diversity indices, in presence/absence of *X. fastidiosa*, have been gathered using a WGS approach.

Strategies for reducing vector populations and transmission of *Xylella fastidiosa* in olive groves.
The main target of the vector control strategies is the juvenile stage of the spittlebug *Philaenus spumarius*, i.e. when the insect populations are more vulnerable and applications more efficacious. In fact, the control of juveniles can contribute significantly to reduce adult populations. This can be achieved through mechanical control of the weeds/groundcover, however, this may not be possible or practical to the extent necessary in many instances. To overcome this limitation, the efficacy of different insecticides, natural or inert substances (sweet orange essential oil, kaolin, zeolite) and synthetic products (deltamethrin, buprofenzin, imidacloprid), sowing different gramineous species to replace the natural ground vegetation and applications of herbicides and pyroherbicides were compared. Among these, soil tillage, pyroherbicides, herbicides, neonicotinoids and pyretroids applied in spring were the most efficacious interventions, able to reduce almost to zero the presence of juvenile spittlebugs.
The experimental work on the insecticidal efficacy against adults of *Philaenus spumarius* showed the highest efficacy and persistence of neonicotinoids and pyrethoids (Dongiovanni *et al*., 2018). Whereas, for organic farming management, applications of kaolin were tested for 4 consecutive years as preventive approach to protect olives from infections. However, even if applied on a calendar basis, its use did not protect the young olives from infections and subsequent symptoms development.
Although, some data are now available on the efficacy of some applications/approaches which will support growers to better target the options for the management of this vector in different agro-ecosystems, it has to be underlined that the control of this highly polyphagous xylem-feeder is still very challenging and novel and sustainable integrated approaches are needed.

Acknowledgements
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A holistic model can be used to explain the symbiotic mitigation of the Olive Quick Decline Syndrome

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Summary

The introduction of a bio-fertilizer (BF), based on symbiotic micro-organisms as agents to promote the yield and health of crops, into the soil is aimed at inducing modifications in the rhizosphere as well as in the plant phenotype. It is here shown that, in *Olea europaea* cv. Ogliarola di Lecce groves affected by Olive Quick Decline Syndrome (OQDS, involving *Xylella fastidiosa* subsp. *Pauca*): i) the vegetative responses to the disease appeared highly variable, but the symptoms were significantly mitigated in two groves out of six and aggravated in only one; ii) the NIR-tomoscopy of hay-litter-bags from non-inoculated soils can be used to forecast the outcome of BF inoculation; iii) a holistic model that gathers differential and compositional analyses of the leaf (pH, crude protein, water) and of the soil (respiration) can explain over 95 percent of the average mitigation response to BF inoculation. The two keys for a successful inoculation have been identified as a high degree of variability of the soil conditions, which is favorable for welcoming the guest BF (lowering the fingerprint of the control litter-bags) and for an enhancement of the homogeneity of the leaves (with increases in the fingerprint of the leaves treated with BF). However, inoculation of BF consortia is far from being the ultimate remedy to mitigate OQDS. Further studies are needed, at a field level, to clarify the soil hosting capacity and to define the mycorrhizal and / or endophytic * plant * pathogen interactions using rapid methods (litter-bags, foliar pH, near-infrared tomoscopy).

Upon an attack by pathogens or insects, plants can "enlist" the help of protective microorganisms and increase their microbial activity to contrast pathogens [1]. However, the delivery of a complex BF, based on microbial consortia (Micosat F ®) [2], can act by modifying the plant's physiology and lowering the in-vivo raw leaf pH, which is a concrete and easy endpoint to measure. Apart from accelerating the metabolism, BF acts on the induction of the genes of resistance present in plants, but which are not expressed without prior contact with pathogens. As a result of the inoculum, a consequent activation or suppression of otherwise silent genes is obtained, which recent studies on the genome of plants have identified as being closely related to contrast and alarm activities toward several phyto-pathologies. Demonstrations of this were pertaining to the recovery of pears heavily affected by *Erwinia amylovora* fire blast [3], and resolving strong outbreaks in coffee Nicaraguans plantations affected by *X. fastidiosa* subsp. *pauca* [4]. Since the above considerations, the present work has been conducted with three objectives: i) to revitalize the root microbiome of the infected plants, that is, to reactivate the symbiotic interactions between the root system of the olive tree and the Arbuscular Mycorrhizae network; ii) to strengthen the defense capabilities of the olive trees by increasing their resilience to the pathogen, through an activation of the latent gene pool; iii) to evaluate simple and accessible techniques to measure the health status of the olive trees as well as the biological status of the soil. This study has involved the use of a BF, which has been defined as "*symbiotic*" because it contains arbuscular mycorrhizal spores, *propaguli*, and other microbial species. The BF was used at a dose of 20 kg ha^{-1} also falling into the framework of precision agriculture, because the inoculum is distributed precisely in the proximity of the secondary roots of adult olive trees affected by OQDS. After three months, treated Symbiotic (S) and non-inoculated Control (C) plants (436 as total) logged in six farms located near Ugento (LE, Italy) were compared to establish their disease severity, by means of a visual appraisal of the Disease Severity Degree (DSD) [0-healty; 1- One dry branch; 2- two÷five dry branches; 3 => five dry branches; 4- plant almost dried; 5- plant totally dry] (Tab. 1). Complementary rapid tests were applied: the published *litter-bags* coupled to NIR-SCIO evaluation [5] (differential and respiratory), and the foliar pH [6, 2], and the new foliar NIR scanning (differential and compositional)[7] (Tab. 1). The Lab-SCIO™ software used the Random Forest algorithm to fingerprint the four classes (CC, CS, SC, SS).

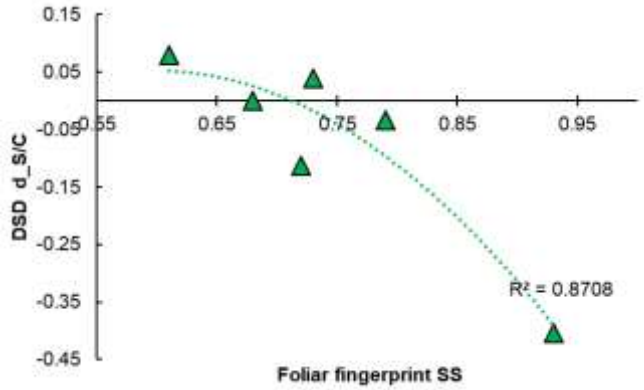
Table 1. Summary table of the disease severity evolution (DSD) observed in the plants (Y) and the results of independent analytical determinations (X) of the foliar pH, foliar NIRS and litter-bags: values of the BF symbiotic effect d_S/C = Ln(S/C).

Farm	Disease Severity	Foliar NIRS fingerprint		Leaf H^+	*Litter-bags*		Soil	Leaf Water	Leaf
	d_DSD	F_CC	F_SS	d_S/C	L_CC	L_SS	d_S/C	d_S/C	d_S/C
A	-3.4%	72.0%	79.0%	-14%	65.0%	87.0%	-1.0%	-0.1%	0.3%
B	0.0%	67.0%	68.0%	-7%	63.0%	70.0%	-11.3%	0.8%	0.7%
C	**-40.4%**	67.0%	93.0%	16%	61.0%	85.0%	3.0%	0.9%	-3.4%
D	**-11.4%**	72.0%	72.0%	5%	73.0%	70.0%	-6.4%	0.0%	-0.7%
E	3.8%	65.0%	73.0%	16%	100.0%	100.0%	37.9%	0.5%	-0.4%
G	**7.9%**	57.0%	61.0%	5%	75.0%	77.0%	-17.3%	0.5%	-0.1%

[1] The one significant non-favorable value is bold red and the two significant favorable values are in bold blue.

A descending parabolic curve (Figure 1) shows that the disease decreased (favourable) when the fingerprint of the S leaves recognized as S was high, and vice versa. Therefore, the symbiotic treatment increased the homogeneity of the leaves.

Figure 1.
Regression of the variation in the disease severity degree (DSD) (Y = d_S/C = Ln(S/C)) on the NIRS fingerprinting of the S-symbiotic olive leaves (X= fingerprint_SS).



Among all the variables, the crude protein in the leaf emerged because of its high correlation (+0.90) with the variation in the disease severity degree, as clearly shown in Figure 2, where a positive relationships linked the two traits, which means that a decrease in the Ln(S/C) of protein favored a reduction in the disease.

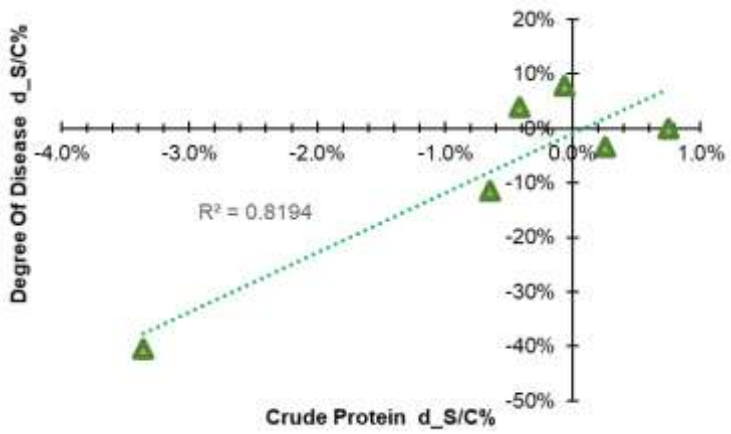
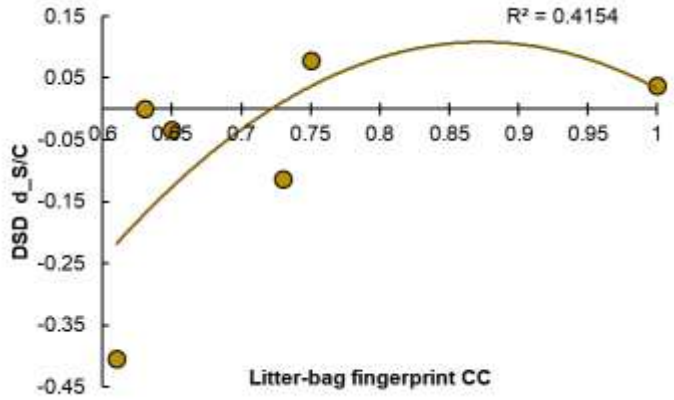


Figure 2.
Regression of the variation in the disease severity degree (Y=DSD d_S/C = Ln(S/C)) on the variation of the mean crude protein content of the leaf (X= CP d_S/C = Ln(S/C).

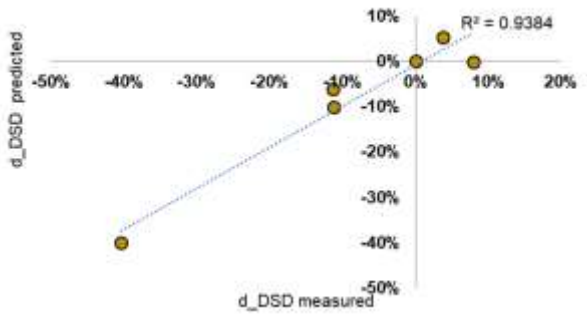
The litter-bags fingerprint appeared very different from the foliar NIRS fingerprint. In fact, an ascending parabolic trend is shown in Figure 3: when the disease decreased, the fingerprint of the Control litter-bags was lower. Vice versa, when the fingerprint of the Control litter-bags increased, the incidence of the disease increased. Therefore, the symbiotic treatment increased the heterogeneity of the litter-bags.

Figure 3.
Regression of the degree of variation of the disease severity (Y, d_DSD) on the fingerprint value of the Control litter-bags (X, Litter-bags_CC).



The average spectra of the litter-bags from the control plots in the six groves were calibrated directly with the effective size Ln(S/C) of the plant response to the disease caused by the pathogen from the soil inoculation. For this purpose, the spectra imported into the WinISI II v1.04 chemometric software were math-treated as 2nd derivates (code SNV, 2, 8, 8, 2), and the observed responses were then fitted to spectra using the modified partial least squares (MPLS) method, in which two latent variables were admitted, and the model was cross-validated. A valuable result was obtained (Figure 4).

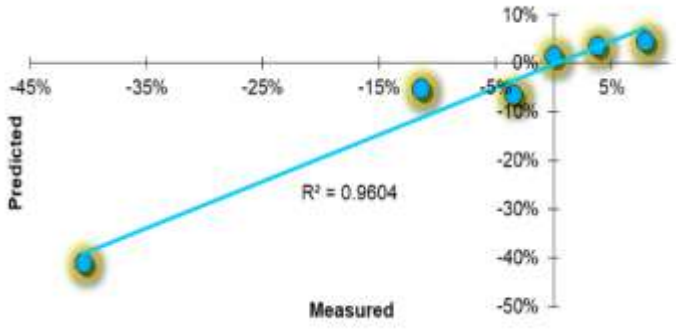
Figure 4.
Fitting of the symbiotic evolution of the disease severity degree (d_DSD) from the average NIR spectra of the Control litter-bags.



A final holistic elaboration, using the Partial Least Squares method (StatBox 6.5, Grimmer Soft, Paris), gathered the most characteristics results from the three main information tools, concerning the plant-soil-BF interactions, in a model [1] that was then used to explain a possible symbiotic mitigation process of the disease:

[1] Ln(S/C) DSD = d_H^+ (-0.155); d_R (-0.209); F_SS (-0.301); L_CC (0.281); Leaf water (-0.133); Leaf crude protein (+0.350). R^2 0.96 (Figure 5) R^2 cross-validated 0.87.

Figure 5.
Linear regression scatterplot of the holistic model solutions for the symbiotic evolution of the disease severity degree (DSD).



In the model [1] there were 6 characteristic factors: 1) the acidity differential, with standardized factor d_H^+ = -0.155, had a negative sign as the factors were opposite: symbiotic BF lowered the pH, raised the H+ and therefore reduced the disease; 2) the fingerprint of the CC litter-bags (L_CC = +0.281) had a positive sign: when the value was reduced, the pathological degree diminished, a sign that the BF had produced some effects; 3) the fingerprint of the SS leaves (F_SS= -0.301) had a high value and a negative sign: when the value was increased, the disease was reduced; 4) the soil respiration had a favorable negative sign (-0.209): when the respiration increased, the incidence of the disease decreased; 5) the water content of the leaves accounted for -0.133 units, which means that a greater quantity of water flowed and remained in the olive leaves during mitigation and recovery; 6) the crude protein accounted for +0.350 units, the highest contribution to the fitting.

Since *X. fastidiosa* subsp. *pauca* damages the vessels and the leaves of plants, why should we pay attention to the roots of these plants? In this work, we have shown that an initial factor (like an *original sin*) can be found in the soil biota. Although we are unaware of the exact etiology of such a favorable response to the inoculation of a small quantity of selected BF, we have described and tested a simple method - litter-bags – which are useful to evaluate the hospitality of a plant-soil complex to the foreign but beneficial BF.

Moreover, we have described and tested a set of rapid analyses to monitor the evolution of the disease, not by means of remote sensing, but through friendly contact with the plant and its earthly world.

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An overview of European regulatory of biopesticides

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Summary
Biopesticides are a contraction of biological pesticides. In the EU, they have been defined as a form of plant protection product based on micro-organisms or natural products; therefore they contain biological control agents (microbials, pheromones, plant extracts, etc.). They are used for pest management through predatory, parasitic or chemical relationship in the agricultural, horticultural and home garden area. The word "biopesticide" covers a wide range of products which can be grouped in the following categories: products containing a micro-organism such as bacterium, fungus, protozoa, virus which are found naturally in the soil, in water; products based on pheromone and other semiochemical, which are communication substances - with no killing effect - found in nature; products based on plant extracts, derived from nature or natural product-like compounds; and then the other biopesticide products such as **macrobials/invertebrates** (insects and nematodes) not registered according to EU regulation.
Biopesticides differ from conventional active substances in various points: by occurring naturally; by being often less stable and rapidly degradable; by being highly host specific (for example microbial insecticides); by having a low toxicity towards non-target organisms and for micro-organisms; by potentially proliferating after release.

Keywords: *Biopesticides, Regulation (EU) 1107/2009, registration process, micro-organisms, semiochemicals, plant extract, natural product-like compounds, regulatory requirements*

The biopesticides registration follows the common process of approval of active substance in Europe according to Reg. (EU) 1107/2009 (Reg.1107): first step, approval of active substance at EU level and second step, registration of product at national level.
Active substances are evaluated through a phased approach. First, an approval application and dossier are submitted by the applicant to a designated rapporteur Member State (RMS). This RMS realises a complete evaluation and produces a draft assessment report (DAR) with the first risk assessment. Specific issues could be discussed with the applicant during this process. The RMS's evaluation dossier is then peer reviewed by EFSA in cooperation with all Member States. At the end of the peer review, EFSA drafts a conclusion report on the active substance. Finally, the European Commission votes a legislative regulation whether or not to include the substance in the European Union's list of approved active substances.
Under the EU rules, it takes at least 2.5 to 3.5 years to obtain the approval of active substance. It could eventually conclude to a low-risk profile, categorising the biopesticide substance as a low-risk substance (criteria set in Com. Reg. (EU) 2017/1432). But because criteria could not be completely fulfilled, expert judgement is often involved.
Within the 484 approved active substances, there are around 40 micro-organisms substances, 20 plant extracts and 20 semiochemicals and others (salts, fatty acids…). Only 14 substances are low-risk substances (ten micro-organisms, three natural extracts and one mineral). Active substances are generally approved for a period of 10 years (7 years for candidate of substitution to 15 years for low-risk substances). After this period, it is possible for an applicant to apply for renewal. The procedure for the renewal of active substance follows the same pathway as for first approval.

When the active substance is approved, the applicant can submit a registration dossier in order to obtain registration of product at national level. A zonal system of authorisation operates in the EU which is divided into three zones; North, Central and South. EU countries assess applications on behalf of other countries in their zone and sometimes on behalf of all zones for indoor and greenhouse uses, post-harvest treatments and seed treatments. Various procedures can be followed to register a product which the principal ones are the first approval (Art. 28-39 of Reg.1107) and the mutual recognition (Art. 40-42, registration of a product already approved with the same use(s) zone under comparable agricultural conditions).

Firstly, the applicant submits a notification to the zRMS around six months before the intended submission date of the dossier. Then the applicant submits the draft registration dossier to the zRMS and to the concerned MS (MS where a registration is also intended). The RMS issues a pre-final registration dossier proposed to the comments of applicant and all the zonal concerned MS. After the commenting phase, the RMS finalises the registration report and its conclusions and edits the registration of the product or informs the applicant of the decision of non-registration. It takes around 1.5 years to register a product at national level.

In order to make sure that products comply with the updated assessment of the active substance following its renewal and with new scientific and technical knowledge, all plant protection products containing renewed active substance must undergo a renewal assessment (Art. 43 of Reg.1107).
As detailed previously, the rules and procedures for authorisation of active substances and PPPs are laid down in Reg.1107 and also in national legislations.
Consequently, biopesticides have the same regulatory framework, but based both on common and specific regulatory requirements. Different concerns may be raised during the European evaluation: for chemicals, they are for environment and ecotoxicology, and also for reproduction toxicity and cancerogenesis; for living organisms, the concerns are the multiplication in the environment, the infectivity, the secondary metabolites and then the antibiotic resistance.
To evaluate and characterise these concerns, within general requirements common with all pesticides, specific demands according to category of biopesticides are mandatory. Therefore, specific guidance documents exist for each category, such as micro-organisms, semiochemicals and botanicals. European Union has edited guidance documents for micro-organisms, semiochemicals and botanicals.
European Data requirements are also listed in the Com. Reg. (EU) 283/2013 and 284/2013, for active substances and products respectively. In these both regulations, Part A applies to chemicals including botanicals and semiochemicals while Part B applies to microorganisms and viruses.
For the submission of authorisation dossier of products containing micro-organisms, specific dRR template has to be used unlike other types of biopesticides (semiochemicals and botanicals). OECD has also emitted specific guidance documents for submission or for generation of supporting data. All these documents guide and help applicants to prepare, compile and submit required data supporting intended approvals of active substance and registration of products containing such active substances.

The registration of these products could be difficult due to the availability of reliable data and the capacities to generate required studies. Required data cover various domains: identity and characterisation; physico-chemistry and analytics; efficacy; toxicology of humans (operator and consumer); residue; behaviour in environment; and then ecotoxicology.

For each domain, all supporting data must be generated following certain standards of quality, reliability and comparability. Therefore, studies should be done according to recognised guidelines (OECD for example) by following GEP (Good Agricultural Practice) or GLP (Good Laboratory Practices) rules as for any substances or products.

However due to the nature of each biopesticide, the accurate characterisation of the substance is not very easy and can be considered as the keystone of the dossier. Indeed, such substance could be difficult to characterise, and to set appropriate and reliable specifications: for micro-organisms it is the strain characterisation and the availability of methods to differentiate a mutant or genetically-modified micro-organism from the parent strain; for semiochemical, it is the volatility of these substances; and for botanicals, it is the availability of specific marker to be followed and the natural variability of botanical material. The characterisation and possibility to follow the active substance during the experiment is important; the purpose is to be sure that the study is done with the right substance throughout the experiment. In the case of biopesticide, these points are particularly difficult to reach also due to the natural presence of similar micro-organisms or compounds. For the demonstration of efficacy, it is also complicated as the guidelines have to be adapted to these specific substances to show principally the practical value of the products (always a case by case). In this framework, the trials have to demonstrate the interest of the product in a combined protection program, i.e. associated with a conventional treatment. Furthermore, the efficacy of biopesticides cannot be compared to the one of conventional products.

Another difficulty is the variability of national rules, as each country has its own criteria to distinguish biopesticides which are not always the same as the ones established at EU level. Indeed, according to

Reg.1107, only plant protection products containing low-risk substances (according to Com. Reg. (EU) 2017/1432) have some advantages which are granted: approval up to 15 years (instead of 10 years); decision of Member States within 120 days (instead of 1 year); data protection up to 13 years; … But regarding the majority of other biopesticide substances, these legal advantages are not applicable; the majority of countries having promoted their expansion by editing specific non-harmonised rules.
For example, in Denmark or Sweden (Northern Zone), only micro-organisms are promoted by application of lower fees for evaluation than conventional products. The other categories are considered as conventional products. In Austria (Central zone), reduced fees are charged for the evaluation, but in this case for the micro-organisms and semiochemicals. Botanicals are not included. For submission of a biopesticides in The Netherlands, applicant should follow a specific evaluation manual with specific data requirements and risk assessment. The fees are also reduced. In France (Southern Zone), a global legislation has been developed to promote biopesticides (Art. L.235-5,6,7 of Code Rural et de la Pêche maritime). This legislation grants reduced fees for all categories of biopesticides according to criteria of Art L.235-5. The legislation grants also reduced evaluation timing. For Italy, only reduced fees are granted for micro-organims, semiochemicals and substances of natural origin non chemically defined. These non-homogenous rules show the complexity of registration of biopesticides in Europe.
The registration of biopesticides is challenging due to the lack of adapted guidelines, due to the variability of national criteria, due to the difficulties to demonstrate efficacy and to assess risk for human, environment and non-target organisms. But in the other hand, these issues do not prevent the expansion of these alternative crop treatment methods through fruitful discussions between applicants and both national and European authorities.

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