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Ph.D. dissertation

Biocontrol Strategies for Effective Kiwifruit Post-Harvest Disease Management

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To my parents:

Your love has fueled my journey to this milestone. This thesis is a testament to your unwaveringsupport. Thank you for being my guiding stars.

Summary

In the early ’90s, the skin-pitting disease was reported in various Italian regions. Initially, its incidence was negligible however, by the year 2000, the percentage of disease was significantly increased (20–30%) in kiwifruit batches coming from the main kiwifruit-producing areas of Italy. For more than a decade, The University of Udine has been dedicated efforts to researching kiwifruit skin-pitting in Friuli Venezia Giulia (FVG) region. The initial focus was on establishing its etiology, followed by the development ofdiagnostic tools.

In 2021, a research was planned with the principal objective of investigating the biological control strategies for the management of this postharvest skin-pitting disease as an eco- friendly approach. Additionally, the study aimed to quantify the population dynamics of causative agent *Cadophora luteo-olivacea*, from the surface of kiwifruit throughout the growing season. The study has been started by the collection of untreated kiwifruits from four different kiwifruit orchards in FVG region followed by weekly monitoring of skin-pitting disease incidence during the postharvest cold storage of these fruits. During this monitoring period, the fungal pathogen *C. luteo-olivacea* was isolated from the surface of symptomatic kiwifruit tissues and molecularly identified through qPCR. Several biological control organisms, including bacteria, yeast, and fungi, were selected to test their antifungal potential against *C. luteo-olivacea* through different in vitro and in vivo assays. Among the tested BCAs, Bacillus spp and *Pseudomonas synxantha* emerged as the most efficient BCAs and selected to subsequent experiments. To understand the mode of action of these BCAs, an in- depth biochemical analysis was conducted using infrared spectroscopy (FT-IR) that revealed the presence of different antifungal compounds where *P. synxantha* showed the most intense peaks (at 988 cm^{-1}), correlating with the highest concentration of antifungal compounds. Considerations the potential antagonistic activity of *P. synxantha*, further biochemical characterization of volatile organic compounds (VOCs) and the effect of single synthetic pure compounds on themycelial growth of kiwifruit postharvest fungal pathogens, *Botrytis cinerea* and *C. luteo-olivacea* was examined. The effect of fruit biofumigation with *P. synxantha* VOCs on the expression of kiwifruit defense-related genes was also studied.

Beyond to postharvest disease management efforts, a comprehensive investigation was conducted into the population dynamics of the fungal pathogen throughout the fruit's growing season. The study was conducted in four kiwifruit orchards situated in the FVG region. The research alsofocused on the potential correlation between the pathogen contamination on the fruit surface and the incidence of postharvest skin-pitting disease.

This multifaceted thesis holds significant implications for the kiwifruit industry, selecting an effective BCA with high antifungal ability and eco-friendly strategies for the effective management of postharvest skin-pitting disease. Use of antifungal VOCs compounds produced by biological agents in postharvest offer novel approaches for biofumigation and disease control. Additionally, this study also provides a holistic understanding of the pathogen's behavior from orchard to postharvest.

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Publications, conferencesand activities

Peer-review articles:

- **Jabeen, F.**, Di Francesco, A., Sadallah, A., Ermacora, P., & Martini, M. (2022). Biocontrol strategies in the management of *Cadophora luteo-olivacea*, skin-pitting agent of kiwifruit. VI International Symposium on Postharvest Pathology: Innovation and Advanced Technologies for Managing Postharvest Pathogens 1363 (pp. 75-80). Acta Hortic. 1363. ISHS 2023. DOI 10.17660/ActaHortic.2023.1363.11

- Di Francesco, A., **Jabeen, F.**, Di Foggia, M., Zanon, C., Cignola, R., Sadallah, A., Ermacora P. & Martini, M. (2023). Study of the efficacy of bacterial antagonists against *Cadophora luteo-olivacea* of kiwifruit. Biological Control, 180, 105199.

- Di Francesco A., **Jabeen F.**, Vall-llaura N., Moret E., Martini M., Torres R., Ermacora P. & Teixidó N (2023). Pseudomonas synxantha volatile organic compounds (VOCs): potential efficacy against *Cadophora luteo-olivacea* and Botrytis cinerea of kiwifruit. *Submitted for publication*: International Journal of Food Microbiology.

- **Jabeen, F.**, Sadallah, A., Di Francesco, A., Cignola R., Ermacora P. & Martini M., (2023). Monitoring the correlation between *Cadophora luteo-olivacea* contamination and incidence of postharvest skin-pitting disease of kiwifruit using a quantitative polymerase chain reaction (PCR). *Under submission*.

Conference attendance:

- VI International Symposium on Postharvest Pathology: Innovation and Advanced Technologies for Managing Postharvest Pathogens, Limassol Cyprus, 2022.

- XXI Jornada de Postcollita (Postharvest technical workshop) IRTA Institute of Agrifood Research and Technology, Parc Agrobiotech, Lleida, Spain, 2023.

- Advances in plant disease and pest management: a holistic approach IRTA Institute of Agrifood Research and Technology, Torre Marimòn, Spain, 2023.

Training activities:

- Emphasis summer school. “Insights on the plant biosystem: enemies, friends or just biomes”: Organized by AAB PhD School board., University of Udine, Italy. June 10th, 2021.

- Emphasis summer school. Nanotechnology in Agriculture - University of Tuscia, 7 April 2022.

- Emphasis summer school. “Plant Health and Quality” coordinated by “Plant pathology and Entomology section” - University of Florence held in “University of Angers, France”, 27th June – 5th July 2023.

Acronyms

AA	Absolute area
BCA	Biological control agent
Bp	Base pair
KA	Kiwi agar
LRI	linear retention index
MNE	Mean normalized. expression
NA	Nutrient agar
NB	Nutrient broth
PDA	Potato dextrose agar
qPCR	Quantitative polymerase chain reaction
RT	Retention time
SPME-GC/MS	Gas chromatography-mass spectrometry method
VOC	Volatile organic compound

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Introduction

Kiwifruit (*Actinidia* spp.) is a deciduous, woody, fruiting vine, belonging to the *Actinidiaceae* family (Qadir et al., 2022). The cultivar *Actinidia deliciosa* 'Hayward' represents about 50% of the kiwifruits produced worldwide (Silva et al., 2023). This fruit contains an array of valuable components including vitamin C, polyphenols such as anthocyanin, polysaccharides as well as alkaloids that are believed to offer antioxidant, anti-inflammatory, and anticancer properties (Li et al., 2023). Kiwifruit is native to China and was initially introduced to Italy in the 1930s,primarily for ornamental purposes (Testolin, 2014). The marked transition from viewing kiwifruit as ornamental to recognizing its potential as a commercially viable crop was started in late 1960s when the first commercial kiwifruit orchards were established in Italy (Costa et al., 2018). The first two experimental orchards were planted in the Latina province in 1971 that gavethe researchers the opportunity to conduct important studies, which helpedin the swift growth of kiwifruit production in the nation (Cacioppo, 2018). Italian scientists were then actively engaged with kiwifruit researchers from different countries especially with New Zeeland fostering collaborations thatcontributed to the understanding of kiwifruit, including its genetics andcultivation practices (Testolin and Ferguson, 2009).

Since the record keeping begins in 1970s, the global production trend of kiwifruit has revealed distinct phases. An incremental growth witnessed in the 1970s, followed by an exponential expansion in the 1980s, a period of consistent production levels in the 1990s, and a sustained, moderate increase over the past decade (Ward and Courtney, 2013). China, Italy, New Zeeland, and Greece have significant kiwifruit production capacities (FAO 2021) (Chen et al., 2023). Out of the worldwide kiwifruit production, China leads with approximately 2.2 million tons followed by New Zealand with approximately 0.62 million tons, Italy with around 0.52 million tons, and Greece with approximately 0.31 million tons. These countries collectively contribute to roughly 85% of the world's kiwifruit production (Bassi et al.,2023). Italy, the world's second/third-largest kiwifruit producer after China and New Zealand, harvested 316,443 tons in 2019, of which 250,000 tons were destined for export (Prencipe et al., 2023). In Italy, kiwifruit is the primary perennial crop after grapevines (Mian et al., 2022). Recently, the 'Hayward' kiwi variety, has dominated global cultivation and become the most grown variety in the world (Purohit et al., 2021).

In Italy, kiwifruits are harvested between October and November and are made available in the market until June (Mian et al., 2022). Kiwifruit belongs to berry fruits, and its ripening physiology continues after harvest.It can be stored in a controlled atmosphere (CA) for a longer period of time, for approximately 4-5 months at 0 ˚C and with 92-95% of relative humidity (Di Francesco et al., 2023). Because of its high-water texture ratio and soft structure, the post-harvest storage may decrease accordingly (Ta¸s et al., 2022). Moreover, kiwifruit is susceptible to a range of fungal (Liu et al., 2018), bacterial (Ares et al., 2021), and viral pathogens (Wanget al., 2020), which can lead to diseases at various stages of growth and even post-harvest. In particular, fungal infections, including those caused by *Botrytis cinerea*, *Penicillium expansum*, *B. cinerea*, *Alternaria Alternata*, *Botryosphaeria dothidea*, and *Diaporthe* spp. leads to significant postharvestlosses in marketable yield (Dai et al., 2022).

Among the postharvest fungal pathogens, *Botrytis cinerea* has consistently posed a significant challenge, particularly with the expansion of production and the need for longer storage durations to meet the year-round demand for kiwifruit by consumers (Li et al., 2022a). *B. cinerea* infect the wound sites of the fruits through its spores and induce rapid deteriorationof fruit (Li et al., 2023b). These post-harvest rots results in substantial economic losses for the global kiwifruit industry (Li et al., 2022c).

Skin-pitting disease: Background

In addition to *B. cinerea*, another re-emerging pathogen of kiwifruit, *Cadophora luteo-olivacea*, has been reported in recent years which is responsible for causing skin-pitting disease during the cold storage period of kiwifruit (Di Francesco et al., 2023). Although skin-pitting was reported in several Italian regions in early '90s. At first, this novel postharvestdisease affected kiwifruit grown in southern Italy and was intermittent and commercially negligible (Gorini 1992, n.d.). Its incidence, however, significantly rise in 1998 and 1999, peaking at 20–30% on kiwifruit batches from the main kiwifruit-producing regions of Italy in the year 2000 (Spadaro et al., 2010). The infection may begin from flowering until harvest where, mummified fruits and wood cankers may also serve as significant sources of inoculum for postharvest infections (Köhl et al., 2018). The symptoms of skin-pitting disease appear in the packing house after different months (mostly after 4 months) of cold storage (Di Francesco et al., 2021). The symptoms characterized by depressed and elliptical areas associated with brown-violet skin and brown pulp and a thin subereous layer can be seen if the fruit peel is removed (Jabeen et al., 2022). However, the incidence of this disease may vary from year to year, showing inconsistency in its appearance. This variability makes it an intriguing disease that can lead to significant economic losses in kiwifruit industry (Jebeen et al., 2022).

Cadophora luteo-olivacea is a fungal pathogen that has been characterized as the responsible agent for skin-pitting. Lagerberg etal. (1927) established the genus *Cadophora*, with *Cadophora* fastigiata designated as the type species. Subsequently, a total of 14 species of *Cadophora* have been described (Maldonado-González et al., 2020). Based on morphology, biology, and ITS sequence, *C. luteo-olivacea* demonstrates the similarity to *C. malorum*. Furthermore, the phylogenetic analysis also indicates a close relationship between *C. malorum* and *C. luteo-olivacea* (Spadaro et al., 2011). This fungal pathogen infects the kiwifruit duringgrowing period and can remain dormant for up to 4-5 months of cold storageperiod and become active as soon as the shelf-life period begins (Di Francescoet al., 2022). The *Cadophora* species are also associated with other plant diseases like Petri disease and esca of grapevine (Maldonado-González et al., 2020), side rot disease of pear fruits (Wenneker et al., 2016), and grapevine trunk disease (Vicente et al., 2020) that results in substantial economic losses for growers and industry worldwide.

In recent times, the management of these pathogens typically involves the pre-harvest application of chemical fungicides (Li et al., 2022). Nevertheless, excessive use of these fungicides has led to a notable rise in pathogen resistance to these chemicals, as well as the presence of fungicide residues on the fruits, which pose risks to both public health and the environment (Chen et al., 2020). But still, it is estimated that the global use of chemicals has risen by over 1500% since 1996 (Clapp, 2021). Consequently, the development of a reliable and eco-friendly alternative forcontrolling post-harvest rot in kiwifruit is of utmost importance to explore. In addition to chemical treatments, there has been a growing interest in recent years in utilizing physical treatments for the management of various postharvest diseases at commercial level. These methods include physical treatments like cold storage (Strano et al., 2017), heat treatment (Sui et al., 2016), hot air treatment (Usall et al., 2016) and ultraviolet-C light (Teraoet al., 2015). However, these physical treatments have certain limitations, such as low persistence, the potential for adverse effects on produce quality,or technological challenges in their commercial application.

Currently, instead to using chemicals or physical methods, there is a growing focus on the utilization of natural compounds as an alternative approach to mitigate postharvest diseases in fruits (Shahbaz et al., 2022). Major postharvest pathogens including *Botrytis cinerea*, *Penicillium expansum*, and *Monilinia fructicola* have been shown to be in vitro inhibitedin their ability to develop radially by using natural components such as volatile molecules, essential oils, and plant extracts (Chowdhury et al., 2022) In the context of combating postharvest diseases, biological control emerges as an effective, sustainable and bio-safe approach, including kiwifruit (Gao et al., 2021). Biological control is based on the natural antagonistic interactions between microorganisms, where one organism suppresses the activity and growth of another organism (Zhao et al., 2023). These biocontrol agents have the ability to compete the pathogens for space and nutrients, produce antimicrobial chemical compounds and cause systemic resistance in fruits (Godana et al., 2023) and they have a crucial role in reducing the prevalence of pre-harvest microbiological infections as well as post-harvest diseases (de Sousa and Granada, 2023). The use of biocontrol agents into disease management procedures could be a comprehensive and ecologically acceptable method of controlling postharvest diseases (Zhang and Showalter, 2020). The objective of research on biocontrol is to identify microorganisms capable of inhibiting pathogen growth without posing any threats to human health or the environment (Leng et al., 2022). So far, the use of biological control agents to manage postharvest diseases in various fruits, including pears, apples, citrus, and vegetables, has been proven highly effective (Zhao et al., 2023a). The application of biological control strategies is crucial in addressing emerging diseases in the kiwifruit industry. One such emerging disease is postharvest skin-pitting of disease of kiwifruit.

Aims of the thesis and workflow

This thesis addresses the emerging challenge of postharvest skin-pitting disease in kiwifruit, using biocontrol strategies as an alternative approach to conventional chemical fungicides for post-harvest disease management. The objectives are structured into four main chapters, each dedicated to distinct facets of the issue.

Firstly, the research aimed to assess the efficacy of several biological control agents (BCAs) in managing the skin-pitting disease through both in vitro and *in vivo* experiments. This work also involved an isolation and understanding of *C. luteo-olivacea*'s pathogenicity characteristics (Chapter 1.)

Secondly, the antifungal activities of four potential bacterial strains belonged to *Bacillus* and *Pseudomonas* spp were evaluated against the pathogen *C. luteo-olivacea* for their effectiveness in in-vitro and in-vivo assays while followed by FT-IR analysis for biochemical characterization of antifungal compounds produced by these antagonists. (Chapter 2).

The third chapter, the efficacy of volatile organic compounds (VOCs) produced by *Pseudomonas synxantha* strain 117-2b against fungal pathogens *C. luteo-olivacea* and *B. cinerea* was investigated. The research also aimed to perform biochemically characterizing the VOCs through of SPME-GC/MS and investigate the impact of fruit biofumigation with *P. synxantha* VOCs on the expression of kiwifruit defense-related genes.
Finally, epidemiology on the pattern of inoculum of *C. luteo-olivacea* contamination over the fruit growing season, and its correlation with the incidence of postharvest skin-pitting disease was studied. (Chapter 4).

The work of the thesis was started by collecting kiwifruit from several kiwi orchards in the Friuli Venezia Giulia region, Italy. The fruits were collected from the same orchards over a span of three consecutive years and the variety was consistent. These orchards were selected with guidance from the FriulKiwi (Rauscedo, Pordenone) technical team, relying on historical insights regarding the presence of skin-pitting within the designated lots. No fungicides were used during vegetative season in any of these orchards, allowing the natural occurrence of skin-pitting disease. The targeted fungal pathogen *Cadophora luteo-olivacea* was isolated from the tissue of symptomatic kiwifruit and subsequently identified molecularly by following the procedure outlined by Martini et al. (2009). The comprehensive and more details about the pathogen isolation and identification is described in chapter 2.

Based on the existing bibliography, 7 biological control agents were selected to examine for their antagonistic efficacy against *C. luteo-olivacea*. These BCAs were purchased from international culture collections except *Pseudomonas synxantha* (117-2b), that belongs to the collection of DI4A- University of Udine. The specifics regarding the biological control agents, including their details, can be found in Chapter 1. After conducting in vitro and *in vivo* assays using selected antagonistic agents against *C. luteo- olivacea*, *P. synxantha* and *Bacillus* spp exhibited the most potent antagonistic activities among the seven chosen biological control agents (BCAs). Due to their high performance, these two BCAs were selected for further experiments aimed at better understanding their mechanisms of action and efficacy. Bacterial antagonists are known to produce bioactive compounds like hydrolytic enzymes, phytohormones, and both volatile and non-volatile metabolites (Di Francesco et al., 2021). To understand the mechanism underlying their antagonistic potential, the selected BCAs were initially tested by in vitro assays utilizing cell filtrates, followed by *in vivo* assessments to determine the efficacy of non-volatile compounds produce by these antagonists, against pathogen. For a deeper understanding of the biochemical landscape, FT-IR (Fourier-Transform Infrared) spectroscopy was performed on bacterial cell filtrates to obtain a rapid and non-destructive characterization of their main antifungal components. The results of showed the effectiveness of BCAs against *C. luteo-olivacea* and FT-IR revealed the presence of several compounds. (Chapter 2). After investigating the impact of non-volatile organic compounds produced by biological control agents (BCAs), another subsequent study was conducted to evaluate the effectiveness of *P. synxantha* volatile organic compounds (VOCs) against *B. cinerea* and *C. luteo-olivacea* in kiwifruit postharvest management using both in vitro and *in vivo* assays. Following the biochemically characterize bacteria VOCs by using headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (SPME-GC/MS), effect of individual synthetic pure compounds was also evaluated on the mycelial growth of the target pathogens. Furthermore, the study also investigated the effects of fruitbiofumigation with *P. synxantha* VOCs on the expression of kiwifruit defense-related genes. The findings revealed a reduction in disease incidence and the upregulation of defense-related genes in the fruit in response to *P. synxantha* VOCs treatment.

This study significantly contributes to our understanding of the antifungal activity of bacterial VOCs and sheds light on the mode of action of bacterial antagonists in the context of kiwifruit postharvest disease management.

Another parallel study was conducted for three years (2020-2022) to address the fourth objective of this research, which aimed to improve epidemiological knowledge's about *Cadophora* and validate the hypothesis proposing an increase in *Cadophora* contamination throughout the fruit's growing season. Over three consecutive years, the fruit's surface was systematically monitored for C. luteo olivacea presence on monthly basis, from July to October. The results demonstrated a significant rise in the pathogen's population density, confirming the initial hypothesis. Although a correlation between pathogen concentration and incidence of skin-pitting disease was also observed however, this correlation was not consistently evident in every instance. (Chapter 4).

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Chapter 1

Biocontrol strategies in the management of *Cadophora luteo-olivacea*, skin-pitting agent of kiwifruit

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Abstract

Skin-pitting causes great economic losses on kiwifruit production, as the symptoms appear on fruits only after 3-4 months of cold storage. Nowadays,very few synthetic active compounds are allowed during the postharvest phase, also because of the related residue problems. For this reason, the goal of the present study was to search for alternative strategies to control skin-pitting symptoms. Thus, the efficacy of bacteria and yeasts strains as biocontrol agents (BCAs) was tested by *in vitro* and *in vivo* assays against *Cadophora luteo-olivacea* strains, the causal agent of skin-pitting disease of kiwifruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson). The efficacy of different BCAs (*Bacillus*, *Pseudomonas*, *Metschnikowia*, and *Aureobasidium* spp.), belonging to our laboratory collection, purchased, and derived from a commercial bio-fungicide, was tested by *in vitro* assays against the fungal mycelial growth. *In vitro* results showed that the strains *Pseudomonas synxantha* (117-2b) and *Bacillus amyloliquefaciens* (FZB24)were the most effective against the pathogen growth through the tested antagonistic strategies. Strains 117-2b and FZB24, as the most active BCAs by *in vitro* experiments, were also tested on kiwifruit. From preliminary results, the antagonists appeared promising in reducing the incidence of skin-pitting symptoms respectively by 73% and 60%, so proposing a valid and sustainable alternative strategy to control *C. luteo-olivacea* during the storage.
Keywords: Antagonistic activity, Postharvest management, Biocontrolagent, Fungal pathogen

1.1 Introduction

The favorable taste, balanced configuration of dietary fibers, high value of vitamin C and polyphenols have made kiwifruit (mainly *Actinidia deliciosa* and *A. chinensis*) popular worldwide (Dai et al., 2021). Kiwifruit can be stored at 0±1 ˚C for up to six months (Spadaro et al., 2010). Unfortunately, kiwifruit is vulnerable to a variety of pathogens at different growth stages as well as after harvesting (Areset al., 2021; Erper et al., 2013) that lead to fruit quality deterioration and losses (González-Estrada et al., 2021). Skin-pitting is a postharvest disease of kiwifruit caused by *Cadophora luteo-olivacea* and reported in recent years in different packing houses (Di Francesco et al., 2021). Symptoms are characterized by depressed and elliptical areas associated with brown-violet skin and brown pulp (Spadaro et al., 2010) that appear after 3-4 months of storage in packaging house. However, the disease incidence may vary year by year, not appearing consistently, making it a very intriguing disease (Di Lenarda, 2010). In the last period, the control of postharvest diseases is shifting from fungicides towards safer and eco-friendly alternatives such as microbial antagonists (Liu et al., 2013). In fact, the aims of this study were: i) to evaluate the efficacy of potential biological control agents (BCAs) for the management of kiwifruit skin-pitting disease by *in vitro* and *in vivo* experiments and ii) to improve the knowledge regarding the virulence characteristics of *C. luteo-olivacea*.

1.2 Materials and methods

1.2.1 Pathogen and antagonists

Two *C. luteo-olivacea* strains were used for *in vitro* experiments. The strain Cad 21, belonging to the mycological collection of DI4A-University of Udine, was isolated from symptomatic kiwifruits and identified by molecular analysis. The fungal strain 141.41 was purchased from Fungal Biodiversity Centre (CBS). *Bacillus amyloliquefaciens* (FZB24) and *Aureobasidium pullulans* (DSM-14940, DSM-1494) strains were acquired from DSM-Deutsche Sammlung von Mikroorganism' collection. In the case of *Bacillus subtilis* (B-21661), *Bacillus pumilus* (B-30087), and *Monilinia fructicola* (Y-27328), the strains were purchased from NRRL-Northern Regional Research Laboratory. The strain *B. amyloliquefaciens* (D747) was isolated from the relative commercial product: Amylo-X®. About *Pseudomonas synxantha* (117-2b), the strain belongs to the collection of DI4A-University of Udine. *C. luteo-olivacea* strains were grown on PDA for two weeks at 25˚C and BCAs on nutrient agar (NA) at 25˚C for 48 hours. Microorganisms were used for obtaining conidial and cell suspensions 105 conidia mL^{-1} and 108 cells mL^{-1}, in distilled water containing 0.05% tween, respectively, by using a hemocytometer.

1.2.2 In vitro assay

C. luteo-olivacea (Cad 21 and 141.41) mycelial plugs (6 mm diameter), derived from 14-days old colonies, were inoculated on PDA (Potato DextroseAgar), in the centre of the plate. One microliter of each BCAs culture (10^8 cells mL-1), previously grown 24-hours in NB (Nutrient Broth), was deposited at the four equidistant peripheral regions of the dish inoculated with the mycelial pathogens, as above-described. Plates inoculated only with fungal pathogens were considered as control. Plates were incubated at 25˚C and 1˚C, respectively. Fungal growth inhibition was determined by measuring the diameter of the colonies after 4 and 8 weeks respectively at 25˚C and 1˚C incubation temperature. Three replicates for each treatment were used, and the experiment was conducted twice.

1.2.3 *In vivo* assay

Kiwifruit cv 'Hayward' were obtained from 2 different orchards located in FVG region (Italy). Fruits were harvested in November 2020, selected homogenous in size, injury-free, and immediately used for *in vivo* experiment. *P. synxantha* (117-2b) and *B. amyloliquefaciens* (FZB24) were used in *in vivo* experiment as the most active isolates in the previous assay. Kiwifruits were washed by sodium hypochlorite solution (0.1% v/v) for 1 minute,rinsed three times with distilled water, and let dried. Fruits were artificially wounded at the equatorial region by using sterile nail (2×2×2 mm) (one wound each fruit). Fruit wounds were inoculated with 15 μL of BCAs suspensions (10^8 cells mL^{-1}) and kept at room temperature. After 1 hour from BCAs inoculation, 15 μL of 10^5 conidia mL^{-1} of pathogen (Cad 21) suspension was inoculated into the same wounds. Fruits inoculated with sterile water represented the positive control;on the other hand, kiwifruit treated with Amylo-X® (105CFU/ g) the negative one. Treated fruits were kept at 1˚C and 95% of R.H. for 4 months. After that period, fruits were maintained 1 week at 20˚C (shelf life). The experiment consisted of three replicates of 15 fruits for each treatment. The experiment was performed twice.

1.2.4 Statistical analysis

Anova One - Way analysis of variance was used to examine the data. Tukey's test was used for the separation of the means (α=0.05).

1.3 Results

1.3.1 *In vitro* assay

Figure 1.1 displayed that *P. synxantha* (117-2b) and *B. amyloliquefaciens* (FZB24) were the most efficient BCAs that inhibited the growth of CBS 141.41 by 73% and 76% and of Cad 21 by 73% and 72%, respectively.However, *B. subtilis* (B-21661), *B. pumilus* (B-30087), and *M. fructicola* (Y- 27328) showed against CBS 141.41 an inhibition of 70%, 59% and 46%and against Cad 21 an inhibition of 70%, 53% and 38%, respectively. A. pullulans (DSM-14940, DSM-14941) showed the least antifungal activity with 33% and 24% of reduction against CBS 141.41 and with 31% and 28% against Cad21,respectively.

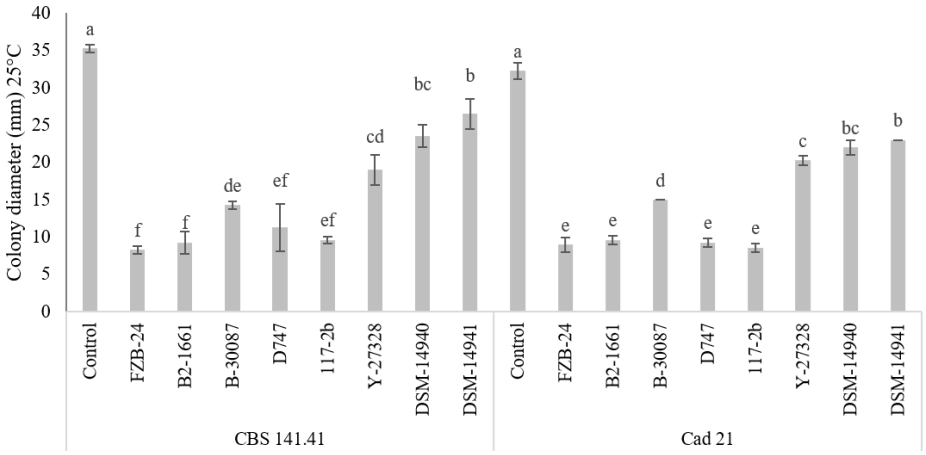

Figure 1.1: Efficacy of selected BCAs (*B. amyloliquefaciens*, *B. subtilis, B. pumilus, P. synxantha, M. fructicola*, *A. pullulans*) on the colony growth of *C. luteo-olivacea* strains (CBS 141.41 and Cad 21). The data are the average of two experiments. Data reporting the same letters were not statistically significant according to Tukey test (α=0.05).

P. synxantha (117-2b)showed the greatest inhibition of mycelial growth of *C. luteo-olivacea* CBS 141.41 and Cad 21 isolates by 68% and 74%, respectively. While,*B. amyloliquefaciens* (FZB24), *B. subtilis* (B-21661), and A. pullulans (DSM-14840, DSM-14941) have shown a reduction of 34%, 39%, 19%, and 19.5% against CBS 141.41 and of 35%, 42%, 24% and 28% against Cad 21, respectively. *M. fructicola* (Y-27328) and *B. pumilus* (B-30087) were the least efficient BCAs at low temperature both with an inhibition rate of 8% against CBS 141.41 and of 13% and 15% against Cad 21. Among all the tested BCAs in *in vitro* assay, *P. synxantha* (117-2b) and*B. amyloliquefaciens* (FZB24) were selected for *in vivo* assay, as the most active.

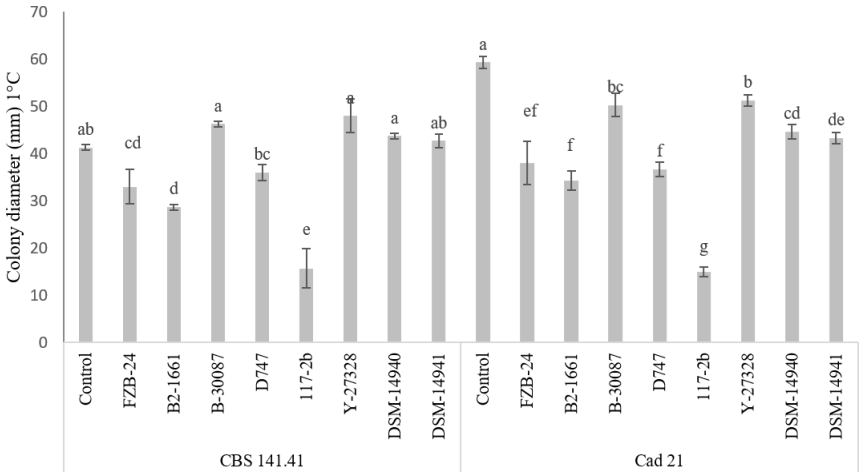

Figure 1.2: Efficacy of BCAs on the colony growth of *C. luteo-olivacea* strains (CBS 141.41 and Cad 21). The data were the average of two experiments. Data reporting the same letters were not statistically significant according to Tukey's test (α=0.05).

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1.3.2 *In vivo* assay

P. synxantha (117-2b) and *B. amyloliquefaciens* (FZB24) significantly reduced the incidence of skin-pitting symptoms respectively by 73% and 60%, with respect to the control, after 4 months of cold storage. The disease incidence on the positive (water) and negative (Amylo-X®) controls was respectively by 90% and 6.6%.

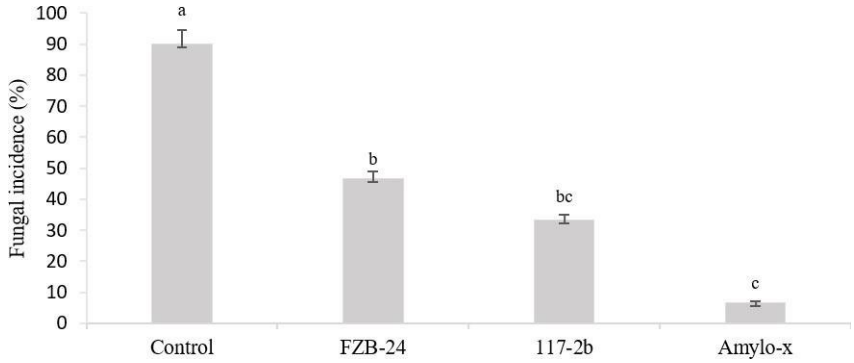

Figure 1.3: Evaluation of the *in vivo* efficacy of *P. synxantha* (117-2b) and *B. amyloliquefaciens* (FZB24) against *C. luteo-olivacea* Cad 21 strain on kiwifruit stored for four months in cold storage. Different letters represented a significant difference according to Tukey Test (α=0.05).

1.4 Discussion

Postharvest treatments of fruits with BCAs have previously been reported in several plant host/pathogen systems (Di Francesco et al., 2018). The present study reported the efficacy of a new BCA *P. synxantha* (117-2b) through *in vitro* and *in vivo* experiments against C. luteo -olivacea strains. *P. synxantha* is already reported as a functional antagonist against fungal pathogen such as *Monilinia fructicola*, without showing side effects on fruits. Moreover, Aiello et al., (2019) reported that *P. synxantha* is an endophytic bacterium, able to move, colonize, and persist inside of the vascular tissues of kiwifruit plant without harming its host. This characteristic should be interesting to develop in the future for the management of the target pathogen whose epidemiology is not still well understood but whose long latency is known and poorly manageable. Also, *B. amyloliquefaciens* previously highly studied for its biocontrol activity (Ji et al., (2013); Chenet et al., (2009) showed an interesting efficacy against skin-pitting symptoms. In fact, the strain used in the present study is the active principle of a bio-product (Taegro®), known to be active against a broad range of plant pathogens. The positive results obtained by the use of a bioformulated strain (FZB-24) and a bioproduct (Amylo-X) could be useful for field-ready treatments. In fact, the management of postharvest diseases by employing BCAs or natural substances has been successfully evaluated on many fruit species and pathogens. However, their efficacy against *C. luteo-olivacea* remain an interesting issue to be developed and deepened by adopting an integrated strategy.

1.5 Conclusion

Our results proved that *P. synxantha* and *B. amyloliquefaciens* could represent a sustainable alternative for kiwifruit skin-pitting disease management Although, further studies will be performed to better understand the mechanisms of action and efficacy of our effective BCAstrain *P. synxantha* (117-2b) against *C. luteo-olivacea* and the most effective application timing in order to optimize its performance.

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Chapter 2

Study of the efficacy of bacterial antagonists against *Cadophora luteo-olivacea* of kiwifruit

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Abstract

Skin pitting currently represents one of the major postharvest diseases of kiwifruit and one of the most difficult to manage in packinghousesdue to its latent behavior and the difficulty in predicting its emergence.Our research demonstrates the potential to use different bacterial strains (*Pseudomonas synxantha* and *Bacillus* spp.) instead of synthetic compounds to preserve kiwifruit from the development of postharvest skin pitting symptoms, following the momentum towards sustainable strategies. The antagonists tested with *in vitro* assays showed different efficacy rates against *C. luteo-olivacea* (strain Cad21) mycelial growth by producing non-volatile metabolites. The biochemical composition of the most active bacterial non-volatile secondary metabolites was described through FT-IR (Fourier-Transform Infrared) spectroscopy. *Pseudomonas synxantha* strain 117-2b emerged as the most active strain in *in vivo* experiments, both as a curative and preventive treatment (63% and 84.7% of inhibition, respectively). In addition to its ability to reduce disease incidence, the biological antagonism exerted by *P. synxantha* strain 117-2b was further demonstrated by qPCR analysis as a reduction in the pathogen's abundance.In view of these results, alternative solutions in the field and during postharvest storage could be considered to control *C. luteo-olivacea* of kiwifruit.
Keywords: Storage, Biological control Skin pitting, Antibiosis, FT-IR.

2.1 Introduction

Kiwifruit is a recently domesticated plant belonging to the genus *Actinidia*, which contains ± 60 species (Zhang et al., 2020). However, two species, A. deliciosa and *A. chinensis*, dominate the kiwifruit industry (Huang et al., 2004). In particular, *Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson) cv. "Hayward", which is planted worldwide in temperate-zone countries (Michaelidis and Elmer, 2000), remains one of the most commercially important cultivars (Choi et al., 2022). The consumption of kiwifruit has been increasing steadily; kiwifruit is one of the most popular and widely consumed fruits due to its flavor and remarkable nutrients (Wang et al., 2022a). Kiwifruit can be stored for about 4–5 months at 0 °C with relative humidity (R.H.) of 92–95% using standard refrigerationor for a longer time in a controlled atmosphere (CA) (Tas¸ et al., 2022). Nevertheless, the fruit is susceptible to different fungal pathogens, among which the best known is Botrytis cinerea (Pers: Fr). It has always been considered one of the most important pathogens for stored kiwifruit (Costa et al., 1991), becoming a serious problem over the last 15 years in kiwifruit production (Michaelidis & Elmer, 2000; Di Francesco et al., 2018). During the last few years, the re-emerging pathogen *Cadophora luteo-olivacea*, which causes the skin-pitting disease of kiwifruit, has been detected in Italian and Chilean packaging houses (Di Francesco et al., 2022; Auger et al., 2018). Skin pitting symptoms (oval dark brown lesions) appear after several months of cold storage (Spadaro et al., 2010; Gilardi et al., 2007). *Cadophora luteo-olivacea* is a fungal pathogen that infects fruits during the developing period, remaining inactive for up to 4–5 months of cold storage (Di Francescoet al., 2022) and can appear immediately during the shelf-life period.

Usually, the risk of fungal postharvest diseases is mitigated with fungicide treatments before and after harvest (Palm and Kruse, 2012). However, issues related to chemical residues in the fruit have recently made these treatments challenging to justify, and this has stimulated the exploration of alternative strategies (Chowdhury et al., 2022). Microorganisms used as biocontrol agents (BCAs) could represent one of the sustainable alternative methods (Wang et al., 2022b) to apply. Among them, bacteria usually show interesting antagonistic mechanisms of action toward fungal pathogens, making them attractive for biological control strategies. Sometimes, different mechanisms can act simultaneously, and it is not easy to establish which individual mechanism contributes the most to the inhibition of fungal pathogen growth (Di Francesco et al., 2016). Among the different species of bacteria used as effective BCAs, *Bacillus*, and *Pseudomonas* spp. have been mainly exploited for their synthesis of extracellular enzymes (Nihorimbere et al., 2011), production of volatile organic compounds (VOCs) (Gotor- Vila et al., 2017; Ni et al., 2022), and induction of resistance responses in the

host (Carmona-Hernandez et al., 2019). All these characteristics could represent a useful starting point for selecting active and effective BCAs.

The objective of the present work was to investigate the antifungal effect of four different bacterial strains belonging to *Bacillus* and *Pseudomonas* spp. against *C. luteo-olivacea* by: i) *in vitro* assays through a coculturing method at different growth temperatures and using agar infusion of bacterial cell filtrates from different growing times; ii) by their biochemical characterization (FT-IR); and iii) via *in vivo* assays on kiwifruit to verify their effectiveness as a preventive or curative treatment.

2.2 Materials and methods

2.2.1 Fruit

Kiwifruit cv 'Hayward' (*Actinidia deliciosa* (A. Chev.) C.F. Liang A. R. Ferguson) were obtained from orchards cultivated under integrated pest management (IPM) production system located in Zoppola (Pordenone province, Google: 45°56 56.91 N, 12°47 15.83 E) and Sedegliano (Udine province: 46°0211.02 N, 12°5746.51 E). Fruits of uniform size and free from lesions were collected and immediately stored at 4 °C with RH 92% until use.

2.2.2 Microorganisms

Cadophora luteo-olivacea strain Cad21 was isolated from the tissue of symptomatic kiwifruit; its fresh mycelium was used for genomic DNA extraction following a procedure by Lecellier and Silar (1994), modified by Martini et al. (2009). The extracted and quantified DNA was diluted in sterile water to obtain a concentration of about 2–5 ng/μl. The fungal strain was molecularly identified by using the specific primers Cad (KSDA)-f2 5′-GTG GCG GGG CTA CCC TAC-3′ /Cad (KSDA)-r1 5′-GCT GGC AAG TAG ACC TAC CG-3′) (Martini et al., unpublished) to amplify a DNA fragment of 485 bp in 25 μL reactions containing 200 μM of each of the four dNTPs, 0.4 μM of each primer, 1.5 mM $MgCl_2$, 0.625 units of GoTaq Flexi DNA Polymerase (Promega, WI, USA) and 1 μL of diluted DNA. The amplification reactions were carried out in a T gradient thermal cycler (MiniAmp Plus, Thermo Fisher Scientific) using the following program: initial denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 40 s, 58 °C for 30 s, 72 °C for 40 s; final extension at 72 °C for 7 min. PCR products were run on 1% agarose gels, stained with ethidium bromide, and visualized under UV light. The amplified DNA was purified with a Nucleospin Extracts II Kit (Macherey–Nagel, Germany) and, finally, Sanger sequenced by BMR Genomics (Padova, Italy). The sequences were compared to those of the GenBank database (https: //www.ncbi.nlm.nih.gov/BLAST/) using the BLASTN search. The fungal colony was maintained on potato dextrose agar (PDA, 39 g L^{-1}, Oxoid - UK) at 4 °C until use.

Regarding the antagonists, the *Pseudomonas synxantha* 117-2b strain belonging to the microbiological collection of DI4A-University of Udine, was originally isolated from the surface of kiwifruits and subsequently molecularly characterized (Di Lenarda et al., 2010). The *Bacillus amyloliquefaciens* FZB24 strain was acquired from the DSM-Deutsche Sammlung von Mikroorganisms collection. The *Bacillus subtilis* QST713 and *Bacillus pumilus* QST2808 strains were purchased from NRRL (Northern Regional Research Laboratory, IL, USA). Two weeks before the experiments, *C. luteo-olivacea* strain Cad21 was grown on PDA (potato dextrose agar, 39 g L^{-1}; Oxoid - UK) at 25 °C. The conidial suspension was prepared by scraping and suspending conidia in sterile distilled water with 0.05% (v/v) Tween 80 and adjusted to the final required concentration (105 conidia mL^{-1}) with a hemocytometer. All the bacterial strains were maintained on nutrient agar (NA, 13 g L^{-1}, Oxoid - UK) at 4 °C until use. For the assays, bacterial cells from two-day-old plates were suspended in potassium phosphate buffer (PPB, 70 mL KH_2PO_4 0.2 M; 30 mL K_2HPO_4 0.2 M and 300 mL deionized water v/v/v pH 65) and adjusted to a final concentration of 106 cells mL^{-1} and 108 cells mL^{-1} according to the experiment.

2.2.3 *In vitro* assays

Two different experiments were performed to test the antagonism of the four bacterial strains. The first, a co-culture assay, was conducted using bacterial cells grown in nutrient broth (NB, 13 g L^{-1}, Oxoid - UK) for 24 h. A mycelial plug (6 mm diameter) of the fungal strain Cad21 derived from 14-day-old colonies was placed in the center of the PDA plate. A loopful (1 μL) of each bacterial culture was deposited at four equidistant peripheral locations on the dish. Plates inoculated only with the fungal pathogen were considered as control. Plates were incubated at 1 °C and 25 °C in dark conditions, and the colony diameters were measured with a ruler after 8 and4 weeks, respectively. Five replicates for each condition were used, and the experiment was conducted twice. The second assay tested the efficacy of bacterial cell filtrates collected at different growing times. A loop of cells of each strain was grown in 100 mL of NB broth at 25 °C in a rotary shaker (250 rpm) for 4 days. A flask was prepared for each sampling time (1 h, 24 h, 48 h, 72 h, 96 h). An aliquot of 50 mL of each sampling time was centrifuged (10,000 rpm at 4 °C for 30 min), and the supernatants were filtered using 0.20 μm Millipore filters (Sigma Aldrich, USA). An aliquot of each collected cell filtrate (50 mL) was infused into 50 mL of PDA and plated onto Petri dishes. Dishes were inoculated with Cad21 strain plugs (6 mm diameter) and kept at 25 °C. After 2 weeks, the colony diameters were measured using a digital caliper (Borletti, Italy). Plates inoculated with the fungal pathogen plug on simple PDA were considered as control. Eight replicates were used for each condition, and the experiment was conducted twice.

2.2.4 FT-IR analysis of bacterial cell filtrates

The most active bacterial cell filtrates were analyzed with FT-IR (Fourier-Transform Infrared) spectroscopy to obtain a rapid and nondestructive characterization of their main antifungal components. Filtrates were obtained as described above in paragraph 2.3. An aliquot (1.5 mL) of each treated sample was collected in sterile tubes (2 mL), stored at - 80 °C, and lyophilized. Infrared spectra were recorded witha Bruker ALPHA series FT-IR spectrophotometer (Bruker, Ettlingen, Germany) equipped with an attenuated total reflectance (ATR-Diamond crystal) apparatus at the standard resolution of 4 cm^{-1}. Three spectra (averaged over 64 measures) were measured for each cell filtrate. The spectra were collected as described by Di Francesco et al. (2021).

2.2.5 *In vivo* assay

For the *in vivo* assay, kiwifruits (6.5° Brix) were divided into two groups according to the applied treatment: curative or preventive. For both treatments, fruits were surface sterilized with sodium hypochlorite (0.1% v/v), rinsed with distilled water for one minute, and air dried at room temperature. Fruits were artificially wounded once at the equatorial line by using a sterile steel nail (2 mm × 2 mm × 2 mm). For curative treatment, 20 μL of pathogen conidial suspension (105 conidia mL^{-1}) were pipetted into each wound. After 24 h, the same wounds were inoculated with 20 μL of *P. synxantha* (117-2b), *B. amyloliquefaciens* (FZB24), *B. subtilis (*QST713), and *B. pumilus* (QST2808) suspensions (108 cells mL^{-1}).
For preventive treatment, 20 μL of each bacterial cell suspension were inoculated first, and after 24 h, the same aliquot of the conidial suspension was added as described above. Treated fruits were kept in plastic trays at 1 °C and 92% relative humidity (R.H.) for 4 months. After the cold storage, fruits were maintained at 20 °C (shelf life) for 1 week. Kiwifruits inoculated with sterile water represented the negative control, while fruits treated with Scholar® (active ingredient: Fludioxonil; Syngenta, Basel, Switzerland) (1.3 mL^{-1}) represented the positive one. The experiment consisted of three replicates of 15 fruits for each treatment, and it was performed twice.

2.2.6 DNA extraction and quantification of the amount offungal DNA by qPCR

The total genomic DNA was extracted from artificially wounded inoculated kiwifruits (as described above in paragraph 2.5) to quantify the amountof fungal pathogen DNA using a qPCR assay. DNA was extracted from 10 portions of kiwifruit tissue (150 mg) for each condition, collected at 1 cm from the inoculation wound following a Doyle and Doyle (1990) protocol

modified by Martini et al. (2009). The quality and quantity of extracted DNA were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and each sample concentration was adjusted to 20 ng/μL by dilution in nucleasefree water. Qualitative qPCR was performed using ITS as a target using the *C. luteo-olivacea* specific primers Cad (KSDA)-f2 5′-GTG GCG GGG CTA CCC TAC-3′/Cad (KSDA)-r2 5′-CGC CAA AGC AAC AAA GGT AGT- 3′ (fragment 105 bp long). qPCRs were performed in 15 μL per reaction in a 96-well Bio- Rad CFX96 RealTime PCR System (Bio-Rad Inc., Hercules, CA, USA). Reaction mixtures contained 0.3 μM of each primer, 1X SsoFast™ EvaGreen® Supermix (Bio-Rad Inc., Hercules, CA, USA), molecular grade H2O; 2 μL of DNA solution containing 20 ng/μL of extracted DNA as a template. Cycling conditions were as follows: initial denaturation at 98 °Cfor 2 min; 50 cycles of 5 sec at 98 °C; 5 sec at 58 °C. A low-resolution melting curve (ramp from 65 °C to 95 °C with 0.5 °C increments and holding timesof 5 s) was programmed at the end of the cycling reaction.

In all positive samples, the amount of pathogen DNA was assessed by qPCR as pg of *C. luteo-olivacea DNA*/mg of kiwifruit tissue to normalize the data. To quantify *C. luteo-olivacea* DNA, a standard curve was prepared with 10-fold serial dilutions of total DNA extracted from a pure culture of the Cad21 strain (quantified by using Qubit® 2.0 Fluorimeter), starting at2 ng/μL and up to 2 pg/μL. PCR mixtures and cycling conditions were performed as described above.

The amount of fluorescence for each sample was measured at the end of each cycle and analyzed via CFX-Manager Software v. 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The baseline was automatically determined, and the fluorescence threshold was set manually to maximize the standard curve efficiency. Each diluted sample and each standard were replicated three times in the experiment. Fungal DNA quantity was expressed as pg of *C. luteo-olivacea* DNA/mg of kiwifruit tissue.

2.2.7 Population dynamics of *Pseudomonas synxantha* strain 117-2b on kiwifruit

To analyze the ability of *P. synxantha (*117-2b) to colonize and proliferate on kiwifruit wound sites, fruits were surface sterilized, as reported in section 2.5. Fruits were wounded at three equidistant points by using a sterile needle. Aliquots of 20 μL of *P. synxantha* strain 117-2b suspension (106 cells mL^{-1}) were pipetted into each wound. Fruits were kept at 0 °C for 4 months. BCA growth was determined at 0 (2 h from the inoculation), 1, 2, 3, and 4 months of storage. Plugs (6 mm, ϕ) of fruit tissue from each wound site were removed with the help of a sterile cork borer. Fruit plugs were transferred into a sterile stomacher bag containing 5 mL of sterile distilled

water and Tween 80 (0.05%). The bag was stomached for 20 min (Bag Mixer 400; Interscience, St Nom, France). The juice (100 μL), diluted in sterile distilled water, was surface-plated on NA, and the Petri dishes were incubated at 25 °C for 2 days. Three fruits representing three replicates wereset up for each sampling time, and the experiment was performed twice.

2.2.8 Statistical analysis

All the experiments were analyzed by one-way analysis of variance (ANOVA). Statistical means were compared by using Tukey's test (α = 0.05). Data were reported as mean values ± SE of two independent experiments. All analyses were performed with the software MiniTab.16.

2.3 Results

2.3.1 *In vitro* assays

Different inhibition values of the fungal pathogen were observed with the *in vitro* assays after the direct application of the bacterial strain or their secondary metabolites. Furthermore, the colony diameter of the control observed at 1 °C showed values that support the ability of the pathogen to grow at low temperatures. Regarding the results of the coculture assay (Fig. 2.1), at 1 °C, *P. synxantha* (117-2b) showed the best antagonistic activity by reducing the growth of the fungal pathogen strain Cad21 by 74.7%. *Bacillus amyloliquefaciens* (FZB24) and *B. subtilis* (QST713) reduced the colony diameter of the *C. luteo-olivacea* strain by 35.9% and 42.1%, respectively. Conversely, *B. pumilus* (QST2808) was the least active strain against fungal pathogen growth, showing a reduction of only 15.1%. Compared to the lower temperature, the BCAs tested have shown a higher antagonism potential at 25 °C. Plates inoculated with *P. synxantha* (117-2b), *B. amyloliquefaciens* (FZB24), and *B. subtilis* (QST713) showed an inhibition of 73.3%, 72.1%, and 70.2%, respectively. *Bacillus pumilus* (QST2808) was the least active strain, with a 53.5% reduction in fungal mycelial growth.

The culture filtrates of the tested bacterial strains were assayed against the growth of the fungal pathogen strain Cad21 and indicated a variable inhibition activity (Fig. 2.2). The effectiveness of *B. amyloliquefaciens* (FZB24) culture filtrates was significantly higher at growing times of 48 h, 72 h, and 96 h, inhibiting fungal growth by 43.6% on average, comparedto the control. *Pseudomonas synxantha* (117-2b) filtrates showed a higher inhibition rate at 72 h and 96 h (9.2% and 24.2%, respectively). Culture filtrates of *B. subtilis* (QST713) had their highest activity at 48 h, 72 h, and 96 h, inhibiting the growth of the fungal mycelium by 31.7%, 41.7%, and 20%, respectively, compared to the control. The tested culture filtrates seemed to be, for all strains, most effective immediately after 48 h of growth. Nevertheless, the activity of *B. pumilus* (QST2808) culture filtrates were confirmed to be

ineffective in inhibiting the growth of *C. luteo-olivacea* strainCad21.

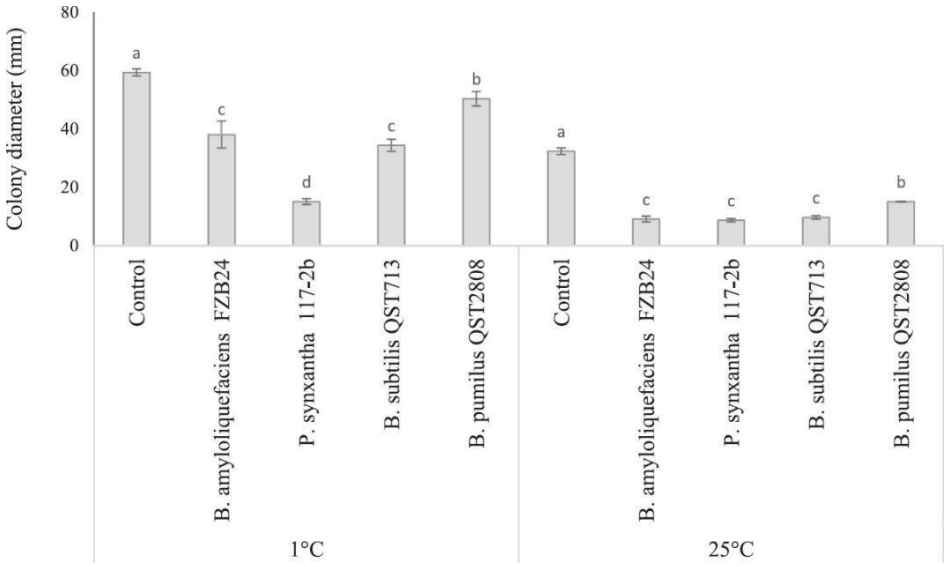


Figure 2.1: *In vitro* co-colture assay. Efficacy of bacterial antagonists at two different temperatures (1 ˚C and 25 ˚C) on the colony growth of *Cadophora luteo-olivacea* (Cad 21). The data were the average of two experiments. Data reporting the same letters are not statistically significant according to Tukey's test (α=0.05).

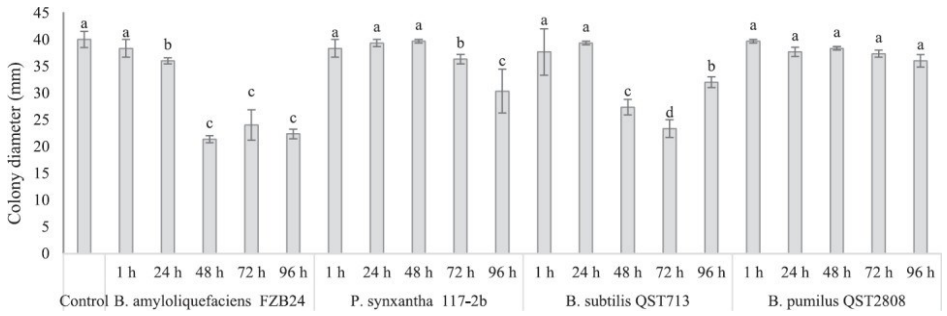


Figure 2.2: Effect of bacterial strain cell-filtrates infused onto PDA plates on the colony growth of *Cadophora luteo-olivacea* (Cad21). Biocontrol agents were grown in nutrient broth at 20 ˚C for one week and filtrates were sampled at 1 h and then every 24 h for up to 96 h. Data are the means of *Cadophora luteo-olivacea* (Cad21) colony growth observed after 14 days at 20 ˚C. For each antagonist different letters indicate significant differences according to Tukey's test (α=0.05).

2.3.2 Biochemical analysis of bacterial cell filtrates

The spectroscopic measurements were carried out to determine the main non-volatile metabolites produced by the different bacterial antagonists. Fig. 2.3 shows the infrared difference spectra between cellfiltrate and the nutrient broth (the spectrum of nutrient broth is reported for comparisonin Figure S1, supplementary material). Difference spectra were considered more valuable for discriminating the metabolites produced by bacteria (positive peaks) and the medium itself (negative peaks). The bands of the nutrient broth medium were attributed thanks to previous data generated from cultures grown on peptonecontaining medium (Trivedi et al., 2015).

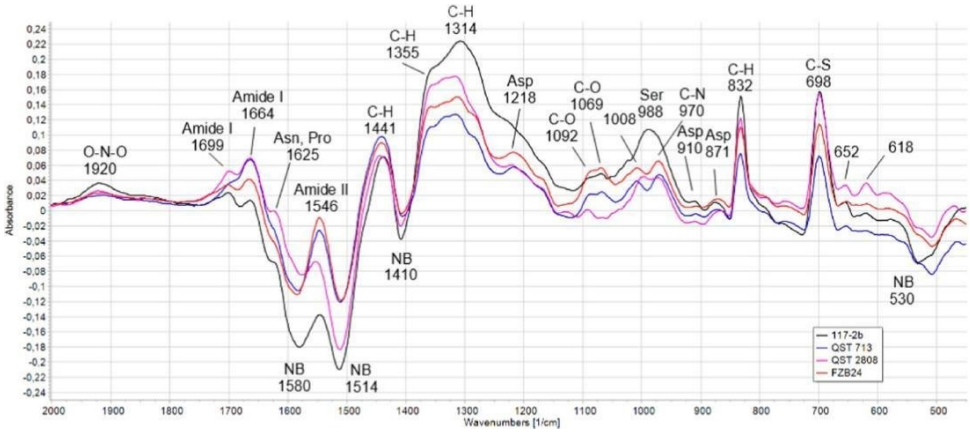


Figure 2.3: Difference FT-IR spectra of bacterial cell filtrates with the main positive bands related to the production of non-volatile compounds discussed in the text. Negative peaks are related to the consumption of the nutrient broth (NB).

The prominent positive peaks were attributed to peptidic compounds: 1699, 1664, and 1625 cm^{-1} bands are located in the amide I spectral region, arising from the C=O and N-H vibration of peptide (and protein) backbones (Besson et al., 1996) and sensitive to their secondary structure.A positive peak in the amide II spectral region at 1546 cm^{-1} (N-H and C-N vibrations) further confirms the peptidic nature of the compounds in the cell filtrate. More specifically, the 1625 cm^{-1} band was attributed to asparagine residues as observed in iturin A, a lipopeptide produced by *Bacillus subtilis* (Besson et al., 1996), while the same authors attributed the 1699 and 1664 cm^{-1} bands to a type II -turn structure adopted by the lipopeptide. This secondary structure is characterized by the presence of selected amino acidssuch as asparagine and proline (band at 1625 cm^{-1}), glycine (band at 698 cm^{-1}), aspartic acid (bands at 1218, 1069, 910, and 871 cm^{-1}) and serine (band at 988 cm^{-1}) (Barth, 2007).

1441, 1355, 1314, and 832 cm^{-1} bands were attributed to C-H vibrations; therefore, they may refer to the hydrophobic side chains of amino acids (i.e., alanine, leucine, etc.) or a lipidic component of an antimicrobial compound as observed in *Bacillus paralicheniformis* (Ahire et al., 2020). The bands at 1218 and 618 cm^{-1} have been previously observed in the IR spectra of linear lipopeptides produced by *Paenibacillus polymixa* (Deng et al., 2011). The 1092 and 698 cm^{-1} peaks were associated with the C-O and C-S bond vibrations in bacitracin, an antibiotic polypeptide produced by *B. subtilis* and *B. licheniformis* (Li et al., 2017). Finally, the 1920 cm^{-1} band could be attributed to the O-NO vibration in an antibiotic peptide produced by B. brevis (Muhammad et al., 2016).

The presence of antibiotic peptides was further supported by the appearance of other bands already reported for *Bacillus megaterius*: the O-N-O band at 1920 cm^{-1} and the C-S band at 698 cm^{-1} are typical of bacitracin, a polypeptide rich in cysteine, glutamic acid, histidine, phenylalanine, lysine, isoleucine, leucine, ornithine, and aspartic acid (Al- Thubiani et al., 2018).

IR spectra reported in Fig. 2.3 show many similarities. However, the differences between the different cell filtrates may reflect a different chemical composition: 1625 (attributed to Asn and Pro amino acids), and the 618 cm^{-1} bands were more intense in the QST2808 filtrate, which had a different profile in the 1080–920 cm^{-1} spectral region. The *P. synxantha* strain 117-2b filtrate was characterized by the most intense peaks, mainly in the 1400–800 cm^{-1} region, with a typical peak at 988 cm^{-1} attributed to Ser (Barth, 2007).

2.3.3 *In vivo* assays

The efficacy of all tested bacterial strains as curative and preventive applications was demonstrated by *in vivo* assays. The wounded fruits treated with *P. synxantha* (117-2b) showed the highest inhibition rate against fungal pathogen strain Cad21 on kiwifruits (63% and 84.3%) in the case of both curative and preventive application, respectively, compared to the other strains and relative to the control (Fig. 2.4).

However, *B. amyloliquefaciens* (FZB24) and *B. subtilis* (QST713) showed low curative activity against the pathogen with reductions of only 13% and 18.5%, respectively, relative to the control. *Bacillus pumilus* (QST2808) had the lowest efficacy against skin pitting disease, with no reduction as a curative treatment and only a 16.3% reduction as a preventive treatment. The synthetic product (Scholar®) was confirmed in both experiments as the most effective treatment. However, the effectiveness of *P. synxantha* (117-2b) was very close to the performance of the chemical treatment, particularly in the preventive application.

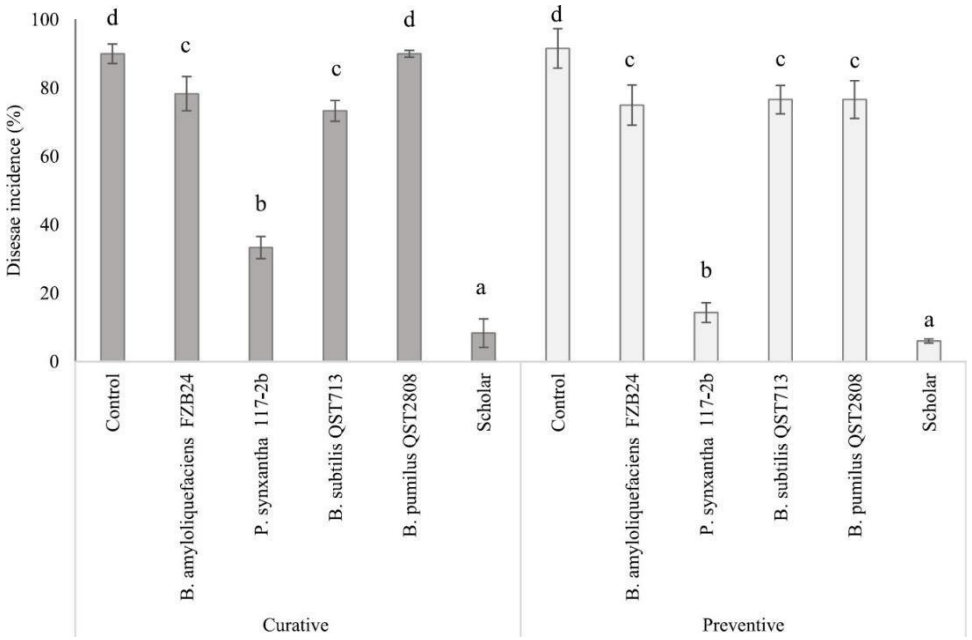


Figure 2.4: Curative and preventive effect of bacterial strains (10^8 cellsmL^{-1}) and Scholar® (1.3 mL^{-1}) on disease incidence on artificially inoculated kiwifruit. Fruits were kept at 1 °C for four months. For the curative effect fruit wounds were first treated with 20 μL of pathogen suspension and after air drying, they were inoculated with 20 μL of each antagonist. For the preventive effect antagonists were inoculated first. Data are the means of fifteen fruits for each antagonist and treatment effect. For each treatment effect different letters indicate significant differences according to Tukey's test (α=0.05).

2.3.4 qPCR analysis

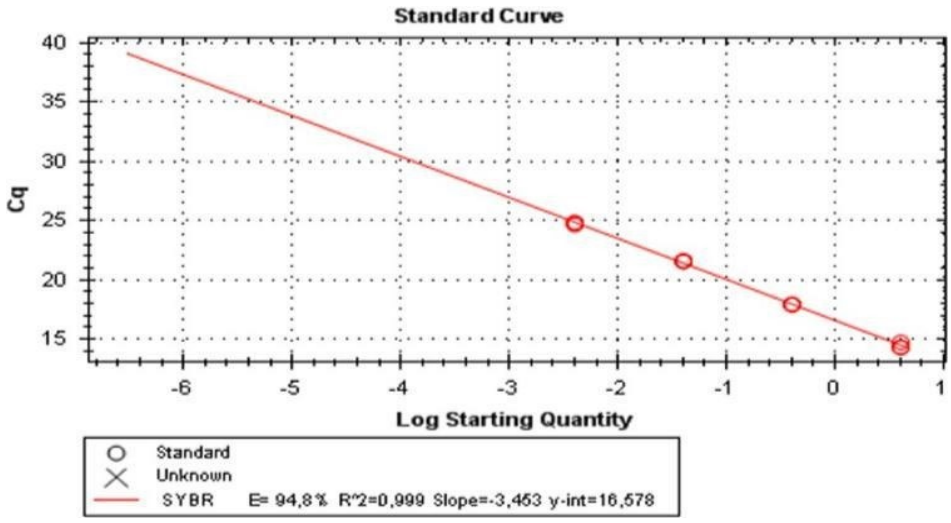
qPCR analysis quantified the amount of fungal pathogen DNA in each portion of the kiwifruit tissue analyzed. The standards were used to construct a standard curve, which presented an optimal efficiency of 94.8%, with $y = -3.453x + 16.578$ and $R^2 = 0.999$ (Fig. 2.5a).

The real-time amplification reaction gave positive results, especially with all DNA samples obtained by processing the portions of untreated fruits (negative controls) and with the majority of DNA samples obtained from *P. synxantha* (117-2b) treated fruits, thus allowing their quantification. The samples' melting temperatures corresponded to 82.5 °C, which was the same as the standards (Fig. 2.5b) and indicated that the amplification was specific. The quantification of *C. luteo-olivacea* in infected samples, measured after 4 months after the artificial inoculation of fruits, was obtained by extrapolating from the standard curve the pg of *C. luteo-olivacea* DNA and normalizing these data with mg of kiwifruit tissue sampled at

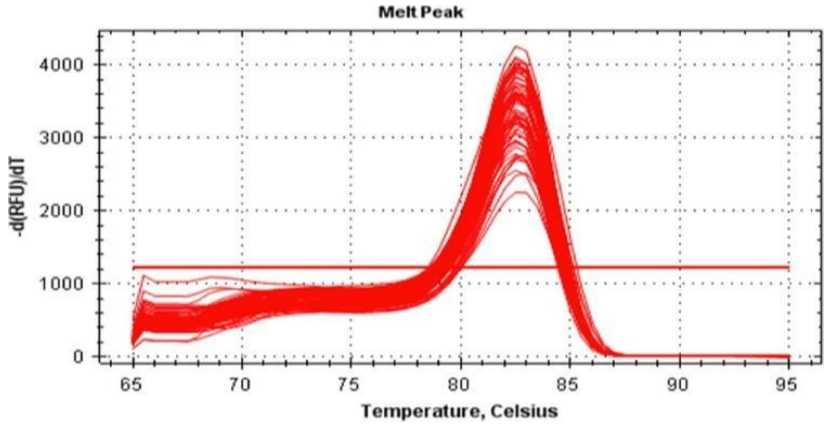
1 cm from the inoculation wound. The results of the quantification are summarized in Table 1. The amount of fungal DNA in the negative control (fungus and water) was estimated at around 30.02 pg/mg. In kiwifruits treated with *P. synxantha* strain 117-2b, selected as the best bacterial strain for controlling the development of skin pitting symptoms, and Scholar®, the amounts of DNA were drastically reduced to 0.13 pg/mg and 0.03 pg/mg, respectively (Table 2.1).

Treatments	Cad21 DNA pg/mg
Control	30.02 ± 9.05 a
P. synxantha 117-2b	0.13 ± 0.02 b
Scholar®	0.03 ± 0.00 c

Table 2.1: *Cadophora luteo-olivacea* (Cad21) quantification (expressed as pg of *C. luteo-olivacea* DNA/mg of kiwifruit tissue) on artificially wounded-inoculated kiwifruits stored for 4 months at 1 °C. Fruits (10 for each condition). were previously treated with sterile water (control) *Pseudomonas synxantha* (117-2b) and Scholar® and successively inoculated with the pathogen conidial suspension. Data reporting different letters are significantly different according to Tukey's test (α = 0.05).



(a)



(b)

Figure 2.5: (a) Standard curve established for the quantification of *Cadophora luteo-olivacea* by plotting the log of the starting quantity (ng)of 1:10 serial dilutions of fungal genomic DNA versus the cycle number (Cq); (b) melting peaks of amplicons generated from 1:10 serial dilutions and genomic DNA samples extracted from artificially wounded-inoculated kiwifruit.

2.3.5 *Pseudomonas synxantha* strain 117-2b population dynamics

The *Pseudomonas synxantha* (117-2b) population dynamics on artificially wounded-inoculated kiwifruit were determined during the 4-month cold storage period (Fig. 2.6). During the first three months of storage, a slight but significant increase in the CFU of the strain was registered, reaching almost 4.9 × 103 CFU wound^{-1}. After the third month of storage, the strain showed a slight but significant decrease with respect to the previous months.

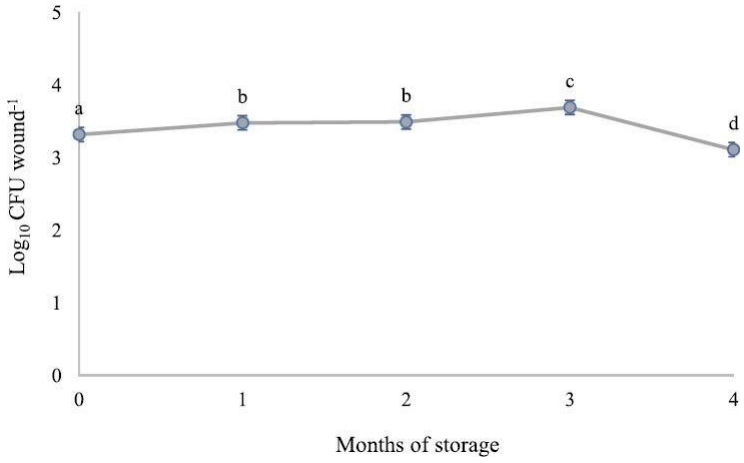


Figure 2.6: Population dynamics of *Pseudomonas synxantha (*117-2b) in previously wounded-inoculated kiwifruit. Fruits were stored at 0 °C in normal refrigeration for 4 months. Each point represents the mean of the number of colonyforming units (CFUs) from four replicates (kiwifruit) for each sampling time. For each sampling time different letters indicate significant differences according to Tukey's test (α=0.05).

2.4 Discussion

Cadophora luteo-olivacea has presented a critical problem in recent years in Italian packaging houses, and the connection between its incidence during the postharvest phase and field conditions at the time of fruit production has made it difficult to manage (Di Francesco et al., 2021). The possible appearance of skin pitting during storage is a challenge for kiwifruit marketing operators and often prevents them from waiting for the best market combination of demand and more profitable prices. This study aimed to find a sustainable solution to preserve kiwifruit from skin pitting losses by using BCAs as an alternative o fungicides. The most intensively studied bacteria for use as BCAs belong to the genera of *Pseudomonas*, *Bacillus*, and *Streptomyces* spp. (Bonaterra et al., 2022). Moreover, many of these have already been registered and marketed as biopesticides.

Among the most interesting, *Pseudomonas* spp. have been studied as potential antagonists against a wide range of fungal pathogens, mainly for their ability to produce active antifungal compounds (Rojas-Solis et al., 2020; Sang & Kim, 2014; Aiello et al., 2019). For example, *Pseudomonas synxantha* produces bioactive compounds such as a biosurfactant effective against several microorganisms (Mukherjee et al., 2014). For this reason, the strain *P. synxantha* 117-2b belonging to our microbiological collection has been studied and tested in the present work as an alternative method tocontrol kiwifruit skin pitting, together with other known bacterial strains.

Among the tested BCAs, *B. amyloliquefaciens* (FZB24), *B. pumilus* (QST2808), and *B. subtilis* (QST713) were assayed for their effectiveness against a broad range of plant pathogens. In fact, these strains are the active components of the bio-products Taegro®, Sonata®, and Serenade ®, respectively, whose effectiveness we also wanted to test against *C. luteo-olivacea* for a possible and valuable field-ready treatment.

It is known that bacterial antagonists can produce bioactive compounds such as hydrolytic enzymes, phytohormones, and volatile and non-volatile metabolites or induce systemic resistance in plants and promote plant growth (Calder´on et al., 2015; de Vleesschauwer et al., 2008; Lo Cantore et al., 2015; Raza et al., 2016; Rojas-Solis et al., 2020). In this regard, non-volatile metabolites, selected as the most active against *C. luteo-olivacea* by *in vitro* assay, were analyzed after different growing times. As noted, all the tested strains, except for *B. pumilus* QST2808, produced active metabolites against *C. luteo-olivacea*, starting from the stationary phase.

The biochemical analysis conducted using infrared spectroscopy on bacterial cell filtrate revealed the presence of peptidic compounds, mainly from the appearance of amide I and amide II bands in 1699–1625 cm^{-1} and 1546 cm^{-1} spectral regions (Fig. 2.3). These compounds could be related to the well-known production of antibiotic peptides and lipopeptides by several bacteria, such as *B. subtilis*, which produces iturin A, a heptapeptide closed in a ring with an amino fatty acid, whose antifungal activity is related to its ability to induce a massive leakage of K^+ and other cell components from the cytosol (Besson et al., 1996). The different profiles observed in the IR spectra could also explain the behavior of the antagonists in the *in vivo* assays. The corresponding *in vivo* effects of the QST713 and FZB24 strains could be related to their similar chemical composition due to the analogous positions and intensities of their peaks (Fig. 2.3). At the same time, the different spectral profile of the QST2808 strain may reflect the production of antifungal compounds that are less effective against the infection. However, the IR data should be interpreted carefully because it is well known that some bacteria, such as various *Pseudomonas* spp., can produce several antagonistic antifungal compounds in the form of peptides, including proteolytic enzymes (i.e., proteases, chitinases, cellulases, pectinases or amylases) or even siderophores (non-proteogenic peptides) (Karmegham et al., 2020). The similar chemical composition of these antagonistic compounds is reflected in their IR spectra, as evidenced in previous papers on non-volatile metabolites in *Aureobasidium pullulans* in which all the previous compounds were detected (Di Francesco et al., 2020; Di Francesco et al., 2021).

Nevertheless, none of the BCA cultures filtrates totally inhibited *C. luteo-olivacea* mycelial growth, even though they were active against B. cinerea mycelium (data not reported). This fact showed a broad spectrum of antagonism against different plant pathogens based on antimicrobial metabolites (Rosell´o et al., 2013).

Despite this, the curative and preventive *in vivo* experiments on fruits showed that *P. synxantha strain* 117-2b had higher effectiveness against *C. luteo-olivacea* (strain Cad21) than the other tested BCAs (Fig. 2.4). This finding was further confirmed by the IR analysis composition of culture filtrates (Fig. 2.3). *Pseudomonas synxantha*'s filtrates showed the most intense peaks (at 988 cm^{-1}), which can be related to the highestconcentration of antifungal compounds. Also, according to the co-culturing assay, the strain had the best adaptation to low temperatures (1°C), permitting long persistence during the storage phase, although, as seen in population dynamics, with a significant cellular decrease. Nevertheless, different studies suggested that high spore concentrations in inoculum were not always connected to the best performance of a BCA (Verma et al., 2007).However, a higher initial cellular concentration will be required for further applications such as in the field.

Indeed, this fact could be connected to the endophytic behavior of *P. synxantha* (Aiello et al., 2019), which can move, colonize, and persist inside its host's vascular tissues. This characteristic should be interesting to develop in the future for managing *C. luteo-olivacea*, whose epidemiologyis not still well understood but whose long latency is known and poorly manageable.

The management of kiwifruit diseases by employing BCAs has been successfully evaluated with many pathogens, but to our knowledge, *C. luteo-olivacea* had not been explored previously. According to the results obtained with qPCR analysis, *C. luteo-olivacea* showed great persistence in kiwifruit despite being inoculated with an antagonist. In fact, the pathogen DNA was detected in inoculated but asymptomatic kiwifruit, although in much smaller quantities than the control. Conversely, in the positive control (fungicide application), almost no traces of the fungal pathogen biomass were detected, confirming the different mechanisms of action between a BCAand a fungicide. Therefore, further studies such as genomic analysis are needed to deeply evaluate the mechanisms of action of *P. synxantha* strain 117-2b. It will be useful to characterize it as a good BCA and for its eventual consideration as an active ingredient in a bioformulation.

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Supplementary data

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Chapter 3

Pseudomonas synxantha volatile organic compounds: efficacy against *Cadophora luteo-olivacea* and *Botrytis cinerea* of kiwifruit

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Abstract

It is known that Volatile Organic Compounds (VOCs), among several other compounds, are responsible for the antagonistic activity exerted by biological control agents (BCAs). In this study, VOCs produced by *Pseudomonas synxantha* strain 117-2b were tested against two kiwifruit fungal postharvest pathogens: *Cadophora luteo-olivacea* and *Botrytis cinerea*, through *in vitro* and *in vivo* assays. *In vitro* results demonstrated that *P. synxantha* 117-2b VOCs inhibit mycelial growth of *C. luteo-olivacea* and *B. cinerea* by 56% and 42.8% after 14 and 5 days of exposition, respectively. Further *in vivo* assay demonstrated significant inhibitory effects, as bacterial VOCs used as a biofumigant treatment during the curing phase, reduced skin pitting symptoms disease severity by 28.5% and grey mold incidence by 66.6%, with respect to the untreated control. Biological control agents (BCA) VOCs were analyzed by SPME-GC-MS and among all the detected compounds, three (decanal, 1-butanol-3-methyl,and 1-nonene) resulted as the most produced in two different nutrientmedia. Their efficacy as pure synthetic compounds were assayed against mycelial growth of fungal pathogens by different concentrations (0.34, 0.56, and 1.12 μL mL^{-1} headspace). The aldehyde decanal had the strongest antifungal

effects on both mycelial fungal pathogens at a concentration of 1.2 μL mL^{-1} headspace. The effect of the application of VOCs as biofumigant was also investigated as expression level of 7 defense-related genes of kiwifruit at different exposition times. The results indicated a significant enhancement of the expression of almost all the genes starting from 3 hours of treatment. These findings described by a multi-approach *P. synxantha* VOCs characteristics and their potential as a promising method to adopt for protecting kiwifruit from postharvest *C. luteo-olivacea* and *B. cinerea* diseases.
Keywords: Biocontrol, Biofumigation, Defence genes, Grey mold, Skin pitting, Volatile metabolites.

3.1 Introduction

In the last years, new strategies are considered for the postharvest fruit management, in line with the new European Plant Health Regulation (EU 2016/2031) jointly with the European Green Deal policy that aim to accelerate the transition to more sustainable systems of food production reducing by 50% the use of pesticides and increasing by 25% land areas used for organic farming by 2030. Due to all these health concerns, biopesticides based on antagonistic microorganisms have been discovered and firstly tested in controlled conditions, and subsequently at commercial and semi/commercial settings (Droby et al., 2016; Droby et al., 2018; Teixidó et al., 2022).

Numerous biological control agents (BCAs), in particular bacteria belonging to Pseudomonas spp. and *Bacillus* spp. are able to produce volatile metabolites known for their antifungal efficacy (Gotor et al., 2017; Toffano et al., 2017). These metabolites, commonly known a Volatile Organic Compounds (VOCs) can act directly or indirectly against fungal pathogens development (Toral et al., 2021). Usually, microbial VOCs are alcohols, alkanes, acids, or ketones, characterized by their low molecular weight (Calvo et al., 2020). Volatile metabolites are produced by microorganism biosynthetic pathways of as integral components of secondary metabolism (Che et al., 2017). VOCs could be potentially used with success as gaseous fruit postharvest treatment in a process defined as 'biofumigation' (Di Francesco et al., 2015).

The present study focused on kiwifruit (*Actinidia deliciosa* (A. Chev) C.F.Liang et A.R.Ferguson) storage management, as fruit that has gained a special attention among consumers for its high nutritional qualities and taste (Xia et al., 2023). Kiwifruit can be stored up to 5 months at 0 °C and relative humidity (R.H.) of 92–95% at normal refrigeration (NR) or for an extended period in controlled atmosphere (CA) (Di Francesco et al., 2018). During the storage period, fungal infections represent the major problem for kiwifruit, causing serious economic losses (Li et al., 2022; Niu et al., 2023).

Botrytis cinerea is the causal agent of kiwifruit grey mold (Li et al., 2023), identified as one of the primary causes of fruit losses (almost up to 30%) mainly during the export phase (Hyun et al., 2022).

Conversely, *Cadophora luteo-olivacea*, a re-emerging fungal pathogen associated to kiwifruit skin -pitting symptom (Di Francesco et al., 2023), or side rot of pears (Wenneker et al., 2016) and apples (Köhl et al., 2018) is considered less harmful for its latent behaviour and sporadic emergence. However, the pathogen can infect fruits during the developmental stage, remaining latent during harvest and appearing after 3-4 months of cold storage causing important economic repercussions for fruit postharvest industry (Di Francesco et al., 2022).

Concerning all these considerations, the present study has the aim to i) evaluate *Pseudomonas synxantha* strain 117-2b VOCs efficacy against kiwifruit postharvest fungal pathogens, *B. cinerea* and *C. luteo-olivacea*, by *in vitro* and *in vivo* assays; ii) biochemically characterize bacteria VOCs by using headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (SPME-GC/MS) and verify the effect of single synthetic pure compounds on the mycelial growth of the target pathogens; iii) check the effect of fruit biofumigation with *P. synxantha* VOCs, combined with curing, on the expression of kiwifruit defense-related genes.

3.2 Materials and Methods

3.2.1 Kiwifruit

Kiwifruits cv 'Hayward' (*Actinidia deliciosa* (A. Chev.) C.F. Liang & A. R. Ferguson) were harvested at commercial maturity (7.2° Brix) from an orchard under integrated pest management (IPM) production system located in Friuli Venezia Giulia (FVG) region (46°02'19.08"N, 12°57'33.66"E), Italy. Fruits were selected homogeneous in size and free of lesions and stored at 0 °C with 92% Relative Humidity (RH) until use.

3.2.2 Microorganisms

Botrytis cinerea (Bc13), *C. luteo-olivacea* (Cad21), and *P. synxantha* (117-2b) belonged to the mycological collection of University of Udine – Di4A. The fungal pathogens were cultivated on Potato Dextrose Agar (PDA, 39 g L^{-1}) (Oxoid, UK) at 25 °C, one and two weeks before the experiments, for *B. cinerea* and *C. luteo-olivacea*, respectively. A strain, *Bacillus pumilus* QST2808, active component of the commercial product Sonata®, was purchased from the NRRL (Northern Regional Research Laboratory, IL, USA) and its effectiveness against the target pathogens was tested in our previous study (Di Francesco et al. 2023) and used as positive control in the

present work. For the assays, both bacterial strains were grown on Nutrient Agar (NA, 13 g L^{-1}) (Oxoid, UK) at 25 °C and the cells from 2-days old NA plates were suspended in potassium phosphate buffer (PPB, 70 mL KH_2PO_4 0.2 M; 30 mL K_2HPO_4 0.2 M and 300 mL deionized water v/v/v pH 6.5) and adjusted to the final concentration of 1×108 cells mL^{-1}.

3.2.3 *In vitro* assay: bacterial VOCs efficacy

To verify *P. synxantha* 117-2b VOCs efficacy against *C. luteo-olivacea* (Cad21) and *B. cinerea* (Bc13) mycelial growth, a double Petri dish assay was performed as reported by Di Francesco et al. (2019). For this purpose, 100 µL of bacterial suspension (1×108 cell mL^{-1}) obtained from 2-days old plates, were spread on new NA plates and incubated at 25 °C for 48 h. Each pathogen mycelial plug (6 mm diameter) was placed in the centre of PDA plates. After removing the covers, plates were immediately joined and sealed with a double layer of Parafilm®. Plates were incubated at 25 °C under darkness conditions for 14 and 5 days for *C. luteo-olivacea* and *B. cinerea*, respectively. *Bacillus pumilus* QST2808 (1×10^8 cells mL^{-1}) was inoculated as above reported and used as positive control. NA plates inoculated with

100 µL of sterile distilled water (SDW) represented the negative control. The sample unit of each treatment was represented by 5 replicates and the experiment was performed twice.

3.2.4 Bacterial volatile compounds analysis

To identify VOCs produced by *P. synxantha* (117-2b), sterile glass vials containing 10 mL of PDA or Kiwifruit Agar (KA) (250 g of fruit pulp, 15 g of agar technical in 1000 mL^{-1} of distilled water) were inoculated with 50µL of *P. synxantha* suspension (1×10^8 cells mL^{-1}). Vials were incubated at 25 °C for 2 days and after, the VOCs were extracted and analyzed by a gas chromatograph (7890B Agilent Technologies, Santa Clara, USA) coupled with mass spectrometer detector (5977A, Agilent Technologies, Santa Clara,USA) equipped with an autosampler for SPME automatic injections (*PAL* RSI 85, CTC). A SPME fiber with 50/30 µm DVB/CAR/PDMS coating was selected and conditioned before the use. The method used for the extraction and absorption of volatiles provided an incubation of the vial at 40 °C for 5 minutes, followed by fiber exposure of 30 min at the same temperature. For the desorption of the volatiles, the fiber was introduced into the injector at 250 °C for 5 min. The separation of analytes was performed on a 60 m long fused silica capillary column, with an internal diameter of 0.50 mmand a polar stationary phase (DB-WAX, Agilent Technologies, Santa Clara,USA) with a film thickness of 0.5 µm. GC oven temperature program was as follows: 40 °C for 3 min, raised from 40 °C to 220 °C by a constant ramp of 4 °C min^{-1}, 220 °C for 1 min, raised from 220 °C to 250 °C at 10

∘C min^{-1}, and 250 ˚C for 1 min. Helium was used as carrier gas with a constant column flow rate of 1.5 ml min^{-1}. The transfer line temperature was maintained constant at 250 ˚C. Ion source and quadrupole were setat 230 ˚C and 150 ˚C, respectively. Upon exiting the column, compounds were ionized via electron impact at 70 eV and detected with a quadrupole mass spectrometer in the range of a mass/charge ratio (m/z) from 35 to 500. Chromatograms were processed and analyzed by Mass Hunter (Agilent, USA). Compounds identification was achieved by comparing the spectra with the NIST Standard Reference Database (NIST2014) and by linear retention indices (LRI) calculated under the same chromatographic conditions, injecting C7–C30 n-alkane series (Supelco, Milan, Italy). VOCs content was reported as absolute peak area (Aprea et al., 2012). The sampleunit consisted of 5 replicates for each condition: controls (NA and KA) and the antagonist strain grown on both medium. The experiment was conducted twice.

3.2.5 Effect of synthetic volatile compounds on *C. luteo-olivacea* and *B. cinerea* mycelial growth

Three synthetic compounds (decanal, 1-butanol-3-methyl, 1-nonene) (Merck, Germany) identified as the main VOCs produced by *P. synxantha* 117-2b were selected and singularly used. For each pure compound, different concentrations (15, 25, and 50 μL, corresponding to 0.34, 0.56, and 1.12 μL mL^{-1} headspace, respectively) were settled on sterile filter paper (Whatman®, 90 mm ϕ), previously placed inside of the Petri dishes (90 mm ϕ) containing PDA inoculated with fungal plugs (6 mm ϕ) of each pathogen). The dishes were immediately double sealed with Parafilm® and incubated at 25 ˚C for 14 and 5 days, respectively for *C. luteo-olivacea* and *B. cinerea*.The effect of each compound was detected by measuring the diameters of each fungal colony. The control was represented by plates containing un-wetsterile filter papers. Each VOC concentration was represented by five plates per pathogen. The experiment was performed twice.

3.2.6 *In vivo* assay: kiwifruit biofumigation

Biofumigation *in vivo* assay was conducted by using sterile plastic boxes (29 $\times$18$\times$10 cm L, W, H) containing 150 mL of NA at the bottom. Six hundred μL of *P. synxantha* 117-2b suspension (1×10^8 cells mL^{-1}) were spread on the medium. The boxes were immediately double sealed with Parafilm® and incubated at 25 ˚C for 2 days. Kiwifruits were surface sterilized for one minute with sodium hypochlorite (0.1% v/v), rinsed with distilled water for one minute, and air dried at room temperature. Fruits were artificially wounded (2 × 2 × 2 mm) at the equatorial part

with a sterile nail and inoculated with 20 μL of conidial suspension of *C. luteo-olivacea* (Cad21) (1×10^5 conidia mL^{-1}) or *B. cinerea* (Bc13) (1×10^5 conidia mL^{-1}). Once dried, fruits were placed on sterile grills inside the boxes to be separated from the medium. Boxes, incubated at 15 °C, were exposed to bacterial VOCs for 96 h. After the biofumigation, treated fruits were removed from the boxes and stored at 0 °C for 3 and 1 months for *C. luteo-olivacea* and *B. cinerea*, respectively. As positive control, *B. pumilus* QST2808 was used as a biofumigant treatment. Boxes containing just NA were considered the negative control. Each sample unit was represented by three boxes (containing 8 kiwifruit) per each pathogen and treatment.

3.2.7 Gene expression analysis

Kiwifruit sampling

Kiwifruit peel was sampled from unwounded fruits treated with *P. synxantha* 117-2b VOCs (as described at section 2.6), and collected at 0, 3, 6, 12, 24, 48, and 96 h of biofumigation treatment using a sterile peeler. Fruit peel samples were immediately frozen in liquid nitrogen, lyophilized byfreeze-drying (FD-10 Freezing Dryer, Lab kits, H.K.) under vacuum (¡20 Pa) at a temperature of -36 °C and freeze-dried for 7 days, and stored at -80 °C until use. The unwounded fruits without *P. synxantha* 117-2b VOCs were considered as control. Three boxes containing 8 fruits were used for each VOCs exposition time.

RNA extraction and cDNA synthesis

Total RNA was extracted from the collected fruit peel samples using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich®, DE, USA), according to the manufacturer's protocol. The total RNA concentration and RNA quality were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, DE, USA). The integrity of RNA was further confirmed with an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). The cDNA synthesis was performed using 1 μg of total RNA and the SuperScript™ IV cDNA synthesis kit (Invitrogen, Thermo Fisher Scientific).

qPCR analysis

The transcription levels of 7 selected fruit defense genes (see Table 3.S in the supplementary material) were assessed by qPCR using a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The reaction mixture consisted of KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, USA), 100nM of each primer, and 9 ng of cDNA. Thermal cycling conditions were as follows: i) 10 min at95 °C for

initial denaturation ii) followed by 40 cycles of 15 s at 95 °C and iii) annealing at 60 °C for 1 min. A final amplification cycle at 95°C for 15 s, 60 °C for 1 min, 95 °C for 30 s and 60 °C for 15 s was applied to determine the melting curve. A non-template control (NTC) was included in each run using DNase free water instead of cDNA. The sequence of each targeted gene was identified in the kiwifruit genome database (https://kiwifruitgenome.org/), and specific primers were designed de novo

(see Table 3.2 in the supplementary material) using NCBI-primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer efficiency was determined by the serial dilution method, using a mix of all cDNA samples as a template. Genes encoding for *ACTIN* and *18S RNA* were checked as independent reference genes, and *ACTIN* was selected for its constant expression among sample conditions. Relative gene expression was expressed as Mean Normalized Expression (MNE) and calculated normalized to the reference gene *ACTIN*. Results were obtained from the average of 3 biological replicates.

3.2.8 Statistical analysis

Data were statistically handled by one-way analysis of variance (ANOVA) using MiniTab 17. Means statistical comparison of the effect of VOCs on the fungal colony diameter, and on the incidence and diseases severity was carried out by using Tukey's HSD Test (α = 0.05). For gene expression analysis, comparison of the means was performed between treatment and control samples for each time point by using the Student's T-test ($\alpha \leq$ 0.05), and between different time points for control and treated samples by conducting Tukey's HSD Test (*$\alpha \leq 0.05$, ** $\alpha \leq 0.01$, *** $\alpha \leq 0.001$).

3.3 Results

3.3.1 Pseudomonas synxantha 117-2b VOCs *in vitro* efficacy against kiwifruit fungal pathogens

The double Petri dish assay displayed the potential *in vitro* efficacy of VOCs produced by *P. synxantha* 117-2b against, *C. luteo-olivacea* and *B. cinerea*. Figure 3.1 reported the results of the experiment that showed a significant inhibitory effect of *P. synxantha* 117-2b and *B. pumilus* QST2808 VOCs against pathogens mycelial diameter. *Pseudomonas synxantha* 117-2b showed an inhibition of 56% and 42.8%, with respect to the relative control, against *C. luteo-olivacea* and *B. cinerea*, respectively. Likewise, *B. pumilus* QST2808 showed a significant inhibition, with respect to the relative control, against both fungal pathogens, of 52% and 44.9%, respectively.

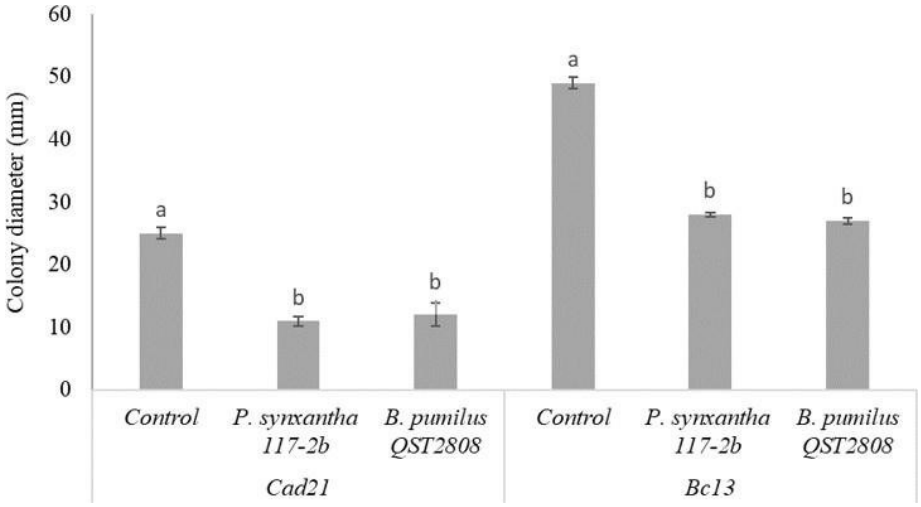


Figure 3.1: Effect of volatile compounds produced by *Pseudomonas synxantha* 117-2b and *Bacillus pumilus* QST2808 on the mycelial growth (mm) of *Cadophora luteo-olivacea* (Cad21) and *Botrytis cinerea* (Bc13). Fungal colony diameters (mm) were measured after 14 and 5 days at 25°C, respectively. Each value is the mean of 5 plates (replicates)±standard error. Different letters represent significant differences among the treatments within each pathogen according to Tukey's HSD Test (α=0.05).

3.3.2 SPME-GC-MS analysis of VOCs from *Pseudomonas synxantha* 117-2b

The volatile profile of *P. synxantha* 117-2b was investigated on NA and KA media through SPME combined with GC–MS technique. VOCs emitted by the bacterial antagonist exhibited a miscellaneous range of chemical compound classes, in particular alcohols (isopropyl alcohol, 1-butanol, 3-methyl, phenylethyl alcohol), alkenes (1-nonene), ketones (2-pentanone), and aldehydes (decanal) (Table 3.1). Detected VOCs resulted more numerous and with a higher absolute area (AA) when the strain was cultured on NA medium. In fact, the aldehyde decanal was detected only on the VOCs profile of the strain grown on KA.

Compound	(AA)		(RT)	
	NA	KA	NA	KA
dimethyl sulfide	3.15E+05	n.d.	5.51	-
1-nonene	3.54E+06	2.38E+04	8.54	8.55
2-Pentanone	8.75E+04	n.d.	9.57	-
Furan 2-propyl	3.39E+05	n.d.	11.18	-
Methyl thiolacetate	1.85E+05	n.d.	11.78	-
s-methyl propanethionate	3.06E+05	n.d.	14.15	-
1-butanol, 3-methyl	1.13E+06	1.04E+04	17.14	17.15
s-methyl 3-methylbutanethioate	1.87E+05	n.d.	17.92	-
Decanal	n.d.	7.42E+05	-	27.28
Dimethyl Sulfoxide	4.14E+04	n.d.	29.81	-
Ethanol, 2-ethoxy	1.05E+05	n.d.	30.93	-
Phenylenthyl alcohol	7.38E+04	n.d.	39.22	-

Table 3.1: Volatile organic compounds (VOCs) produced by *Pseudomonas synxantha* 117-2b at 25 °C after 48 h on Nutrient Agar (NA) and on Kiwi Agar (KA). The values (AA, absolute area; RT, retention time) represent the average of the same compound analysed on four vials and not detected on control vials. (n.d., not detected).

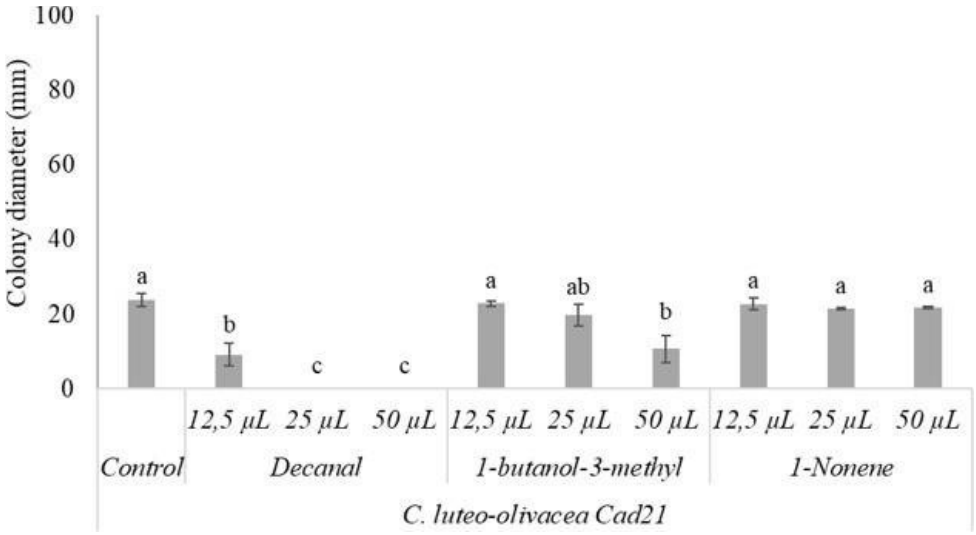
3.3.3 Effect of synthetic volatile compounds on pathogens mycelial growth

Among the detected VOCs, those with a higher AA or present on both agar medium such as 1-butanol-3-methyl, 1-nonene, and decanal, were selected for assessing their effect on *C. luteo-olivacea* (Figure 3.2a) and *B. cinerea* (Figure 3.2b) growth. Decanal, detected only on KA medium, exhibited the higher inhibition of the colony growth of both pathogens with respectto the other selected compounds. Specifically, decanal, at the lower tested concentration (0.34 μL mL^{-1} headspace), showed an inhibition of 61.7% of *C. luteo-olivacea* mycelial growth. The complete inhibition of the fungal growth was displayed with 0.56 and 1.12 μL mL^{-1} headspace. Regarding *B. cinerea*, a significant inhibition of 31% and 100% was obtained when decanal was applied at the concentrations of 0.56 and 1.12 μL mL^{-1} headspace,

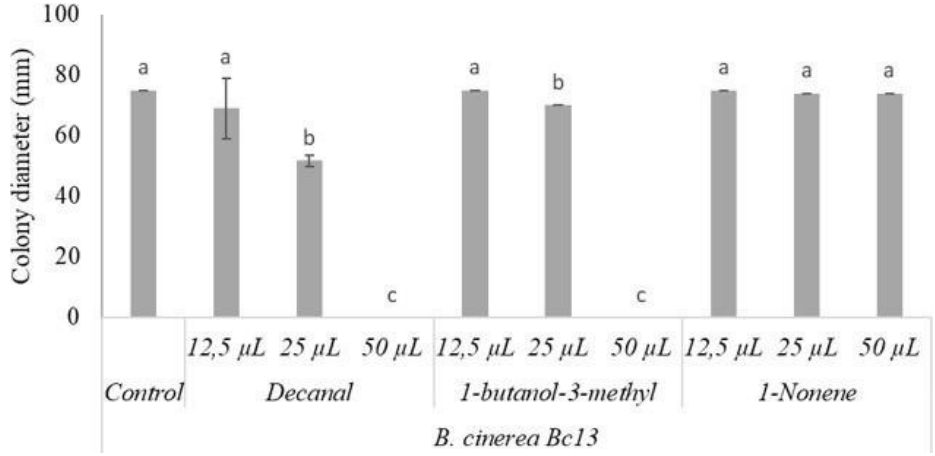
respectively. The compound 1-butanol-3-methyl resulted effective only at 1.12 μL mL^{-1} headspace, showing an inhibition of 55.3% and 100% of *C. luteo-olivacea* and *B. cinerea*, respectively. The alkene 1-nonene did not exhibit any significant inhibitory effect against both fungal pathogens.

3.3.4 In vivo assay: efficacy of bacterial VOCs against kiwifruit postharvest molds

VOCs produced by *P. synxantha* 117-2b showed a significant inhibitory effect against the targeted pathogens. Regarding *C. luteo-olivacea*, results were reported as severity (mm) of the disease (Figure 3.3a), and for *B. cinerea* as percentage (%) of the disease incidence (Figure 3.3b), after 3 and 1 month, respectively, of cold storage after 96 h of bacterial VOCs biofumigation. Regarding the skin pitting symptoms, *P. synxantha* VOCs reduced the disease severity by 28.5% with respect to the control. For *B. cinerea*, the displayed inhibition of the disease incidence was 66.6% with respect to the control. VOCs produced by the reference strain *B. pumilus* QST2808, showed, in the case of skin-pitting, the same efficacy of the tested BCA, and conversely significantly higher in the case of grey mold incidence (90.3%). Regarding *C. luteo-olivacea*, the incidence inhibition of the symptoms on fruits was not reported.



(a)



(b)

Figure 3.2: Effect of 96 h of exposition to VOCs produced by *Pseudomonas synxantha* 117-2b and *Bacillus pumilus* QST2808 on *Cadophora luteo-olivacea* severity (mm) (a) and *Botrytis cinerea* (b) disease incidence (%) on kiwifruit after 3 months and 1 month of cold storage (0 °C), respectively.

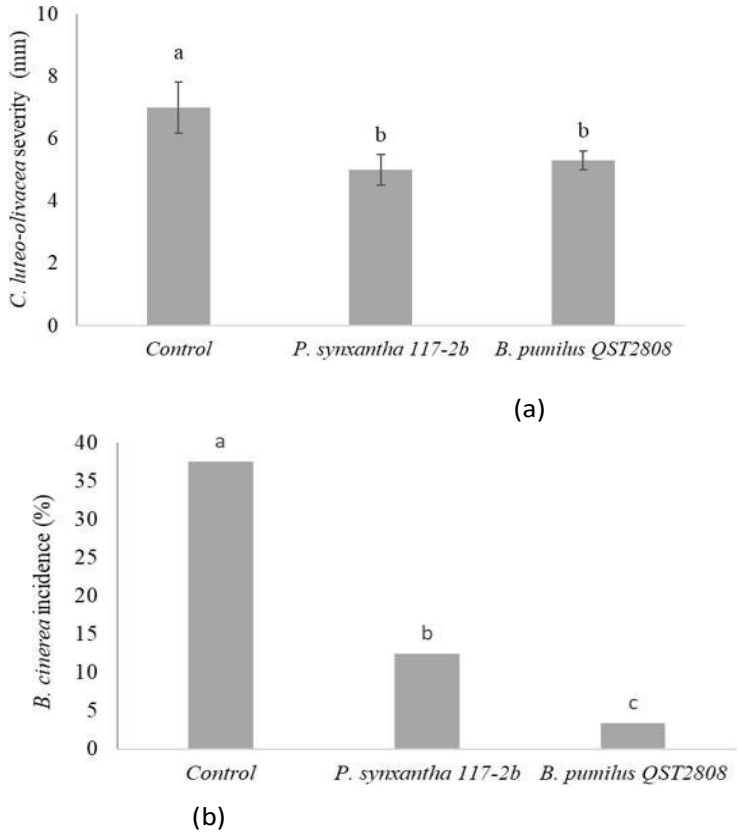


Figure 3.3: Effect of 96 h of exposition to VOCs produced by *Pseudomonas synxantha* 117-2b and *Bacillus pumilus* QST2808 on *Cadophora luteo-olivacea* severity (mm) (a) and *Botrytis cinerea* (b) disease incidence (%) on kiwifruit after 3 months and 1 month of cold storage (0 °C), respectively.

3.3.5 Gene expression analysis of kiwifruit exposed to *P. synxantha* 117-2b VOCs.

With the aim of determining the expression pattern of defense-related genes activated in kiwifruit as response to *P. synxantha* 117-2b VOCs exposition, the expression levels of several genes (see see Table 3.2 in the supplementary material) at six different time points (3, 6, 12, 24, 48,and 96 h) after biofumigation were analyzed. Results revealed that thebacterial VOCs, in general, promoted the up regulation of the target genes (Figure 3.4). Specifically, the results for *CAT* expression levels (Figure 3.4a) revealed a significant increase in its expression at 3, 24, 48, and 96 h from the treatment with bacterial VOCs, compared to the control. Notably, the highest expression level was observed at 48 h post-treatment, being 1.2-fold higher the expression in the treated sample compared to the control. However, no significant differences in *CAT* gene expression compared to the

control were observed after 6 and 12 h from the treatment. Moreover, a significant increase in the expression level of *CAT* gene was also observed in the control samples from 24 to 96 h. Similarly, the expression of *CHI* gene (Figure 3.4b) started increasing significantly at 3 h from the treatment and continued to rise until 12 h. However, after the 12-hour time point, the expression levels of *CHI* gene were sustained, although a significantly higher expression occurred at 24 and 96 h, compared to the control. In the control fruit, *CHI* gene showed a higher expression after 48 and 96 h compared to the earlier time points. In the case of *GLU* (Figure 3.4c), a significant increase in the expression was observed at 6 h from the treatment compared to the untreated fruit, increasing its expression until 96 h. No significant increase was detected in the control samples over time. The results for *NPR* gene expression level (Figure 3.4d) revealed a significant increase along with the treatment time points and the maximum induction took place after 96 hours from the treatment compared to the control. Conversely, no significant differences in *NPR* gene expression were observed at 6 and 12 h with respect to the control. The exposition of fruits to bacterial VOCs caused a significant increase in *POD* gene expression (Figure 3.4e) at 6 h, 24 h, 48 h and 96 h compared to the control. Specifically, a notable upregulation occurred at 96 h, being 2.3-fold higher compared to the control. However, if compared to the control, no significant differences in *POD* expression at 3 and 12 h were detected. Regarding *SOD* gene (Figure 3.4f), the treatment induced its expression levels after 12 h, and this pattern was sustained until 96 h. *SOD* gene expression increased in the control samples at 96 h compared to 0h time point. Fruit *PAL* gene (Figure 3.4g) was notably upregulated after bacterial VOCs exposition at 24, 48, and 96 h compared to the earlier time points. In contrast, no significant differences in *PAL* expression were observed in control samples, except for 96 h, in which gene expression levels were upregulated compared to the other time points.

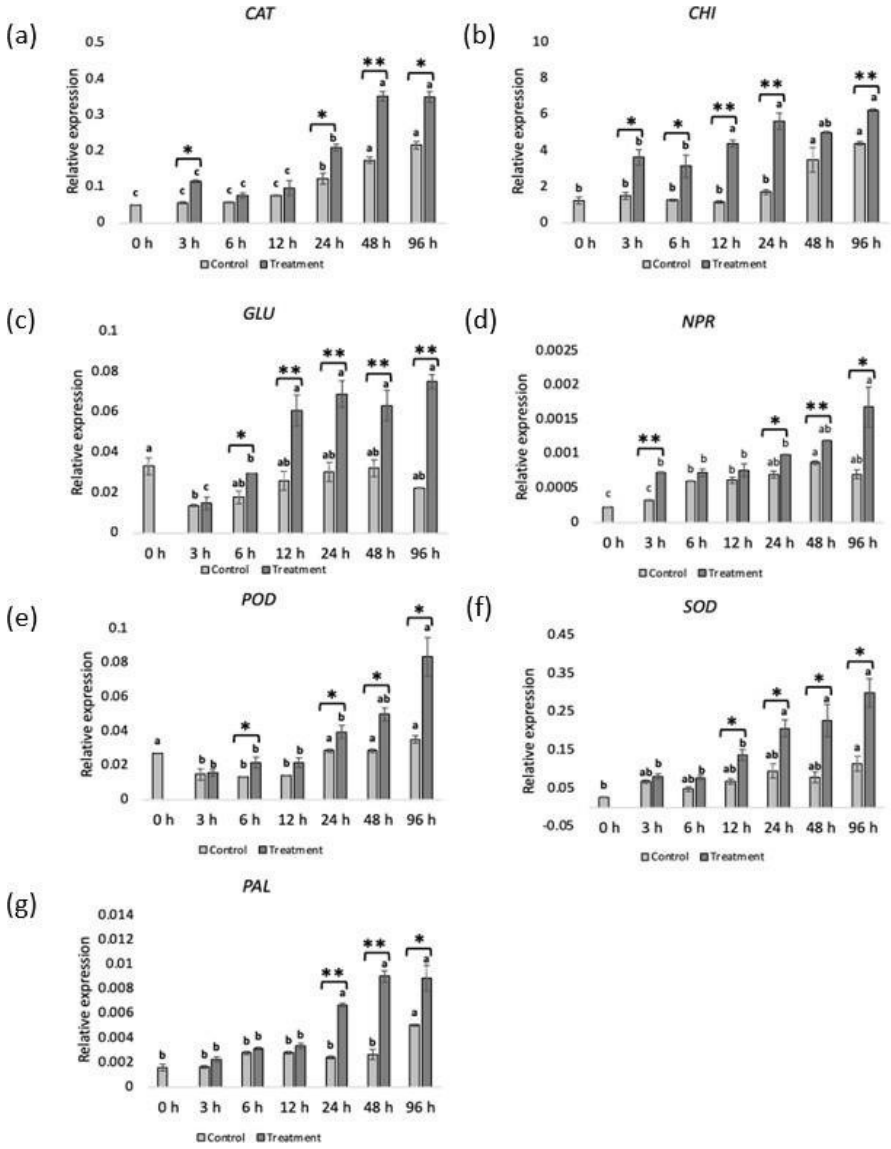


Figure 3.4: Effect of volatile organic compounds (VOCs) produced by *Pseudomonas synxantha* 117-2b on the expression level of *CAT* (a), *CHI* (b), *GLU* (c), *NPR* (d), *POD* (e), *SOD* (f) and *PAL* (g) genes in kiwifruit sampled after different time points (3, 6, 12, 24, 48, and 96 h) from bacterial VOCs exposition. Data are expressed as the mean of 3 biological replicates ± standard error. Different letters indicate significant differences along time for each control and treated samples according to analysis of variance (ANOVA) and Tukey's HSD test ($\alpha < 0.05$). Asterisks denote significant differences between control and VOCs treated samples (*$\alpha \leq 0.05$, ** $\alpha \leq 0.01$, *** $\alpha \leq 0.001$).

3.4 Discussion

The occurrence of fruit postharvest diseases always represents a great challenge for market, especially because they are often unmanageable and unpreventable. In fact, kiwifruit can be stored up to 6 months in cold storage, thus increasing the possibility of reporting fungal infections and also decreasing the nutraceutical features (Xia et al., 2016). The present study aimed to find a sustainable solution to use during kiwifruit storage, for its preservation from skin pitting and grey mold diseases by using a bacterial BCA instead of a synthetic fungicide. Many microorganisms including bacteria, yeasts, and fungi are VOCs producers, and their biocontrol activity has been studied (Guo et al., 2023; Gotor et al. 2017; Di Francesco et al 2020). Among bacteria, *Pseudomonas* has been studied as a potential BCA against a wide range of fungal pathogens, mainly for its ability to produce antifungal compounds (Rojas-Solis et al., 2020; Aiello et al., 2019;Di Francesco et al 2023; Sang et al., 2014). In particular, *Pseudomonas cepacia* and *Pseudomonas fluorescens* have been tested for the control of pome fruits diseases (Janisiewicz and Roitman, 1988; Wallace et al., 2017) or *Pseudomonas syringae* for the control of citrus fruit fungal pathogens (Panebianco et al., 2015). Mukherjee et al. (2014) also reported *P. synxantha* as producer of bioactive compounds, including volatile compounds, effective against several microorganisms. In the last years, many studies have been conducted on the different modes of action exerted by BCAs in the inhibition of pathogenic fungi. Recently, the essential role of VOCs has been given more consideration (Toffano et al., 2017), specially for the potential use during the fruit postharvest phase as biofumigants. The present study has displayed the efficacy of the strain *P. synxantha* 117-2b against skin pitting and grey mold of kiwifruit by producing VOCs, as it was already reported as an effective antagonist against *C. luteo-olivacea* through other modes of action (Di Francesco et al., 2023). In fact, *P. synxantha* 117-2b by double Petri dish assay showed a significant inhibition of the mycelial growth of the targeted pathogens *C. luteo-olivacea* (Cad21) and *B. cinerea* (Bc13) after the exposition to VOCs, reaching an inhibition of 56% and 42.8%, respectively. Its effectiveness was compared to *B. pumilus* QST2808, a strain selected for its high efficacy against *C. luteo-olivacea* in a previous study (Di Francesco et al., 2023). In *in vitro assays*, both treatments showed the same efficacy against the two target pathogens, making *P. synxantha* 117-2b a promising candidate for further investigation and potential application. The outcomes of our study are partially in agreement with the antifungal effect of VOCs produced by *P. synxantha* strain DLS65 applied against *Monilinia fructicola* and *Monilinia fructigena* in stone fruits (Aiello et al., 2019). In fact, Aiello et al. (2019) reported that the best performances of *P. synxantha* DLS65 were determined mainly by the mechanisms of action that involved the presence of bacterial living cells. In our case, VOCs produced by *P. synxantha* 117- 2b resulted effective also in *in vivo* assays where an incidence reduction of

almost 70% was registered for *B. cinerea*. In fact, the experiment combined two different strategies, curing and biofumigation, that can also contribute to an enhancement of kiwifruit disease resistance. Firstly, it was necessary to determine the *P. synxantha* 117-2b VOCs profile by SPME-GC-MS analysis. Results revealed an array of volatile compounds belonging mainly to alcohols, aldehydes, and alkanes classes. Among the identified compounds, 1-nonene, and 1-butanol-3-methyl resulted the main compounds produced on NA medium, and at the same time decanal, the main produced compound on KA medium. These results showed how microorganism VOCs production can vary depending on the growth media composition, highlighting the importance of the substrate on the antifungal volatiles production (Gotor-Vila et al., 2017).

Usually, the principal group of microorganism metabolites was formed by alcohols as reported in previous studies (Stoppacher et al., 2010; Rouissi et al., 2013). However, the applied methodology to collect and detect VOCs, and also the growth medium, can strongly influence the results and often hinder the comparison between studies (Martins et al., 2010; Di Francesco et al., 2015). In fact, in our study, the antagonistic strain produced decanal only on KA medium, the most active volatile when tested as individual compound against both kiwifruit pathogens. In relation to aldehydes, they were reported as active nematocidal (Huang et al. 2010) and fungicidal (Fernando et al., 2005) molecules. Similarly, Fernando et al. (2005) displayed that decanal can play an important role in the inhibition of fungal growth, limiting spore production, and reducing disease severity. Results reported by He et al. (2020) supported our finding, since decanal inhibited *Alternaria alternata* germ tube formation and *B. cinerea* mycelial growth. Comprehensively, *P. synxantha* VOCs application as a biofumigant treatment during the curing phase slightly reduce *C. luteo-olivacea* symptoms without controlling the infection, so in this case, the treatment could be part of an integrated strategy to control skin pitting. Instead, for *B. cinerea*, *P. synxantha* VOCs notably reduced the pathogen incidence (66.6%) compared to the untreated control. Probably, this effectiveness can be in part attributed to the overexpression of different fruitdefence-related genes, such as *CAT*, *CHI*, *GLU*, *NPR*, *POD*, *SOD*, and *PAL*.

In particular, the antioxidant role of genes such as *CAT*, *POD*, and *SOD* has been proven to be crucial for fruit disease resistance, also contributing to the response to biotic and abiotic stresses (Pan et al., 2020). Recent evidences suggest that plants treated with resistance-inducing substances (e.g. chitosan) exhibit faster and stronger defense responses when infected by pathogens (Maya et al., 2013; Zhang et al., 2018). Our results showed that the bacterial VOCs exposition increased the expression level of all targeted genes in treated kiwifruits. However, the different genes showed different behavior patterns, and the higher expression level at different time points. Kiwifruit *CHI*, *NPR*, *GLU*, and *CAT* genes showed an early response to bacterial VOCs exposition starting their induction at 3 h from

the treatment. Among the selected defense genes, *CHI* gene represents one of the main pathogenesis-related *(PR)* members (Zhang et al., 2018) responsible for plant defence against pathogenic fungi by breaking down their cell walls (Li et al., 2013). Like *CHI* gene, VOCs exposition also triggered the expression of *GLU* gene in kiwifruit. *β*-1,3-glucanase plays a direct role in fungal defense by hydrolyzing fungal cell walls and promoting the formation of elicitors (Ebrahim et al., 2011). Our study reported that the increased expression of kiwifruit *GLU* gene by VOCs treatment was principally detected after 6 h compared to the control.

Regarding *NPR* gene, it has been identified as a positive controller of defense response mediated by salicylic acid (SA) (Hong et al., 2016). In certain plants like Arabidopsis and grapes, the activation of *NPR* genes can occur within a day when exposed to SA or analogs, or to specific pathogens (Le Henanff et al., 2009). In our case, results showed a significant increaseof *NPR1* expression levels after 3 h from treatment exposition, such asoccurred for *CHI*, *GLU*, and *CAT* genes. In fact, a significant upregulation of key defense-related genes like *CAT*, *CHI*, *GLU*, *NPR*, *POD*, but also *SOD*, and *PAL*, supports the hypothesis that *P. synxantha* 117-2b VOCs induced defense mechanisms in kiwifruit. However, the findings of our study demonstrated that the activation of each defense-related genes is strictly dependent on the different treatment times. Moreover, we also observed a gradual increase in the expression of *CAT*, *CHI*, *POD*, and *PAL* genes also in the untreated samples over time. This phenomenon is likely due to the possible kiwifruit endogenous processes such as natural senescence process that triggers the upregulation of defence related genes, probably stimulated by the curing period (Wurms et al., 1997). Overall, our results demonstrated that VOCs produced by *P. synxantha* strain 117-2b can elicit a robust defense response in kiwifruit during the curing phase supporting the obtained *in vivo* results, where the treatment reduced postharvest fungal pathogen incidence in the case of grey mold, and severity in the case of skin pitting. Further studies are needed to better investigate the molecular mechanisms involved in the activation of defense response in kiwifruit and to implement the practical application of biocontrol agents and volatilemetabolites in postharvest phase to manage fruits through a sustainable strategy.

3.5 Conclusion

The findings of the present study provide new insights into the effects of VOCs produced by the strain *P. synxantha* 117-2b against *C. luteo-olivacea* and *B. cinerea*, both fungal postharvest pathogens of kiwifruit. The results showed how the production of VOCs play an essential role in the antagonistic activity of a BCA and how their production can be strongly influenced by growth substrates. Also, the biofumigant treatment of kiwifruits by *P. synxantha* 117-2b notably promoted the expression levels of fruit defense genes. For this reason, this BCA may be applied with the purpose to inhibit the pathogens growth, and at the same time, increase the fruit defense responses. However, further investigation is necessary to understand the exact contribution of *P. synxantha* 117-2b in the control of kiwifruit postharvest pathogens, to improve its efficacy which was variable depending on the pathogen and the experimental conditions.

Acknowledgements

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Supplementary material

Gene	Accession no.	Primer sequence $5' \rightarrow 3'$	Product size (bp)
CAT	Ach21g051741.2	F: ACAAGGCCGGTAAAGCACAT	92
		R: TCCGACCTTAACCGACTCCT	
CHI	Ach08g341891.2	F: AATCGCTGCTTTCTTCGCAC	71
		R: CGATTTCCGGGTTGTTGAGC	
GLU	Ach24g103301.2	F: ACCAGAACCTCTTTGACGCC	145
		R: TAAGTGGCTGCGTTGTCGAT	
NPR	Ach01g326501.2	F: GCCTTCTCCGATTCCAACGA	110
		R: CAGCGAAGGGATGCGATTTC	
POD	Ach01g373131	F: GGGGTGTGATGCTTCGGTTA	97
		R: GTCAAATCCATCTCCGGCCA	
SOD	Ach03g419431.2	F: ACTACAACAACGCCCTCCAG	108
		R: TGATATGACCTCCGCCGTTG	
PAL	Ach08g066341.2	F: ATCGTCGACCAAGTCCATCG	143
		R: TTGCTAGACGGGTGTTGTCC	
18s RNA	AB253775	F: GAATGACCCGCGAACTTGTC	140
		R: CGGGATTCGTTGTTTGACCG	
ACTIN	EF063572.1	F: CATCGTCCACAGGAAGTGCT	193
		R: GGCAAGGAGAGCCATCACAT	

Table 3.S: Primers used in qPCR analysis. Gene name, accession number, primer sequence and product size are specified. Target genes were identified from the kiwifruit genome database (https://kiwifruitgenome.org/). Both reference genes, *ACTIN* and 18s RNA were identified from NCBI (https://www.ncbi.nlm.nih.gov/genbank/).

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Chapter 4

Quantification of *Cadophora luteo-olivacea* on kiwifruit surface throughout the growing season and its correlation with postharvest skin-pitting incidence: A quantitative PCR approach

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Abstract

Skin-pitting disease caused by *Cadophora luteo-olivacea* is an important postharvest disease of kiwifruit in Italy. Although the pathogen inoculation occurs in the field, symptoms appear after about three or more months of cold storage. This study systematically monitored the pathogen inoculum density on fruit surfaces in four kiwifruit orchards during the growing season of 2021 and 2022. Enrichment steps were carried out through filtration and centrifugation after washing and sonication of the fruits, whereas qPCR assay was used to efficiently quantify the dynamics of fungal inoculum on surface of kiwifruits from the selected orchards during the 4 months of growing season (Jul – Oct). Different patterns of pathogen amount were observed within these orchards but a significant increase in pathogen concentration was observed in all orchards during the season. Furthermore, in this study we also attempted to establish a possible correlation between inoculum concentration of *C. luteo-olivacea* on kiwifruit surface and incidence

of skin-pitting. However, a correlation between pathogen concentration and incidence of skin-pitting disease was evident only in some cases.

Keywords: *Actinidia deliciosa* – epiphytic microflora – Inoculum dynamics – Diseaseincidence – Postharvest cold storage.

4.1 Introduction

Kiwifruit (Actinidia deliciosa) is a delicious and nutrient-rich fruit that has gained worldwide acclaim for its exceptional taste, vibrant green flesh, and characteristic small black seeds (Q. Zhao et al., 2023). This fruit offers a multitude of health benefits to humans as it is rich in vitamins C and E, folate, potassium, and antioxidants (Xiaojiao Li et al., 2022). Over the past decade, kiwifruit cultivation area and its production have witnessed substantial global growth rates, with a remarkable increase of 71.25% in cultivation area and 55.58% in production. (Xiaopeng Li et al., 2022).

Kiwi is a climacteric fruit, and its ripening physiology continues after harvest (Taş et al., 2022). It can be stored at 0 °C for about 4–5 months with relative humidity (R.H.) of 92–95% by using conventional refrigeration methods if the fruits are handled properly before harvest (Jabeen et al., 2022) However, due to its soft and succulent composition characterized by high water content, kiwifruit is vulnerable to infection by various pathogenic fungi (Zhao et al., 2023). This susceptibility can lead to the softening and rotting of the fruit, causing economic losses for fruit growers and food safety concerns (Li et al., 2023).

Over the last few years, the lesser-known fungal pathogen *Cadophora luteo-olivacea*, responsible for skin-pitting disease in kiwifruit, has been reported in packaging houses located in Italy and Chile (Di Francesco et al., 2023c; Jabeen et al., 2023). This pathogen is known to initiate the infection during the fruit's development stage, remaining quiescent for a duration of approximately 3–4 months in cold storage before becoming active and manifesting symptoms during the subsequent shelf-life period. (Di Francesco et al., 2023). Skin pitting symptoms, characterized by oval-shaped dark brown lesions, manifest following an extended period of cold storage (Spadaro, 2010). Incidence of skin-pitting disease is variable within the orchards that makes the study of this disease more complicated (Di Lenarda et al., 2011)

Knowledge about the epidemiology of skin-pitting caused by latent (quiescent) infections during long-term storage is still limited; unlike diseases caused by wound pathogens, such as Botrytis cinerea. Since their control relies on the routine use of fungicide applications; due to the growing concern over the use of synthetic fungicides, alternative control measures are strongly advocated. Over the past years the use of

physical treatments, natural compounds, biocontrol agents, and more recently the fruit microbiome (Droby and Wisniewski 2018) have been investigated as alternatives for controlling latent postharvest diseases. In a recent review about latent postharvest pathogens of pome fruit and their management, it is debated to approach such diseases as complex problems that require multiple interventions at different stages of the disease process (Wenneker & Thomma, 2020). Consequently, a deep understanding of the epidemiology of latent postharvest pathogens in the orchard together with the knowledge on fruit defence mechanisms against pathogens, and the molecular biology of their interactions are required in order to develop novel integrated disease control methods.

In order to improve the knowledge on *C. luteo-olivacea* epidemiologyin the orchard, one of the main aims of this manuscript was the estimation of the *C. luteo-olivacea* concentration on the surface of kiwifruit and its monitoring during the growing season (July-October) of 2021 and 2022 by using a protocol developed in the present work. The protocol was based on washing of kiwifruit surface, sonication and filtration to obtain a pellet of epiphytic microflora and on the applicationof molecular techniques such as total DNA extraction from the pellets and quantitative polymerase chain reaction (qPCR), which has demonstrated its worth in identifying and quantifying pathogens in various environmental samples because of being precise and rapid compared to conventional techniques (Maldonado-González et al., 2020). Furthermore, another objective of this work aimed to determine a quantitative relationship between the incidence of postharvest skin- pitting disease and inoculum concentration at harvest time for three consecutive years (2020-2022).

4.2 Materials and methods

4.2.1 Kiwifruit collection

The kiwifruits cv Hayward were meticulously sampled from four distinct kiwifruit farms in Friuli Venezia Giulia (FVG) region, Italy. In 2020, the fruits were collected in October at harvest time to design and set up the experimental procedure for determining the quantity of *C. luteo-olivacea* onthe surface of the fruits. In the following years, 2021 and 2022, fruit sampling was carried out on a monthly basis in all the selected orchards during the vegetative period from July to October. The orchards were selected under the guidance of the FriulKiwi (Rauscedo, Pordenone) technical team, relying on historical insights regarding the incidence of skin- pitting on fruits from the designated farms. No fungicides were used during the vegetative season in any of these orchards, allowing the natural occurrence of skin-pitting disease.

4.2.2 Separation of the epiphytic microflora from the kiwifruit surface

Upon harvest, the kiwifruits were transported to the Plant Pathology laboratory of University of Udine to separate the epiphytic microflora from the fruit surface following a protocol which was developed based on the studies conducted by Hamilton et al. (2020). The fruits from each orchard were divided into two or three biological replicates of 24 fruits each. The 24 fruits of each repetition were processed in groups of eight fruits of the same size, which were placed in a clean polyethylene bag containing 1 L of phosphate potassium buffer with Tween 20 (KH2PO4 [0.05 M], K2HPO4 [0.05 M], and 0.05% [w/v] Tween 20, pH 6.5). The bag was placed on an orbital shaker (KS 250, IKA, Germany) for 10 minutes at 60 rpm for 20 min atroom temperature followed by an ultrasonic cleaner (Elma Transonic T460, Burladingen, Germany) for 4 minutes at 36 kHz. The last steps were repeated two more times, each time replacing the group of 8 fruits with a new one in the same bag. The washing suspensions from the two-three biological replicates were filtered through a sterile cotton gauze in a beaker to eliminate the coarser material and left to decant for 3 hours at 4°C.

Aliquots of 100 mL were taken and filtered with a vacuum filtration apparatus through 2 distinct hydrophilic nylon membranes (pore diameter 0.22 µm, Millipore, USA). The filters were then washed in 50 ml Falcon tubes (VWR, USA) with 20 mL of phosphate buffer with Tween 20 and centrifuged at 16,000 X g for 10 min. The supernatant was discarded, and the pellet was stored at -80 °C until the next step.

4.2.3 Total genomic DNA extraction from the pellet and Quantification of the amount of *C. luteo-olivacea* DNA by qPCR

DNA was extracted from the pallets using DNA extraction kit (Quick-DNA™ Fecal/Soil Microbe Miniprep, Zymo Research) according to manufacturer's protocol and eluting DNA with 50 µL od DNA Elution Buffer. DNA quantity and quality were analyzed by using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and each DNA sample was diluted to 2 ng/µL in nuclease-free water. In order to determine the presence/absence of *C. luteo-olivacea* propagules a qualitative qPCR was performed as described in Di Francesco et al. (2023) using ITS as a target and the *C. luteo-olivacea* specific primers Cad(KSDA)-f2/Cad (KSDA)-r2 (fragment 105 bp long).

Successively, in all positive samples, the amount of pathogen DNA was assessed by qPCR as pg of *C. luteo-olivacea* DNA through the establishment of a standard curve with 10-fold serial dilutions of total DNA extracted from a pure culture of the Cad21 strain and quantified by using Qubit® 2.0 Fluorimeter (Thermo Scientific, Wilmington, DE,

USA), starting from 2 ng/µL to 20 fg/µL. PCR mixtures and cycling conditions were performed as described in Di Francesco et al. (2023). The amountof fluorescence for each sample was measured at the end of each cycle and analyzed via CFX-Manager Software v. 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The baseline was automatically determined, and the fluorescence threshold was set manually to maximize the standard curve efficiency. Each diluted sample and each standard were replicated three times in the experiment. Fungal DNA quantity was expressed as pg of *C. luteo-olivacea* DNA/ng of total genomic DNA extracted from the pellets to normalize the data.

4.2.4 Incidence of skin-pitting disease

Part of the fruits harvested in October during the years 2020-2022 (about 100 kiwifruits), were immediately stored at 0°C for a duration of four months. Throughout this extended period of cold storage, the fruits were monitored for the incidence of skin-pitting disease on a weekly basis. This observational study spanned three consecutive years, enabling acomprehensive evaluation of the long-term effects of postharvest cold storage on disease development. The correlation between pathogen concentration on fruit surfaces and the occurrence of postharvest skin-pitting disease was also assessed.

4.2.5 Statistical analysis

Statistical analysis of the data involved a one-way analysis of variance (ANOVA) using JMP® (v. 13.1, SAS Institute Inc., Cary, NC) to detect the quantity of *C. luteo-olivacea* DNA concentration on the fruit surface during each month (Jul – Oct) of the growing season. When the analysis was statistically significant, the Tukey's HSD test at the level $p \leq 0.05$ was performed for comparison of means along different months of kiwifruit development period. The reported data represents mean values ± standard error (SE) across two independent years (2021, 2022) of experimentation. The relationships between skin-pitting incidence and *C. luteo-olivacea* DNA concentration were subjected to linear regression analysis. The analysis of variance procedure was used to determine significance among regressions.

4.3. Results

4.3.1. Separation of epiphytic microflora from theKiwifruit surface and quantitative qPCR

The results obtained from the spectrophotometric analysis of the extracted DNA revealed a substantial quantity, falling within the range of 140 to 150 ng/µL, and acceptable quality indices demonstrating that the protocol, based on washing with solutions containing a surfactant component, incorporating a sonication step and a vacuum filtration step, allowed for consistent, high-yielding DNA recovery from the entire kiwifruit surface.

Qualitative qPCR gave positive results with all DNA samples extracted from the pellets containing the epiphytic microflora and obtained by washing the surface of the fruits. The melting temperatures of the samples corresponded to 82.5°C, the same as the standards. This indicates that the amplification was specific, as the standards contained only *C. luteo-olivacea* DNA (CBS 141.41). These results therefore indicated that this fungus is a constituent of the epiphytic flora constantly present in all selected orchards during the vegetative season from July to October of 2021 and 2022.

4.3.2. Quantification of the amount of *C. luteo-olivacea* DNA on kiwifruit surface during the vegetative season (July-October).

In order to accurately quantify the concentration of *Cadophora* DNA in our samples, a standard curve was generated using known concentrations of target DNA. The standard curve exhibited a linear relationship across the specified concentration range, with an optimal efficiency of 95%, with $y = -3.4416x + 10.665$ and $R^2 = 0.9994$ (Fig. 4.1).

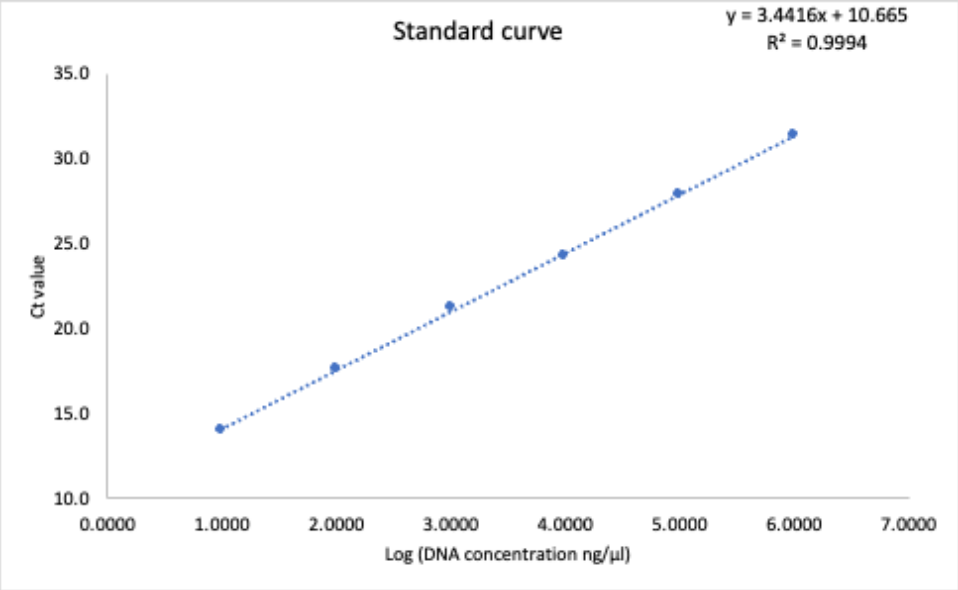

Figure 4.1: Standard curve for the quantification of *Cadophora luteo-olivacea* DNA established using the logarithmic plot of the starting quantity (ng) of 1:10 serial dilutions of fungal genomic DNA against the cycle number (Cq).

The quantification of *C. luteo-olivacea* on kiwifruit surface, was obtained by extrapolating from the standard curve the pg of *C. luteo-olivacea* DNA and normalizing these data with ng of total genomic DNA. The results of the quantification during 2021-2022 growing season in each orchard are shown in Fig. 4.2. The quantitative results indicated a statistically significant progressive increase in the concentration of *C. luteo-olivacea* on the fruit surface in each sampling month during the growing season, reaching the peak in October. The increase exhibited during the vegetative season from July to October in the two years was on average of 1.2 times. Surprisingly,no statistically significant differences of *C. luteo-olivacea* concentration were observed among the various orchards studied. Similarly, there were no significant differences detected between the years.

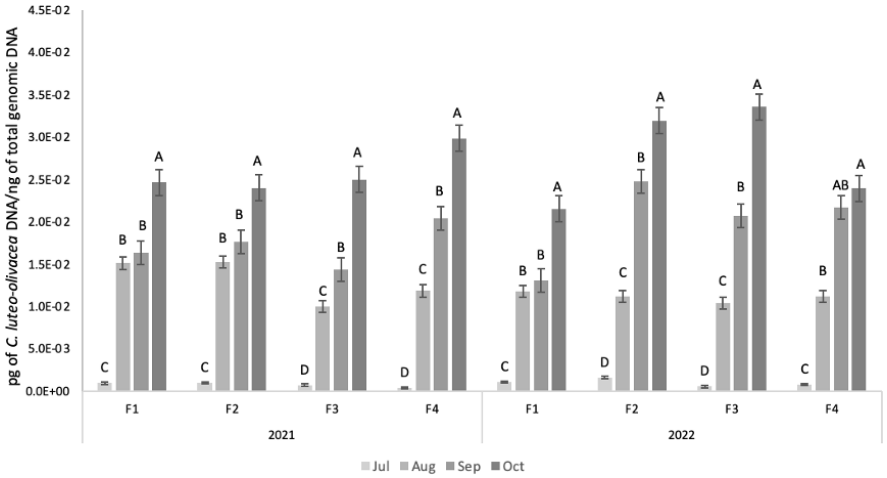

Figure 4.2: Population dynamics of *C. luteo-olivacea* on kiwifruit surface (expressed as pg of *C. luteo-olivacea* DNA/ng of total genomic DNA) recorded from July to October in 2021 and 2022 across four kiwifruit orchards (F1-F4) in FVG region, Italy. The data, estimated using qPCR, represent the average of two/three replicates for each orchard at each monthly sampling. For each month, different letters indicate significant differences according to Tukey's test (α = 0.05).

Nevertheless, in 2020, a marked statistically significant variation in pathogen concentration among farms was evident during the harvest period. Farm 1 displayed the lowest pathogen load, while the highest concentration was observed in Farm 4 (Fig. S1) with a difference of approximately 7.6 times in pathogen concentration between the two farms.

4.2.6 Post-harvest disease incidence of skin-pitting and possible correlation with the amount of *C. luteo-olivacea* at the time of harvest

The results of postharvest skin-pitting disease incidence analysis revealed varying levels of disease across the studied farms ranged from 1% to 10%. In certain cases, a positive correlation was observed between pathogen quantity on the fruit surface at harvest and the incidence of postharvest skin-pitting disease, with variations observed across different farms (Fig 4.3a & 4.3b). For instance, farms 2 and 3 exhibited a substantial inoculum quantity on fruits during the growing season and concurrently, they showed a quite high skin-pitting incidence during the storage period. However, the relationship between pathogen load and disease incidence differed in the case of farms 1 and 4.

In farm 1, despite the increase of the *C. luteo-olivacea* quantity on the fruit surface at harvest in both 2021 and 2022 compared to 2020, the post-harvest disease incidence of skin-pitting remained consistently low in all three years. Particularly, in 2022, the disease appeared even less frequently.

For farm 4, the correlation between the pathogen quantity on the fruitsurface and the incidence of postharvest skin-pitting disease was not statistically significant. In 2020 and 2021, the amount of the fungus on the surface was the highest among the farms, whereas the disease incidence presented an intermediate percentage value. Nevertheless, the quantity of the *C. luteo-olivacea* on the fruit decreased at harvest time, from 2020 to 2022, and this trend was also reflected in the decrease in the disease incidence that in 2022 was exceptionally low compared to previous years.

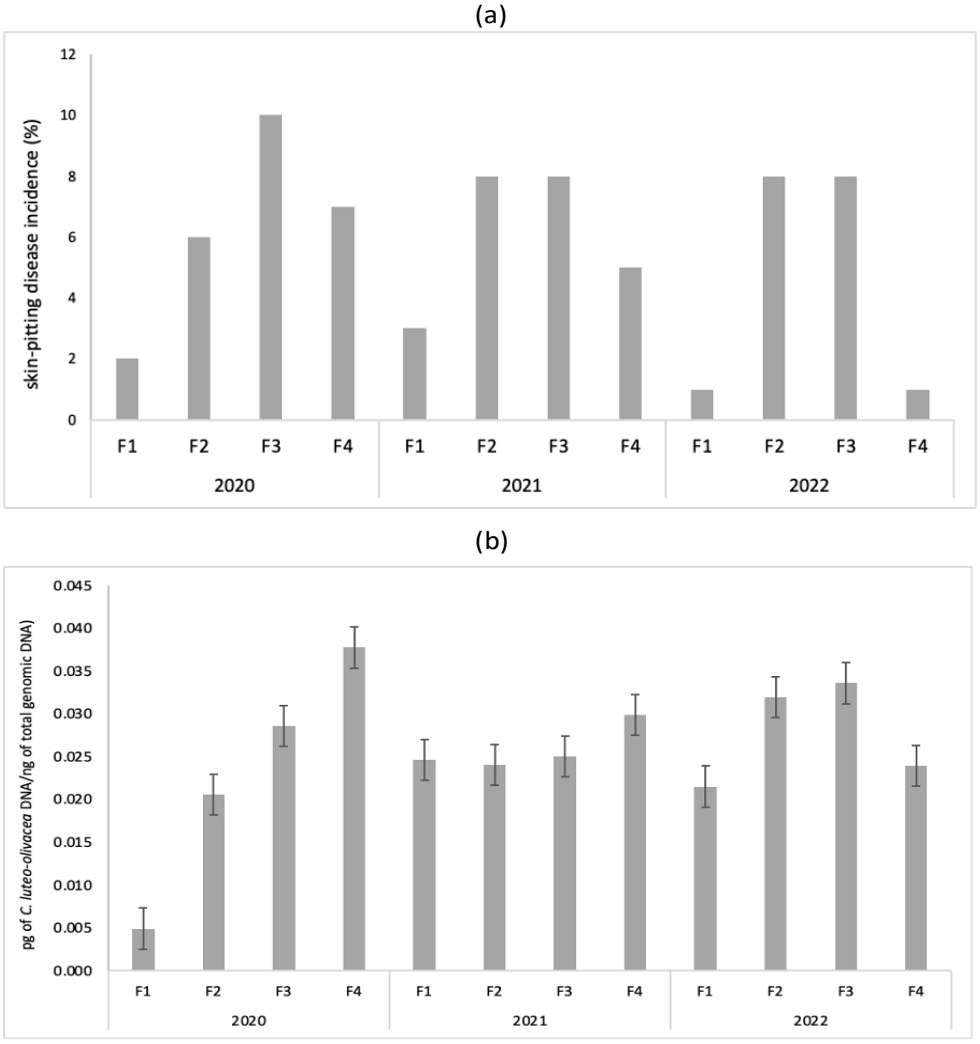

Figure 4.3: (a) Postharvest skin-pitting disease incidence (%) over three years (2020-2022) across four kiwifruit orchards (F1-F4). Pearson's correlation coefficient (r = 0.493, p = 0.002) indicates a moderate positive correlation between disease incidence and amount of *C. luteo-olivacea* DNA (Fig. 3b). Amount of *C. luteo- olivacea* DNA on kiwifruit surface (expressed as pg of *C. luteo-olivacea* DNA/ng of total genomic DNA) recorded in October 2020-2022 across four kiwifruit orchards (F1-F4) in FVG region. The data represent the mean of two/three replicates for eachorchard.

4.3 Discussion

To date, various methodologies including microscopy, imaging and non-imaging hyperspectral techniques, spore trap catches has been used for quantifying the fungal pathogens. In this study, we introduced a novel methodology, a reproducible method useful to concentrate and quantify fungal DNA from kiwifruit surfaces. The method was developed from some previous ones (Angeli et al., 2019; Hamilton et al., 2020) established for the study of the microbiome on the carposphere, which normally poses a significant barrier to high-quality microbiome research due to its low abundance of microorganisms, particularly on the fruit surface, due to environmental conditions including ultraviolet light exposure, limited nutrients and desiccation, and the physiological status of the plant (Hamilton et al., 2020). Enrichment procedures are required to increase microbial populations and to ensure that adequate DNA quantities are available for downstream amplification. In the present work, enrichment steps were carried out through filtration and centrifugation after washing and sonication of the fruits.

Additionally, a qPCR method was used to detect and quantify *C. luteo-olivacea,* the causal agent of postharvest skin-pitting disease, on the pellets obtained from kiwifruit surface through washing, sonication, and concentration steps. In the present investigation, a dynamic pattern in the inoculum density of *C. luteo-olivacea* on kiwifruit surface was studied using fruits from various orchards and years. A progressive increase in pathogen concentration during the growing season with peak levels in October during harvest, highlights the pronounced seasonal dynamics of this fungal pathogen.

Since the precise infection period of *C. luteo-olivacea* is not yet known and may occur during the entire growing season, from flowering to harvest (Köhl et al., 2018), an increase of pathogen inoculum on kiwifruit surface represents a serious risk of disease development in postharvest, especially if the infection can occur at fruit skinlevel. Interestingly, in a recent study by Di Francesco et al. (2022), a unique interaction between the pathogen and kiwifruit, characterized by the production of subepidermal mycelial stroma during the initial months of storage at 0 °C was described on fruits that were inoculated by wounds with *C. luteo-olivacea* conidial suspension. This stroma exhibits lytic activity on adjacent healthy tissues, remaining imperceptible until the fourth month.

The control of this complex disease trough preventive measures aims to reduce disease pressure; therefore, it will be necessary in the future to verify what the main sources of inoculum are in a kiwifruit orchard. In apple

orchards for example, highest concentrations of *C. luteo-olivacea* were found in apple leaf litter, mummies, and dead weeds, whereas in pear orchards *C. luteo-olivacea* was found in highest concentrations in pear leaf litter and in dead weeds (Köhl et al., 2018).

Interestingly, the lack of significant differences among orchards and between years suggests a uniform response of the pathogen to environmental conditions, emphasizing its resilience and adaptability. Only in October 2020, a significant variation among the farms was observed at harvest. This farm-specific variation indicated that some abiotic factors may have contributed to variations in pathogen concentration during the final stages of fruit development. Rodríguez et al. (2020) investigated the impact of meteorological factors on the development of *Botrytis cinerea* in two North-West vineyards, shedding light on the intricate relationship between environmental conditions and pathogen dynamics.

The quantification of *C. luteo-olivacea* populations on kiwifruit surfaces, determined by DNA quantification using qPCR, also served as a basis for assessing a possible correlation with skin-pitting incidence during prolonged cold storage. However, we observed variability in the correlation between *C. luteo-olivacea* population on fruit surfaces and postharvest disease incidence across different kiwifruit orchards. A positive correlation was shown in two farms which suggests a potential link between the pathogen load on the fruit surface and the development of postharvest disease in these farms. Our findings align with the research of Spotts et al. (2008) who reported statistically significant relationships between *Botrytis cinerea*,

Penicillium expansum, and stem end decay of pear fruit. Another study by Gell et al. (2008) presented a positive correlation between the occurrence of latent infection by *Monilinia* spp. and the development of post-harvest brown rot in peach and nectarine orchards in Spain.

In contrast, the remaining two farms exhibited lower incidence despite the pathogen's presence on fruit surfaces. It will be interesting in the future to analyze the dry matter and nitrogen content of the fruits at the end of their storage, since according to Spadaro et al. (2010) both characteristics are significantly associated with the disease incidence.

Overall, this study has paved the way for understanding *C. luteo-olivacea* dynamics on the kiwifruit surface, unveiling valuable insights into its seasonal patterns and inoculum density. While our focus was on the external aspects of pathogen presence, future investigations should delve deeper into what happens at the level of the flower and fruit tissues to unravel the complete spectrum of host-pathogen interactions. The main finding of this study emphasizes that to minimize the incidence of postharvest latent

infection of skin-pitting it is necessary not only to eliminate sources of primary inoculum but also to reduce the pathogen load on fruit surfaces before storage. Additionally, disease management programs should consider addressing the sources of airborne conidia of *C. luteo-olivacea.*

Supplementary material

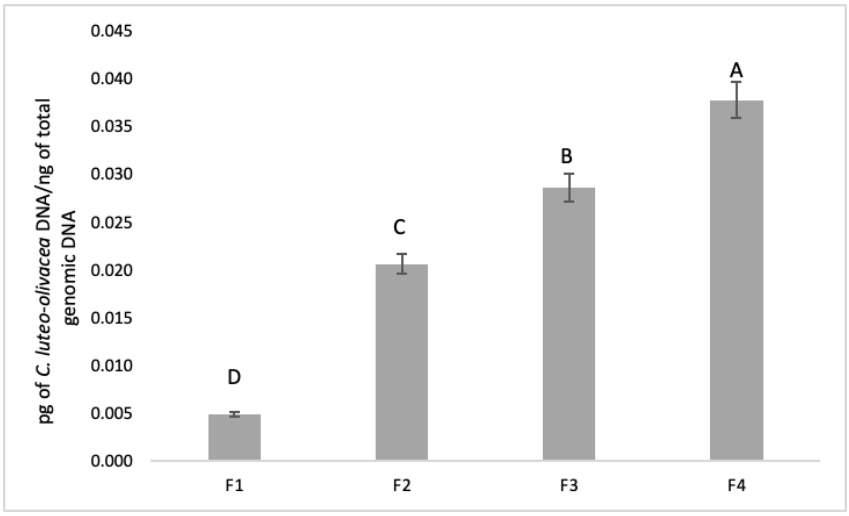

Figure 4.S: Amount of *C. luteo-olivacea* DNA on kiwifruit surface (expressed as pg of *C. luteo-olivacea* DNA/ng of total genomic DNA) recorded in October 2020 across four kiwifruit orchards (F1-F4) in FVG region. Data represent the mean of three replicates for each orchard. Different letters indicate significant differences between orchards according to Tukey's test ($\alpha = 0.05$).

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Final dissertation

Our research embarked a significant advancement to manage the postharvest skin-pitting disease of kiwifruit through the exploration of efficient biological control agents (BCAs) against the fungal pathogen *Cadophora luteo- olivacea* as an alternative of conventional fungicides. The European Green Deal aims to reduce the use and risk of chemical pesticides, as well as the use of more hazardous pesticides, by 50% by 2030 (Schneider 2023). In this context the multifaceted approach of this study provides a detailed understanding of the interactions between BCAs, targeted pathogens, and kiwifruit, offering efficient postharvest disease management strategies.

The initial phase of activity in 2021 involved isolating the fungal pathogen *C. luteo-olivacea* from symptomatic fruit tissues during postharvest cold storage (Chapter 1). This step aimed to address the poorly understood epidemiology of the target pathogen, known for its long latency and challenging manageability (Di Francesco et al., 2022). The efficacy of several bacterial and yeast strains was then evaluated as BCAs against the fungal pathogen, with *Pseudomonas* and *Bacillus* spp identified as the most efficient antagonists. While these strains are well-studied for their antagonistic activities on various fruit species and pathogens (Ji et al., 2013; Aiello et al., 2019), our focus was on their efficacy against *C. luteo- olivacea*. This integrated strategy seeks to further develop and deepen our understanding of their effectiveness in managing our specific pathogen.

However, the mechanism of action of these efficient BCAs should have been studied. The *in vitro* assays using these BCAs demonstrated different efficacy rates against *C. luteo-olivacea* (strain Cad21, isolated from kiwifruit) mycelial growth by producing non-volatile metabolites (Chapter2). The biochemical composition of the most active bacterial non-volatile secondary metabolites was analyzed via FT-IR (Fourier-Transform Infrared) spectroscopy, revealing the presence of peptide compounds, particularly from the amide I and amide II bands. These compounds are likely associated with the known production of antibiotic peptides and lipopeptides by various bacteria. Our results were aligned to many studies (Caldero et al., 2015: Rojas-Solis et al., 2020) where BCAs are known to produce these bioactive compounds.

Despite this, *i n vivo* assays, both curative and preventative, identified *P. synxantha* as most active candidate to inhibit the mycelial growth of *C. luteo-olivacea* on fruit surface. So, the biological antagonism exerted by *P. synxantha* strain 117-2b was further affirmed through qPCR analysis, showcasing a reduction in the pathogen's abundance. Asymptomatic fruits inoculated with BCAs exhibited lower fungal pathogen biomass compared to the control.

Recognizing *P. synxantha* as the most promising candidate, it's population dynamics was also studied during the four months of storage at0 ◦C. The results highlighted a significant increase in CFU during the initial three months, followed by a slight but noteworthy decrease after the third month of storage. This observation underscores the remarkable adaptation of *P. synxantha* to low temperatures, enabling prolonged persistence duringthe storage phase.

In addition to non-volatile compounds, the antagonistic activity of biological control agents (BCAs) is attributed to Volatile Organic Compounds (VOCs). Focusing on *P. synxantha* strain 117-2b as the most potential, it's VOCs were tested against two kiwifruit fungal postharvest pathogens: *C. luteo-olivacea* and *Botrytis cinerea* (Chapter 3). The results demonstrated a significant mycelial inhibition for fungal pathogen after the *in vitro*exposition to P. *synxantha* VOCs. Biofumigation of kiwifruits with these VOCs further showcased a substantial reduction in skin-pitting incidence caused by *C. luteo-olivacea* and grey mold caused by *B. cinerea*.
P. synxantha VOCs were analyzed by SPME-GC-MS analysis and pure synthetic compounds were tested against the mycelial growth of both fungal pathogens. These VOCs indicated a significant enhancement of the expression level of 7 defense-related genes of kiwifruit when used as biofumigant.

This comprehensive study shed new light on the effects of VOCs produced by the strain *P. synxantha* against *C. luteo-olivacea* and *B. cinerea*,both fungal postharvest pathogens of kiwifruit. The results underscored the essential role of VOCs in the antagonistic activity of a BCA and highlighted how their production can be significantly influenced by growth substrates. Moreover, the biofumigant treatment of kiwifruits with *P. synxantha* 117-2b notably enhanced the expression levels of fruit defense genes. The findings described a multi-approach *P. synxantha* VOCs characteristics that holds promise for inhibiting pathogen growth while simultaneously boosting fruit defense responses.

Although the symptoms of skin-pitting appear after three or more months of cold storage, the pathogen inoculation takes place in the field. A systematical monitoring of the pathogen inoculum densities on kiwifruit surfaces was performed across four kiwifruit orchards annually during the growing period for three consecutive years (Chapter 4). The underlying hypothesis posited that the concentration of pathogen inoculum increases during the growing season, serving as a significant source of postharvest skin-pitting disease. To effectively quantify the dynamics of inoculum density in these orchards, a real-time PCR assay was developed and deployed. Different patterns of *C. luteo-olivacea* density were observed within these orchards but a significant increase in pathogen concentration was observed in all orchards during the season. However, a correlation between pathogen concentration and the incidence of postharvest skin-pitting disease was also noted, though it was not consistently evident in every instance.

While this Ph.D. thesis has provided valuable insights into the development and biocontrol strategies for postharvest kiwifruit diseases, particularly skin-pitting, there remains a need for additional investigations to fully comprehend the precise contribution and practical application of biological control agents, with a particular focus on P. synxantha 117-2b, in mitigating kiwifruit postharvest pathogens. The findings and knowledge gained from this thesis lay a foundation for future studies in this field, contributing to the ongoing pursuit of effective strategies for managing postharvest diseases in kiwifruit.

Future perspectives

Although substantial progress has been achieved in this study in understanding the biocontrol potential of antagonistic organisms and the antifungal properties of bacterial strains, this study also highlights several directions for future investigations. Specifically, an in-depth genomic investigation of the underlying molecular mechanisms of actions of biological control agents, particularly regarding the activation of defense response in kiwifruit after the treatment of volatile organic compounds (VOCs), and non-volatiles could be an exciting direction for future research. Evaluating the combination of the most potent antifungal VOCs and their practical application is essential, as such strategies have the potential to reduce the risk of disease development and help to improve the fruit shelf life during storage. However, the practical application of these VOCs and their impact on fruit flavor and quality presents a rich area for future research. Additionally, this study underscores the importance of preharvest strategies, indicating a potential shift towards more proactive and targeted controlof postharvest diseases. Furthermore, analysis of the microbiome presents on the fruit surface and highlight of microbial community differences with high or low presence of *Cadophora* could address research of potential biocontrol agents. Potential of recent molecular tools in DNA sequencing have improved the capacity to investigate the composition and dynamics of complex microbial communities. Samples obtained in Chapter 4 could be a starting point in this kind of analyses about the compressive microbiome of fruit surface. Biocontrol agents isolated from the surface of kiwifruit could have the advantage of being adapted to colonize that surface and therefore potentially stable over time after they artificial application.

Nevertheless, the multifactorial nature of skin-pitting is challenging, and a significant research gap exist due to limited epidemiological knowledge of kiwifruit skin-pitting disease. Investigating the factors contributing to the inconsistent correlation between pathogen concentration and the incidence of disease which is responsible for irregular disease pattern is

essential. The disease incidence is likely linked to climate change so, a more detailed study on the external factors that might be influencing the disease outbreaks becomes essential. While our investigation centered on pathogen concentration on the fruit surface, future research should shift its focus to unraveling the complete spectrum of host-pathogen interactions within the fruit tissues.

For a healthier and more resilient kiwifruit industry, the implementation of advance and rapid strategies during harvest become crucial for early detection of pathogen. This can help to predict the susceptibility to skin pitting on kiwifruit and ensures effective management during fruit storage. A key point for future research involves the collaboration with industrystakeholders to create a knowledge-sharing network for continuous improvement. Simultaneously, engaging with kiwifruit growers to promote best practices is essential, making sure they know effective ways to manage diseases.

Finally, as this thesis addressed the postharvest disease control strategies for kiwifruit, knowledge derived from this thesis will certainly prove helpful in achieving the future perspectives mentioned above.

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